INVESTIGATION ON GALLIUM MALTOLATE PHARMACOKINETICS AND EFFICACY, AS ANTIMICROBIAL ALTERNATIVE IN AN EQUINE PROLIFERATIVE ENTEROPATHY INFECTION MODEL.

A Dissertation Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Veterinary Biomedical Sciences
University of Saskatchewan
Saskatoon

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PREFACE

This dissertation has been organized as a series of manuscripts which were or will be submitted for publication to peer-reviewed scientific journals. Thus, some repetition of introductory or methodology material is unavoidable.

ABSTRACT

Lawsonia intracellularis causes proliferative enteropathies in juvenile mammals. The porcine (PPE) and equine (EPE) diseases are worldwide. Rabbits and hamsters are naturally susceptible, the latter being a classic modeling-host for PPE. None is known for EPE, besides foals. An *in vitro* evaluation of antimicrobial efficacy against *L. intracellularis* is difficult. This study aimed to validate a laboratory animal EPE model and to investigate pharmacokinetics (PK) and efficacy of gallium maltolate (GaM) as an alternative antimicrobial therapy. Infected animals were inoculated with cell-cultured *L. intracellularis* and infection was verified with clinically utilized diagnostic tests.

Initially, 2 groups of EPE-infected rabbits were compared to 1 uninfected group. After inoculation (PI), EPE-infected rabbits showed mild clinical signs; detectable seroconversion, fecal shedding, gross lesions in intestinal tissues (IT), and early immuno-histochemistry labeling of *L. intracellularis* antigen. Thus, a humane EPE-rabbit model was achieved. Subsequently, EPE-infected hamsters were compared to uninfected and PPE-infected hamsters; whereas, PPE-infected rabbits were compared to EPE-infected rabbits. EPE-hamsters did not develop infection, unlike PPE-infected controls; and PPE-rabbits did not develop IT lesions or seroconversion comparable to EPE-rabbits.

Therefore rabbits were chosen as the EPE modeling-host for the GaM studies. First, GaM PK and IT concentrations of Ga and Fe were measured. Then, GaM efficacy was compared to a current EPE antimicrobial treatment. During sampling, the intra-arterial catheters in the rabbits' ears were protected with a novel moleskin-cover, allowing repeated sampling while minimally restrained.

The PK study was based on the comparison of EPE-infected and uninfected rabbits, after a single treatment with GaM, collection of serial blood samples and IT samples. The only differing PK parameter, between groups, was a decrease in the terminal phase rate constant of the EPE-rabbits, so a 48h dosing interval was chosen for the efficacy study.

In the efficacy study, 3 groups of EPE-infected rabbits were treated with GaM, doxycycline and a placebo, respectively. No differences were noted between treatments, in terms of lesions and fecal shedding. GaM appears no more efficacious than doxycycline in EPE-rabbits. In conclusion, albeit GaM tolerance appeared adequate in rabbits, results do not support its use in EPE-infected animals.

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to my supervisor Dr. Don L. Hamilton for his support through every step of my growth, as a laboratory researcher, critical thinker, statistician and better writer. To Dr. Julie Thompson I extend my thanks for having introduced and guided me to the unknown world of Synchrotron Radiation Analysis, trying to merge high-end technology and inorganic science with biomedical disciplines. To Drs. Chris R. Clark and Trisha M. Dowling, go my deep respect and gratitude for having introduced me to a different approach to pharmacology, teaching and antimicrobial drugs in veterinary medicine. My most sincere thanks go also to Dr. Jane Alcorn, as her help and dedication have been fundamental in transforming an equine medicine clinician into a researcher with a passion for laboratory animal models and pharmacokinetics. Last but not least, a special thanks to Dr. Gillian Muir for her supportive encouragement and a well thought-out opinion always available. As there are just so many ways in English to say "thanks" for their time, their help and for having put up with the turns my life has taken during my doctoral studies, I'm happy to say that I will never forget their commitment and example. I hope one day, as a sign of thanks to them, I will be able to pass on the grace they bestowed upon me to the people I will train and guide in scientific research.

The accomplishment of this dissertation includes people that were not directly involved in my advisory committee, but due to their dedication, constant presence and support they felt like they were. Thus, my gratitude goes to Dr. Andrew Allen (University of Saskatchewan, Saskatoon, SK), who guided me through those pathology branches that I've never appreciated so deeply, as a clinician, as I do now; To Drs. Connie Gebhart and Fabio Vannucci (University of Minnesota, St. Paul, MN, USA) who taught me about *Lawsonia intracellularis* and its genetics, the immunohistochemistry, serology techniques, cell cultures and infection model challenges, more than I ever imagined; To Drs. Nicola Pusterla and Samantha Mapes (University of California Davis, Davis, CA, USA) I express my gratitude for their precious contribution of extensive qPCR analysis and neverending support in "our bunny" research.

Special thanks go to my family and to those friends without whom it would have never been possible to drive this project to completion, much like thanking fellow riders through an endurance ride: Drs. Katherine R. Ball, DVM, Rekha T. Orchard, DVM, Joseph Rubin, DVM, Kim A. Tryon, DVM, Kelly M.N. MacLellan, DVM, Jasmine M Dhillon, DVM, Sheryl Gow,

DVM, Murray Jelinski, DVM and finally my husband COL. Kevin C. Smith, DO. A very special "thank you" goes to a veterinary student, Aphroditi J. Antonopoulos, who has been an incredible asset to my research: her presence and enthusiasm made everyone's day better (including the animals'). Thanks to their support and knowledge, most conversations became learning experiences and many complicated steps of the research were simplified, or at least eased.

I would like to express my gratitude to Dr. Colette Wheler (University of Saskatchewan, Saskatoon, SK) for training me for appropriate laboratory animals handling and always being open to lend instruments or advice; Dr. Ernest Olfert for helping with experienced advice about pertinent literature on rabbit models and rabbit catheterization. At this stage, it is fundamental to thank the Animal Care Unit personnel that helped during experiment time: Ms. Monique Burmester, Mrs. Paula Mason, Mel Browning and E.D. Parsons. Without their expertise and encouragement, the project would have been a daunting challenge. Equally my thanks go to Mrs. Cathy Coghlin, Darlene Hall, Cheryl Hack and Sandra Rose for their precious help in shipping samples to Minnesota or California: thanks to their professionalism and organization every sample collected arrived at destination in due time.

Also, for the submission of the 5 data chapters as articles in peer-reviewed journals I would like to acknowledge Ms. A.T. Gebhart, for the attentive editing provided and the many suggestions.

These studies were funded by a grant from the 2009 Equine Health Research Fund of the Western College of Veterinary Medicine and (in part) by a 2008 NSERC Grant ("Synchrotron microanalysis of gallium as a potential therapy for antimicrobial-resistant urinary tract infections"). As a Fellow Trainee I think it is my duty to acknowledge the CIHR-THRUST (Canadian Institutes of Health Research - Training Grant in Health Research Using Synchrotron Techniques) and all the supporters within the Synchrotron community at the Canadian Light Source, particularly Drs. Robert Blyth, Lucia Zuin and Renfei Feng.

Finally I would like to acknowledge the animals (hamsters and rabbits) that were used for these experiments, hoping that our experimental results will encourage other researchers, near and far, to be respectful of what animal models can provide and teach.

DEDICATION

This dissertation is dedicated to several people in my life, as their constant presence made it possible for me to achieve such a prestigious goal with passion and determination.

To my husband, Kevin, as he kindly supported me through every step of this adventure with patience, encouragement and love.

To my parents, Giuseppe and Rita, as their encouragement and unconditional love never faltered through a far distance and… the third thesis;

To my in-laws, Charles and Norma, as their support and understanding came unconditioned and total from the day I've met them;

To my Alabamian family, Jeanne, Jerry, Peggy and Bob, for they supported me as their own daughter and niece with love and advices.

To my brother and sister, Raffaele and Cristiana, and to the brothers and sisters of my heart, Chris and Kim, Sam, Tommy and Nichole, Wes and Sandra, Rita and Kim, Mala, and Lucia, as they always pushed me with a smile to never give up, even when things were difficult.

Last but not least, this dissertation is dedicated to my yet unborn son, to show him that commitment runs in the family and I'll be committed to him the same way I'm committed to good science. All my life.

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LIST OF ABBREVIATIONS

AFMA	Americans for Medical Advancement
AM(s)	Antimicrobial(s)
AUC	Area under the curve
Cl_O	Oral clearance
C_{max}	Maximum concentration obtained in serum after drug exposure
DMT-1	Divalent metal transporter-1
DPI or PI	Days after inoculation or Post infection
ELISA	Enzyme Linked Immuno-sorbent Assay
EPE	Equine proliferative enteropathy
Fe(II)	Ferrous Iron
Fe(III)	Ferric Iron
[Fe]	Iron concentration
FISH	Fluorescent in-situ hybridization
[Ga]	Gallium concentration
Ga(III)	Elemental Gallium
GaM	Gallium maltolate
GIT	Gastrointestinal tract
H&E	Haematoxylin and Eosin staining
IFAT	Indirect Fluorescent Antibody Test
IHC	Immunohistochemistry
IPMA	Immunoperoxidase monolayer assay
IPX	Immunoperoxidase
IRE	Iron responsive elements
IRP	Iron regulatory protein (1, 2,…)
ISH	In-situ hybridization
IT	Intestinal tissues
K_m	Michaelis-Menten affinity constant
mM	Millimole
MRT	Mean residence time
OGAT	Oral glucose absorption test
PCR	Polymerase chain reaction
PE	Proliferative enteropathy (in general, for every species)
PHE	Porcine hemorrhagic enteritis
PIA	Porcine intestinal adenomatosis
PK	Pharmacokinetics
PPE	Porcine proliferative enteropathy
PT	Post treatment
qPCR	Quantitative PCR

$t_{½}$	Half-life
T_{max}	Time to reach maximum concentration after drug exposure
V_{max}	Maximum initial rate of an enzyme (in this case) catalysed reaction
VESPERS	Very Sensitive Elemental and Structural Probe Employing Radiation from a Synchrotron
vs.	versus
WS	Warthin-Starry staining
λ	Elimination rate constant (non-compartmental analysis)
μM	micromole

CHAPTER 1

INTRODUCTION

"Give a man a horse he can ride. Give a man a boat he can sail."

J. Thomsen

Equine proliferative enteropathy, EPE, reported since the early 1980's in sporadic and isolated cases, is now an important equine gastrointestinal illness, with frequent reported outbreaks of economic significance. As recently reviewed, EPE is caused by *Lawsonia intracellularis*, an intracellular bacterium affecting the small intestine of many other mammalian and avian species, both among domestic and wildlife animals (Pusterla & Gebhart, 2009). *Lawsonia intracellularis* is now the focus of attention in many centers of the equine industry around world (*e.g.*, Kentucky, USA or Ontario, Canada etc.). A few review publications about porcine proliferative enteropathy (PPE) showed that EPE in horses parallels what was observed since 1931 in juvenile pigs. The bacterial cause was only suspected in the early 1970's, and taxonomically classified in 1995 (Lawson & McOrist, 1993; Lawson & Gebhart, 2000).

The characteristic enteropathy generated by this bacterium appears similar in all the species affected (not only horses and pigs, but also hamsters). The typical lesions reported in susceptible animals are: a similar pattern of mucosal hyperplasia, lack of cell differentiation, malabsorption and reduced inflammatory response. Thus far, humans seem not to be part of the broad target of this bacterium (Michalski *et al.*, 2006). *Lawsonia intracellularis* customarily targets young animals undergoing stressful situations (*e.g.* weaning, deworming, commingling and traveling), and very seldom adult animals, where it usually constitutes a secondary pathogen (*e.g.*, complicated salmonellosis or digestive tract lymphoma).

The intracellular location of this pathogen affords it with protection from the effects of humoral and cellular immune-response and classical first line antimicrobial drugs. As reports of cases of treatment failure in light of exacerbated clinical signs, and the economic impact on the

industry are increasing (Frazer, 2008), the need for novel antimicrobial alternatives is acknowledged both in research and clinical settings.

In the context of a potential antimicrobial alternative, the compound gallium maltolate, appears as an obvious candidate. It has already been safely administered, orally, to multiple animal species: rodents, dogs, horses and humans. Gallium has iron mimicking abilities in regards to its metabolism, but it is virtually unable to complete redox reactions (Bernstein *et al.*, 2000). This feature could be utilized against iron dependent bacteria, hopefully without setting up a situation for future antimicrobial resistance.

The general objective of this project was to test a laboratory animal infection model for *L. intracellularis*, to estimate appropriate PK parameters for gallium, before an efficacy study can occur, which would compare gallium maltolate and the current clinical treatments against EPE.

CHAPTER 2

LITERATURE REVIEW

"…there is a touch of divinity…and a special halo about a horse that should forever exempt him from indignities."

H. Melville

2.1: Equine Proliferative Enteropathy: *Lawsonia intracellularis* infection in horses and present knowledge of the disease

The gastrointestinal tract of horses is the object of many life-threatening ailments, mechanical or inflammatory, affecting the small or large intestine. They might also present as combined disturbances, whose clinical appearance is complicated by inter-twined pathogeneses of different lesions. This dissertation will focus on an emerging bacterial infection of the small intestine, Equine Proliferative Enteropathy (EPE), caused by *Lawsonia intracellularis* a pathogen that recently is receiving appreciable attention in equine medicine.

2.1.1. Gastro-enteric diseases in foals

Gastrointestinal (GI) inflammatory disorders are amongst the most important causes of morbidity and mortality in horses of all ages. GI conditions challenge clinicians' knowledge, diagnostic procedures and therapy, as reviewed by Feary & Hassel, in 2006. Even more complicated is the gastroenterology of young horses (younger than 8 - 10 months of age), with multifaceted presentations predominant at different pediatric ages, and clinical signs common between many etiologies. Usually these diseases develop rapidly and can result in acute or chronic debilitation, due to diarrhea, fever, colic, gastric reflux, and culminating potentially with shock and death (Feary & Hassel, 2006).

Infectious diarrhea is a clinical condition that may require veterinary intervention at least once for ~80% of foals, within their first 6 months of life (Urquhart, 1981). Foal diarrhea can be due to infectious and non-infectious causes. In foals and weanlings, dietary indiscretion, over-feeding and lactose intolerance, nutritional intoxications (*e.g.*, cantharidin toxicity, related to alfalfa hay feedings), non-steroidal anti-inflammatory drugs (NSAIDs) toxicity, sand enteropathies, and gastro-duodenal ulceration, should be considered as differential diagnoses (Urquhart, 1981, Sweeney, 1987; Magdesian, 2005).

Several non-infectious causes can be excluded through detailed history and appropriate diagnostics (Baverud, *et al.*, 1998; Frank, *et al.*, 1998; Feary & Hassel, 2006). Also, the age of the patient is a determinant factor in diagnosis of foal-heat diarrhea and neonatal asphyxia-associated gastro-enteropathies, as are typical of neonates, much younger than the EPE's target population (Urquhart, 1981; Magdesian, *et al.*, 2002; Magdesian, 2005; Frazer, 2008; Lavoie & Drolet, 2009, Pusterla & Gebhart, 2009).

Many infectious agents are implicated in diarrhea and malabsorption processes in foals, whether as single or combined pathogenic causes. The most common infectious causes are *Salmonella spp.* and *Clostridium spp.; Rhodococcus equi* and *Lawsonia intracellularis* for bacteria*; Adenovirus, Rotavirus* and *Coronavirus* for viruses; and nematodes and a few protozoan species for parasites (Feary & Hassel, 2006; Frederick, *et al.*, 2009). A brief mention of each group of differential etiologies follows below, as the reasoning process of the attending clinician should include these possibilities when admitting foals with colic, diarrhea, marked weight loss and/or ventral edema.

Rotavirus, Adenovirus and *Coronavirus* infections typically present with profuse diarrhea and cannot be confirmed, or excluded, solely on the basis of foal age and history (Browning, *et al.*, 1991). Adenoviral infection may cause severe diarrhea and in some cases lead to intussusceptions and death. In some cases, *Adenovirus* and *Rotavirus* are cofactors in causing infectious diarrhea, but pure rotaviral infections in foals are common and can cause severe acute diarrhea, with a very brief incubation period (hours) after inoculation. Investigation on adult horses showed that the world-wide seroprevalence is 100% and vaccination of mares has been possible since the late 1990s (Powell, *et al.*, 1997). It appears more common in younger foals (up to 2.5 months of age) than coronaviral infections, whose role within enteric diseases needs

yet to be clarified (Browning, *et al.*, 1991; Guy, *et al.*, 2000). Currently an effective and reliable ELISA test for *Rotavirus* is available (Frederick, *et al.*, 2009), whereas *Coronavirus* diarrhea outbreaks in horses are more difficult to demonstrate, although a PCR technique has been recently reported (Frederick, *et al.*, 2009; Pusterla, *et al.*, 2013).

For parasitic infections, *Strongyloides westerii* is probably the most common cause of colitis and diarrhea in foals younger than 1 month of age, but ascarids (*Parascaris equorum)* are considered the more pathogenic causing a variety of signs, from unthrifty appearance, to colic and death, in foals younger than 6 months of age. This holds particularly true, if foals are kept on a poor deworming program, or raised in areas where anti-helmintic resistance is possible (*e.g.*, Kentucky, USA) (Feary & Hassel, 2006; Lyons, *et al.*, 2006). Removal of these parasites by use of parasiticides before maturation (*e.g.*, *P. equorum* has a minimum pre-patent period of about 8-10 weeks), lessens the possibility of pathologic effects. Modern anti-helmintic regimens have this common aim, but they also have caused the emergence of several sources of resistance, such that small strongyles (or cyathostomes) are often a cause of colitis, acute and chronic diarrhea, particularly in horses between 1-6 years of age (Feary & Hassel, 2006). In temperate areas, cyathostomiasis' occurrence is typical of late winter and spring months, whereas in South-Eastern USA, and other subtropical regions, it is more characteristic of late fall and winter (Feary & Hassel, 2006). Interestingly, this pattern matches the typical period of EPE's onset in weanlings in the Northern Hemisphere (Frazer, 2008). Testing for fecal parasitic egg-counts can lead to exclusion of parasitoses, or to the understanding of their role as co-factors in the symptomatology. Foals on good anti-helmintic treatment routines, typically present only small strongyle *larvae* (Lyons & Tolliver, 2003). Recently, a study conducted on Thoroughbred breeding farms, in central Kentucky, reported that the percentage of parasite eggs in feces of foals younger than 12 months of age (*i.e.*, the age range for EPE) was 39% for *P. equorum*, 32% for *Strongyles spp.*, 2% for *Strongyloides westerii*, and 28% for the protozoan *Eimeria leuckarti* (Lyons, *et al.*, 2006). More than one foal harbored *S. westeri* infections in 29% of the tested farms, whereas 86% of foals harbored *P. equorum* and small *Strongyles spp*. Again, *E. leuckarti* was detected in foals' feces on 86% of farms year around, except December (Lyons, *et al.*, 2006). Last, but not least, *Crytptosporidium spp.* should be considered, but it is less common and can be excluded upon negative results of fecal material examination (Frank, *et al.*, 1998).

With regards to bacterial infections, the most common pathogens for foals of suckling/weaning age remain *Salmonella spp*. and *Clostridium perfringens*, while *Clostridium difficile* seems more common in foals between the ages of 2 weeks to 4 months. However, *Salmonella spp.* and *Clostridium spp.* are the most common pathogens isolated from horse fecal material at any age (East, *et al.*, 1998; Magdesian, *et al.*, 2002; Frederick, *et al.*, 2009). Their diagnosis rests on repeated culture tests for the bacterial isolation (at least 5 in 5 consecutive days, for suspected salmonellosis) or PCR techniques. Specifically for *Clostridia*, PCR techniques are utilized to isolate not only the bacterial DNA, but also the enterotoxins. Unfortunately, culture tests are not very sensitive (20%, in *Salmonella* cases) and even PCR testing sometimes fails to detect clostridial enterotoxin, or signals false positives (Palmer, *et al.*, 1985). Indirect tests, although non-specific, may support the diagnosis of salmonellosis, as in the case of WBC count in fecal smears (>10 cells/high power field) (Morris, *et al.*, 1983). *Rhodococcus equi* occasionally causes enteritis and colitis, and so does *Neorickettsia risticii*, but only in rare cases in this age range (Feary & Hassel, 2006). Finally, EPE is now a well described emerging disease, which in the past 20 years has reached world-wide distribution and whose case presentations have been extensively reviewed (Lawson & Gebhart, 2000; Magdesian, *et al.*, 2002; Feary & Hassel, 2006; Lavoie & Drolet, 2009; Pusterla & Gebhart, 2009).

2.1.2. Equine proliferative enteropathy: from sporadic beginnings to outbreaks.

The first EPE case reported was a 6-month-old Arabian filly admitted in a semi-comatose condition to the Veterinary Teaching Hospital at University of California – Davis, in 1982 (Duhamel & Wheeldon, 1982). The filly had a 4-day-long history of anorexia, depression and black loose feces, while at pasture with its dam. Despite intensive treatment, the filly died 12 h after admission and was necropsied shortly after. On *post-mortem* examination, large hemorrhagic gastro-duodenal ulcers and extensive, irregular mural thickening (up to 12 mm) with prominent corrugation of the mucosal surface was detected in the distal duodenum, jejunum and ileum. Also, flattened nodules in the mucosa were noted in the proximal to mid-ileum, some 3-7 mm in diameter, along with sparse large colon mucosal inflammation. On histopathology,

intestinal crypts appeared hyperplastic, elongated and branched, containing occasionally purulent material (Duhamel & Wheeldon, 1982).

Epithelial cells of the enteric crypts' characteristically contained comma-like rod shaped bacteria localized at the apex. The bacterial structures, located within the cytoplasm, were faintly visible with Levaditi-Manovelian stain, but were clearly detected on electron microscope examination (Duhamel & Wheeldon, 1982). Negative culture and staining were obtained for routine gastro-intestinal (GI) bacteriology, as well as for *Salmonella spp.* and *Mycobacterium spp.* A McMasters' egg-count was also negative and consistent with recent deworming. The bacteria detected were identified as *Campylobacter sputorum* subsp. *mucosalis,* the "at the time" suspected bacterial etiologic agent of porcine intestinal adenomatosis (later renamed porcine proliferative enteropathy – or PPE), and also reported as a cause of disease in sheep, hamsters and the blue fox (Wagner, *et al.*, 1973; Rowland & Lawson 1975; Vandenberghe & Hoorens, 1980; Landsverk, 1981; Duhamel & Wheeldon, 1982). In the 1980's, the bacterium did not fit the complete *Campylobacter* definition and testing. It indeed constituted a new genus and species, *Lawsonia intracellularis,* was finally identified and coded in the early 1990's, after being renamed a few times (Jones, *et al.*, 1993a; Gebhart, *et al.*, 1993; McOrist, *et al.*, 1995a).

In 1996, the second case of EPE was detected in a 5-month-old filly at the Veterinary Diagnostic Laboratory of the University of Kentucky, with a week-long history of depression, lethargy, anorexia and profuse watery diarrhea (Williams, *et al.*, 1996). The etiology of this case was ascribed to a *L. intracellularis*-like bacterium on the basis of necropsy results. The thickening and corrugated lesions were confined to jejunum, with a multifocal, discoidal pattern and a diffuse thickening, and an even more pronounced rugose pattern in the ileum (Williams, *et al.*, 1996). Bacteria were consistently noted in the apex of the epithelial cells of the enteric crypts through Warthin-Starry silver impregnation method and confirmed through electron microscopy and other DNA sequencing tests (See Chapter 2.2). Bacterial colonization was not demonstrated anywhere else (Williams, *et al.*, 1996).

The third case was reported in 1998 in a 6-month-old Quarter Horse colt admitted for diarrhea of 3 days duration at the Veterinary Teaching Hospital at Purdue University (Frank, *et al.*, 1998). The foal, recently vaccinated and dewormed, showed the first clinical signs while at a horse show. The foal had fever, profuse projectile diarrhea, depression and mild dehydration. It

was in good body condition and had normal cardiac and respiratory rates. Hyperfibrinogenemia, leucocytosis with left shift, hypoproteinemia with hypoalbuminemia, azotemia and hyponatremia were detected (Frank, *et al.*, 1998). Several infectious diseases were excluded by microbiology and parasitology test results. Infiltrative bowel diseases (*i.e.*, granulomatous enteritis, eosinophilic gastroenteritis and intestinal lymphosarcoma) were considered in the differential diagnoses list (Frank, *et al.*, 1998). The foal underwent supportive treatment with fluid-therapy and plasma transfusions, but no antimicrobial (AM) therapy. After the fever abated, corticosteroid therapy was initiated in an attempt to treat the suspected infiltrative disorder. Following clinical improvement, the colt was discharged, but re-admitted within a few weeks. On the second admission, its physical and clinico-pathological condition had relapsed and worsened. Euthanasia was elected due to poor prognosis (Frank, *et al.*, 1998). At necropsy, thickening and multi-nodular corrugation of the intestinal mucosa, associated with sub-mucosal edema, was detected in duodenum, jejunum and ileum, along with small *foci* of mucosal ulceration and necrosis, covered with fibrin. Histologically, hyperplastic enteric crypts, elongated, tortuous, and, at times, "herniated" in the sub-mucosa, were detected. Intestinal villi appeared blunted and fused together and the crypts' epithelium appeared multi-layered and lacking mucous vacuoles (*i.e.*, showing sign of immaturity). The area of ulceration showed granulation tissue replacing the damaged mucosa. Rod-shaped microorganisms were noted again at cells' apex and immunohistochemistry (IHC) and polymerase chain reaction (PCR) techniques, previously described for PPE (see Chapter 2.2), were used to confirm the diagnosis on the tissues of the affected foal (McOrist, *et al.*, 1987; Cooper, *et al.*, 1997a).

A miniature foal, admitted for weight loss and diarrhea to the Veterinary Teaching Hospital at Iowa State University and deceased shortly after, was diagnosed only after *post-mortem* examination, adding to the list of fatalities during that time period (Brees, *et al.*, 1999). Until 1999, *L. intracellularis* infection in foals was considered debilitating and invariably fatal and through journal editorials, clinicians advocated for an early diagnostic technique, to deal in a timely fashion with an infection that was gaining notoriety and knowledge only in necropsy settings (Duhamel & Wheeldon, 1982; Williams, *et al.*, 1996; Frank, *et al.*, 1998; Smith, 1998; Brees, *et al.*, 1999).

In 2000, EPE came to international attention, and was officially defined and named according to similarities to PPE's presentation (Lawson & McOrist 1993; McOrist, *et al.*, 1995a;

Lavoie, *et al.*, 2000; Lawson & Gebhart, 2000; Lavoie & Drolet, 2009). Lavoie and coworkers reported the findings and the outcomes of 3 outbreaks in 3 different farms, 2 of which were in Quebec and one in Ontario, Canada. The former 2 farms were Arabian horse breeding operations, related by transport and commingling of foals; the third one dealt with Thoroughbreds horses and different routines. The 2 outbreaks in Quebec occurred in large farms, where the number of broodmares and weanlings recently increased. Weanlings were housed together in pens of 4, during the day, and paired 2 per stall, at night. On those premises, not only EPE was detected, but also other infective stressors were diagnosed (*e.g., R. equi* and *S. equi*, among others). Two farms were not close to piggeries, but were bordered by a forest, with access to wildlife, and a creek running through the pastures was used for watering purposes. However, one Québécoise farm was not far from a piggery and a dog moved unrestrictedly between the 2 structures (Lavoie, *et al.*, 2000). All the index cases died, despite treatment. Two foals died on the first and 1 on the second Quebec farm, and 1 on the Ontario farm. The 5th foal died on the first farm 6 weeks after, and despite, an initial clinical recovery. All deceased foals were cachectic and affected by other diseases concomitantly to EPE, such as: ascaridiasis (1/5), gastric ulcers and gastritis (3/5) and concurrent pulmonary or broncho-pulmonary pathology (all 5), similarly to many surviving foals (Lavoie, *et al.*, 2000).

The typical presenting signs were depression, poor body condition, despite a good appetite, profuse diarrhea, ventral edema and colic (as reported for EPE by other authors (Duhamel & Wheeldon, 1982; Williams, *et al.*, 1996; Frank, *et al.*, 1998; Lavoie, *et al.*, 2000; Bihr, 2003)). Consistent blood work findings were: marked mature leucocytosis, hyperfibrinogenemia and hypoproteinemia. Only in 2 cases total serum proteins were above 60 g/L, as foals were concomitantly affected by *S. equi*. The main management difference between deceased and survivor foals appeared due to the AM therapy (see Chapter 2.3). Supportive therapy alone did not lead to survival (Lavoie, *et al.*, 2000). The diagnosis of EPE was achieved through the *post-mortem* examination of the deceased foals. Diagnosis was obtained through gross and histopathologic evaluation of the bowels (particularly as lesions were confined to distal jejunum and ileum, or ileum alone) and ileal tissue PCR – although the bench tests were not always in agreement (2/5 cases). Survivors, treated on the basis of the diagnosis achieved at necropsy, were tested for fecal PCR (negative in most cases) and for seroconversion, which

never yielded high titers (1:120 max). In conclusion, with the appropriate AM therapy, 24 out of 29 affected foals recovered, in the first EPE outbreak (Lavoie, *et al.*, 2000).

Shortly after Lavoie's report, a case of surgical and medical management of EPE was published. A 3-month-old Arabian filly was admitted to Auburn University Veterinary Teaching Hospital, after recurrent abdominal pain of a week's duration (Schumacher, *et al.*, 2000). The only bout of diarrhea reported in her history was of a couple weeks from birth and it was seemingly unrelated to the clinical condition on admission. Despite normal vital parameters, the filly appeared markedly underweight. The ultrasonographic examination allowed visualization of thickened small intestine, with loss of discernible architecture of the three-layered wall's (Schumacher, *et al.*, 2000). The filly underwent exploratory laparotomy, as abdominal pain was unrelenting. Distal jejunum and ileum were severely thickened and the GI sections proximal to the mural thickening were distended, hypomotile and fluid filled, explaining the origin of pain. Surgeons collected a diagnostic biopsy from the thickened tracts, and by-passed the affected small intestine with a side-to-side anastomosis between normal jejunum and cecum. Diagnosis was achieved through special stains and AM therapy was changed accordingly, leading to a full recovery and conditions comparable to unaffected herdmates, within a couple of months (Schumacher, *et al.*, 2000).

Since these latter reports, clinical EPE cases have been reported in multiple localities of North America, Europe, Brazil, Australia, Japan and South Aftrica, suggesting that EPE has worldwide distribution, either presenting as isolated cases or outbreaks (Bihr, 2003; McClintock & Collins, 2004; Deprez, *et al.*, 2005; Dauvillier, *et al.*, 2006; Sampieri, *et al.*, 2006; Feary, *et al.*, 2007; Frazer, 2008; Guimarães-Ladeira, *et al.*, 2009; Lavoie & Drolet, 2009; Merlo, *et al.*, 2009; Shimizu, *et al.*, 2010).

2.1.3. Epidemiology and clinical appearance of EPE

In the past 20 years, *L. intracellularis* has been reported in red deer, foxes, grey wolves, dogs, calves, hedgehogs, rabbits, hamsters, ostriches, macaques and a giraffe, but it seems not to constitute a zoonosis, although such debates have been heated for a while (Collins, *et al.*, 1983;

Hotchkiss, *et al.*, 1996; Cooper, *et al.*, 1997b; Duhamel, *et al.*, 1998; Klein, *et al.*, 1999; Herbst, *et al.*, 2003; Tomanova, *et al.*, 2003; Michalski, *et al.*, 2006; Jacobson, *et al.*, 2010). Clinical *L. intracellularis* infection most commonly occurs in foals between 4 and 7 months of age, but range from 2 to 13 months, with no gender or breed predisposition (Lavoie, *et al.*, 2000; Frazer, 2008; Lavoie & Drolet, 2009). Age is also a contributing factor in other species, including pigs, hamsters and deer (Jasni, *et al.*, 1994a; Drolet, *et al.*, 1996). Both pigs and horses are affected as nursing or weanling animals, and, although it is suspected that immunosuppression plays a major role in the pathogenesis, any kind of stress can constitute a predisposing factor, including vaccination, anti-helmintic therapies, transport, overcrowding and commingling, ration changes and/or intensive training (Smith, 1998; Lavoie, *et al.*, 2000; Dauvillier, *et al.*, 2006).

L. intracellularis is now considered endemic in North America, with clinical disease in foals observed primarily between August and January (Frazer, 2008; Pusterla, *et al.*, 2009a). Exposure is related to the per-oral contamination with feces, so infected animals represent a *reservoir* for infection for their herd-mates (Lawson & Gebhart, 2000; Pusterla & Gebhart, 2009; Pusterla, *et al.*, 2010a). Pigs and foals are known to shed *L. intracellularis* for up to 12 weeks post-infection (Guedes & Gebhart, 2003a; Pusterla, *et al.*, 2010a). Debate exists about the potential of cross-contamination between pigs and horses, as the first outbreak was reported in the vicinity of a piggery (Smith, 1998; Lavoie, *et al.*, 2000). However, *L. intracellularis* becomes inactivated by direct exposure to atmospheric oxygen, making cross-contamination through inanimate objects unlikely (Smith, 1998), even though it was shown that PPE *L. intracellularis* could survive almost 2 weeks in the environment.

The genomic similarity of equine and porcine strains is approximately 98%, on the basis of results of 16S rDNA sequencing (Cooper, *et al.*, 1997b; Lawson & Gebhart, 2000), and hamsters and other rodents could be infected with a porcine strain with variable success (Cooper, *et al.*, 1997b; Smith, 1998; Smith & Lawson, 2001; Vannucci, *et al.*, 2010). Furthermore, the possibility of cross-infection from *reservoirs* other than pigs has been suggested, ranging from animals living on the farms' premises (*i.e.*, dogs, cats, mice), or the surrounding wildlife (*e.g.*, deer, rabbits, hares, etc.) (Cooper, *et al.*, 1997b; Duhamel, *et al.*, 1998; Lawson & Gebhart, 2000; Herbst, *et al.*, 2003; Pusterla, *et al.*, 2008a; Pusterla, *et al.*, 2008b; Collins, *et al.*, 2011; Pusterla, *et al.*, 2012a). However, whether cross-infection is possible thus far remains speculative, and

complicates the assessment and control plans for EPE in horse farms (Pusterla, *et al.*, 2008a; Pusterla, *et al.*, 2009a).

The severity of clinical EPE varies between affected horses within a group, as some may exhibit fever, repeated episodes of colic, whether or not complicated by persistent diarrhea and tenesmus (Duhamel & Wheeldon, 1982; Williams, *et al.*, 1996; Frank, *et al.*, 1998; Lavoie, *et al.*, 2000; Bihr, 2003). Most affected subjects present with marked ventral edema (pot-bellied appearance) and hypoproteinemia, overt weight loss, depression and inappetance (Duhamel & Wheeldon, 1982; Williams, *et al.*, 1996; Frank, *et al.*, 1998; Lavoie, *et al.*, 2000, Bihr, 2003). In other cases, fever is absent, clinical GI signs, and edema, are less obvious, but body condition is generally poor and debilitation is chronic (Lavoie, *et al.*, 2000; Sampieri, *et al.*, 2006; Lavoie & Drolet, 2009). Some horses seroconvert and shed *L. intracellularis* in feces without ever showing clinical signs (Pusterla, *et al.*, 2008a; Lavoie & Drolet, 2009). Recently, 3 EPE clinical forms have been identified (similarly to PPE): an acute presentation; a classical form and a subclinical form. In addition, a very recent report about a fulminant enteritis form, associated with DIC, has been published (Page, *et al.*, 2011a; Page, *et al.*, 2012). Clinically affected horses may die, or be euthanized, due to severe enteropathy and accompanying biochemical imbalances, dependent on small intestinal malabsorption (Wong, *et al.*, 2009), or signs of colic may be so severe to warrant exploratory surgery (Schumacher, *et al.*, 2000; Sampieri, *et al.*, 2006; Frazer, 2008). In affected foals, both jejunal and ileal walls are grossly thickened and corrugated, and, on ultrasonographic examination, intestinal loops have a typical "doughnut-like" appearance, which today is considered a pathognomonic sign of EPE (Schumacher, *et al.*, 2000; Sampieri, *et al.*, 2006; Frazer, 2008).

2.1.4. Pathogenesis of EPE

EPE is by definition an enteropathy, since inflammatory infiltration with reticulo-endothelial cells was typically seldom detected on histological examination (McOrist, *et al.*, 1995a; Lavoie, *et al.*, 2000; Lawson & Gebhart, 2000). Contamination and infection occur through the fecal-oral route and infected herd-mates have a pivotal role as *reservoirs* in propagating the infection to naïve foals (McOrist, *et al.*, 1993; McOrist, *et al.*, 1995a). In horses,

the progression of EPE pathogenesis has been confirmed through epidemiological evaluation of outbreaks and the reproduction of a *L. intracellularis* infection model in foals. (Lavoie, *et al.*, 2000; Lavoie & Drolet, 2009; Pusterla, *et al.*, 2010a) Experimental incubation is reportedly 2 to 3 weeks in pigs and horses, and is likely similar for other species (Pusterla, *et al.*, 2010a). Pathological lesions in horses are similar to those found in pigs and hamsters with PPE and PPE-derived enteropathy, respectively, as reviewed in 2 classic publications (Lawson & Gebhart, 2000; Smith & Lawson 2001).

Development of *L. intracellularis* infection is widely influenced by the presence of an active intestinal bacterial flora, as pathogen-free piglets inoculated with *in vitro* grown *L. intracellularis* do not develop infection, whereas piglets infected with filtered mucosal homogenates – wherein *L. intracellularis* is not the only pathogen – develop disease easily (McOrist, *et al.*, 1993; McOrist, *et al.*, 1994). Similarly, gnotobiotic piglets infected with *in vitro* grown *inoculum* develop infection (McOrist & Lawson, 1989).

As initial studies were not performed with purified bacterial cell culture, but with filtered mucosal homogenates containing an undefined bacterial flora along with *L. intracellularis*, the first phases of infection and its early cellular effects are still in the process of being clarified (Frisk & Wagner, 1977; Jacoby, 1978; Johnson & Jacoby, 1978). Initially, it was controversial whether *L. intracellularis* had a flagellum (which would facilitate movement towards, or within, the intracellular space) as no typical remnants were detected in the cell wall. It is now confirmed that the bacterium is a flagellated microorganism (McOrist, *et al.*, 1995a). One of the fundamental steps of infection for obligate intracellular bacteria is the phase of attachment to the cell surface. However, micrographic description of *L. intracellularis* is very limited (McOrist & Lawson, 1989; Jasni, *et al.*, 1994a). Such findings were controversial, because the luminal-attached pathogens did not always stain as expected, when tested with immunological reagents, suggesting either that they were not *L. intracellularis*, or that *L. intracellularis* expresses phase-variable antigens (McOrist, *et al.*, 1989).

In one study, the process of adhesion and entry in cultured rat cells appeared rapid (minutes to hours), with only a small percentage of bacteria not entering cells or causing damage to the cell wall (Lawson, *et al.*, 1995). In another study, crypt enterocytes appeared damaged at the site of entry, although the infection process was centrifuge-assisted and potentially the

damage was caused by centrifugation (McOrist, *et al.*, 1995b). More recent studies in pigs (one in ligated intestinal loops *in vitro*, and one *in vivo*) confirmed *L. intracellularis* and brush-border interactions within 3-6 h, and up to 12 h, from exposure, moving into the *lamina propria* and connective tissue, hypothesizing an active role of the *lamina propria* in the spread of infection (Boutrup, *et al.*, 2010a; Boutrup, *et al.*, 2010b). Nonetheless, polyclonal serum and monoclonal antibodies only stunt, but don't stop, the infection process in cell monolayers, suggesting that multiple entry pathways could be possible (Smith & McOrist, 1997; Smith & Lawson, 2001).

After attachment, *L. intracellularis* enters within the enterocytes singularly in vacuoles, or membrane bound vesicles, through a process similar to phagocytosis observed for other intracellular bacteria (*i.e.*, *Chlamydia spp., Shigella spp., Yersinia spp.* and *Cl. piliformis)* (Jasni, *et al.*, 1994b; Smith & Lawson, 2001). The ingress in the cell appears associated not with bacterial viability, but rather with host-cell susceptibility, requiring cellular metabolism and actin polymers re-arrangements (Lawson, *et al.*, 1993; Lawson, *et al.*, 1995). Once within the infected enterocytes, bacteria escape the cell vacuoles and replicate freely, in proximity of the cell's apical pole, where they are able to escape the intervention and destruction by phago-lysosomes (Gebhart, *et al.*, 1993; McOrist, *et al.*, 1995a; McOrist, *et al.*, 1995b). Several bacterial species (*i.e.*, *Listeria spp., Shigella*, *Rickettsia* and *Cl. piliformis*) also utilize a vacuole-mediated escape system within the cytoplasm and do not associate with any intracellular organelles, producing toxins to exit the vacuole. Seemingly, *L. intracellularis* might use a similar system, although the type of toxin produced is yet undetermined. A documented haemolytic activity *in vitro* has been extensively reviewed (Smith & Lawson, 2001).

L. intracellularis replication is concomitant to the replication of infected host-cells, and we assume that they are tightly linked. Within the intestinal wall structure, the location of tubular crypts is typically at the base of the epithelial layer, and here cells undergo an active mitotic phase. The most developed enterocytes, ready to exert their nutrient absorption's function are the most superficially (closer to the lumen) located within the crypts. The tropism of *L. intracellularis* for crypts' deeper cells is known, and appears to be one of its physiological requirements, as it affords the infection to use the continuous hyperactive mitotic activity not only for boosting its own replication process, but also to maximize and mediate *in vivo* the rapid colonization of the intestinal epithelium (Lawson, *et al.*, 1993; McOrist, *et al.*, 1995b). Crude

estimates from *in vitro* evaluation show that *L. intracellularis* is a highly specialized intracellular pathogen, which can divide *q* 8 h, and in the presence of a synthetic peptide, in the culture systems, it can divide or spread infection even faster. Bacterial division occasionally occurs by septation, but the transfer from cell to cell has not been reported, implying that the spread of infected cells is not based on migration of bacteria, but on migration of colonized cells (Gebhart, *et al.*, 1993). As a result, the first structural changes in the intestine derived from *L. intracellularis* infection is a profound mucosal hyperplasia, predominantly caused by proliferation of crypt epithelium, which starts locally, in infected islands, where, amidst normal epithelium, only infected cells are proliferative (Gebhart, *et al.*, 1993). Eventually, in a segmental fashion, infection extends to the entire distal jejunum and ileum, and potentially to the duodenum (Duhamel & Wheeldon, 1982; Williams, *et al.*, 1996; Frank, *et al.*, 1998). Such pattern of replication and infection explains the continuous mitotic activity, generating hyperplastic crypts composed of immature enterocytes, with stunted villi and an almost atrophic brush border, along with concomitant decreased enzymatic and absorptive capacities, progressing to a stage of adenomatosis, as they become elongated, frequently branched and potentially "herniating" in the submucosa (Frank, *et al.*, 1998). The *lamina propria*, especially around crypts, appears expanded by moderate numbers of macrophages, lymphocytes, plasma cells and, occasionally, eosinophils (Schumacher, *et al.*, 2000).

Although the molecular and genetic basis for the enhanced proliferation and the rapid bacterial division is still unknown and under investigation, *in vitro* and *in vivo* experiments suggest that infected enterocytes divide at a rate 4-fold faster than non-infected cells, but once hyperplasia develops, the proliferation rate appears to wane (Lawson, *et al.* 1993; McOrist, *et al.*, 1994b). In the chronic stages of EPE, the mucosa can be corrugated, with ulcers and focal submucosal erosions, necrosis and hemorrhages (uncommon), and edema (Williams, *et al.*, 1996; Frank, *et al.*, 1998; Smith & Lawson, 2001). Occasionally, mononuclear inflammation and muscular hypertrophy are reported in affected intestinal segments. Rapid enterocyte proliferation with a lack of differentiation is associated with the clinical signs, ultrasonographic changes and macroscopic appearance on gross pathology, as well as the microscopic findings.

In EPE, diarrhea develops due to malabsorption and increased small intestinal permeability (See Sub-chapter 2.5), particularly because small intestinal disaccharidase activity

is reduced. When small intestinal absorption is reduced, along with reduced uptake of protein, lipids and minerals, too many carbohydrates reach the large colon, where they are metabolized by intestinal flora and produce an osmotic type of diarrhea (Sweeney, 1987). This is more probable than large intestinal inflammation due to EPE, as *L. intracellularis* infection in this latter tract of foals' intestine is rare, although it was demonstrated in rabbits, and occasionally in pigs and hamsters (Hotchkiss & Merritt, 1996; Hotchkiss, *et al.*, 1996; Lawson & Gebhart, 2000; Vannucci, *et al.*, 2010).

The protein losing enteropathy process leads to the hallmark signs of hypoproteinemia (< 50 g/L) and ill-thrift appearance of affected foals (Duhamel & Wheeldon, 1982; Williams, *et al.*, 1996; Frank, *et al.*, 1998; Lavoie, *et al.*, 2000; Bihr, 2003). Hypoproteinemia arises from the conjunction of increased intestinal permeability and overall decreased absorption of amino acids, while normal systemic protein catabolism continues, or is increased, depending on disease stage. Hypoalbuminemia, in particular, leads to low plasma colloid-osmotic pressure and formation of ventral edema (Frank, *et al.* 1998; Sampieri, *et al.*, 2006). In some EPE-affected foals anemia develops, due to the lack of nutrients and possibly to a systemic response to the infection (Lavoie, *et al.*, 2000).

The insight on the role of humoral, mucosal and cellular immunity and their phases of development of EPE is limited, as studies on *L. intracellularis* foals models and vaccination investigations are in progress (Pusterla, *et al.*, 2009b; Pusterla, *et al.*, 2010a; Pusterla, *et al.* 2010b). It is clear that foals can mount a humoral response versus *L. intracellularis* (see Sub-chapter 2.3.4), but whether serum antibodies are protective remains to be elucidated (Pusterla, *et al.*, 2010a).

"Il n'existe pas de sciences appliquées mais seulement des application de la science."

"There are no such things as applied sciences only applications of science."

L. Pasteur

2.2. *Lawsonia intracellularis*: the challenges of its discovery.

The clinical presentation of proliferative enteropathy was first reported in 1931 in swine (Porcine Proliferative Enteropathy, PPE), and more recently in horses (Equine Proliferative Enteropathy, EPE), while the causative etiologic agent, *Lawsonia intracellularis* was not detected, within the cells, until the early 1970s (Biester & Schwarte 1931; Rowland, *et al.*, 1973). Regardless of when it was recognized, today PPE causes major economic losses worldwide, in the porcine production industry, and losses associated with EPE appear to follow a similar trend (McOrist & Gebhart, 1999; Lawson & Gebhart, 2000, Frazer 2008).

Lawsonia intracellularis, named in honor of its discoverer, G. H. K. Lawson (McOrist, *et al.*, 1995a), is a gram-negative, non-spore-forming, straight to slightly curved rod bacterium, 1.25 - 2 μm long, and 0.25 - 0.43 μm wide. It is flagellated and non-pigmented, with cellular sections typical of a gram-negative prokaryote, exhibiting a wavy, tri-laminated, outer cellular wall structure and dense cytoplasmic granules (McOrist, *et al.*, 1995a). Characteristically, *L. intracellularis* is detected, in the small intestinal epithelium layer, free-floating in the cytoplasm, at the apex of enterocytes, where it survives and replicates as an obligate intracellular bacterium (McOrist, *et al.*, 1995a). Its multiplication occurs by septum formation, or by fission, and requires an almost anaerobic atmosphere for growth (Gebhart, *et al.* 1993; Lawson, *et al.*, 1993).

L. intracellularis retains carbol fuchsin, when stained by modified Ziehl-Neelsen method, exhibiting acid-fast staining in smears of intestinal epithelium and enterocytes (McOrist, *et al.*, 1995a). It was first isolated from porcine intestinal epithelial cells affected by PPE and, apparently it does not infect cells, unless a normal intestinal flora is present (See Chapter 2.1.4.) (McCartney, *et al.*, 1984; Boosinger, *et al.*, 1985; McOrist & Lawson, 1989). Having found the bacteria in the diseased animals; reproduced the bacterial colonization in pure cell-culture, starting from infected tissues; and having obtained PPE lesions in porcine intestine from the

cultured organism, researchers fulfilled Koch's postulates and warranted the denomination of *L. intracellularis* as a pathogenic species (Koch, 1890; McOrist, *et al.*, 1995a).

Although not completely understood at the time, the first strain was deposited in 1989 (1482/89) in the National Collection of Type Cultures, London, UK and named as *strain NCTC 12656T*. It was cultured in a rat enterocyte cell-line monolayer after infection with a bacterial suspension, from a natural case of porcine disease. Strain NCTC 12656T reproduces effectively, but causes no noticeable cytopathic effect in rat enterocytes, nor is a threat to culture survival. Another strain deposited in 2000, PHE/MN1-00, was collected in Minnesota from a pig affected with proliferative hemorrhagic enteropathy (PHE), and its full genomic sequence has been deposited (http://www.straininfo.net/strains/681612).

The exact taxonomy of *L. intracellularis* took decades to be completely identified, as its characterization and understanding kept eluding researchers and empirical therapies for a long while (Gebhart, *et al.*, 1993; McOrist, *et al.*, 1993; McOrist, *et al.*, 1995a). Its etiology was ascribed to different genera, or species, in the 1980s (*e.g.*, *Campylobacter hyointestinalis*, *Campylobacter sputorum* subsp. *mucosalis*) and it was temporarily named *Ileal Symbiont intracellularis* in 1993, until it could be better defined (Duhamel & Wheeldon, 1982; Gebhart, *et al.* 1983; McCartney, *et al.*, 1984; Boosinger, *et al.*, 1985; Gebhart, *et al.*, 1993). Initially, the investigation of this infective pathogen was hampered by the reduced supply available for taxonomy studies, because *L. intracellularis* cell-free cultures failed and successful growth *in vitro* was cumbersome (Gebhart, *et al.*, 1993; Lawson, *et al.*, 1993). Cultivation *in vitro* of individual strains is now feasible, although it mandatorily requires enterocyte cell-culture and "microaerophilia" to succeed (Lawson, *et al.*, 1993; Guedes & Gebhart, 2003b; Yeh, *et al.*, 2006; Watarai, *et al.*, 2008; Pusterla, *et al.*, 2010a). Microaerophilia indicates an anaerobic condition with nitrogen and carbon dioxide replacing the evacuated room air, to obtain a gas mixture with 8% O_2 and 7% CO_2. Initially, isolated strains were maintained in IEC-18 and ATCC CRL1589 cell cultures, for at least 20 passages (Lawson, *et al.*, 1993).

The identification of *L. intracellularis* in tissue culture cells and in the naturally affected porcine ileal epithelia occurred through the species-specific recombinant DNA probes reported by Gebhart and coworkers (1991) and, later, by McOrist and colleagues (1995). The bacterium had novel immunological and DNA probe reactions, compared with *Campylobacter* spp.

(McOrist, *et al.*, 1987), and, through 16S ribosomal DNA (rDNA) amplification and sequencing, it was shown that it belonged to the δ subdivision of the class *Proteobacteria* (Gebhart, *et al.*, 1993). Moreover, the DNA sequences, determined from each purified bacterial preparation, were most similar to the sulfate-reducing proteobacterium *Desulfovibrio desulfuricans* (Gebhart, *et al.*, 1993). At that stage, *L. intracellularis* was believed to colonize the apex of the cytoplasm of mammalian enterocytes only, particularly pigs and hamsters (Rowland & Lawson 1974, Lawson, *et al.*, 1985). Today it is determined that *L. intracellularis* has a broad range of host-susceptibility, with high likelihood for infection across species (Cooper, *et al.*, 1997a; Herbst, *et al.*, 2003), and it is known to infect a variety of mammalian and avian species (Drolet, *et al.*, 1996; Cooper, *et al.*, 1997a; Cooper, *et al.*, 1997b; Lemarchand, *et al.*, 1997; Duhamel, *et al.*, 1998; Frank, *et al.*, 1998; Klein, *et al*., 1999; Herbst, *et al.*, 2003).

To determine beyond doubts that *L. intracellularis* constituted a new genus and species, McOrist and colleagues (1995) compared it, through fermentative and biochemical taxonomic tests, with *Rickettsia spp.* and *Desulfovibrio desulfuricans*, among others (McOrist, *et al.*, 1995a). Although they are obligate intracellular bacteria, occurring free in epithelial cells cytoplasm, *Rickettsia spp.* were excluded because they have different rDNA sequencing data, structure and habitat. Moreover, one of the Rickettsiales' requirements is an insect-host during the life cycle (*e.g,.* may-fly for *Neorickettsia risticii*) (Moulder, 1985; Weisburg, *et al.*, 1991). The differences with *Desulfovibrio spp.* were more subtle. Although the 2 species appeared similar for morphology (91% DNA similarity) and habitat (mammalian GIT) (Gibson, *et al.*, 1993), electrophoretic protein profiles, DNA probe and monoclonal antibody reactions, confirmed the unique novelty of *L. intracellularis* (McOrist, *et al.*, 1995a).

2.2.1. The challenges of a timely-fashioned clinical diagnosis: past and present techniques.

As described in the first EPE case reports (See chapter 2.1.2.), achieving *ante-mortem* diagnosis represented the prime challenge (Duhamel & Wheeldon, 1982; Williams, *et al.*, 1996; Frank, *et al.*, 1998), but when EPE resulted to be the equine equivalent of PPE, diagnostic techniques and therapeutic approaches were readily borrowed from the porcine industry's

experience (Williams, *et al.*, 1996; Lavoie, *et al.*, 2000). Unfortunately, *L. intracellularis* isolation from feces, intestinal mucosa, or intestinal content is impractical, laborious and not 100% reliable, due to the capricious behavior of the bacterium, and, hence, it is clinically impractical (Lawson, *et al.*, 1993; McOrist, *et al.*, 1993).

Today, immediate clinical assessment and tentative *ante-mortem* diagnosis of EPE are possible on the basis of specific GIT clinical signs that are accompanied with hypoproteinemia and hypoalbuminemia, and detection, through ultrasonography, of a thickened small intestinal wall (Schumacher, *et al.*, 2000; Sampieri, *et al.*, 2006). Regardless of the technique used, both PPE and EPE have been diagnosed typically by the presence of bacteria within the apical cytoplasm of actively proliferating crypts' cells in *post-mortem* histopathology, or in surgical biopsy material (Schumacher, *et al.*, 2000; Guedes, *et al.*, 2002a). To date, utilization of routine endoscopic-guided duodenal biopsies for IHC or PCR analysis is not common practice in horses (or pigs) (Schumacher, *et al.*, 2000). Rather, the clinical diagnosis is corroborated with non-invasive tests such as serology, PCR analysis on fecal material, or both (Lavoie, *et al.*, 2000; Schumacher, *et al.*, 2000; Sampieri, *et al.*, 2006). Today 3 main types of laboratory diagnostics are used to diagnose PPE/EPE in live animals: serology, immunochemical-staining on tissues and polymerase chain reaction (PCR) on fecal samples (Lawson & Gebhart, 2000). Serology provides information about historical exposure to *L. intracellularis*; histology (through immunoperoxidase – IPX; or immunohistochemistry – IHC) demonstrates the presence of bacterial antigen; and PCR analysis determines if the presence of bacterial DNA in feces (fecal shedding), or tissues, is a source of disease propagation, even during treatment (Guedes, *et al.*, 2002a).

2.2.2. Serology

Serology is a relatively inexpensive and universally applied tool, used to investigate "disease status" and epidemiology of *L. intracellularis*, among other infectious agents (Holyoake, *et al.*, 1994; Knittel, *et al.*, 1998; Guedes, *et al.*, 2002a; Guedes, *et al.*, 2002b). For PPE, the presence of detectable antibodies against *L. intracellularis* correlated closely to the presence of lesions, and not always to mere exposure to infection (Lawson, *et al.*, 1988; Guedes,

et al., 2002a). In an attempt to achieve optimal results, several techniques (Enzyme-linked immunosorbent assay, ELISA; indirect fluorescent antibody technique, IFAT; immunoperoxidase monolayer assay, IPMA) were developed over the years and a chronological description of serology techniques is listed in **Table 2.1 (Serology techniques)**, whereas their diagnostic value is briefly discussed below.

Table 2.1 Serology Techniques

Table 2.1. Serological techniques used for diagnosis of proliferative enteropathy exposure in porcine (PPE) equine (EPE) and lapine species (rabbits)**.**

Technique	Species	Antigen	Country	Year	Reference
ELISA	PPE	Percoll-gradient purified-PPE antigen from intestines of naturally infected pigs	Australia	1994	(Holyoake, *et al.*, 1994)
IFAT	PPE	Pure cell-culture of *L. intracellularis*	Ames, IA, USA	1998	(Knittel, *et al.*, 1998)
IFAT	PPE	Pure cell-culture of *L. intracellularis*	St. Paul, MN, USA	2002	(Guedes, *et al.*, 2002a)
IMPA	PPE EPE	Pure monolayer cell culture *L. intracellularis*	St. Paul, MN, USA	2002	(Guedes, *et al.*, 2002c, Guedes, *et al.*, 2002d)
ELISA	PPE rabbits	Antiserum against synthetic peptides of *L. intracellularis*-surface	Japan	2004	(Watarai, *et al.*, 2004)
ELISA	PPE	*L. intracellularis* lipopolysaccharide, and through percoll-purified antigen-coated plate	Ames, IA USA	2005	(Kroll, *et al.*, 2005)
ELISA	PPE	Sonicated pure culture of *L. intracellularis*	St. Paul, MN, USA	2008	(Wattanaphansak, *et al.*, 2008)
ELISA	EPE	Modified methodology from (Wattanaphansak, *et al.*, 2008)	Lexington, KY, USA	2011	(Page, *et al.*, 2011b)

In the table, from left to right, are listed the serological techniques, the species targeted, the country, the year of publication and the bibliographic reference that first communicated the use of such a technique in regards of *L. intracellularis.*

2.2.2.1. ELISA techniques.

In the 1990s, an enzyme linked immuno-sorbent assay (ELISA) was used with good results for IgG detection, but its specificity and sensitivity were not demonstrated, as the antigen used to coat the plates was harvested from naturally PPE-infected pigs, and the supply was limited (Holyoake, *et al.*, 1994). Interestingly, higher titers were obtained in pigs infected with unfiltered homogenates, confirming the requirements of a normal intestinal flora for PPE to develop successfully, but also leaving room for cross-contamination with other hypothetical pathogens (McOrist & Lawson, 1989; McOrist, *et al.*, 1993; Holyoake, *et al.*, 1994). Lack of sensitivity and specificity information was obvious in evaluating a Japanese study, where the adaptation of an antiserum against synthetic peptides of *L. intracellularis*-surface, previously used in IHC analysis, was reported in *L. intracellularis* infected rabbits (Watarai, *et al.*, 2004). In 2005, Kroll and colleagues finally shed light on these attributes of ELISA testing used for serology, showing a sensitivity and specificity of 88.7% and 93.7%, on the basis of *L. intracellularis*-lipopolysaccharide plates-coating (Kroll, *et al.*, 2005).

In 2008, a further modification of the antigen coating for the plates (sonication, So-ELISA), was utilized to provide an even more accurate estimate of sensitivity and specificity in PPE infection, finding that on 332 samples from naturally and experimentally infected animals (compared to 350 uninfected control samples) the diagnostic value was 89.8% and 99.4%, respectively (Wattanaphansak, *et al.*, 2008). An adaptation of this So-ELISA technique was later studied (2011) to explore EPE's seroprevalence in Thoroughbred farms of central Kentucky, but sensitivity or specificity were not considered (Page, *et al.*, 2011).

2.2.2.2. IFA technique.

An indirect fluorescent antibody test was reported first in 1998 (Knittel, *et al.*, 1998), and over the years it has been used not only on serum, but also on feces and tissues (See below, subheadings 2.2.3 and 2.2.4). This assay positively detected the infection with the bacterium 3-4 weeks post inoculation in 90% of experimentally infected pigs. Unfortunately, the IFA technique showed 2 main methodology limitations: 1) the need to maintain *L. intracellularis in vitro* to provide a source of antigen (only a few laboratories worldwide have access to established *in vitro* pure cell-cultures); and 2) the need to store acetone-fixed cultures of *L. intracellularis* in 96-well-plates, at -20°C conditions until use (Knittel, *et al.*, 1998). Although specificity data were not available, IFA was considered analytically very accurate, as no cross-reactivity of

hyperimmune sera of pigs experimentally infected with different enteropathogenic bacteria was detected (Knittel, *et al.*, 1998). A different study, comparing IFA serology tests versus PCR analyses in fecal samples, showed that IFA had higher sensitivity for detection of experimentally infected pigs, but, assuming a virtual PCR specificity of 100%, the drawback was its diagnostic sensitivity, as the reported sensitivities were 90% - 91% versus 39% - 67% for serology and for PCR in feces, respectively (Jones, *et al.*, 1993b; Knittel, *et al.*, 1998). In 2002, Guedes and colleagues, reproduced the technique with a modified preparation (Guedes, *et al.*, 2002a), as the study compared a tissue-IFA test to a glass-slide IFA test, along with other diagnostics' comparisons. The IFA serology positive results matched the presence of PPE lesions with a 78% and 83% agreement, for the tissue IFA and the glass-slide IFA, respectively (Guedes, *et al.*, 2002a). The advantage of the glass-slide IFA was an easier transport, record keeping, and commercialization, as glass-slides coated with pure *L. intracellularis* can be fixed, dried and stored without a freezer. However, IFA still holds the disadvantages of a complicated antigen preparation method and the necessity of a highly experienced fluorescence test-reader (Guedes, *et al.*, 2002a).

2.2.2.3. IPMA technique.

In 2002, IPMA was developed as a potentially more practical approach to infection detection (Guedes, *et al.*, 2002b; Guedes, *et al.*, 2002c; Guedes, *et al.*, 2002d). IPMA had similar sensitivity to IFA for PPE screening and showed undeniable advantages over IFA that could expedite and simplify analysis, such as: 1) did not require the use of a fluorescent microscope; 2) it was easier to interpret; and 3) it maintained stable color reactions for months (easier record keeping for extended time) (Guedes, *et al.*, 2002b). A large cohort comparison of PPE-infected pigs and control pigs demonstrated that the sensitivity of IPMA was only 88% with 100% specificity. Thus, the test could be used for screening purposes, but not for definitive testing (Knittel, *et al.*, 1998; Guedes, *et al.*, 2002a; Guedes, *et al.*, 2002b; Guedes, *et al.*, 2002c). Negative IPMA results were confirmed through PCR analysis, and positive IPMA results were confirmed by PCR and IHC analysis (Guedes, *et al.*, 2002c). Also, with the aim of improving the results of IPMA and IHC diagnostics in pigs, the same authors investigated the production of mono- and polyclonal antibodies to apply to those techniques (Guedes & Gebhart, 2003c).

Serologic studies in foals started much more recently, as initially the techniques utilized for pigs were simply "adopted" for diagnostic purposes, on the basis of their availability, and regardless of the expected diagnostic value (Lavoie, *et al.*, 2000; Schumacher, *et al.*, 2000; Sampieri, *et al.*, 2006). Specific validation processes have just started due to the rising interests in a vaccine production (Page, *et al.*, 2011a; Page, *et al.*, 2011b). However, also for EPE, serology (regardless of the specific technique) appears more sensitive than PCR on fecal material (Lavoie, *et al.*, 2000; McClintock & Collins, 2004). Positive seroconversion may be observed in foals a couple weeks after experimental inoculation, suggesting that serological evaluation could be a useful screening tool for EPE (Pusterla, *et al.*, 2010a). Furthermore, a serological response can occur in naïve foals, in contact with experimentally inoculated foals, and serum antibodies can persist for up to 2 months, whereas in naturally infected foals antibodies can be found for up to 6 months (Pusterla, *et al.*, 2010a). Today in North America, IPMA is widely and reliably utilized to screen or compare for anti-*L. intracellularis* antibodies in horses and pigs (Sampieri, *et al.*, 2006; Frazer, 2008; Pusterla, *et al.*, 2010a; Page, *et al.*, 2011a). In the rest of the world, it is more common to observe the use of indirect IFA or ELISA testing, depending on the availability of *L. intracellularis* cell-cultures (Boesen, *et al.*, 2005; Nathues & Beilage, 2008; Guimarães-Ladeira, *et al.*, 2009; Boutrup, *et al.*, 2010a).

2.2.3. Fecal smears analysis: from immunology tests to PCR analysis

In the late 1980s, fecal material from PPE-affected pigs was analyzed with an indirect immunofluorescence technique (IFA) and monoclonal *L. intracellularis*-specific antibodies, showing that clinically affected animals usually shed the pathogen in feces (McOrist, *et al.*, 1987). Since then several techniques (indirect fluorescent antibody technique, IFAT; immunoperoxidase IPX; Polymerase chain reaction, PCR) were developed over the years and a chronological description of the techniques is listed in **Table 2.2 (Diagnostic techniques for fecal samples)**, whereas their diagnostic value is briefly discussed below.

Table 2.2. Diagnostic techniques in fecal samples.

Table 2.2. Diagnostic techniques utilized to detect *L. intracellularis* in fecal or tissue samples in the porcine (PPE), equine (EPE) or other species.

Technique	Species	Antigen	Country	Year	Reference
IFA	PPE (tissue/ feces)	Bacterial smears stained with homologous antisera	St. Paul, MN, USA	1984	(Chang, *et al.*, 1984)
IFA	PPE (feces)	Bacterial smears stained with homologous antisera	United Kingdom	1987	(McOrist, *et al.*, 1987)
IPX	PPE (feces)	Monoclonal antibody *L. intracellularis*-specific and red chromogen aminoethyl-carbazole	St. Paul, MN, USA	2002	(Guedes, *et al.*, 2002a)
Conventional PCR	PPE (feces)	375-base pair (bp) segment of a DNA fragment from *L. intracellularis* DNA clone p78	St. Paul, MN, USA	1993	(Jones, *et al.*, 1993b)
Nested PCR	PPE (tissue/ feces)	375-base pair (bp) segment of a DNA fragment from *L. intracellularis* DNA clone p78 DNA-DNA hybridization (for amplification)	United Kingdom	1994	(McOrist, *et al.*, 1994c)
Multiplex PCR	PPE	Purified DNA from pure cultures of *L. intracellularis, Serpulina hyodysenteriae, Salmonella spp.* Can detect DNAs from a number of different intestinal pathogens concurrently, in the one test sample.	Lincoln, NE, USA	1997	(Elder, *et al.*, 1997)
PCR enzyme-linked oligosorbent assay (PCR-ELOSA)	PPE	Involved procedures of labeling PCR products with biotin, hybridization with an amine-modified internal oligonucleotide probe, addition of peroxidase–streptavidin complex, and measurement of spectrophotometric signal (results reported as optical density values).	St. Paul, MN, USA	2000	(Zhang, *et al.*, 2000)
Real time PCR	PPE EPE wildlife	A specific oligonucleotide probe labeled with 2 fluorescent dyes, allowing amplification and detection of an emission signal to occur simultaneously (Lindecrona, *et al.*, 2002).	Davis, CA, USA	2002 2008 2009	(Pusterla, *et al.*, 2008; Nathues, *et al.*, 2009; Pusterla, *et al.*, 2009a; Wattanaphansak *et al.*, 2010)

In the table, from left to right, are listed the techniques used for feces and tissues, the species targeted, the country, the year of publication and the bibliographic reference(s) that first communicated the use of such a technique in regards of *L. intracellularis.*

2.2.3.1. IFA technique.

Used and described formerly as a serology diagnostic, initially this technique was used to stain tissues and fecal smears, although a better result was claimed on the debris rather than the cells (Chang, *et al.*, 1984; McOrist, *et al.*, 1987). However, the advent of PCR techniques replaced this diagnostic almost worldwide (see below).

2.2.3.2. IPX technique.

In 2002, IPX testing of fecal smears showed sensitivity close to 90% at the peak of the disease, 21 days post inoculation (PI), but needed clarification with regards to specificity. In comparison to IFA, IPX reduces background scatter and affords clear visualization of the bright red antigen-labeled *L. intracellularis*, although the limitation of a non-specific background remains, as it is unavoidable with fecal smears, and can increase the number of false positives (Guedes, *et al.*, 2002). Even in this case, as PCR reagents are more widely available, IPX has been somewhat replaced in the past 10 years (see below).

2.2.3.3. PCR technique.

The specificity offered by the emerging and newly developed PCR techniques in 1983, did not go unnoticed among the *L. intracellularis* investigators. Even though the immunologic tests held the advantage of not being hampered by those same inhibiting factors, that can hinder DNA amplification within fecal specimens, as demonstrated in a human *Rotavirus* study, the new technique had soon a lot of appeal (Wilde, *et al.*, 1990). Thus, a few years after the initial PCR studies, 2 joined research groups claimed that as few as 10 *L. intracellularis* bacteria could be detected by PCR technique in DNA extracted from infected mucosal filtrate and feces (Jones, *et al.*, 1993b; McOrist, *et al.*, 1994c). In PPE samples, different PCR techniques have provided, either in feces and tissues or in feces only, variable sensitivities (36 - 100%) and specificities (50 - 100%) (Pedersen, *et al.*, 2010), depending on the diagnostics used as terms of comparison (*i.e.*, IHC rather than IFAT or Warthin-Starry staining) or the time of sampling in relation to the peak of disease, for example 3 vs. 3.5, or 4 weeks PI (Knittel, *et al.*, 1998; Guedes, *et al.*, 2002a). However, Guedes and coworkers in 2002, reported a PCR sensitivity higher than previously published on 2 weeks PI (71.05%) and 3 weeks DPI (60.5%), although it decreased (37.5%) by 4 weeks PI (end of experiment). A further explanation for the diagnostics differences has been

ascribed to the different infection model used by Guedes (ileal mucosal scrapings homogenate from PPE affected pigs) versus the one used by Knittel (pure cell-culture) (Knittel, *et al.*, 1998; Guedes *et al.*, 2002a). Reportedly, animals infected with mucosal homogenates develop more severe clinical signs and gross lesions, than the ones challenged with pure cell-culture, with the amount of bacterial DNA shed in feces closely related to the severity/extension of intestinal lesions (Guedes, *et al.*, 2002). A better test sensitivity was advocated, in part due to the necessity of assessing the degree of fecal shedding without results being hampered by false-negatives; in part because PCR's specificity for detecting *L. intracellularis* fecal shedding is virtually close to 100% (Jones, *et al.*, 1993; McOrist, *et al.*, 1994c). To date, PCR is not used as a sole diagnostic tool for PE diagnosis (Knittel, *et al.*, 1998; Lavoie, *et al.*, 2000; Zhang, *et al.*, 2000; Guedes, *et al.*, 2002, Guedes & Gebhart, 2003a; Jacobson, *et al.*, 2004; McOrist & Smits, 2007; Guimarães-Ladeira, *et al.* 2009; Merlo, *et al.*, 2009; Pusterla, *et al.*, 2009a; Pusterla, *et al.*, 2009b; Pusterla & Gebhart, 2009*;* Pusterla, *et al.*, 2010a; Pusterla, *et al.*, 2010b; Page, *et al.*, 2011b), particularly if samples are collected after the start of AM therapy, as *L. intracellularis* fecal shedding apparently decreases or stops (Dauvillier, *et al.*, 2006; Nathues & Beilage, 2008).

In 2003, an internal standard, called "mimic", was developed to use along with *L. intracellularis* PCR technique, the "mimic" differed in size from the target template, but used the same primer recognition sites, hence affording the mimicking action through PCR amplification (Jacobson, *et al.*, 2003). The goal of using the "mimic" is based on demonstration of PCR-inhibitory factors in fecal samples and facilitating the differentiation between false-negative and real-negative results, thus overcoming the main limitation of background "noise" on fecal samples (Jacobson, *et al.*, 2004). In such study, 10^2 mimic molecules per 0.1 gram of feces were elected as a detection cut-off by nested-PCR (which uses a second set of primers – re-amplifying the reaction of the conventional PCR), also considering that *L. intracellularis* fecal shedding is estimated as equal to 10^8/g. Through this PCR technique, DNA detection was sufficiently accurate to confirm PPE disease or exposure, but not lack of exposure (Jones, *et al.*, 1993b; Jacobson, *et al.*, 2003).

Lately, a quantitative real time PCR (or qPCR) has been prompted for the use on both equine and porcine tissues (embedded and fresh) and feces (Pusterla, *et al.*, 2008a; Pusterla, *et al.*, 2009a; Drozd, *et al.*, 2010; Richter, *et al.*, 2010; Wattanaphansak, *et al.*, 2010), with a sensitivity surpassing previous PCR methodologies. Richter and colleagues reported an ability

of detection of *L. intracellularis* up to 4 amplicons/well in fresh and formalin-fixed-paraffin-embedded tissues, versus the 18 amplicons/well in fecal material, as the inhibitory factors in feces remain a diagnostic hamper (Richter, *et al.*, 2010). Today, qPCR (SYBR Green principle) is applied successfully on quantitation procedures of experimental infectious inocula (Wattanaphansak, *et al.*, 2010), and more recently real-time PCR (TAqMan principle) in the equine field, was utilized to confirm fecal shedding in animal modeling (Pusterla, *et al.*, 2010a), as well as epidemiology investigations on horses and wildlife in stud farms (Pusterla, *et al.*, 2008b; Pusterla, *et al.*, 2009a; Pusterla, *et al.*, 2009c; Pusterla, *et al.*, 2010b; Pusterla, *et al.*, 2010c). Real-time PCR is utilized today, in porcine research, to investigate infection, or co-infection, "status" involving *L. intracellularis*, among other bacteria (Molbak, *et al.*, 2008; Willems & Reiner, 2010; Collins, *et al.*, 2011).

Interestingly, a systematic review by Pedersen and coworkers (2010) examined the diagnostic value of PCR analyses for *L. intracellularis* on porcine fecal samples (Pedersen, *et al.*, 2010). Inclusion criteria were multifactorial, as they varied from the PCR principle and type of primer used, to the type and number of samples tested. The study examined the statistical analysis provided in over 21 studies, and attempted to provide a uniform predictive value (from 15 to 90%) for all the PCR tests proposed in the last 20 years. Seven different sets of primers were reported, with five different PCR principles applied **(See table 2.2)**. The most sensitive method was the nested PCR, but the complicated methodology reduces its applicability for clinical needs (Jacobson, *et al.*, 2004). Pedersen distinguished elegantly the concepts of diagnostic and analytical sensitivity and specificity for PCR tests applied to feces: analytically, PCR tests were able to discern *L. intracellularis* from a list of enteric and non-enteric pathogens with an almost perfect specificity and a sensitivity (or limits of detection) that detected 10^2-10^5 bacteria/g of feces. Epidemiologically, we appreciate the attempt to extrapolate sensitivity and specificity available for different sample sizes to a unique similar scale. This is said despite the challenge of relating to a single interpretation studies conducted by different groups, in different time-frames and different settings. The report showed that the 3 PCR tests with diagnostic specificity <100% and higher chance of reporting false-positive results were preferable to the 4 PCR tests with diagnostic sensitivity <100% (3 <50%), as they had higher chance of reporting false-negatives, despite having histological lesions validated by IHC (Pedersen, *et al.*, 2010). To

date, a similar report on PCR assays' diagnostic specificity and sensitivity in EPE cases is not available.

2.2.4. Immunohistochemistry: achieving the gold standard.

Several histologic techniques have been described in the literature: staining with haematoxylin and eosin (H&E) (Rowland & Hutchings, 1978), Warthin–Starry (WS) silver stain and modified Ziehl–Nielsen (ZN) stain (Pedersen, *et al.*, 2010), *in situ* hybridization (ISH) (Gebhart, *et al.*, 1994), and immunohistochemistry (IHC) (McOrist, *et al.*, 1987), but until 10 years ago no technique could be considered the "gold standard" diagnostic method for *L. intracellularis*-infected animals. Firm diagnosis of PPE infected and non-infected animals requires a combination of tests, such as a positive fecal PCR test and a positive result by IHC in ileum sections (Guedes, *et al.*, 2002a; Guedes, *et al.*, 2002c). This combination was elected on the basis of their high specificity (100%) (McOrist, *et al.*, 1987; Jones, *et al.*, 1993c).

On *post-mortem* examination, histological diagnostics are necessary to confirm both EPE and PPE beyond doubt, despite the presence of typical intestinal lesions. Moderate to severe lesions are readily detected on H&E preparations, as enterocytes' proliferation is obvious; but detection of milder (or subclinical) cases is unreliable. In the first cases of EPE, organisms were feebly visualized with Manovelian – Levaditi and then better detected with Steiner or WS methods, the latter becoming quickly the tissue staining of choice (Duhamel & Wheeldon, 1982, McOrist, *et al.*, 1995a; Schumacher, *et al.*, 2000). Unfortunately WS is not specific for *L. intracellularis* and not very sensitive when applied to autolysed or necrotic tissues (Jensen, *et al.*, 1997).

Immunohistochemistry (IHC) techniques, with murine monoclonal anti-*L. intracellularis* antibodies, have been used successfully to diagnose PPE since 1987, when results were first published about the utility of monoclonal antibodies for diagnosis of porcine ileitis (McOrist, *et al.*, 1987). According to comparison studies, WS staining and IHC detected similarly *L. intracellularis* infection in severely affected tissues (macroscopically visible), with 95% and 98% detection, respectively, but they differed profoundly when lesions were not macroscopically evident (Jensen, *et al.*, 1997). Further comparisons between WS and H&E techniques, and IHC labeling on 3 adjacent sections in 38 PPE-positive pigs showed that H&E had the lower sensitivity, followed by WS staining, compared to IHC (33 positive compared to 14 (H&E) and

19 (WS)) (Guedes, *et al.*, 2002a). The higher sensitivity of IHC is due to the specific binding of the monoclonal antibody to the outer membrane protein of *L. intracellularis*, and such bond is neither affected by severe necrosis, or autolysis, nor in the phase of recovery, when the bacterial antigen is found only in the cytoplasm of mononuclear cells in the *lamina propria*. Thus, detection of positive antigen labeling and presence of PPE macroscopic lesions were in agreement (82.5%) even during remission or resolution (28 days PI) (Guedes, *et al.*, 2002a).

Veterinary diagnostic laboratories typically use H&E and WS stains for histology, as they are readily available, and corroborate the findings with a fecal PCR analysis (or on fresh intestine). Ideally the high specificity of IHC and fecal IPX make these highly desirable tests. However, their need for the monoclonal antibody against *L. intracellularis*, limits their widespread use, although IHC has the ability to detect antigen labeling in tissues even at the end of the clinical phase (28 days PI), when fecal shedding falls below the limits of detection of the best technique used, IPX or PCR (Guedes, *et al.*, 2002a).

Today, IHC is acclaimed as the "golden standard" diagnostic test on available tissues. Most laboratories have a dichotomous classification system - either positive or negative. In 2003, a classification system was published, with lesions categorized on a scale from 0 to 4, where 0 equals no IHC labeling (or no infection) and 4 corresponds to 100% of IHC labeled enterocytes in the crypts (Guedes & Gebhart, 2003b).

To conclude, it is worth mentioning an *in-situ* hybridization (ISH) technique, studied for PPE detection even in absence of specific monoclonal antibodies (Ladinig, *et al.*, 2009). The first study (1994) that reported the use of ISH for *L. intracellularis* diagnostic purposes was conducted in the USA (Gebhart, *et al.*, 1994). Later, a Danish research group reported their fluorescent ISH (or FISH), targeting 16S ribosomal RNA, on formalin-fixed-paraffin-embedded tissues of 15 PPE-affected pigs (and 10 negative controls). The oligonucleotide probe utilized was specific for histological detection of *L. intracellularis* (Boye, *et al.*, 1998). In 2007, a ISH digoxigenin-labeled oligonucleotide-probe was used in PPE cases, comparing the ISH technique to PCR and WS staining, but not IHC. Results were highly favorable (71% agreement with PCR) for ISH, so that it was recommended as the second best test, after IHC (Weissenböck, *et al.*, 2007). Several studies followed, within the same European research groups, utilizing FISH and ISH as an ancillary diagnostic, along with q-PCR or RT-qPCR in the context of infection modeling experiments (Molbak, *et al.*, 2008; Ladinig, *et al.*, 2009, Boutrup, et al., 2010b; Jensen,

et al., 2010, Richter, *et al.*, 2010). Particularly 2 studies compared ISH and FISH diagnostic specificity and sensitivity to other techniques. The first one, showed a lower sensitivity, but a higher specificity than PCR analysis on feces, because of a high possibility of false-positives. However, when compared to IHC for routine diagnosis, 66% sensitivity and 99% specificity and 54%, sensitivity and 100% specificity were found, for IHC and ISH respectively (Ladinig, *et al.*, 2009). The second study was a comparison between FISH and IFA, which yielded 91% sensitivity for FISH, and 100% for IFA, although both methods had 100% specificity. Furthermore, IFA was much less susceptible than FISH to the effects of autolysis and cryopreservation (Jensen, *et al.*, 2010).

"Principiis obsta: sero medicina paratur, cum mala per longas convaluere moras.

Stop it at the start, it's late for medicine to be prepared when the disease has grown strong through long delays." *Ovid*

2.3. Current therapeutic approaches and EPE management in horses

In the first cases ascribed to *L. intracellularis*, either non-specified or no AM treatment was implemented (Duhamel & Wheeldon, 1982, Williams, *et al.*, 1996, Frank, *et al.*, 1998). Foals either received supportive intravenous fluid-therapy and nonsteroidal anti-inflammatory treatment (Duhamel & Wheeldon, 1982), or corticosteroids (Frank, *et al.*, 1998). In all cases, the foals died shortly after institution of therapy. Although corticosteroids initially improved fecal consistency and the apparent clinical condition of the foal, therapy-induced immunosuppression obviously inhibited long-term recovery. It was thus confirmed that administration of dexamethasone prior, or concurrently, to inoculation with *L. intracellularis* increased infectivity, or complicated recovery with secondary infections, as detected through experimental investigation in pigs (Holyoake, *et al.*, 1994; Frank, *et al.*, 1998) and hamsters (*e.g.*, *C. difficile* overgrowth) (Drs. Vannucci and Gebhart, University of Minnesota, personal communications). Interestingly, in a recent study where EPE presentations were investigated in a foal model, the

authors did not demonstrate any worsening effect, after injectable dexamethasone was used to mimic stress (Page, *et al.*, 2011c).

Recently, 3 distinct forms of EPE have been described in foals and yearlings on the basis of the symptomatology and pathological lesions: classic, acute and subclinical, although, previously, only 1 clinical presentation was recognized (Page, *et al.*, 2011c). This is similar to the distinction in 4 clinical PPE presentations: 1) porcine intestinal adenomatosis (PIA); 2) necrotic enteritis; 3) regional ileitis and 4) proliferative hemorrhagic enteropathy (or PHE). Other authors divide PPE into 2 major clinical presentations: acute hemorrhagic diarrhea and death in young adult pigs; or chronic diarrhea and reduced growth performance in growing pigs (Lawson & Rowland, 1974; Rowland, 1975; Lawson, *et al.*, 1979; Lawson & McOrist, 1993; Cooper & Gebhart, 1998; Lawson & Gebhart, 2000). Over the years, the various manifestations of PPE in pigs have controversially been considered self-limiting at herd level, but in regards to EPE, where the single animal is the focus of production, the current perception is that AM administration should be recommended, and provided early in the course of disease (Cooper & Gebhart, 1998; Lavoie, *et al.*, 2000). Nonetheless, the economic impact of EPE is not only related to the clinical infection, but also due to costs of the prolonged recovery. Horses surviving EPE have lower ability to absorb nutrients and poorer growth rates, when compared to breed averages, for several months after clinical disease (Frazer, 2008; Lavoie & Drolet, 2009; Merlo, *et al.*, 2009, Wong, *et al.*, 2009). An example of the economic impact of *L. intracellularis* infection is clearly depicted in an age-matched comparison, where prolonged convalescence and unthrifty appearance, due to EPE, translated into lower sale prices (68%) for Thoroughbred yearlings in Kentucky (Frazer, 2008).

Regardless of the drug, AM therapy for EPE usually lasts several weeks and is often accompanied by intense, and sometimes lengthy, hospitalization and care. Starting with Lavoie's report of the first outbreak (2000), hospitalized foals were administered oral cimetidine or sucralfate, intravenous plasma transfusion or colloid solution infusions, along with parenteral nutrition and balanced polyionic solution therapy (Lavoie, *et al.*, 2000). Moreover, close monitoring, through repeated blood work and diagnostic tests, was used to assess the response to therapy. In some cases, diet was also enriched with vegetable oil and multivitamin supplements (Lavoie, *et al.*, 2000; Sampieri, *et al.*, 2006; Frazer, 2008).

When treatment was directed to EPE and other concurrent enteric infections (*e.g.*, salmonellosis), and clinical disease was more severe, treatment was more challenging, with further prolonged hospitalization and recovery, poorer prognosis and higher expenses at discharge (Frazer, 2008). Occasionally, changes in AM therapy are required, to successfully complete EPE treatment, suggesting that *L. intracellularis* may become more susceptible to certain drugs or may develop AM resistance, although neither is clearly demonstrated (Sampieri, et al., 2006; Frazer, 2008).

2.3.1. Current antimicrobial therapy in EPE affected foals.

On the basis of the results presented by the only *in vitro* work available at the time, the advantage of the intracellular "sanctuary-like" location of *L. intracellularis* (McOrist et al., 1995a; McOrist, *et al.*, 1995c) appeared successfully overcome by the treatment with erythromycin during the first EPE outbreak, so that the drug became immediately the treatment of choice (Lavoie, *et al.*, 2000). In that report, it was clinically demonstrated that oral erythromycin estolate (15–25 mg/kg BW *q.* 6–8 h), alone or in combination with oral rifampin (7–10 mg/kg BW, *q.* 12 h), rapidly improved foals' clinical condition (*i.e.*, attitude, weight gain, reduction in colic signs or diarrhea) (Lavoie, *et al.*, 2000). Despite the appropriate AM therapy, complete recovery and normalization of plasma protein levels was later proven to take months (Wong, *et al.*, 2009). In Lavoie's report, only 2 foals were switched to chloramphenicol (50 mg/kg BW *q.* 6 h), due to the recurrence of diarrhea and the desire to avoid *C. difficile* overgrowth, underlining the possible limitations to a successful erythromycin therapy, as already feared in treatment of *R. equi* (Baverud, *et al.*, 1998, Lavoie, *et al.*, 2000).

Nonetheless, in another case report, a filly underwent colic surgery because of unrelenting pain. Neither the perioperative parenteral procaine penicillin G (22000 IU/kg BW IM, *q*12 h) and gentamicin sulfate (5 mg/kg BW, IV *q.* 24 h) treatment, or the 5 days of postoperative oral trimethoprim-sulfametoxazole (15 mg/kg BW *q.* 12 h) resolved her abdominal discomfort. Following the histopathology diagnosis of *L. intracellularis* infection, AM treatment was successfully changed to oral erythromycin stearate (25 mg/kg BW, *q.* 6 h) and continued for 6 weeks leading to rapid improvement and full recovery in 2 months (Schumacher, *et al.*, 2000). Due to its almost infallible success, the AM combination of erythromycin (estolate or stearate)

and rifampin was considered the only resolutive treatment, even though expensive, for EPE for several years (Bihr, 2003; McClintock & Collins, 2004; Dauvillier, *et al.*, 2006; Feary & Hassel, 2006).

A clinical case review by Sampieri and colleagues (2006) demonstrated the efficacy of intravenous oxytetracycline (6.6 mg/kg BW *q.* 12 h) during the hospitalization phase of treatment, followed by oral doxycycline (10 mg/kg BW *q.* 12 h) during the convalescence phase at home (Sampieri, *et al.*, 2006). Such a combination was chosen on the basis of publications regarding *in vitro* studies and the success obtained with tetracycline compounds in pigs, both in USA and Europe (Collins, *et al.*, 1999; Kyriakis, *et al.*, 2002). To date, macrolides, rifampin and tetracycline are the most frequently used antimicrobial classes in weanling or yearling horses affected by EPE, although newer macrolides (oral clarithromycin, 7.5 mg/kg BW *q.* 12 h) or azalides (oral azithromycin,10 mg/kg BW *q.* 24 h) often in association with rifampin (5 mg/kg BW *per os q.* 12 h) also have been documented (Feary, *et al.*, 2007; Frazer, 2008; Lavoie & Drolet, 2009; van den Wollenberg, *et al.*, 2011).

Recently, the veterinary community has expressed increasing concern that *L. intracellularis* infection seems to have extended its morbidity spectrum to adult horses, which are otherwise debilitated, or concurrently affected by other enteric pathogens (*e.g., Salmonella spp., R. equi*) or ailments (*e.g.*, digestive tract lymphoma) (Dr. C. Gebhart, personal communication). A case of EPE in a 26-month-old Thoroughbred colt in Japan, showed that the combination of *R. equi* and *L. intracellularis* was indeed fatal (Shimizu, *et al.*, 2010). In such cases, attending veterinarians resort, sometimes fruitlessly, to AMs such as fluoroquinolones, metronidazole (combined with tetracycline, in 16/46 cases, at an oral dose of 10 – 15 mg/kg BW *q.* 8 -12 h) and chloramphenicol (44 mg/kg *per os q.* 6-8 h). This has been reported in affected foals in Kentucky, between 2005 and 2007 (Frazer, 2008). Such reports leave room for debate, regarding *L. intracellularis* treatment, as to whether bacteria are developing AM resistance or have varying susceptibility.

2.3.2. Experimental background to the clinical therapy

As the bacteria eluded every attempt at growing on agar plates (Roberts, *et al.*, 1977; Lawson, *et al.*, 1993; McOrist, *et al.*, 1993), AM susceptibility investigations based on Kirby-Bauer method were not applicable, thus initially, PPE treatment was empirical and variably successful (Greenwood, 1981, McOrist, *et al.*, 1995c). Also, limited information was available, in general, for intracellular treatment of pathogens (*e.g.*, *Chlamydia pneumoniae, Rickettsia spp.*, *Bartonella spp.* and *Anaplasma phagocytophylum*), until Minimum Inhibitory Concentrations (MIC) studies were established through complicated cell-culture systems (Gnarpe, *et al.*, 1996; Ives, *et al.*, 2000; Horowitz, *et al.*, 2001).

In the 1980s, when *L. intracellularis* taxonomy was still unclear, 2 studies showed the effectiveness of tetracycline (and its derivatives) against PPE, one being a controlled field trial in affected pigs, and the other a comparison between tetracycline hydrochloride, neomycin and dimetridazole in a hamster infection model (La Regina, *et al.*, 1980). Both trials showed that tetracycline was effective at inhibiting *L. intracellularis* (La Regina, *et al.*, 1980), and over the years, the efficacy of tetracycline against several intracellular bacteria was demonstrated, although obligate intracellular bacteria should be considered as a separate group altogether (Moulder, 1985).

Several years later (1995), an *in vitro* study successfully tested more AMs against porcine *L. intracellularis*, applying the MIC method to cell cultures (McOrist, *et al.*, 1995c). In the 1990s, not only the bacterium's taxonomy was clarified, but also cell-culture methodologies were consistently standardized; moreover the bacteria produced *in vitro* were proven to be virulent for live hosts (pigs) (Lawson, *et al.*, 1993; McOrist, *et al.*, 1993; McOrist, et al., 1994a; Lawson, et al. 1995; McOrist, *et al.*, 1995a; McOrist, *et al.*, 1995c). Thus, because of the difficulties encountered in understanding *L. intracellularis*, clearly McOrist's *in vitro* work (1995) became a stepping stone in understanding unexpected treatment failures, or successes, in PPE cases in the field (McOrist, *et al.*, 1995c).

In order to determine appropriate concentrations for extracellular and intracellular AM activity, *L. intracellularis* cultures were incubated for 5 days, either in the presence or absence of various AMs' concentrations, in order to afford unequivocal detection of inhibition of bacterial growth. Each AM's MIC was defined as the lowest concentration consistently inhibiting growth (equal to 0.1% of the growth of controls) and considered evidence of bacteriostatic activity only,

as Minimum Bactericidal Concentration (MBC) would have required testing *in vivo* (McOrist, *et al.*, 1995c). Penicillin, erythromycin, chlortetracycline, along with difloxacin and virginiamycin, were the most active AMs against *L. intracellularis* with a MICs ≤1 μg/ml; tilmicosin and tiamulin had a MICs ≤ 4 μg/ml; lincomycin and tylosin had a MIC ≥ 32 or 64 μg/ml; and bacitracin-zinc, avoparcin, and all aminoglycoside, or aminocyclitol, had MIC >128 μg/ml (McOrist, *et al.*, 1995c). Importantly, it was recognized that *in vivo* responses could have differed, due to specific drugs' pharmacokinetics and pharmacodynamics. A candidate drug requires for efficacy the ability of killing/inhibiting a Gram-negative intracellular bacterium located in the lower bowel, where the oxygen concentration is very low, yet constitutes a thriving condition for *L. intracellularis* (Lawson, *et al.*, 1993; Wattanaphansak, *et al.*, 2009a). Truthfully some drugs are simply unable to penetrate into enteric cells, or they only target inadequate cellular locations (Easmon & Crane, 1984).

In regards to clinical EPE therapeutic approach, no novel information has been reported since 2008, when suspected resistant cases, also called "slow burners", were treated with a plethora of AM (from metronidazole to chloramphenicol), in addition to the macrolides and rifampin combination, or the tetracycline class (Frazer, 2008). Also these same drugs have proven useless in a fulminant form of EPE recently reported (Page, *et al.*, 2012). Thus, in the quest for an alternative solution, *in vitro* experiments are explored, although they are few and yet not specific for EPE.

Two conceptually similar studies showed MIC investigations on multiple porcine *L. intracellularis* isolates, obtained from North American, European and Asian strains (Wattanaphansak, *et al.*, 2009a; Yeh, *et al.*, 2011). The Asian study is the first publication on AM susceptibility of Asian isolates, wherein the MIC of 16 AM drugs were assessed for 2 isolates recovered in South Korea: one from a finisher pig affected by PHE; the other from a grower pig affected by porcine intestinal adenomatosis (PIA). Tylosin and tilmicosin were the most active AMs both intracellularly (MIC, 0.25 -0.5 μg/ml and 0.125 μg/ml, respectively); and extracellularly (MIC, 0.25 -0.5 μg/ml and 1 μg/ml), depending on the type of isolate (Yeh, *et al.*, 2011).

However, the study conducted in North America, to date, is the most complete *in vitro* evaluation of AM susceptibility for *L. intracellularis* (Wattanaphansak, *et al.*, 2009a). Carbadox,

chlortetracycline hydrochloride, lincomycin hydrochloride, tylosin tartrate, tiamulin hydrogen fumarate and valnemulin hydrochloride were tested against 10 PPE *L. intracellularis* field isolates, collected between 1983 and 2006 in North America (6 strains), United Kingdom (3) and Denmark (1). All strains were grown in murine fibroblast-like McCoy cells, new/clinical (or low passage) and well-established (or high passage) isolates alike, as intracellular bacteria cultured for extended time become "immortalized", hence able to survive indefinitely, but potentially unable to react "normally" to AMs, due to the genomic mutations developed overtime (Drancourt & Raoult, 1993; Wattanaphansak, *et al.*, 2009a). Again, MIC was expressed for both intracellular and extracellular AM activities. For the intracellular MIC assessment, a bacterial suspension was inoculated onto one-day-old McCoy cells 24 h prior to AM exposure, providing *L. intracellularis* a sufficient time-frame to invade the host-cells, and then maintained for 3 days, in optimal culture conditions (McOrist, *et al.*, 1995; Guedes & Gebhart, 2003a; Wattanaphansak, *et al.*, 2009a). The extracellular MIC assessment was designed to mimic the AM effects on *L. intracellularis*, when bacteria are free in the gut lumen, before infecting the intestinal cells. Therefore the incubation with AM solutions was conducted on cell-cultures, at body temperature (37.8 C), in optimal PPE growth conditions (8.0% O_2, 8.8% CO_2, and 83.2% N_2 atmosphere), only for 24 h. After that, the bacterial suspension was transferred to infect one-day-old McCoy cells, but the medium was replaced with a new, blank, culture medium, to underline in this case that AM exposure occurs only in the gut lumen, but not within the cells (McOrist, *et al.*, 1995c; Wattanaphansak, *et al.*, 2009a). Results showed that *in vitro* carbadox, tiamulin and valnemulin are most active, chlortetracycline and tylosin are intermediately active, and lincomycin is the least active, although different *L. intracellularis* isolates express a variety of AM sensitivity patterns. Specifically, North American isolates showed higher AMs MIC (intra- and extracellular), than the European ones. Obviously additional PPE and EPE isolates should be tested before making any speculations about geographic differences and AM sensitivities changing over time, but those results could serve as a guideline for the range of AM responses. Clinically, in fact, it would be unlikely to isolate and test an *L. intracellularis* strain during an ongoing PE outbreak, regardless of the species affected (Wattanaphansak, *et al.*, 2009a).

Again the pharmacodynamic and pharmacokinetic limitations of an *in vitro* study were underlined, but until 2009, no AMs' MIC breakpoints for intracellular organisms in cell-culture were available, thus the understanding of the full implications of those results has been

complicated (Wattanaphansak, *et al.*, 2009a). Although *in vitro* sensitivity is helpful, the limitations of its different set-up need to be considered in comparison to an *in vivo* (or a field) study, before proclaiming a verdict of efficacy. In an *in vitro* study, researchers have the ability of focusing on one question at the time, such as whether the AM concentration at the infection site is the determining factor for strain susceptibility, without concerns of other *in vivo* variables, even though it is currently unknown whether AMs would target differently extracellular or intracellular *L. intracellularis* (Wattanaphansak, *et al.*, 2009a). For example, it could be arguable that intracellular carbadox might be appropriate for all isolates, as no difference was detected in activity levels at its lower concentrations; or that chlortetracycline's intracellular MICs large range indicates that some *L. intracellularis* isolates may be less susceptible, or that therapeutic difficulties, or even failures, should be easily expected. Interestingly, the novel approach at evaluating AMs' MICs showed that for all drugs extracellular MICs were higher than the correspondent intracellular ones, as the study accounted for the shorter contact time allowed for extracellular bacteria (1 day versus 3 days). Such a conceptual difference may be difficult to quantify *in vivo*, beyond the reaching of a steady state condition in the cell-culture system (Wattanaphansak, *et al.*, 2009a). However, the main conclusion was that on the basis of the extracellular AM exposure, a single extracellular treatment (equal to a single-dose treatment) could be insufficient to inhibit bacterial growth long enough to resolve the infection. Also the lower intracellular MICs could be due to AM accumulation within the cells, thus enhancing the chemotherapeutic activity, showing that most AMs tested can bind to internalized *L. intracellularis* sufficiently to exert their action (Wattanaphansak, *et al.*, 2009a).

2.3.3. Current use of immunization

EPE's epidemiology is not yet completely clarified and no specific measures have been established for preventing the disease in horses. In pigs, the current prevention strategy is achieved mostly using AMs in feed (prophylactic chemotherapy) and a commercially available *L. intracellularis* modified-live vaccine (prophylactic immunization). In horses, single case treatments and metaphylactic chemotherapy (clinical treatment) usually lead to a cure, but is occasionally unsuccessful (Lavoie, *et al.*, 2000; Sampieri, *et al.*, 2006; Frazer, 2008; Pusterla &

Gebhart, 2009; Page, *et al.*, 2012). However, if the example to follow is PPE, whose exposure incidence is purported to be 97% worldwide, the many difficulties in controlling its effects and *sequelae*, through therapy and vaccinal prophylaxis, point to a need for early immunization (Lawson & Gebhart, 2000; Kroll, *et al*., 2004; Almond & Bilkei, 2006; McOrist & Smits, 2007).

Initial investigations of the impact of *L. intracellularis* vaccination in weanling foals have shown detectable antibody titers, following oral or rectal administration of the modified-live *L. intracellularis* vaccine commercially available for pigs, Enterisol Ileitis (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, USA) (Guedes, *et al.*, 2002c; Pusterla, *et al.*, 2009b). The vaccine was a frozen avirulent live *L. intracellularis*, administered twice intra-rectally, as 50 mL vaccine, regardless of age and weight. Later, this formulation was compared in foals to a lyophilized avirulent live vaccine, leading to comparable findings of safety, onset and duration of fecal shedding and serological response (IgG), even with a smaller (30 mL) dosing volume (Pusterla, *et al.*, 2009b; Pusterla, *et al.*, 2009c; Pusterla, *et al.*, 2010b)

In the first experimental evaluation of the vaccine, 3 groups of naïve foals, aged 4-6 months, underwent 2 administrations, 3 weeks apart: 1) oral immunization, following omeprazole pre-medication; 2) intra-rectal immunization; and 3) oral immunization, without omeprazole pre-medication. No foal had detectable adverse effects due to the vaccine, regardless of the route of administration. In foals vaccinated intra-gastrically with omeprazole pre-medication, no *L. intracellularis*' DNA was detected at any point in the entire study. PCR analysis of serial rectal swabs demonstrated fecal shedding, starting at 11–15 days following the first intra-rectal vaccine administration. It lasted for 1–12 days thereafter, whereas in foals vaccinated intra-gastrically, without omeprazole pre-medication, *L. intracellularis*' DNA was detected only for the first 2 days following the first vaccination (Pusterla, *et al.*, 2009b; Pusterla, *et al.*, 2009c). All the foals vaccinated intra-rectally seroconverted after the first vaccine, whereas the intra-gastrically vaccinated foals demonstrated seroconversion only after omeprazole pre-medication, with an earlier, and stronger, detectable humoral response. The non-pre-medicated foals never seroconverted (Pusterla, *et al.*, 2009b).

Subsequently, a study investigating the humoral response and the duration of fecal shedding was conducted in a group of EPE-naïve, healthy, pregnant broodmares, to determine whether specific maternal anti-*L. intracellularis* antibodies could be passively transferred to foals

through the colostrum (Pusterla, *et al.*, 2009c). Mares were divided in 2 groups, a vaccinated group (8 mares), and sentinel group (4). The first group was immunized intra-rectally, 3 to 5 weeks ahead of the expected due date, with the protocol previously used in foals, as it produced the strongest seroconversion response, whereas sentinel mares were just "housed" together with vaccinated mares (Pusterla, *et al.*, 2009b). All mares were monitored for the 12 weeks following vaccination. After foaling, dam's colostrum and foal's serum were collected before and after nursing, to determine antibody titres in foals, and thereafter their titers increments were monitored for 8 weeks (Pusterla, *et al.*, 2009c). Vaccination against *L. intracellularis* in mares was safe, and fecal shedding was documented in only 3 vaccinated mares, with a pattern similar to the first study, but a lower magnitude and shorter duration (2 – 3 days) (Pusterla, *et al.*, 2009b; Pusterla, *et al.*, 2009c). Fecal dilution may explain the lower detection rates in brood mares (adult horses produce a larger volume of fecal material relative to foals), but fecal shedding of *L. intracellularis* DNA, within a couple of weeks from vaccination, suggests active mucosal replication of the organism.

Sentinel mares never seroconverted, whereas vaccinated mares seroconverted 2 to 4 weeks after immunization, with the highest titres between 1:120 to 1:240 and *L. intracellularis* antibodies detectable for 6 to 10 weeks (Pusterla, *et al.*, 2009c). However, colostral antibodies (with titres of 1:240) were detected only in 25% of the seropositive mares that seroconverted before parturition. Another 25% of mares had no detectable serum antibodies before parturition (1 mare seroconverted post-foaling; 1 mare, on the day of foaling). The remaining 50% did not have measurable colostral antibodies, despite seroconversion occurring between 4 and 17 days before foaling (Pusterla, *et al.*, 2009c).

All foals' transfer of passive colostral antibodies was adequate (IgG ≥800 mg/dl). Passive transfer of immunity against *L intracellularis* was documented in 6 foals (titres between 1:60 and 1:120), and were detectable for 1.5 to 8 weeks. The reasons for the lack of detectable antibodies in the dam's serum, and the lack of transferred antibodies in foals, were not clear. Potentially, broodmares with undetectable serum antibodies may have colostral antibodies below the assay's limits of detection, as a foal born from a sero-negative mare had measurable *L. intracellularis* antibodies for 8 weeks after birth.

In 1 case, it is arguable that the mare had not the chance of mounting a proper peripheral immune response, as the foaling occurred less than 2 weeks after vaccination, but in the other,

the foal was born from a mare with high serum and colostrum titres. Thus, it was hypothesized that, despite an adequate transfer of passive immunity for all foals, individual variability in ingestion, or absorption (or both) of specific colostral antibodies played a role. In other words, although IgG transfer was normal, *L. intracellularis* specific IgG may have not been significant, and protection against EPE could have been conferred by mucosal IgA, together with the cell-mediated immune response, which apparently can be associated with satisfactory protection against *L. intracellularis* infection (Metzger, *et al.*, 2006; Pusterla, *et al.*, 2009c).

The results of the EPE immunization studies are in agreement with a body of work determining the immune response in pigs challenged with an attenuated *L. intracellularis* vaccine strain. A cautioning opinion was expressed in a few studies versus considering, or expecting, the anti-*L. intracellularis*-IgG in serum to be protective against a disease located within the intestinal wall. Mucosal IgA levels and cell-mediated immune responses could have a more probable role in protection against infection (Guedes, *et al.*, 2002a). The larger role of IgA against PE is ascribed to the ability of IgA to survive digestion more effectively, and to its larger concentration on a mucosal surface. This concept has been supported for years, although a complete demonstration still has to be determined (Holyoake, *et al.*, 1994; Pusterla, *et al.*, 2010c; Page, *et al.*, 2011c).

One study demonstrated improved growing and finishing performance in addition to a strong humoral response in vaccinated piglets (Almond & Bilkei, 2006) and another study conducted on sows naturally exposed to *L. intracellularis,* documented that gilts developed a high serum immune response, for up to 3 months after the original exposure, thus corroborating the potential usefulness of mares' vaccination (Guedes, *et al.*, 2002d; Guedes & Gebhart, 2003a; Pusterla, *et al.*, 2009b), although in piglets, the transfer of maternally derived antibodies against *L. intracellularis* had low titres that lasted for only 3 weeks. (Holyoake, *et al.*, 1994; Guedes, *et al.*, 2002d)

Vaccine protection against *L. intracellularis* in foals definitely warrants more research, to both determine the presence of different classes of colostral antibodies (IgG and IgA) for *L. intracellularis* in seropositive mares; and to correlate those findings with immunity in foals. To conclude, it is interesting that the sentinel mares did not show a humoral response. In adult horses it could be due to the absence of exposure, as vaccinated herd-mates did not constitute a

risk of contamination for the environment, differently than foals, due to their longer fecal shedding and different behaviour (*e.g.*, some degree of coprophagy is typical of young foals). Also, the controlled environment of an experimental setting differs from the normal foal rearing conditions, in a pasture. In fact, not only were the vaccinated mares "shedders", and allowed to commingle with the sentinel mares, but also all the other sources of contamination were kept under control, such as exposure to other animals and wildlife and uncontrolled and potentially contaminated feed (Pusterla, *et al.*, 2008b; Pusterla, *et al.*, 2009b).

"A desperate disease requires a dangerous remedy"

G. Fawkes

2.4. Pharmacokinetics and pharmacodynamics of AM drugs potentially useful against EPE

Today, with the enhanced knowledge of *L. intracellularis* biology and the new methodologies available for research, it is simpler to understand successes, or failures, of past treatments for PPE or EPE infections. Coupling that understanding to the knowledge of pharmacokinetic and pharmacodynamic principles at the cellular level of the major AM classes against obligate (and facultative) intracellular bacteria, allows a more rational discussion and selection of the therapeutic choices available (Carryn, *et al.*, 2003).

2.4.1. Pharmacokinetic considerations

The pharmacokinetic characteristics exhibited by an AM drug once administered to the affected pig, rodent or foal need to be considered to assess the appropriate therapy regimens, as each AM standard dose could only provide a cautious estimate of the likelihood of achieving a useful MIC *in vivo* (McOrist, *et al.*, 1995c). Interestingly, erythromycin, tetracycline, and penicillin, AM drugs of choice to treat cell-dependent micro-aerobic organisms, are the same drugs that were successful against *L. intracellularis* in the 1995 *in vitro* study. Their adequacy to treat pathogens such as *Treponema pallidum*, *Rickettsia spp*. and *Chlamydia spp*., for example,

was confirmed by *in vitro* cell-culture systems, and their clinical application in human medicine, although initially challenging, has been standardized for several years now (Norris & Edmondson, 1988; Hammerschlag, 1994). For *L. intracellularis*, instead, the current successful treatments have been achieved mostly through clinical experience and very limited *in vitro* investigation. Thus exploring potential alternative AM therapy for this bacterium, may require some evaluation of past studies, along with new compounds. In the attempt to summarize the relevant literature regarding AM pharmacokinetics at the cellular level, the β-lactam class will be the first one described, as it is the more commonly used AMs in equine medicine (F. Sampieri *et al.*, unpublished data)**.**

2.4.1.1. β-lactams.

Beta-lactams are unable to accumulate intracellularly, in phagocytic or non-phagocytic cells alike, and, at equilibrium, intracellular concentrations are much lower than extracellular levels (Prokesch & Hand, 1982; Nix, *et al.*, 1991; Carryn, *et al.*, 2002). All β-lactams display a free carboxylic functional group (or an equivalent proton donor-group), essential to exert their activity. A low intracellular concentration was ascribed to their inability to cross cell membranes and penetrate within cells, but that is probably an incorrect perception as, through modeling studies of trans-membrane distribution, most β-lactam drugs cross biologic membranes reasonably well (Tipper & Strominger, 1965; Carryn, *et al.*, 2002). Moreover, **cephalosporins** and **penicillins** are known today to be substrates of the transporter OCTN2, the same transporter of carnitine and acylcarnitine, 2 zwitterions that require a Na^+-dependent transport within cells for function. This mechanism may facilitate the β-lactams transport within cells of the tubular system in kidneys and in enterocytes, pointing to the fact that several mechanisms of intracellular absorption may be related, and conditioned, not only by the genetics of the patient, but also by the presence of specific co-substrates (Ganapathy, *et al.*, 2000).

The β-lactams are weak organic acids: because the intracellular *milieu* is slightly more acidic than the extracellular one, β-lactam accumulation within cells, according to some authors, is prevented by an issue of pH balance, rather than their ability to cross cell membranes (Renard, *et al.*, 1987), and masking the free carboxylic group with a basic moiety, would be all it takes to allow intracellular accumulation (Renard, et al., 1987). Such a theory could be a possibility in macrophages (*i.e.*, they have a lower pH), although it may be different in relation to different

cells. The newer generation **cephalosporins** and **zwitterionic β-lactam**s (*e.g.*, ampicillin) are unable to accumulate within cells for the same reasons. Furthermore, the presence of antibiotic efflux pumps, able to actively extrude β-lactams from the cells, may explain the low intracellular concentration of β-lactams (Van Bambeke, *et al.*, 2003).

2.4.1.2. Aminoglycosides.

Aminoglycosides are thought to localize within membrane-bound portions of exposed cells and, normally, are inactive against obligate anaerobic bacteria, due to their need for aerobic transport of the AM into bacterial membranes. Probably the best example of such a limitation is the variable success of clinical therapy with aminoglycosides for intracellular *Listeria monocytogenes*, despite it being active against the bacterium *in vitro* (Mates, *et al.*, 1983; Tulkens, 1991; Carryn, *et al.*, 2002). Prolonged incubation (*i.e.*, days) of macrophages and fibroblasts with aminoglycoside solutions have shown that such cells can achieve a ratio between intracellular to extracellular concentrations equal to 2:4 (Tulkens & Trouet, 1978), leaving the question open about whether or not such a ratio is sufficient to kill intracellular bacteria. Also, aminoglycosides are found almost exclusively in the lysosomes, explaining their slow rate of accumulation and why it is incorrect to believe that they lack ability to penetrate within cells (Tulkens & Trouet, 1978; Ford, *et al.*, 1994).

2.4.1.3. Macrolides.

Typically macrolides accumulate within all types of cells, with a few exceptions (Carryn, *et al.*, 2002; Carryn, *et al.*, 2003). As weak organic bases, these compounds accumulate within intracellular acidic environments (2/3 in lysosomes and phagosomes, and 1/3 in cytosol) at the site of infection, mainly due to their pH drive, with chemical structure differences between derivatives, being lower for erythromycin and much higher for those macrolides that carry more than 1 basic function. Both the uptake and efflux of macrolides are usually rapid, with the exception of azithromycin, as its binding to cellular structures (*e.g.*, phospholipids) may play a critical role (Van Bambeke, *et al.*, 1998; Montenez, *et al.*, 1999). Studies on clarithromycin uptake by fibroblasts showed that intracellular steady state is reached within 15 minutes (Chou & Walters, 2008). This said, once the pH drive is satisfied accumulation stops and macrolide drugs distribute from cytosol to lysosomes (more neutral pH) (Mandell & Coleman, 2001; Carryn, *et al.*, 2002; Perletti, *et al.*, 2011). Recent studies (one of which in foals) showed the ability of

clarithromycin, azithromycin and gamithromycin to accumulate with ranges of over 10 to 100-fold higher in the intracellular space of neutrophils and pulmonary macrophages, compared to the epithelial lining fluid and to plasma, respectively (Togami, *et al.*, 2011, Berghaus, *et al.*, 2012). Also, a study on erythromycin and its metabolites showed that intracellular accumulation to inhibitory and sub-inhibitory levels is much higher than plasma concentration (up to 40-fold) and tissue distribution and equilibration is delayed, likely due to cell uptake and accumulation processes (Krasniqi, *et al.*, 2012). Drug transporters linked to Ca^{2+} or Ca^{2+}-channel-operated mechanisms may be involved in macrolide uptake, although it appears limited to certain cell types. Also an efflux transporter, modulating macrolide accumulation at equilibrium has been evidenced in rats' macrophages, but it seems related only to erythromycin and azithromycin (Carryn, *et al.*, 2003).

2.4.1.4. Fluoroquinolones.

Fluoroquinolones are known to rapidly accumulate in the cytosol up to 4- to 10-fold higher than the extracellular concentration (Carryn, *et al.*, 2002). In veterinary research, enrofloxacin improved the potency of pulmonary neutrophils against a battery of porcine pulmonary pathogens. As well, enrofloxacin and marbofloxacin accumulated easily in canine neutrophils and in alveolar macrophages, respectively, suggesting that these AM's may have improved treatment outcomes in cases of canine systemic inflammation and lung infection (Schoevers, *et al.*, 1999; Boothe, *et al.*, 2005; Boothe, *et al.*, 2009). Although the mechanisms for such accumulation are not completely defined, a specific transport pathway (phorbol myristate acetate activated transporter) may be involved and uptake may require activation of protein kinase C and even simple diffusion processes (Cao, *et al.*, 1993; Walters, *et al.*, 1999; Hotta, *et al.*, 2002).

Cell maturation was believed to play a role in fluoroquinolone uptake by human monocytes and macrophages, but a French study demonstrated that cell activation due to inflammation, rather than maturation, was responsible (Dorian, *et al.*, 2001). Cell-associated fluoroquinolones have been consistently detected in the final supernatant of cell fractionation studies. Such findings suggest a rapid efflux of fluoroquinolones from a sub-cellular compartment, or that fluoroquinolones, which generally localize in the cytosol, easily diffuse to various sub-cellular components (Carlier, *et al.*, 1990; Seral, *et al.*, 2003a).

2.4.1.5. Tetracycline.

The pharmacokinetics of tetracylines at the cellular level is still largely uncharacterized, aside from an indirect, or partial, evidence of their ability to penetrate cell membranes and accumulate within cells (Najar, *et al.*, 1984). Initially it was believed that tetracycline penetrated cells, bacterial or otherwise, through a passive diffusion mechanism. However, recent studies with cell-associated fluorescence clarified that tetracycline, and its derivatives, enter cells rapidly using a Na^+-dependent active transport mechanism, with further regulation of intracellular levels by efflux pumps, such that intracellular drug concentrations exceed extracellular levels up to 40-fold, particularly if the drug contains lipophilic moieties (Katiyar & Edlind, 1991; Schnappinger & Hillen, 1996; Brayton, *et al.*, 2002; Walters, 2006). Within neutrophils, tetracycline can be observed in the nucleus within 1 h of addition to the cell-culture medium, and the intracellular concentration appears unaffected by 2 pH unit variation and by cell activation with phorbol mirystate acetate (Kirkwood, *et al.*, 2004; Walters, 2006). The rate limiting step is transport across the plasma membrane, as equilibration within cytoplasm and liposomes is achieved in less than 1 h for tetracycline and their derivatives not displaying the carboxamide group (Sigler, *et al.*, 2000). Interestingly, reductions in extracellular levels trigger efflux pumps to extrude intracellular tetracycline (Walters, 2006).

2.4.1.6. Other antimicrobials.

In regard to other AM drugs pharmacokinetic activity at intracellular level, the information available is fairly limited. For **lincosamides**, lincomycin exhibits poor intracellular accumulation (thus explaining Wattanaphansak *et al.*, 2009a's *in vitro* findings), but clindamycin extensively accumulates within cells due to its basic nature (similar mechanism described for macrolides) and to the involvement of a nucleoside transporter (Hand & King-Thompson, 1982; Prokesch & Hand, 1982; Easmon & Crane, 1984).

Among **ansamycins**, rifampin accumulates intracellularly, up to 2- to 10-fold according to the available literature, whereas other compounds of the same class may accumulate for up to 60-80 fold (rifapentine). Unfortunately, even for this class the accumulation mechanism is unknown (Johnson, *et al.*, 1980; Hand, *et al.*, 1984; Pascual, *et al.*, 1987).

For **glycopeptides** and **polypetides** (*e.g.*, vancomycin and bacitracin), which are not active against Gram-negative bacteria, because of their actions against peptidoglycans,

intracellular kinetics studies have been limited too. Vancomycin shows typically a slow and modest uptake in macrophages (up to only 8-folds in 24 h period) and, supposedly, accumulates within lysosomes, but those results are based on a study *in vivo* (Beauchamp, *et al.*, 1992). Recent studies in regards of a novel lipoglycopeptide, confirmed *in vitro* a low accumulation of this class of AM drugs at the lysosomal level (Barcia-Macay, *et al.*, 2008).

2.4.2. Pharmacodynamic considerations

From the standpoint of pharmacodynamic activity, clinical studies are particularly difficult yet extremely important, because they ultimately provide one overall global answer, to what is affected by the combination of complex extracellular and intracellular pharmacokinetic variables. The equally large array of microbial and host-response variables and the simultaneous presence of infection, both in intracellular and extracellular foci, should be examined; furthermore, one must keep into account the dose, dosing interval and duration of therapy. Such points are exemplified with studies on rifamycin activities, and, to a broader extent, about AM therapy against *Mycobacteria* (Burman, 1997; Burman, *et al.*, 2001). A recent comparison of several AM from different classes against intracellular *S. aureus* showed that dicloxacillin, cefuroxime, gentamicin, azithromycin, and rifampin (rifampicin), were tested in a new *in vivo* sepsis murine model, to distinguish between their extra- and intracellular effects. The intracellular effects of the AMs could be ranked as the mean change in the log(10) number of CFU/ml (Delta log(10) CFU/ml), between treated and untreated mice, after 4 h of treatment: the higher ranked CFU was dicloxacillin (with 3.70 Delta log(10) CFU/ml) and the lower was azithromycin (0.21 Delta log(10) CFU/ml), with cefuroxime, rifampin and gentamicin, respectively, in between (Sandberg, *et al.*, 2009). As stated above, factors such as size, number and frequency of doses; time of exposure and timing of treatment initiation, during testing of intracellular activity *in vivo* are determinants for therapeutic success, as intrinsically a poor correlation between the intracellular accumulation of AMs and the actual intracellular effect was detected (Sandberg, *et al.*, 2009).

These factors explain why it is difficult to delineate precisely the pharmacodynamic properties where the specific intracellular AM activity is concerned and why discussions on this purpose generally lead to conflicting opinions between researchers. Some prefer to rely on predictions made through intracellular pharmacokinetics of an AM and its MICs, where some

other researchers focus on the results of *in vivo* models (Cappelletty, 2007; Sandberg, *et al.*, 2009). Moreover, a few studies aiming to compare *in vitro* and *in vivo* results of AM intracellular activity (particularly for active drugs such as fluoroquinolones and ansamycins) often are complicated by unexpected outcomes, further obfuscating an unclear clinical perception (Shandil, *et al.*, 2007; Vallet, *et al.*, 2011).

Despite continuous improvement and standardization of better methodologies, the assessment of pharmacokinetic–pharmacodynamic properties in the context of intracellular infections (*e.g., Myc. tuberculosis, Staph. aureus, Str. pneumonia,* etc.) are greatly lacking when compared to studies and assessments about extracellularly localized diseases (Carryn, *et al.*, 2003). Nevertheless, a generalized consensus about macrolides, fluoroquinolones, tetracyclines and ansamycins is their ability to exert their action against intracellular bacteria, as these drugs were used repeatedly and successfully to treat such infections, whether generated by obligate, or facultative intracellular bacteria. A key question is whether this activity is optimal or sub-optimal, and whether cellular accumulation and sub-cellular disposition are related to the level of activity (Carryn, *et al.*, 2003).

However, there is agreement about β-lactams and aminoglycosides showing poor intracellular activity, although once again, an attentive analysis of the studies reporting such results should be prompted. Often the exposure of the microorganism to the drug has only been for a short-term treatment (Carryn, *et al.*, 2003). Pharmacodynamically, the mechanism of action of such drugs differs by class. The aminoglycosides and fluoroquinolones are considered concentration-dependent at the intracellular level too, whereas β-lactams, which exhibit time-dependent and concentration-independent bactericidal activity on an extracellular level, should be considered equally active intracellularly, in a time-dependent fashion (Craig & Ebert, 1992; Carryn, *et al.*, 2002). Thus, the main pharmacodynamic tenets for those classes of AM still hold valid, although it is puzzling to find certain paradoxes, or contradictions, reported in the literature, regarding treatment successes or failures, particularly in regards of human infections with *Staphylococcus aureus*, *Chlamydia spp.*, *Listeria monocytogenes* and *Legionella spp*. The most interesting examples being listeriosis and penicillin and tuberculosis and gentamicin (Carryn, *et al.*, 2003).

Possibly, certain episodes of fluoroquinolone failure could be ascribed to suboptimal action toward intracellular bacteria despite high intracellular concentrations, whereas the failure of macrolide therapy could be due to their bacteriostatic properties, rather than to inadequate intracellular accumulation (Craig & Ebert, 1992; Carryn, *et al.*, 2002). Furthermore, the time-dependent β-lactams showed that *in vitro* failures depended mainly on a short exposure times, rather than on inadequate drug potency, hence explaining why repeated, and frequent, treatments reported clinical cure in diseases considered "off limits" for the "good old-fashioned" penicillin G (Carryn, *et al.*, 2003). The multiple and frequent administrations allowed achievement of an intracellular critical concentration condition for effective therapeutic outcomes with these AMs (Michelet, *et al.*, 1994; Michelet, *et al.*, 1997; Kutlin, *et al.*, 1999; Somani, *et al.*, 2000; Paillard, *et al.*, 2002; Seral, *et al.*, 2003b).

In this context, the pharmacokinetic and pharmacodynamic characteristics and the results obtained *in vitro* by McOrist's (1995), explains the lack of *in vivo* efficacy of the aminoglycosides, bacitracin or vancomycin, as the AM's oxygen demand is far higher than the microaerophilic environment, wherein *L. intracellularis* thrives (Lawson, *et al.*, 1993). Moreover, it should be considered that β-lactams, aminoglycosides and lincomycin accumulate intracellularly, but with difficulty (Carryn, *et al.*, 2002; Carryn, *et al.*, 2003; Wattanaphansak, *et al.*, 2009a). In contrast, macrolides, tetracyclines, pleuromulins, and virginiamycin localize in the cytosol of exposed cells, wherein *L. intracellularis* resides, and act by selectively blocking protein synthesis in ribosomes, a process that is independent of oxygen concentration (Cocito, 1979; Tulkens, 1991). Particularly, in regards to tetracycline's mechanism of action, its definition is becoming more multifaceted. Tetracyclines are bacteriostatic and inhibit bacterial protein synthesis, which destroys bacterial membranes, but these compounds also inhibit matrix metallo-proteinases and cause apoptosis in a variety of cells, which renders these drugs potentially helpful in other therapeutic fields (*e.g.*, cardiovascular therapy) (Schnappinger & Hillen, 1996; Sagar, *et al.*, 2010; Castro, *et al.*, 2011).

2.4.3. An alternative antimicrobial prospect: gallium maltolate

Having reviewed the mechanism of action of the available drugs against Gram-negative bacteria, the therapeutic options explored empirically for *L. intracellularis* over the years appear appropriate. However, lately EPE was unresponsive to treatment, or was suspected to have a variable susceptibility to AMs, in a few cases across North America (Sampieri, *et al.*, 2006; Frazer, 2008; Page, *et al.*, 2012). In usual EPE cases the disease and its management costs are dreaded, as much as its financial impact on the industry, especially in the areas where the disease is endemic and the concentration of horses is high (Lavoie, *et al.*, 2000; Frazer, 2008; Pusterla, *et al.*, 2009a). Thus, in the context of this dissertation project, EPE was exposed to a completely different compound with an hypothesized AM activity: the novel gallium-based molecule, gallium maltolate (Bernstein, *et al.*, 2000).

2.4.3.1. Gallium compounds available

Gallium (Ga(III)) is a post-transition metal belonging to the group IIIA of the periodic table. Chemically it is similar to ferric iron (Fe(III)), aluminium (Al), zinc (Zn) and indium (In) but its chemical behavior is particularly close to the one of Fe (III), in terms of electric charge, ionic radius, valence and electronic configuration (Collery, *et al.*, 2002). Unlike iron, Ga (III) is unable to attain a divalent state (*e.g.*, ferrous iron - Fe(II)) under physiologic conditions, and it cannot participate in redox reactions, or enter mammalian Fe(II) binding molecules (*e.g.*, heme) (Logan, *et al.*, 1981; Bernstein, 1998), although it apparently has a strong affinity for transferrin (an iron transporter, or siderophore – see below) (Vallabhajosula, *et al.*, 1980; Rudnev, *et al.*, 2006; Chikh, *et al.*, 2007), binding to it slowly, but tightly (Harris & Pecoraro, 1983). It was estimated that in humans administered a Ga(III) product, the total gallium in plasma could be 99.9% transferrin-bound.

In human medicine, gallium salts have been studied and employed for years to normalise bone metabolism through down-regulation of osteoclastic degranulation, and the pertinent research has been abundant. Suffice to say that gallium was found capable of inhibiting osteoclast reabsorption, when added to the culture medium at concentrations equal to, or higher than 0.39 μM (Hall & Chambers, 1990); also, it reversibly inhibited osteolysis induced by parathormone and tumor necrosis factor in a dose-dependent manner, when added in a culture of viable osteoclasts (Bockman, 1991). Its main effects were believed to be on mature osteoclasts (Blair, *et al.*, 1992), due to inhibition of enzymatic synthesis and degranulation (starting from 1 μM and increasing concentrations), but recent studies showed that gallium dose-dependently (0-100 μM)

inhibited the *in vitro* reabsorption activity of RBC and induced a significant decrease in the expression level of transcripts coding for osteoclastic markers, as it affects the regulatory gene transcription (at concentrations at least equal to 2.5 μM). Furthermore, it dramatically reduced the formation of TRAP-positive multinucleated cells. The dose-dependent down-regulation was clear for the expression of the transcription factor NFATc1, a master regulator of RANK-induced osteoclastic differentiation, but did not affect the viability or activity of primary and MC3T3-E1 osteoblasts (Verron, *et al.*, 2010).

Gallium salts (particularly chloride and nitrate) dissociate easily in aqueous solution, becoming often unsuitable for oral or parenteral administration, but due to one of the dissociation by-product, gallium hydroxide, which is insoluble and severely limits the bioavailability in the intestines (Bernstein, 1998). Thus far, only an injectable solution of sodium citrate-chelated gallium nitrate (Ganite^{R}) is approved in the United States for the treatment of human cancer-related hypercalcemia, and it is administered as continuous IV infusion once diluted in saline or dextrose solution, to avoid the nephrotoxic effects reported with IV bolus (Martens, *et al.*, 2007a). This injectable gallium nitrate solution is able to acutely normalise calcemia (at concentrations of 0.92 μg/ml) in human patients affected by paraneoplastic hypercalcemia (Warrell, *et al.*, 1986).

Several studies on gallium compounds, in both veterinary species and humans, show a fairly long elimination kinetics, which varied depending on the species. It is claimed to be potentially due to the slow absorption (Harrington, *et al.*, 2006; Martens, *et al.*, 2007a; Fecteau, et al., 2011; Pollina, *et al.*, 2012), although several studies did not implement intravenous administration. Probably, the main factors impacting the slow absorption are the high precipitation rate of by-products, gallates, ($Ga(OH)_4^-$), which are rapidly excreted by the kidneys; and the slow, but almost complete, binding to transferrin (Harris & Pecoraro 1983). Furthermore, in early studies of gallium based compounds (Bruner, *et al.*, 1953a; Bruner, *et al*., 1953b), elimination was shown to occur mainly by renal excretion within the first 24 h post-treatment, or by intestinal excretion, in the following days, as by then it mostly exists in plasma as transferrin-bound gallium. However, the main reason of our interest is that, regardless of the specific compound vectoring it, Ga(III) binds to siderophores i*n vitro* and is able to compete with Fe (III) for bacterial uptake, Gram-negative and Gram-positive alike.

2.4.3.2 Gallium maltolate

Gallium maltolate (GaM), as **visible in Figure 2.1**, is a coordinated complex consisting of a Ga (III) ion surrounded by 3 maltol ligands, and different from other compounds, it is deemed safe when orally administered to humans, mice, rats, dogs, foals (Bernstein, *et al.*, 2000; Martens, *et al.*, 2010), and guinea pigs (L.R. Bernstein, personal communication), making it suitable for treating ambulatory patients. Thus far, no nephrotoxic effects of GaM are known, likely because all the Ga(III) from GaM administered orally becomes protein bound in blood (Bernstein, *et al.*, 2000). Although the characteristics of the replication pathways specific for *L. intracellularis* are still incompletely known, we assume that GaM, should adequately convey Ga(III) in proximity of the bacteria, particularly the ones located in the enterocytes' brush border (Bernstein, 1998).

Figure 2.1. Gallium maltolate chemical structure.

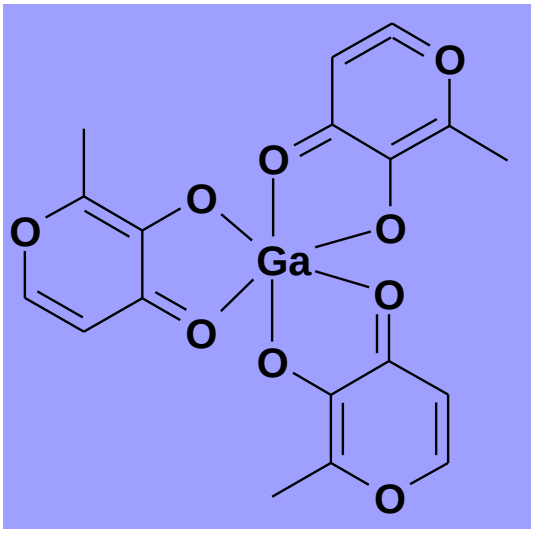

Figure 2.1. Gallium maltolate structure. Depiction of the chemical structure of the trivalent gallium (at the center), with the 3 maltol ligand molecules connected in a triangular shape.

In regards to its use in foals, GaM was first tested *in vitro* and *in vivo* (mice) against another equine intracellular pathogen, *Rhodococcus equi (R. equi),* with encouraging results (Harrington, *et al.*, 2006). Subsequently, GaM pharmacokinetics was investigated in foals, as a novel AM approach to treat rhodococcal pneumonia, particularly because the achieved serum concentrations exceeded the MIC determined *in vitro* (Harrington, *et al.*, 2006; Martens, *et al.*, 2007a, Martens, *et al.*, 2007b, Martens, *et al.*, 2010), both with single and multiple oral administration, via water solution and gel suspension (Chaffin, *et al.*, 2010). *R. equi* (gram-positive) and *L. intracellularis* (gram-negative) are structurally and genetically different bacteria, but both are successfully treated with macrolides, or azalides, and rifampin or doxycycline (Jacks, *et al.*, 2003; Giguere, *et al.*, 2004; Womble, *et al.*, 2007; Chaffin, *et al.*, 2008). Despite these differences, its use against *L. intracellularis* shows promise, as also this bacterium shares common intracellular bacteria characteristics, in that proliferation and virulence depends on the iron concentration in the host cell environment.

Normal biological function of proteins containing Fe(III) depends on the reduction of the metal, and when Fe(III) is replaced by Ga(III), reduction cannot occur and this is hypothesized to disable the proteins, disrupting cellular metabolism and causing cell death. A key study *in vitro* ascribed beyond doubt the efficacy of the Ga(III) moiety in impairing *Mycobacteria* replication and survival, clarifying the role of both moieties (Olakanmi, *et al.*, 2000). An even stronger demonstration of the iron mimicking strategy (also called "Trojan horse" mechanism) behind the gallium compounds was obvious when organic moieties (*i.e.* using a siderophore) compounds were used. Recently gallium desferrioxamine topical applications were implemented against a *Pseudomonas* spp. infection in a rabbit uveitis model. The desferrioxamine moiety vectored, better than others, Ga(III) to success, likely because many iron-dependent mechanisms, potentially too slow to access for Ga(III), were bypassed by the desferrioxamine (Banin, *et al.*, 2008). An additional favorable pharmacologic feature of Ga(III) is its apparent accumulation at sites of inflammation, which underlies its use as a diagnostic imaging agent (Berry, *et al.*, 1984; Bernstein, 1998), but it could be useful as a chemotactic feature to the site of infection.

2.4.3.3. The rationale for using an iron mimicking compound

The body needs iron for multiple functions, the most known being the role of heme and hemoglobin for oxygen transport. Iron also plays a fundamental role in several transport proteins,

cell membrane transporters, metallo-enzymes, DNA replication processes and xenobiotic metabolism. It is transported into cells by transferrin-mediated endocytosis, but other absorption mechanisms likely exist as the transport proteins (*e.g.*, apotransferrin) are insufficient to fully account for dietary iron absorption (Gunshin, *et al.*, 1997). In monogastrates, the iron balance is dependent almost entirely on dietary intake, as it is absorbed in the intestinal tract and internalized by enterocytes as ionic ferrous iron (Fe(II)) (Ma, *et al.*, 2002; Ma, *et al.*, 2006; Chua, *et al.*, 2007; Kolachala, *et al.*, 2007) (See sub-chapter 2.5).

Iron is essential not only for mammalian functions, but it is also required for a variety of metabolic processes in virtually all microorganisms. Its ability to cycle between Fe(II) and Fe(III) represents one of the limiting steps of metallo-mediated functions in mammalian physiology, whereas bacteria preferentially uptake Fe(III). Now in an aerobic, neutral pH environment the concentration of free Fe(III) is limited to 10^{-18}M by the insolubility of Fe(OH), which is well below the required concentration for cells survival (Girijavallabhan & Miller, 2004). However, the concentration of Fe(III) available for bacteria is even lower, as mammalian sequester and bind Fe(III) through serum proteins, such as transferrin and lactoferrin (Raymond & Dertz, 2004), thus many bacteria circumvent the Fe(III) nutritional limitation by producing the "siderophores". These are chelating low-molecular-weight agents, secreted under iron-limited conditions, which strongly and specifically bind, solubilize and deliver Fe(III) to bacterial cells through specific cell surface receptors (Raymond & Dertz, 2004). The virulence of certain intracellular bacteria (*e.g.*, *E. coli*, *Shigella spp.* and *Salmonella spp*.), is coupled with the siderophores production (Reigstad, *et al.*, 2007; DeLeon, *et al.*, 2009). Some bacteria produce only 1 siderophore, while others have many, thus increasing the chances to utilize available Fe(III). For example, *E.coli* siderophore-system has been extensively studied and it is paradigmatic for bacterial siderophore–dependent iron uptake (Earhart, 2004). Iron assimilation in *E.coli* occurs by means of the "enterobactin-system", which is also typical of most enteric Gram-negative and intracellular bacteria, such as *L. intracellularis* (Payne & Mey, 2004). In latently infected epithelial cells iron uptake systems are expressed up to a 100-fold more than in normal or early infected cells.

Thus, targeting bacterial iron uptake mechanisms and their metabolism could constitute good targets for novel AMs compounds. By a larger definition, siderophores can be indicated as those

molecular structures, or proteins, that literally "carry" or transport iron. These latter are currently investigated as therapeutic aids in different medical branches, to either vector antimicrobial drugs within bacteria, or, as, for example, desferrioxamine mesylate, used to reduce iron availability in cases of patients with thalassemia, or subjected to multiple transfusions (Girijavallabhan & Miller, 2004). Regardless of the carrier that Ga(III) can use to interact with the Fe(III) pathway, its ability to bind to transferrin (and the likes) has been documented and becomes of great pharmacokinetic relevance in our study. Transferrin receptors are present on many epithelial cells, including epithelial cells of small and large intestine and intestinal crypt cells (Jeffrey, 1996), as that gains a route of entry into enterocytes for Ga(III); moreover its accumulation in lysosomes (mature endosomes) of neoplastic tissues, renal tubular cells and macrophages has been reported too, providing other routes to convey it to the site of infection (Berry, *et al.*, 1984, Bernstein, 1998; Bernstein, *et al.*, 2000).

"Il faut manger pour vivre et non pas vivre pour manger"

"One should eat to live, and not live to eat."

Molière

2.5 Gastrointestinal function.

A brief review of the GIT absorption processes follows, as *L. intracellularis* infection can be a useful model for alimentary tract diseases, due to the typical pathogenetic "malabsorption" traits. In humans, malabsorption defines altered, or deficient, structure or function (or both) of the small intestine, alone or together with disturbances of liver, biliary ducts and pancreas. In equine medicine, it defines malfunction of both small and large intestine (Roberts, 2004). Its diagnosis is complicated and, regardless of the affected site, malabsorption often exhibits diarrhea as a feature, not as a cause (Roberts, 2004), as absorption disturbances in the small intestine lead to altered microflora and fermentation products in the large intestine, predisposing the horse to maldigestion, diarrhea (see below) and weight loss, or more complex disturbances, such as colic, the leading cause of mortality in horses (Dyer, *et al.*, 2002).

The most important function of the gastrointestinal tract (GIT) is to transform orally ingested food into absorbable substrates and energy sources for the body functions. The key point is in the ability of absorbing and transporting substances through the mucosal barrier of enterocytes to the lymphatic system and the blood. To these ends, the GIT transforms diet's contents, mainly represented by polymers (*e.g.*, cellulose, starches, proteins and triglycerides), into monomers or specially reconstituted polymers (*e.g.*, chylomicrons), in the only form of utilizable and transportable substrate, in order to vector it to the blood stream, and from there, wherever necessary (Caspary, 1992).

2.5.1. Physiology of intestinal absorption

Nutrient absorption occurs both in the small and large intestine. The large intestine absorption mechanisms are briefly approached first, as the small intestinal absorptive function is more detailed (and pertinent to the dissertation's subject) and will be addressed more in detail. For this section the main body of literature cited is constituted by GIT physiology reviews and GIT physiology book chapters, although, where appropriate, original research papers were consulted for novel or specific details.

The horse large intestinal functions consist mainly of: 1) absorbing (and reabsorbing) water (up to 95%) from the chyme originating from the ileum and the large intestinal secretions; 2) absorbing electrolytes; and 3) providing a site of slower transit for microbial digestion (Argenzio, *et al.*, 1974a). Specifically to compensate their incomplete colonic functions, younger foals, rabbits and other rodents resort to coprophagy, the former often ingesting the dam's feces to expedite the large intestine bacterial colonization (Crowell-Davis & Houpt, 1985; Crowell-Davis,1986); the others ingesting their own feces to compensate the partial absorption obtained through the stationary haustral contractions and retrograde propulsion, as one-pass only is insufficient to absorb the microbial digestion by-products (*e.g.*, vitamin B complex) (Pickard & Stevens, 1972).

In a series of studies conducted in ponies, a considerably slower transit through the large colon was demonstrated with radio-marked particles (chromium radioisotopes) injected at the level of stomach, cecum and large colon (Argenzio, *et al.*, 1974a; Argenzio, *et al.*, 1974b; Argenzio & Stevens, 1975). *Digesta* were observed to move very quickly from the stomach to

the cecum, and then to the proximal colon. While the cecum absorbs large quantities of water, especially between meals, the large colon sees large water volumes exchanged through the colonic mucosa, through cyclic periods of net influx and efflux. Such a conspicuous exchange between *digesta* and plasma content apparently is associated primarily with cyclic changes in osmolality, resulting from a cyclic microbial digestion (Argenzio, *et al.*, 1974a). Resident microorganisms' digestion not only affects the *digesta*'s electrolyte contents, but also the fermentation of vegetable fiber. The result is the formation of short chain fatty acids, which provides a large portion of the horse's energy requirements. Thus, variations of the fermentation pattern lead to osmotic imbalances, and to osmotic diarrhea.

Today's horses' nutrition regimens contain much more concentrate feed than they did 50-60 years ago and the absorption of such high levels of starches (*i.e.*, grain) is managed by the small intestine, providing a substantial fraction of energy for metabolism (Alexander, 1955; Argenzio & Hintz, 1972; Dyer, *et al.*, 2002). The small intestine of non-ruminant herbivores, such non-suckling horses, absorbs large amounts of carbohydrates, and proportionally less, proteins, lipids, electrolytes and water, due to the driving force that sodium ions (Na^+) exert on the small intestinal uptake of other electrolytes and substances. Enterocytes can absorb Na^+ through 3 major processes.

1) The first process is active absorption through a "conductance pathway", which is electrogenic, though uncoupled with other substance's uptake, as Na^+ initiates an inward electrochemical gradient between extracellular and intracellular spaces. The gradient is maintained in exchange of energy, as it requires catalysation of ATP, via hydrolysis, by the Na^+-K^+-ATP-ase "pump" located on the enterocyte's basolateral membrane. The "pump" exchanges Na^+ "out" for potassium ions (K^+) "in", thus establishing the steep electrochemical gradient that leads to Na^+ absorption from the lumen. Under these conditions, particularly in the duodenum and ileum, chloride ion (Cl^-) absorption accompanies Na^+ in its inward transition. Supposedly it occurs through a paracellular mechanism, and marginally, by the transcellular route. This is not the only mode of entry for Na^+ and Cl^-, although the uncoupled absorption of Na^+ is perhaps the major route of Na^+ entry in the mammalian colon (Fondacaro, 1986). One should note that throughout the whole intestine, water follows the so-constituted electrolyte gradient and is

absorbed almost entirely by diffusion through the intestinal wall, obeying classical osmosis laws. (Guyton & Hall, 2005a).

2) The second process of Na^+ mucosal absorption is through a coupling to other organic solutes, as Na^+ is required for, and is co-transported with, the absorption of glucose, amino-acids, bile acids (in the ileum), water and water-soluble vitamins. The driving force, even in the face of uphill movement, is the electrochemical gradient discussed above, created at the level of the baso-lateral membrane (Fondacaro, 1986).

3) The last process is a neutral, carrier-mediated, electrically silent one-for-one Na-Cl co-transport system, at the level of the villous brush border, in the small intestine. This process, energized by the inwardly directed Na^+-dependent electrochemical gradient, provides the bulk of absorbed Na^+ and Cl^- in the mammalian small intestine. More importantly, this coupled entry seems not to be an unidirectional co-transport, or "symport", but involves coupling of Na^+-H^- and Cl^--HCO_3^-, or Cl^--OH^- exchange (Liedtke & Hopfer, 1982a; Liedtke & Hopfer, 1982b; Gunther & Wright, 1983, Knickelbein, *et al.*, 1983, Fondacaro 1986). The exchange of Cl^- for HCO_3^- is an equally active process. In the duodenum and jejunum, a high HCO_3^- concentration occurs as a result of bile and intestinal and pancreatic secretions. The HCO_3^- absorption process is more complicated than an electric gradient absorption, as it implies an exchange of H^+ for Na^+ (Guyton & Hall, 2005a).

Calcium ions (Ca^{++}) are absorbed through active transport mechanisms into the blood, especially in the duodenum, and the absorbed amount of Ca^{++} is closely regulated by a finely tuned interaction of parathyroid hormones and vitamin D (Caspary, 1992). K^+, magnesium (Mg^{++}) and phosphate (P^-) ions, are absorbed through the small intestinal mucosa, following the general rule that, monovalent ions are easily absorbed in great quantities, whereas bivalent ions are normally absorbed in smaller amounts (*e.g.*, Ca^{++} in humans is absorbed at a ratio of 1:50 compared to Na^+) (Caspary, 1992; Guyton & Hall, 2005).

2.5.1.1. Carbohydrate absorption

The detailed explanation of the role of Na^+ in absorption clarifies that virtually no carbohydrate could be absorbed in absence of Na^+ co-transport. In non-ruminant species, digestible carbohydrates are hydrolysed within the lumen by pancreatic α-amylase and

disaccharidases of the brush-border membrane, such as sucrase, maltase and lactase, to the 3 main monosaccharides: D-glucose, D-fructose and D-galactose. These are vectored through the brush-border membrane by specific monosaccharides transporters (Shirazi-Beechey, 1995). The expression of sucrase, maltase and lactase in the brush-border membrane and the Na^+-glucose transporters' distribution in the small intestine of mature grass-fed horses (< 4 years of age) was elegantly illustrated by Dyer and coworkers (2002). Dyer evaluated the digestive and absorptive ability of duodenum, jejunum and ileum both at cellular and molecular levels, in terms of abundance and level of activity of transporters and enzymes. Sucrase apparently is highly concentrated in the proximal small intestine, whereas maltase is expressed all along the small intestinal brush border membrane, with the findings in horses much higher than in other non-ruminant species (1 μM/min/mg protein in horses vs. 0.013-0.054 μM/min/mg protein in man) (Dyer, *et al.*, 2002). Opposite to the general belief of the mid 1970's, lactase is expressed also in mature horses, at higher concentrations in duodenal and jejunal brush-border than the ileal one, confirming that non-nursing animals can still digest the disaccharide lactose (Roberts, 1975).

A similar pattern of expression was reported for the Na^+-dependent-D-glucose-transport protein (SGLT), which is a bifunctional carrier and works optimally when both sites - one for monosaccharides (both glucose and galactose are accepted) and one for Na^+ - are occupied (Caspary, 1992; Dyer, *et al.*, 2002). Its kinetics was investigated in tissues, testing increasing D-glucose concentrations (0.01-2.5 mM), with the result that the D-glucose uptake conformed to Michaelis-Menten kinetics, with K_m (0.49 ± 0.06 mM) similar in the 3 small intestine sections, but with a significantly higher V_{max} in duodenum (1312 ± 141.9 pM/s/mg protein) (Dyer, *et al.*, 2002). As inhibitors selectivity (and aminoacid sequence) is similar to the one of other species, the equine SGLT co-transporter, is believed to be the isoform 1 (Wright, 1993; Dyer, *et al.*, 2002). As a note of interest for the purpose of animal modeling and comparisons (see chapter 2.6), the Equine SGLT1 shares the same hypervariable regions as SGLT1 in other species, although it's the only one together with the rabbit, showing a lower number (662) of constituting amino acids and 2 amino acid deletions at positions 592-593 (Dyer, *et al.*, 2002).

Once in the intracellular space, glucose, and almost identically galactose, is facilitated through the baso-lateral membrane towards the bloodstream. Differently, fructose never uses the

Na^+-co-transport mechanism, as it is absorbed by facilitated diffusion along the whole intestine. (Caspary, 1992).

2.5.1.2 Protein absorption

The ingested proteins first undergo denaturation by pepsins in the acid gastric environment, although a large amount of polypeptides still enters the duodenum (Caspary, 1992). In the intestinal lumen, those polypeptides are hydrolyzed as di-peptides, tri-peptides and few free amino-acids, so that they can be absorbed through the enterocytes' luminal membrane (Guyton & Hall, 2005a; Guyton & Hall, 2005b).

The assimilation of peptides occurs mainly in the proximal jejunum, and to a lesser extent in the distal jejunum and proximal ileum, which is a key in understanding the reasons behind the protein-losing aspect of EPE, as in the majority of cases affects terminal jejunum and ileum. The final phase of the proteins absorption follows 2 energy mediated mechanisms: one is the active Na^+-co-transport, which takes care of absorbing di- and tri-peptides. The other is the process managing the larger peptides, cleaved at the level of the brush border membrane and subsequently trans-located through specific carrier-mediated transport-systems for neutral, basic and acidic amino acids (Caspary, 1992). Only a few free amino-acids are transported inside the cells through facilitated diffusion, similarly to fructose (Guyton & Hall, 2005a; Guyton & Hall, 2005b).

2.5.1.3. Lipid absorption

The absorption of fats consists of 2 phases: a luminal and a mucosal phase. In the luminal phase the complex food lipid aggregates are solubilized as "micellar" dispersions of monomeric lipids, by the interactions of several mechanical and emulsifying agents. The emulsification process takes place partially in the stomach, thanks to the contribution of salivary lipase, although this is a minor step in the complex of reactions of emulsification. Subsequently, enzymatic hydrolysis of lipid esters ensues by pancreatic lipase in the duodenum, preparing the lipid substrate for the last step of the conversion of lipolytic products from insoluble to hydrosoluble status, so enterocytes can absorb it through the membrane.

In the duodenum, the low pH triggers the release of secretin, which triggers the release of HCO_3^-, as a chain reaction, and due to the rise in pH, fat droplets rise to surface and perform a

more efficient emulsification. The release of cholecystokinin–pancreozymin substances triggers biliary and pancreatic enzyme secretion.

Bile's main constituents are bile salts and bile lipids (phospholipids), which as micelles, act on the basis of their detergents properties and increase the surface area of the fat droplets. A large surface area is a requirement for fat digestion: the hydrolytic steps of pancreatic lipase/colipase system and the interaction of esterases, phospholipase-A_2 and cholesterolase. The obtained mixed micelles have a very small size (3-6 nm), are highly charged and form an aqueous suspension which is easily absorbed by the mucosal cells (Carey, *et al.*, 1983; Caspary,1992). The mucosal phase is the actual absorption step in which fatty acids, monoglycerids, cholesterol and lipid-soluble vitamins are transported from jejunum to blood. The bile micelles constitute the key step for intestinal absorption of fats (with bile micelles about 97% of fat is absorbed, without bile micelles only 40-50% is absorbed) (Guyton & Hall, 2005a; Guyton & Hall, 2005c), as their absorption depends solely on the rate of penetration through 2 barriers: 1) the unstirred water layer, at the surface of mucosal cells membranes; and 2) the lipid membrane into the mucosal cell.

Fat absorption starts in the distal duodenum and it is complete by the proximal part of the jejunum, whereas the bile salts are reabsorbed in the terminal ileum (entero-hepatic recirculation of bile salts) (Caspary, 1992). However, in horses, the absorption of short-chain fatty acids derived by resident microfloral fermentation is reported to occur in the large intestine, particularly in animals whose nutrition is grass-based (Alexander, 1955; Argenzio & Hintz, 1972). Permeation of fatty acids through the brush border membrane occurs through a specific carrier-mediated Na^+-co-transport, which is energy dependent. Once in the intracellular space, short-chain fatty acids are absorbed with more ease than long-chain ones, although micelles are disassembled and the fatty acids are re-synthesized at the level of SER and Golgi apparatus where are packaged in low-density lipo-protein and chylomicrons, and then released again in the cytoplasm to migrate in the systemic bloodstream, via the lymphatic system, as lipids are pushed in through the villi's pumping contractions (Guyton & Hall, 2005a; Guyton & Hall, 2005c).

2.5.2. Pathogenesis of malabsorption.

After the brief review of principal nutrient groups' absorption, it is clear that a diagnosis of malabsorption is complicated and, in horses, it is often a diagnosis of exclusion, particularly in those cases that present for chronic weight loss and wasting. If the lesions are located in the small intestine (*e.g.*, EPE lesions), the absorption of carbohydrates, proteins, fats, oligominerals and vitamins is profoundly changed, whereas the changes in absorption of electrolytes and water are marginal. However, the chyme presenting to the large intestine is substantially altered, containing larger amount of unabsorbed substrates, which change the pH altogether. These changes subsequently complicate the chyme's fermentation in the large intestine, impacting abnormally both caecal and colonic microflora, the cardinal point of equine digestion.

The reason why it is difficult to accurately diagnose malabsorption in horses, is due to their herbivorous condition, with an eminent role of the large intestine and a limited exocrine pancreatic activity (*i.e.*, compared to humans or small animals). Nonetheless, maldigestion and malabsorption can undoubtedly contribute to chronic weight loss, which is significant during severe infiltrative disease of the small intestine, with partial or total villous atrophy (Roberts, 2004). In foals, such an impaired digestive process may exacerbate diarrhea, wherein bile salts concentration may be reduced because of chyme dilution, hepatic or ileal dysfunction.

2.5.3. Tests to evaluate intestinal integrity and absorption ability.

In the last 35 years, several testing methodologies were investigated and are now routinely available, allowing us to evaluate the function and potentially the integrity, the structural appearance and mobility of the intestine, so as to improve the therapeutic approach without resorting to exploratory celiotomy (Schumacher, *et al.*, 2000). Until recently, suspected malabsorption was confirmed upon histopathology examination of tissues, whether obtained surgically, or necroscopically, regardless of the causal dysfunction (Kemper, *et al.*, 2000).

Through ultrasonographic examination, wall-thickening lesions (> 3 mm) and changes in architectural structure of the intestine can be discerned, this is not a specific diagnostic for malabsorption, as thickening of the intestinal wall is typical not only for EPE (Schumacher, *et*

al., 2000), but also for other infiltrative/inflammatory disorders (PE in general), or even of strangulating accidents leading to edema or blood stasis (Alderton, *et al.*, 1992; Kemper, *et al.*, 2000; van den Wollenberg, *et al.*, 2011). In EPE affected foals, the jejunal and ileal walls are grossly thickened and corrugated. Typically intestinal loops have a "doughnut-like" appearance when detected transversally through ultrasonographic examination, as their thickness is often in the range of 0.5-0.8 cm, or larger, compared to the normal intestinal thickness of < 0.3 cm. Today such intestinal wall thickening and its ultrasonographic appearance are considered a pathognomonic EPE clinical sign (Schumacher, *et al.*, 2000; Sampieri, *et al.*, 2006; Frazer, 2008), yet not specific enough to justify therapy initiation on their own (Page, *et al.*, 2011a).

Thus far, the best tests to assess for suspected intestinal malabsorption mechanisms are the D-glucose test and the D-xylose test (Roberts & Hill, 1973; Roberts, 1974, Roberts & Norman, 1979; Mair, *et al.*, 1991; Brown, 1992), and in younger foals, it can be helpful to utilize the lactose tolerance test as well (Sweeney, 1987). The D-xylose test technique is typical of research settings, although both D-xylose and D-glucose tests have similar protocols, yet different analytical objectives (glucose versus xylose). The protocols use, after an overnight fasting, the oral administration of either glucose (or dextrose) at 0.5 -1 g/Kg BW in a 20% water solution, for the oral glucose absorption test (OGAT); or D-xylose at 0.5 -1 g/Kg BW in a 20% water solution, with the smaller dose suggested for ponies (Roberts & Hill, 1973, Brown 1992). Either test provides information on the intestinal ability to digest and absorb nutrients, with the monosaccharides easier to test. Samples are taken *q* 30 minutes, from time 0 to 6 h after administration (Roberts & Hill, 1973; Mair, *et al.*, 1991).

2.5.3.1. Oral glucose absorption test (OGAT).

OGAT is a valid test for small intestinal absorption capacity, hepatic glucose uptake and endocrine pancreatic function (Sweeney, 1987; Mair, *et al.*, 1991; Firshman & Valberg, 2007). In healthy horses it consists of 2 phases: the first one sees the blood glucose concentration peaking by 120 minutes after oral administration; the second one sees the return to baseline glycaemia in 4-6 h from administration (Roberts & Hill, 1973; Mair, *et al.*, 1991). A modified, faster and cheaper, version is reported with only 2 blood samples, 1 as a baseline, and 1 120 minutes later (Murphy, *et al.*, 1997). However, most reports use, or suggest, the 6 hours sampling time-frame (Mair, *et al.*, 1991; Kemper, *et al.*, 2000; Firshman & Valberg, 2007) for

better results. The results of the OGAT test define complete malabsorption if the blood glucose peak is <15% above the resting levels by 120 minutes after glucose administration; whereas partial malabsorption is defined by a peak between 15 and 85% above the resting level at 120 minutes (Roberts & Hill, 1973; Mair, *et al.*, 1991). OGAT is a sensitive, but non-specific test, as it identified correctly complete malabsorption in horses, but it was accurate in identifying the partial condition in only 72% of horses with a structural small intestinal disorder; or only in 75% of horses that suffered lymphocytic-plasmacytic enterocolitis, as per microscopic diagnosis (Roberts & Hill, 1973; Mair, *et al.*, 1991; Kemper, *et al.*, 2000). Gastric emptying, intestinal transit time and length of fasting can affect cellular uptake of glucose, as well as metabolism and endocrine function (i.e., insulin release) (Freeman, *et al.*, 1989; Mair, *et al.*, 1991).

OGAT was performed in 4 EPE affected foals (Wong, *et al.*, 2009). Three of them were tested at presentation, and then repeated OGATs were performed to follow-up their recovery. The fourth case was not tested prior to treatment, due to the profound debilitation and the critical conditions on arrival (*i.e.*, recumbency), requiring immediate initiation of total parenteral nutrition (Wong, *et al.*, 2009). As weight loss continued (40 Kg) through the first month of treatment/convalescence, the OGAT at that stage confirmed a complete condition of malabsorption. The clinical observations on those foals reported 3 out of 4 animals with complete malabsorption, as the glucose curves were flattened (normal glucose range: 4.5 -10.9 mM/L) (Bauer, *et al.*, 1984), whereas one foal showed partial malabsorption. During recovery samples were taken at different time points, but only 2 foals showed a return to normal OGAT conditions within 3 months from admission, whereas the other 2 only showed partial improvement from complete to partial malabsorption, in the same time frame. To confirm it, serum total protein (TP) and albumin (ALB) concentrations were still far below normal range (TP: 50-61 g/L; ALB: 26 – 32 g/L, in foals 1-6 month-old) for the duration of the follow-up (Lumsden, *et al.*, 1980; Wong, *et al.*, 2009). This report was the first one to assess directly the degree of impaired intestinal absorption, as previous clinical series assessed the suspicion of malabsorption based on clinical signs and blood-work abnormalities (hypoproteinemia and anemia) (Lavoie, *et al.*, 2000; Bihr, 2003; Dauvillier, *et al.*, 2006). Wong's observations (2009) clarified why the complete recovery is so prolonged in EPE affected foals and why, even after several months, the disease effects on growth rate are still compromising the weanlings/yearlings general appearance (Frazer, 2008).

2.5.3.2. D-xylose absorption test.

The D xylose test has some advantages over OGAT, because xylose is not metabolized in the small intestine, and its absorption is not affected by insulin. Identically to D-glucose, D-xylose is affected by GIT motility, length of fasting (*i.e.*, the longer the fasting, the flatter the curves) and intestinal bacterial overgrowth, but it is also affected by renal function, as it is cleared by the kidneys (Freeman, *et al.*, 1989; Brown, 1992). Normal D-xylose absorption test results should peak between 20 and 25 mg/dl at 60 and 120 minutes after dosing (Brown, 1992). Decreased D-xylose absorption has been observed in cases of extensive small intestinal resection and any cause of villous atrophy, along with the causes for low OGAT results.

2.5.3.3 Other tests

Another test to evaluate the common event of maldigestion in foals with diarrhea, or recovering from diarrhea, is **D-lactose test,** to evaluate the ability to tolerate the lactose contained in the mare's milk. D-lactose, dosed naso-gastrically at 1g/kg BW, in normal cases should lead to doubling of blood glucose level by 60 minutes after dosing and in intolerant animals it should detrimentally induce osmotic diarrhea (Roberts, 1975). However, lactose intolerance seems more typical of young nursing foals affected *by Clostridium spp., Rotaviruses* and *Coronaviruses*, rather than others infectious agents. Also, opposite to OGAT and D-xylose tests, the lactose test has not been proved to increase disorder and recovery information in EPE foals and weanlings (Wong, *et al.*, 2009).

2.5.4 Iron absorption.

For the purposes of this dissertation objectives, it is important to emphasize the small intestine's active transport mechanism in absorbing iron ions, Fe(II) and Fe(III). Mammalian organisms need iron for multiple functions, the primary being oxygen transport in blood (*i.e.*, heme and hemoglobin), but other roles are not less important, such as the catalysis reactions of multiple metallo-enzymes and cytochromes, DNA replication processes and transport proteins, needed both for generating energy and drug metabolism (Guyton & Hall, 2005a; Naigamwalla, *et al.*, 2012).

As described by many textbooks and thoroughly summarized in a recent review by Naigamwalla (2012), functional iron is chiefly found in hemoglobin (heme), and to a smaller

extent in myoglobin and cytochromes (Naigamwalla, *et al.*, 2012). Non-functional iron is largely stored in the liver, wherein iron transport proteins are produced, as **ferritin**, the primary iron storage protein, ubiquitous and soluble; and **hemosiderin**, that has a similar structure, but is insoluble, with more iron component relative to the protein one. Also the reticulo-endothelial cells of bone marrow (with the exception of cats) and spleen are storage locations as well. Mammalian cells are capable of utilizing iron red-ox functions (*i.e.*, donation or acceptance of an electron to/from the surrounding elements), in a uniquely advantageous manner for transport across membranes, as iron exists as a reduced ferrous (Fe(II)), or an oxidative ferric state (Fe(III)).

Iron is transported into cells by transferrin-mediated endocytosis, and absorbed and internalized by cells as Fe(II) (Ma, *et al.*, 2006; Chua, *et al.*, 2007; Kolachala, *et al.*, 2007). However, other absorption mechanisms likely exist, as the transport proteins (*e.g.*, **apotransferrin**) are insufficient to fully account for dietary iron absorption (Gunshin, *et al.*, 1997). The mechanism of iron absorption and homeostasis in humans has generated copious research in murine models, as dietary imbalances, intoxications, and genetic disorders are frequent and severe (Andrews, 2000; Pietrangelo, 2010). This absorption process is mediated by the **divalent metal transporter-1 (DMT1/SLC11A2)** across the apical membrane of enterocytes and it is regulated by several enzyme and protein complexes (Gunshin, *et al.*, 2005; Thompson, *et al.*, 2007). The complete role and function of the DMT1/SLC11A2 transporter is currently under investigation with radioactive iron and IHC techniques, and age differences, in absorption of non-heme iron, are becoming better defined (Thompson, *et al.*, 2007).

In monogastrics, the iron balance is dependent almost entirely on diet, as the non-heme iron is absorbed by the body solely through diet. It is absorbed in the intestinal tract with duodenum playing a primary role in it (Ma, *et al.*, 2002; Ma, *et al.*, 2006; Chua, *et al.*, 2007, Kolachala, *et al.*, 2007). Iron is then transported across the enterocytes apical membrane by **DMT-1**. According to recent publications (Harvey, 2008; Crichton, 2009), iron is then transferred across the enterocyte to the basolateral membrane through a yet unknown mechanism, but it is exported across the enterocyte's basolateral membrane by **ferroportin**, to be subsequently bound to transferrin in the plasma and then vectored in target organs and/or storage (Naigamwalla, *et al.*, 2012).

The total body iron stores are finely tuned and their regulation is strict, to provide an adequate availability of iron for cellular needs, yet preventing excess and toxicity. Excess of iron storage, in human hepatocytes, leads to escalating pathological lesions, such as hemochromatosis, cirrhosis and fibrosis (Harvey, 2008; Weiss, 2010; Naigamwalla, *et al.*, 2012). Thus, iron homeostasis is tightly controlled to limit intestinal iron uptake, through an impaired efflux from enterocytes, as the body is not provided of an efficient mechanism for excretion of iron excess. Recently, the hormone regulating iron efflux was discovered, and named **hepcidin**. When iron stores are adequate to high, hepatocytes release hepcidin, which binds intestinal ferroportin, causing its internalization and destruction. A reduced availability of ferroportin, induces the storage of absorbed dietary iron within the enterocyte, where it is lost with the enterocyte by the time this latter sheds out (in normal conditions, 3-4 days). However, if or when iron stores are low, hepcidin production and secretion are suppressed, thus allowing the ferroportin system to proceed with the facilitation of the increased iron efflux from enterocytes to the blood. In other words, whereas the down-regulation of iron absorption is complex if not impossible, despite a very limited capability of the duodenum of absorbing dietary iron; the up-regulation of iron absorption is feasible in situations of iron deficiency and depletion of body iron stores.

Two cytoplasmic homologous **iron regulatory proteins, IRP-1 and IRP-2**, are found in liver and duodenal cells, where they can be "switched on" to the uptake function by **iron responsive elements (IREs)** of ferritin and **transferrin** receptors (Schumann, *et al.*, 2004; Starzynski, *et al.*, 2004). Electrophoretic mobility shift assay (EMSA) showed IREs and IRP-1 presence in murine duodenal mucosa, and confirmed it within hepatocytes of rabbits, cattle, goats, and pigs (Schumann, *et al.*, 2004; Starzynski, *et al.*, 2004). This is important because one should not plan on restoring adequate iron homeostasis after chronic blood loss and subsequent iron deficiency only counting on up-regulation of iron absorption, because it may be inappropriate.

Iron deficiency can be the result of either inappropriate dietary intake, when intestinal absorption or feed iron content does not meet the body's requirements, or in case of a chronic non-resorptive blood loss (*e.g.,* severe ectoparasites infestation or a chronic bleeding in the lumen of the GIT). The dietary iron requirement for adult dogs and cats is estimated at 80 mg/kg

dry matter, in horses is 300 mg/day, regardless of being at rest or in exercise (note that it is higher in young animals, due to their rapid growth (Dzanis, 1994, Rooney, 2004)). Inadequate intake is rare in animals, except in nursing ones, due to the low concentration of iron in milk (300-350 mg in foals and 350-450 mg in weanlings) (Weiss, 2010; Naigamwalla, *et al.*, 2012). High iron content is found in meat products (obviously not relevant to horses feeding), but also in brewer's yeast, wheat germ, and some fruits; furthermore green-leaf vegetables, cereals, have an intermediate amount of iron, whereas, similarly to milk, most non-green vegetables contain low iron (Harvey, 2000; Naigamwalla, *et al.*, 2012).

However, little research focus specifically on iron absorption patterns of monogastrate herbivores, such as horses and rabbits. Disorders and fatalities related to iron deficiency or toxicity are reported in large animals, but the knowledge of potential interaction of dietary iron with orally administered drugs, or the extent of iron absorption during gastrointestinal disease, is limited, or extrapolated from other species, particularly murine models and findings in humans (Somers, 1947; Ruhr, *et al.*, 1983; Mullaney & Brown, 1988).

Leaving aside the diseases leading to chronic blood loss that can onset iron deficiency derived disorders, such as anemia, one should mention that such disorders don't usually start from, or during, acute blood loss, because body iron stores are generally sufficient to restore erythropoiesis activity. Nursing animals are particularly prone to developing iron deficiency anemia, not only because of the low iron content of a milk based diet, but because of lower body iron stores and larger requirements due to growth (Stockholm & Scott, 2002; Abrams-Ogg, 2010; Naigamwalla, *et al.*, 2012). Quite intuitively, surgical resection of the entire duodenum will result in iron malabsorption, and it is worth mentioning that iatrogenically induced iron deficiency anemia is possible, through excessive phlebotomies of blood donor animals, considering that a regular blood donation of 450 mL removes approximately 200 mg of iron from the body (Giger, 2005). Similarly, particular care should be taken in bleeding smaller animals, as iron deficiency limits can be reached with harvested blood volume in excess of 1% of the animal's body weight per week (Diehl, *et al.*, 2001; Giger, 2005; Naigamwalla, *et al.*, 2012). It is worth mentioning that rare genetic defects in ferroportin and hepcidin regulation have been reported to cause iron refractory iron deficiency anemia in humans, but these have not been reported in animals, aside from a single case of iron-refractory-iron-deficiency-anemia in a

cocker spaniel recently mentioned, yet unpublished, by Naigamwalla and colleagues, which was identified as a defect in the hepcidin regulator, *Tmprss6* (Naigamwalla, *et al.*, 2012).

"All animals are equal, but some animals are more equal than others."

G. Orwell

2.6 Animal models.

An elegant way to introduce the concept of using animal models for research could be a quotation by R. Virchow (1821-1902) "*Between animal and human medicine there is no dividing line — nor should there be. The object is different, but the experience obtained constitutes the basis of all medicine*" (Klauder, 1958).

Today many organizations and charity funds have formed to protect research animals, insure the quality of scientific projects, and the quality of life offered to experimental animals, ultimately determining whether using live animals in a variety of biomedical fields is necessary. In Canada the supervising role of such institutions is covered by the Canadian Council of Animal Care (CCAC). Correspondent regulatory organs are found in many Western countries. Not every country in the world adopts the same beliefs and stands by the same rules, and that is recognizable in the research allowances of studies conducted in some countries, but not in others. The role of CCAC, among others, is to bridge the needs of research, yet maintaining animal wellbeing, care and respect as a priority. To fulfill that priority, in 1989, the fundamental Policy Statement on the Ethics of Animal Investigation was approved and the Fund for Replacement of Animals in Medical Experiments promoted the use of the **"Three Rs" tenet**: replacement, reduction and refinement of animal use (Fenwick, *et al.*, 2009; Greek & Shanks, 2009; Shanks, *et al.*, 2009). The CCAC adopted the program and has been recommending it for the multiple branches of biomedical research that use animal models: from orthopaedic surgery to neuropsychiatry, to every branch of internal medicine, in subjects of various ages, gender and genetic background. More importantly, biomedical research had, and still has, the need for live animal studies in fields such as pharmacology, toxicology or infectious diseases. A reviewed

analysis of the "Three R's" tenet, and the programs to which it is applicable, has been recently published, showing the commitment to improve the practical aspects of it and the applicability of the concepts to provide ethical support to well-structured research, so as to protect animals and yet to not completely hamper researchers (Fenwick, *et al.*, 2009; Griffin, 2009). Currently, neither the reduction or replacement concepts apply to the financial aspect of research. For example, replacing an animal species with another for the purpose of veterinary experimentation is not considered a true fulfilling of the tenet, as it is perceived as driven only by economic rationales (Fenwick, *et al.*, 2009). Admittedly, although very useful in supporting the concept of research on animals, the current program needs perfecting for all branches of research (Fenwick, *et al.*, 2009).

In countries such as Canada and the USA the thoughts about biomedical research on animals divides the public understanding, regardless of engaging into such a debate with well-informed scientists or the general public. In other western countries (*e.g.*, Italy, United Kingdom and Switzerland), the drive to ban research on animals is rising frantically, in the attempt to please the masses (Drs. D. Panzani and A. Rota, School of Veterinary Medicine, University of Pisa, Italy; Dr. K.J. Smith, University of Cambridge, United Kingdom; and Dr. C.C. Schwartzwald, Universität Zürich Vetsuisse-Fakultät, personal communications).

However, sustainers of the "*One medicine: One health*" concept support the research on animal models as they see it useful to develop basic science knowledge prior to its application to clinical trials, not only in human medicine, but also in veterinary species (Sundberg & Schofield, 2009; Sundberg, *et al.*, 2009). For example, the laboratory mouse has been the species of choice for many animal models of human diseases and for basic research areas. It is due not only to the low cost of the subjects, but most importantly because of the long-standing genetic resources available for that species. Moreover, today, the development of powerful new techniques for generating mutations, analysis of complex traits, and high through-put data-gathering render mice the preeminent animal model (Schofield, *et al.*, 2004; Rosenthal & Brown, 2007). In parallel, there is the increasing demand for laboratory animal specialists and "mouse pathologists", with either veterinary or medical training, who can interpret the histopathologic changes (so-called phenotyping) that occur primarily in genetically engineered inbred strains of mice (Cardiff, *et al.*, 2008), as well as specialists that can deal with cloned animal clinical

presentations (Fecteau, *et al.*, 2005). Animal models more pertinent to this dissertation will be addressed below.

The counterpart opposing to animal research, however, seems further divided. Several associations have risen, besides the extreme violent outbursts of some anti-vivisectionists movements (*e.g.*, PETA), several national associations are active in North America to those ends (*e.g.*, National Anti-Vivisection Society etc.) (Shanks, *et al.*, 2009). However, not all the opponents operate on the mere principle of "ostracism" to animal model research. Some groups, Americans for Medical Advancement (AFMA) for example, are interested in improving the availability of therapeutics, in particular, for human diseases and they mainly refute the concept of prediction extendable from animal models to humans, with somewhat reasonable scientific arguments (Greek & Shanks, 2009; Shanks, *et al.*, 2009). They advocate the use of animal models only as "heuristic devices", rather than predictive models where findings can be extrapolated to higher species, specifically humans. Their critique is based on the alleged misinterpretation of the word "prediction", and its statistical meaning, in the scientific literature, as according to them not even animals of the same species could fulfill such a requirement, as shown by different studies in humans, and monozygotic twins in particular (Knight, *et al.*, 2006a; Bruder, *et al.*, 2008; Weiss, *et al.*, 2008). According to AFMA, animals and humans are complex systems evolved along separate lineages over time. Thus, the very mechanisms and outcomes of evolution are precisely why animals cannot be used to predict human responses. Their assertion is that, as in genetics, context of expression is everything, organisms belonging to different species, or even different strains of the same species, may show different responses to the same stimuli because of differences, with respect to: 1) the genes present and the versions (alleles) of those genes; 2) mutations in individual genes, gene expression and gene regulation; 3) proteins and protein activity and protein-protein interactions; 4) genetic networks (*e.g.*, robustness and pleiotropy); 5) environmental exposures and evolutionary histories (Knight, *et al.*, 2006; Wall & Shani, 2008; Shanks, *et al.*, 2009).

Among other referenced pharmacology or toxicology studies, the lack of risk demonstration in the use of thalidomide in pregnant women is widely used by the AFMA activists, proving that even with all the studies "after the fact" such a teratogenic disaster could not have been prevented (Runner, 1967). Approximately 10 strains of rats, 15 strains of mice, 11 breeds of rabbits, 2 breeds of dogs, 3 strains of hamsters, 8 species of primates, and in other such

varied species as cats, armadillos, guinea pigs, swine and ferrets thalidomide was tested and, teratogenic effects have been induced only occasionally (Staples & Holtkamp, 1963; Shardein, J., 1976, "*Drugs as teratogens*" cited in Shanks, *et al.*, 2009). More than one example is used in their argumentations, among others: 1) troglitazone (Rezulin™), withdrawn for 1% incidence of liver failure (unnoticed in animal models); 2) rofecoxib (Vioxx™), withdrawn because it caused < 1% incidence of heart attack, or stroke (unnoticed in animal models); and 3) an open critique to the work of Olson on multiple pharmaceutical companies toxicity controls (Olson, *et al.*, 2000; Topol, 2004; Masubuchi, 2006), to prove that the animal model testing would not have been accurate enough for society's standards. "*Medical practice*", they stated, "*does not tolerate risks that are instead acceptable for laboratory experiments*", since "*getting the answer wrong in medical practice has consequences; people die*" (*sic*) (Shanks, *et al.*, 2009).

However, the AFMA manifest's conclusions, published on the web (http://www.afma-curedisease.org) or on scientific journals (Greek & Shanks, 2009; Shanks, *et al.*, 2009), leave a disheartening feeling in a knowledgeable readership. Partially, because they are biased in addressing only a portion of the experiments currently in process (*i.e.*, excerpts wisely chosen of pharmaceutical and toxicological research, only); partially, because they are confuting the value of experiments on models. However, they don't support fully the *in vitro*, *ex vivo* and *in silico* studies either, prospecting no specific solution, but to use "*intraspecific rather than interspecific studies*", almost advocating for self-experimentation and micro-dosing experiments for toxicology studies in humans (Shanks, *et al.*, 2009).

In summary, the conclusions, or lack thereof, of the groups opposed to the use of animal models in modern biomedical science, bring us back to the fact that specific clinical problems require a deeper understanding *in vivo*. As exposing people to un-trialed experiments is ethically unacceptable, the judicious use of animal models (often on mammalians) is, to this point, the only reasonable answer, before clinical trials can be condoned, and undertaken, with a margin of safety.

2.6.1. The beginnings of the research on animal models.

The first study that can thoroughly document a certain type of "modeling" is likely the collection of observations of the cow-pox (or better, horse-pox) vaccine for human small-pox,

which Dr. Jenner carefully compiled in 1798 (Jenner, 1798). His experiments were based on clinical situations, as he was trying to understand the disease without compromising, or threatening, further human lives. Jenner observed that passing the unknown etiologic agent from horse's greasy heels (he defined it horse-pox) to cattle's udder (cow-pox) caused the typical lesions, when the horse attendants were employed in the cattle barn (e.g. in the busier times of the year) (Jenner, 1798). He described vividly his results, reporting all what he deemed important about the enrolled volunteers. Men and women alike, often desperate, choose to test the scarification technique on themselves, or their children, hoping for protection from the disease. At the time, he used no privacy statements (aside of some name omission) or informed consent to enroll "experimental" subjects. Careful annotations about probable previous exposure, and an apparent healthy physical condition were the only objective criteria in order to proceed with the scarification.

The success of small-pox vaccination is an historical milestone in medicine, and many are examples followed. Particularly the studies conducted by L. Pasteur, and his collaborators, on fowl cholera, the anthrax vaccine, and, most prominently, the rabies vaccine, opened the route to the understanding of immunology and microbiology, validating the use of animal models in research (Campbell, 1915). During his work on the bacteria responsible for the fowl cholera in chickens, Pasteur and Chamberland found that the culture used to experimentally infect chickens had spoiled. Thus it failed to induce the overt disease, although mild clinical signs and short term recovery, without fatalities, were noticed (Sternberg, 1901). Upon re-using the same healthy chickens, Pasteur discovered he could not infect them, even with fresh bacteria, as chickens developed a "sort of immunity" (Sternberg,1901; Walsh, 1913; Ullmann, 2007). Similar results were obtained in cattle treated with a "vaccine" consisting of oxygen inactivated (via potassium dichromate) bacilli, following the initial steps taken by Dr. Toussaint, a veterinarian from Toulouse. In the 1870s, Pasteur applied this immunization method to cattle, and the success aroused interest in combating other diseases (Sternberg, 1901). However, it is the rabies vaccine, initially created by Dr. Roux, Pasteur's colleague, who had the French scientist hailed as a hero. The killed vaccine was produced by desiccating the spinal cords of infected rabbits and, when Pasteur utilized successfully the vaccine on a 9-year old boy, mauled by a rabid dog, it had been tested only on 11 dogs (Walsh, 1913; Campbell, 1915). The boy survived, Pasteur's place in history became assured.

Although several types of research progressed on animal models across the world, a few dilemmas were encountered in those studies, for which either the transmission route or the pathogen itself could not be recognized, as in the case of Yellow Fever, which brought several expeditions from Europe and North America to their knees. Christopher Columbus's second expedition appears to be the 1st report; the decimation of Napoleon's contingent sent to Louisiana and Mississippi, USA, at the beginning of the 1800, is another historical example; and, at the turn of the 20th century, the majority of US Army troops sent to Cuba suffered the same fate (Pierce, 2011). While the physician "in charge" of the experimentation, MAJ (Dr.) Walter Reed, was struggling to use a guinea pig model, to reproduce the disease from mosquitoes fed onto diseased soldiers; Dr. Lazear, one of his collaborators, proceeded to infect directly a volunteer and himself. Although in Lazear's laboratory book every change observed in the "guinea pigs" was annotated, MAJ Reed understood that there were no animals involved, but rather Lazear was writing about his self-inflicted disease. In that study, started against military orders, the soldier recovered, but Dr. Lazear died, demonstrating that Yellow Fever needed several days' "extrinsic" incubation within the mosquitoes, before being contagious for men. A volunteer-based research started shortly after, utilizing for the first time: 1) an informed consent, 2) a privacy statement, and 3) a monetary retribution, as the concept of experimentation on people was already considered inappropriate and unwelcome to the public (Pierce, 2011). Fortunately, the precautions against mosquitoes derived from Reed's experiments impacted significantly both Yellow Fever's morbidity and mortality, to be praised beyond the coasts of US and Cuba (*e.g.*, Panama) within 6 months, even though *Flaviviridae* (the Yellow Fever viral agent) were not identified for another 30 years (Pierce, 2011).

This brief historical introduction describes where the western biomedical science and research community started, and today still stands, considering the use of animals for research a privilege to use with ethic deontology, respect and appropriate statistics, to maximize its value. Unfortunately there are branches of biomedical research that cannot rely on systems (*in vitro*, *in silico*, or *ex vivo*) other than *in vivo* animal modeling.

2.6.2. Rabbits and hamsters as animal models.

The volume of literature retrievable, thanks to computerized search engines, in regard to "rabbits" or "hamsters" used as animal models is enormous, with 26379 hits for rabbits animal models in general, and 2906 hits for rabbit infection animal models specifically (http://www.ncbi.nlm.nih.gov/pubmed?term=rabbit%); 9894 hits for hamster animal models in general, and 963 hits for hamster infection animal models specifically (http://www.ncbi.nlm.nih.gov/pubmed?term=hamster%).

For this dissertation's purpose, the focus will be on the above mentioned animal species used as infection models. It should not be disregarded that both species have long been used, alone or "in tandem" with other species, in every type of research, from pharmacology (Chambers ,2006; Moon, *et al.*, 2006), to cancer pathogenesis studies (Vanderhyden, *et al.*, 2003; Brandsma, 2005; Chen & Lin 2010), to, even, infant sudden unexplained death investigations (Blood-Siegfried & Shelton, 2004) and the literature cited are just recent limited examples of specific studies or reviews of standardized models. Experimental infection models have the distinct advantages of controlling: 1) the unpredictability of naturally occurring infection; 2) external non-interventional factors (*i.e.*, age, sex, feeding and housing); and 3) host characteristics which would require a large number of animals to separate the interventional effect from the natural chance in the disease/treatment (intervention) progression.

For several human diseases (*e.g.*, tuberculosis, cryptococcosis, anthrax etc.) multiple excellent infection models exist, and rabbits have been used often, depending on the infection's aspect that the model is intended to clarify (Carroll, *et al.*, 2007; Yee, *et al.*, 2010; Jassal, *et al.*, 2011). Depending on the infection, rabbits showed a good correlation between their response to treatment and the human disease outcomes (Carroll, *et al.*, 2007; Yee, *et al.*, 2010). Each model-host species confers specific advantages, and limitations, for modeling disease in another species (*e.g.*, from humans). Often, starting with a species naturally susceptible to the disease may be advantageous, for therapy research, or even more relevant for vaccine development (Carroll, *et al.*, 2007), or, as of lately, to prevent bioterrorism (Chapman, *et al.*, 2010; Yee, *et al.*, 2010), even though bioterrorism is not a novel warfare method. For example, ignoring those *Orthopoxvirus* disease's (and immunity's) details, known and studied today in *Rabbitpox* infection models (Chapman, *et al.*, 2010; Rice, *et al.*, 2011), in 1763 did not stop soldiers from decimating Native American populations, by distributing smallpox-contaminated blankets, during the French and Indian wars (Henderson, *et al.*, 1999). Thus, in the development of a new

rabbit infection model for EPE, it is important to know that rabbits are naturally susceptible to *L. intracellularis* infection, as observed since before the pathogen was defined in 1995 (Moon, *et al.*, 1974; Schoeb & Fox, 1990; Watarai, *et al.*, 2004; Horiuchi, *et al.*, 2008; Watarai, *et al.*, 2008).

On the other hand, choosing a species naturally resistant to the infection (*e.g.*, rabbits and cryptococcosis), may be needed for different reasons (*e.g.*, epidemiology), although it is undeniable that immune-suppression is mandatory (Carroll, *et al.*, 2007). Also, a model-host species may be targeted to develop a type of symptomatology (or a treatment to the disease), rather than others, although it may need previous preparation (*e.g.*, bacterial sensitization) (Jassal, *et al.*, 2011). For example, a large size animal, such as the rabbit, can be useful in meningo-encephalitis infections because the size and accessibility of the brain; whereas a mouse-model might be a better fit for a lung study, as more of its genomic needs and characteristics are known with respect to pulmonary pathophysiology (Carroll, *et al.*, 2007).

Regardless of the chosen model host, its role in the study of complex-host pathogen interactions may vary, depending on the study's objective (Basaraba, 2008). We can distinguish research approaches as "host-centric" and as "microbe-centric". The former approach focuses on the immune-response and the inflammatory contributions typical of that species when challenged with a standardized microbial load, or inoculum (Casadevall, 2005). The latter, is typically oriented at investigating the disease-causing factors related to the pathogen in a uniform model host system (Carroll, *et al.*, 2007). Such concepts may merge in the future, depending on the level of manipulation achieved, not only on model hosts (*e.g.*, the transgenic mice), but also and more specifically on the infectious agents utilized for modeling.

2.6.2.1. Rabbits.

Probably the first claim to fame for rabbits used in an infection model was their use to produce the rabies vaccine that Roux and Pasteur discovered (Walsh, 1913). Today several species of rabbits are used in a variety of infection models, depending on the specific research needs. The rabbits used in animal models are breeds belonging to the *Oryctolagus cuniculi* species, specifically the New Zealand white, the Dutch Belted, and the Old English (less commonly used today). Old English rabbits appeared to have the highest immune-response, in comparison to other breeds, (*i.e.*, increase for up to 46 days of E-type prostaglandins, total

leucocyte counts and free acid phosphatase activity) when challenged identically at the level of the knee joint, as it was reported in an older study on prostaglandins and arthritic inflammation (Blackham, *et al.*, 1974).

Rabbits have been long known to: 1) develop quickly an appropriately high immune-response, whether it is needed for the production of diagnostics based on antibodies (*e.g.*, monoclonal and polyclonal antibodies for plate coatings) (Guedes & Gebhart, 2003c); 2) construct allergy-based (asthma) laboratory models (Keir & Page, 2008); or 3) for the need to mount an immune (Breau, *et al.*, 2012; Spaulding, *et al.*, 2012) or autoimmune response (Dvorak & Waksman, 1962; Zhu, *et al.*, 2003; Wei, *et al.*, 2012).

With the focus on the infection models that have used, and still use, rabbits, a few points should be underlined, as those are the characteristics to emulate when aiming to build a new infection model. From the practical side, all rabbits (unless neonates are used) have a fairly large body weight (*i.e.*, 2 - 3 kg) and usually a fairly high body temperature (39 - 39.5C) (Bergman, 1966), with a few variations due to age and breed. A number of routes of infection are easily accessible aside of the oral and parenteral routes: retinal, and ocular in general, via carotid artery (Fujita, *et al.*, 1983); intra-cisternal (Perfect, *et al.*, 1980); intra-tracheal (Nessa, *et al.*, 1997), intra-testicular (Bergman, 1966), being utilized for those infectious agents requiring a slightly lower body temperature. Their large body size allows repeated body fluid sampling (*i.e.*, blood, but also CSF) and drug administration (Zak & Sande,1999), although they also require large doses of corticosteroids for immunosuppression (similarly to humans) (Zak & Sande, 1999), and depending on the route of infection, large inocula (Perfect, *et al.*, 1980). Thus far, their genetic information is incomplete (Zak & Sande,1999), but their purchase and maintenance costs are elevated, compared to other rodents (Bonner, *et al.*, 1976) and (http://www.criver.com/en-S/ProdServ/ByType/ResModOver/ResMod/Pages/NZWRabbit.aspx). Rabbits today are utilized commonly for ophthalmology infection studies (Altmann, *et al.*, 2011), as a disease model for humans and veterinary species (Schulz, *et al.*, 2001; Campo, 2002; Hayashi, et al., 2002; An, *et al.*, 2006; Clemons, *et al.*, 2007; Stanford, *et al.*, 2007; Mitchell & Taggart, 2009; Ramirez-Iglesias, *et al.*, 2011); for the production of vaccines (Nalca & Nichols 2011; Breau, *et al.*, 2012); and for the observation of preclinical, or basic, development of specific lesions (Basaraba, 2008), along with research in drug development, either for antimicrobial or anti-neoplastic therapy (Banin, *et al.*, 2008; Kohno, *et al.*, 2008; Zhou, *et al.*, 2011).

2.6.2.1. Hamsters

The species more commonly used is the *Mesocricetus auratus*, and the breed is the Syrian golden hamster. Hamsters usually are of small size with body weight between 100 and 150 g (depending on the breeding colony), although pet breeds can be larger (or smaller). The approved routes of drug administration and sampling in hamster are the oral, intraperitoneal, sub cutis and intravenous routes. The latter is suggested through the saphenous vein and, in anesthetized animals, the sublingual vein, the tip of the tail and the retrobulbar plexus, similarly to mouse and rat (Diehl, *et al.*, 2001). However, the use of the retrobulbar plexus is not supported any longer by the CCAC guidelines, as much as the bleeding through cardiocentesis is not advised for repeated sampling, but only for terminal blood collection, due to the incidence of fatalities (Diehl, et al., 2001).

The use of hamsters has been more limited than other rodents, dogs or minipigs. In the past, hamsters were more widely used for evaluation of carcinogenicity, in part to understand the original cause and the similarity with human adenocaricinomata (Jonas, *et al.*, 1965), but particularly because diethylstilbesterol administration to hamsters resulted in neoplasms of the *reproductive* tract (both in males and females), liver and kidney (Jacobs 2006), which rendered them a very reliable model. Today, hamsters are scarcely used for drug carcinogenicity studies, although they can be appropriate when species-specific effects arise in other rodent species, and their use is approved by Center for Drug Evaluation and Research (CDER)/FDA (Chen & Lin, 2010).

In general studies in hamster are recommended for evaluation of oral cavity pharmaceuticals, as their buccal cavity and cheek pouches are a good model for studies on non-keratinized skin (http://www.fda.gov/cder/guidelines.htm 2005; Chen & Lin, 2010). Hamsters may be used as models for effects of inhaled particles or administered intratracheally. For some drugs, hamsters have been preferred because of higher bioavailability compared to rats (>10-times greater in hamsters) (Jacobs, 2006). Model-host studies in hamsters may be useful to evaluate not only a variety of drug administrations, but also infectious disease models (Haake, 2006; Moon, *et al.*, 2006; Nambiar, *et al.*, 2006; Zuerner, *et al.*, 2011) and diabetes investigation (Morand, *et al.*, 2005). Several parasitic diseases have been successfully modeled in hamsters and gerbils (Fenoy, *et al.*, 2001), as mice are resistant to *Entoamoeba spp.* infection (Tsutsumi &

Shibayama, 2006), along with other human (Fox, *et al.*, 2009; Reuter & Schneider-Schaulies, 2010), and animal infectious diseases (Garg & Dube, 2006; Flisser, *et al.*, 2010).

In our specific case, hamster have been long reported as models for *L. intracellularis* infection, as they are naturally and experimentally susceptible to the PE agent, usually known as "wet tail" (Jacoby, *et al.*, 1975; Amend, *et al.*, 1976; Frisk & Wagner, 1977; Jacoby, 1978; Cooper, *et al.*, 1997), with typical lesions of the ileum, from mild to severe and hyperplastic, and explains why the almost neoplastic appearance was initially confused with adenocarcinomatosis and compared to the human ileal neoplasia (Jonas, *et al.*, 1965; Boothe & Cheville, 1967).

2.6.3. Animal models for *Lawsonia intracellularis*

Although *L. intracellularis* is an obligate intracellular bacterium, it is apparently not host-restricted and it is able to produce infection in animals different than the species of origin, as it was detected in multiple species: horse, hamster, deer, dog, fox, rabbit, macaque, ostrich, emus and a giraffe (Drolet, *et al.*, 1996; Hotchkiss, *et al.*, 1996; Cooper, *et al.*, 1997; Lemarchand, *et al.*, 1997; Klein, *et al.*, 1999*;* Herbst, *et al.*, 2003; Pusterla, *et al.*, 2008b).

The first trials in reproducing the disease in pigs were published in 1977, with ileal mucosa scrapings from diseased subjects, as the etiological agent was still unclear and it could not be isolated on bacteriology plates (Roberts, *et al.*, 1977). In the following years a few pathogenesis characteristics were understood through *in vivo* modeling, as the disease failed to reproduce successfully in pathogen free piglets challenged with pure cell culture, although the reproduction was successful in gnotobiotic pigs, with both ileal scrapings and pure cell culture (McCartney, *et al.*, 1984; McOrist & Lawson, 1989; McOrist, *et al.*, 1993). Yet again a full understanding of the bacterium characteristic remained out of grasp for a few more years (Gebhart, *et al.*, 1991; Gebhart *et al.*, 1993; Lawson & McOrist, 1993; McOrist, *et al.*, 1995a). The bacterium was revealed to be very complicated to maintain *in vitro* and absolutely unable to grow on traditional bacteriology plates (Lawson, *et al.*, 1993). Since its classification was determined, multiple studies were conducted for *L. intracellularis in vivo* (McOrist, *et al.*, 1994a; Guedes & Gebhart, 2003b; Guedes, *et al.*, 2003; Jordan, *et al.*, 2004), to determine the impact of pure cell culture-induced disease versus mucosal-scrapings-induced disease, along with the

presence, or absence, of a normal bacterial flora; the impact of the inoculum dose on infection; or the effect of a seeder sentinel pig in a herd. Also, research was conducted on *ex vivo* ileal loops explanted from pigs (Collins, *et al.*, 2000; McOrist, *et al.*, 2006; Boutrup, *et al.*, 2010a; Boutrup, *et al.*, 2010b), contributing to a better understanding of its behavior, both for the requirements of diagnostics and vaccine development. Lately, studies investigating the level of naturally-acquired immunity were performed, serologically testing or re-challenging pigs exposed to infection in the past (Guedes, *et al.*, 2002; Collins & Love, 2007).

For epidemiology interest, the *L. intracellularis* infection has been reproduced in mice starting with isolates obtained from pigs and rabbits, although lesions are not detectable for long, so the species cannot be employed as an *in vivo* model (Murakata, *et al.*, 2008). Similar results have been achieved in rats and chickens (Collins, *et al.*, 1999).

Among rodents, probably the best cross-infection model for pigs *in vivo* is represented by the hamster, as its disease was noted early in the 1960's (see above) and connected to the porcine clinical disease in the early research years (1970-1980s) (Frisk & Wagner, 1977; Johnson & Jacoby, 1978; Lawson, *et al.*, 1985; McOrist & Lawson, 1987; McOrist, *et al.*, 1989). The first successful hamster species-specific model was reported around 1991 (also including the study on a cultured *Chlamydia spp*. by mistake) (Stills, *et al.*, 1987; Stills & Hook, 1989; Stills, 1991; Stills, *et al.*, 1991). From the porcine strain, a cross infection model was obtained, only a few years later, with a reduced symptomatology if it was caused by pure cell culture rather than mucosal scrapings (Jasni, *et al.*, 1994a; Jasni, *et al.*, 1994b). To this day, the only hamster models that produce severe clinical signs are the ones obtained with ileal mucosal scrapings, wherein the small intestine absorption and histo-morphometry are markedly abnormal (Vannucci, *et al.*, 2010).

Among rabbits and hares, the disease was found naturally (see above) or their fecal shedding was detected in horse farm surroundings (Pusterla, *et al.*, 2008b; Pusterla, *et al.*, 2012a), but no rabbit has ever been used to reproduce the disease, either from other species or from species-specific infectious material (Duhamel, *et al.*, 1998).

In horses the only autologous model that has been published is the one by Pusterla and collaborators in 2010 (Pusterla, *et al.*, 2010a), and reproduced by the group at University of

Kentucky (Page, *et al.*, 2011c). To this day, such a model is the only one available for EPE and may have serious clinical manifestations that render it risky for the animals (C. Gebhart, University of Minnesota, personal communication). It is currently utilized for the investigations in process about developing a vaccine for EPE at University of California-Davis (See chapter 2.3).

With the intention of bearing in mind every possible alternative, before embarking on a new animal model validation, consideration was given to the *ex vivo* "Ussing chambers" already used for studies on equine intestinal tissues permeability and drug absorption (Davis, *et al.*, 2006). However, with the difficulties in maintaining the bacteria alive in the tissue harvested (Lawson, *et al.*, 1993), the risk is that the model may lack validity before starting the tests on drug absorption, as bacteria may die regardless of treatment.

CHAPTER 3

OBJECTIVE AND HYPOTHESES

"Is the nature of an hypothesis, when once a man has conceived it, that it assimilates everything to itself, as proper nourishment"

L. Sterne

OBJECTIVE: To demonstrate that gallium maltolate is an appropriate metal based antimicrobial therapeutic to treat Equine Proliferative Enteropathy (EPE) in a laboratory animal infection model.

3.1 Animal Model Validation – Hypotheses (H)

Rationale: *L. intracellularis* is the obligate intra-cytoplasmic agent of EPE, Porcine Proliferative Enteropathy (PPE) complex and enteropathies in various animal species. Its replication and maintenance *in vitro* are easily challenged by minimal variations in oxygen tension and carbon dioxide concentration, and the necessity of inducing disease in animal models has been demonstrated since the initial phases of its investigation. *L. intracellularis* has a 98% genomic similarity (16s rDNA sequencing) between species' isolates (horse, pig, hamster and deer). Infection has been transmitted experimentally from pigs to hamsters since 1991 and it is suspected to cross infect to horses.

3.1.1 - H1) Cross-infection of juvenile rabbits, with a pure cell culture of equine strain of *L. intracellularis*, will lead to mild to moderate clinical signs of disease and intestinal lesions, together with fecal shedding of bacteria and serologic immune-response, detectable by gold standard diagnostics (*i.e.*, immuno-histochemistry on tissues, quantitative PCR on feces and immuno-peroxidase monolayer assay on serum).

3.1.2 - H2) Cross-infection of weanling hamsters, with a pure cell culture of equine strain of *L. intracellularis*, will lead - similarly to what is reported in hamsters infected with PPE - to mild clinical signs, but typical intestinal lesions, together with fecal shedding of bacterial DNA and serologic immune response.

3.1.3 - H3) Cross-infection of juvenile rabbits, with a pure cell culture of PPE *L. intracellularis* will lead to mild to moderate clinical signs of disease, typical intestinal lesions - as the ones published in PPE-infected hamsters – together with fecal shedding and serologic immune-response.

3.2 Therapeutic hypotheses

Rationale: As a novel antimicrobial candidate, gallium maltolate (GaM), is the core of our research projects. It is claimed to: be "highly" bioavailable; easily administered *per os;* able to release elemental gallium (Ga(III)) for absorption within cells. The novelty of this idea rests with the iron-mimicry abilities of Ga(III) compared to ferric iron (Fe(III)), as Ga(III) has the same chemical radius and "footprint" of Fe(III), although incapable of undergoing a red-ox reaction. This phenomenon, known as "Trojan horse mechanism", is selectively safe for mammalian versus protozoan cells, as bacteria preferentially uptake iron as Fe(III) (ferric iron), whereas mammalian cells prefer Fe(II) (ferrous iron). Due to the incapability of red-ox reacting with the bacterial organelles, gallium maltolate treatment should lead to the destruction of *L. intracellularis*, or to the inhibition of its replication, hence accomplishing its antimicrobial goal.

3.2.1. - H1) After oral administration Ga(III) will be detected both in healthy and EPE-infected rabbits intestinal tissues through hard x-ray fluorescence (VESPERS beamline at the Canadian Light Source), demonstrating its presence within the target tissues.

3.2.2. – H2) After oral administration in healthy rabbits, Ga(III) will be detectable in serum, for pharmacokinetic evaluation, and within intestinal tissues both at 24 h and at the completion of a full pharmacokinetic study, lasting at least 3 estimated half-lives.

3.2.3. - H3) After oral administration in EPE-infected rabbits, elemental gallium will be detectable in serum, for pharmacokinetic evaluation, and within intestinal tissues at 24 h and at completion of the full pharmacokinetic study, lasting at least 3 estimated half-lives.

3.2.4. - H4) The comparison between the healthy and EPE-infected rabbits will show a marked reduction in concentration of gallium and iron, both in blood and in intestinal tissues of the latter group, due to absorption difficulties of infected animals.

3.2.5. - H5) The concentration of Ga(III) in serum and tissue in infected hamsters will be sufficient to exert antimicrobial activity, which is hypothesized to be superior to current clinical therapeutic treatments for EPE.

CHAPTER 4

THE RABBIT AS AN INFECTION MODEL FOR EQUINE PROLIFERATIVE ENTEROPATHY.*

Relationship of this study to the dissertation objectives.

In this chapter, the results of a preliminary study on a rabbit infection model for EPE are reported. Rabbits had never been utilized as a modeling-host for *Lawsonia intracellularis* infection, although they are naturally susceptible to the infection. The development of the model was a necessary step-stone in this entire research project. Thus, prior to investigation of the pharmacokinetics of gallium maltolate in this lagomorph species, the infection model was tested after inoculation with EPE bacteria cultured *in vitro*. The success of this modeling experiment was of fundamental importance for the comparison between the pharmacokinetics of the compound of interest in healthy and EPE-diseased animals.

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* *This material is to be published as:* Sampieri, F., Allen, A.L., Pusterla, N., Vannucci, F.A., Antonopoulos, A.J., Ball, K.R., Thompson, J., Dowling, P.M., Hamilton, D.L. & Gebhart C.J. (2013). The rabbit as an infection model for equine proliferative enteropathy. *Canadian Journal of Veterinary Research* 77: 000-000. *In press.*

4.1. Abstract

This study demonstrates the susceptibility of rabbits to Lawsonia intracellularis obtained from a clinical equine proliferative enteropathy (EPE) case, and thus provides a potential animal model for investigations on pathogenesis and therapy of this disease in horses. Nine does were equally assigned to 3 groups. Animals in 2 groups (Group 1 and Group 2) were orally inoculated with different doses of cell-cultured *L. intracellularis*. Controls (Group 3) were sham inoculated. Feces and blood were collected prior to infection and at 7, 14, 21 days post infection (DPI). Serum IgG titers were measured using an immuno-peroxidase monolayer assay (IPMA) and fecal samples were analyzed with quantitative PCR (qPCR). A doe from each group was euthanized at 7, 14, 21 DPI for collection and evaluation of intestinal samples. Tissues were stained by routine H&E and immunohistochemistry (IHC) with L. intracellularis-specific mouse monoclonal antibody. Serologic responses were detected beginning at 14 DPI in both infected groups, which maintained high titers through 21 DPI. On 7 DPI, L. intracellularis DNA was detected in the feces of Group 2 and on 14 DPI was detected in both infected groups. Gross lesions were apparent in Group 1 and Group 2 on 14 DPI. IHC confirmed L. intracellularis antigen within cells of Group 1 and Group 2 rabbits on 7, 14, 21 DPI. No lesions, serologic response, shedding or IHC labeling were found in Group 3 rabbits. This study describes an EPE rabbit model that simulates natural infection, as typical lesions, immune response, and fecal shedding were present.

4.2. Introduction

Lawsonia intracellularis is a Gram-negative, obligate intracellular bacterium affecting a wide range of domestic, wildlife, avian and laboratory animal species (McOrist, *et al.*, 1995a; Hotchkiss, *et al.*, 1996; Cooper, *et al.*, 1997b; Lawson & Gebhart, 2000; Pusterla, *et al.*, 2008b). Principally, *L. intracellularis* is known as the etiologic agent of porcine proliferative enteropathy (PPE) and equine proliferative enteropathy (EPE), with approximately 98% 16S rDNA gene similarities reported between strains (Cooper, *et al.*, 1997a; Lawson & Gebhart, 2000; Pusterla & Gebhart, 2009). Associated with *L. intracellularis* intra-cytoplasmic replication is the patent proliferation induced in the host's enterocytes of either the small or large intestine, which

reduces nutrient absorption, resulting in malabsorption, diarrhea, depression, weight loss, abdominal pain, and even death (Wong, *et al.*, 2009; Vannucci *et al.*, 2010). Typically, EPE affects the enterocytes of the distal portion of the jejunum and ileum of weanling foals. Several individual, isolated cases of EPE were reported before 1999 (Duhamel & Wheeldon, 1982; Williams, *et al.*, 1996; Brees, *et al.*, 1999; Schumacher, *et al.*, 2000). The first large outbreaks in foals occurred in Quebec and Ontario, Canada, in 1999, and since then clinical EPE has acquired growing worldwide importance (Lavoie, *et al.*, 2000; Frazer, 2008; Pusterla & Gebhart, 2009).

L. intracellularis is a challenging organism to isolate and maintain *in vitro*, as its growth requires an intracellular environment and a specific microaerophilic atmosphere (Lawson, *et al.*, 1993). Thus, multiple studies about *L. intracellularis* susceptibility in pigs targeted not only bacterial culture *in vitro*, but also experimental reproduction of PPE in animal models. Hamsters are the main laboratory species that can be naturally affected by *L. intracellularis*, and the disease in this species is commonly and non-specifically addressed as "wet tail" (Lawson & Gebhart, 2000; Vannucci, *et al.*, 2010). Hamsters were the first model species in which information about lesion progression was experimentally gained, as disease was induced through laborious filtration of porcine infected ileal tissue scrapings (Jasni, *et al.*, 1994b). Since the early 1990's hamsters, more than mice, have been considered a potentially advantageous model for PPE because they afforded lower cost and larger statistical power of experiments (Stills, *et al.*, 1987; Stills & Hook, 1989; Stills, 1991; Stills, *et al.*, 1991; Murakata, *et al.*, 2008). Reproduction of the disease in laboratory species using intestinal mucosa homogenate or pure cell cultures has been traditionally limited to pigs or hamsters, either infected (from strains collected from animals of the same species) or cross-infected (from strains collected from different animal species) (Stills, 1991; Boutrup, *et al.*, 2010a; Boutrup, *et al.*, 2010b). Thus, although a few investigators have successfully produced typical lesions of PE in mice, hamsters continue to provide a reliable infection model to investigate PPE using porcine ileal scraping homogenates as inocula (Jasni, *et al.*, 1994a; Murakata, *et al.*, 2008; Vannucci, et al., 2010).

In foals, EPE has only been experimentally reproduced after oral inoculation of an equine strain of *L. intracellularis*, originating from the intestine of an affected foal that succumbed to EPE (Pusterla, *et al.*, 2010a). However, there are no published reports of EPE reproduced in a different species and the question arises about the susceptibility of other animals to the equine *L.*

intracellularis strain. Two preliminary infection trials in hamsters with a virulent pure cell culture equine *L. intracellularis* strain did not yield EPE lesions. However, classical microscopic lesions were clearly observed in positive control hamsters infected with a pure cell culture porcine *L. intracellularis* strain (Chapter 5).

Interestingly, natural *L. intracellularis* infection is also recognized in rabbits, although less frequently than in hamsters. Clinical signs are similar to the ones noted in other species, although the intestinal lesions are also commonly found in the cecum and large colon (Schoeb & Fox 1990; Horiuchi, *et al.*, 2008; Murakata, *et al.*, 2008; Watarai, *et al.*, 2008). Furthermore, fecal shedding has been observed through quantitative polymerase chain reaction (qPCR) of fecal material recovered from wild rabbits which were populating the grounds of equine stud farms, wherein EPE was endemic (Pusterla, *et al.*, 2008b).

This present study aimed to reproduce EPE lesions in rabbits due to the anatomical and physiological similarities of their gastrointestinal systems to the horse (Harcourt-Brown, 2002a). This is the first report of experimental infection in rabbits with a pure cell culture of *L. intracellularis* derived from an EPE clinical case, and it represents a valuable preliminary step in developing a rabbit infection model for studying EPE in horses.

4.3. Materials and Methods

4.3.1 Animals

Nine, 7- to 8-wk-old female New Zealand white rabbits (Charles River Canada, Pointe Claire, QC, Canada) were used (spp. *Oryctolagus cuniculus,* strain -052). They originated from colonies tested, at regular intervals, for absence (VAF) of *Reovirus, Rotavirus, L. intracellularis, Helicobacter spp., CAR Bacillus, Salmonella spp., Pasteurella spp., P. aeruginosa, Treponema Helmints spp., C. piliforme, Eimeria spp.* amongst other viral, bacterial and parasitic diseases. Upon arrival to the Animal Care Unit facilities at the University of Saskatchewan's Western College of Veterinary Medicine, the 9 does were mandatorily acclimated to the room facilities for 1 week. At the beginning of the experimental trial they were weighed (range: 1.8 to 2.3 kg), examined and tattooed on the right ear after local

anesthetic treatment (EMLA Cream, Astra Zeneca Canada Inc., Missisauga, ON, Canada). Rabbits were randomly assigned to 3 groups of 3 animals each (Group 1, Group 2 and Group 3), group-housed in isolated pens in a Containment Level 2 Room, fed rabbit pellets (Co-op Whole Earth* Rabbit Ration, Federated Co-op. Ltd. Saskatoon, SK, Canada) *ad libitum* and maintained using standard husbandry conditions (12/12 h light – dark cycle, and 20 ± 2^{o}C room temperature). All groups were housed, within the same room, but managed separately, with particular care to assess Group 3 (uninfected controls) first. Animals were bedded on ventilated shavings. The base of the pen was separated and isolated from other pens by PVC compartments (40-50 cm tall), whereas a stainless steel mesh was used at the top, impeding direct contact between rabbits of adjacent pens' and the mixing of bedding. Cleaning and disinfection procedures were standardized for the facilities and a peroxide-based disinfectant (Peroxigard, Bayer Healthcare, Animal Health Division, Bayer Inc., Toronto, ON Canada) was used. This study was approved by the Animal Research Ethics Board of the University of Saskatchewan and conducted according to Canadian Council on Animal Care (CCAC) guidelines.

4.3.2. Inoculum preparation

L. intracellularis inocula were prepared as previously described (Pusterla, *et al.*, 2010a). The infectious material was harvested during necropsy of a foal diagnosed with EPE, wherein clinical signs were confirmed by histopathology and IHC findings. Subsequently, the *L. intracellularis* harvested from the ileal mucosa were cultured on McCoy cells (ATCC CRL 1696), as previously described (Lawson, *et al.*, 1993; Guedes & Gebhart, 2003b). Two different doses of inocula were prepared and each was suspended in 3 mL of buffered sucrose/phosphate/glutamate (SPG) medium, to be administered to Group 1 and Group 2 rabbits (Guedes, et al., 2003). Sham treatment (SPG medium only) was administered to Group 3 (controls). A SYBR Green-based PCR quantitation assay was used to determine the bacterial concentration of the inocula. This assay targets the aspartate ammonia lyase gene (*aspA* gene), which is represented just once on the *L. intracellularis* chromosome, so that each *aspA* gene copy corresponds to one organism (Wattanaphansak, *et al.*, 2010). Since a lower culture passage appears related to higher *L. intracellularis* virulence, passage cultures lower than 20 (passages 9

and 10, for Group 1 and Group 2, respectively) were used to infect the rabbits, but with different doses, to potentially compensate for the difference in passage. Thus, Group 1 received a dose equivalent to 1.3×10^8 bacteria/rabbit; and Group 2 received a dose equivalent to 2.5×10^8 bacteria/rabbit. These infecting doses, obtained from the same equine infected isolate previously utilized in the foal model, are approximately 150-200 times smaller than the doses used to infect foals; and 6-10 times larger than the dose used to infect hamsters (Pusterla, *et al.*, 2010a; Chapter 5).

4.3.3. Inoculation procedures

Rabbit inoculation with *L. intracellularis* was performed on the first day after the acclimation period (a week from arrival). Prior to the infection, pooled fecal samples were collected from each separate pen, each rabbit was weighed and its general health was assessed, and a blood sample was collected from the left ear's central artery, which was previously prepped with a local anesthetic cream (EMLA Cream, Astra Zeneca Canada Inc., Missisauga, ON, Canada). A local lidocaine gel block (Xylocaine Gel 2%, Astra Zeneca Canada Inc., Missisauga, ON, Canada) was applied inside the right nostril; and a 5 Fr. wide, 40 cm long feeding tube (Kendall Sovereign, Tyco Health Care Group LP, Mansfield, MA, USA) was inserted via the nose into the stomach. Each rabbit received naso-gastrically either a 3 mL *L. intracellularis* inoculum or a 3 mL SPG medium-only treatment.

4.3.4. Collection of samples

Body weight changes were monitored daily throughout the study (21 days). Demeanor, gross appearance, fecal consistency and quality, appetite, and self and mutual grooming were monitored twice daily with a 4 step grading system, a score of 0 corresponding to normal and 3 to severely abnormal findings. All rabbits readily adapted to handling and their interest could be easily stimulated with carrots and apples (treats). Treats were offered twice daily to check appetite, as in the group-housing setting it is difficult to assess each individual's food intake. Lack of interest in treats was considered a sign of decreased well-being (Johnson-Delaney, 2006).

Blood samples for serology were collected from the central artery of the left or right ear of each rabbit once weekly and at the time of euthanasia. Also, pooled fecal samples were collected from each separate pen once weekly, and individual fecal samples were collected at the time of euthanasia. One doe per week per group was randomly selected and humanely euthanized with an intravenous overdose (720 mg/rabbit) of pentobarbital (Euthanyl, Bimeda MTC, Animal Health Inc., Cambridge, ON, Canada). Necropsies were performed to observe evidence of gross lesions and to collect multiple intestinal tract samples (1.25 to 2.5 cm long) for microscopic examination. Samples were immediately placed in phosphate buffered 10% formalin solution.

4.3.5. Sample analysis

4.3.5.1. Macroscopic examination.

The abdominal cavity was opened shortly after euthanasia, the mesentery and mesocolon were dissected, and the organs were examined visually, from stomach to rectum. Changes in thickness, discoloration or content of the intestinal tract were observed. The sample of duodenum was collected 2.5 cm distal to the pylorus; one jejunal sample was collected at an approximate mid-distance between the end of duodenum and the beginning of ileum, whereas the terminal jejunal sample was collected proximal to the last jejunal Peyer's patch. The ileal sample was collected proximal and adjacent to the ileal Peyer's patch, approximately 5 cm proximal to the ileocecal valve. The ileocecal valve was also collected in its entirety, including the *ampulla coli* and *sacculus rotundus*. The sample of cecum was collected in the transitional area between cecum and *appendix ceci*, and the terminal portion of the *appendix ceci* was also collected. The sample of large colon was collected adjacent and distal to the cecum, whereas the sample of terminal colon was collected at a mid-distance between the end of the large colon and the rectum.

4.3.5.2. Histology and immunohistochemistry.

Two adjacent formalin-fixed paraffin-embedded sections per sample were cut and stained by haematoxylin-eosin method (H&E) and streptavidin method, using anti-*L. intracellularis*-specific murine monoclonal antibody (IHC), to observe for typical proliferative lesions of the intestinal epithelium and for the presence of the antigen within the cells (Guedes & Gebhart,

2003b; Guedes & Gebhart 2003c). The *L. intracellularis*-specific antigen in the enterocytes was blindly evaluated with a 5 grade IHC scoring system, as follows: grade 0, for no antigen labeling in the tissue; grade 1, for up to 25% of the crypts labeled; grade 2, for 26 - 50% crypts labeled; grade 3, for 51-75% crypts labeled; and grade 4, for 76-100% crypts labeled (Guedes & Gebhart, 2003b). For each rabbit, the negative control for each tissue section consisted of a correspondent tissue section IHC-labeled, except for the primary antibody. Furthermore, pig ileal tissues, known to be negative and positive for *L. intracellularis* infection, were labeled with the murine anti-*L. intracellularis* monoclonal antibody to confirm the antibody's specificity and sensitivity, respectively.

4.3.5.3. Serology analysis.

In serum, anti–*L. intracellularis*–specific IgG concentration was measured by an immuno peroxidase monolayer assay (IPMA), as reported previously (Guedes, *et al.*, 2002c). Positive serum samples were end-point titrated starting with a dilution of 1:30 up to 1:1920. Control samples consisted of serum from a rabbit prior to (negative control) and after (positive control) hyperimmunization with *L. intracellularis*, purified from cell culture. Also, serum total protein concentration was measured with the refractometer method to investigate if changes in serum total protein concentration were comparable to the values reported in foals naturally and experimentally infected with EPE (Pusterla, *et al.*, 2010a).

4.3.5.4. Quantitative PCR Analysis.

Quantitative PCR (qPCR) analysis was conducted on fecal samples, as previously reported (Pusterla, *et al.*, 2010a). The purified DNA was analyzed by qPCR for presence of *L. intracellularis aspA* gene copies (Pusterla, *et al.*, 2008b; Pusterla, et al., 2009a). For each target gene, two primers and an internal, fluorescent labeled TaqMan probe (5´end, reporter dye FAM (6-carboxyflourescein), 3´end, quencher dye TAMRA (6-carboxytetramethylrhodamine)) was designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) **(Table 1)**. TaqMan PCR systems were validated using 2-fold dilutions of gDNA testing positive for the target genes. Dilutions were analyzed in triplicate and a standard curve was plotted against the dilutions. The slope of the standard curve was used to calculate amplification efficiencies using the formula $E = 10^{1/-s}-1$. Each system needed to be greater than 95% efficiency to be considered. The detection limit for "L.intra system" is between 5-10 copies/µl DNA (determined with plasmid DNA). The "L.intra system" detects all *L. intracellularis*, regardless of the host

species. Known positive controls and no template controls were run on every plate, and met previously standardized values. DNA quality was determined by the "PanBacteria system", with a control value under 30, to pass quality control (Leutenegger, *et al.*, 1999).

Table 4.1 PCR analysis details

System	Forward primer	Reverse primer	Probe	target	Accession number	Efficiency
L.intra	bcL.intra-114f CACTTGCAAA CAATAAACTT GGTCTTC	bcL.intra-263r CATTCATATTTGT ACTTGTCCCTGCA	bcL.intra-201p TCCTTGATCAATTTGT TGTGGATTGTATTCAA GG	Aspartate ammonia lyase	EU127293. 1	95.6%
Pan-bacteria	PB.283f GGATGATCA GCCACACTGG A	PB.352r CCAATATTCCTCA CTGCTGCC	PB.305 CCCGTAGGAGTCTGG ACCGTGTCTCA	16S rRNA	Multi sequence alignment	98.2%

Table 4.1: Specific forward and reverse primers sequences, along with probe, target gene and accession numbers for the technique of PCR utilized in the diagnosis of *L. intracellularis* fecal shedding in rabbits, after infectious challenge with pure cell bacteria. In the last column of the table, note the rate of efficiency.

4.3.5.5. RT-reaction and real-time TaqMan PCR.

Each PCR reaction contained 20x primer and probes for the respective TaqMan system with a final concentration of 400 nM for each primer and 80 nM for the TaqMan probe and commercially available PCR Mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems, Foster City, CA, USA), containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction; 0.25 U AmpErase UNG per reaction; and 1 μl of the DNA sample in a final volume of 12 μl. The samples were placed in 384 well plates and amplified in an automated fluorometer (ABI PRISM 7900 HTA FAST, ABI). ABI's standard amplification conditions were used: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Fluorescent signals were collected during the annealing temperature and CT values extracted with a threshold of 0.1 and baseline values of 3-12 for all samples using SDS 2.2.1 (Applied Biosystems, Foster City, CA, USA). Absolute quantitation was calculated by a standard curve and expressed as copy numbers of the *L. intracellularis aspA* gene per gram of feces.

4.3.6. Statistics

In general, only descriptive statistics were used in this study, as sample numbers were insufficient for statistical analysis. However, bodyweight and serum total protein concentration were analyzed with a Kruskal-Wallis non-parametric test and Dunn post-hoc testing for multiple comparisons between groups. Commercial software (GraphPad Prism 5.4 Software, CA, USA) was used, with p–value set at ≤ 0.05.

4.4. Results

4.4.1. Clinical signs.

During the entire duration of this study, no evidence of diarrhea, overt signs of pain (*e.g.*, bruxism), or prostration were detected in the rabbits. Minor details of incomplete grooming around the tail region were noted in rabbits of all groups, but these were observed sporadically

and deemed not relevant. No remarkable changes were noted in any group during the first 7 DPI, aside from a transient (1 day's duration only) weight loss, or a weight gain smaller than half the individual's average daily gain. This weight loss affected control and infected rabbits alike on 1 DPI and was ascribed to the stressful procedures on the day of infection (day 0). As the rabbits were still growing, their bodyweight gain should have ranged between 100 to 200 g/week (or 14.2 to 28.5 g/day), as per the provider's indications (Charles River Canada, Pointe Claire, QC, Canada). All rabbits' averaged daily weight gains were within limits, with 2 rabbits well above that range (52 g and 49.1 g for Group 1 and Group 2, respectively). Negative weight changes were not statistically significant (p–value = 0.87) in any of the groups throughout the 21 DPI (**Figure 1**). However, between 12 to 17 DPI in Group 1 and Group 2, infected rabbits' weight losses, or gains smaller than half the individual's average daily gain, were noted. The only animal that showed depression and dullness between 12 (rated 1) and 14 (rated 2) DPI was the Group 1 doe euthanized at 14 DPI, which showed marked progressive weight loss and lack of appetite. Additionally, although general self-grooming appeared normal, the normal residues of cecotrophes on the perineum of this doe were not cleaned (abnormal behavior).

Figure 4.1.

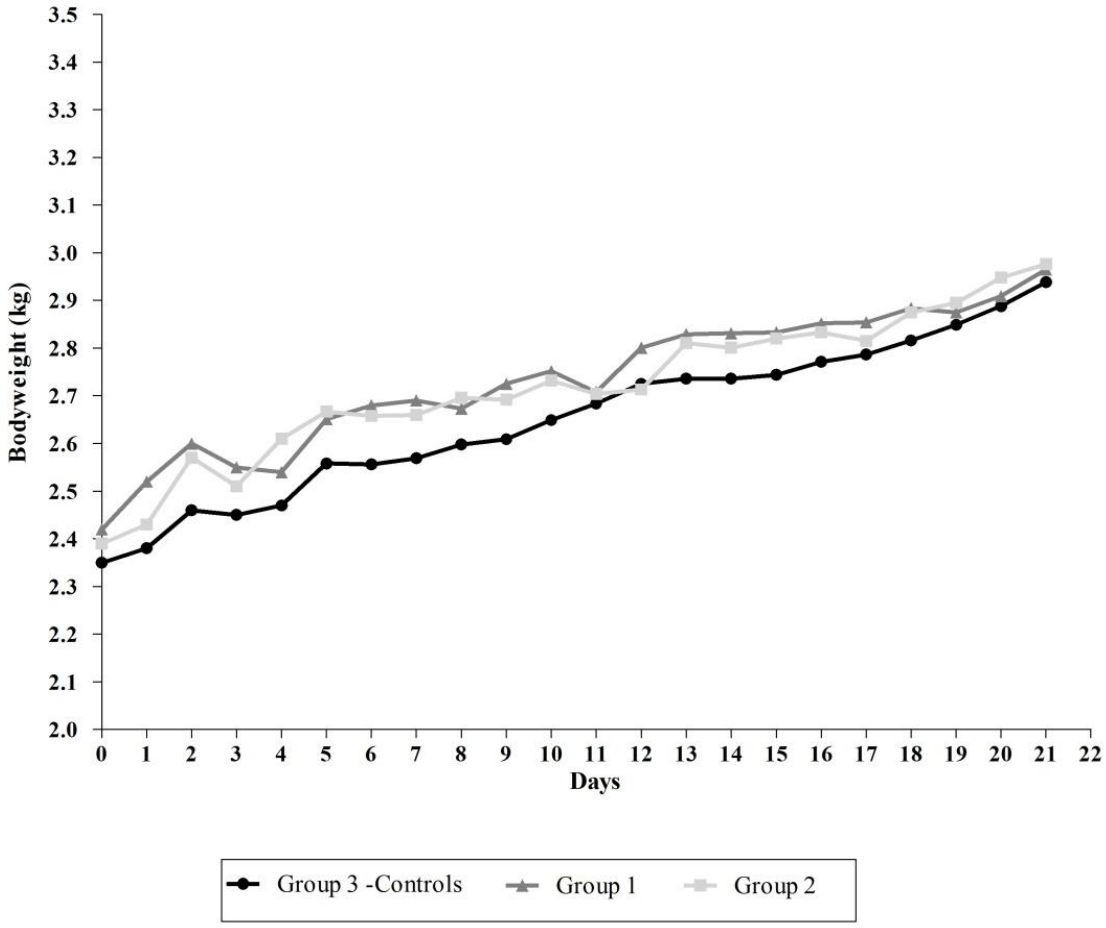


Figure 4.1: Bodyweight (in Kg) changes in Rabbits infected with *L. intracellularis.*

Graphical representation of the weight gains during the whole duration of the experiment (21 DPI). In the 2 infected groups a trend to reduced weight gain and marginal weight loss can be noted, although these were statistically not different than the control weight gains (p=0.87).

4.4.2. Necropsy findings.

Gross lesions were not evident in any of the rabbits examined on 7 DPI, but on 14 DPI, Group 1 and Group 2 infected rabbits showed hyperemia and edematous corrugation of the serosal surface of the jejunum (such as a "cobblestone-like" appearance) (**Figure 2a**) compared to controls (**Figures 2b and 2d**). Additionally, typical PE lesions were observed in the mucosal surface (**Figure 2c**). The ileum showed a similar "cobblestone-like" appearance, particularly close to the ileocecal valve. These gross changes were consistent with proliferative enteropathy lesions, as described in rabbits, pigs and horses (Lavoie, *et al.*, 2000; Lawson & Gebhart, 2000; Horiuchi, *et al.*, 2008). The cecum of the Group 1 infected rabbit appeared generally hyperemic once opened, and it was deemed non-specific for EPE. Hyperemia was not noticeable in the cecum of Group 2 or Group 3 does.

By 14 DPI, the Group 1 rabbit that was euthanized had lost over 200g in 3 days. In contrast, the rabbits in Group 2 and Group 3 gained marginal or no weight but did not lose weight. Also, the Group 1 rabbit's terminal colon and rectum were mostly empty, although cecum and colon content did not differ from the control doe.

By 21 DPI, only mild, non-specific changes were noted on gross pathology at necropsy. These changes included hyperemia and mild wall thickening (edema) of the serosal surface of the jejunum of both Group 1 and Group 2 rabbits, and were more pronounced in Group 1.

Figure 4.2.1.

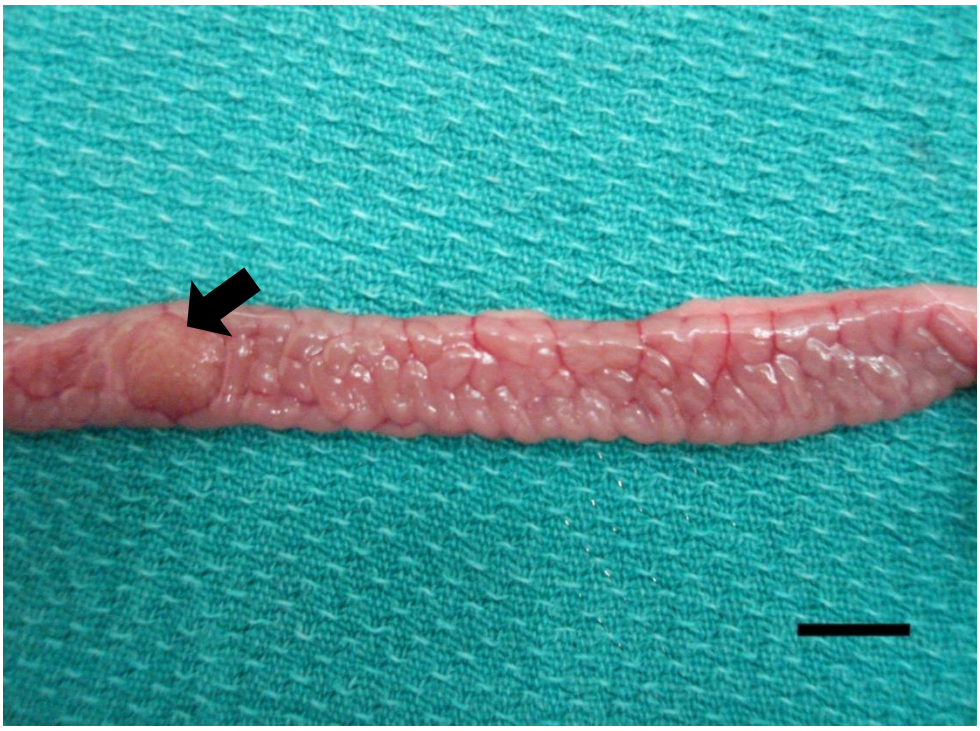


Figure 4.2.2.

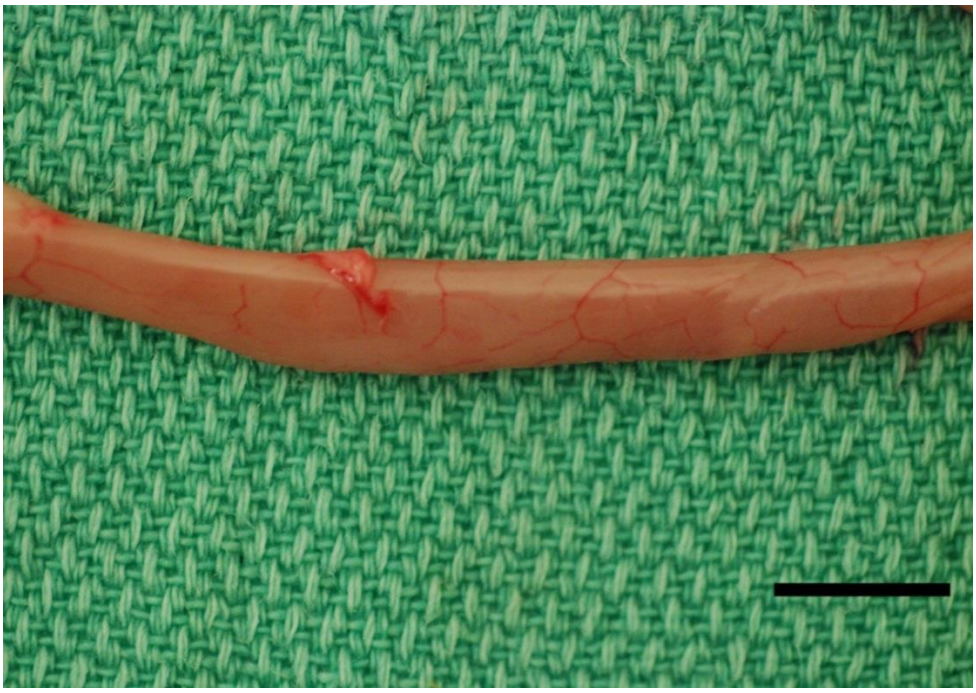


Figure 4.2.3.

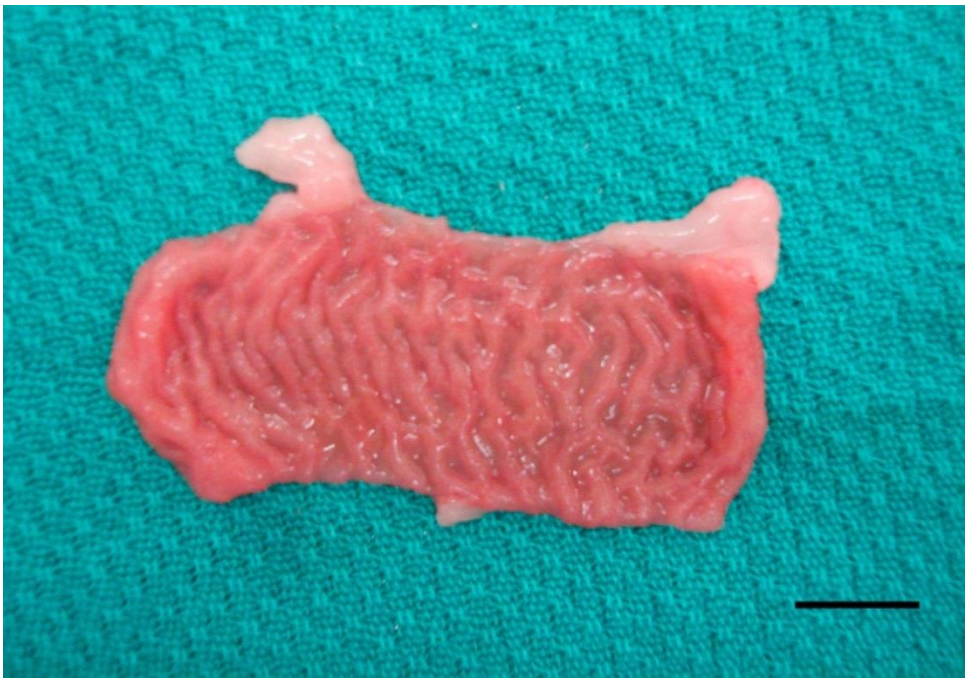


Figure 4.2.4.

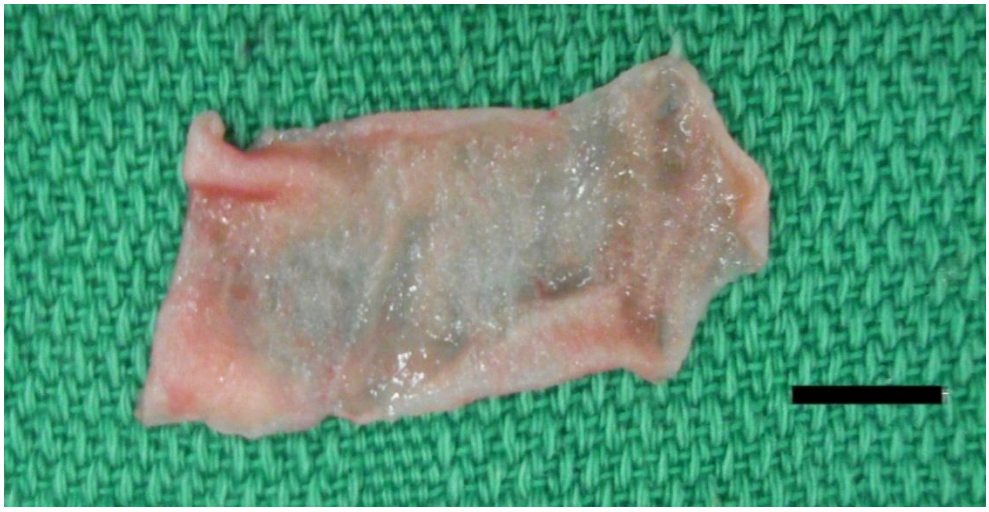


Figure 4.2: **Comparisons of infected and uninfected jejunum in a rabbit model for *L. intracellularis* infection.** Scale bar, 1 cm. Description: **Figure 4.2.1**: Serosal surface of the mid-jejunum from an infected rabbit (Group 1 inoculum) at 14 DPI. Note the cobblestone-like appearance of the serosal surface. A round jejunal Peyer's patch is notable on the left side (arrow). **Figure 4.2.2.**: Serosal surface of a mid-jejunum section of a control rabbit. **Figure 4.2.3.**: Luminal surface of the mid-jejunum from an infected rabbit (Group 1 inoculum) at 14 DPI. Note the rugae of the mucosa. **Figure 4.2.4**: Luminal surface of the mid-jejunum from a control uninfected rabbit. Note the smooth surface.

4.4.3. Histology and immunohistochemistry.

Typical histological lesions characterized by hyperplasia of immature enterocytes and absence of goblet cells were observed by H&E staining, but no inflammation was detected. These proliferative changes were consistently associated with the presence of intracellular bacteria identified by IHC (**Figure 4.3.1. and 4.3.2.**). Detailed results of IHC analysis on intestinal sections are **summarized in Table 4.2**.

Table 4.2. Immunohistochemistry (IHC) results in *L. intracellularis* orally inoculated rabbits.

Section	Group 3 Rabbits (Control)			Group 1 Rabbits			Group 2 Rabbits		
	7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days
Duodenum	0	0	0	0	0	0	0	0	0
Mid jejunum	0	0	0	0	+3	0	+1	+1	0
End jejunum	0	0	0	+1	+1	0	+2	+2	0
Ileum	0	0	0	0	+1	0	+2	+1	0
Cecum	0	0	0	+2	+2	+1	+2	+1	0
Appendix ceci	0	0	0	0	+1	0	+1	+1	0
Ileocecal valve	0	0	0	0	+1	0	+2	+1	0
Colon	0	0	0	+1	+2	0	+2	+2	0
Terminal colon	0	0	0	0	0	0	0	0	0
Rectum	0	0	0	0	0	0	0	0	0

Table 4.2: Results are separated by group and by DPI (7, 14, and 21). Numbers in the table refer to IHC grading from 0 to 4, grade 0 is no antigen labeling detected (no shading), grade +1 (light gray shading) equivalent to 25% antigen labeled, grade +2 (gray shading) equivalent to 50%, grade +3 (dark gray shading) to 75%, and grade +4 (very dark gray shading) equivalent to 100% antigen labeled. The groups consisted of 3 rabbits each, which were euthanized once weekly, one per group. Group 1 was infected orally with 1.3 x 10^8 bacteria/rabbit and Group 2 with 2.5 x 10^8 bacteria/rabbit. Group 3 was inoculated with medium only. Bacterial virulence of inocula was verified by challenge of natural host (4 foals) with the same lot of bacteria in different studies (Pusterla, *et al.*, 2010a; Pusterla, *et al.*, 2012a).

IHC analysis was conducted on intestinal sections from duodenum to rectum at 7, 14, and 21 DPI. At 7 DPI, *L. intracellularis* antigen was found as multifocal (mild to diffuse) labeling in the apical cytoplasm of enterocytes in the jejunum, caecal tip and colon of the Group 1 infected rabbits, and in the jejunum, ileum, cecum and colon of the Group 2 infected rabbit (**Figure 4.3.1.**). The labeled antigen in caecal epithelium is **shown in Figure 4.3.2.** At 7 DPI, all stained antigen was in the enterocytes and no *L. intracellularis* antigen was detectable in the *lamina propria* of any of the intestinal sections.

Figure 4.3.1.

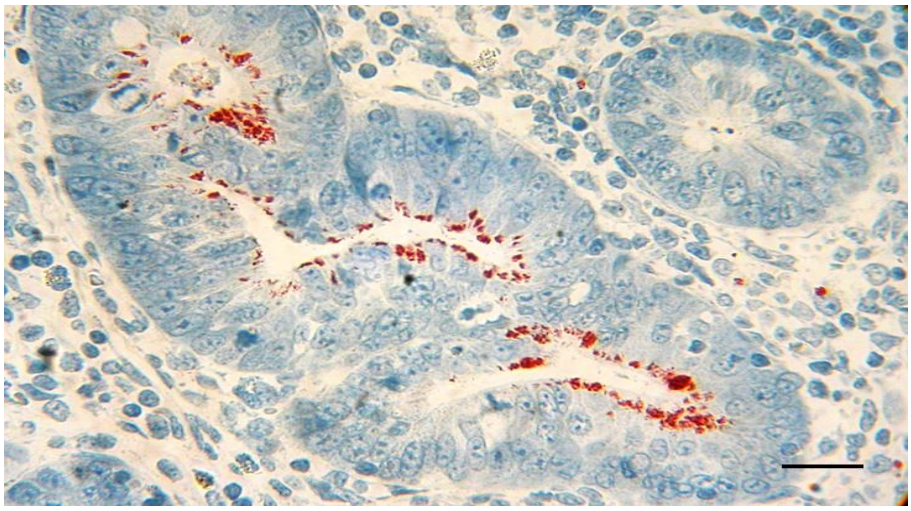


Figure 4.3.2.

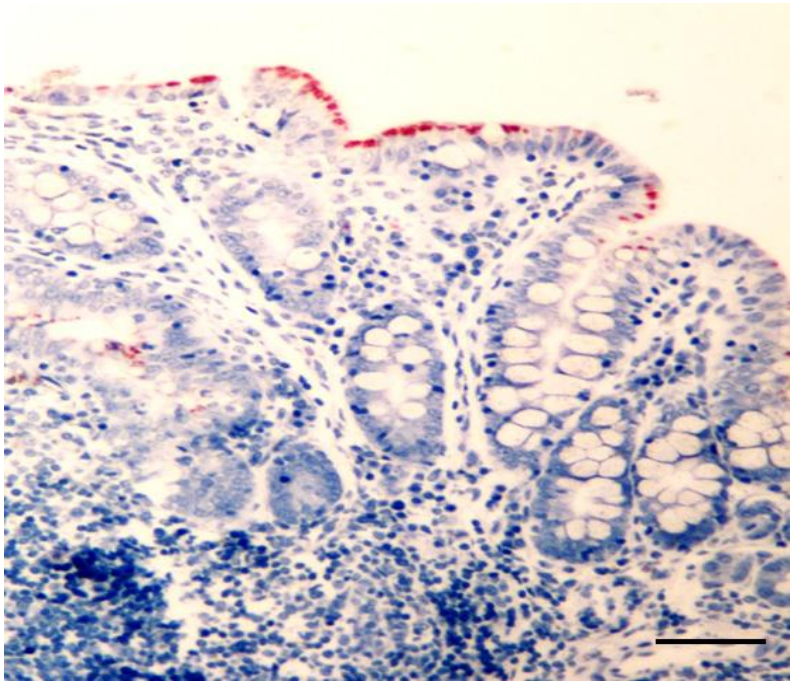


Figure 4.3: **Immunochemistry stains of infected rabbit intestine.** *L. intracellularis* antigen-specific staining with AEC substrate-chromogen and counterstained with Mayer's haematoxylin. **Figure 4.3.1**: note ileal crypts of an experimentally infected rabbit (14 DPI). Note the hyperplasia of immature enterocytes associated with bacterial infection (apical membrane). Scale bar, 10 µm. **Figure 4.3.2**: note the caecal mucosa of an experimentally infected rabbit (14DPI), showing labeled antigen in the superficial epithelium. Scale bar, 100 µm.

At 14 DPI, the antigen was found in epithelium and *lamina propria* as focal to multifocal and diffuse labeling, and the extent of the microscopic lesions previously observed in the H&E staining was associated with the increased IHC grading (grades 2 or 3). In the Group 2 infected rabbit, *L. intracellularis* antigen was not found in the *lamina propria* of terminal jejunum, *appendix ceci* or colon samples, but the grading was higher due to the higher frequency of antigen detection in the epithelium.

At 21 DPI, IHC analysis showed no detectable antigen in any of the infected groups except a focally labeled area noted in the *lamina propria* of the caecal tip of the Group 1 infected rabbit. Thus, IHC labeling and histologic analysis demonstrated that the rabbits had lesions from about 7 through 21 DPI, with highest severity noted at 14 DPI. No presence of labeled *L. intracellularis* antigen was detected in the duodenum, terminal colon, or rectum of any groups, nor was antigen detected in the intestinal tract of Group 3 (control rabbits) at any point in the experiment.

4.4.4. Serum analyses.

As **displayed in Figure 4**, serologic responses were detectable by IPMA analyses in the infected groups at 14 DPI. *L. intracellularis* infection generated a high serologic response in both infected groups, coinciding with the severity of lesions. With regards to total protein concentrations in sera, no significant differences ($p = 0.72$) were found between groups.

Figure 4.4. Serologic response in *L. intracellularis* infected rabbits

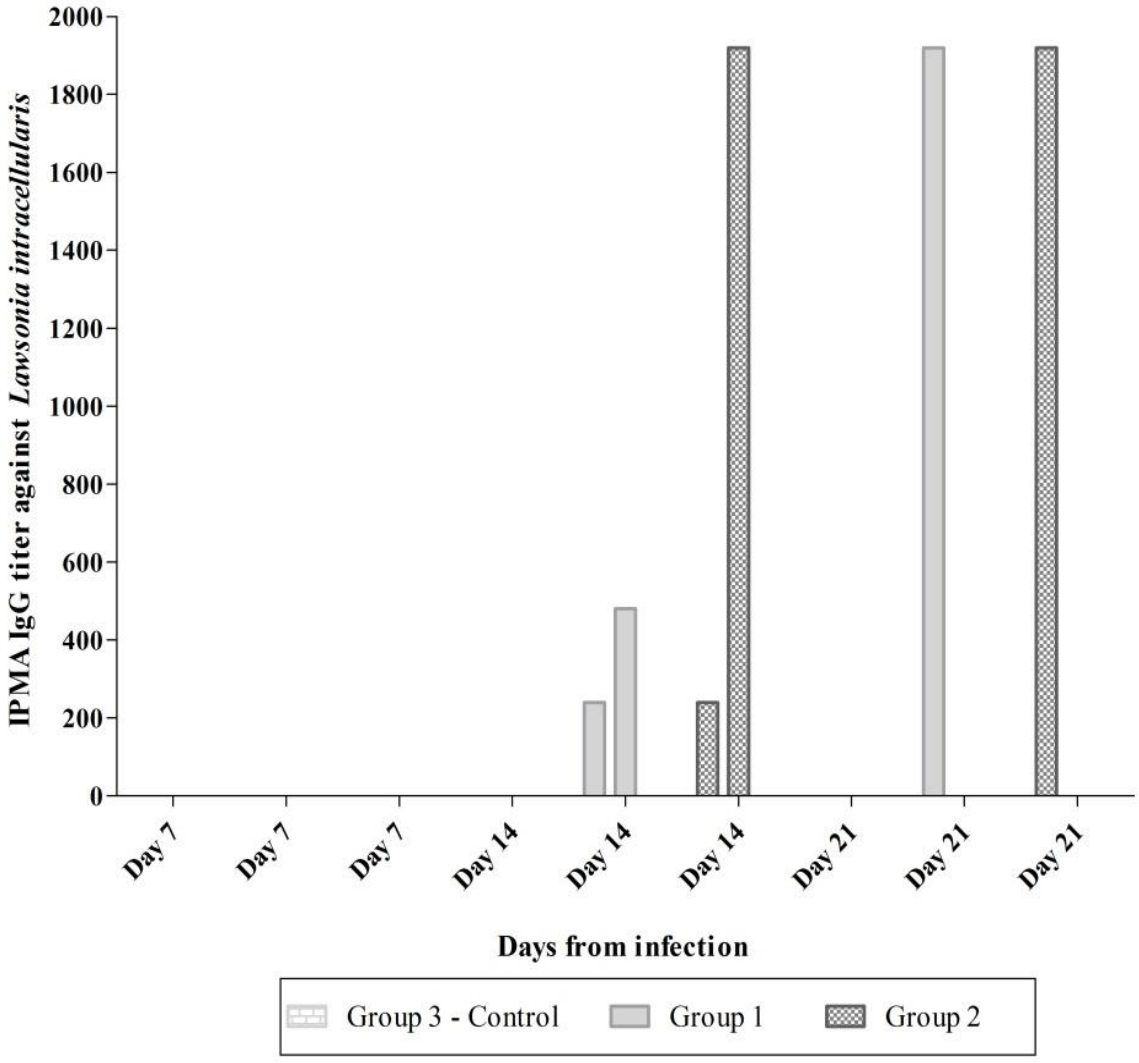


Figure 4.4: Serologic response detected in experimentally infected and uninfected control rabbits after oral challenge with an equine strain of *Lawsonia intracellularis*. The rabbits were infected with a strain proven virulent in foals and did not show any circulating antibody until 14 DPI, showing that the immune response requires a minimum time to show and does not manifest equally in every individual (note the different titers in different animals).

4.4.5. Quantitative PCR.

The results of the qPCR analysis are **displayed in Figure 5**. *L. intracellularis* was detected in feces of Group 2 infected rabbits at 7 DPI and was detected in both Group 1 and Group 2 rabbits at 14 DPI, becoming undetectable at 21 DPI. In Group 3 rabbits (controls), *L. intracellularis* organisms were never shed in feces throughout the experiment.

Figure 4.5

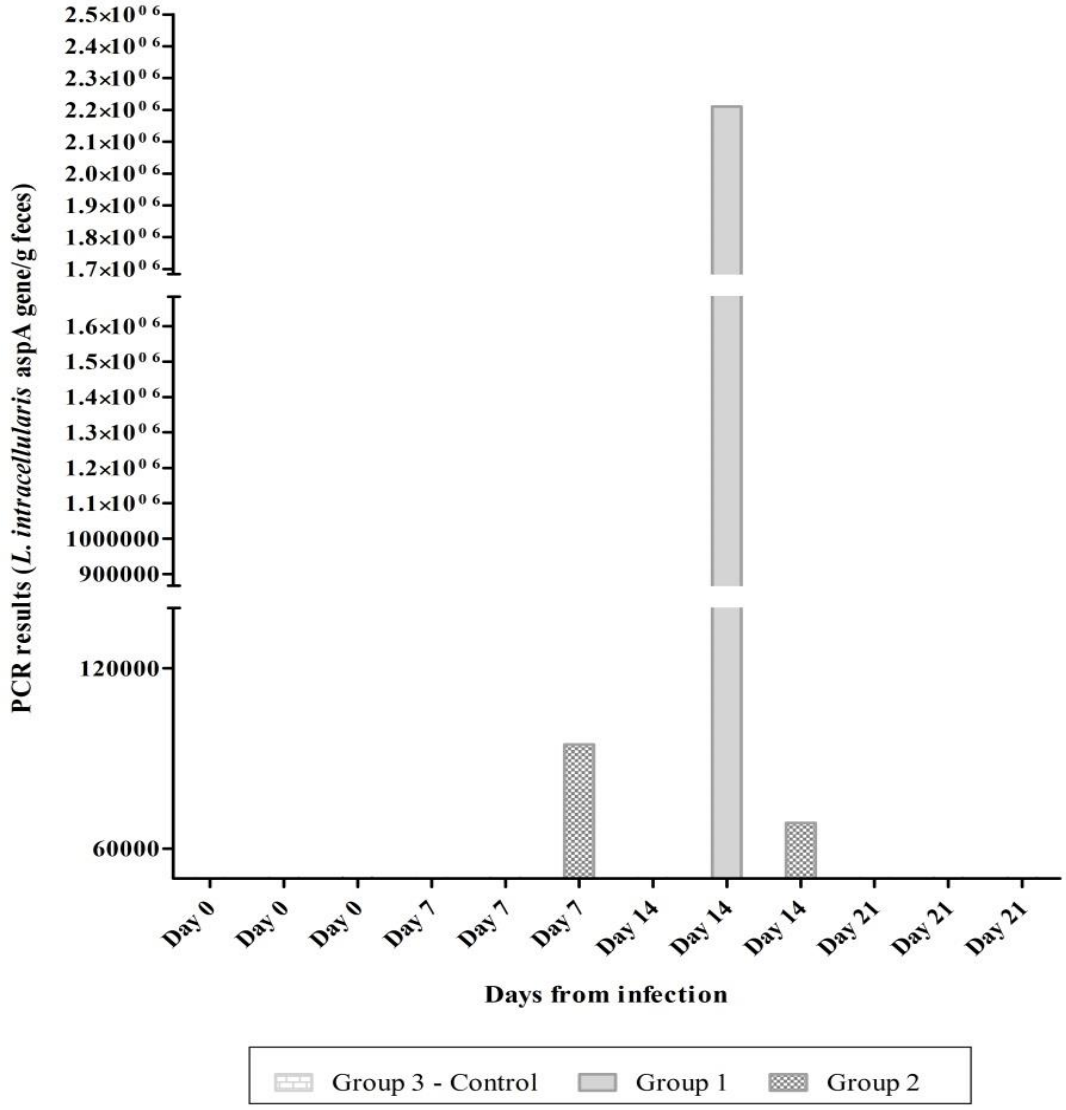


Figure 4.5: **Quantitative PCR results.** Quantitative PCR results (represented by *L. intracellularis* DNA gene copies/g feces) in rabbits experimentally infected with equine strain of *Lawsonia intracellularis* and in uninfected controls. Note that the represented fecal shedding was obtained for each group not for each individual. From these preliminary results the fecal shedding of *L. intracellularis* after cross infection from horses to rabbits, can be noted as early as 7 DPI, but apparently resolves by 21 DPI.

4.5. Discussion

This study describes the initial development of a rabbit infection model to investigate EPE in horses. Results show that there is merit in substituting rabbits for horses in infection model studies for EPE, as infection in juvenile rabbits was achieved after inoculation with a pure cell culture equine strain of *L. intracellularis*. Such findings provide a basis for pathogenesis studies, as this EPE infection model can be obtained within a relatively short incubation period and lesions can resolve quickly without subjecting animals to excessive discomfort. The equine strain of *L. intracellularis*, although purified through growth in cell culture, was pathogenic for rabbits. The infection did not cause marked clinical illness, but macroscopic and microscopic lesions were detected and confirmed by histopathology and IHC. Such findings were comparable to the ones observed in foals naturally and experimentally infected with EPE (Pusterla & Gebhart, 2009; Pusterla, *et al.*, 2010a). Similarly, in the infected rabbits, fecal qPCR findings and IPMA serology showed fecal shedding consistent with bacterial active replication and marked immune response, respectively.

A trend of reduced growth performance was noted in Group 1 and Group 2 rabbits around 12 and 17 DPI, which approximately corresponded with the time of the infection's peak (14 DPI) as lesions appeared more severe on gross pathology and IHC labeling at that time. Moderate weight loss and dullness, along with decreased appetite, were the most remarkable clinical signs noted. Overall, the clinical signs observed were mild; likely, the rabbits experimentally infected in this model could survive through the acute phase of the infection. Diarrhea was never noted, contrary to what is reported in both naturally infected rabbits and naturally and experimentally infected foals (Horiuchi, *et al.*, 2008; Pusterla, *et al.*, 2010a). In this study, the clinical condition of infected does appeared normal on 21 DPI, although the surviving Group 1 rabbit (the most clinically-affected group) did not appear to gain weight at the rate exhibited prior to the peak of disease (or less than half the average daily weight gain). Even with no clinical support, recovery appeared to occur by 21 DPI, as evidenced by improved weight gain and the absence of major IHC labeling. Mild IHC labeling (grade 1) in the cecum of the Group 1 rabbit could be consistent with ongoing recovery. This differs from reports about rabbits exhibiting clinical disease, after natural exposure, wherein clinical signs consist of marked diarrhea, weight loss and dehydration; or from sub-clinically affected rabbits, as they are

commonly reported to be exposed to the disease (Duhamel, *et al.*, 1998; Horiuchi, *et al.*, 2008; Pusterla, *et al*. 2008b; Watarai, *et al.*, 2008).

Previous reports and personal communications regarding hamsters' infection with porcine strain *L. intracellularis* grown in cell culture noted that clinical signs are usually subtle or absent, yet characteristic histopathology lesions were present (Jasni, *et al.*, 1994a; Vannucci, *et al.*, 2010). In the rabbits of this study, in spite of mild clinical signs, gross lesions typical of EPE extended from the mid-jejunum to the terminal ileum (Lavoie, *et al.*, 2000; Schumacher, *et al.*, 2000; Pusterla & Gebhart, 2009). Characteristically, *L. intracellularis* is found free within the apical cytoplasm of small intestinal crypt cells, particularly in the distal jejunum and ileum, in both horses and pigs, as previously reviewed (Lawson & Gebhart, 2000). Caecal hyperemia was noted in the Group 1 rabbit, at the disease's peak, and deemed a non-specific change, although caecal and colonic proliferative lesions can typically be found in naturally infected rabbits (Schoeb & Fox, 1990). Possibly, in this cross-infection model, lesion location may be related to the bacterial isolate involved, rather than the animal species affected. In other words, EPE lesions, even in rabbits, appear preferentially located in those sites typical for horses, such as jejunum and ileum, whereas only mild, non-specific lesions are found in sites observed only in overtly diseased rabbits, such as cecum and colon (Duhamel, *et al.*, 1998; Horiuchi, *et al.*, 2008). However, further studies are warranted to confirm this suspicion.

Lesions were confirmed through IHC labeling starting at 7 DPI, and their severity increased at 14 DPI, but decreased dramatically by 21 DPI. Such a finding is quite novel, as the incubation period is longer in experimentally infected horses and pigs (Lawson & Gebhart, 2000; Pusterla, *et al.*, 2010a). Furthermore, presence of antigen in the *lamina propria* was noted, particularly in the Group 1 infected rabbit, suggesting an infection at an advanced stage or, later on (21 DPI), resolution of the lesions, as antigen was observed in the macrophages in the *lamina propria* (Jensen, *et al.*, 1997).

The dose of bacterial challenge, administered orally as it is the natural infection route, differed slightly in the 2 infected groups, but this did not seem to have a major impact on results, as infection was obtained in both groups. For both inocula, the bacterial virulence was verified by challenge of natural host (4 foals) with the same lot of *L. intracellularis*, in a different study,

in which foals developed clinical EPE, with severe manifestations (3 out of 4) and demise (1 out of 4) (Vannucci, *et al.*, 2012).

On gross inspection the passage 9 inocula (Group 1) caused marked lesions and temporary weight loss, whereas the passage 10 inocula (Group 2) caused milder lesions and even milder clinical signs. Although studies in pig models show that increased (10-fold) concentrations of inoculum are related to increased severity of clinical signs, different virulence did not appear related to a 2-fold increase in concentration in the rabbit model (Guedes, *et al.*, 2003). Moreover, severity of clinical appearance in this model could be related to a low passage isolate, which somehow compensated for a lower concentration of the inoculum. At this stage, further investigation is needed, but it is clear that a low passage isolate can cause lesions in rabbits.

This *L. intracellularis* cross infection study shows fecal shedding in infected rabbits starting at 7 DPI and increasing by 14 DPI. TaqMan PCR technique detects the presence of genetic material regardless of whether it is viable or inactivated, and so it is of interest that in rabbits, fecal shedding appears limited to the presence of active infection (based on IHC lesions). Shedding was not detectable at 21 DPI, when clinical signs had already subsided and IHC labeling was drastically reduced. In the foal infection model the fecal shedding is reported to last longer than 21 DPI, but a specific relation between duration of fecal shedding, presence of lesions and IHC labeling in the equine model is not available, as the foals were enrolled in a survival study (Pusterla, *et al.*, 2010a). The information gathered about serologic conversion is limited, in this initial experiment, due to the short experimental timeline. Rabbits manifested a high serologic response 14 DPI, and even higher responses (>1920) in both diseased groups, by the final week. Such a high immune response showed that this EPE infection model in rabbits could be considered a "time-compressed" course of infection, in comparison to the equine EPE infection model, as such high titers were achieved in foals only by 28 or 35 DPI (Pusterla *et al.*, 2010a). Contrary to what is found in foals, a clinically significant hypoproteinemia was not found in the rabbits of our model, nor was edema of the limbs or abdomen ever detected, though these *sequelae* would normally occur after 21 DPI. Further investigations with larger numbers of rabbits and throughout a longer time period are needed to investigate the onset of hypoproteinemia and hypoalbuminemia in this rabbit model.

In conclusion, on the basis of clinical, diagnostic, gross pathology and IHC findings, the incubation period in rabbits infected with an equine *L. intracellularis* isolate appears to be shorter than it is for pigs and hamsters challenged with a pig isolate or for the EPE infection model (Lawson & Gebhart 2000; Pusterla *et al.*, 2010a; Vannucci, *et al.*, 2010). Moreover, intestinal lesions appeared to resolve by 21 DPI. These time factors could constitute an experimental advantage for the model in rabbits. Furthermore, infection is reproducible with no discomfort or overt disease. In pharmacology research, such a finding would represent an optimal model for drug preclinical testing. Further, rabbits, like horses, are hindgut fermenters, and unlike laboratory rodents offer the advantages of a larger bodyweight and blood volume, and accessible blood vessels for collection of multiple blood samples. Further studies with this infection model could be used for pathogenesis and epidemiology studies, particularly when associated with such a remarkable serologic response. In fact, the ability to overcome infection quickly, and without overt disease, can represent an epidemiologic risk, or may explain the limited success of prevention measures, if the equine *L. intracellularis* strain can be carried sub-clinically by rabbits populating the horse farm grounds (Pusterla, *et al.*, 2008b). Finally, the success of this cross-infection experiment and the failure to generate infection in hamsters, which were previously the animal model of choice for proliferative enteropathies, suggest a selective, secondary host adaptation of a given *L. intracellularis* strain. Further studies are now necessary to define the details of such a discovery and its multiple implications.

CHAPTER 5

SPECIES-SPECIFICITY OF EQUINE AND PORCINE *L. INTRACELLULARIS* ISOLATES IN LABORATORY ANIMALS*.

Relationship of this study to the dissertation objectives.

In this chapter the results of a preliminary study on a hamster infection model for EPE are reported. As hamsters have been considered the model-host for excellence, with regards to *Lawsonia intracellularis* infection, the fact that the first trial failed to confirm infection, generated a few question marks in the structure of the whole research and in the species specificity of a bacterium that was considered equally pathogenic for multiple animal species, mammalian and avian. However, with the success of rabbit as a model-host for EPE and the future of the project secured, we explored a few microbiology concepts that brought us well beyond the expectations of this Doctoral dissertation. Through our initial failures and the repetition of cross-infection of the 2 known strains of *L. intracellularis* (EPE and PPE) we demonstrated that an unsuspected secondary species-specificity restriction for each strain exist in laboratory animals, as it does in pigs and horses.

**This material is to be published as*: Sampieri, F., Vannucci, F.A., Allen, A.L., Pusterla, N., Antonopoulos, A.J., Ball, K.R., Thompson, J., Dowling, P.M., Hamilton, D.L. & Gebhart, C.J. (2013). Species-specificity of equine and porcine *L. intracellularis* isolates in laboratory animals. *Canadian Journal of Veterinary Research. In press.*

5.1. Abstract

L. intracellularis infection causes proliferative enteropathies in many mammalian species, the porcine (PPE) and equine (EPE) ones being known worldwide. Hamsters are a well-published animal model for PPE infection studies in pigs. There is no laboratory animal model, currently, for EPE infection studies and it is unknown whether there is species-specificity for equine or porcine isolates of L. intracellularis in animal models. In 2 separate trials, 4-wk-old and 3-wk-old weanling golden Syrian hamsters were challenged with EPE strains and compared to uninfected (both trials) and PPE infected controls (trial 2 only). Concurrently, 6 female New Zealand white juvenile rabbits were infected with PPE strain and observed concomitantly to 8 similar rabbits infected with EPE strain (for a different experiment requirement). Hamsters and rabbits were observed for 21 to 24 days post-infection (DPI), depending on the experiment. Neither infected species developed clinical signs. Presence of disease was assessed with diagnostic techniques classically used for pigs and horses: immune-peroxidase monolayer assay on sera; quantitative PCR detection of molecular DNA in feces; haematoxylin-eosin stain and immunohistochemistry (IHC) on intestinal tissues. Our results showed that EPE-challenged hamsters do not develop infection when compared with PPE controls (IHC p 0.009; qPCR p 0.0003). Conversely, PPE-challenged rabbits do not develop typical intestinal lesions in comparison to EPE-challenged rabbits, with serological response at 14 DPI being significantly lower (p 0.0023). In conclusion, PPE and EPE strains appear to have different host-specificities for hamsters and rabbits, respectively.

5.2. Introduction

Lawsonia intracellularis is a Gram-negative, obligate intracellular bacterium infecting the enterocytes of large or small intestines of a wide range of domestic, wildlife, avian and laboratory animal species (McOrist, *et al.*, 1995a; Hotchkiss, *et al.*, 1996; Lavoie, *et al.*, 2000; Lawson & Gebhart, 2000; Pusterla, *et al.*, 2008b). An intense proliferation is induced by *L. intracellularis* intra-cytoplasmic replication in the host's enterocytes, principally in the jejunum and ileum, wherein the hyperplasic activity corresponds to loss of function. Such lesions cause a variety of clinical signs, acute and chronic, in affected individuals ranging from malabsorption,

diarrhea, depression, weight loss, abdominal pain and even death (Pusterla & Gebhart, 2009; Wong, *et al.*, 2009; Vannucci, *et al.*, 2010). *L. intracellularis* is known to cause porcine and equine proliferative enteropathies (PPE and EPE, respectively): the former represented a challenge for the swine industry for over 50 years; the latter emerged more recently, and is now diffused worldwide, after sporadic and isolated beginnings (Gebhart, *et al.*, 1993; Lawson & Gebhart, 2000; Frazer, 2008; Pusterla & Gebhart, 2009). Proliferative enteropathies reflect negatively on both the porcine and equine industries, as their impact goes beyond the presence of challenging clinical signs, as slow and long recoveries, or severely reduced growth performance are common consequences (Lavoie, *et al.*, 2000; Frazer, 2008; Pusterla & Gebhart, 2009).

Proliferative enteropathy has been studied experimentally in pigs for years, either utilizing porcine *L. intracellularis* isolates grown *in vitro* or porcine tissues (Guedes & Gebhart, 2003a; Boutrup, *et al.*, 2010b). Similarly, amongst laboratory animals, hamsters are known to be naturally, and experimentally, affected by the disease, since long before *L. intracellularis* was defined and named in 1995 (Stills, *et al.*, 1987). Naturally affected hamsters undergo diarrhea (also known as "wet-tail") and weight loss, with severe hyperplasia of the ileal segment in the small intestine, similar to lesions observed in pigs (Stills, 1991). Thus, hamsters have been used as an animal model for PPE studies, whether infection is produced through ileal homogenates, harvested from naturally-diseased pigs, or bacteria grown in pure cell culture (McOrist, *et al.*, 1989; Stills & Hook, 1989; Stills, 1991; Jasni, *et al.*, 1994a; Jasni, *et al.*, 1994b; Cooper, *et al.*, 1997a; Vannucci, *et al.*, 2010). A few researchers attempted, with variable success, infection on other rodents (mice and rats) and chickens (Collins, *et al.*, 1999; Murakata, *et al.*, 2008; Collins, *et al.*, 2011). Contrarily to PPE studies, the research on EPE animal models is limited to a recently established foal model and to the preliminary results of a rabbit model, both obtained from the same virulent EPE strain (Pusterla, *et al.*, 2010a; Vannucci, *et al.*, 2012; Chapter 4).

Due to the increasing costs of research for the main species affected - horses and pigs-, replacing the large animal species with laboratory animal models could be useful to reduce the expenses, yet obtaining standardized welfare conditions, and maximising environmental and infection control over larger populations at one time, thus potentiating the statistical value of results. Although the Canadian Council for Animal Care (CCAC) "3R's" tenet (replacement, reduction and refinement) is not aimed to reduce experimental costs, but to responsibly control

animals use in research, animal models are advocated when *in vitro* models have great limitations, as in the case of *L. intracellularis* (Lawson, *et al.*, 1993; Fenwick, *et al.*, 2009). The studies here described were part of a larger project investigating EPE in relation to a novel pharmaceutical compound, which contains the post-transition metal gallium, whose environmental impact, after excretion, has not been clarified (Fecteau, *et al.*, 2011). To that end, modeling EPE on smaller surrogate animals, was considered liberally in agreement with the "3R's" tenet.

Our interest was to verify whether *L. intracellularis* exhibits a species-restricted host-susceptibility, or if one given strain could generate infection in multiple species (Lavoie, *et al.*, 2000; Pusterla, *et al.*, 2008b). Also, understanding the role of cross-infection in the pathogenesis of proliferative enteropathy would be epidemiologically useful to define which species may amplify the bacterial shedding in the environment. Importantly, such an understanding could help in determining to what extent and for how long these species represent a challenge for horse, or pig, farms' epidemiology boundaries. Even more importantly, clarifying the ability of *L. intracellularis* to cross-infect, from one host-species to another, will help researchers to choose appropriate animal models, hence reducing costs, refining experimental methods and, ultimately, sparing animals. The specific objectives of the present study were to determine whether it is possible to generate typical EPE lesions in hamsters, after inoculation with an equine strain of *L. intracellularis* (EPE strain), and whether it is comparatively possible to generate PPE lesions in rabbits, after inoculation with a porcine strain of *L. intracellularis* (PPE strain).

5.3. Materials and methods

All the studies described were approved by the Animal Research Ethics Board of the University of Saskatchewan and conducted according to CCAC guidelines.

5.3.1. Animals: Hamsters

5.3.1.1. Trial 1.

Twenty-nine pathogen-free golden Syrian hamster (*Mesocricetus auratus*) weanlings (Strain 049 - VAF Hamsters – Charles River Canada, Pointe Claire, QC, Canada) were born at the Animal Care Unit - Western College of Veterinary Medicine - University of Saskatchewan. The hamsters originated from immuno-competent animal colonies monitored by serology, PCR, bacteriology, parasitology or gross pathology tests, for absence (pathogen free and virus antibody free, or VAF/Plus hamsters) of *Reovirus, Sendai virus*, *Lymphocytic choriomeningitis virus, Bordetella bronchiseptica, Strep. pneumonia, Campylobacter jejuni, Cl. piliforme, Corynebacterium kutscheri, Encephalitozoon cuniculi, Helicobacter spp., Myc. pulmonis, Salmonella spp.,* β*-hemolytic Streptococcus spp. (serogroups A, B and G); all intestinal helmints (Syphacia spp., Hymenolepis spp. etc.) and protozoa species (Coccidia spp., Trichomonadaceae, Entamoeba spp., etc.);* dermathophytosis and all arthropods ectoparasites *spp.* (Strain 049 - VAF Hamsters – Charles River Canada, Pointe Claire, QC, Canada). They were housed, with their dams, in rodent cages in a Containment Level (CL)-1 Room until weaning (21 days from birth), bedded on dust free shavings, fed rodent chow pellets (5P00, Prolab RMH 3000, LabDiet - PMI Nutritional International, LLC, Brentwood, MO USA) and fresh water *ad libitum* and maintained in standard husbandry conditions (12/12 h light – dark cycle, and $20^{o}C \pm 2^{o}C$ room temperature). Newborn hamsters were visually monitored once daily, for the first 5 days of life, and then twice daily until weaning. At weaning, they were weighed, ear punched and randomly assigned to 2 groups: uninfected controls (9 hamsters) and EPE-strain infected (20 hamsters). At that time, hamsters were separated into pairs, or triplets, according to group (controls vs. EPE-infected) and sex. Inoculation with *L. intracellularis* occurred 1 week after weaning. After inoculation, hamsters were housed in rodent cages in a CL-2 Room, bedded on dust-free shavings in identical husbandry conditions and provided with the same feed and water sources, as described above.

5.3.1.2. Trial 2.

An additional 24 pathogen-free and VAF golden Syrian hamsters (Strain HsdHanTM AURA – Harlan Laboratories –, Indianapolis, IN, USA) were born at the same Animal Care Unit facility described for Trial 1. Both for experimental subjects and their dams, housing, husbandry and daily monitoring conditions were identical to that described for Trial 1. The health

monitoring of the immuno-competent originating hamster colonies included serology, gross pathology and microscopic analysis tests to verify the absence of pathogens and related antibodies for *Reovirus, Sendai virus, Lymphocytic choriomeningitis virus, Simian Virus 5, Bordetella bronchiseptica, Cl. piliforme, Corynebacterium kutscheri, Salmonella spp., Campylobacter jejuni, L. intracellularis, Helicobacter spp., Klebsiella spp., Myc. pulmonis Proteus mirabilis, Pseudomonas aeruginosa, Pneumocystis spp., Pasteurella pneumotropica, β-hemolytic Streptococcus spp., Strep. pneumonia, Staph. aureus;* ectoparasites and endoparasites, *Demodex spp., Encephalitozoon cuniculi* (Strain HsdHanTM AURA – Harlan Laboratories –, Indianapolis, IN, USA). On day 21, hamsters were weaned, ear punched, weighed and randomly assigned to 3 groups: controls - uninfected (6 hamsters); PPE-strain group – inoculated with a porcine strain of *L. intracellularis* (9 hamsters); and EPE-strain group – inoculated with an equine strain of *L. intracellularis* (9 hamsters). Trial 2 hamsters were inoculated at weaning, to increase the impact of stress on disease reproduction.

5.3.2. Animals: Rabbits.

Similarly to what described in Chapter 4 for rabbits, 6 female, New Zealand white pathogen-free rabbits (Strain - 052 VAF Rabbits - Charles River Canada, Pointe Claire, QC, Canada) (*Oryctolagus cuniculus)* were used, differing from Chapter 4 does only in age, as they were 4- to 5- weeks old. Animals originated from colonies tested regularly by serology, gross and histo- pathology, bacteriology and parasitology tests for absence of pathogen agents and related antibodies (VAF) (*Lymphocytic choriomeningitis virus, Parainfluenza virus 1 and 2, Rabbit hemorrhagic disease virus, Reovirus, Rotavirus, Bordetella bronchiseptica, CAR-Bacillus, Cl. piliforme, Campylobacter jejuni, Corynebacterium kutscheri, Helicobacter spp., Klebsiella spp., L. intracellularis, Myc. pulmonis P. mirabilis, Ps. aeruginosa, Pneumocystis spp., Past. pneumotropica, Salmonella spp., β-hemolytic Streptococcus spp., Strep. pneumonia, Staph. aureus, Encephalitozoon cuniculi, Giardia spp., Toxoplasma gondii, Treponema cuniculi,* pinworms and several tests for typical rodent diseases, but used annually in rabbits by the provider for internal control purposes - Strain - 052 VAF Rabbits - Charles River Canada, Pointe Claire, QC, Canada), as described for the previous study on the *L. intracellularis* rabbit model

(Chapter 4). Also, 8 further does of the same age, weight and origin (Strain - 052 VAF Rabbits - Charles River Canada, Pointe Claire, QC, Canada) were used as a comparative control to the PPE-strain study, as they were EPE-infected for a different experiment (Chapter 8). The 2 studies were purposefully conducted at the same time, to compare the lesions at the peak of infection (14 DPI), and no sham-challenged rabbits were used in this experiment, as unexposed rabbits have previously been shown to not develop infection (Chapter 4). The receiving and identification procedures and the husbandry conditions in CL-2 Room were already described in detail in Chapter 4, as animals were group-housed in two separate and isolated pens. Both EPE- and PPE-infected rabbits were inoculated concurrently and managed identically, with strict reverse isolation procedures maintained for the PPE-strain group.

5.3.3. Inoculum preparation.

Control inoculum (medium only) consisted of buffered sucrose/phosphate/glutamate (SPG) solution and was administered to the uninfected hamster groups in both trials (controls), but not to the rabbits.

5.3.3.1. EPE strain.

Hamsters in Trials 1 and 2 and the EPE-infected rabbits were challenged with the equine strain of *L. intracellularis* E40504, prepared as previously described (Pusterla, *et al.*, 2010a; Vannucci, *et al.*, 2012). The *L. intracellularis* infectious material originated from ileal mucosal scrapings of a foal that succumbed to EPE. It was subsequently cultured on McCoy cells (ATCC CRL 1696) in a defined environment (Lawson, *et al.*, 1993; Guedes & Gebhart, 2003b). Infectious virulent isolate from low passage pure cell-cultures was prepared and suspended in buffered SPG medium for all the EPE infected animals, as previously described (Pusterla, *et al.*, 2010; Vannucci, *et al.*, 2012). In the hamster trials, inoculum concentration was between 4.5 x 10^7 bacteria/ml and 7.5 x10^7 bacteria/ml (Guedes, *et al.*, 2003). For the positive control, EPE infected rabbits were inoculated at a dose of 5.5 ml/rabbit, to give a final inoculum between 1.3 and 2.5 x 10^8 bacteria per rabbit (Chapter 4).

5.3.3.2. PPE strain.

For the Trial 2 hamsters and PPE-infected rabbits, inocula were prepared from PPE lesions harvested from an infected pig's ileal mucosa (PHE/MN1-00) and cultured on McCoy cells (ATCC CRL 1696), in a defined environment (Lawson, *et al.*, 1993; Guedes & Gebhart, 2003b; Vannucci, *et al.*, 2012). Infectious inocula were prepared as described above, from low passage pure cell-culture isolate. The main difference between hamster's and rabbit's inocula was the final concentration and the volume, which for hamster's inocula was a concentration of 3.2×10^7 bacteria/ml and a volume as close as possible to 1 mL (see below); whereas rabbits' inocula were 8.5 mL per rabbit (target dose of inoculum was 4.25×10^8 bacteria per animal).

For Trial 1 hamsters, a total dose of 0.45 mL/hamster (approx. 3.375×10^7 bacteria\hamster), suspended in commercial fruit flavoured pudding (Jell-O, Kraft Foods, Canada) was administered to each animal once, on day 0. In trial 2, an equal inoculum dose was given, but no palatable medium was added. Subjects were 1 week younger and of lighter body weight (BW), thus the total target volume (1 mL) was divided into 3 administrations over 2 days (once on day 0, twice on day 1). The dosing volume calculations were based on a maximum volume of 20 ml/kg BW and repeated inoculation was used to maximize exposure (Morton, *et al.*, 2001). All challenge material was stored in a -20^{o}C freezer and thawed overnight at 4^{o}C, 12 hours prior to the experiments.

5.3.4. Inoculation procedures.

5.3.4.1. Hamsters.

Inoculation occurred by 2 different techniques. In Trial 1, hamsters were fed the inocula through a syringe, because of the palatable medium added. In trial 2, hamsters were inoculated via oral *gavage*, to ensure that the intended dose was entirely administered to the smaller animals.

5.3.4.2. Rabbits.

Rabbit inoculation occurred after a 1 week mandatory acclimation with identical procedures and husbandry protocols, as described in Chapter 4.

5.3.5. Collection of samples

5.3.5.1. Hamsters.

In Trial 1, BW changes were monitored daily for 21 DPI. In Trial 2, BW changes were monitored daily for 24 DPI. In both trials, demeanor, gross appearance, fecal consistency and quality, as well as self and mutual grooming were monitored twice daily with a 4 step grading system, 0 corresponding to normal and 3 to severely abnormal findings (**Table 5.1**). Treats (apples and small amounts of fruit flavored pudding - Jell-O, Kraft Foods, Canada) were used to check individual animals' appetite and awareness, twice daily. Lack of response to treats and to researchers' voices was considered a sign of decreased well-being (Johnson-Delaney, 2006). In both trials, pooled fecal samples from the cages were collected once weekly and on the predetermined days of euthanasia, when individual samples were collected from the sacrificed animals.

Blood samples for serologic evaluation were collected at the time of euthanasia via intracardiac puncture. In Trial 1, 4 EPE-infected hamsters were euthanized at 7, 11, 14, 17, 21 DPI; 1 control was euthanized at 7, 11 and 21 DPI, and 3 controls were sacrificed at 14 and 17 DPI. In Trial 2, 8 hamsters (3 from EPE group; 3 from PPE group; and 2 controls) were euthanized at 17, 21 and 24 DPI. In both trials, euthanasia was achieved with an inhalatory anesthetic overdose (isofluorane - Isoflurane, 99.9%, Halocarbon Products Corporation, River Edge, NJ, USA) in a glass chamber. Within each group, selection for euthanasia was randomized. At necropsy, for evaluation of the presence of proliferative enteropathy lesions, sections of the ileum, jejunum, cecum and colon were harvested, prepared within histology cassettes and immediately placed in 10% phosphate buffered formalin solution, for histopathologic (haematoxylin & eosin – H&E) and immunohistochemical (IHC) examination (Vannucci, *et al.*, 2010).

Table 5.1. Humane Intervention Monitoring Parameters for the hamster studies.

EXPERIMENTAL DAY:	DATE:	HAMSTER ID CODE:
	Variable	Individual score
	Daily Feed Intake	Am
0	Same or increased from previous day	
1	Slight decreased from previous day	
2	Marked decrease from previous day	Pm
3	Complete anorexia	
	Daily Water Intake	Am
0	Same or increased from previous day	
1	Slight decreased from previous day	
2	Marked decrease from previous day	Pm
3	No water taken in	
	Body Weight (average weight gain for young hamsters is 1-2g/day or ≈10g/wk.)	Am
0	Increasing weight ~ 20%/wk.	
1	Increasing weight ~ 10% /wk.	(Calculated gain from
2	Maintaining weight, no weight gain	last BW measure)
3	Rapid weight loss (20-25%/wk.) or losing weight for more than 2 days	
	Stool	Am
0	Normal feces	
1	Feces formed but softer than usual	
2	Feces not formed, but not liquid	Pm
3	Liquid feces (for >2days) leading to emaciation	
	Physical Appearance	Am
0	Normal	
1	Lack of grooming	
2	Rough coat, nasal/ocular discharge,	Pm
3	Very rough coat, abnormally hunched posture, distended abdomen (>3days)	
	Behavior	Am
0	Normal, alert, playful, comes to front of cage	
1	Hyperactive, agitated	
2	Decreased activity, minor depression, unwilling to move	Pm
3	Depressed, head turned to corner of cage, still moves when stimulated	

Table 5.1: This "Humane Intervention Monitoring Parameters" format was used for the hamsters enrolled in the infection animal model for porcine and equine proliferative enteropathy, during phase of validation. Each animal had this table as a daily monitoring spreadsheet, where twice daily clinical observations were recorded, except for BW (assessed only once daily).

5.3.5.2. Rabbits.

BW changes were monitored daily. Demeanor, gross appearance, fecal consistency and quality, appetite, and self and mutual grooming were monitored twice daily with a 4 step grading system, 0 corresponding to normal and 3 to severely abnormal findings, as previously described (**Table 5.2**) (Chapter 4). Again, lack of interest in treats (apples and carrots, in this case) was considered a sign of decreased well-being (Johnson-Delaney, 2006).

Blood samples (1 ml each) for serology were collected with a sterile needle and syringe once weekly and at the time of euthanasia, from the ear's central artery, after a local block and disinfection were applied, as previously described (Chapter 4). Also, pooled fecal samples were collected from the pen once weekly, and individual fecal samples were collected at the time of euthanasia. Two PPE-infected rabbits per week were humanely euthanized with an intravenous overdose (720 mg/rabbit) of pentobarbital (Euthanyl, Bimeda MTC, Animal Health Inc., Cambridge, ON, Canada). To observe for evidence of lesions, the same collection procedures and storage protocols were followed as described in Chapter 4.

Table 5.2. Humane Intervention Monitoring Parameters for the rabbit studies.

EXPERIMENTAL DAY:	DATE:	RABBIT ID CODE:
	Variable	Individual score
	Daily Feed Intake	Am
0	Same or increased from previous day	
1	Slight decreased from previous day	
2	Marked decrease from previous day	Pm
3	Complete anorexia	
	Daily Water Intake	Am
0	Same or increased from previous day	
1	Slight decreased from previous day	
2	Marked decrease from previous day	Pm
3	No water taken in	
	Body Weight (average weight gain for young rabbits is 200	Am
0	g/wk.)	
1	Increasing weight ~ 200 g/wk.	
2	Increasing weight ~ 100 g /wk.	(Calculated gain from last BW
3	Maintaining weight, no weight gain - Losing weight for more than 2 days	measure)
	Stool	Am
0	Normal feces	
1	Feces formed but softer than usual	
2	Feces not formed, but not liquid	Pm
3	Liquid feces	
	Physical Appearance	Am
0	Normal	
1	Lack of grooming	
2	Rough coat, nasal/ocular discharge,	Pm
3	Very rough coat, abnormal posture, ears low	
	Behaviour	Am
0	Normal, alert, comes to front of cage	
1	Minor depression, but still moves when stimulated	
2	Grinding teeth, depressed, head turned to corner of cage	Pm
3	Very depressed, almost unresponsive	

Table 5.2: This "Humane Intervention Monitoring Parameters" format was used for the rabbits enrolled in the rabbit animal model for porcine (and equine) proliferative enteropathy, during phase of validation. Each animal had this table as a daily monitoring spreadsheet, where twice daily clinical observations were recorded, except for BW (assessed only once daily).

5.3.6. Sample analysis.

5.3.6.1. Macroscopic examination.

For both hamsters and rabbits, regardless of group and trial, shortly after euthanasia the alimentary tract was examined visually from stomach to rectum. The sampling protocol for hamsters consisted of collection of the terminal jejunum, ileum, ileocecal valve, caecal *ampulla* and colon, as previously reported (Vannucci, *et al.*, 2010). Fecal pellets were collected from the terminal colon and rectum. The sampling protocol for rabbits was identical to what described in a previous study (Chapter 4).

5.3.6.2. Immunohistochemistry.

Formalin-fixed sections were paraffin embedded, cut and stained by streptavidin method, including anti-*L. intracellularis* specific mouse monoclonal antibody (Guedes & Gebhart, 2003c). The positive labeled *L. intracellularis*-specific antigen in the crypts was evaluated with a 5 grade IHC scoring system, previously published (Guedes & Gebhart, 2003c; Vannucci, *et al.*, 2010). For each animal, the negative control for each tissue section consisted of a correspondent tissue section IHC-labeled, but omitting the primary antibody. Furthermore, pig ileal tissues, known to be negative and positive for *L. intracellularis* infection, were labeled with the murine anti-*L. intracellularis* monoclonal antibody to confirm the antibody's specificity and sensitivity, respectively.

5.3.6.3. Serology analysis.

Anti–*L. intracellularis*–specific IgG in serum was measured by an immuno-peroxidase monolayer assay (IPMA), as reported previously (Guedes, *et al.*, 2002c). Positive serum samples were end-point titrated from 1:30 up to 1:1920. Control samples consisted of serum from a rabbit prior to (negative control) and after (positive control) hyperimmunization with *L. intracellularis*, purified from cell culture. Also, serum total protein concentration was measured, with the refractometer method, to investigate changes overtime.

5.3.6.4. Quantitative PCR Analysis.

Quantitative PCR analysis was conducted on fecal samples as previously reported (Pusterla, *et al.*, 2010a). The purified DNA was analyzed by qPCR for presence of *L. intracellularis aspA* gene copies (Pusterla, *et al.*, 2008b; Pusterla, *et al.*, 2009a). For each target gene, two primers and an internal, fluorescent labeled TaqMan probe (5´end, reporter dye FAM

(6-carboxyflourescein), 3´end, quencher dye TAMRA (6-carboxytetramethylrhodamine)) was designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). The "L.intra system" forward primer and reverse primer were "bcL.intra-114f" (CACTTGCAAACAATAAACTTGGTCTTC) and "bcL.intra-263r" (CATTCATATTTGTACT TGTCCCTGCA), respectively, with the Aspartate Ammonia Lyase (AspA) gene as target, and "bcL.intra-201p" (TCCTTGATCAATTTGTTGTGGATTGTATTCAAGG) as probe. The "Pan-Bacteria system" forward primer and reverse primer were "PB.283f" (GGATGATCAGCCACACTGGA) and "PB.352r" (CCAATATTCCTCACTGCTGCC), respectively, with 16S rRNA as a target, and "PB.305" (CCCGTAGGAGTCTGGACCGTGTCTCA) as a probe. TaqMan PCR systems were validated using 2-fold dilutions of gDNA testing positive for the target genes. Dilutions were analyzed in triplicate and a standard curve was plotted against the dilutions. The slope of the standard curve was used to calculate amplification efficiencies using the formula $E = 10^{1/-s} - 1$. Each system needed to be greater than 95% efficiency to be considered. The detection limit for "L.intra system" is between 5-10 copies/µl DNA (determined with plasmid DNA). The "L.intra system" detects all *L. intracellularis*, regardless of the host species. Known positive controls and no template controls were run on every plate, and met previously standardization values. DNA quality was determined by the "PanBacteria system", with a control value under 30, to pass quality control (Leutenegger, *et al.*, 1999). A real-time PCR assay targeting a universal sequence of the bacterial 16S rRNA gene was used as quality control (*i.e.*, efficiency of DNA purification and amplification) and as indicator of fecal inhibition (Mapes, *et al.*, 2007).

5.3.6.5. RT-reaction and real-time TaqMan PCR.

Each PCR reaction contained 20x primer and probes for the respective TaqMan system with a final concentration of 400 nM for each primer and 80 nM for the TaqMan probe and commercially available PCR Mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems, Foster City, CA, USA), containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction; 0.25 U AmpErase UNG per reaction; and 1 µl of the DNA sample in a final volume of 12 µl. The samples were placed in 384 well plate and amplified in an automated fluorometer (ABI PRISM 7900 HTA FAST, ABI). ABI's standard amplification conditions were used: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Fluorescent signals

were collected during the annealing temperature and CT values extracted with a threshold of 0.1 and baseline values of 3-12 for all samples using SDS 2.2.1 (Applied Biosystems, Foster City, CA, USA). Absolute quantitation was calculated by a standard curve and expressed as copy numbers of the *L. intracellularis aspA* gene/g of feces.

5.3.7. Statistics.

5.3.7.1. Hamster trials.

BW findings were analyzed through a Mann-Whitney test, as Trial 1 had 2 uneven groups; and Kruskal-Wallis test with Dunn`s *post hoc* testing, as Trial 2 had 3 uneven groups. IHC results were analyzed through contingency tables with Fisher Exact Test, to compare the 2 categorical outcomes (lesions versus no lesions) and to estimate odds ratio and relative risks. For qPCR results analyses, a one-way ANOVA, with Bonferroni's *post hoc* testing was used.

5.3.7.2. Rabbit trial.

For PPE-infected rabbits only descriptive statistics were used, as the sample number was insufficient for statistical analysis; however, where the comparison was applicable to the EPE-infected animals, Mann Whitney, Kruskal-Wallis and unpaired t- tests were used (BW and serum total protein concentration). For all statistical analyses in hamsters and rabbits, a commercial software (GraphPad Prism 5.4 Software, CA, USA) was used, and alpha was set at 5%.

5.4. Results

5.4.1. Clinical appearance.

5.4.1.1. Hamsters.

No hamster showed sign of diarrhea, throughout either trial, and their enthusiastic interest in treats was never diminished (Johnson-Delaney, 2006). The only behavioural change was a mild to moderate hyperactivity, noted after 14 DPI, as some PPE-infected animals rearranged the entire cage`s content within a few hours, in the daylight hours, suggesting abnormal behaviour. In regards to daily BW gains, there was no difference between infected and uninfected hamsters

in either trial (*p*: 0.12 for Trial 1; 0.88 for Trial 2). Comparisons between 3- and 4-wk-old hamsters showed no difference (*p*: 0.14), due to age (3 versus 4 weeks old) or breeding colony (comparison of Trial 1 hamsters (Strain 049 - VAF Hamsters – Charles River Canada, Pointe Claire, QC, Canada) versus Trial 2 hamsters (Strain HsdHanTM AURA – Harlan Laboratories –, Indianapolis, IN, USA) providers), in infected and uninfected hamsters (*p*: for EPE group: 0.77; and for EPE and PPE combined group: 0.6).

5.4.1.2. Rabbits.

For the PPE-infected rabbits, no clinical abnormalities were noted during the study. Signs of aggression toward other pen-mates were noted in 2 does: 1 improved over time, 1 developed it toward the end of the trial. No significant depression, nor BW gain suppression, was noted in any PPE-infected rabbit, not even around 14 DPI (peak of disease), as observed in EPE-infected rabbits (Chapter 4). Three PPE-infected rabbits had negative BW gains at different stages, unrelated to each other and quickly compensated (within 1 day) as the total BW gain of PPE-infected rabbits (7 DPI: 171 – 309g; 14 DPI: 506 – 667g; 21 DPI: 781 - 871g) was above the average recommended by the provider (200g/week) (Strain - 052 VAF Rabbits - Charles River Canada, Pointe Claire, QC, Canada). A comparison between daily BW gain averages in the EPE and PPE groups at 14 DPI **(Figure 1)** showed that EPE-does had a BW gain suppression (*p* 0.036).

Figure 5.1.

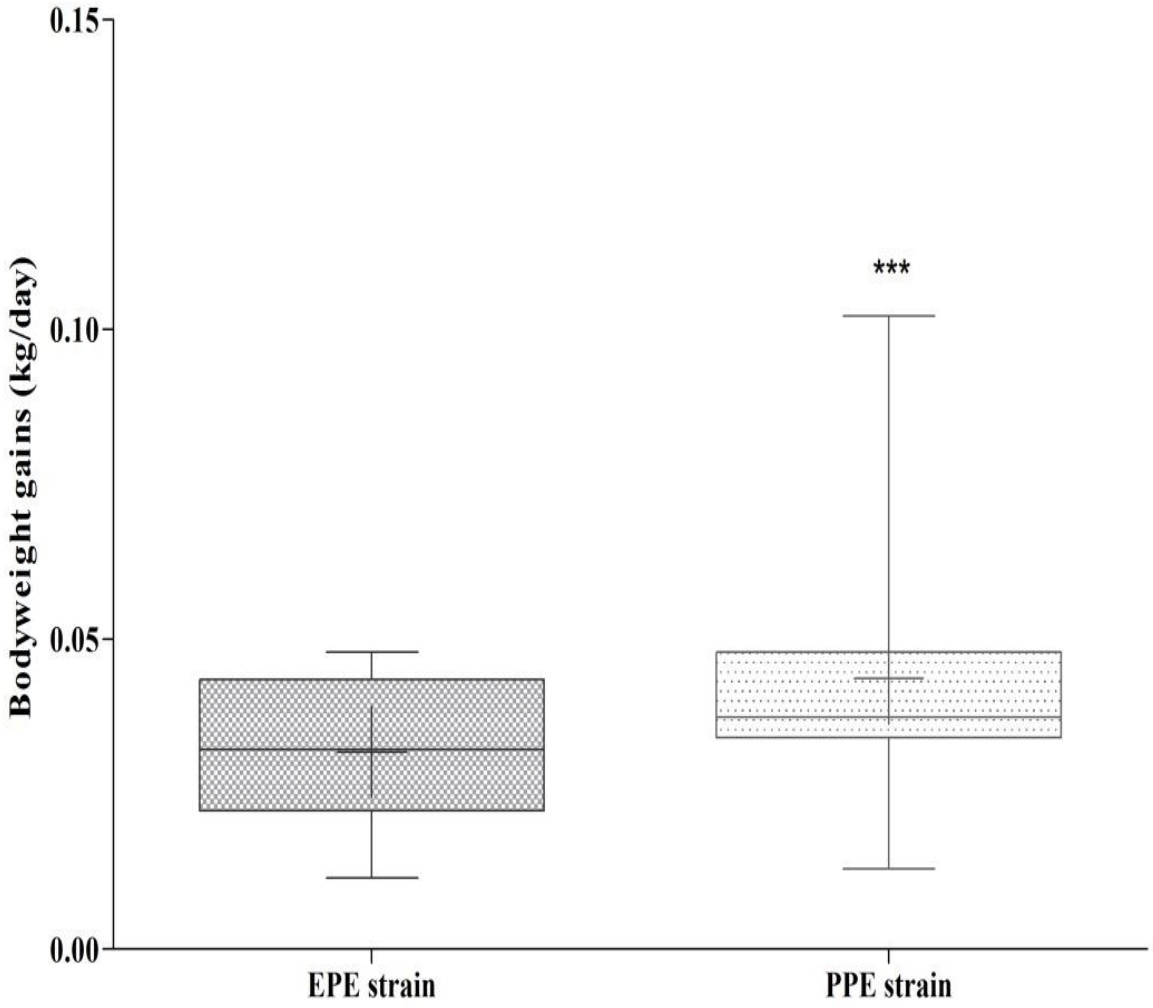


Figure 5.1: Comparison of bodyweight changes in infected rabbits. Comparison between infection with the EPE-strain and the PPE-strain of *L. intracellularis* in 5-wk-old rabbits. The PPE-infected rabbits gained overall more weight than the EPE-infected counterparts by 14 DPI (p 0.04). The mean in the boxplot is represented by a cross hair, whereas the whiskers represent the 5 – 95 percentile interval. One should note that the preliminary phases of the EPE-strain of *L. intracellularis* infection model in rabbits show a trend of reduced growth performance around the infection peak (approximately 14 DPI), not matched by the PPE-infected rabbits.

5.4.2. Gross pathology.

5.4.2.1. Hamsters.

In either trial there were no gross lesions in the intestines. A larger amount of abdominal fat was noted in older animals (Trial 1). Intestinal content appeared adequate for the tracts examined and, upon measurement of the diameter of the intestinal sections, trial 1 controls had smaller ilea (*p* 0.0011) than EPE-infected hamsters, at the level of the ileocecal valve (1.5 -2.5 mm vs. 2-3 mm). However, in Trial 2, hamsters, jejunum and ileum diameters were not different in EPE-, PPE- and control-groups (*p* 0.9 and 0.26, respectively).

5.4.2.2. Rabbits.

In PPE-infected rabbits, mild edematous changes of the serosal layer of the terminal portion of jejunum and ileum were noted and were comparable to EPE-infected rabbits serosal findings **(Figure 5.2)**. However, no *rugae* were observed in the mucosa and by 14 DPI, only the serosal and mucosal edema was apparently increased.

Figure 5.2.

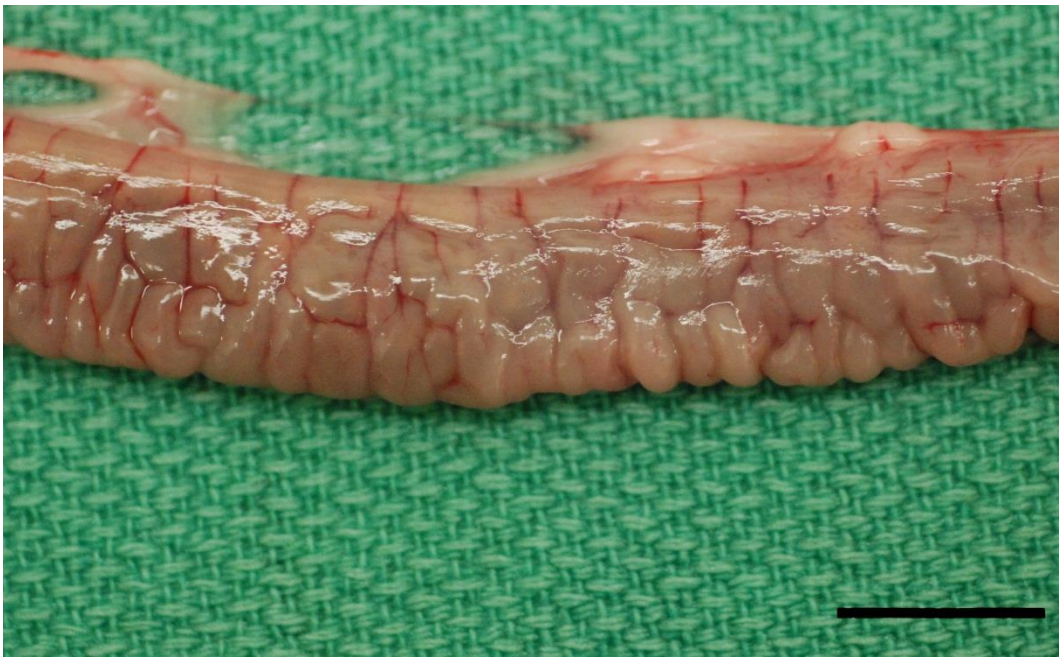


Figure 5.2: Infected rabbit jejunum. Example of the macroscopic appearance of a tract of jejunum, approximately 3.5 cm in length (bar's size: 1 cm), in rabbits infected with *L. intracellularis* PPE-strain. Note that the serosal edema and "cobble-stone" appearance on the anti-mesenteric aspect of the sample is reminiscent of the macroscopic lesions of the EPE infection model in rabbits, however such serosal appearance was not related to mucosal lesions in the PPE-infected rabbits.

5.4.3. Immunohistochemistry.

5.4.3.1. Hamsters.

In Trial 1, only 1 out of the 20 EPE-infected hamsters showed mild (grade 1) IHC labeling, which statistically was not different (*p* 1.0) from controls. The odds ratio showed 2.586 higher chances of getting a hamster infected when challenged with the inoculum versus an uninfected control. In Trial 2, a statistical difference (*p* 0.013) was noted between the infected and uninfected hamsters, with greatly reduced (0.04) odds ratios of having PE lesions in uninfected animals. However, comparing the presence of IHC labelled lesions between PPE-infected hamsters and uninfected controls increased the statistical significance (*p* 0.0003), whereas the comparison of PPE-strain and EPE-strain groups showed that the PPE-strain had higher ability than EPE-strain to infect hamsters (*p* 0.009) **(Figure 5.3.1. and Figure 5.3.2.)**. The relative risk of inducing IHC detectable lesions in hamsters after PPE-strain inoculation was 3 times higher than after EPE-strain inoculation, with an odds ratio of 35.29 in favor of PPE-strain infection. The results of the challenge in Trial 2 are **represented in figure 4**.

One last observation about EPE-strain is that there was no difference (*p* 0.28) found in either trial regarding age (3-wk-old versus 4-wk-old), level of stress (inoculated a week after weaning versus inoculated at weaning) or method of inoculation (oral feeding versus oral gavage), even though the relative risk appears 3.3 times higher in younger, stressed hamsters.

Figure 5.3.1.

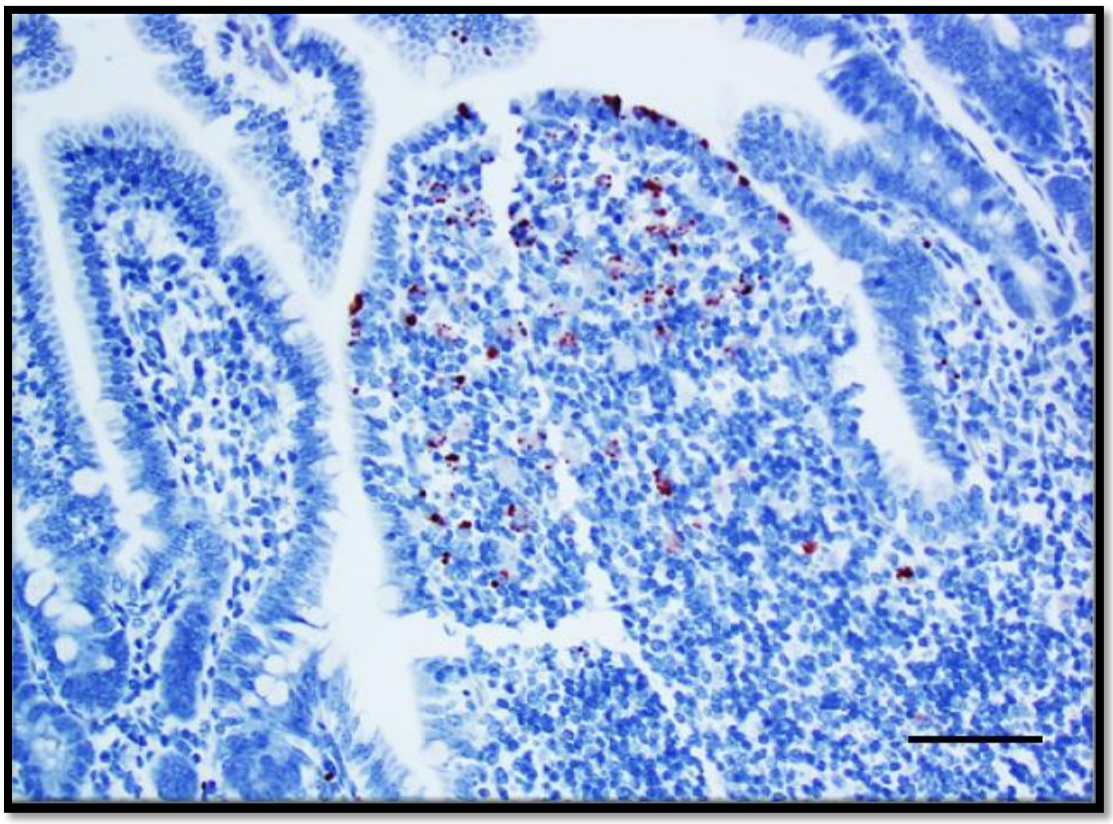


Figure 5.3. Immunohistochemistry (IHC) results in hamsters. Comparison of IHC labelling in the EPE-strain and the PPE-strain infected hamsters. Staining: streptavidin method, including anti-*L. intracellularis* specific mouse monoclonal antibody, with 3-amino-9-ethylcarbazol (AEC) substrate-chromogen and counterstained with Mayer's haematoxylin to observe for the presence of the antigen within the cells. **Figure 5.3.1: IHC of the ileum of an EPE-infected hamster**. Note that distribution of antigen (red dots) in the mucosal enterocytes of ileal villi is very limited and no antigen is detectable within the enteric crypts. Scale bar, 100µm.

Figure 5.3.2

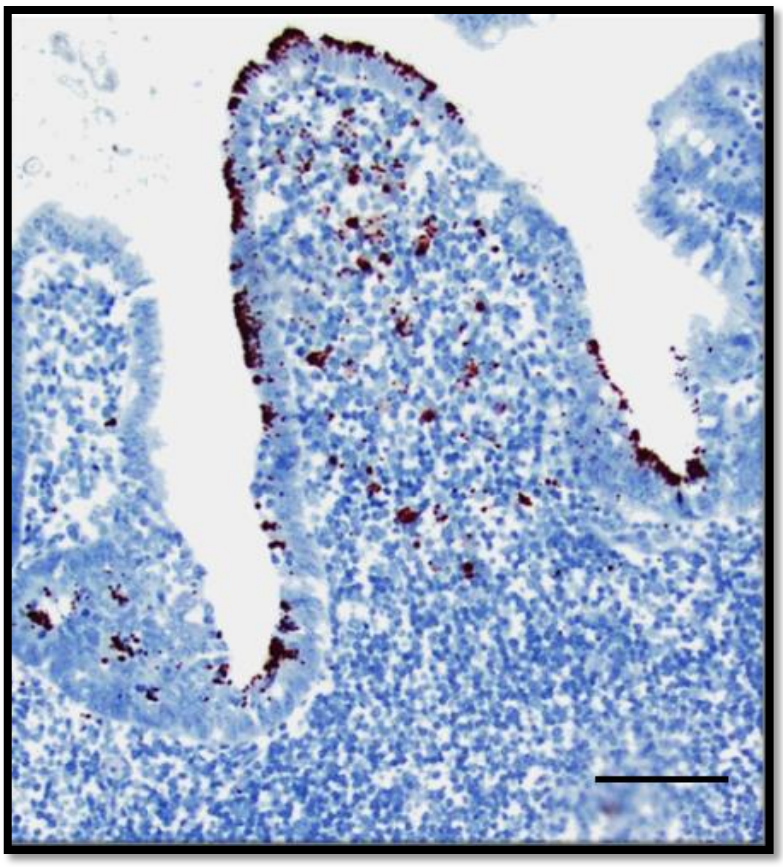


Figure 5.3.2: IHC of the ileum of a PPE-infected hamster. Note the much larger distribution of detectable antigen in the mucosal enterocytes. Scale bar, 100µm.

Figure 5.4.

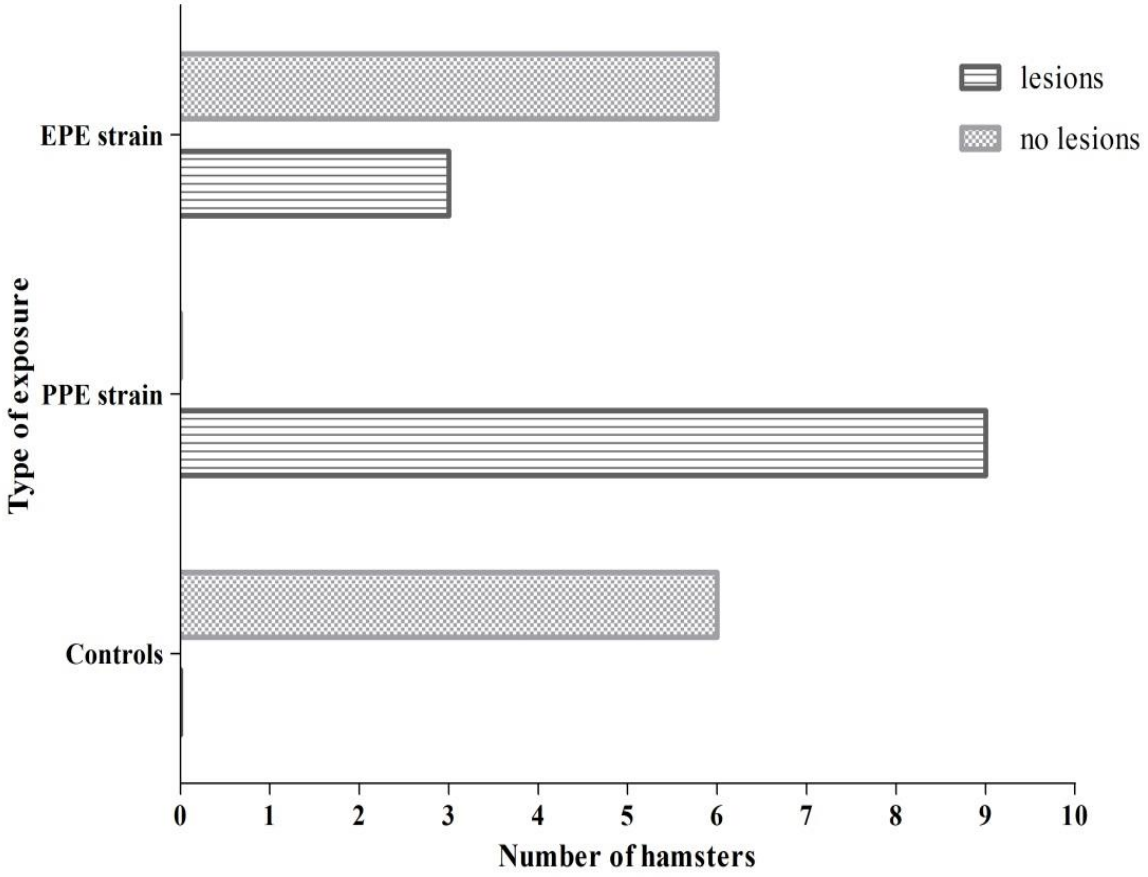


Figure 5.4.*:* Comparison between strains infection in hamsters. In this graph of Trial 2, the predominance of infection obtained in hamsters inoculated with the PPE-strain of *L. intracellularis* versus the EPE-strain and uninfected controls is shown. Note that all the PPE-challenged hamsters were showing IHC detectable antigen in typical lesion sites (enteric crypts and mucosal enterocytes), whereas only 3 out of 9 hamsters showed mild EPE lesions (not in the intestinal crypts) after the EPE-strain challenge.

5.4.3.2. Rabbits.

In the PPE-infected rabbit experiment, no characteristic lesions were detected through IHC labeling, at any stage of the study. Only fragments of reactive DNA at the level of the *lamina propria* were noted (**figures 5.5.1 and 5.5.2**). In the EPE-infected rabbits, euthanized at the same time, IHC detectable lesions were found in several rabbits in at least 2 GIT sections (jejunum and ileum) (Chapter 8), mirroring the initial model development results (Chapter 4).

Figure 5.5.1

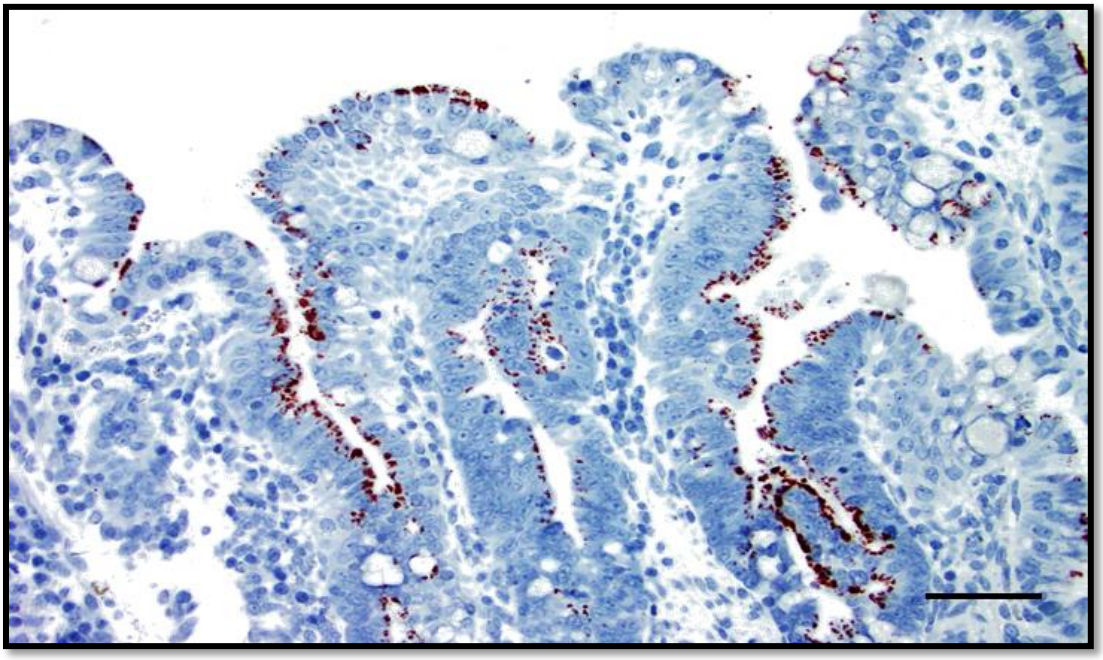


Figure 5.5: Comparison of IHC labelling in EPE- and PPE-infected rabbits. Staining: streptavidin method, including anti-*L. intracellularis* specific mouse monoclonal antibody, with 3-amino-9-ethylcarbazol (AEC) substrate-chromogen; counterstained with Mayer's haematoxylin to observe for typical proliferative lesions of the intestinal epithelium, and for the presence of the antigen within the cells. **Figure 5.5.1**: **EPE-infected rabbit, example of ileum at 14 DPI.** Note the wide distribution of IHC detectable antigen in the mucosal enterocytes over ileal villi and within enteric crypts. Scale bar, 100 µm.

Figure 5.5.2

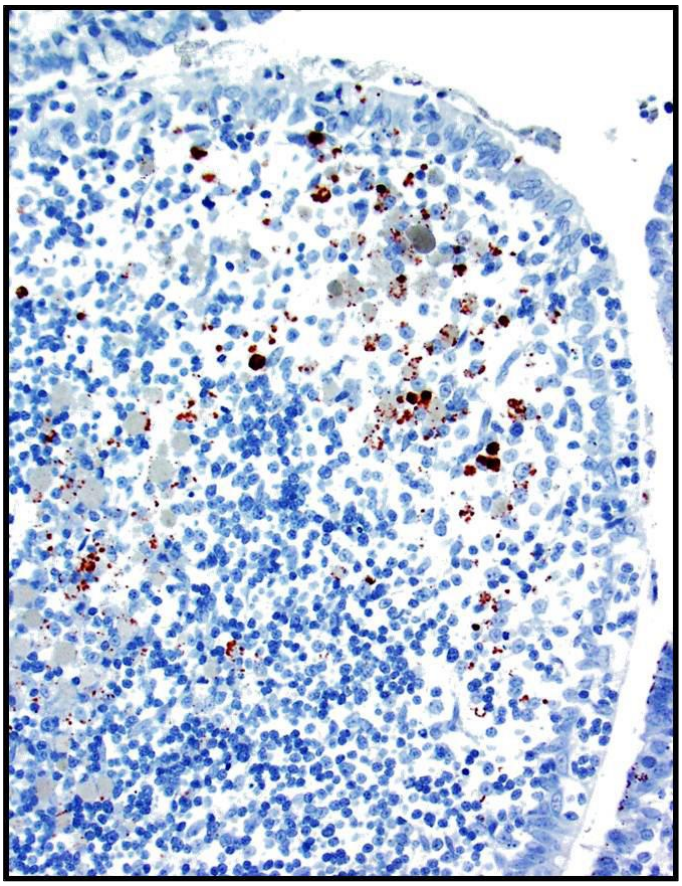


Figure 5.5.1*:* PPE-infected rabbit, example of cecum at 14 DPI. Scale bar, 100 µm. An ileal tissue counterpart for the PPE strain was unavailable as no antigen was detected anywhere, except in the cecum. Note that not only the distribution of detectable antigen in the mucosal enterocytes is minimal to none, but also the antigenic material appears scattered at the level of the *lamina propria*, wherein it is destroyed after being engulfed and processed by macrophages.

5.4.4. Serology.

Control samples for the IPMA procedure consisted of serum from a rabbit prior to (negative control) and after (positive control) hyperimmunization with *L. intracellularis*, purified from cell culture. No IPMA serology results consistent with immune response, were noted in hamsters in either trial, whether they were terminated at 21 or 24 DPI.

For the PPE infected rabbits, no antibody titers were evident until 14 DPI. The IgG titers at that time **(Figure 6)** appeared significantly lower (*p* .0023) in PPE-infected rabbits (range: 60 - 960) than the EPE-infected group (range: 480 - 1920) of rabbits of same age, sex, litter and housing. Serum total protein concentration was measured to investigate changes, as decreased values are reported in foals naturally and experimentally affected by EPE (Lavoie, *et al.*, 2000; Pusterla, *et al.*, 2010a). No statistical difference was noted overtime (*p* 0.88, as it ranged from 49 - 60 g/L on day 0; 45 – 53 g/L at 7 DPI; 40.5 – 49 g/L at 14 DPI and 47.5-53.2 g/L on 21 DPI) in PPE infected rabbits, although around 14 DPI, a decreased concentration in at least 4 rabbits was noted, soon to be recovered by the 2 survivor rabbits by 21 DPI.

Figure 5.6

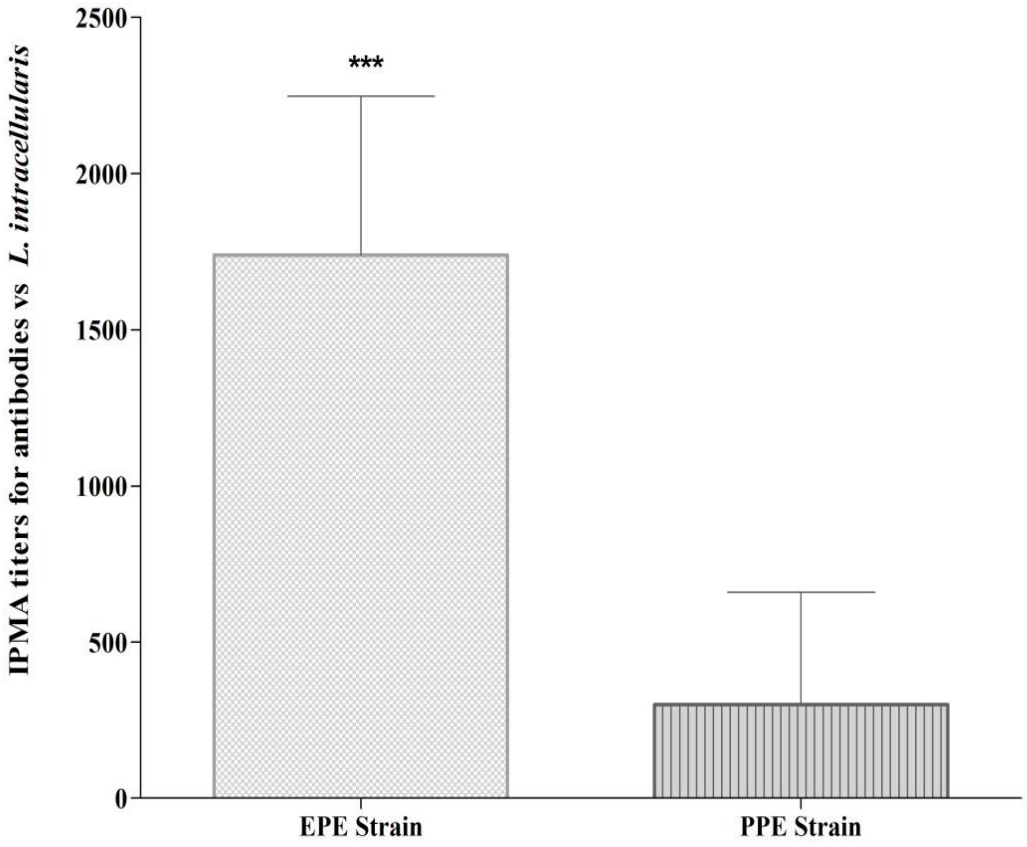


Figure 5.6: Serology results. Immuno-peroxidase monolayer assay (IPMA) findings indicating antibody response in EPE-strain and PPE-strain infected rabbits 14 DPI with *L. intracellularis*. The serological response at 14 DPI in EPE-infected rabbits is visibly different (*p* 0.0023) compared to PPE-infected ones. The bars' whiskers indicate standard deviations.

5.4.5. Quantitative PCR.

5.4.5.1. Hamsters.

On Trial 1, on day 0: 5 hamsters from the EPE-infected group showed positive fecal qPCR - one being quite high (693609.1 *aspA* gene copies/g feces - **see day 0 column on Figure 5.7.1.**). For Trial 1, qPCR results showed no difference (p 0.051) over time, when compared to the "day 0" group, although at the limits of significance **(Figure 5.7.1)**. However, by 21 DPI, 3 out of 4 infected hamsters were still shedding *L. intracellularis.* After infection with EPE-strain *L.intracellularis,* a comparison between 3 and 4-week-old hamsters, showed that younger hamsters have a higher likelihood of shedding *L. intracellularis* in feces (p <0.0001).

Conversely, in Trial 2 **(Figure 5.7.2)**, despite an initial higher fecal shedding of *L. intracellularis* for EPE-infected hamsters by 7 DPI, the overall comparison of the EPE-strain and PPE-strain over time, from day 0 to 24 DPI, showed higher shedding in the PPE-strain group (p 0.0003) related to both time and infection factors **(Figure 5.7.3)**.

Figure 5.7.1.

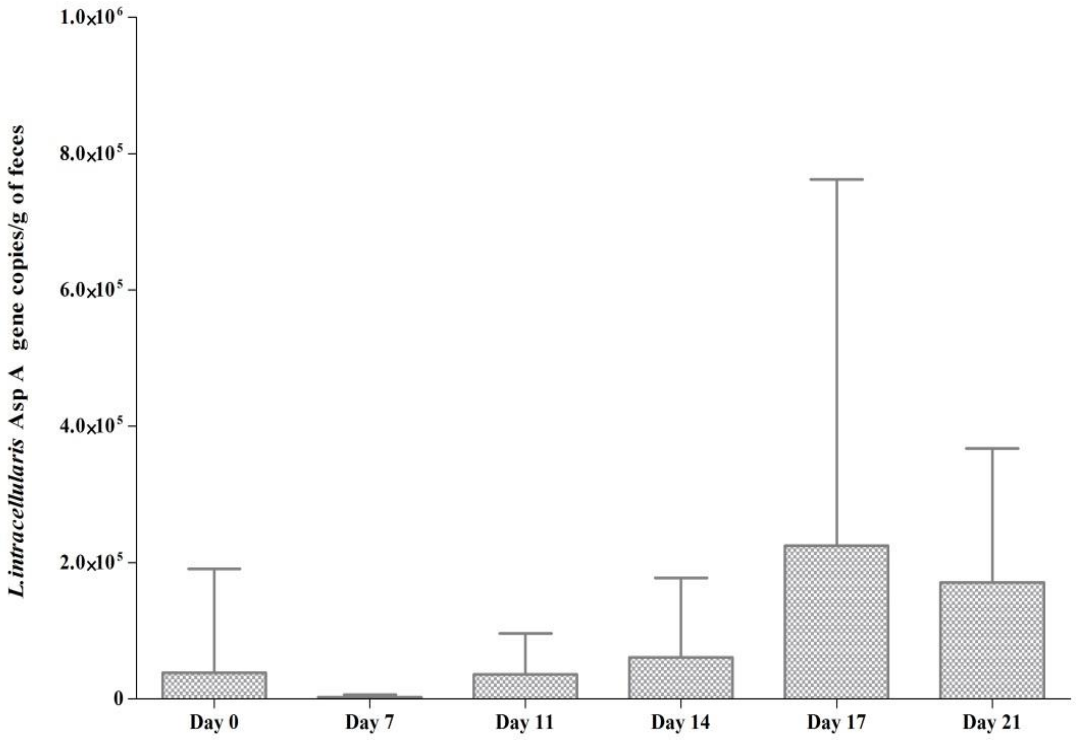


Figure 5.7. Detection of *L. intracellularis* DNA gene copies (*aspA* gene) through qPCR analysis in feces (grams) of experimentally infected weanling hamsters. For all 3 graphs, the bars' whiskers indicate the standard deviations. In **figure 5.7.1. fecal shedding for EPE-strain *L. intracellularis* in hamsters**, in the course of Trial 1, shown from day 0 to 21 DPI. On day 0 some unexplained technical contamination occurred while feeding orally the infectious inocula and its responsible for the measurable *AspA* gene in the material collected on inoculation day. The shedding was minimal at 7 DPI and then increased over time. Because of the technical contamination observed, the method of inoculation was changed for Trial 2. Note that the concentration of the *aspA* gene is barely in the range 10^5-10^6, particularly when compared to the PPE-infected hamsters (10^7-10^8 range).

Figure 5.7.2.

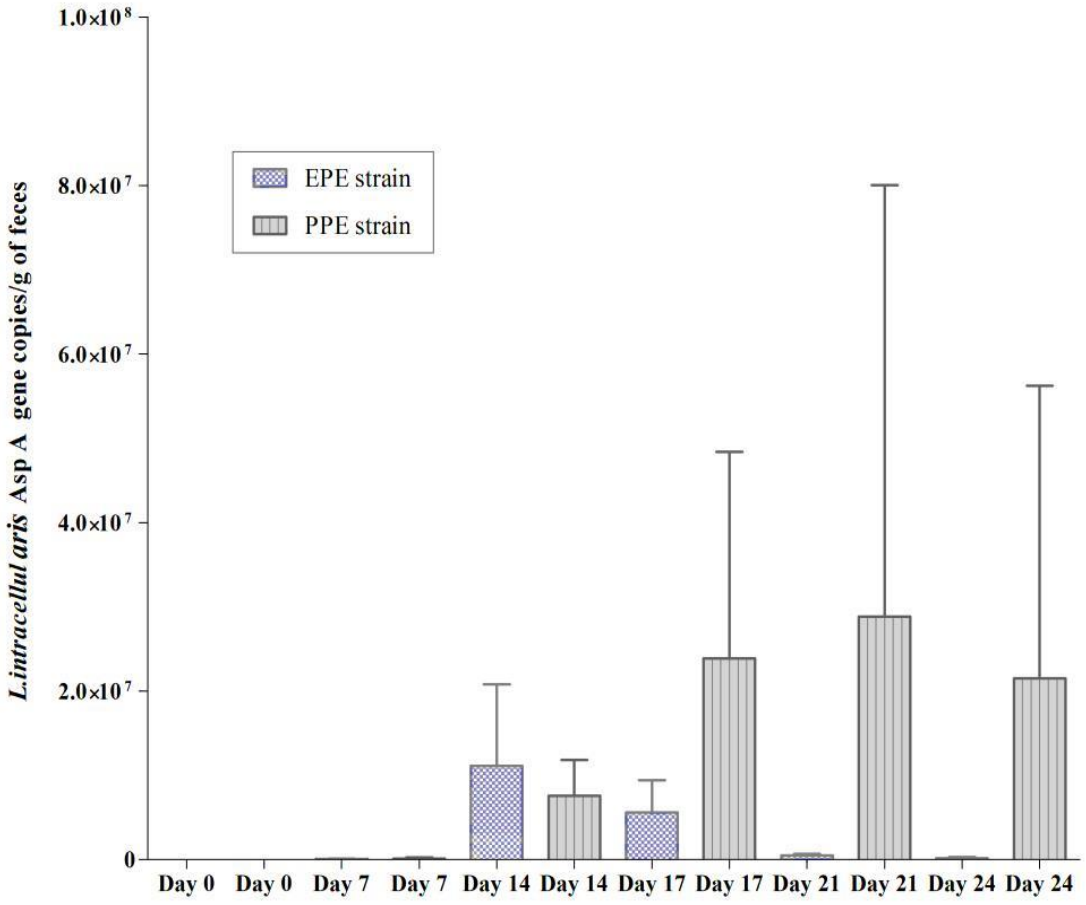
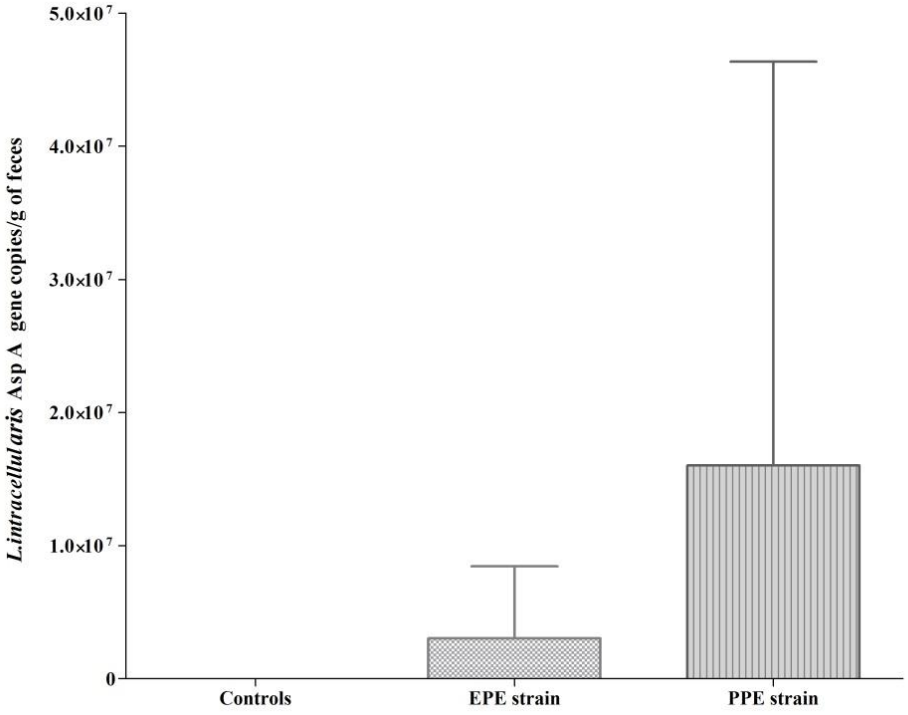


Figure 5.7.2. Fecal shedding in hamsters, for EPE-strain and PPE-strain *L.intracellularis* in the course of Trial 2, shown from day 0 to 24 DPI. While the PPE-strain fecal shedding increases exponentially over time, the EPE-strain fecal shedding is highest 14 DPI and then decreases significantly over time (*p* 0.003) to the end of the experiment (24 DPI).

Figure 5.7.3.



In **figure 5.7.3. Overall comparison between strain group in hamsters** (uninfected controls, EPE and PPE). The different amounts of shedding amongst groups are visible (*p* 0.033).

5.4.5.2. Rabbits.

A comparison between the detection of PPE-strain versus EPE-strain *L. intracellularis* is **shown in figure 5.8.**, where higher fecal shedding is noted in the EPE-infected rabbits (*p* 0.0089).

Figure 5.8.

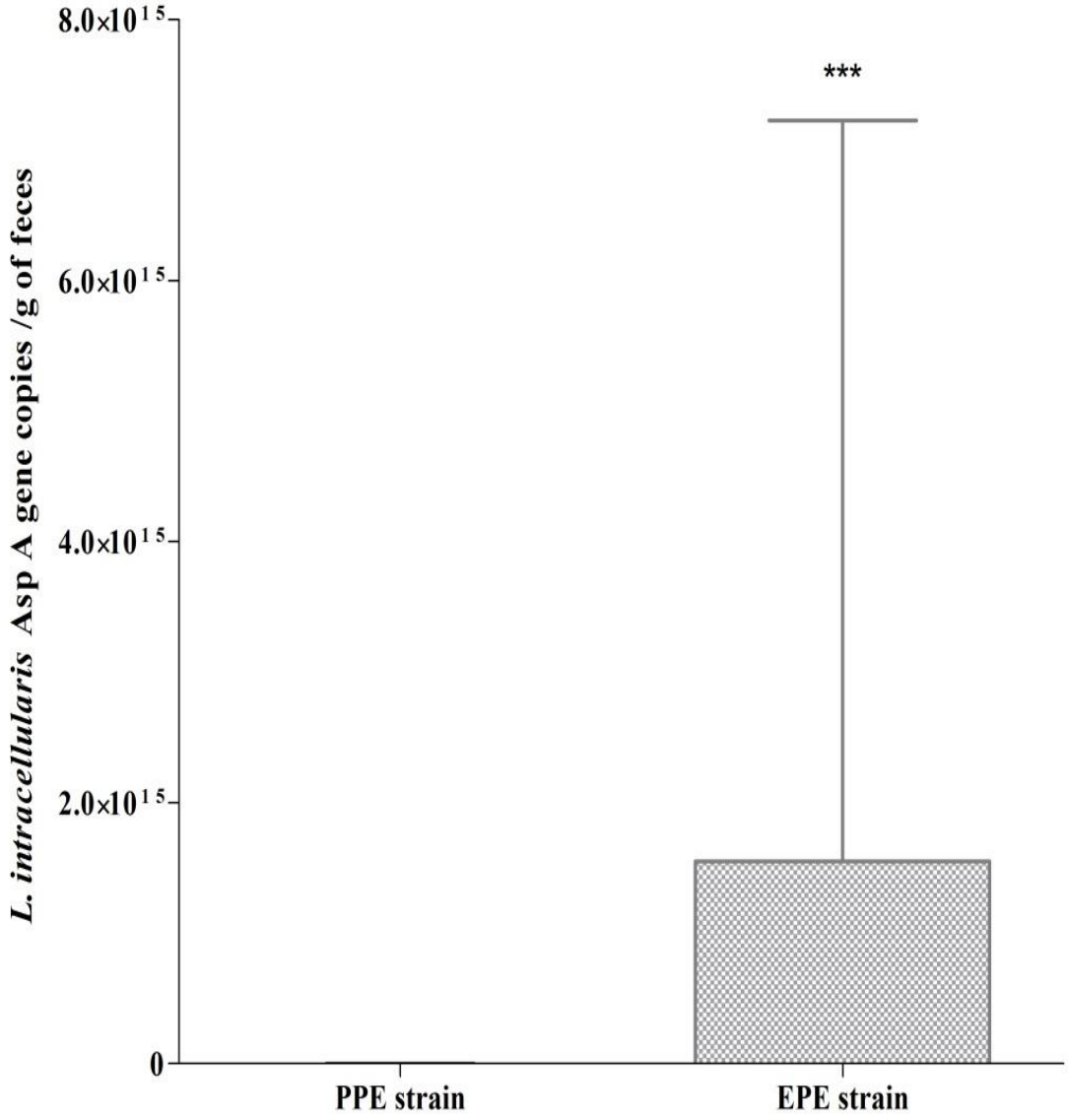


Figure 5.8. qPCR results in rabbits. Comparison of *L. intracellularis aspA* gene copies/g of feces obtained through qPCR between EPE-infected versus PPE-infected rabbits (*p* 0.0089). The bars' whiskers indicate the standard deviation. Note that the concentration of the *aspA* gene in EPE-infected rabbits' feces is so high that the PPE-strain infected group values don't appear in the graph, as they remained several orders of magnitude lower (10^6 or 10^7, at the most).

5.6. Discussion

These studies show that *L. intracellularis* cross-infection in laboratory animals can occur, but not with ease. The goal was to reproduce PE disease, or lesions, from equine and porcine strain isolates, so to individuate a surrogate species small enough to be cost effective, yet sufficiently large to endure humane research procedures, as, for example, repeated blood samples.

The PPE infection model in hamsters was considered the term of comparison of our hamsters' studies. Replication of infection in hamster weanlings, from a pure cell culture of PPE-strain of *L. intracellularis*, isolated from naturally diseased pigs, was in agreement with previous publications, as inoculation of hamsters yielded a typical PE infection (Stills, 1991; Jasni, *et al.*, 1994a; Jasni, *et al.*, 1994b; Vannucci, *et al.*, 2010). In the infected hamsters of these studies, no clinical signs were noted, as is commonly reported with pure cell culture inoculation, both in hamsters and pigs (Jasni, *et al.*, 1994b; Guedes & Gebhart, 2003b; Vannucci, *et al.*, 2010). The only exception was some degree of hyperactivity during daylight hours, atypical for hamsters, as they are nocturnal animals. In addition, intestinal lesions were detected by H&E and confirmed with IHC labelling. Quantitative PCR showed that *L. intracellularis* DNA was shed in feces in exponentially increasing concentrations throughout the study (24 DPI), demonstrating active replication of the bacteria in the host's intestine (McOrist, *et al.*, 1989; Stills, 1991; Cooper, *et al.*, 1997a; Cooper, *et al.*, 1997b; Vannucci, *et al.*, 2010).

The leading hypothesis supporting the idea of the EPE-strain of *L. intracellularis* being capable of infecting hamsters was based not only on the similarities of the bacterial genomic characteristics (98% between PPE and EPE strains), but also on how easily the PPE-strain can cross-infect from pigs to hamsters (Jasni, *et al.*, 1994b; Cooper, *et al.*, 1997a; Cooper, *et al.*, 1997b; Vannucci, *et al.*, 2010). Furthermore, GIT similarities between hamsters, rabbits and horses, such as hind-gut fermentation and a proportionally large cecum, were considered advantageous, even in the absence of evolutionary correlations (Argenzio, 1984). The cross-infection of hamsters with the EPE-strain, starting from a pure cell culture isolate, virulent for both horses and rabbits, was attempted in Trial 1 in young susceptible animals (4-week-old) and, since it failed to generate detectable IHC lesions, was tried again on younger and potentially

more vulnerable subjects (3-week-old) (Pusterla, *et al.*, 2012a; and Chapter 4). One should note that in Trial 1, a contamination of the fecal material on day 0 appeared evident in 5 of the 20 inoculated hamsters **(see figure 5.7.1., day 0)**, despite having followed rigorous experimental procedures in handling each subject. Although such an outcome was unexpected, potential contamination of the feces with fresh inoculum was a plausible explanation. Interestingly, *L.intracellularis* DNA material reached values higher than the ones detected in the first 11 DPI, with maximum values in the range of $2.0x\ 10^5$ AspA gene/g. In the whole experiment, the DNA concentration never rose above the order of magnitude of 10^5, in contrast to the concentrations detected in PPE infected hamsters (largely over 2 orders of magnitude higher at weeks 2 and 3 PI).

In Trial 2, the component of stress (carefully avoided in Trial 1) as well as young age, was added as hamsters were infected immediately after weaning. Despite 2 attempts, EPE-strain cross-infection of hamsters was unsuccessful, aside from a mild and inconsistent fecal shedding, which, even at its maximum value, was never as high as the PPE-infected hamsters' shedding (at disease peak between 17 and 24 DPI). This is particularly striking in comparison to the textbook-like development of fecal shedding and lesions in PPE-infected hamsters. Although our results originated from a small population, the fact that PPE-challenged hamsters have over 35 times more chances to develop lesions than the EPE-challenged ones is an obvious limitation. It should discourage the further use of hamsters to replace horses in EPE infection models. On a wider perspective, these results constitute evidence to reconsider the ability of the EPE-strain of *L. intracellularis* to cross-infect and cause lesions into other rodents, contrarily to what was seen for the PPE-strain, whether it depends on their resistance to infection, or on specific bacterial tropism for specific hosts (Murakata, *et al.*, 2008; Vannucci, *et al.*, 2010).

As no serologic response was noted in either hamster trials, it is arguable that hamsters may need a longer time to develop a detectable immune response to *L. intracellularis* infection: partially, due to the *L. intracellularis* privileged location, remote from processing, opsonizing and exposing units of the reticulo-endothelial system; partially, because weanling hamsters may not be as immuno-competent as adults are. Older subjects, in other infection models, have been shown to develop an IgG response in a much more timely fashion (1 week PI) (Zuerner, *et al.*, 2011). Based on lack of seroconversion and of limited fecal shedding, the question now is

whether EPE-challenged rodents can become healthy short-term carriers for the disease, and in what capacity they contribute to the amplification of infection.

In this context, failing to reproduce a viable PPE-infection in rabbits is significant, as no clinical signs (even as reduced growth performance), nor IHC lesions were observed in our PPE-challenged rabbits (Chapter 4). A transient IHC labelling was noted in the *lamina propria* of only 1 sample out of 6 whole GIT sets of samples (42 samples total), consistent with destruction of the bacteria, but not with typical active lesions, which should be located at the apex of enterocytes, in the intestinal crypts. This shows that the PPE-strain *L. intracellularis* is eliminated swiftly by rabbits, despite inoculation with almost twice the dose used in the EPE-challenged rabbits (Chapter 4).

A recent publication showed that the EPE-strain *L. intracellularis* is unable to cross-infect age-susceptible pigs and the PPE-strain is unable to cross-infect age-susceptible foals, thus the results of this study on laboratory animals acquire even more relevance (Vannucci, *et al.*, 2012). The incapability of the EPE-strain to cause demonstrable infection in hamsters mirrors the results of cross-infection onto pigs, although it is clearly capable of infecting and inducing clinical disease and lesions in young horses, as well as cross-infecting rabbits, with mild clinical discomfort, typical intestinal lesions, limited fecal shedding and high serologic response (Vannucci, *et al.*, 2012; and Chapter 4). On the contrary, the PPE-strain *L. intracellularis* is capable of infecting and causing overt (in some case) disease in pigs and hamsters, but does not cause infection in rabbits nor horses, although in rabbits a limited immune-response and fecal shedding were detected as well, reminiscent of some exposure results in sentinel rabbits used in piggeries (Duhamel, *et al.*, 1998; Guedes & Gebhart, 2003b; Vannucci, *et al.*, 2010). Perhaps a secondary host-adaptation, or species-specificity, exists for different *L. intracellularis* strains (Friedman, *et al.*, 2008; Vannucci, *et al.*, 2012; and Chapter 4). In hamsters cross-infected with PPE-strain, the most severe clinical signs were caused by inoculation with homogenates of porcine mucosal ileal scrapings (Stills, *et al.*, 1989; Jasni, *et al.*, 1994a; Jasni, *et al.*, 1994b; Vannucci, *et al.*, 2010). Thus, future studies may determine whether the role of cell culture passages of the infectious material through cell cultures hampers the virulence in EPE-strains too, as we know that higher passages of PPE-strain in cell cultures definitely immortalize *L. intracellularis*, but reduce its virulence.

In conclusion, although using only a small number of animals, our investigations help clarify whether different strains of *L. intracellularis* have different secondary host-specificities. Such findings should help to exclude hamsters from modeling EPE-strain infection and rabbits from modeling PPE-strain infection. However, as EPE modeling has been achieved in rabbits, 2 important concepts may be extrapolated from our results: first, studying the infection caused by *L. intracellularis* may necessitate more than one animal model; and, second, different wildlife species, potentially, could be a connecting link between differently adapted infectious strains (Pusterla, *et al.*, 2008b).

CHAPTER 6:

PROTECTING SHORT-TERM EAR CATHETERS IN HEALTHY RABBITS.

Relationship of this study to the dissertation objectives.

In this chapter the results of a technique's refinement study are presented. When the pharmacokinetic study (Chapter 7) was started, several difficulties in maintaining auricular catheters in rabbits were noted, hindering the timeliness and the feasibility of sample collection. As the subjects in the study, albeit infected, were not sick or depressed, and behaved not at all differently than healthy rabbits, the catheter's bandaging, usually appropriate for sick animals, proved to be inadequate. The bandaging technique described in this chapter not only secured the catheter to the ear and allowed repeated and timely samples for the pharmacokinetic study; but also allowed us to release the rabbits in their pen between samplings, promoting normal behavior and decreasing stress.

This material has been published as: Sampieri, F., Orchard, R.N., Antonopoulos, A.J. & Hamilton, D.L. (2012). Protecting short-term intravascular ear catheters in healthy rabbits. *Lab Animal*, **41**, 44-48.

6.1. Abstract

Often in rabbits used for research, the catheterization of the ear's vessels is chosen, because it requires minimal restraint. Healthy rabbits are challenging with regards to maintaining indwelling catheters in their ears, as they tend to scratch catheters out or cause large hematomas that impair blood sampling. This case study was generated by the necessity of collecting, for 12-hour terms, multiple blood samples in rabbits enrolled in a pharmacokinetics study. Nine juvenile does, divided in 3 groups of 3, had an intra-arterial short-term catheter placed in the ear. The novel aspect of this technique consisted of protecting the indwelling catheter, by covering it with a section of moleskin, in the same shape as the ear. Catheters remained patent for repeated sampling in 5 rabbits for 12 hours; in 2 rabbits for 8 hours, 1 rabbit for 6 hours, and 1 rabbit for 4 hours. This method, using a lightweight bandage, resulted in minimal interference to the rabbits.

6.2. Introduction

Healthy rabbits, more commonly as research subjects than patients, might need an intravascular catheter for a short period of time, so that repeated blood samplings can be performed with minimal restraint and distress. The common target of indwelling catheters in rabbits, for both clinicians and researchers, is to preserve the patency of the main blood vessels (arteries or veins) and to avoid trauma, but the necessary approach is perceived in a dichotomous way.

The recommendation of clinicians is to collect blood through venipuncture of either the jugular or the saphenous veins, leaving the cephalic vein as a privileged site for potential insertion of an intravenous indwelling catheter (Graham, 2006). Other authors indicate that, for a slow rate administration of drugs or intravenous fluids, the ear's marginal vein is the easiest to approach, particularly in sick and critical patients (Harcourt-Brown, 2002b). However, commonly it is suggested not to use the ear's central artery and marginal vein, as there is a high incidence of hematomas, potential arterial damage and subsequent skin sloughing at the ear's tip (Harcourt-Brown, 2002b; Graham, 2006). Undoubtedly, it is better for the clinician facing a critical patient, to attempt blood sampling from a large vessel, rather than trying to obtain a

significant amount of blood from small vessels on the ear's delicate surface (Paul-Murphy, 2007). However, blood sampling from the ideal sites listed above requires the presence of a technician, or an experienced handler. Appropriate restraint is the main limiting factor to a careful sampling procedure, in critical patients to minimize their stress; and in healthy research subjects, to limit their normal grooming and scratching reactions (Graham, 2006).

The researchers working with rabbits target the ear's marginal vein and ear's central artery, as such approach requires minimal restraint and causes less stress, and are therefore commonly accepted as sampling sites (Diehl, *et al.*, 2001). In research, it is necessary to satisfy many experimental needs, not least, the fact that samplings often need to be feasible without assistance (*e.g.*, night sampling). Research rabbits usually are not sick and depressed, as clinical patients can be. They get accustomed over time to mechanical restraint devices and are comfortable when so kept, for short periods of time (Suchow & Douglas, 1997). In other studies, restraint jackets are utilized with multiple functions, beside restraint, such as protection for bandages, or carriers for drug administering pumps (Clifford, *et al.*, 2003). In these rabbits the main challenge is to maintain an indwelling catheter, regardless of the period's length, because they are mostly healthy and behave accordingly. Rabbits usually resist indwelling catheters and their protections, by scratching or biting them. Thus, they often have to wear Elizabethan collars, which are a notable source of stress for the rabbits, as shown by the changes in their glycaemia (Knudtzon, 1984). Furthermore the presence of an Elizabethan collar, as it limits both regular feeding and cecotrophy, can lead to inappetence and anorexia, unless rabbits are acclimated to wear it, before the experiments begin (Harcourt-Brown, 2002b; Clifford, *et al.*, 2003; Graham, 2006).

During the initial phase of a pharmacokinetic study, while working with 3 healthy rabbits, this research team encountered many difficulties in maintaining working catheters, preventing trauma to the ears and bandaging ears and catheters in a scratch-proof manner. In the experiments, multiple, small volume (1 ml), blood samples were needed at specific time-points, initially concentrated within a 12-hour time-frame. Thus, this study aimed to improve the management of ear catheters in the short term, catheterizing the animals only once and preventing multiple punctures of the vessels. This method was sought to provide rabbits with

their customary freedom of movement and to allow researchers the peace of mind of a working catheter.

6.3. Materials and methods

6.3.1. Animals.

Nine New Zealand White female rabbits (Charles River Canada, Pointe Claire, QC, Canada), 8 to 10 weeks of age, body weight range of 2.1 - 2.8 kg, were enrolled in this research case study. The catheterization study was conducted on 3 groups of 3 does, each equally handled and treated, which participated in a pharmacokinetic study (same does of the experiments described in Chapter 7).

6.3.2. Ear Preparation.

After cleaning and drying the outside aspect of the ear's pinna, a topical anesthetic cream (EMLA Cream Astra Zeneca Canada Inc., Mississauga, ON, L4Y 1M4 Canada) was applied in the area above the ear's central artery, for two thirds of its length, excluding the tip, as the catheter was placed in the rostral portion of the artery. A 30-45 minute interval was allowed to obtain appropriate anesthetic effects.

The proper placement of an arterial catheter requires typical animal restraint, to minimize the chances of damaging the artery. A mechanical restraint device **(visible in Figures 6.1 to 6.5)** was used. The ear selected for catheterization was gently directed laterally from the rabbit's head, to allow full access to the artery. The fur on the pinna's outer surface was shaved with clippers (blade #40 - Oster Golden A5,J. Tarnow Company Ltd., Toronto, ON, M6A 2X5 Canada), assuring that no hair was left on the ear's portion to be covered by the catheter protection **(visible in Figure 6.1.1.)**.

Figure 6.1.1.

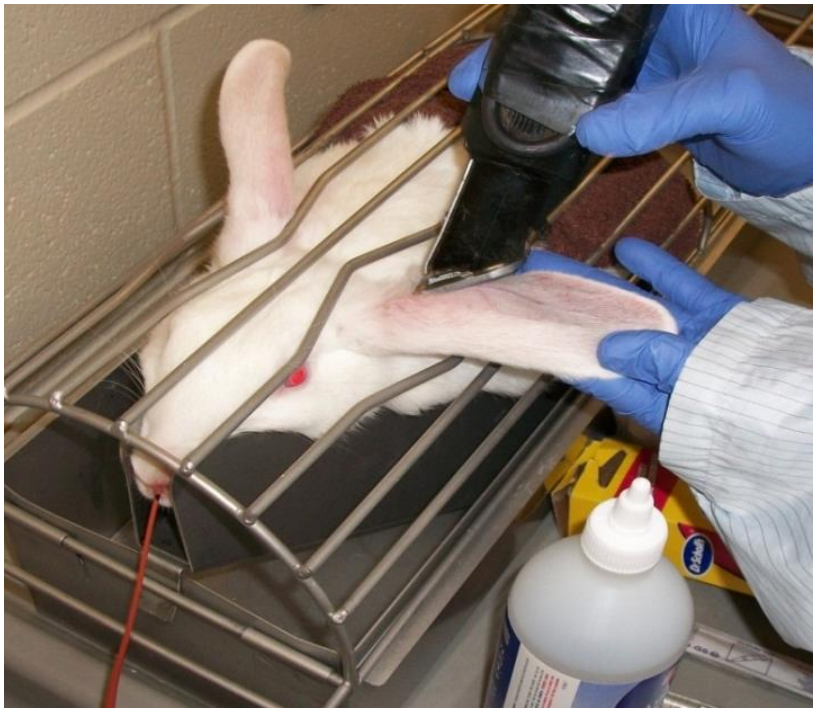


Figure 6.1.1. **Ear's preparation.** Once the rabbit is properly restrained and the ear's skin above the artery has been locally anesthetized, the fur can be shaved off.

6.3.3. Preparation of supplies

Moleskin (Dr. Scholl's Extra Soft Moleskin. Imported by Schering Canada Inc., Pointe Claire, QC, H9R 1B4 Canada): It was chosen to cover the catheter and the catheter's insertion site, with the idea of somehow simulating a "skin-embedded" injection port. The moleskin coverage was used as it holds and protects the catheter, while still allowing the rabbit free, unhindered movement of the ear. The size of the moleskin section was pre-measured and pre-cut in the shape of the ear **(Figures 6.1.2. and 6.1.3.)**, so that the moleskin's edges did not extend beyond the ear's margins. The moleskin covered from the base of the ear, to 2 – 3 cm from the tip.

Catheter (BD Insyte - Becton Dickinson Infusion Therapy Systems Inc. Sandy, UT, 84070 USA)**:** After opening the package and ensuring that the catheter moved freely from its stylet, the catheter was pre-flushed with heparinized saline.

Tissue glue (3M Vetbond - 3M Animal Care Products, St. Paul, MN, 55144 USA): Although the underside of Dr Scholl's moleskin is prepared with an adhesive layer, tissue glue was used to reinforce sealing and adhesion of the cover to the skin. In the absence of tissue adhesive, Krazy Glue (Instant Krazy Glue - Imported by Elmer's Products Canada Corp., Toronto, ON, M1P 28B Canada) can be used, although a greater thermal reaction would be expected.

Figure 6.1.2.

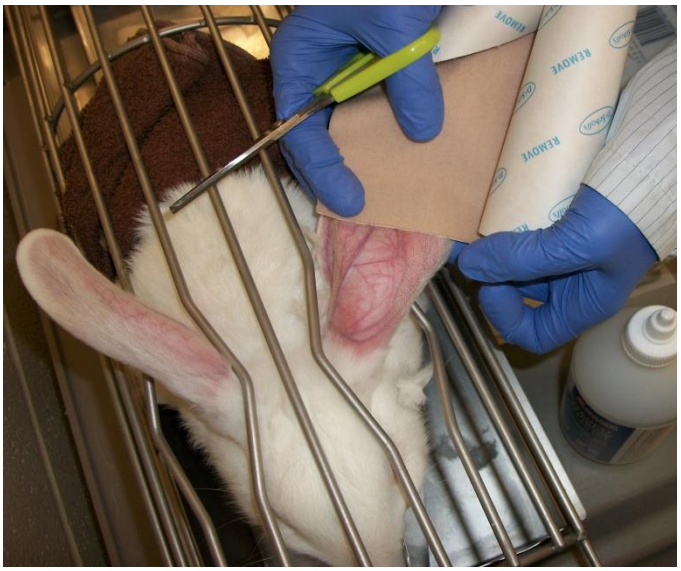


Figure 6.1.2. Preparation of the moleskin: Step 1. The moleskin width is measured to the breadth of the rabbit's auricular pinna.

Figure 6.1.3.

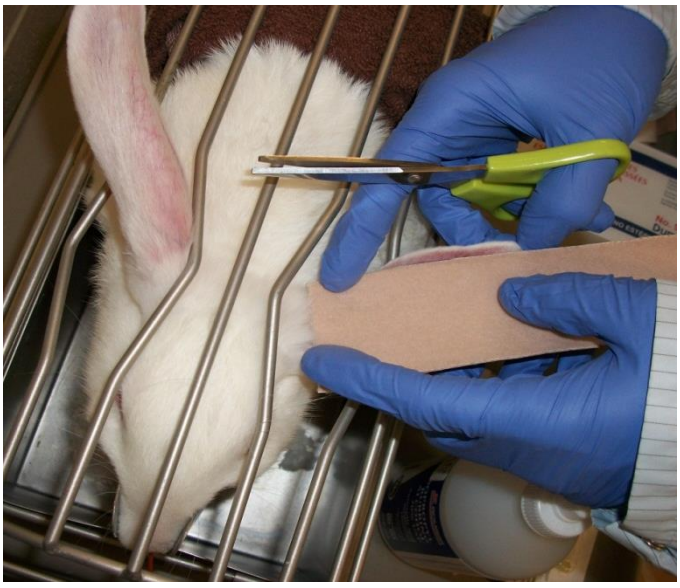


Figure 6.1.3. Preparation of the moleskin: Step 2. Measure the moleskin length to the desired length on the rabbit's auricular pinna. Paper scissors are more appropriate for this initial preparation step.

6.3.4. Placement of the catheter and protection cover

The entire procedure is **illustrated in Figures 6.2.1 through 6.2.6**. The catheter was aligned directly with the artery, near the ear's midpoint, taking care of not placing it too far close to the tip of the ear, as the weight of the catheter could contribute to the ear flopping. As the skin must be dry for the moleskin to adhere, the catheter was inserted using gauzes to catch any blood spills, before the placement of the injection port. Keeping the catheter in line with the artery, the catheter's hub was glued to the ear with tissue glue.

The pre-cut moleskin was placed near the ear's base, after covering the adhesive side with tissue glue, which was applied to the rest of the moleskin's adhesive surface, particularly near the edges. It was then laid over the entire catheter's hub and injection port (Intermittent Infusion Plug - Kendall Argyle TYCO Healthcare Group LP. USA) **(Figures 6.3.1. to 6.3.2.)** and all the moleskin edges were pressed down. Careful not to perforate the injection port or the ear below the moleskin, a small hole was cut in the moleskin, with a #11 scalpel blade, to allow the injection port to "peep" out, for the purpose of sampling. Thus, the effect of "skin embedding" was obtained.

Figure 6.2.1.

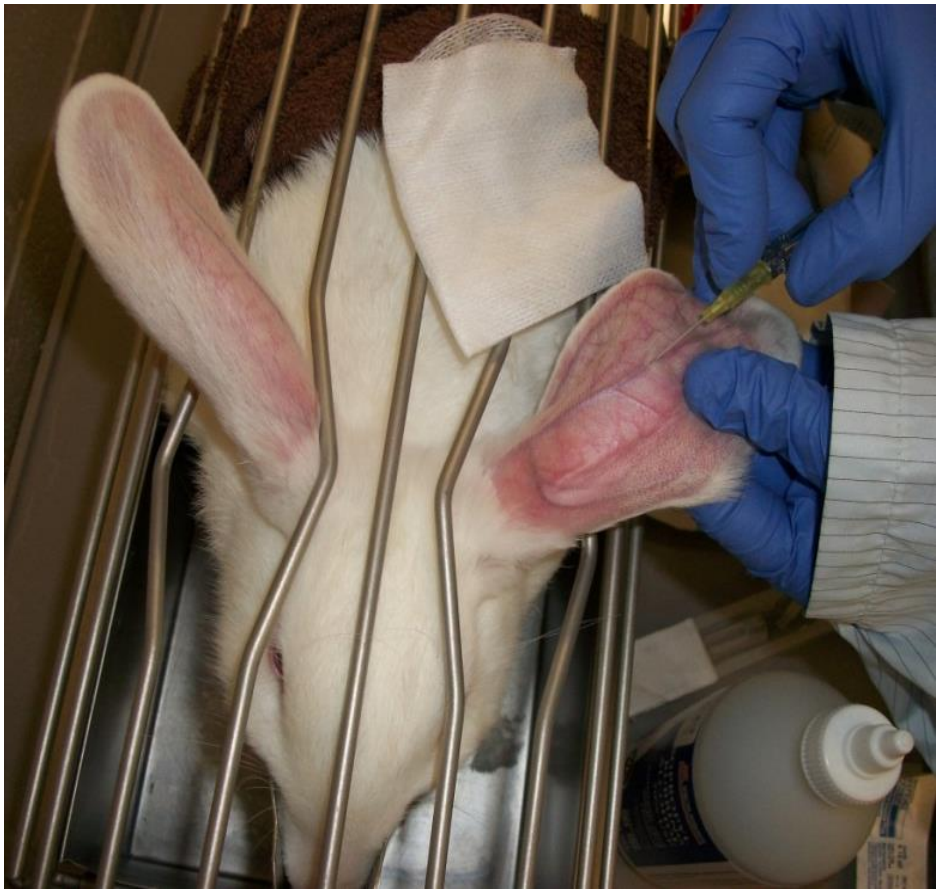


Figure 6.2.1. Catheter insertion: Step 1. An arterial catheter is inserted after the skin over the ear's central artery has been locally anesthetized, shaved and scrubbed. Prior to insertion, the catheter is filled with sterile heparinized saline.

Figure 6.2.2.

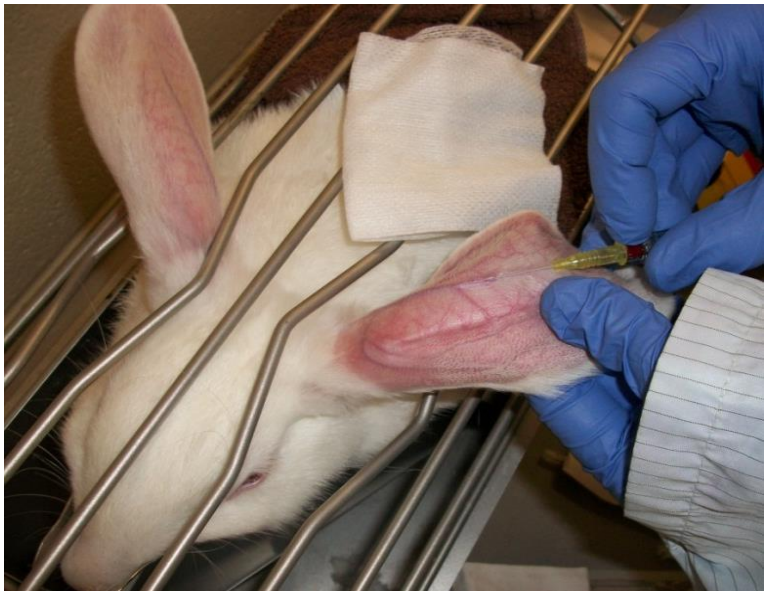


Figure 6.2.3.

Figures 6.2.2. and 6.2.3. Catheter insertion: steps 2 and 3. As soon as blood is noted in the hub of the catheter, gauze is used to prevent the area surrounding the catheter from getting wet. A key point of this technique is to have the ear's skin clean and dry throughout the procedure.

Figure 6.2.4.

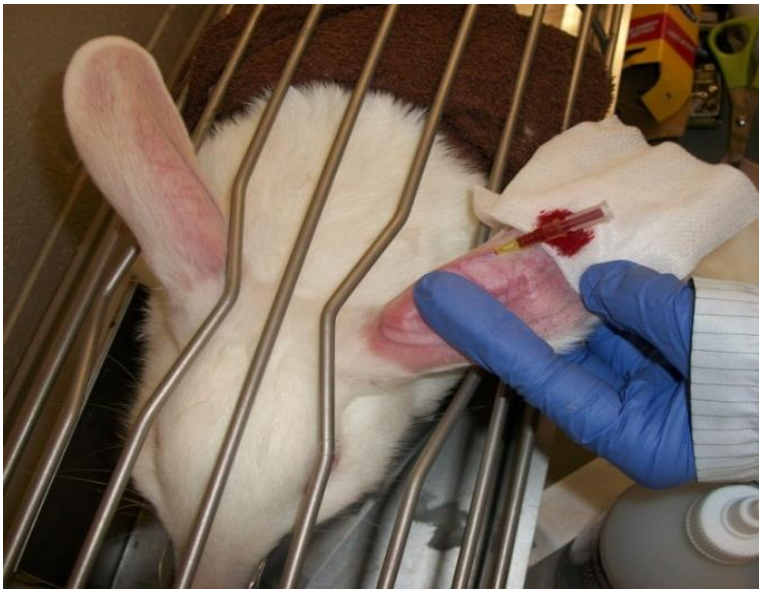
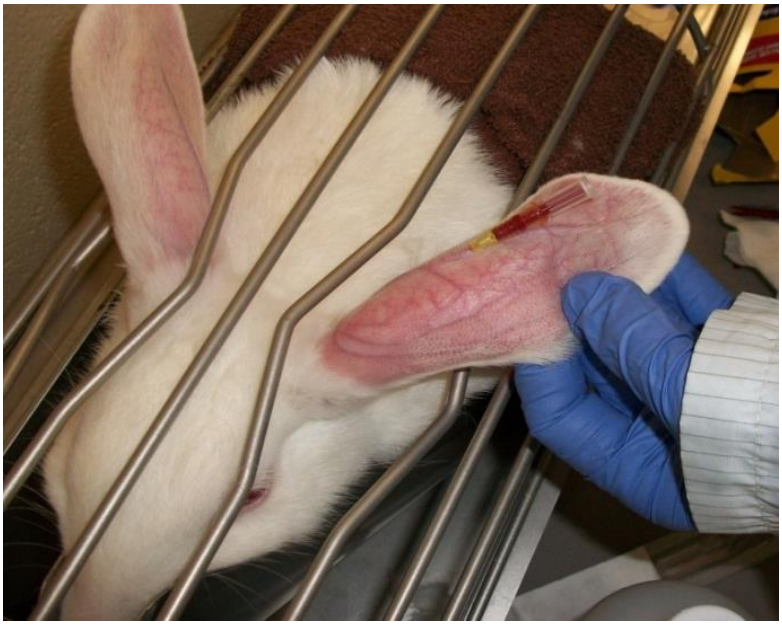


Figure 6.2.5



Figures 6.2.4. and 6.2.5. Catheter insertion: Steps 4 and 5. The catheter is capped with a temporary cap.

Figure 6.2.6.

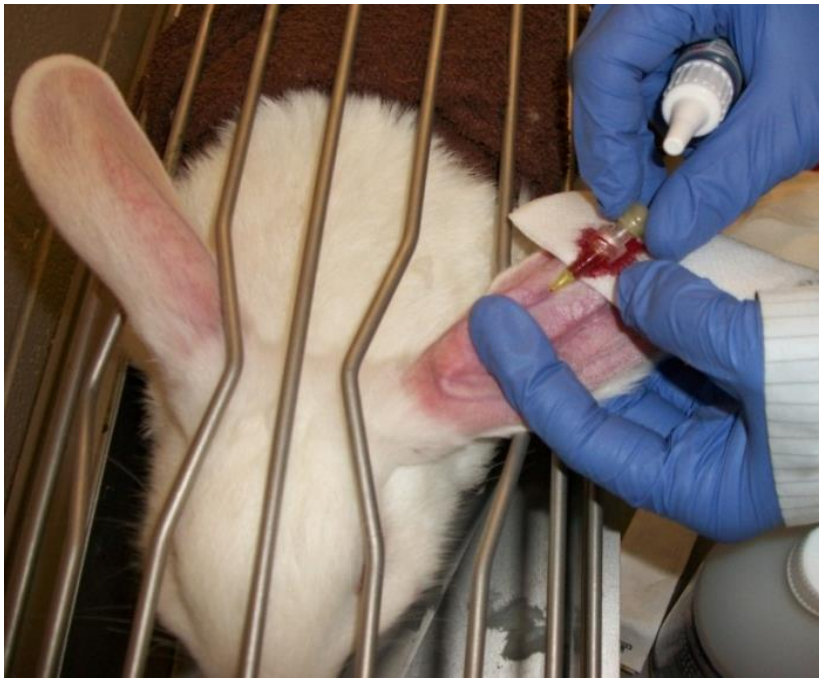


Figure 6.2.6. Catheter insertion: Step 6. After changing the gauze to a clean (and dry) one, a screw-on injection port is attached to the catheter (2f).

Figure 6.3.1.

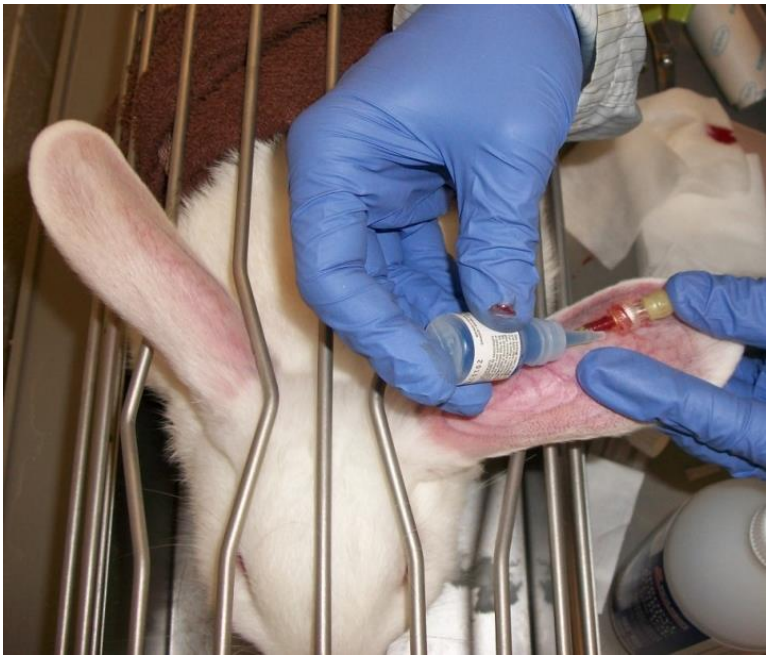


Figure 6.3.1. Securing the catheter and the moleskin: Step 1. Tissue glue is distributed around the catheter's hub and crown, so it stays in place.

Figure 6.3.2.

Figure 6.3.2. Securing the moleskin: Step 2. The moleskin paper protection is removed and the moleskin is placed appropriately on the rabbit's auricular pavilion. Tissue glue is distributed on the sticky surface of the moleskin, in particular close to the edges.

Figure 6.3.3.

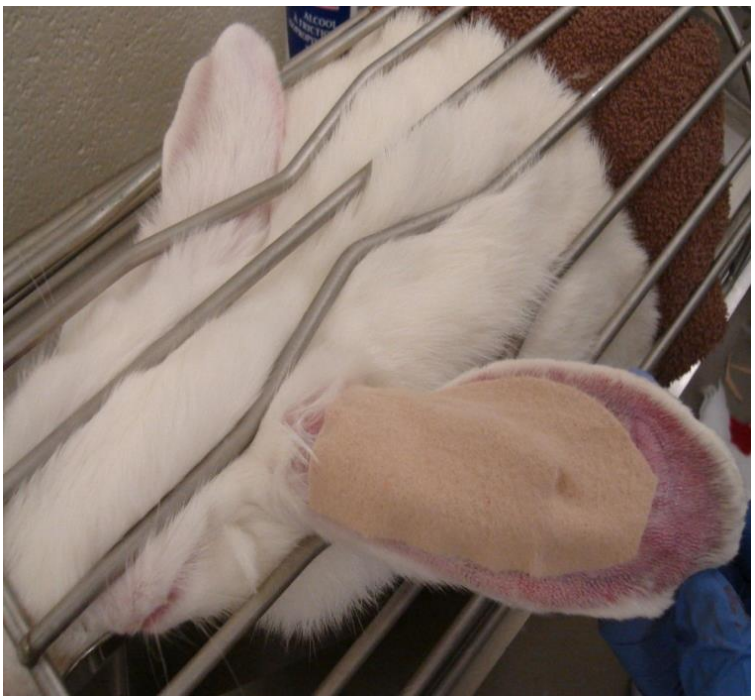


Figure 6.3.3. Moleskin in place. This illustrates the final appearance of the moleskin coverage on the catheter.

Figure 6.4.

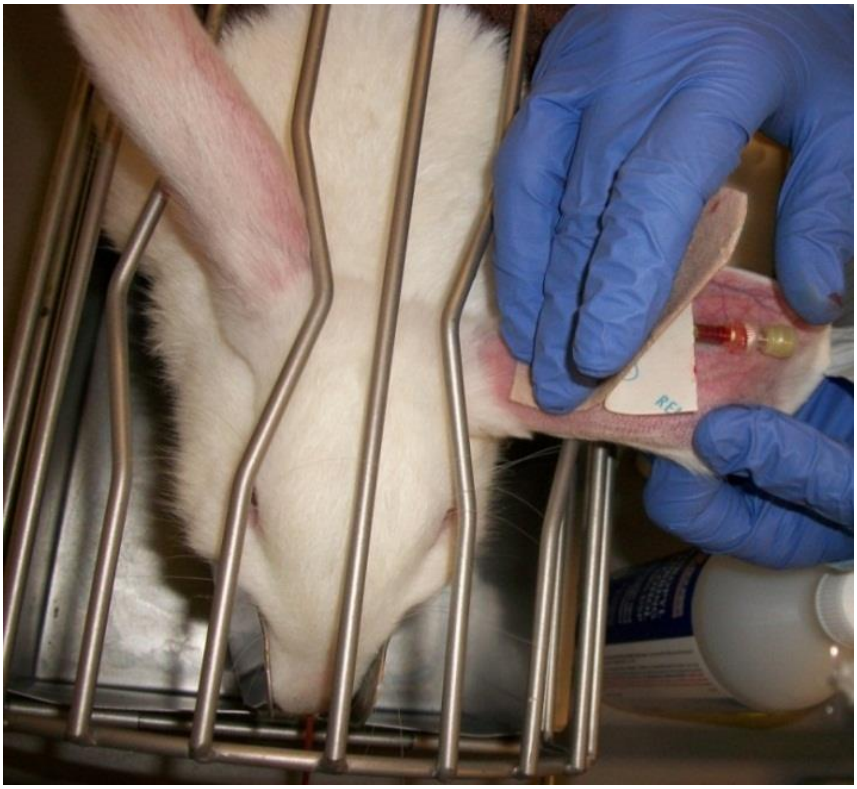


Figure 6.4. Troubleshooting. Removing the paper protection of the moleskin as it gets attached to the ear is not convenient, because it can get hung on the catheter's hub. The risk is moving or offsetting the catheter.

6.3.5. Maintenance of the Catheter: repeated sampling.

After catheter placement or sample collection, a small volume of heparinized saline (3000 IU of sodium heparin in 500 ml of 0.9% sodium chloride solution) was injected to maintain the patency of the catheter **(Figure 6.5)**. The heparinized catheter's "lock" (a small amount of heparinized saline to maintain the catheter's hub clear and patent and prevent clotting) was achieved by flushing the catheter after sampling, with 0.3-0.5 ml of heparinized solution. At the time of collection, the "lock" was removed and discarded, along with a small amount of blood. A new syringe and needle were used for each sample collection.

Figure 6.5.

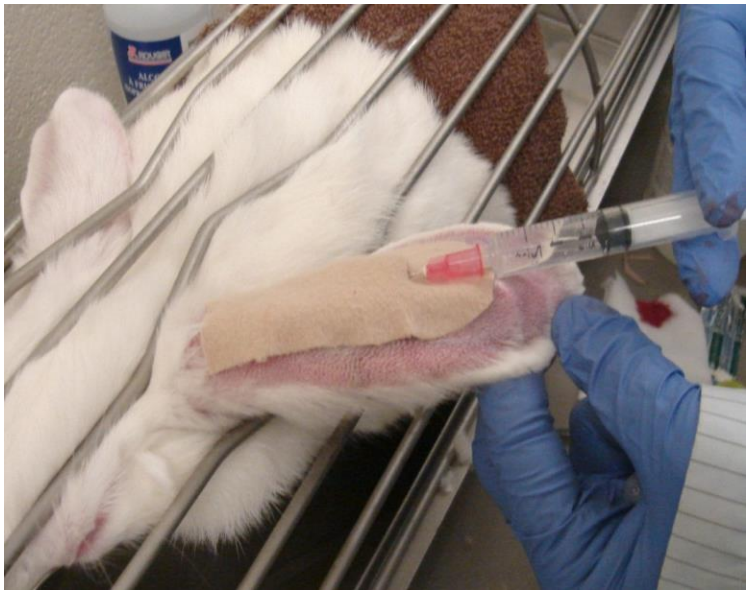


Figure 6.5. Catheter Flush. Flushing of the catheter, which is completely embedded under the moleskin.

6.3.6. Removal of the catheter and recovery of the ear

The moleskin protective cover was soaked with a biological adhesive remover (Remove* - Imported by Smith and Nephew Inc. St Laurent, QC H4R 2P9 Canada) and gently peeled off at the end of the 12-hour period, or at the time of catheter's failure **(Figure 6.6.1. to 6.6.3.)**. The catheter was removed and compression was applied on the puncture site of the vessel for up to 5 minutes, to avoid arterial bleeding and hematomas. The remaining adhesive, in many cases, were easily peeled off, after removing the protection cover and the catheter. Smaller residual areas peeled off over time, once dried. Ears were cleaned with alcohol and dried gently to not remove the recently formed clot. Antiseptic cream (Hibitane –Wyeth Santé Animale – Division de Wyeth Canada, Guelph, ON, N1K 1E4 Canada) was applied over the ear, after the removal of the catheter **(Figure 6.7.2.)** and, thereafter daily after cleaning the area, until hematomas or signs of skin irritation were resolved.

Figure 6.6.1.

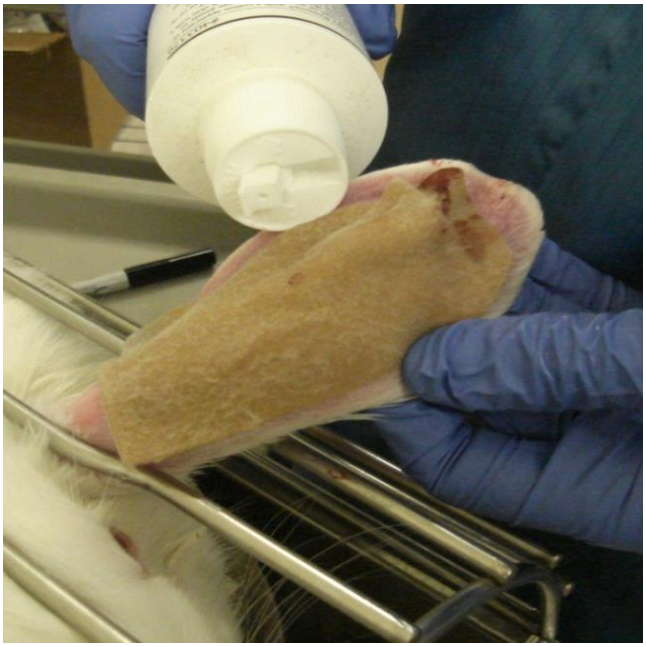


Figure 6.6.1. Removal of the moleskin: Step 1. The moleskin is carefully soaked, so to not wet the rabbit's head (and eyes) with adhesive remover.

Figure 6.6.2.

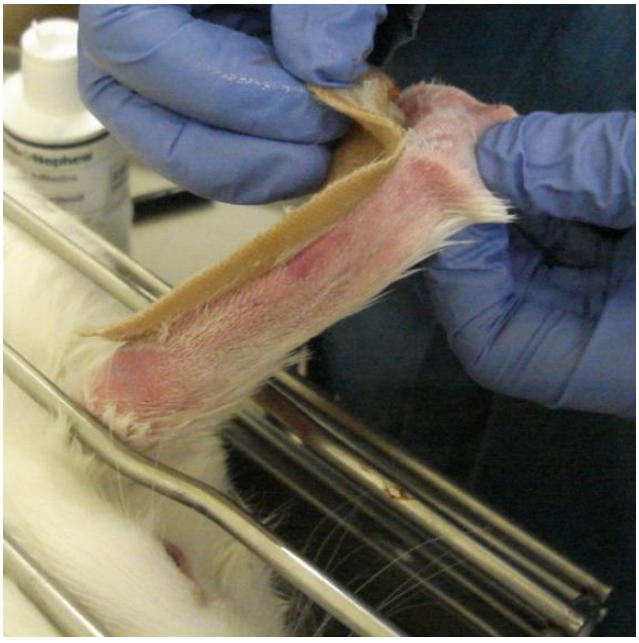


Figure 6.6.2: Removal of the moleskin: Step 2. The edges of the protective cover are lifted carefully.

Figure 6.6.3.

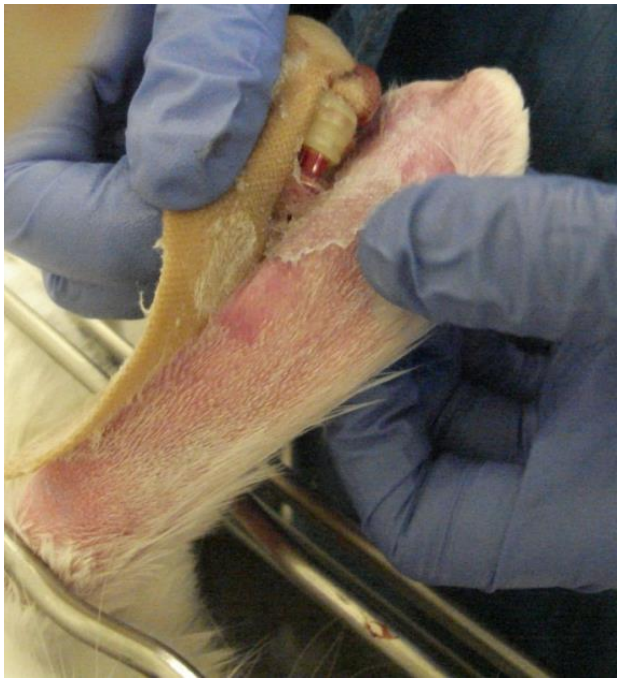


Figure 6.6.3.: Removal of the moleskin: Step 3. The moleskin is carefully removed to maintain hemostasis, paying attention to not pull the catheter and induce bleeding and subcutaneous hematomas.

Figure 6.7.1.

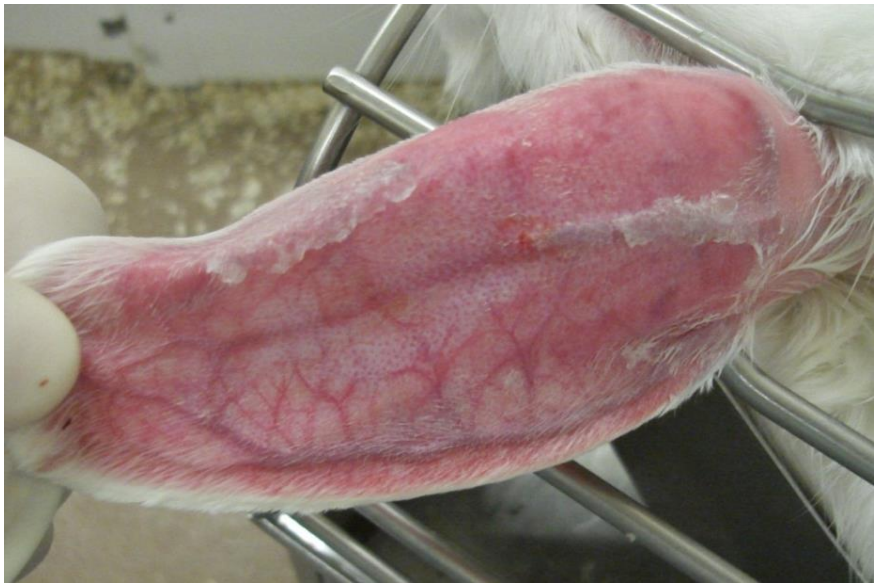


Figure 6.7.1. Appearance of the catheterized ear after removal of the catheter. Appearance of the auricular pinna in the rabbit, after removing the moleskin and the catheter utilized for repeated sampling within 12 hours.

Figure 6.7.2.

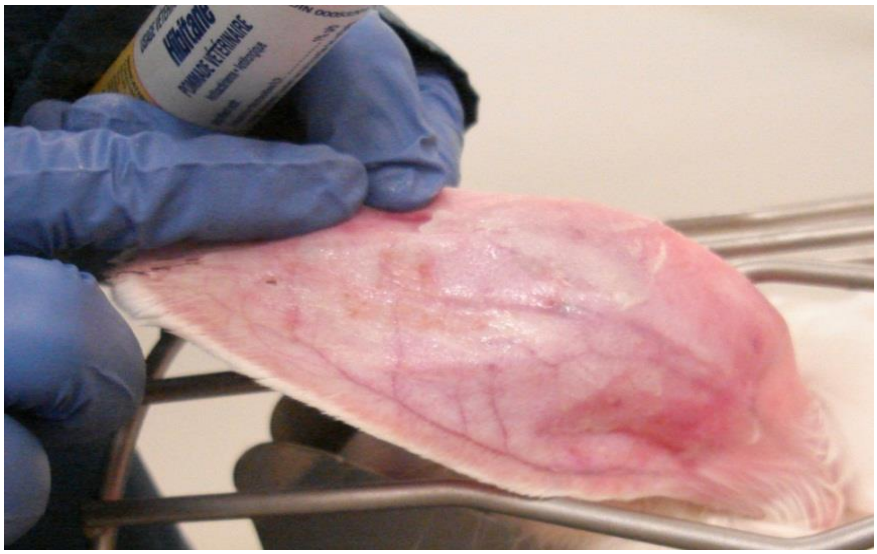


Figure 6.7.2. Aftercare of the rabbit's ear, after the ear has been cleaned and dried. Antiseptic cream is applied daily on the area previously covered with the protective moleskin.

6.4. Results

Out of 9 placed catheters, multiple samples were collected successfully for 12 hours in 5 animals. Of the remaining 4 catheters inserted, 2 failed to yield successful samples after 8 hours from insertion, 1 failed after 6 hours and 1 failed after 4 hours. This technique was perfected over time and a better catheter's performance was obtained in the last 6 rabbits, particularly when heparinized solution flushes were applied every 2 hours, regardless of sample collection. As the technique improved, the length of aftercare decreased. This simple technique allowed precision and timeliness in the collection of samples for the pharmacokinetic study, compared to conventional methods used for catheterization and bandaging techniques.

Three rabbits could be caught, prepared for sampling in the restraint devices, sampled and released in less than 10 minutes, accounting for an average handling time of 3 minutes per rabbit. Between samples, the rabbits were released in their pens; free to eat and drink *at libitum* and able to interact normally with their pen mates **(Figure 6.8.)**.

Figure 6.8.

Figure 6.8. Rabbits with catheter and moleskin protection cover. They are released with their peers in their pen. On the rabbit on right, the light tan color of the moleskin is visible on the ear's outer surface; the edges of the moleskin protection cover are visible on the rabbit on the left, whereas in the rabbit in the center of the picture, the moleskin protection cover is perceivable from the front as a dark shape. It could look like a big ear bruise from the inner aspect of the ear!

Pictures of the outcome after removing the moleskin after 12-hours sampling are **illustrated in Figure 6.7.1 (shown on page 21)**, as well as pictures of recovery, after 3 and 7 days, following catheterization **(Figures 6.9.1, 6.9.2. and 6.10)**. The ear's central artery and the surrounding skin successfully recovered from the catheterization and the application of the glued-on protective moleskin.

Figure 6.9.1.

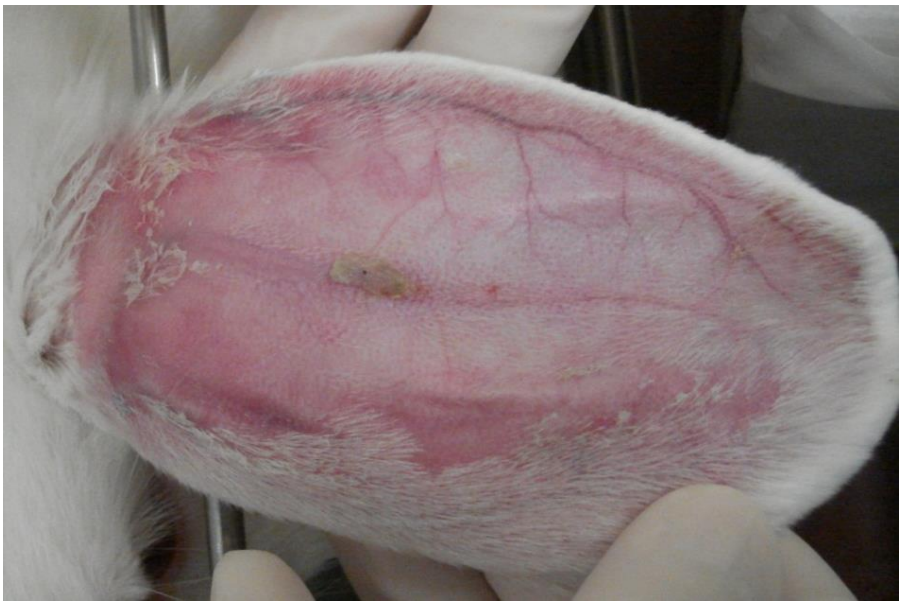


Figure 6.9.1: **Ear's recovery 3 days after** the catheter and the moleskin protective cover was removed. Small residues of dried tissue glue are visible, but little irritation is detectable.

Figure 6.9.2.

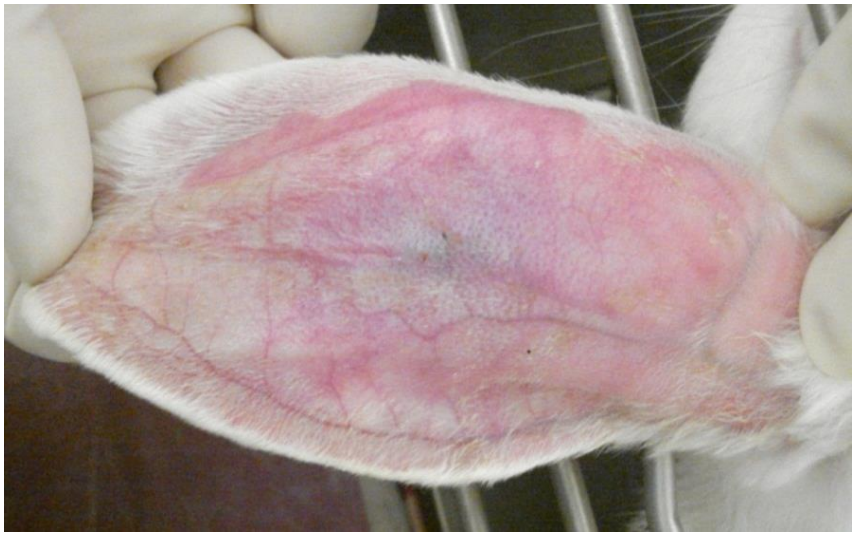


Figure 6.9.2. Ear's recovery 3 days after catheter removal. Another ear's recovery 3 days after the catheter and moleskin were removed. Small residues of dried tissue glue are visible, as well as a small hematoma over the insertion site.

Figure 6.10.

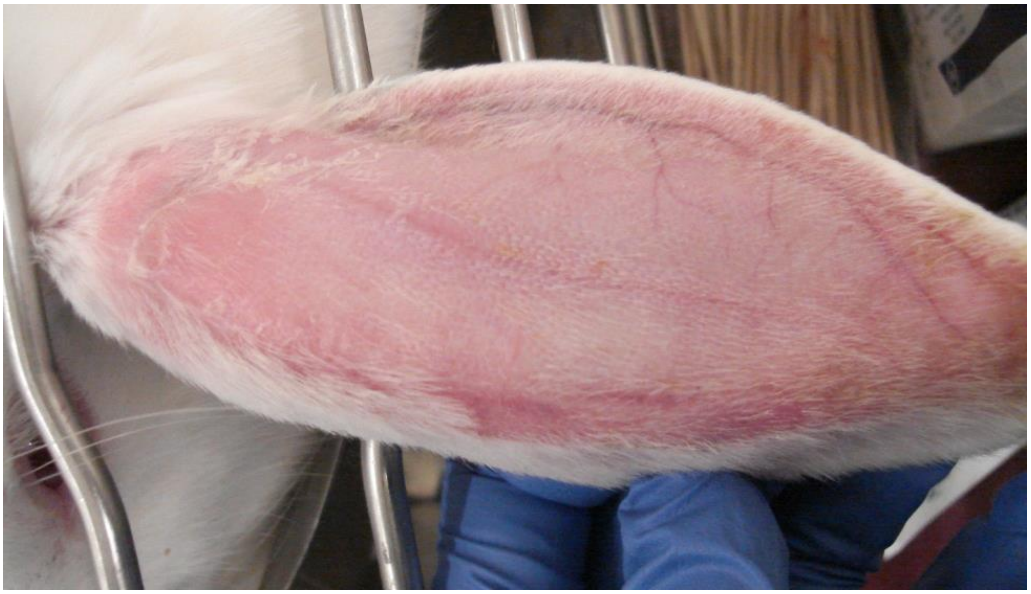


Figure 6.10: **Ear's recovery 7 days after the catheter and moleskin were removed.** The artery is clear and the growing hair is noticeable.

6.5. Discussion

The results of this case study demonstrate a very simple approach to maintaining a short term intra-arterial catheter, for multiple blood samples in research rabbits. The novelty consists in the application of the protective moleskin over the catheter site and the ear, rather than the catheterization itself. The full coverage of the catheter and the light weight of the moleskin cover are key-points to the technique, as this ear shaped moleskin provides protection from scratching and improves stability of the catheter, yet it allows the rabbit to move the ear freely and to carry it in a normal position. As the base of the pinna is thicker, the moleskin functioned as a semi-rigid splint to the ear itself. The tissue glue reinforced the splinting effect, ensuring an all-around better seal. Had the moleskin been placed only near the ear's tip, it would have caused the ear to flop and would have been irritating to the rabbit.

The tissue glue dried quickly and caused minor thermal and chemical irritation to the rabbits' ears. In absence of tissue glue, Krazy glue has been used, although it appears to develop more of a thermal reaction, hence irritating the rabbits' ears more than tissue glue. The use of superglue to stabilize a butterfly needle that is inserted in the ear's marginal vein, was previously reported, this technique was advised for critical, or moribund, patients, as they probably would not attempt to scratch their catheters (Harcourt-Brown, 2002b). In comparison to the previously suggested superglue method, our technique can be applied safely to healthy and normally active animals. Some irritation would be expected during the 12-hour period, but the rabbits were observed to not scratch the catheterized ear with a higher frequency than the bare ear. During grooming, the rabbits' paws, glided swiftly over the protective moleskin, more so when the injection port "peeped" halfway through the moleskin, than when it was at the end of the protective cover, as the surface exposed to snagging and scratching was minimized.

One of the advantages of this method is the ability, without assistance, for a veterinarian or a technician to take repeated blood samples, provided that the rabbit is adequately restrained in a mechanical device; the material is pre-organized; and that the operator is experienced at inserting small arterial catheters. Having said that, a "bunny-wrap" method of restraint can also be used, but such a technique would require an assistant, to hold the wrap.

Although the results of this protective cover were encouraging, and the catheter failures decreased with experience, some flaws were also observed. First, the catheters' patency was at times dependent on its position within the vessel, as the angle at which the ear was catheterized, at the time of stylet insertion, needed to be exactly repeated to obtain blood flow through the catheter, at the time of blood collection. The reason could be due to the tip of the catheter being too close against the vascular wall, or the decreased pressure surrounding the catheter itself. A second flaw was the incapability of inspecting the catheter directly, throughout the experiment; and finally, the inaccessibility of the injection port for cleaning and disinfection. These are the main reasons why such a technique is not ideal for long term repeated sampling, as contamination of the catheter can occur and initiate arterial damage or infection.

Despite the few limitations listed, this protected catheterization technique provided an easy access for short term repeated blood sampling and was followed by a quick recovery of the catheterized ear's arteries and skin. Moreover, the appealing aspect consisted in being able to utilize the same artery for repeated sampling within a few days from catheterization. In conclusion, this protective technique could be a valuable procedure to bear in mind for both pharmacokinetics and endocrinology studies on rabbits, both as research animals and as patients.

CHAPTER 7

PHARMACOKINETICS OF GALLIUM MALTOLATE IN *LAWSONIA INTRACELLULARIS*-INFECTED AND UNINFECTED RABBITS.

Relationship of this project to the dissertation objectives.

In this chapter the methodologies and the results of a pharmacokinetic study regarding the compound Gallium maltolate are reported. The pharmacokinetic analysis of this novel compound was one of the main goals of this doctoral project. For this purpose the rabbit infection model was validated, to investigate the impact of gastrointestinal disease on the total body exposure to the drug administered at 50 mg/kg BW orally once. The results of this study were utilized to calculate a dosing interval for the other main goal of the project, the evaluation of efficacy of the compound against gastrointestinal infection.

7.1 Abstract

Oral gallium maltolate (GaM) pharmacokinetics (PK) and intestinal tissue (IT) concentrations of elemental gallium ([Ga]) and iron ([Fe]) were investigated in a rabbit model of equine proliferative enteropathy (EPE). New Zealand white does (uninfected controls and EPE-infected, n=6/group) were given a single oral GaM dose (50 mg/kg). Serial blood samples were collected from 0–216 h post-treatment (PT) and IT samples after euthanasia. Serology, qPCR and immunohistochemistry confirmed, or excluded, EPE. Blood and IT [Ga] and [Fe] were determined using inductively coupled plasma–mass spectrometry. PK parameters were estimated through non-compartmental approaches. For all [Ga] and [Fe] statistical comparisons were set at α=5%. The Ga log-linear terminal phase rate constant was lower in EPE-rabbits versus uninfected controls (0.0116 ±0.004 (SD) vs. 0.0171 ±0.0028 h^{-1}) (p=0.03); but half-life (59.4 ± 24.0 vs. 39.4 ± 10.8 h) (p=0.12); C_{max} (0.50 ±0.21 vs. 0.59 ±0.42 µg/mL) (p=0.45); t_{max} (1.75 ±0.41 vs. 0.9 ± 0.37 h) (p=0.20); and oral clearance (6.743 ± 1.887 vs. 7.208 ± 2.565 L/h) (p=0.74) were not. IT's [Ga] and [Fe] were higher (p<0.0001) in controls. In conclusion, a 48 h GaM dosing interval is appropriate for multi-dose studies in EPE-rabbits, wherein both IT [Ga] and [Fe] are reduced.

7.2 Introduction

Equine proliferative enteropathy (EPE) is an emerging disease in weanling horses caused by *Lawsonia intracellularis,* an obligate intracellular gram-negative bacterium (McOrist, *et al.*, 1995a; Pusterla & Gebhart, 2009). The disease causes hyperplasia of enterocytes and enteric crypts, primarily in the distal jejunum and ileum, and occasionally in the cecum and colon, affecting the intestinal ability to absorb nutrients in multiple domestic and wildlife animal species (Herbst, *et al.*, 2003; Horiuchi, *et al.*, 2008; Pusterla & Gebhart, 2009; Wong, *et al.*, 2009). In weanling foals, EPE's clinical signs range in severity and chronicity from generalized wasting, pronounced ventral edema (along with hypoproteinemia), to profuse diarrhea, fever, colic, acute shock and death (Lavoie, *et al.*, 2000; Pusterla & Gebhart, 2009; Page, *et al.*, 2012). Similarly to its impact on the swine production industry worldwide, *L. intracellularis* infection in weanling horses is a significant economic and welfare burden for the equestrian industry, with

economic losses due to animal mortality, poor growth rates, and costly therapeutic interventions. Moreover, EPE typically long convalescence and recovery constitute a sale-price drawback, up to a year after clinical manifestation (Guedes, *et al.*, 2002d; Frazer, 2008).

L. intracellularis infections pose a challenge for antimicrobial drug (AMs) therapy due to the bacteria's privileged intracellular location within enterocytes. This "sanctuary-like" location protects the bacteria from the host defenses and from the common first-line AMs adopted by equine clinicians. Currently, the therapeutic management of EPE relies on the oral employment of macrolides or azalides, often combined with rifampin, or the tetracycline class, either through parenteral or oral administration (Lavoie, *et al.*, 2000; Schumacher, *et al.*, 2000; Sampieri, *et al.*, 2006, Feary, *et al.*, 2007; Frazer, 2008). No new alternative AMs have been proposed for EPE therapy since 2006. A few reported cases of treatment failures suggest a need for alternative therapeutic approaches, but the potential for *L. intracellularis* to develop a specific susceptibility pattern, or even an AM resistance, although currently unknown, provides increasing motivation to identify other AM treatments (Frazer, 2008). Strain susceptibility studies *in vitro* have not yet been conducted on the EPE strain. For the porcine proliferative enteropathy (PPE) strains, the limited number of studies conducted thus far suggests a potential for geographical strain differentiation (McOrist, *et al.*, 1995c: Wattanaphansak, *et al.*, 2009a). Interestingly, most therapeutic knowledge has been achieved through clinical experiences, rather than laboratory trials, due to intrinsic difficulties in maintaining *L. intracellularis* in a pure cell-culture environment (Lawson, *et al.*, 1993). A few studies involving exposure of cultured bacteria *in vitro* to a variety of AMs and disinfectants showed the clear technical limitations of *in vitro* research for intracellular organisms (Wattanaphansak, *et al.*, 2009a; Wattanaphansak, *et al.*, 2009b). To overcome these limitations, in the present study the use of a rabbit infection model was elected (Chapter 4), to obtain a valuable preclinical characterization of the drug during disease, for the needs of EPE therapy.

A potential alternative AM therapy for EPE is the novel, gallium-based compound, gallium maltolate (GaM). It is hypothesized that gallium may reach intracellular bacteria through typical mechanisms of oligo-element absorption (specifically iron, zinc and aluminum) in the small intestine (Gunther & Wright, 1983; Caspary, 1992). Given its similarity in electric charge, ionic radius, valence and electronic footprint to ferric iron (Fe(III)), elemental gallium (Ga(III))

may function through a "Trojan horse" mechanism replacing ferric iron (Fe(III)), a metabolically essential element to most pathogens' survival, and thereby reduce the risk for development of AM resistance (Bernstein, 1998; Collery, *et al.* 2002). Gallium is predominantly detected in plasma bound to Fe(III)-sites of transferrin. It is preferentially taken up by phagocytic cells during the inflammation process entering such cells through mechanisms dependent and independent of transferrin (Tsan, 1986; Chitambar & Zivkovic, 1987). Selectivity must occur because bacteria take up ferric iron, while healthy mammalian cells tend to heavily recycle iron locally or, in the case of erythropoietic cells, take up ferrous iron (Fe(II)), which subsequently undergoes intracellular redox reactions (Logan, *et al.*, 1981; Bernstein, 1998). In the presence of sufficiently high Ga(III) concentration, then, bacteria may take up large amounts of Ga(III), altering the first step of the bacterial ferric metabolism (Olakanmi, *et al.*, 2000). Unlike iron, gallium is unable to complete a redox-cycling reaction (from valence III to valence II) and thus cannot be incorporated or function in many iron metabolic pathways and enzymes (e.g. ribonucleotide reductase) necessary for DNA replication and cell function (Bernstein, 1998). The ability of Ga(III) to block these iron-dependent metabolic steps has been demonstrated in *Mycobacteria* and hypothesized in *R. equi*, and it has been clearly ascribed to the gallium moiety, not to the particular salt or ligand utilized in the formulation (i.e. nitrate, chloride or maltolate) (Olakanmi, *et al.*, 2000; Harrington, *et al.*, 2006). Inhibition of bacterial iron metabolism may lead to AM effects, as it may ultimately reduce bacterial survival and replication (Bernstein, 1998).

The AM activity of several gallium-based compounds has been explored for a few intracellular bacteria in veterinary medicine (Harrington, *et al.*, 2006; Martens, *et al.*, 2007a; Fecteau, *et al.*, 2011). Only an injectable solution of citrate-chelated gallium nitrate is approved in United States for the treatment of human cancer-related hypercalcemia, and is administered as a continuous IV infusion, to avoid the nephrotoxic effects reported with IV bolus (Martens, *et al.*, 2007). GaM is deemed safe when orally administered to humans, mice, rats, dogs and foals (Bernstein, *et al.*, 2000; Martens, *et al.*, 2010). Unlike with gallium salts (i.e. gallium chloride or nitrate) there are no reports of GaM nephrotoxicity, as it is apparently highly protein-bound in the bloodstream; it is thus expected to convey Ga(III) to the bacteria located in the intestinal brush border membrane (Collery, *et al.*, 1989; Bernstein, *et al.*, 2000).

In the present study, the potential differences induced by EPE on GaM pharmacokinetics (PK) parameters were investigated in rabbits, prior to exposing foals affected by *L. intracellularis* to treatment. Using a bolus dose (50 mg/kg BW once intra-gastrically) previously utilized in a mouse model for *R. equi*, for a preclinical trial (Harrington, *et al.*, 2006; Martens, *et al.*, 2010; Chaffin, *et al.*, 2011), the present study aimed to obtain sufficient information on the PK characteristics of this novel compound to determine the feasibility of a multiple oral dose GaM efficacy study in a rabbit infection model.

7.3 Material and Methods

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and it adhered to the Canadian Council on Animal Care guidelines for humane animal use.

7.3.1. Animals

Eighteen, 8 to 9-week-old, 2.01 - 3.07 kg BW, New Zealand white female rabbits (Charles River Canada, Pointe Claire, QC, Canada) were used. The infected does were managed, fed and housed as described in Chapter 4; the uninfected controls were treated equally but, as they were not infected, they were housed in the Animal Care Unit facilities in a Containment Level (CL)-1 room. Experiments started after a mandatory week of acclimation. Treats (apples and carrots) were offered to promote socialization, facilitate handling, and monitor individual appetite, alertness and brightness, particularly in group-housing conditions (Johnson-Delaney, 2006; Chapter 4). The rabbits monitoring followed daily or more often (in the case of multiple samples in a day) the protocol previously reported (Chapters 4 and 5).

7.3.2. *L. intracellularis* rabbit infection model

The protocol described for inducing equine strain *L. intracellularis* lesions in juvenile rabbits was followed to achieve infection in the EPE-infected group (Chapter 4). Each rabbit was inoculated via nasogastric intubation with a low passage pure cell-culture inoculum of E04504 *L. intracellularis* harvested after isolating the bacteria from a deceased foal's ileal

mucosa. Inocula were prepared and suspended in 3 mL of buffered sucrose/phosphate/glutamate medium, for a total inoculating dose of 2.5 x 10^8 bacteria/rabbit (Chapter 4), administered with a chasing volume of 3 mL of distilled water. A SYBR Green-based PCR quantitation assay was used to determine the bacterial concentration of the infectious material, as previously published (Wattanaphansak, *et al.*, 2010). Onset of infection, or its absence in the controls, was detected *ante mortem* through diagnostics used currently in clinical practice for EPE, such as immune-peroxidase monolayer assay (IPMA) serology and TaqMan qPCR on fecal material (Guedes, *et al.*, 2002c; Chapter 4). The *post mortem* assessment was based on gross pathology examination, haematoxylin-eosin (H&E) staining, and immunohistochemistry (IHC) labeling of specific lesions based on murine anti-*L. intracellularis* antibody on samples fixed in 10% buffered formalin at collection time (Guedes, *et al.*, 2002a).

7.3.3. Study design

Rabbits were divided into 2 groups of 9 animals each: the uninfected control group (controls) and EPE-infected group (EPE-infected). In each group, 3 does were randomly selected for a pilot – limit of detection – experiment. Those rabbits underwent euthanasia and full necropsy 24 h post-treatment (PT).

The remaining 6 rabbits of both groups were enrolled in the PK study: the uninfected control and the EPE-infected groups were studied in 2 phases a few days apart, so that only 3 animals at a time were treated, sampled and euthanized, to maximize accuracy in sample collection and animal monitoring, throughout the experiment.

Gallium maltolate (provided by LRB, Terrametrix, Menlo Park, CA, USA) was dissolved in sterile double-distilled water, immediately prior to administration, achieving a concentration of 8 mg/mL, with a solution's final pH = 5.8 - 6 (Harrington, *et al.*, 2006). Rabbits were administered a single intragastric dose of GaM solution, 50 mg/kg BW, on day 0 for the controls and on day 7 post infection (PI) for the EPE-infected rabbits. Such timing was elected to evaluate the ability of small intestine to absorb the solution when the EPE lesions start to appear, as the disease peak in the rabbit model is reported to be around 12-14 days PI.

A nasogastric tube was used for intragastric administration of GaM, due to the relatively large administered volume. Briefly, an anesthetic gel (Xylocaine Gel 2%, Astra Zeneca Canada Inc., Mississauga, ON, Canada) was applied medially on both nostrils. Approximately 30 to 45 minutes later, a 5 Fr. wide, 40 cm long feeding tube (Kendall Sovereign, Tyco Health Care Group LP, Mansfield, MA, USA) was inserted via the nose into the stomach. Serial blood samples were taken from a catheter in the ear central artery. Briefly, a local skin block above the ear's central artery was achieved with a minute amount of local anesthetic cream (EMLA Cream, Astra Zeneca Canada Inc., Missisauga, ON, Canada). The rabbits were placed within restraint cages and the catheterization of the ear's central artery was performed following a previously published protocol (Chapter 6) with an indwelling sterile catheter (BD Insyte - Becton Dickinson Infusion Therapy Systems Inc. Sandy, UT, USA) affixed to an injection port (Intermittent Infusion Plug - Kendall Argyle TYCO Healthcare Group LP. USA). The injection port, along with the catheter hub, was glued temporarily to the skin with tissue glue (3M Vetbond - 3M Animal Care Products, St. Paul, MN, USA). Subsequently, the whole ear was bandaged with moleskin (Dr. Scholl's Extra Soft Moleskin, imported Schering Canada Inc., Pointe Claire, QC, Canada) in such a way that the bandage mimicked a skin embedded port, affording timely repeated samples (Chapter 6).

7.3.4. Sample collection .

7.3.4.1. Blood.

Arterial blood was collected (1 mL/sample) from time 0 h (t_{first}), immediately after GaM administration, and then at time 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 36, 48 h, and subsequently every 24 h, until time 216 h (9 days PT, t_{last}). The first 9 samples were collected through the injection port bandaged in place, as described above. Starting with the sample at 24 h PT to the t_{last} sample, blood was collected off the needle, after local anesthesia was applied on the skin over the ear's central artery.

7.3.4.2. Tissue samples.

A full necropsy examination was conducted in all 18 rabbits. A published, sampling protocol (Chapter 4) was followed for visual examination of the organs, from stomach to rectum, for thickness, discoloration or type of content as *L. intracellularis* lesions are not located in any other organ. Samples of duodenum, mid-jejunum and terminal jejunum, ileum, ileocecal valve

(including *ampulla coli* and *sacculus rotundus)*, cecum (transitional area between cecum and caecal appendix*)*, terminal portion of the caecal *appendix*, large colon (proximal to the *fusum coli)*, terminal colon and rectum were collected (Chapter 4).

7.3.5. Verification of infection.

7.3.5.1. Histology and immunohistochemistry.

Two adjacent, formalin-fixed and paraffin-embedded sections per sample were cut and stained by H&E and IHC with streptavidin method using murine anti-*L. intracellularis*-specific monoclonal antibody to detect proliferative lesions of the intestinal epithelium and the presence of the antigen within the cells (Guedes & Gebhart, 2003c). The *L. intracellularis*-specific antigen in the enterocytes was blindly evaluated with a 5 grade IHC scoring system, as previously reported (Guedes & Gebhart, 2003b). For each rabbit, the negative control for each tissue section consisted of a correspondent IHC-labeled tissue section, except for the primary antibody. Furthermore, pig ileal tissues known to be negative and positive for *L. intracellularis* infection were labeled with the murine anti-*L. intracellularis* monoclonal antibody to confirm the antibody's specificity and sensitivity, respectively.

7.3.5.2. Serology.

In serum, anti–*L. intracellularis*–specific IgG concentration was measured by an IPMA as reported previously (Guedes, *et al.*, 2002c) and in Chapters 4 and 5.

7.3.5.3. Quantitative PCR Analysis.

Quantitative PCR analysis was conducted on fecal samples as previously reported, including a general bacteriology 16 sRNA for quality control (Pusterla, *et al.*, 2010a; Chapter 4). The purified DNA was analyzed by qPCR for presence of *L. intracellularis aspA* gene copies (Pusterla, *et al.*, 2008; Pusterla, *et al.*, 2009).

7.3.6. Elemental Iron and Gallium tissue concentration

Serum and intestinal tissue (IT) concentrations of elemental gallium ([Ga]) and iron ([Fe]) were determined through Inductively Coupled Plasma - Mass Spectrometry (ICP-MS). The maltol portion of the initial compound was not detectable due to its rapid metabolism, hence the tissue analysis focused on elemental metal concentrations (Bernstein, *et al.*, 2000). The

methodology was titrated prior to each batch of sample testing with rabbit blank serum, standard samples spiked with known [Ga] and [Fe], and verified on a batch of test tissues (liver). Analysis was repeated in duplicate.

7.3.7. Pharmacokinetic analysis.

Serum [Ga], or C_s, versus time data was analyzed using the non-compartmental approach (NCA), and the analysis was carried out using GraphPad Prism 5.4 Software (GraphPad Software, Inc., La Jolla, CA, USA). The NCA was used to provide robust estimates of area under the curve from time 0 to time 216 h ($AUC_{0\rightarrow 216}$), area under the curve extrapolated to infinity ($AUC_{0\rightarrow\infty}$), elimination rate constant (λ), mean residence time (MRT), terminal half-life ($t_{1/2}$), and oral clearance (Cl_o). The observed maximum plasma concentration (C_{max}) and observed time to maximum concentration (t_{max}) were determined by visual inspection of the serum concentration versus time profiles. The AUC was determined using the trapezoidal rule with the extrapolated area calculated from the ratio of the last calculated concentration and λ. Oral clearance (Cl_o) was calculated from the ratio of oral dose and AUC extrapolated to infinity ($AUC_{0\rightarrow\infty}$); MRT was calculated as the ratio of the area under the first moment curve ($AUMC_{0\rightarrow\infty}$) to the $AUC_{0\rightarrow\infty}$; and half-life was obtained from the product of 0.693 and MRT.

7.3.8. Statistics.

Descriptive statistics (average and standard deviation, among others) were calculated for each NCA derived parameter. For comparison of the PK parameters, a Student t-test was used. For the [Ga] and [Fe] in intestinal tissues a one-way ANOVA was used; whereas a two-way ANOVA for repeated measures was used for PCR comparisons in relation to GaM treatment. For both ANOVAs, a Bonferroni *post-hoc* test was implemented. When it was more appropriate not to assume a Gaussian distribution (uneven groups), a Kruskal Wallis analysis with Dunn's *post-hoc* test was used. Alpha was set at 5% for all the comparisons.

7.4. Results

7.4.1. Clinical appearance.

All rabbits tolerated well the single oral GaM dose. During the 24 h pilot study, no abnormalities were detected in either uninfected control or EPE-infected rabbits. In the pharmacokinetic studies, no change was evident in either group, for the duration of the experiments for appetite, fecal consistency, self or mutual grooming, and behavior. Only a marginal weight loss was observed on day 1 PT due to blood sampling loss and stress associated with multiple blood sampling (9 samples in 12 h). In all cases, BW rapidly recovered within a day **(Figures 7.1.1. and 7.1.2.)**. A decrease in BW gain trend (indicating a clear weight loss, or a BW gain smaller than half the average daily BW gain observed throughout the experiment) was observed in EPE-infected rabbits **(visible in Figure 7.1.2.)** on or around 12 days PI, although not statistically significant (p=0.97).

Figure 7.1.1.

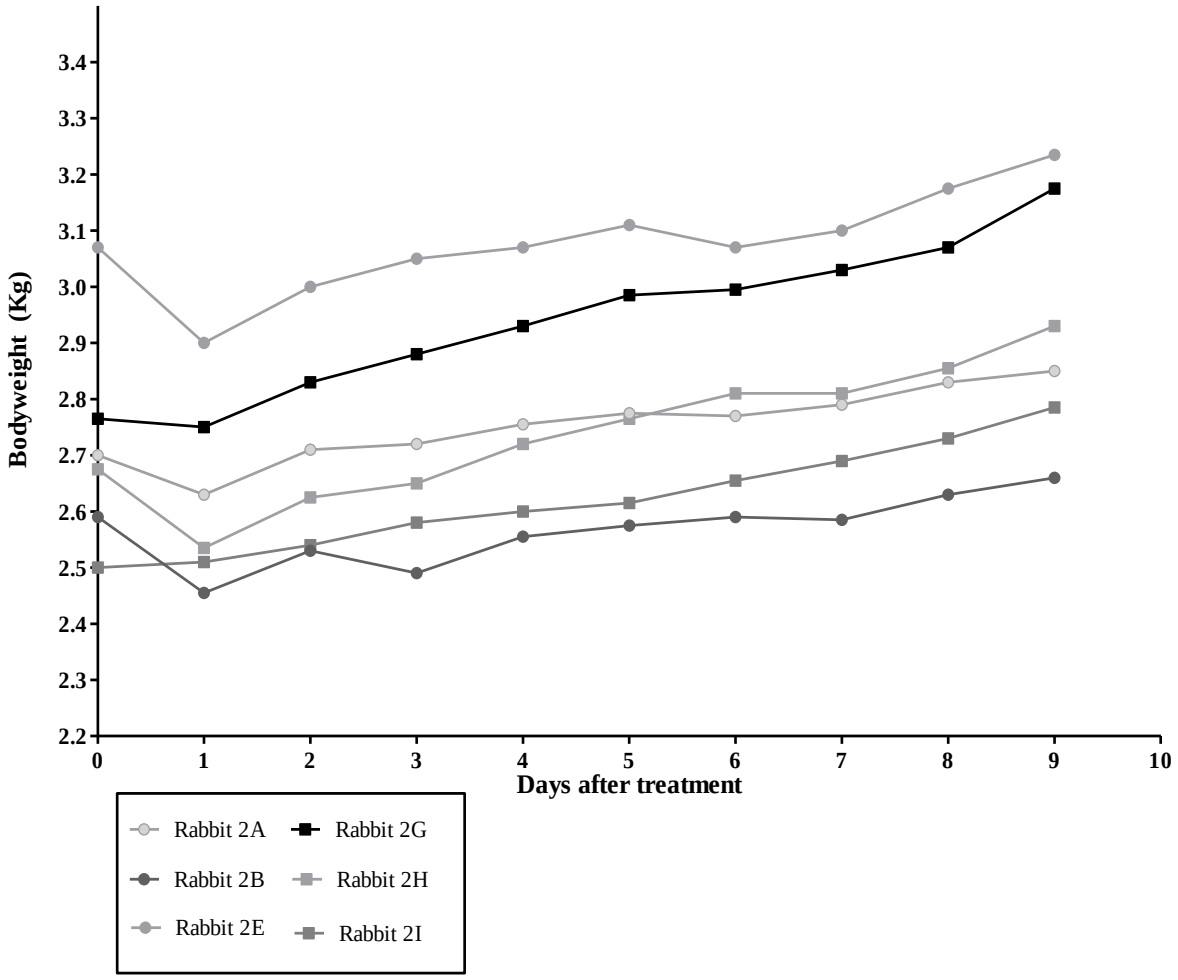


Figure 7.1.1. Body weight changes in uninfected rabbits administered gallium maltolate. Body weight (Kg) changes in 6 uninfected controls rabbit (PK study only) administered a single oral dose of gallium maltolate (50 mg/Kg) on day 0, with serial blood sampling until day 9 post treatment (PT). BW loss on day 1 PT is ascribed to the stress of dosing and blood sampling on day 1 PT.

Figure 7.1.2.

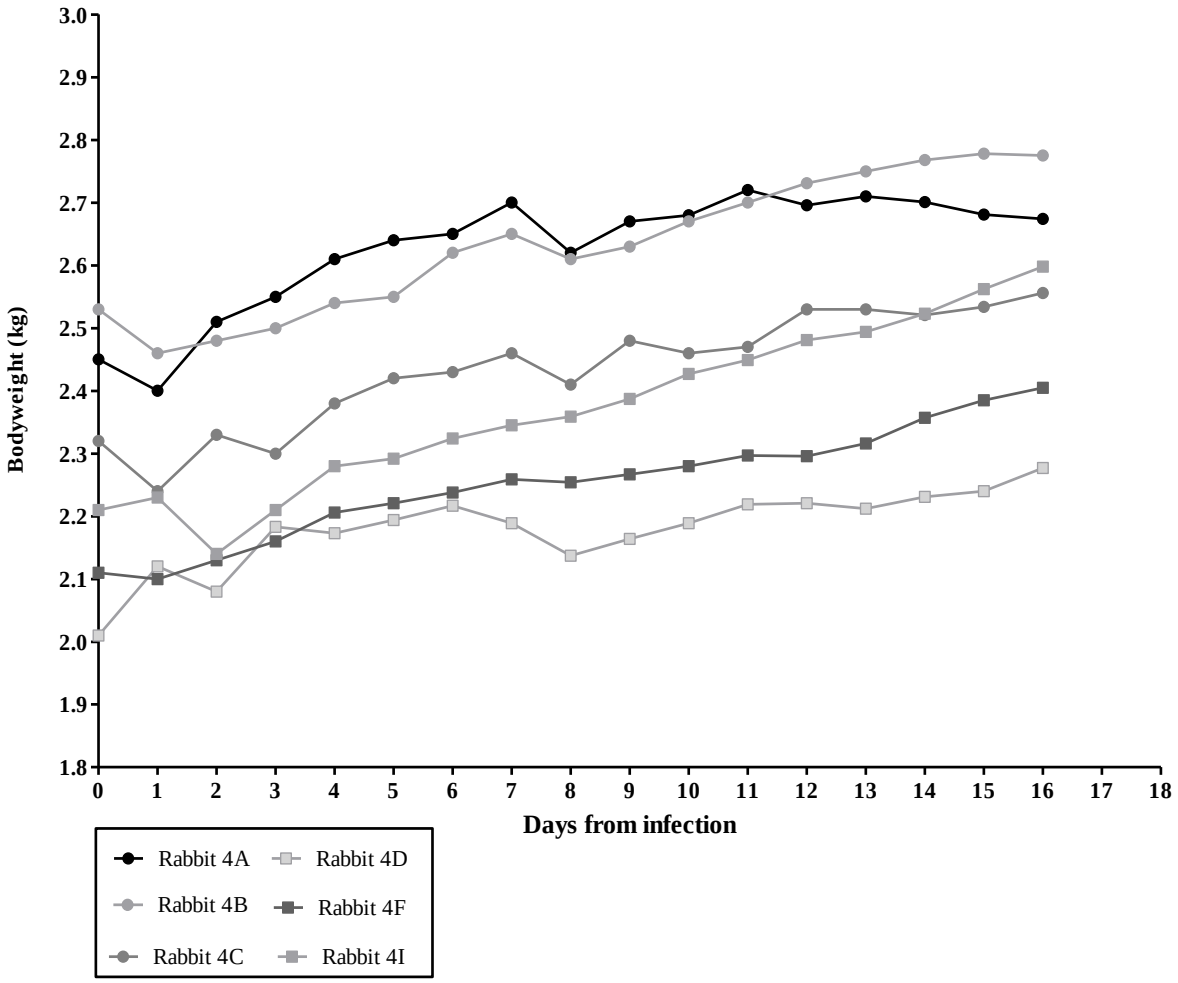


Figure 7.1.2. Body weight changes in infected rabbits administered gallium maltolate. Body weight (Kg) changes in 6 EPE-infected rabbits (PK study only) inoculated with *L. intracellularis* on day 0 and administered a single oral dose of gallium maltolate (50 mg/Kg) on day 7 post infection (PI). Rabbits underwent blood sampling until day 9 PT, or 16 PI. Note that the BW loss on day 8 PT is ascribed to the stress of dosing and serial blood sampling, but a trend towards BW gain suppression is noted around day 12 PI and is consistent with EPE infection.

7.4.2. Pharmacokinetics.

Gallium was rapidly absorbed, with a minimal to nonexistent lag phase **(Figures 7.2.1 and 7.2.2.)**. The mean of the first detected C_s immediately after nasogastric administration was 57.3 μg/L in controls and 110.2 μg/L in EPE-infected rabbits, increasing to 389.5 μg/L and to 533.75 μg/L, respectively, within 30 minutes. In control does, C_{max} ranged between 248 μg/L and 849.5 μg/L (mean 500.43 ± 214.07 μg/L), and it peaked with t_{max} in 1-12 h (mean 3.41 ± 4.22 (SD) h). In EPE-infected rabbits, C_{max} ranged between 238 μg/L to 1415 μg/L (590.7 ± 416.07 μg/L), and t_{max} had a range of 0.5 - 1.5 h (0.9 ± 0.37 h).

Elemental gallium half-life in rabbits (uninfected controls and EPE-infected alike) is prolonged, particularly in the EPE-infected rabbits. The elimination rate constants, **λ,** were 0.0171 ± 0.0028 h^{-1} and 0.0116 ± 0.004 h^{-1}, elimination half-lives of 39.38 ± 10.78 h and 59.36 ± 24.02 h; and oral clearance of 6.743 ± 1.887 L/h and 7.208 ± 2.565 L/h, respectively, in uninfected controls and EPE-infected rabbits. In both groups, the slope calculations were verified through the fitting of the line calculations and the evaluation of residuals, which showed a random distribution and close proximity to the fitted line.

The total body exposure to the drug showed a mean AUC extrapolated to infinity ($AUC_{0\rightarrow\infty}$) of 21069.47 ± 6263.01 [(μg/L) x h] and 19146.35 ± 9403.13 [(μg/L) x h], respectively, in uninfected controls and EPE–infected rabbits. Averaged and detailed C_s values from each rabbit are reported in **Table 7.1** for control and **Table 7.2** for EPE-infected rabbits. The only statistically significant parameter between the healthy and EPE-infected rabbits was the elimination rate constant λ (p=0.03), whereas no parameter differences were found within the 2 groups, as **indicated in Table 7.2**. Individual log-linear C_s versus time curves are **represented in Figures 7.2.1. and 7.2.2.** for healthy and EPE-infected rabbits, respectively. In several cases, cecotrophes ingestion, typical of normally behaving rabbits, could not be prevented, despite close monitoring, and it is clearly visible in the figures.

Figure 7.2.1.

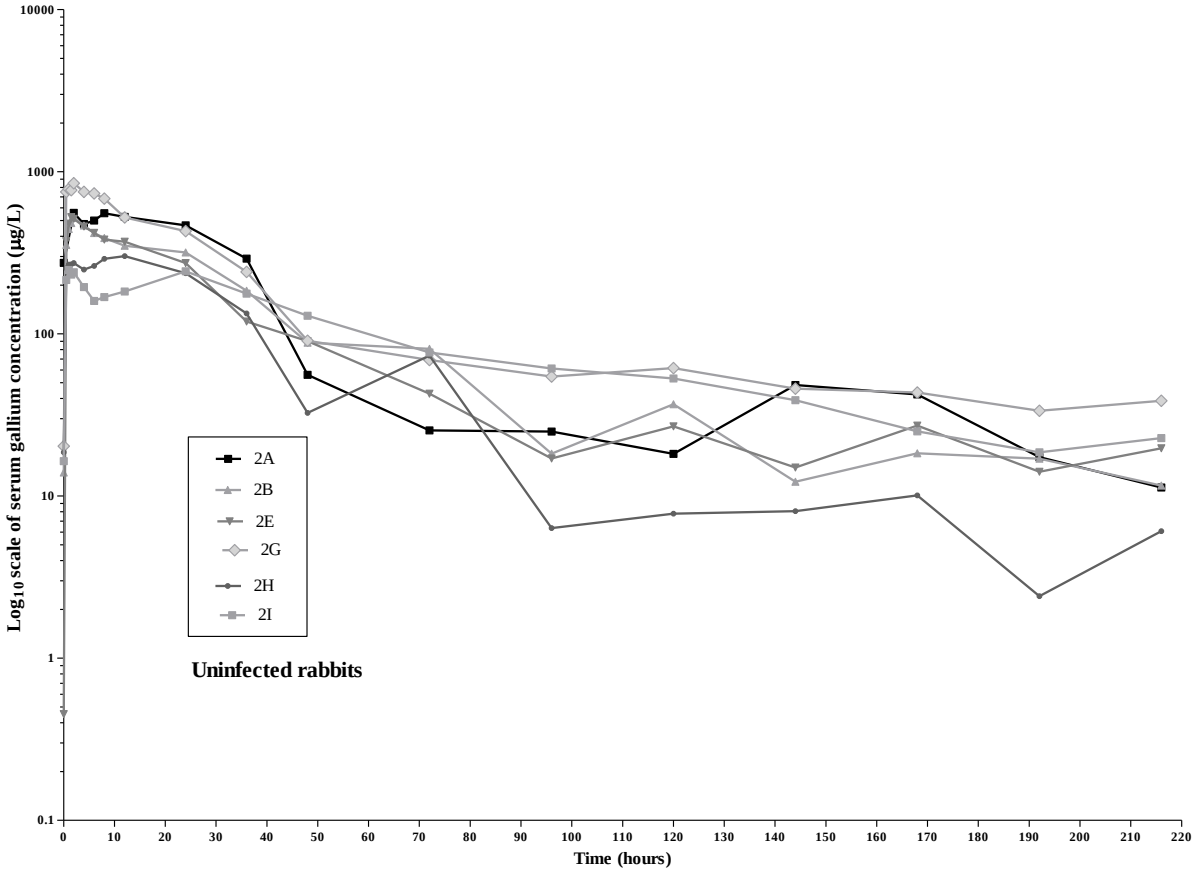


Figure 7.2.1. Concentration versus time curves for uninfected rabbits administered gallium maltolate. Representation on log-linear scale of gallium serum concentrations in 6 uninfected rabbits, treated orally once with gallium maltolate (50 mg/kg). Blood sampling lasted for 9 days (216 h) PT.

Figure 7.2.2.

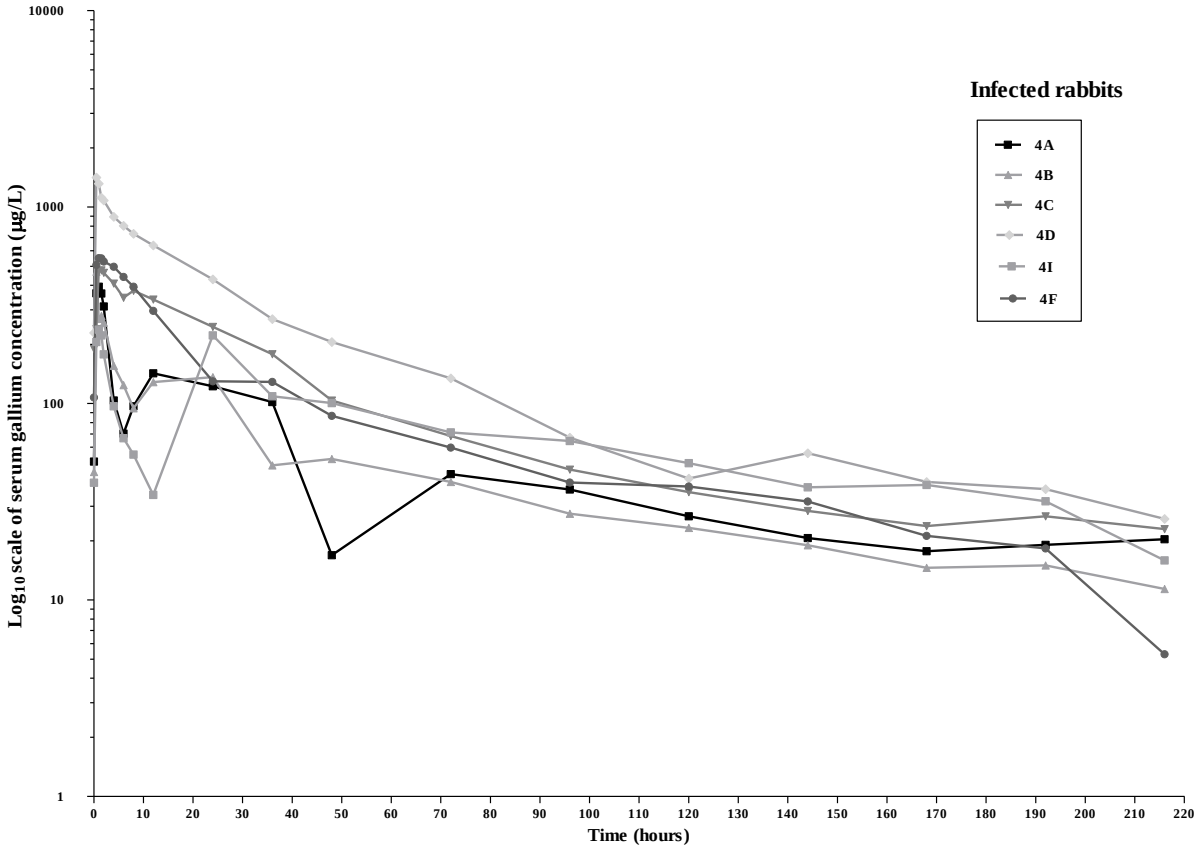


Figure 7.2.2. Concentration versus time curves for the infected rabbits administered gallium maltolate. Representation on log-linear scale of gallium serum concentrations in 6 rabbits infected with *L. intracellularis* and treated orally once with gallium maltolate (50 mg/kg). Blood sampling lasted for 9 days (216 h) PT. Note in both graphs that in the first 24 h cecotrophy was not completely prevented in all rabbits; hence, a second peak in the curves is noticeable.

Table 7.1. Pharmacokinetic parameters in uninfected control rabbits.

	Rabbits							
PK parameters (units)	2A	2B	2E	2G	2H	2I	**Mean**	**SD**
C_{max} (µg/L)	557	519	525	849	302	248	**500.4**	**214**
t_{max} (h)	2	2	1.5	2	12	1	**1.7**	**0.4**
λ (h^{-1})	0.0168	0.0188	0.0178	0.0155	0.021	0.0127	**0.0171**	**0.0028**
$AUC_{(0 \rightarrow \infty)}$ (µg·h /L)	24419.2	19816.1	18318.4	31480.9	13047.0	19335.0	**21069.4**	**6263**
$AUMC_{(0 - \infty)}$ (µg·h^2/L)	1170142	979910	1033179	2098963	532047	1616948	**1238532**	**547063**
Cl_o (L/h)	5.5	6.4	8.2	4.0	9.9	6.3	**6.7**	**1.8**
MRT (h)	47	49	56	66	40	83	**56.8**	**15.5**
$t_{½}$ (h)	32.5	33.9	38.8	45.7	27.7	57.5	**39.3**	**10.7**

Table 7.1. Pharmacokinetic parameter estimates for healthy uninfected rabbits after a single oral bolus of gallium maltolate (50 mg/kg BW). The individual values, mean and SD are reported.

Table 7.2. Pharmacokinetic parameters in EPE-infected rabbits

	Rabbits								
PK parameter (units)	4A	4B	4C	4D	4F	4I	**Mean**	**SD**	**p-value**
C_{max} (μg/L)	393	468	477	1415	549	238	**590.4**	**417**	**0.45**
t_{max} (h)	1	0.5	1.5	0.5	1	1	**0.9**	**0.3**	**0.20**
λ (h^{-1})	0.0059	0.0087	0.0139	0.0168	0.014	0.0105	**0.0116**	**0.004**	**0.03**
$AUC_{(0-\infty)}$ (μg·h /L)	13382.6	10664.3	20949.6	36990.6	17023.5	15867.2	**19146.3**	**9403**	**0.53**
$AUMC_{(0-\infty)}$ (μg·h^2/L)	1965962	980040	1467763	2039778	922321	1564630	**1490082**	**472689**	**0.13**
Cl_0 (L/h)	10.1	10.4	5.8	2.8	6.6	7.4	**7.2**	**2.5**	**0.74**
MRT (h)	146	91	70	55	54	98	**85.6**	**34.6**	**0.12**
$t_{½}$ (h)	101.1	63	48.5	38.1	37.4	67.9	**59.3**	**24.0**	**0.12**

Table 7.2. Pharmacokinetic parameter estimates for 6 EPE-infected rabbits after a single oral bolus dose of gallium maltolate (50 mg/kg BW). The individual values, mean and SD are reported. In the last column the p-value derived from t-test comparison between uninfected controls and EPE-infected rabbits are reported indicating the level of significance. Significantly different parameters are shaded in grey in the table.

Our estimates of the $AUC_{0\rightarrow\infty}$ were considered reliable; in the control group the tail region estimate was always below 20% and in 3 rabbits was below 5%, whereas, in the EPE-infected group, all but one doe (4A) had a tail region estimate below 20%, of which 2 does (4D and 4F) were below 5%.

7.4.3. Gross pathology.

In the limit of detection pilot study, rabbits were euthanized 1 day PT (8 days PT in EPE-infected rabbits). In the PK study, euthanasia was performed 9 days PT (16 days PI in EPE-infected rabbits), after collection of the last blood sample. No abnormalities were observed in the gastrointestinal tract (GIT) and in any other organ in the uninfected control rabbits. Lesions consistent with *L. intracellularis* infection, as described in Chapter 4, were noted in the GIT of EPE-infected rabbits, but no other gross abnormalities were detected in any other organ.

7.4.4. Intestinal tissue Fe(III) and Ga(III) concentrations.

A three order of magnitude of difference between [Fe] and [Ga] was detected in intestinal tissues with [Fe] detected in ppm (mg/kg) and [Ga] in ppb (μg/kg). In healthy rabbits euthanized 1 day PT (pilot study), no significant difference was observed between the six different GIT sections collected for [Ga] (p=0.26) or [Fe] (p=0.58). In uninfected control rabbits, euthanized 9 days PT, a significant difference in [Ga] and [Fe] (p <0.0001, for both) was observed between the 6 different GIT regions collected. For [Ga] the greatest concentration was determined in the caecal appendix and lowest in mid-jejunum, cecum, and colon. Similarly, for [Fe] the greatest concentration was found in the caecal appendix. A comparison between uninfected control rabbits sacrificed 1 day PT versus 9 days PT indicates a significantly lower [Ga] and [Fe] in GIT sections of subjects euthanized 9 days PT (p<0.0001 and p=0.0011, respectively) with the comparisons between the 2 sets of *ceca* and *cola* showing the greater differences for [Ga], but no specific differences for [Fe].

Among the EPE-infected rabbits euthanized 1 day PT, significant differences were observed between the six different GIT sections collected for [Ga] (p =0.031) and [Fe] (p =0.015), with greater concentrations in the cecum and colon and lower concentrations in the caecal appendix for both metals (**Figures 7.3.1 and 7.3.2**, respectively for gallium and iron).

Among the EPE-infected rabbits euthanized 9 days PT, a significant difference in [Ga] and [Fe] (p <0.0001, for both) was observed between the 6 different GIT regions collected, with the greatest concentration in the caecal appendix and the lowest in the colon for [Ga], but the highest in the cecum and lowest in the caecal appendix for [Fe]. In the comparison of the uninfected and EPE-infected rabbits, euthanized 9 days PT, [Ga] and [Fe] were significantly different in the 2 groups (p <0.0001, for both) with the greatest differences observed in the end-jejunum, ileum and caecal appendix for [Ga] (**Figure 7.4.1.**), but only in the cecum for [Fe] (**Figure 7.4.2.**).

Figure 7.3.1

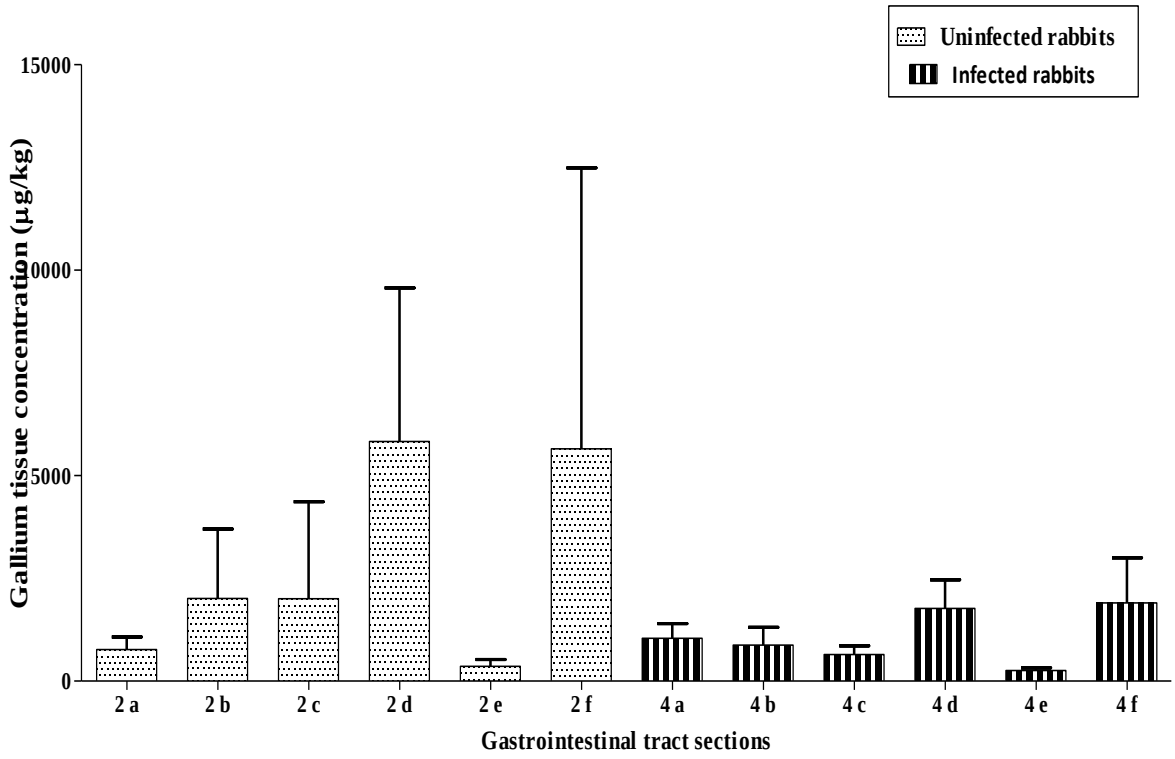


Figure 7.3.1. Comparison of elemental gallium concentration in EPE-infected rabbits and uninfected controls at 24 h PT. Comparison of [Ga] (in µg/kg) in sections of the small and large intestine of 3 uninfected controls and 3 EPE-infected rabbits, treated with a single oral dose of gallium maltolate (50 mg/kg) and sacrificed 1 day PT (8 days PI, for the EPE-infected subjects). Each bar graph represents the averaged value of each intestinal section (n=3): a = mid jejunum; b = end jejunum; c = ileum; d = cecum; e = caecal appendix; f = colon.

Figure 7.3.2.

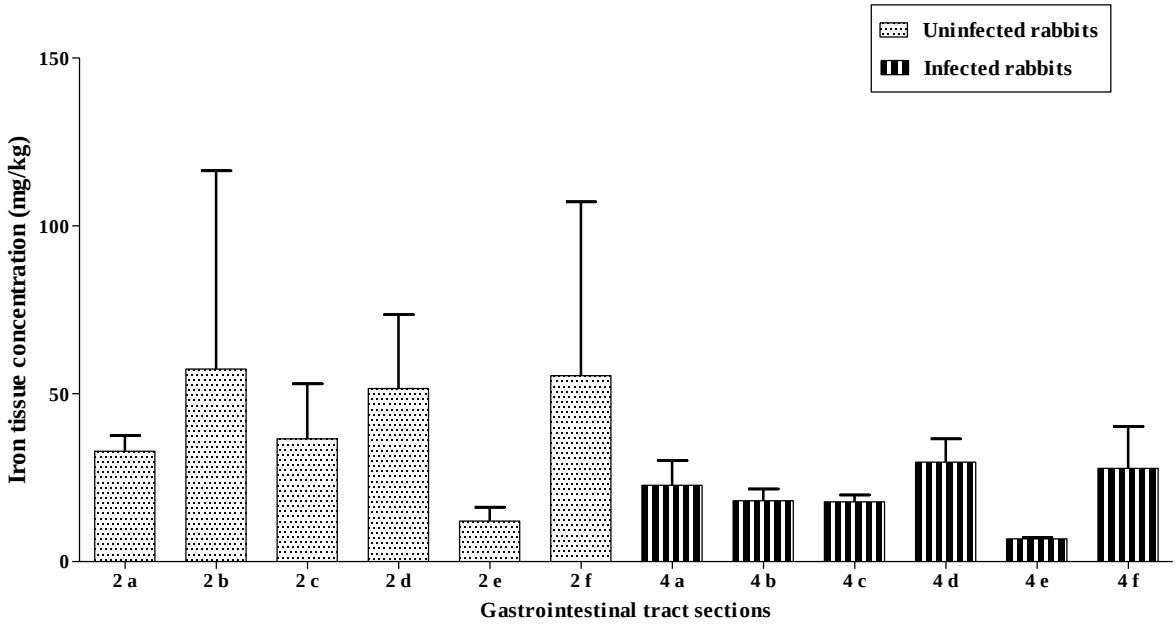


Figure 7.3.2. Comparison of elemental iron concentration in EPE-infected rabbits and uninfected controls at 24 h PT. Comparison of [Fe] (in mg/kg) in sections of the small and large intestine of 3 uninfected controls and 3 EPE-infected rabbits, treated with a single oral dose of gallium maltolate (50 mg/kg) and sacrificed 1 day PT (8 days PI, for the EPE-infected subjects). Each bar graph represents the averaged value of each intestinal section (n=3): a = mid jejunum; b = end jejunum; c = ileum; d = cecum; e = caecal appendix; f = colon.

Figure 7.4.1.

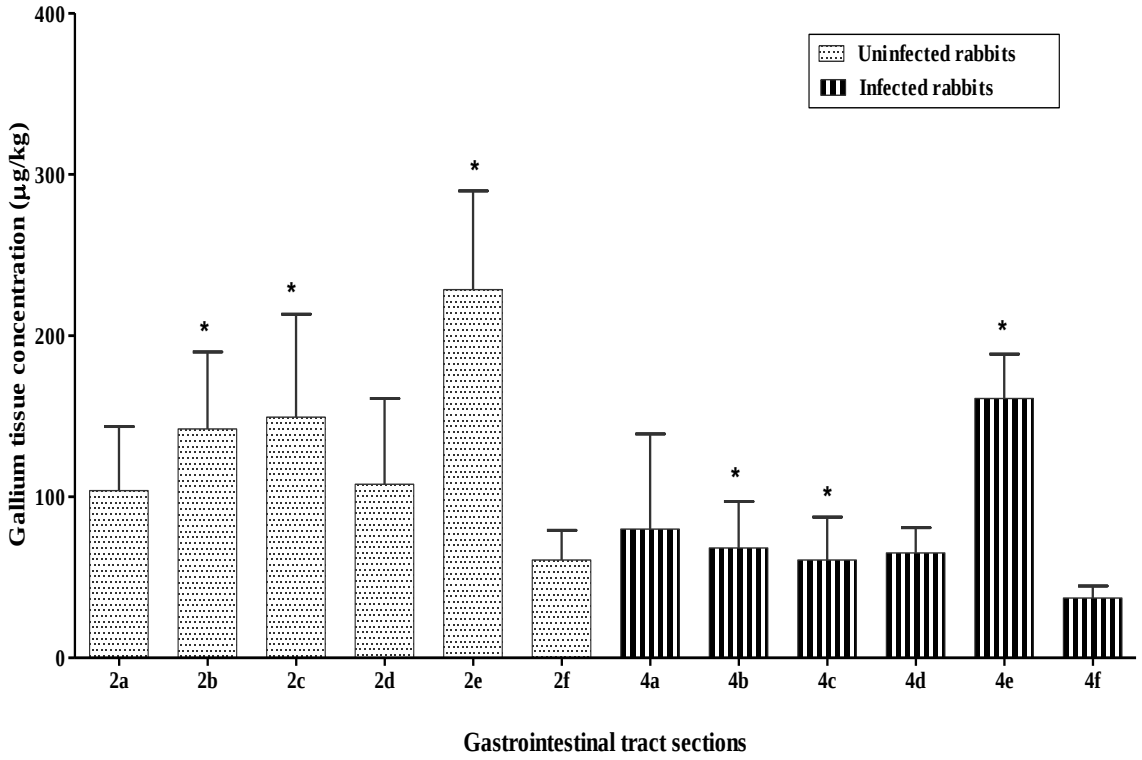


Figure 7.4.1. Comparison of elemental gallium concentration in EPE-infected rabbits and uninfected controls at 9 days PT. Comparison of intestinal tissue [Ga] (µg/kg) in 6 uninfected controls and 6 EPE-infected rabbits after a single oral gallium maltolate dose (50 mg/kg BW). Samples collected 9 days PT (or 16 days PI). In both graphs, each bar graph represents the averaged value of each intestinal section (n=6): a = mid jejunum; b = end jejunum; c = ileum; d = cecum; e = caecal appendix; f = colon. The asterisks indicate statistical significance ≥ 0.05.

Figure 7.4.2.

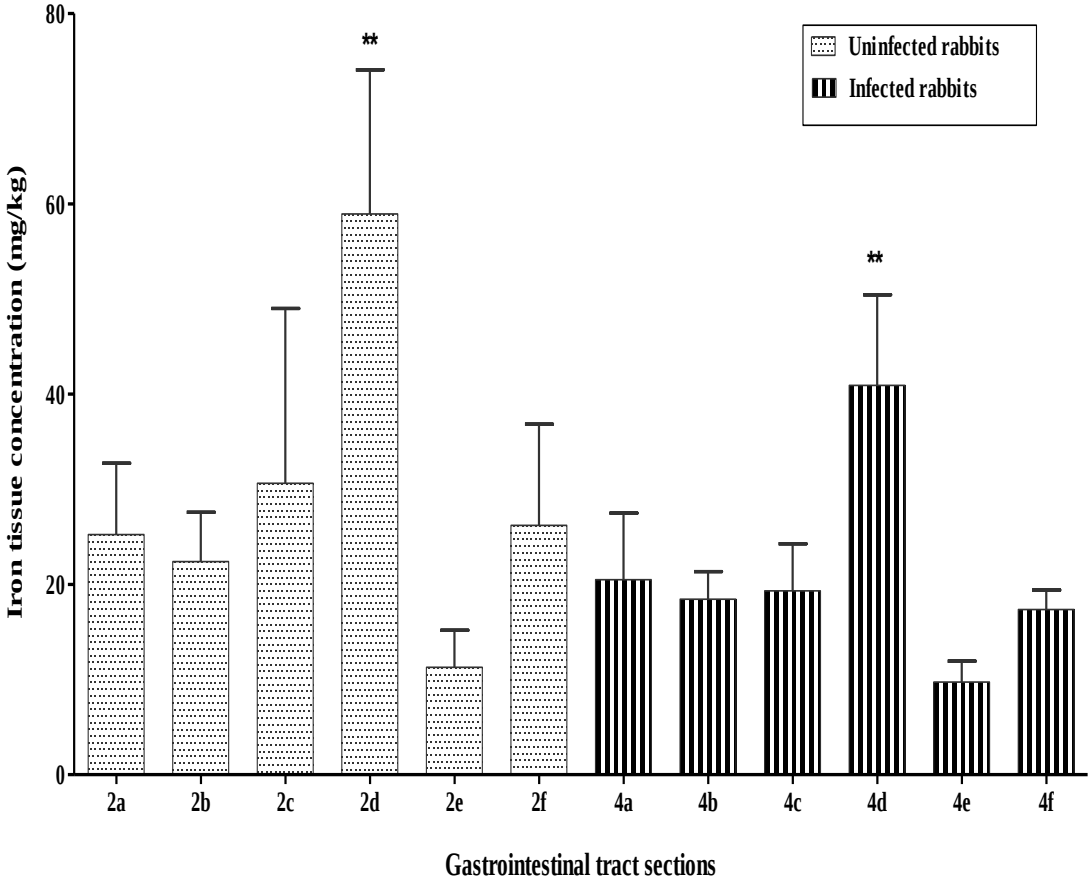


Figure 7.4.2. Comparison of elemental iron concentration in EPE-infected rabbits and uninfected controls at 9 days PT. Comparison of intestinal tissue [Fe] (mg/kg) in 6 uninfected controls and 6 EPE-infected rabbits, after a single oral gallium maltolate dose (50 mg/kg BW). Samples collected 9 DPT (16 DPI). In both graphs, each bar graph represents the averaged value of each intestinal section (n=6): a = mid jejunum; b = end jejunum; c = ileum; d = cecum; e = caecal appendix; f = colon. The asterisks indicate statistical significance ≥ 0.05.

7.4.5. Serology.

The uninfected control rabbits' serology was negative for specific anti-*L. intracellularis* antibodies. For EPE-infected rabbits, titers were negative at day 7 PI, started rising at day 14 PI, and increased even further by day 16 PI (9 days PT, and time of euthanasia), confirming presence of infection and reflecting data previously published (Chapters 4 and 5) .

7.4.6. Histology and immunohistochemistry.

In uninfected controls, no alterations of the intestinal structure were noted and no IHC labelling with specific murine anti-*L. intracellularis* antibodies was detected, in the prime areas usually infected by *L. intracellularis* (jejunum, ileum and cecum). In EPE-infected rabbits the IHC labelling confirmed typical lesions in 7 out of 9 animals, with no statistical difference between lesions detected in 1 versus multiple tracts of the GIT (p =0.07), such as jejunum, ileum and cecum.

7.4.7. PCR.

A complete absence of *L. intracellularis* shedding was confirmed in feces of uninfected control rabbits (p<0.0001). In the EPE-infected rabbits fecal shedding of *L. intracellularis* was confirmed **(Figures 7.5.1 and 7.5.2)** at around day 4 PI, in partial agreement with previous reports, as it started earlier than day 7 PI and lasted for the duration of the experiment (Chapter 4). No differences were noted with regards to comparisons between rabbits (p=0.53), between days (p=0.21) or between fecal and caecal content (at necropsy, p=0.7).

Figure 7.5.1.

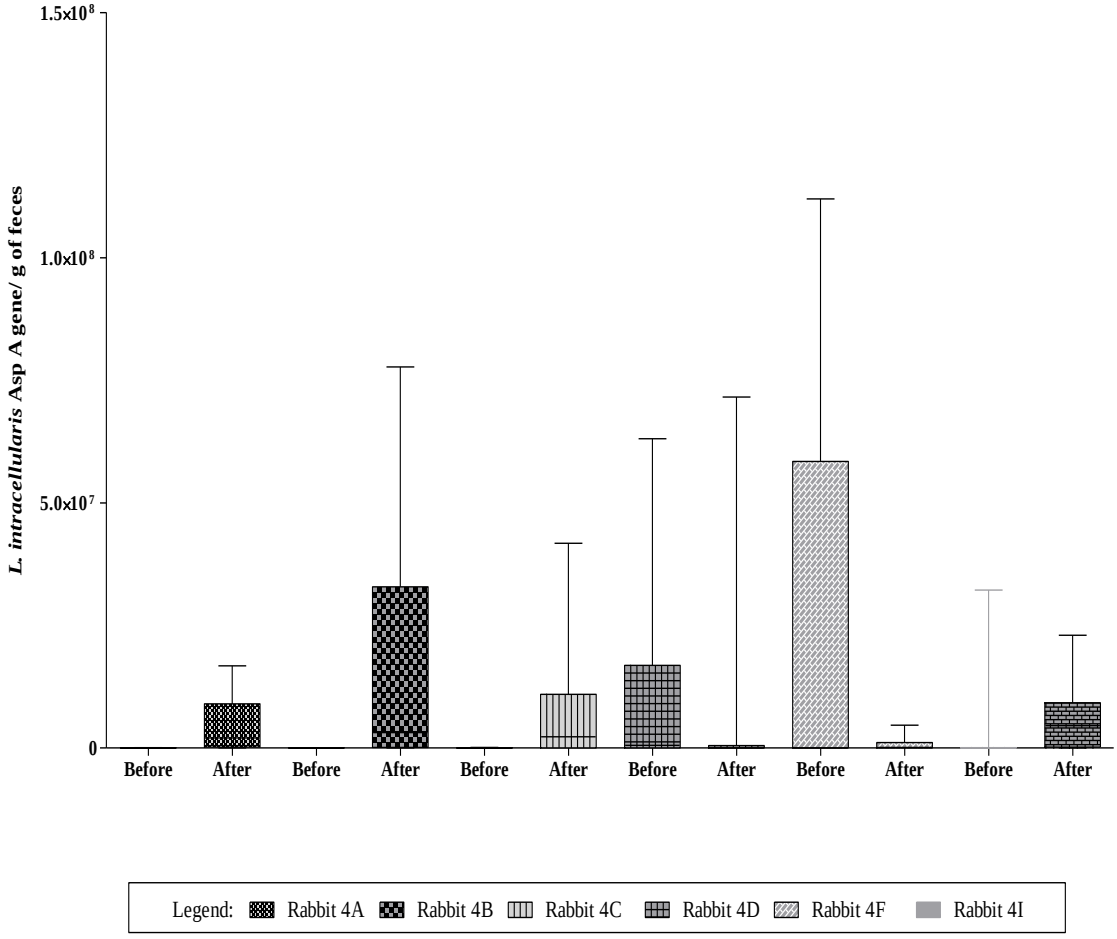


Figure 7.5.1. PCR results before and after gallium maltolate treatment in EPE-infected rabbits. *L. intracellularis* DNA (*AspA* gene)/gram of feces following *L. intracellularis* qPCR analysis in feces of experimentally infected rabbits before and after a single oral dose of gallium maltolate (50 mg/kg). Each pair of bars represents an individual rabbit, in the days before and after the treatment.

Figure 7.5.2.

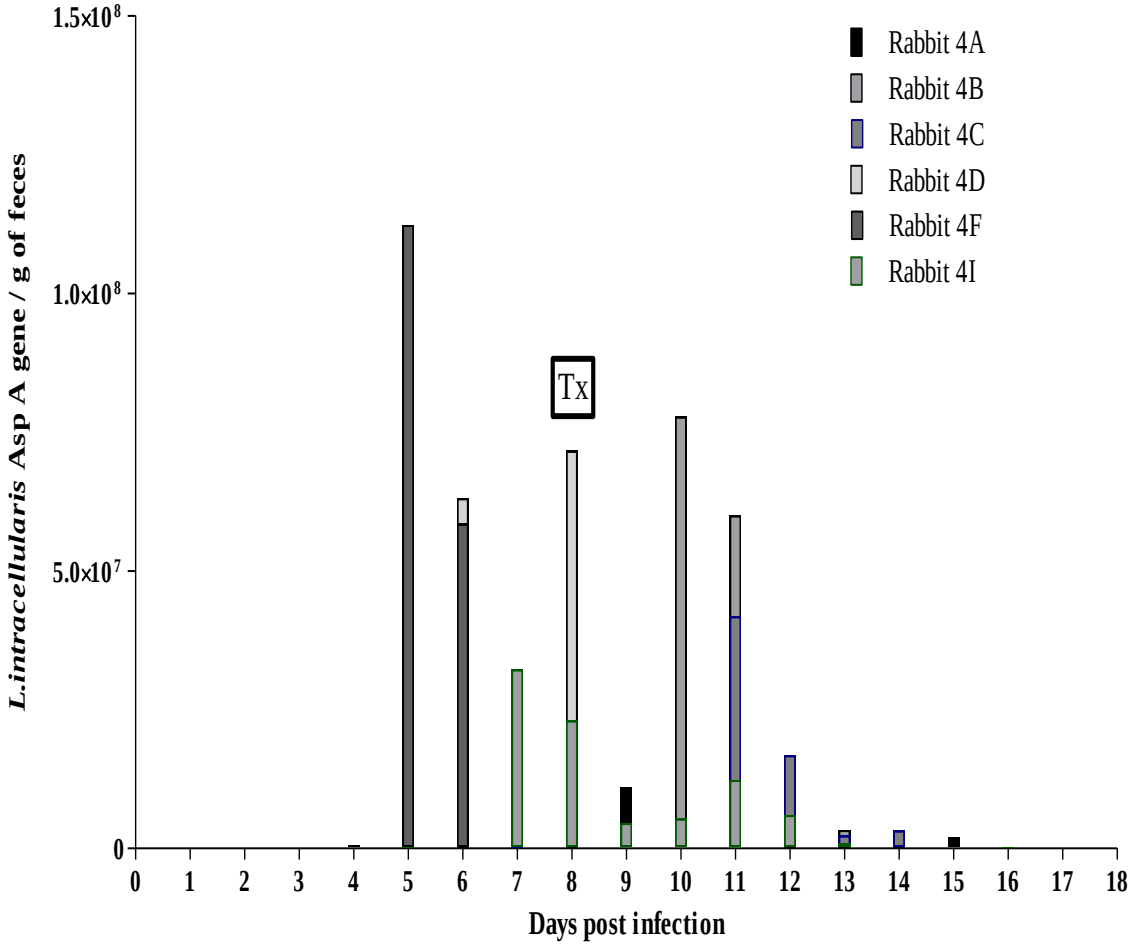


Figure 7.5.2 PCR results indicating fecal shedding after EPE infection in rabbits: time of onset and subjects variability. *L. intracellularis* DNA (AspA gene)/gram of feces following *L. intracellularis* qPCR analysis in feces of experimentally infected rabbits treated with a single oral dose of gallium maltolate (50 mg/kg). Superimposed bars represent each individual rabbit concentration and "Tx" indicates the day of treatment.

7.5. Discussion

In this study a single oral bolus dose of GaM was readily tolerated by uninfected and EPE-infected rabbits. However, EPE infection caused prolongation of the terminal half-life ($t_{½}$) without alteration of other PK characteristics. Given the long half-life, a GaM dosing interval of once every 48 h should lead to sufficient concentrations in rabbits for AM activity of GaM. This dosing interval was deemed safe to test in an efficacy study, although the potential for elemental [Ga] accumulation and environmental impact requires further consideration (Fecteau, *et al.*, 2011).

The *in vitro* susceptibility studies for *L. intracellularis* clarified that multiple AM dosing is necessary for therapy, due to the variable distribution and differentiation state of the infected cells in the enteric crypts (Wattanaphansak, *et al.*, 2009a). Furthermore, no clinical study has shown efficacious AM therapy against EPE following a single dose or short duration AM dosing regimen (Lavoie, *et al.*, 2000; Sampieri, *et al.*, 2006; Frazer, 2008). The 48 h dosing interval was derived from estimates for the elimination half-life in EPE-infected rabbits, as half-life is the only factor determining the time to steady state concentrations. As well, assuming linear pharmacokinetics and according to the principle of superposition, our single GaM dose's AUC estimate suggests that the 48 h dosing interval is adequate to assure appropriate exposure for AM therapy. These assumptions are necessary as multiple dosing studies of gallium-based compounds for PK purposes are not common in animals, with only one study for GaM reported in foals (Martens, *et al.*, 2010), and a multiple dosing regimen estimated for gallium nitrate in adult horses based on a projected study *in silico* (Pollina, *et al.*, 2012). We did not administer an intravenous dose to rabbits, which precluded the calculation of absolute GaM's bioavailability, but in healthy humans GaM is reported to provide a Ga(III) bioavailability of $\geq$ 25% to 57% and exhibits linear absorption and elimination kinetics (Bernstein, *et al.*, 2000). Presuming similar bioavailabilities of GaM in rabbits, such data also supported our decision of a 48 h dosing interval for GaM in EPE infected rabbits.

Our study using the rabbit model is intended to be a preclinical study to help in determining the clinical GaM treatment of foals. An effective GaM therapy against *L. intracellularis* in an infection rabbit model of EPE might allow allometric scaling of a GaM dose for a clinical trial in weanling foals. Also, despite the anatomical similarities between rabbits

and horses (*e.g.*, herbivores, hind-gut fermenters with a small stomach and large cecum), we would still expect to observe significant interspecies variation and, hence, the need to conduct dose titration studies in foals (Bernstein, *et al.*, 2000). Certainly, the design of our rabbit study using a 9 day sampling period was premised on information available in humans ($t_{½}$ ≈5 days), dogs ($t_{½}$ ≈3 days), rather than foals ($t_{½}$ ≈1.5 days), and a consideration of body-scaling in rabbits and the putative effect of EPE infection on GaM absorption and disposition characteristics (Bernstein, *et al.*, 2000; Martens, *et al.*, 2007a).

It is relevant to compare our findings to what Martens and colleagues (2007) estimated in healthy neonatal foals where MRT was 39.5 ± 17.2 h, and $t_{½}$ was 26.6 ± 11.6 h (Martens, *et al.* 2007a). The MRT and $t_{½}$ in juvenile rabbits were much longer. Interestingly, in 2010 the same researchers reported several differences in the PK parameters estimates of GaM administered to adult horses (Arnold, *et al.*, 2010). Sampling lasted 5 days and the main PK estimates were generally more fitting with the rabbits' PK findings, being C_{max}: 280 ± 90 µg/L, t_{max}: 3.09 ± 0.43 h, lag-time: 0.26 ± 0.11 h, and apparent elimination $t_{½}$:48.82 ± 5.63 h (Arnold, *et al.*, 2010). The differences in half-lives may relate to ontogenic differences in the degree of maturation of the drug clearance mechanisms between the neonate foal and the adult horse. Usually, drug clearance mechanisms are underdeveloped in neonatal stages resulting in longer half-lives relative to the adult. The study reported by Martens and coworkers utilized a 48 h blood sampling period, which may have been insufficient to adequately describe the terminal disposition rate constant, on the concentration versus time profiles of the foals in that study (Martens, *et al.*, 2007a). Furthermore, our juvenile rabbits gave PK estimates compatible with results reported in mice treated with the same dose once daily for 10 days (Harrington, *et al.*, 2006), but the estimates were nearly 4 times lower than the canine ones, after a single dose (Bernstein, *et al.*, 2000).

In addition to identifying an appropriate dosing interval for GaM in rabbits, we wished to understand the influence of EPE infection on the PK of GaM. The EPE rabbit model demonstrated clearly the impact of GIT disease on [Ga] and [Fe] following a single dose of a metal-based compound. In order to mimic a natural infection in foals (Lavoie, *et al.*, 2000, Sampieri, *et al.*, 2006; Frazer, 2008), the delay between the onset of infection (inoculation) and treatment was purposefully designed in our preclinical rabbit model and current diagnostics were utilized to confirm the status of disease. It was expected that a single GaM dose would not affect

the fecal shedding of *L. intracellularis* DNA and the immune-response (Chapter 4); thus, serology, PCR analysis and IHC tests confirmed the EPE-infected status in rabbits. In particular, IHC showed that only 2 out of 6 total EPE-infected does had no detectable lesions at disease peak (14 days PI) and the 3 EPE-infected rabbits euthanized at 8 days PI had confirmed lesions too, despite a typical EPE appearance of the serosal and mucosal layers of jejunum and ileum on gross pathology (Chapter 4). Furthermore, without bacterial inoculation, no infection occurs (p=0.002), with an odds ratio of developing infection 57 times higher after exposure to *L. intracellularis*.

It is difficult to determine if the apparently faster (day 16 PI) cell turnover/healing (usually noted at 20-21 days PI in the original infection model) was due to the natural evolution of the lesions (Chapter 4) or GaM effects on macrophage activity, similar to the action of other gallium compounds on various cell-lines of the reticulo-endothelial system (Bockman, 1991). However, intracellular [Ga] in activated macrophages was not assessed in this lapine model, or in equine experiments described thus far (Martens, *et al.*, 2007; Arnold, *et al.*, 2010). In the rabbit model, we assessed [Ga] and [Fe] in targeted GIT sections and not in macrophages for 2 reasons: to assess the impact of disease on the tissue content of these oligo-elements and because EPE is uncommonly associated with large inflammatory reactions. In the equine studies, both foals and horses utilized were clinically normal (Martens, *et al.*, 2007; Arnold, *et al.*, 2010).

In uninfected control rabbits, a single oral GaM administration did not cause major differences in tissue accumulation within a day PT. However, in rabbits sacrificed 9 days PT, [Ga] and [Fe] in tissues tended to show reverse correspondence in specific GIT sections: gallium preferentially accumulated in the caecal appendix tissue, where reticulo-endothelial tissue is mostly concentrated, but this finding was true in reverse for [Fe]. Whether this phenomenon is dependent upon Ga(III) sequestration by macrophages is not known.

Not surprisingly, EPE infection led to decreased tissue [Ga], particularly where lesions were more pronounced (*i.e.*, terminal jejunum and ileum), as the intestinal absorption is severely impaired (Wong, *et al.*, 2009). Moreover, [Fe] was decreased throughout the entire GIT in the EPE-infected rabbits. Reductions in [Fe] may be due to competition with GaM for tissue uptake and disruption in iron absorption due potentially to saturation of Fe(III) transporters (*e.g.*, transferrin, siderophores). It is important to note that [Fe] was decreased in the cecum, despite

the prolonged residence of *digesta* in that GIT section, and a single GaM dose can alter both iron absorption and catabolism *equilibria*, despite normal iron dietary levels.

In conclusion, our study allowed the first evaluation of GaM PK in uninfected and in EPE-infected rabbits, along with an assessment of iron tissue contents achieved after a single GaM administration. A dosing interval of *q* 48 h was estimated to sufficiently maintain therapeutic concentrations during EPE infection in rabbits. Nonetheless, dose titration studies and assessment of safety of such a dosing regimen (50 mg/kg *q* 48 h) should be carried out in foals, before employing this therapy in clinical patients. Finally, it should be considered that EPE-related malabsorption may result in decreased Ga and Fe GIT tissue concentrations in EPE-infected animals. Thus, the use of GaM as AM alternative therapy warrants further assessment, not only for its ability to compete with the essential nutrient iron, but also for its impact on recovery from infection.

CHAPTER 8

EFFICACY OF GALLIUM MALTOLATE AGAINST *LAWSONIA INTRACELLULARIS* INFECTION IN A RABBIT MODEL.

Relationship of this study to the dissertation objectives.

In this chapter we report the study and its results in regards of gallium maltolate efficacy in a rabbit infection model for EPE. This study was the ultimate goal of the doctoral project in an animal model phase, where the novel compound gallium maltolte was compared to the leading treatment (doxycycline) in equine medicine for EPE cases and a negative untreated control. This study needed all of the research studies reported throughout the dissertation. In the future, it may be helpful in constructing further studies whether in animal models or in clinical trials.

8.1 Abstract

Antimicrobial efficacy against *Lawsonia intracellularis* (LI) is difficult to evaluate *in vitro*, thus gallium maltolate's (GaM) effects were investigated in a rabbit model for equine proliferative enteropathy (EPE). Juvenile (5-6 week old) does were infected with 3.0×10^8 LI/rabbit and divided into 3 groups (n=8). One week post-infection, one group was treated with GaM, 50 mg/kg; one, with doxycycline, 5 mg/kg; and one with a sham-treatment (control). Feces and blood were collected daily and weekly, respectively, to verify presence of LI fecal shedding using qPCR and seroconversion using immunoperoxidase monolayer assay. Rabbits were sacrificed after one week of treatment to collect intestinal tissues (IT) with focus on EPE-affected sections. In IT, lesions were confirmed via immunohistochemistry (IHC) and gallium ([Ga]) and iron ([Fe]) concentrations via inductively coupled plasma-mass spectrometry. Chi-square tests and one-way ANOVA were used (α=5%), respectively, for the comparison of lesions, the results of other diagnostics in the 3 groups, and for comparing [Ga] and [Fe] in IT. No difference was noted between treatments regarding EPE-lesions in jejunum (p=0.51), ileum (p=0.74), and cecum (p=0.35), or in LI fecal shedding (p=0.64). GaM appears to be no more efficacious than doxycycline against EPE in infected rabbits, but multiple treatments led to higher [Ga] and [Fe] in the cecum (p=0.0064, <0.0001, respectively).

8.2 Introduction

The obligate intracellular gram-negative bacterium *Lawsonia intracellularis* is the etiological agent of proliferative enteritis, or enteropathy (PE), an emerging disease in weanling horses known as equine PE, or EPE (McOrist, *et al.*, 1995a; Pusterla & Gebhart, 2009). Among the multiple domestic and wildlife species affected, PE is of prime importance in pigs (porcine PE, or PPE) due to the significant economic and welfare losses incurred with this pathogen (Lawson & Gebhart, 2000). *L. intracellularis* causes different degrees of enterocytic hyperplasia in the distal jejunum and ileum (occasionally cecum and colon), and affects the intestinal ability to absorb nutrients (Herbst, *et al.*, 2003; Horiuchi, *et al.*, 2008; Pusterla & Gebhart, 2009; Wong, *et al.*, 2009). In foals, EPE's clinical signs range in severity and chronicity from generalized wasting and pronounced ventral edema (along with altered colloid-osmotic pressure and

hypoalbuminemia) to profuse diarrhea, fever, colic, acute shock and death (Pusterla & Gebhart, 2009; Page, *et al.*, 2012). Thus, *L. intracellularis* infection is a significant burden for the equestrian and swine industries, with economic losses due to animal mortality, poor growth rates and costly therapeutic interventions, as well as lost revenue from recovered animals (Frazer, 2008).

The current antimicrobial (AM) treatment for EPE relies either on oral macrolides, or azalides, often combined with rifampin (Lavoie, *et al.*, 2000; Schumacher, *et al.*, 2000; Feary, *et al.*, 2007), or on the parenteral or oral administration of tetracyclines, with no further alternative AMs proposed since 2006 (Sampieri, *et al.*, 2006; Frazer, 2008). Those AMs are lipophilic drugs, capable of permeating cell membranes to reach intracellular microorganisms that are otherwise inaccessible to the common water-soluble AM utilized in equine therapy (*i.e.*, beta-lactams and gentamicin) (Sampieri, *et al.*, unpublished data). *L. intracellularis* presents a challenge for AM therapy as these bacteria use the "sanctuary-like" location of enterocytes to avoid AM activity and the local and cellular host immune defenses. Although not specifically evaluated, treatment failures (Page, *et al.*, 2012; Frazer, 2008), indicate the potential for *L. intracellularis* to develop a specific AM susceptibility pattern, or even resistance (Frazer, 2008).

To date, antimicrobial susceptibility studies for EPE have not been conducted *in vitro*, but several studies were designed to evaluate PPE strain susceptibility to a variety of AMs. Although they attempted to plausibly mimic clinical conditions, these studies demonstrated clear technical limitations, possible geographical strain differentiation for the 10 PPE strains known today, and the intrinsic difficulty of maintaining *L. intracellularis* in pure cell-culture (Wattanaphansak, *et al.*, 2009a; McOrist, *et al.*, 1995c; Lawson, *et al.*, 1993). In the early years of PE research, two clinical studies showed the effectiveness of the tetracycline class in affected pigs and in a hamster PPE model (La Regina, *et al.*, 1980). Furthermore, tetracycline AMs demonstrated efficacy against several other intracellular bacteria (Moulder, 1985; McOrist, *et al.*, 1995c), but for EPE most therapeutic knowledge has been achieved through clinical experience.

It is postulated that a novel, gallium-based compound, gallium maltolate (GaM), might be capable of reaching intracellular bacteria through the oligoelement absorption mechanism (specifically iron, zinc and aluminum) in the small intestine, without generating specific grounds for AM resistance (Gunther & Wright, 1983; Berry, *et al.*, 1984; Caspary, 1992). Elemental

gallium (Ga(III)), a post-transition metal belonging to the periodic table's group IIIA, seems ideal for the so-called "Trojan horse" mechanism, as its electric charge, ionic radius, valence and electronic footprint are similar to ferric iron (Fe(III)), which is metabolically essential to pathogen survival (Bernstein, 1998; Collery, *et al.*, 2002). Gallium is preferentially absorbed by phagocytic cells during the inflammatory process and appears to copiously converge onto sites of infection, entering macrophages through mechanisms dependent and independent of the iron-transporter transferrin (Tsan, 1986; Chitambar & Zivkovic, 1987). Bacteria take up Fe(III), whereas mammalian cells and structures (*e.g.*, heme) use ferrous iron (Fe(II)) and subsequently process it through various red-ox reactions (Logan, *et al.*, 1981; Bernstein, 1998). Thus, at sufficiently high concentrations, Ga(III) can be absorbed by bacteria, altering the first step of the bacterial ferric metabolism (Olakanmi, *et al.*, 2000). Unlike iron, gallium is unable to complete a redox-cycling reaction in several iron metabolic pathways and enzymes (*e.g.*, ribonucleotide reductase) necessary for DNA replication and cell functions (Bernstein, 1998). The blockage of these iron-dependent metabolic steps has been clearly ascribed to elemental gallium, not its salt forms, in *Mycobacteria,* and it was hypothesized in *R. equi* (Olakanmi, *et al.*, 2000; Harrington, *et al.*, 2006). Ga(III)'s activity appears sufficient to reduce bacterial survival and replication (Bernstein, 1998). In the United States, gallium is used for treatment of human cancer-related hypercalcemia (citrate-chelated gallium nitrate) and for diagnostic imaging (as the radioisotope Ga^{67}) (Chitambar, 2010).

Recently, the AM activity of several gallium-based compounds, such as gallium maltolate and gallium nitrate, was explored for several other intracellular bacteria of veterinary interest (Harrington, *et al.*, 2006; Fecteau, *et al.*, 2011; Arnold, *et al.*, 2012). Gallium salts have poor solubility in aqueous solution, becoming often unsuitable for oral or parenteral administration due to the formation of insoluble by-products (*e.g.*, gallium hydroxide, gallate), which limits enteral absorption and bioavailability or causes nephrotoxicity (Bernstein, 1998). On the contrary, GaM is safe when orally administered to humans, mice, rats, dogs and foals (Bernstein, *et al.*, 2000; Martens, *et al.*, 2007a; Martens, *et al.*, 2010) and no nephrotoxic effects of GaM are reported to date (Bernstein, *et al.*, 2000). Although the replication pathway characteristics specific for *L. intracellularis* are still incompletely known, it can be assumed that GaM should convey Ga(III) in proximity of the bacteria, particularly the ones located at the intestinal brush border membrane (Bernstein, 1998).

In this study, the potential differences in efficacy between doxycycline (at 5 mg/kg), the current most common AM treatment in EPE, and GaM (50 mg/kg) relative to an untreated control were tested following a multiple oral dosing regimen in a rabbit EPE infection model (Chapter 4). The dosing regimens are based on our understanding of doxycycline pharmacokinetics in horses (2006) (Davis, *et al.*, 2006) and GaM pharmacokinetics in healthy and EPE-infected rabbits (Chapter 7), as well as literature support for doxycycline efficacy against multiple intracellular bacteria (*e.g.*, *N. risticii* and *R. equi*) (Womble, *et al.*, 2007).

8.3 Material and Methods

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

8.3.1 Animals.

The same species and provider for rabbit was used (*Oryctolagus cuniculi,* Strain - 052 VAF Rabbits; Charles River Canada, Pointe Claire, QC, Canada), as previously described for Chapter 4, 5 and 7. The main difference from the described protocol was the 24 does were 5 to 6-week-old, 1.2–1.5 kg BW. The rabbits health status report, their identification procedure, the housing, the husbandry conditions, feeding, environmental enrichment and monitoring were identical to the ones previously described (Chapter 4). The rabbits were allowed a one-week acclimatization period, during which time treats (apples and carrots) were offered to promote socialization, facilitate handling, and monitor individual appetite, alertness and brightness, and to help the animals to ingest the oral medication more willingly (*e.g.*, bananas specifically in the doxycycline group). Does were group-housed in separate pens according to group (controls, GaM and doxycycline).

8.3.2. *L. intracellularis* rabbit infection model.

The protocol previously described for inducing lesions with the equine strain *L. intracellularis* in juvenile rabbits, was followed to achieve infection (Chapter 4). Onset of infection was detected *ante mortem* through current diagnostics used for EPE, such as immunoperoxidase monolayer assay (IPMA) serology and TaqMan qPCR on fecal material

(Guedes, *et al.*, 2002c; Pusterla, *et al.*, 2009a; Chapter 4). The *post mortem* assessment was based on gross pathology examination, haematoxylin-eosin (H&E) staining, and immunohistochemistry (IHC) labeling of *L. intracellularis* using murine anti-*L.intracellularis*-antibody on samples previously fixed in 10% buffered formalin (Guedes, *et al.*, 2002a).

8.3.3. Study design.

Gallium maltolate (provided by L.R.B., Terrametrix, Menlo Park, CA, USA) was dissolved in sterile double-distilled water until a clear solution was achieved immediately prior to administration at a concentration of 8 mg/mL and final pH = 5.8 - 6 (Harrington, *et al.*, 2006).

Rabbits were divided into 3 treatment groups of 8 animals: control group, doxycycline group and GaM group. Drug treatments were started on day 7 post-infection (PI), a time when EPE lesions begin to appear, with a disease peak around 12-14 days PI in the rabbit model (Chapter 4). The control group received a mixture, in 6 mL suspension volume, of water and Jell-O (Kraft Foods, Canada), administered orally *q* 48 h. The doxycycline group received an oral suspension of doxycycline at 5 mg/kg *q* 24 h dose (10 mg/ml). The GaM-group was administered the GaM solution via a nasogastric tube (5 Fr. wide, 40 cm long feeding tube (Kendall Sovereign, Tyco Health Care Group LP, Mansfield, MA, USA)), as previously described in Chapter 7.

8.3.4. Sample collection.

Pooled fecal samples were collected daily from each group and frozen at -20°C until analysis. In all does, arterial blood (1 mL) was collected at the time of infection and then once weekly until the day of euthanasia (day 14 PI) to detect an immune response. For pharmacokinetic purposes the GaM group rabbits were sampled at time 0 h (t_{first}), at 24 h post dose (peak), and at 48 h (trough), immediately before the next GaM administration. Due to a need to examine *L. intracellularis* lesions at disease peak, euthanasia coincided with day 8 of treatment and GaM was therefore administered only 4 times. As the concentration achieved with 4 administrations was not sufficient to expect steady-state, the data collected were not analyzed for PK parameters estimates (Riviere, 2011). Intestinal sections were collected from all rabbits.

One rabbit in the GaM-group died within less than 18 after dosing. Necropsy indicated that cause of death was aspiration pneumonia.

8.3.5. Verification of infection

8.3.5.1. Histology and immunohistochemistry.

Two adjacent, formalin-fixed and paraffin-embedded sections per sample were cut and stained by H&E and IHC with streptavidin method, using murine anti-*L. intracellularis*-specific monoclonal antibody, to detect proliferative lesions of the intestinal epithelium and the presence of the antigen within the cells, respectively. The *L. intracellularis*-specific antigen in the enterocytes was blindly evaluated with a 5 grade IHC scoring system as previously reported (Guedes, *et al.*, 2002a). For each rabbit, a negative control for each intestinal section consisted of a corresponding IHC-labeled tissue section with the exclusion of the primary antibody. Furthermore, pig ileal tissues known to be negative and positive for *L. intracellularis* infection were labeled with the murine anti-*L. intracellularis* monoclonal antibody to confirm the antibody's specificity and sensitivity, respectively.

8.3.5.2. Serology.

In serum, anti–*L. intracellularis*–specific IgG titer was measured by an immunoperoxidase monolayer assay (IPMA), as reported previously (Guedes, *et al.*, 2002c). Positive serum samples were end-point titrated starting with a dilution of 1:30 up to 1:1920. Control samples consisted of serum from a rabbit prior to (negative control) and after (positive control) hyperimmunization with *L. intracellularis* purified from cell-culture.

8.3.5.3 Quantitative PCR Analysis.

Quantitative PCR analysis was conducted on fecal samples, as previously reported (Pusterla, *et al.*, 2010a; Chapter 4). The purified DNA was analyzed by qPCR for presence of *L. intracellularis aspA* gene copies (Pusterla, *et al.*, 2009a).

8.3.5.4 Gallium and iron elemental analysis

Serum and intestinal tissue (IT) concentrations of elemental gallium ([Ga]) and iron ([Fe]) were determined through Inductively Couple Plasma - Mass Spectrometry (ICP-Mass Spec) as already described in Chapter 7. The maltol portion of the initial compound was not

detectable, due to its rapid metabolism through the liver, hence the tissue analysis focused on metals' concentrations (Bernstein, *et al.*, 2000).

8.3.6. Statistics

Statistical analysis was conducted through a commercial software program (GraphPad Prism 5.4, GraphPad Software, Inc., Software, La Jolla, CA, USA). Contingency tables with Chi-square tests were used for comparison of the IHC lesions in different IT sections and for trend. For [Ga] and [Fe] in intestinal tissues, bodyweight gains, qPCR analysis and serology comparisons in relation to GaM treatment a one-way ANOVA with Bonferroni *post-hoc* test was implemented. Alpha was set at 5%.

8.4 Results

8.4.1. Clinical appearance.

All rabbits tolerated the infection, the stress of handling and multiple GaM treatments well. Rate of body weight gain decreased around 11 and 12 DPI (repeatedly observed in the replication of the rabbit EPE model), although this was not significant (p=0.98). In all groups, no significant changes in food intake, fecal consistency, grooming, and behavior were noted for the duration of the experiments. A marginal decrease in body weight gain (a BW gain smaller than half the average daily BW gain observed throughout the experiment) in the GaM-group was observed on day 8, 10, 12, 14 PI and was attributed to the dosing volume (often >10 mL), as it was earlier observed during the PK experiments (Chapter 7) **(Figures 8.1.1 and 8.1.2)**. This was attributed to the sensation of satiation and hunger suppression induced by the stomach distension, after administration of large amounts of fluid (GaM and water solution).

Figure 8.1.1.

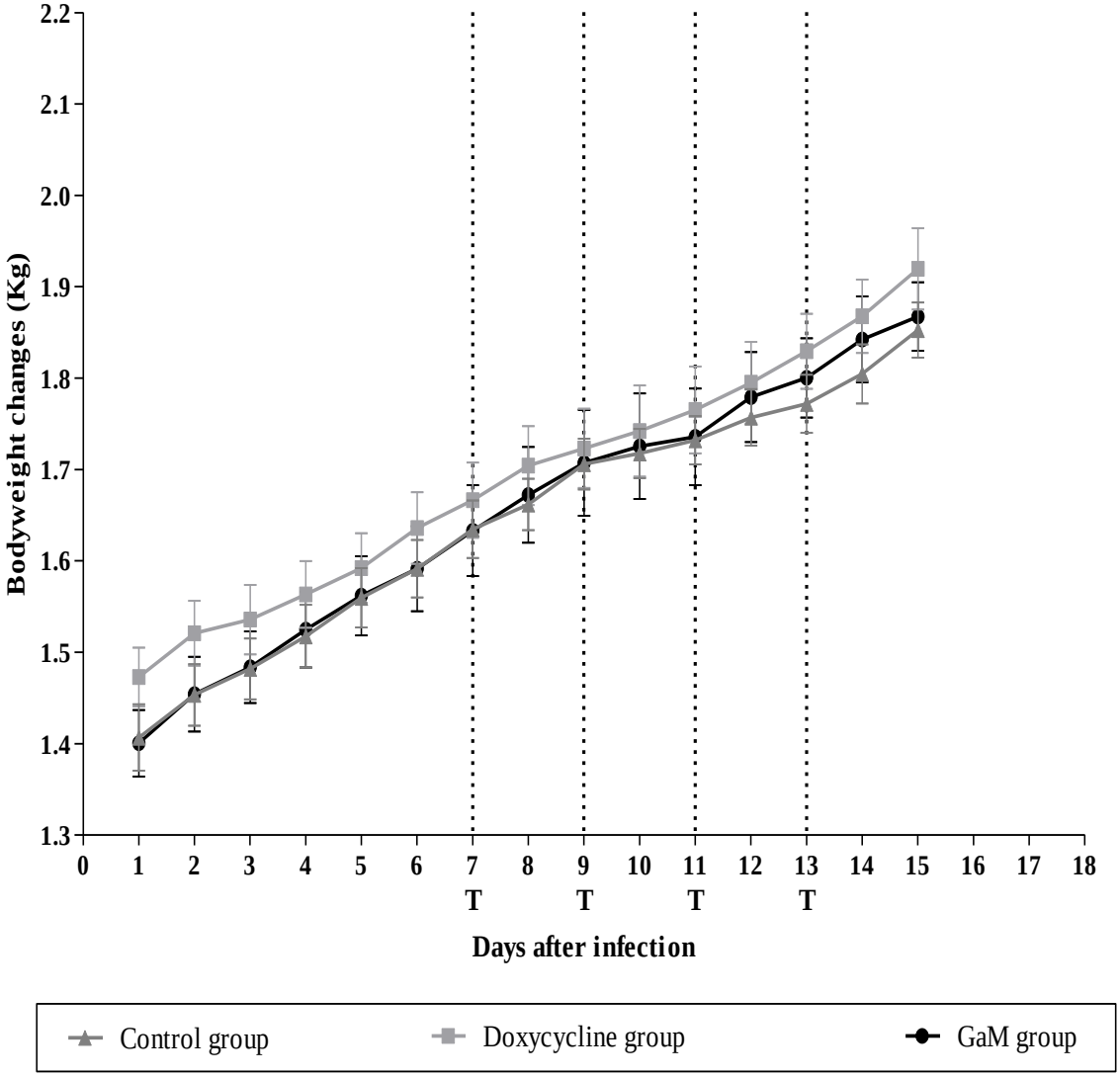


Figure 8.1.1. Bodyweight changes in EPE infected rabbits undergoing 3 different AM treatments. Mean ± SD body weight gain (kg) (n=8/group) of rabbits inoculated orally with *L. intracellularis* and treated with vehicle-only (controls) (6 mL every second day), doxycycline (5 mg/kg once daily) or gallium maltolate (50 mg/kg every second day) by nasogastric tube beginning 7 DPI. No statistically significant difference was noted between the 3 groups. In all groups a reduction of BW gain was noted between day 8 and day 12 PI. The dashed vertical lines and "T" indicate gallium maltolate or control treatment.

Figure 8.1.2.

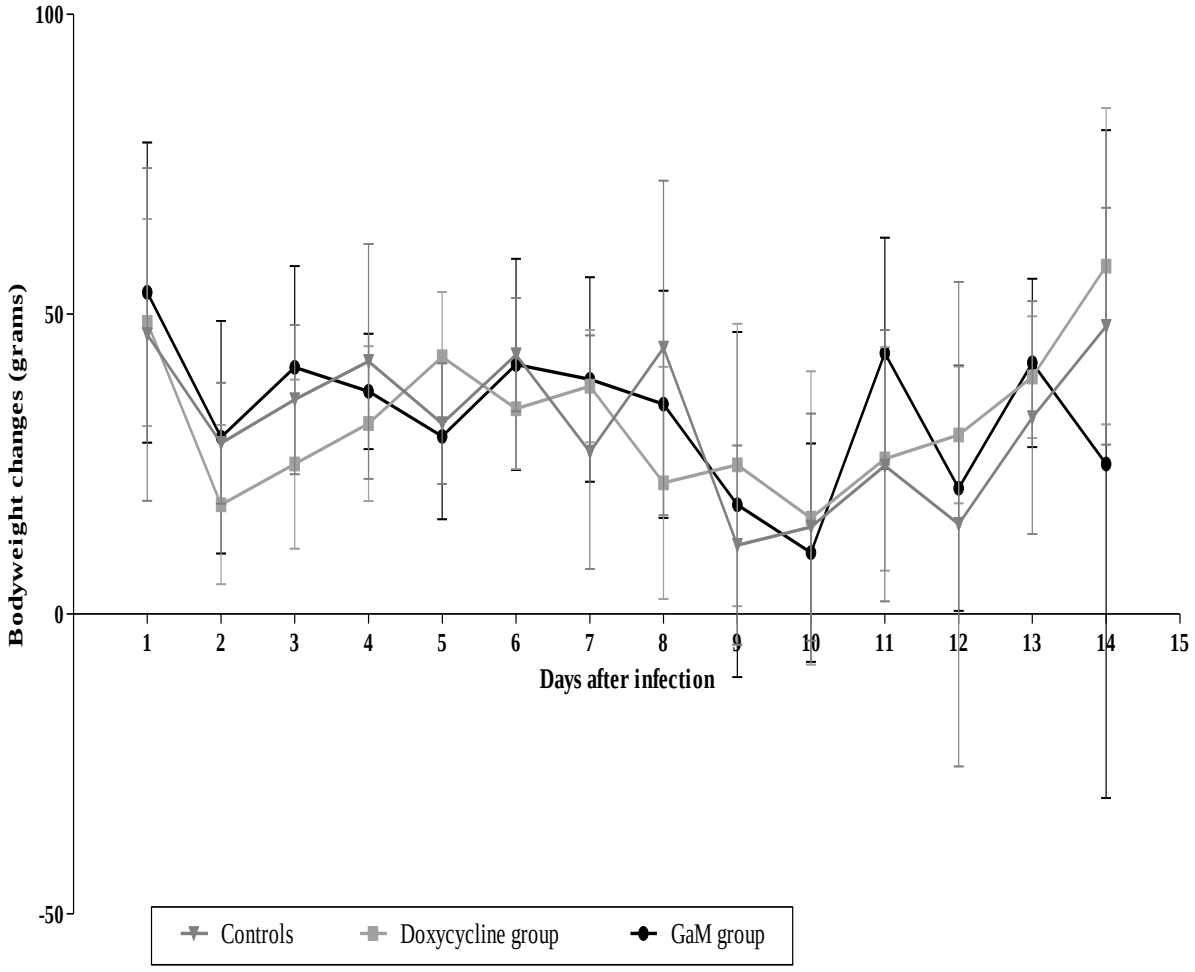


Figure 8.1.2. Daily variation in bodyweight in 3 groups of EPE-infected rabbits undergoing 3 different AM treatments. Mean ± SD daily body weight changes (in grams) in rabbits (n=8/group) inoculated orally with *L. intracellularis* and treated with vehicle-only (controls) (6 mL every second day), doxycycline (5 mg/kg once daily) or gallium maltolate (50 mg/kg every second day) by nasogastric tube beginning 7 DPI. Note the trend in suppression of BW gain between 8 and 12 DPI.

8.4.2. Gross pathology.

Euthanasia was performed 14 days PI, after collection of the last blood sample. Lesions consistent with *L. intracellularis* infection were visible in jejunum and ileum, but were less pronounced in the doxycycline group, with a notable return towards normal in the appearance of the lesions from proximal to distal jejunum.

8.4.3 Serology.

All IgG titers, except for 1 GaM treated rabbit, were negative at 7 DPI (low titer: 60). Seroconversion was demonstrated in all rabbits by 14 days PI (with reciprocal titers ranging between 480 to >1920 in the control group, 120 to >1920 in the doxycycline group, and 480 to >1920 in the GaM group), but no difference (p=0.56) was detected between groups, merely confirming presence of infection and immune-response, reflecting data previously published (Chapter 4).

8.4.4. Histology and immunohistochemistry.

Evaluation of IHC labeling with specific murine anti-*L. intracellularis* antibodies was performed by a blinded examiner on jejunum, ileum and cecum samples, the principal lesion sites of *L. intracellularis* infection (Chapter 4). Typical lesions were confirmed in jejunum (controls: 2; doxycycline: 3; GaM: 1, respectively), ileum (controls: 1; doxycycline: 2; GaM: 1, respectively) and cecum (controls: 0; doxycycline: 0; GaM: 1, respectively) and no statistically significant differences were detected between the treatments, with p=0.51, for jejunum; p=0.74 for ileum; and p=0.35 for cecum.

8.4.5. PCR.

Fecal shedding of *L. intracellularis* was noted by 5 DPI (although inconsistently observed starting at 2 DPI in 2/3 groups). On daily assessments, no statistically significant differences were noted between groups (p=0.61) **(Figure 8.2)**. Shedding persisted for the entire experiment, as previously seen in Chapter 4. Also, no difference was noted in fecal shedding amongst the three treatment groups, considering the amount of *L. intracellularis* DNA detected

in the feces (p=0.64) **(Figure 8.3)** and in the caecal content (p=0.32) collected at the time of euthanasia **(Figure 8.4)**.

Figure 8.2.

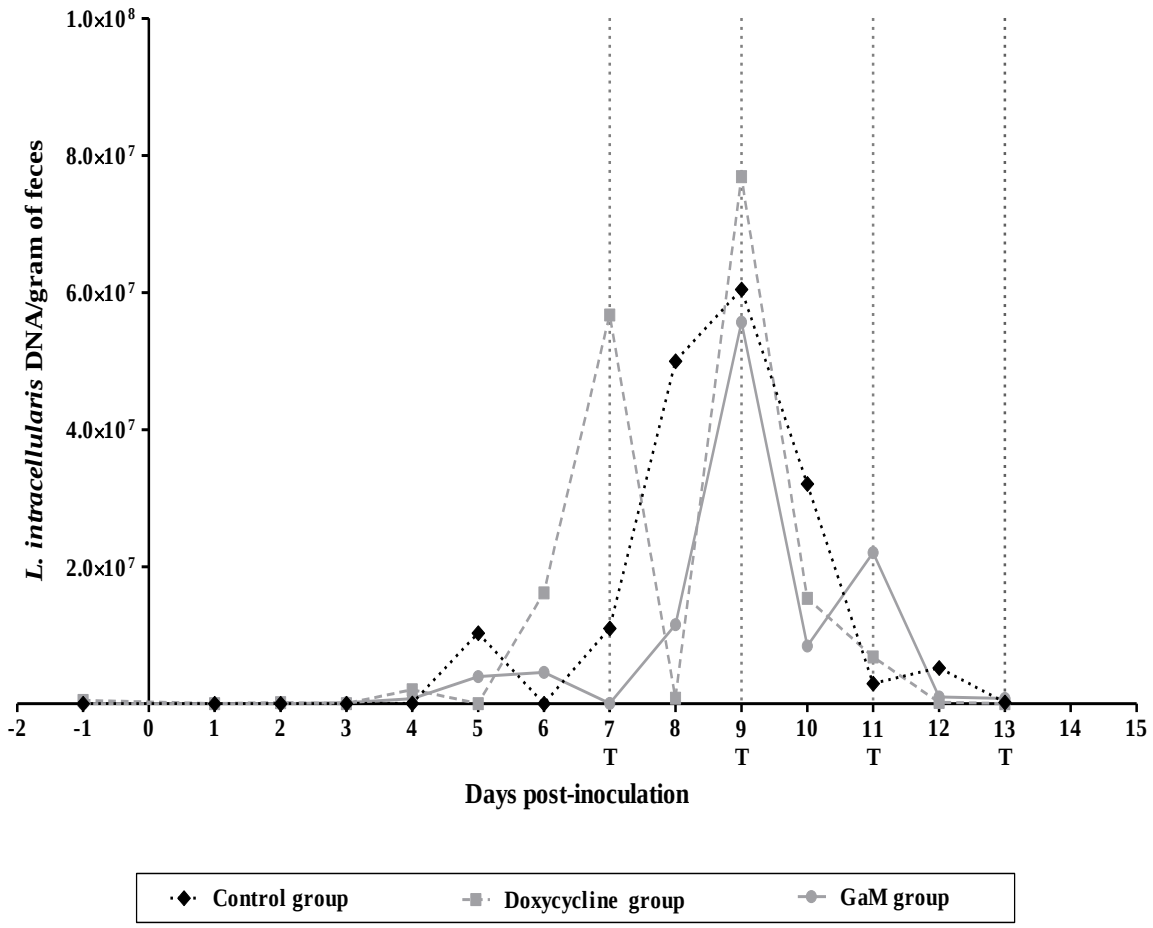


Figure 8.2. Daily progression of fecal shedding in EPE-infected rabbits undergoing 3 different AM treatments. qPCR results reporting daily fecal *L. intracellularis* DNA levels, in rabbits inoculated orally with *L. intracellularis* and treated with vehicle-only (controls) (6 mL every second day), doxycycline (5 mg/kg once daily) or gallium maltolate (50 mg/kg every second day) by nasogastric tube beginning 7 DPI. Fresh feces were collected as pooled samples for each group. The dashed vertical lines and "T" indicate gallium maltolate or control treatment.

Figure 8.3.

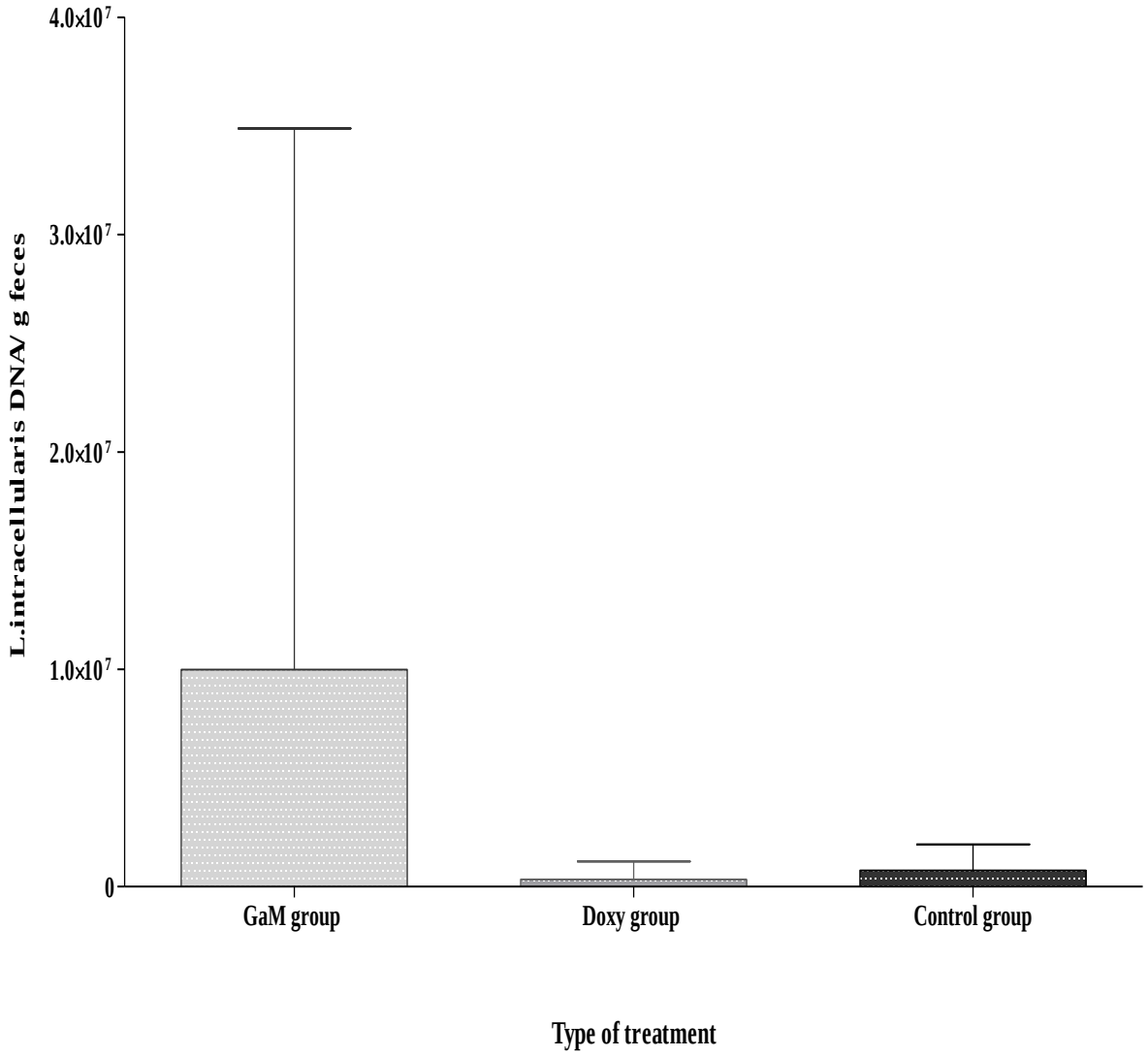


Figure 8.3: Comparison of *L. intracellularis* fecal shedding between groups (averaged values). Mean ± SD of qPCR results of fecal *L. intracellularis* DNA concentrations collected at necropsy in 3 groups of 8 rabbits each, after inoculation with *L. intracellularis* and 7 days treatment with vehicle-only (control) (6 mL every second day), doxycycline (5 mg/kg once daily) or gallium maltolate (50 mg/kg every second day) by nasogastric tube. Although not statistically different, fecal *L. intracellularis* DNA concentration for both doxycycline and control groups were lower than GaM, but still in the range 10^6 to $>10^7$.

Figure 8.4.

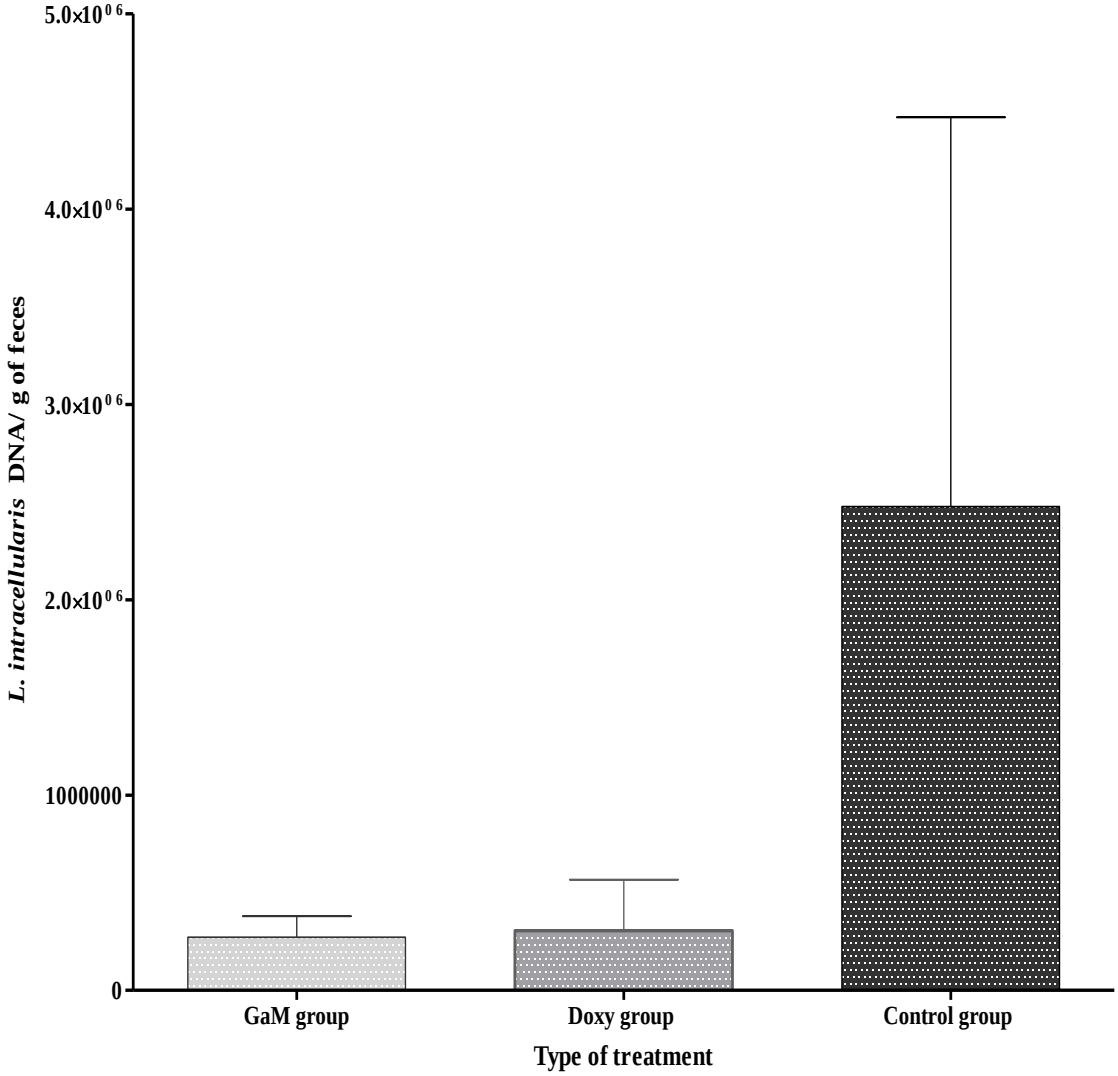


Figure 8.4: Comparison of L. intracellularis caecal content between groups (averaged values on day of euthanasia). Mean ± SD of qPCR results of caecal content *L. intracellularis* DNA concentrations collected at necropsy in 3 groups of 8 rabbits each, after inoculation with *L. intracellularis* and 7 days treatment with vehicle-only (control) (6 mL every second day), doxycycline (5 mg/kg once daily) or gallium maltolate (50 mg/kg every second day) by nasogastric tube. Although not statistically different, caecal *L. intracellularis* DNA concentration for both doxycycline and GaM groups were lower than untreated controls, but still in the range 10^5 to $>10^6$, showing that untreated animals may carry (and shed) live bacteria in their intestines contaminating the environment with low to undetectable amounts of bacteria for a long time.

8.4.6. Gallium and Iron elemental analysis.

Similarly to the PK study conducted by our research group, [Ga] and [Fe] differed by an order of magnitude, [Fe] in ppm (μg/g) and [Ga] in ppb (ng/g) (GaM-group only). After repeated dosing, comparison between GIT sections of GaM-group rabbits demonstrated higher [Ga] in cecum vs. jejunum and caecal appendix (p=0.0064); whereas for [Fe], the same comparison yielded the highest values in cecum, but the lowest in the caecal appendix (p<0.0001). A comparison between experiments (Kruskall-Wallis test with Dunn's *post hoc* test) of the data obtained from does treated once (limits of detection study) to the ones treated for 7 days, demonstrated a [Ga] higher (p=0.0016) in cecum, caecal appendix and, colon in the latter ones. However, in the same comparison [Fe] is significantly lower (p<0.0001) in every GIT section after multiple dosing. Finally, a comparison of the data obtained from does treated once (PK study) to does treated for 7 days, demonstrated that, as expected, [Ga] is higher (p<0.0001) in the repeated treatment rabbits, but [Fe] is significantly lower in every GIT section, excluding caecal appendix and colon (p<0.0001) as **visible in figures 8.5.1. and 8.5.2.**

Figure 8.5.1.

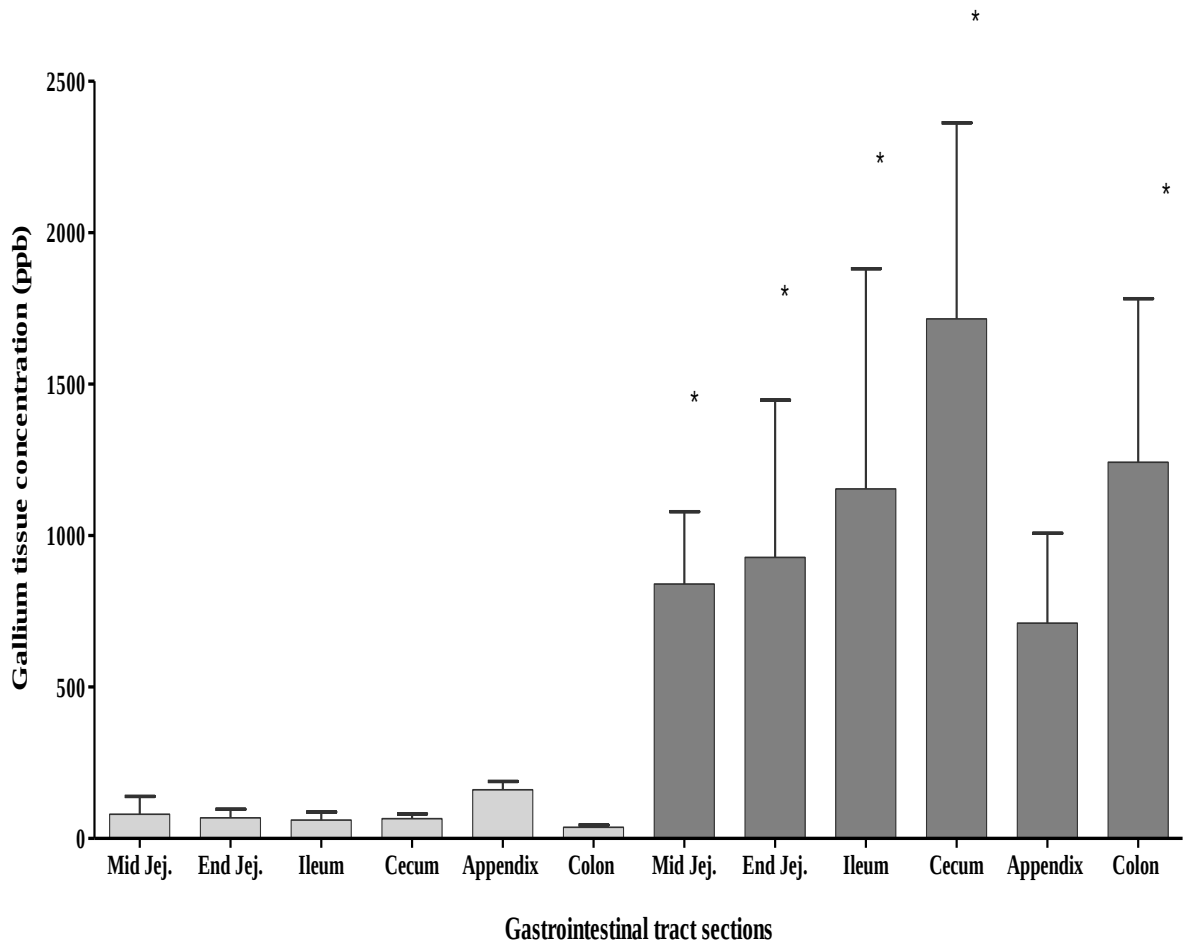


Figure 8.5.1. Accumulation of elemental gallium (in ppb = ng/g) in intestinal tract's sections of EPE-infected rabbits, comparing one single oral GaM treatment (light gray bars) and multiple (n=4) oral GaM treatments (dark gray bars) 24 h after the last dose. The bars indicate the average values and the skewers represent the SD for each intestinal section: light gray bars indicate values in 3 rabbits, for the single oral treatment with gallium maltolate (described in Chapter 7); the dark gray bars indicate value in 8 rabbits treated multiple times.

Figure 8.5.2.

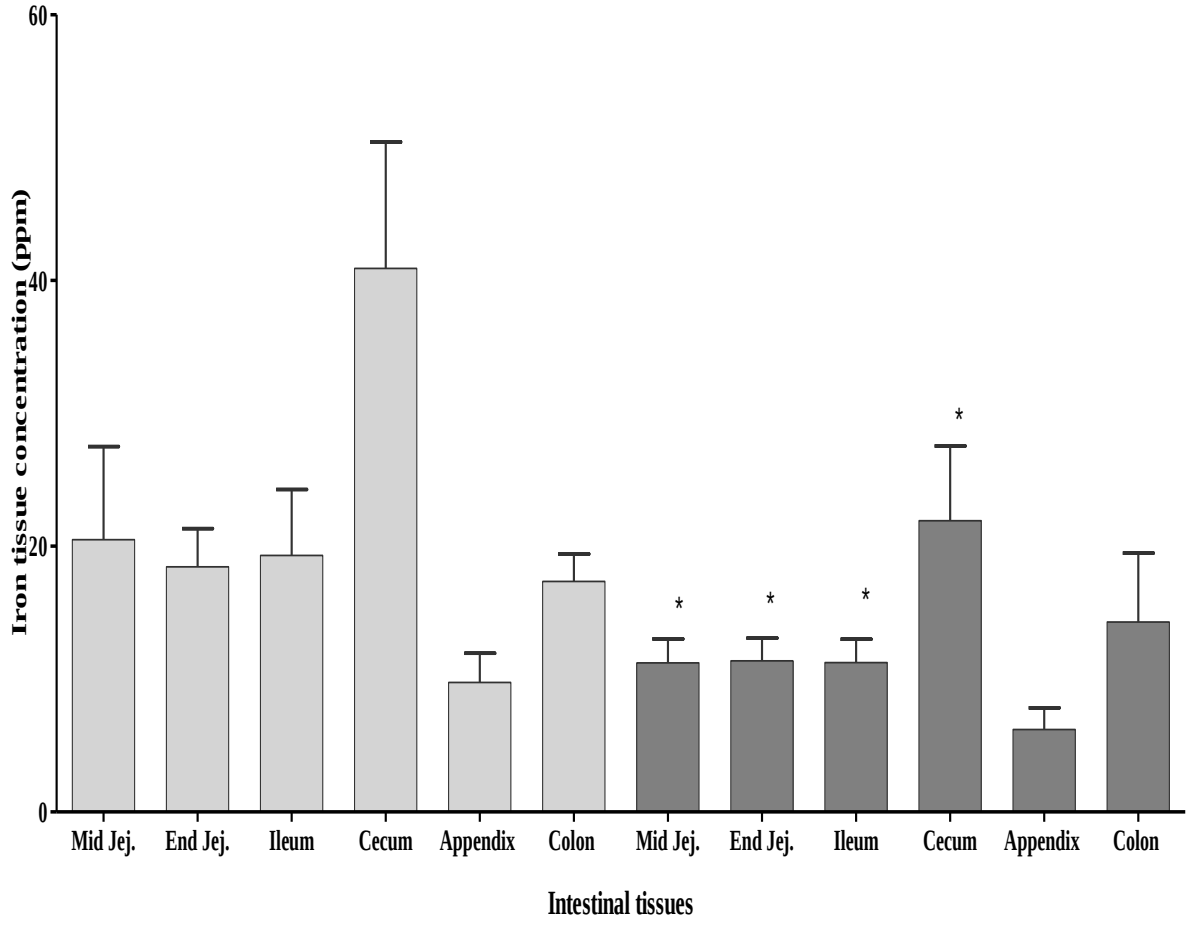


Figure 8.5.2. Concentration of elemental iron (in ppm = μg/g) in intestinal tract's sections of EPE-infected rabbits, comparing one single oral GaM treatment (light gray bars) and multiple (n = 4) oral GaM treatments (dark gray bars) 24 h after the last dose. The bars indicate the average values and the skewers the SD, for each intestinal section: light gray bars indicate values in 3 rabbits, for the single oral treatment with gallium maltolate (described in Chapter 7); the dark gray bars indicate value in 8 rabbits, treated multiple times.

8.5 Discussion

This efficacy study is the first reported antimicrobial (AM) comparison for EPE treatment in an infection model. Multiple oral bolus doses of GaM were clinically well tolerated by EPE-infected rabbits. However, no difference was detected through several diagnostic methodologies between treatment outcomes, between GaM, doxycycline, and a vehicle-only control. Our data in the EPE-infected rabbit model suggests GaM is no more efficacious than doxycycline against EPE. Also, an iron reduced tissue content was noted in several intestinal sections, confirming previous suspicions in regards of risks related to GaM oral administration (Chapter 7) and (Fecteau, *et al.*, 2011).

In the establishment of the EPE-infected rabbit model, we have repeatedly observed a suppression in BW gain on or around the 12th day PI, a time that corresponds to peak infection (Chapter 4). In the present study, we expected the added stress associated with the multiple dosing regimen (*i.e.*, repeated nasogastric intubation and dosing) to affect BW gain more significantly (*i.e.*, remaining well below ≈28.5 g/day in 5 to 6-week-old does (Charles River Canada, Pointe Claire, QC, Canada)), but this was not observed. Furthermore, treatment with GaM or doxycycline failed to reverse this suppression in BW gain around the peak infection time, demonstrating that, regardless of treatment, EPE infected animals still lose body condition (Frazer, 2008; Wong, *et al.*, 2009).

We used the rabbit model to compare AM efficacy against EPE infection because the rabbit represents a "time-compressed" model, as specific IgG antibodies against *L. intracellularis* are typically detectable in rabbits within 2 weeks PI, and occasionally by day 7 PI (Chapters 4 and 5), unlike the foal EPE model (Pusterla, *et al.*, 2010a). Unlike our previous work, the present study used younger rabbits (3 weeks younger than the rabbit model described in Chapter 4), as younger animals were hypothesized to develop more severe lesions, due to their immature gastrointestinal tract and lower immune-competence (Pakandl, *et al.*, 2008). More severe lesions, in turn, would have allowed more readily observable positive treatment outcomes. Once an immune-competent age is achieved, weaned rabbits are known to mount a sufficient immune response when challenged with infectious agents, although this depends on infection type and load (Guedes & Gebhart, 2003c; Pakandl, *et al.*, 2008). Changes in immune-

competence induced by age in suckling rabbits, in relation to *L. intracellularis*, though, are currently unknown. However, rabbits in all treatment groups developed a very high immune response (IgG) to *L. intracellularis* infection, with titers comparable, or higher, than the levels observed in the original EPE infection model rabbits (8-9 weeks old). This may explain the low incidence of severe lesions on pathological and immunohistochemical analysis.

Similarly to the effect on serology, multiple GaM doses failed to decrease fecal shedding of *L. intracellularis* DNA (Figure 3). For all groups the, fecal shedding rates were similar to rates identified in our original infection model (Chapter 4).

Interestingly, DNA concentration in feces from untreated control rabbits was not significantly different than the treatment groups, although the GaM showed somewhat increased values. Quantitative PCR detects DNA material (live and dead bacteria alike), confirming the occurrence of bacterial replication within the intestine (concentrations range: 10^5 to 10^7) during AM therapy with varying concentrations in different intestinal sections (see the differences between feces and caecal contents). Higher fecal shedding of DNA in AM treated rabbits may be related to faster bacterial inactivation and subsequent expulsion, following therapy. However, this finding disagrees with the reported association of AM treatment and decreased bacterial fecal shedding, which prompts clinicians to diligently collect fecal samples before therapy initiation, so to increase diagnostic accuracy (Dauvillier, *et al.*, 2006). In rabbits, the higher caecal concentrations of *L. intracellularis* of untreated controls could be related to *L. intracellularis* colonization in the cecum as reported in the literature (Duhamel, *et al.*, 1998; Horiuchi, *et al.*, 2008), or it may depend on prolonged residence of *digesta* in the cecum relative to other sections of the intestinal tract. A better detailing of such differences between the rabbit model and horses would be useful to understand the true implications of therapeutic responses or lesion (and clinical signs) development. At present, a detailed understanding of differences in bacterial concentrations of the foal's gastrointestinal tract sections is not available (Pusterla, *et al.*, 2010a).

In this study, lesion remission occurred more rapidly than our previous study (14 versus 21 DPI). This observation may relate to the age difference of the rabbits used in each study. Possibly, younger rabbits exposed to EPE proceed more quickly from "onset" to "remission" than older does (9-10 weeks), showing fecal shedding and seroconversion as expected, but

"compressing" even further the timeframe of lesion appearance (Chapter 4). This concept may have epidemiological relevance for the contamination of horse premises (Pusterla, *et al.*, 2012b), but it is as an unwanted complication for our present study. This required us to examine more closely the results obtained through the gold-standard IHC analysis (Guedes, et al., 2002), along with the above-discussed serology and PCR, in the context of the EPE model.

Our statistical comparison of gut lesion counts (or the percentage of rabbits affected) between groups failed to identify significant differences between treatments or between treatments and untreated controls. However, the location of the IHC-labeling within the gut mucosa should be discussed as well, in view of the further time-compression exerted by age on this infection model. Lesion location, allows us to approximate the time of lesion appearance closer to either the time of onset, or the time of recovery. This type of differences is not applicable to statistics, but underlines differences between the affected groups (Chapter 4). Only 37.5% of control rabbits demonstrated observable lesions (counting all 3 sections with IHC positive lesions), but IHC labeling was detected at the level of the *lamina propria*, where bacteria are processed and destroyed. This suggests that rabbits were in the recovery phase, which is typically observed around week 3 PI, in the original model using 9 - 10 weeks old rabbits. In the doxycycline and the GaM treatment groups, rabbits demonstrated observable IHC-labeled lesions in clusters, in 62.5% and 37.5% of cases, respectively. Lesions were principally located in the mucosa and still progressing toward the *lamina propria.* These observations suggest an important delay in lesion development and progression, ascribable to AM therapy. This may explain the enhanced *L. intracellularis* fecal shedding in the GaM group at time of euthanasia, as active lesions remained present in the gut, unlike with untreated controls. In our model, timing of euthanasia with duration of treatment is a significant consideration in the study design and in understanding lesion progression and fecal shedding of *L. intracellularis,* after the initiation of AM therapy.

An additional consideration is the age of the animal. The variability observed between our previously reported EPE model and the present study may suggest a potential difference between the maturation of the IgG system versus the intestinal mucosal immunoglobulin systems (A and E) and the way it affects the response to infectious insults in recently weaned rabbits. Undoubtedly, such a consideration warrants further research (not only in rabbits), as similar

findings were obtained with AM treatments in porcine models of PPE used in efficacy studies (personal communication of Dr. C.J. Gebhart - University of Minnesota). Interestingly, gross pathological examination of the intestines suggested that doxycycline-treated rabbits appeared to show a faster return to normal of the affected digestive tract. Gross reductions of serosal edema and wall thickness were observed moving progressively from proximal to distal locations within the small intestine, and this effect of doxycycline should be further investigated.

Our comparative AM analysis in an EPE model calls into question the use of GaM as a potential AM treatment for EPE. Recent studies on GaM's efficacy against *R. equi* infection showed its failure to prevent disease, after a prophylactic treatment prior to experimental infection at a dose capable of achieving the minimum inhibitory concentration (MIC) necessary to inhibit rhodococcal growth (Harrington, *et al.*, 2006; Coleman, *et al.*, 2010; Chaffin, *et al.*, 2011). For *L. intracellularis*, where *in vitro* susceptibility studies are very difficult (Lawson, *et al.*, 1993), our use of a rabbit model of EPE clearly indicated a delay in lesion development with GaM therapy suggesting that any expectation for obtaining a better result with a GaM prophylactic treatment are low, unlike the expectations associated with GaM research on *R. equi* affected foals (Chaffin, *et al.*, 2011). Until *in vitro* studies clearly demonstrate *L. intracellularis* sensitivity to GaM, we suggest that further studies *in vivo* are not warranted and would conflict with the 'Three Rs' tenet of animal experimentation (Fenwick, *et al.*, 2009).

Furthermore, multiple GaM doses in EPE-rabbits, expectedly, led to increased IT [Ga], when compared to a single dose, but also caused a decreased [Fe] in IT, except in caecal appendix and colon (where Fe absorption is reduced, as per normal physiology). Interestingly, multiple GaM dosing highlighted a preferential location for this transitional metal, similarly to the findings of the rabbits examined 9 days PT in the PK study (Chapter 7), as Ga(III) tends to accumulate in the caecal appendix, mostly constituted by reticulo-endothelial tissue, and to affect negatively [Fe] in the same tissue. At this stage, it can only be hypothesized that the low [Fe] could depend on Ga(III) sequestration in macrophages. Regardless of the reason, these findings confirm that GaM hinders iron absorption, competing for the same absorption pathways and transporters (*i.e.*, transferrin and siderophores). The impact of this process, and the altered regulation of iron absorption (demonstrated by reduced tissue content), appears to complicate the already fine equilibrium of oligo-elements in a diseased intestine. This is hypothesized as due to

the further absorption competition created by the presence of Ga(III), particularly in spite of a normal dietary iron concentration. Overall, our model sets a valid mimicry situation of an impaired intestinal absorption. In fact it demonstrates that if the effects of GaM may reduce considerably the ability of the intestine to absorb iron, the results are not only that it would limit the bacterial metabolism, for which it was advocated initially, but also that it may contribute to defects in host's iron metabolism, if treatment is sufficiently protracted (Wong, *et al.*, 2009).

In conclusion, multiple dosing of GaM, at 50 mg/kg (2.5 times the dose used in foals), although clinically safe, is not a valid alternative to the current AM therapy employed in EPE-affected animals. Although our rabbit model represents a mild form of EPE-infection, no treatment advantage was observed with GaM. We conclude that our study offered no evidence to support the use of multiple dosing of GaM, at 50 mg/kg (2.5 times the dose used in foals) as an alternative treatment to current AM therapy employed in EPE-affected animals. Given the lack of supportive evidence in a preclinical model of EPE, further investigations with GaM in foals infected with EPE is not warranted at this time. *In vitro* MIC studies may demonstrate a need to re-evaluate GaM *in vivo* in the foal, but until such studies are completed we recommend that EPE infected foals be treated with the current standard of care. Moreover, prolonged treatment with GaM may become a hindrance into the normal iron metabolism in the host, potentially adding complications and prolonging either the appearance of disease, or the progression of recovery.

CHAPTER 9

GENERAL DISCUSSION

"Be calm in arguing; for fierceness makes
Error a fault and truth discourtesy."

G. Herbert

This project was structured in 2 phases, and for clarity, the phases will be discussed in order: the animal modeling phase and the pharmacology phase. The combined results of the animal modeling experiments changed the structure of the pharmacology experiments, but broadened the perspective for the future use of animal models in research involving *L. intracellularis*, the agent of equine and porcine proliferative enteropathy (EPE and PPE, respectively).

9.1. Animal Modeling Phase

9.1.1. Rabbit Model

The first objective was to determine whether EPE infection can be achieved after challenging juvenile rabbits (*Oryctolagus cuniculi*) with an equine strain of *L. intracellularis*, as lagomorphs are naturally susceptible to infection (Duhamel, *et al.*, 1998; Horiuchi, *et al.*, 2008). As the infection route is typically fecal-oral, the rabbits were inoculated intragastrically with a pure low-passage cell-culture of E40504 *L. intracellularis*, harvested from a deceased foal (Pusterla, *et al.*, 2010). Results were consistent with a humane model. EPE caused detectable lesions in jejunum and ileum (prime sites for EPE lesions), by gross pathology, histopathology and immunohistochemistry (IHC) (Guedes, *et al.*, 2002a). Also, large fecal shedding of *L. intracellularis* DNA, closely connected with the presence of lesions, was detected, along with high serological response (high IgG concentration). However, no overt disease (*e.g.*, diarrhea)

was observed. Clinically, we noted only what appeared to be a consistent trend in body weight (BW) gain suppression, although never statistically significant, both in the first (p = 0.87) and in all the subsequent experiments (total p = 0.23) involving EPE- infected rabbits. Out of 42 infected-rabbits in total, only one rabbit manifested overt depression, lack of appetite and weight loss, around 12-14 days post infection (DPI) (See Chapter 4).

The rabbit infection model, established for the first time, permitted the planned pharmacokinetic study on Gallium maltolate (GaM) in rabbits, both in healthy and in EPE-infected animals, as well as supplying a reliable alternative, when the experiments on hamsters failed to deliver the expected results (See Chapter 5).

9.1.2. Hamster Model

The second objective of this study was to reproduce EPE from an equine strain of *L. intracellularis* in weanling hamsters (*Mesocricetus auratus*), as they have been successfully utilized, for over 20 years, to model PPE and they are naturally susceptible to proliferative enteropathy ("wet tail") (Jasni, *et al.*, 1994a; Vannucci, *et al.*, 2010). The results in 2 groups of weanling hamsters, challenged again with the *L. intracellularis* E40504 strain, were consistent with this species being apparently resistant to developing detectable intestinal lesions, serological response, and clinical disease; despite a low and inconsistent level of fecal shedding of bacterial DNA, detected throughout the experiment (See Chapter 5).

A few factors were discussed and evaluated while trying to understand why, during the experiments, the hamster appeared to be EPE-resistant: breeding colony differences (*i.e.*, tested from 2 different providers); BW and age at the time of inoculation (*i.e.*, 3 vs. 4 weeks old at the time of exposure); and stress factors, as one group was infected after a post-weaning acclimation period, but the other one was infected right after weaning (*i.e.*, same day). To test such difference during the second trial, along with the EPE infection, a concomitant infection with PPE strain (PHE-MN-01) was performed and used as a positive control. As per textbook description, PPE caused lesions and exponentially higher fecal shedding of DNA, although no seroconversion was demonstrated. Such results confirmed that PPE strain adapts well to the *Cricetinae* species and that pure cell-culture-derived inoculum does not cause overt and severe clinical signs (i.e.

diarrhea and prostration), compared to ileal scrapings from diseased animals (Jasni, *et al.*, 1994b; Vannucci, *et al.*, 2010).

On the other hand, a suspicion took shape that a secondary host-restriction, or species-specificity of the strain (not the bacterium), could exist. In order to pursue this concept, a small group of rabbits (n = 6) were infected with PPE strain and compared to the EPE-infected group. The results obtained in rabbits were opposite to the ones obtained in hamsters. Rabbits developed the EPE-infection (as described above – See chapter 4), but not the PPE-one, even though transient low levels of fecal shedding and low titers of serological response were detected (See Chapter 5).

9.1.3. Considerations and Discussion

On the basis of these findings, multiple directions can be considered for future studies on *L. intracellularis*. First and foremost the rabbit EPE model appears to be a "time-compressed" infection model when compared to experimentally infected foals. This has been observed both in terms of onset and duration of lesions (2 weeks versus 4-5 weeks), but also immune-response and fecal shedding, although these latter aspects can be considered from different viewpoints.

The compressed lesion and shedding time in rabbits could allow a better focus, for a shorter time, on the research regarding EPE pathogenesis and the recovery pathophysiology. However, at this stage it is unknown how long the immune response could last, because of the short duration (only up to 3 weeks) of our experiments. In foals, the longer incubation, development of clinical signs and lesions is likely tightly related to the development of hypoproteinemia and hypoalbuminemia, 2 very important features in clinical treatment, but in the rabbit model that aspect could not be analyzed, as experiments were only 21 days long.

Very importantly this model is more humane and self-limiting than the one achieved in foals, where a few horses may manifest low grade clinical signs, a few are completely subclinical, but others present the full-blown acute disease, which leaves no alternative but euthanasia, as the experimentally induced disease can be unresponsive to current therapeutic intervention (Page, *et al.*, 2011c; Vannucci, *et al.*, 2012).

The first source of future investigation could be a longitudinal study in rabbits, to understand how long they are really susceptible to infection, and to what extent their immune-response may rise. The researchers (including extramural collaborators) involved in the modeling experiments are aware that laboratory experimental conditions differ from wildlife exposure and natural course of infection. However, observing the whole course of disease and recovery may clarify several of the epidemiology steps that lead to the differences of cross-infection, behind such a peculiar secondary host-specificity.

Dr. Vannucci (University of Minnesota), in his PhD thesis, is demonstrating that infection transfer from finisher pigs to weanling foals, and vice versa, can occur (also investigated subsequently to our hamster trials – see Chapter 5). Although no clinical disease was obtained in both porcine and equine bacterial cross-specific challenges, increase in serology titers and fecal shedding were positive findings (Vannucci, *et al.*, 2012). Those results showed a partial host-adaptation, whereas ours showed a partial host-restriction: in summary both studies provided grounds to the hypothesis of several *L. intracellularis* subspecies. Currently it is unknown how many subspecies exist and whether every subspecies is able to infect across species, but *L. intracellularis* genomics investigation is underway (F.A. Vannucci and C.J. Gebhart, University of Minnesota -- personal communication).

From the longitudinal study we could derive a better understanding of *L. intracellularis* survivability and infectivity, after being shed in the feces, as wild rabbits often populate the environment where horses live (*i.e.*, pastures) and their feed is stored (*i.e.*, haymows) (Pusterla, *et al.*, 2008b; Pusterla, *et al.*, 2012a). Indeed, Dr. Pusterla (University of California-Davis) has reproduced *L. intracellularis* exposure in foals fed grain contaminated with EPE-infected rabbits' feces, obtaining serological response and fecal shedding of the bacterium, although no overt clinical disease (Pusterla, *et al.*, 2012c). As EPE targets mainly young animals, a shorter and more intense (larger litters) reproductive cycle in rabbits and rodents than in horses, contributes to enhance the potential for sub-clinical carriers, for contamination of feed stuff and thereby transmission of the infection.

Further uses of the rabbit model could be to investigate the clinico-pathological combination hypoalbuminemia/ hypoproteinemia, a natural EPE clinical feature in horses. As the rabbit model is "time-compressed", additional clinical changes could be more focused within a

short time frame, to gain knowledge about the processes leading to EPE and also to understand the natural step to remission and recovery, which are apparently normal in rabbits.

One question may arise regarding immune-competence and age as limiting factors for the EPE model in rabbits. A quicker lesion progression and resolution was unexpectedly observed during the efficacy experiment (See chapter 8). The study was conducted on does 4 weeks younger than the original model ones, as older subjects were not available at the time. Immuno-competence and age-dependent factors are not known specifically in relation to *L. intracellularis* infection in rabbits, regardless of the strain; thus, it could be questionable whether our subjects were appropriately immuno-competent, yet too young, for developing normally progressing lesions and that might have complicated the results of the study, not offering a solid term of comparison to infected treated rabbits investigated (Chapter 8). Studies on parasitosis rabbit models, such as nematode or coccidiosis infections (for example), report differences in regards of the immune response and the onset of disease depending on age, and different models are established at different ages (suckling rabbits versus > 3 weeks rabbits, and versus juvenile/prae-pubertal rabbits) targeting different responses. My opinion is that infecting rabbits 8-9 weeks old was a good timeframe to get both immune response and lesions. However, it is to consider in the future whether or not, similarly to those parasitosis rabbit models, infection in suckling rabbits does not lead to disease or immune-response; infection in weaned rabbits (around 3-4 weeks old) leads to very high immune-response with questionable development of lesions; and if infection in 2-3 months old subjects leads to a decent immune-response and disease manifestation (Pakandl, *et al.*, 2008). In the case of parasitology models the intervening immune-response factors may be different than bacterial infection, such as *L. intracellularis*, but such a consideration could clarify not only some use of the model, but also what could be the age categories to target in rabbits, in order to control the impact of EPE carriers on horse premises.

Aside from epidemiological, bacterial genetics and potentially clinical benefits realized, further developments and use of the rabbit model could target the investigation of serological responses in challenged animals. Rabbits are known to mount an intense, and in some cases, prolonged immune response, for which they have already been used in the production of polyclonal antibodies in diagnostic techniques for *L. intracellularis* research (Guedes & Gebhart, 2003c). The possibility of using this model to investigate future development of vaccinal

products could be of support, and benefit, to some of the studies already in progress in the foal model (Pusterla, *et al.*, 2009b; Pusterla, *et al.*, 2009c; Pusterla, *et al.*, 2010a; Pusterla, *et al.*, 2012b; Pusterla, *et al.*, 2012d). For example: 1. reduced purchase and husbandry costs compared to foals; 2. reduced starting inoculum dose (as reproducing sufficient material to inoculate rabbits is less time- and resource-consuming, than producing it for foals) (Pusterla, *et al.*, 2010a); 3. reduced time necessary to detect immune-response (See Chapter 4).

Finally, to try and conclude on the potential developments of this novel (and mild) infection model, it would be interesting to reproduce EPE in a rabbit model starting from equine ileal/ jejunal scrapings rather than from pure cell-culture, as, if we think of a parallel with the PPE strain, both in pigs and hamsters, there is the potential to obtain a more clinically aggressive model from the fresh "street-strain" than from the low-passage cultures (McOrist, *et al.*, 1993; Jasni, *et al.*, 1994a; Jasni, *et al.*, 1994b; Guedes & Gebhart, 2003a; Guedes & Gebhart, 2003b; Vannucci, *et al.*, 2010). The interest tied to this type of study is derived from the initial experiments, as the higher concentration/lower cell-culture passage produced the only "somewhat" clinically affected rabbit (See Chapter 4). Moreover, knowledge gained from the field experiments (Dr. C.J. Gebhart -- University of Minnesota -- personal communication) on vaccines, noted that lower-passage cultures provided a better immunity response, although those same cultures are harder to maintain *in vitro*, whereas the higher the passage in culture, the more immortalized they become. Nonetheless, developing a more aggressive facet to the rabbit EPE model may provide better grounds both for the testing of vaccines and antimicrobial drugs.

Last but not least, the infection modeling phase indicated also that the use of hamsters should be avoided for experimental EPE modeling, unless there is only the aim to account for the fecal shedding of infectious material into the environment. Not only do they not develop clinical disease and intestinal lesions, but the serological response appears to be minimal to none when challenged either with EPE or PPE *L. intracellularis* (See Chapter 5). At this stage, is not clear and may warrant further investigation, whether the lack of a strong immune-response is related to the low immune-competence of weanling hamsters; or due to the effects of the intracellular location, where *L. intracellularis* is protected from the processing steps of the immune system, humoral or cellular. It is arguable, however, that another and more challenge-responsive species, such as the rabbit model, may offer a better insight.

9.2. Pharmacology phase

The pharmacology phase of this project involved the use of the EPE rabbit model in a pharmacokinetic (PK) study and in an efficacy study for the novel gallium-based compound gallium maltolate (GaM). The EPE-infected rabbits were compared to healthy subjects in the PK study, to assess the possible effects of intestinal absorption impairment on subsequent plasma concentrations of elemental gallium. Gallium and iron concentrations in intestinal tissues were compared between EPE-infected and healthy rabbits, both 24 h and 9 days after one single oral bolus.

9.2.1. Ear catheterization and timely sample collection

The first challenge encountered in this phase was merely technical, as healthy rabbits tend to scratch their ears and remove the indwelling catheters placed in the central artery, complicating blood sampling procedures. The problem was solved with a moleskin-glued bandage, in the shape of the ear, transforming the catheter hub and the injection port into a smooth surface, where rabbits' paws could readily glide over. This skin-embedded-like injection-port was utilized up to 9 times in 12 h, simplifying rabbit preparation and restraint, and keeping sampling timely (See Chapter 6). This technical contribution refined the project and reduced stress, both on the rabbits as well as the researchers. It can be easily applied to any situation requiring multiple and rapid sampling within a small time-frame in rabbits (*i.e.*, PK or endocrinology studies).

9.2.2. Pharmacokinetic (PK) study

The PK study demonstrated that rabbits can readily tolerate a single GaM dose (50 mg/kg BW p.o.). PK parameters were estimated in both healthy and EPE-infected animals (See Chapter 7), with estimates more similar to healthy adult horses (C_{max}, t_{max}, elimination rate constant and half-life) than neonatal foals. Although those experiments in horses used a smaller dose than ours (20 mg/kg BW p.o.), they also utilized neonatal foals, or adult horses. We used juvenile rabbits (7-9 weeks old), as they were more comparable to the susceptible age for EPE, although they have a different physiology and body composition than neonates (Martens, *et al.*, 2007a; Arnold, *et al.*, 2010; Chaffin, *et al.*, 2010). In the rabbit model, one bolus dose of GaM did not appear to alter gross pathology appearance, histopathology and IHC of tissues of EPE-infected animals,

and it did not change the serological response and the fecal shedding after infection. However, the concentrations of gallium and iron in the intestinal tissues showed an expected decrease of tissue content of both metals, due to disease. This was particularly apparent for iron, despite it being available in the diet, throughout the experiment, and GaM being administered only once (See Chapter 7).

9.2.3. Synchrotron Radiation Analysis

An accompanying experiment to this second phase was based on synchrotron radiation analysis, to compare gallium, copper, zinc (κ-alpha and κ-beta), and iron's (κ-alpha and κ-beta) fluorescence and spectroscopy values, in intestinal tissues of healthy and EPE-infected rabbits treated with GaM. This study failed to yield results due to the beam-line restricted "limits of detection" abilities and microscopic staging malfunction. The concentrations for gallium were in the range of ppb (ng/g); and for iron in the range of ppm (μg/g), but as many oligoelements in biological matrices, their location is diffuse within cells and tissues, rather than focused in only one point. I suggest that, for synchrotron radiation analysis on specific anatomical structures, a better method of slides' preparation needs to be tested. Specific targets (*e.g.*, epithelial cells) cannot be analyzed without a few reference points (*i.e.*, visible staining or cross wires). Also beam-lines' scientists could invest in a few methodology-development studies, so that reference "limits of detection" can be available to appropriately design investigation of biological material of animal origin and anatomic tissue (See Appendix A).

9.2.4. Efficacy Study

Of the potential uses for the GaM PK study results, one represented the last part of this dissertation's project (See Chapter 8). A safe dosing interval of the same dose of GaM used in the PK study (50 mg/kg BW p.o., *q* 48 h) was derived for EPE-infected rabbits from the estimated PK parameters (See Chapter 7) to administer multiple times in an efficacy study utilizing the rabbit model.

GaM was compared to the leading clinical treatment for EPE, doxycycline (positive control), and to a sham-treatment (negative control). Although all rabbits appeared to tolerate the multiple treatments, no changes were observed in the serological response and the fecal shedding, but, more importantly it led to no better IHC grading for EPE lesions, when compared

to doxycycline, or sham treatment. Actually, lesions consistent with *L. intracellularis* infection were visible in jejunum and ileum of all rabbits, but were much less pronounced in the doxycycline group, particularly with a notable improvement progressing from proximal to distal, in the jejunum (See Chapter 8). However, as the presence of lesions was not a clear cut between infected treated animals and infected non-treated controls, our findings cannot be as definitive as initially we had wished. The considerations about possible age limitations of the rabbit models have been prospected above in the paragraph 9.1. and should be born in mind if a future efficacy study on the EPE model is prospected.

9.2.5 Considerations and Discussion

In light of these results, along with a worsened iron tissue content and apparent sequestration of gallium in the highly lymphatic tissue of the caecal appendix, due to prolonged GaM administration, the options of pursuing further research on GaM in animal models (surrogate species or same species) should probably be reconsidered, or avoided (Fenwick, *et al.*, 2009). In fact, GaM not only failed to affect the *L. intracellularis* infection in this study, but also *R. equi* and a few *Staphylococcus spp.* in other studies (Martens, *et al.*, 2007b; Coleman, *et al.*, 2010 ; Arnold, *et al.*, 2012).

Recently, research results on GaM's efficacy versus *R. equi* were published, showing a failure to prevent disease, as a prophylactic treatment was started even before infection, trying to achieve a desired serum concentration, matched to the minimum inhibitory concentration (MIC) adequate to inhibit bacterial growth (Coleman, *et al.*, 2010; Chaffin, *et al.*, 2011). Thus, unless a MIC study *in vitro* for *L. intracellularis* elucidates a few steps in dose choice or formulation, and supports further investigation, GaM antimicrobial research may have a limited future. Moreover, prolonged treatment with GaM may interfere with the normal host's iron metabolism. This interference could potentially add complications and exacerbation to the disease, or delay the progression of recovery (See Chapter 8).

CHAPTER 10

CONCLUSIONS

"Securus iudicat orbis terrarum"

"The verdict of the world is conclusive"

St. Augustine

10.1.1. Findings: Rabbits infected with equine strain *L. intracellularis* constitute a mild and humane infection model for Equine Proliferative Enteropathy (EPE), without overt disease, but a possible transient weight loss, with detectable immunohistochemistry (IHC) lesions, marked fecal shedding of *L. intracellularis* DNA and detectable specific antibodies (IgG) in serum.

10.1.2. Future implications: The rabbit EPE infection model could be used to substitute for foals in pharmacological and vaccine studies, and to clarify the epidemiological role of wildlife subclinical carriers, in the context of EPE geographical distribution.

10.2.1. Findings: Contrary to the published reports of successful cross- infections after challenging hamsters with a porcine strain of *L. intracellularis* (agent of Porcine Proliferative Enteropathy - PPE), hamsters infected with EPE strain, do not develop evidence of clinical disease, seroconversion (IgG), gross pathology or IHC detectable lesions. Similarly rabbits infected with a PPE strain do not develop evidence of clinical disease or gross and IHC detectable lesions, although they showed a mild to moderate seroconversion and fecal shedding of bacterial DNA.

10.2.2. Future implications: Hamsters should not be used to model EPE infection and rabbits should not be used to model PPE disease in future research, but their ability to shed *L. intracellularis* increases the importance of subclinical and resistant carriers in the environment.

However, such a host-specificity restriction could depend on the specific genetic subspecies of *L. intracellularis*, which will need further genetic investigation

10.3.1. Findings: A moleskin "ear-shaped" adhesive bandage, applied over the ear of a catheterized central artery was developed, simulating a skin embedded injection port. It simplified and refined (according to the "Three Rs" CCAC's tenet) repeated blood sampling, restraint and timeliness in the rabbits' pharmacokinetic study, but it could be applied to any other study that requires repeated blood sampling in rabbits.

10.3.2. Future implications: This technique was intended for short-term 12 h study only. However it could be modified to maintain a catheter for longer than 12 h.

10.4.1. Findings: Gallium maltolate (GaM) was safe to administer orally as a single bolus dose (50 mg/kg) to healthy and EPE-infected rabbits. Pharmacokinetic parameters related to the total body exposure to the drug, and its disposition, were estimated. A dosing interval for EPE-infected rabbits (*q* 48 h) was derived. Our results were closer to estimates in adult horses (treated with 20 mg/kg), than the ones in neonatal foals. GaM administration significantly decreased iron concentration in several intestinal sections' tissue. This decrease was greater in EPE infected rabbits, as tissue oligoelement concentrations were overall reduced.

10.4.2. Future implications: As no absorption and bioavailability parameters could be estimated, due to the restrictions of an oral dosing only, an intravenous administration study should be carried out before initiating any further investigation. Knowledge, of the real bioavailability of this compound will be fundamental for future studies, should the need arise.

10.5.1. Findings: GaM was safe to administer in multiple oral administrations (50 mg/kg *q* 48 h) to EPE-infected rabbits. GaM was compared to control medium-only and doxycycline administrations in EPE infected rabbits. When this occurred, IHC lesions, fecal shedding and serological response did not show any improved efficacy of GaM. Moreover, repeated treatment caused significant reduction of intestinal iron tissue concentration.

10.5.2. Future implications: GaM efficacy and minimum inhibitory concentration (MIC) studies should only be tried on pure cell-culture *L. intracellularis*, as the trials performed

in this project (and in other studies) showed that its efficacy is no better, or worse, than the current clinical therapies, and the only justification to use GaM *in vivo* again should be on the grounds of promising MIC findings.

CHAPTER 11

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APPENDIX A

DATA FROM SYNCHROTRON RADIATION ANALYSIS

"Even the longest journey starts with a single step."

Attributed to Lao Tzu

Experiments for the limits of detection of Gallium maltolate (GaM) in intestinal tissues after oral treatment were conducted in 6 New Zealand White female rabbits, 2.2-2.7 Kg BW. Three does were uninfected prior to treatment and 3 were infected with *Lawsonia intracellularis* inocula a week before GaM treatment, as described in Chapters 4 and 7. Gastro-intestinal tissue samples were collected, as described in Chapter 4, with 10 μm sections mounted on pure quartz slides to be analyzed at the Canadian Light Source third generation synchrotron, VESPERS beamline (Jun $2 – 4^{th}$ 2010 and Sept $14 – 18^{th}$ 2010).

Due to time restrictions, synchrotron radiation data were collected from one section of mid-jejunum/rabbit and one section of caecal tip/rabbit and compared to a blank section of mid-jejunum and caecal tip, respectively, from an infected untreated rabbit (RABBIT 1G).

Results were such that rarely the tissue anatomical structures were discernible and in every case they appeared as moved from the original area, initially chosen on the basis of the inside-the-hutch camera references and H & E stained adjacent reference section. During experiments a skip-stepping default was experienced repeatedly due to a glitch in the sample stage motors for fine tuning of the sample in front of the detector. More importantly, lack of staining of our samples (as required by the analytical technique) rendered orientation most difficult in paraffin embedded samples, where no exact landmarks are visible, unless under bright direct light source.

The findings demonstrated that the amount of elemental gallium in tissue of treated animals is very low, in the order of ppb measurements, as confirmed by Inductively Plasma Coupled (IPC) Mass Spectrometry (MS) analysis on the same tissues. Our experiments confirmed that detection through hard x-ray technique is difficult, more so because a confirmation of the finding through x-ray absorption spectroscopy for such small quantities is not provided by the capacity of the VESPERS beamline.

The reasons behind the lack of success of these experiments can be related to either:

1) Low sensibility for small elemental quantities in the beamline instruments (detector), or

2) Necessity of higher synchrotron energy for detection of the element specific fluorescence, when present in tissues in such small amounts, particularly when easily overshadowed by other elements, such as Iron, Zinc or Copper that are present in ranges of entire orders of magnitude greater than what applicable for gallium. The statistical analysis in the biomedical terms could not be carried out as the number of the findings were exiguous (1 sample/rabbit with 3 rabbits/group and some findings were not relevant to the analysis).

The fluorescence map analysis was conducted through SMAK software (Sam's Microprobe Analysis Kit – Webb, Software and documentation. available at: http://www-ssrl.slac.stanford.edu/~swebb.); whereas the statistical analysis in regards of the values of the single elements collected, through fluorescence maps and spectra was conducted through Principal Component Analysis (PCA) (Jolliffe, 2002).

PCA was invented in 1901 by Karl Pearson, is a mathematical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of uncorrelated variables called principal components (Pearson, 1901) The number of principal components is less than, or equal to, the number of original variables. This transformation is defined so that the first principal component accounts for as much of the variability in the data as possible, whereas each following component in turn has the highest variance possible under the constraint that it be uncorrelated to the previous components (orthogonal). Principal components are guaranteed to be independent only if the data set is jointly normally distributed. PCA is sensitive to the relative scaling of the original variables, and it has different nomenclature depending on the field of application (discrete Karhunen–Loève transform (KLT), the Hotelling transform or proper orthogonal decomposition (POD)).

Currently it is mostly used as a tool in exploratory data analysis and for making predictive models. PCA can be done by eigenvalue decomposition of a data covariance matrix or singular value decomposition of a data matrix, usually after mean centering the data for each attribute. The results of a PCA are usually discussed in terms of component scores (the transformed

variable values corresponding to a particular data point) and loadings (the weight by which each standardized original variable should be multiplied to get the component score) (Shaw, 2003).

PCA is the simplest of the true eigenvector-based multivariate analyses. Often, its operation can be thought of as revealing the internal structure of the data in a way which best explains the variance in the data. If a multivariate dataset is visualised as a set of coordinates in a high-dimensional data space (1 axis per variable), PCA can supply the user with a lower-dimensional picture, a "shadow" of this object when viewed from its (in some sense) most informative viewpoint. This is done by using only the first few principal components so that the dimensionality of the transformed data is reduced (Jolliffe, 2002). PCA is closely related to factor analysis; indeed, some statistical packages deliberately conflate the two techniques. True factor analysis makes different assumptions about the underlying structure and solves eigenvectors of a slightly different matrix (Jolliffe, 2002).

The SMAK program offers a software to perform PCA on the data collected through the VESPERS beamline and allowed us to analyze the findings of each samples. Unfortunately, not having detected, beyond reasonable doubt in any of the samples the element of gallium, further analysis was unnecessary and the major data obtained is herein collected for archival purposes. Furthermore although the data appears in a non-summarized form, such a complete collection was deemed necessary, as no common grouping criteria could be found for each sample.

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A.1. Beamline set-up

Energy was set up at 12 keV

Integration time: 20 seconds/point

Stepsize: 10 μm (June experiment) 5 μm (September experiment)

Region of interest set-ups (for both session of experimental analysis) [X-RAY DATA BOOKLET -- Center for X-ray Optics and Advanced Light Source -- Lawrence Berkeley National Laboratory, 2009] as **illustrated in the Table A.1.**

Table A.1. Region of interest (ROI) set ups for Gallium analysis

Element	Level analyzed	Lower limit (eV)	Higher limit (eV)
Gallium	**Ga k α**	9100	9400
Calcium	**Ca k α**	3500	3900
Copper	**Cu k α**	7800	8200
Iron	**Fe k α**	6200	6600
Iron	**Fe k β**	6800	7200
Zinc	**Zn k α**	8440	8840
Zinc	**Zn k β**	9410	9770
-	**Background low**	4800	5200
-	**Background high**	14900	15100

Table A.1. ROI's definition provided for the elements of interest (Calcium, Copper, Gallium, Iron k α and k β, Zinc k α and k β) a lower limit of detection (in eV) and an upper limit of detection, including the typical fluorescence and x-ray absorption of energy of each given element. This defines the elemental "footprint", which is unique for each element of the periodic table. To ensure that background scatter was excluded adequately from our analysis, a low and a high set-ups for background scatter were also defined prior to analysis.

A.2 Sample set-up

The following pages will report in detail, in the same order for each sample, the data collected in the form of fluorescence maps (8 images per sample) as each ROI - **indicated in Table A.1.**- is reported. For each sample the size of the area to map ranged between 250 μm x 250μm and 300 μm X 300 μm, as the long integration time for each step analysed would have rendered the analysis impractical in the time allotted. A colour code system has been set for each map image as "Jet": with red range = high concentration; yellow range = medium-high concentration; green range = medium-low concentration; and blue range = low concentration. The full burgundy field indicates an oversaturation of fluorescence due to mix signals reading.

Also a table reporting the correlation analysis is available, to define statistically the chances of having detected Gallium vs. the other elements selected. Finally the PCA analysis images show the main component curves with particular attention to indicate where the Gallium should have projected a visible curve/jump on the graph (corresponding to the ROI previously defined).

The samples scanned are jejunum sections in the first group of analysis and cecum tip in the second group of analysis. In order of listing therefore will follow: fluorescence maps, correlation to gallium detection tables and PCA analysis for each sample. Data will be commented in the figures' or tables' legends. In order:

a) Negative control (n=1, Rabbit 1G), infected untreated samples: **a.1.)** jejunum fluorescence maps **(Figures A.1.1. to A.1.8.)**; Correlation analysis results are represented in **Table A.2.**; PCA results are represented in **Figures A.1.9. and A.1.10. a.2)** cecum fluorescence maps **(Figure A.8.1. to A.8.9.)**. Correlation analysis results are represented in **Table A.9.**; the PCA analysis results are represented in **Figure A.8.10 - A.8.12.**

b) Positive control (n=3, Rabbits 2C, 2D, 2F) uninfected treated samples:

b.1.) jejunum fluorescence maps **(Figures A.2.1. to A.2.8.** for Rabbit 2C; **Figures A.3.1. to A.3.9.** for Rabbit 2D; and **Figures A.4.1. to A.4.9.** for Rabbit 2F**)**; Correlation analysis results are represented in **Table A.3.** for Rabbit 2C; **Table A.4.** for Rabbit 2D; **Table A.5.** for Rabbit 2F; PCA results are represented in **Figures A.2.9. - A.2.11.** for Rabbit 2C; **Figures A.3.10. - A.3.11.** for Rabbit 2D; **Figure A.4.10.** for Rabbit 2F.

b.2) cecum fluorescence maps **(Figures A.9.1. to A.9.9.** for Rabbit 2C (plus **addendum A.9.14 through A.9.16**); **Figures A.10.1. to A.10.9.** for Rabbit 2D; and **Figures A.11.1. to A.11.9.** for Rabbit 2F (plus **addendum A.11.10.- A.11.12)**; Correlation analysis results are represented in **Table A.10.** for Rabbit 2C; **Table A.11.** for Rabbit 2D; **Table A.12.** for Rabbit 2F; PCA results are represented in **Figures A.9.10. - A.9.13.** for Rabbit 2C; **Figures A.10.10. - A.10.13.** for Rabbit 2D; **Figures A.11.13. - A.11.16.** for Rabbit 2F.

c) Target sample (n=3, Rabbits 4E, 4G, 4H) infected and treated samples:

c.1.) jejunum fluorescence maps **(Figures A.5.1. to A.5.9.** for Rabbit 4E; **Figures A.6.1. to A.6.9.** for Rabbit 4G; and **Figures A.7.1. to A.7.9.** for Rabbit 4H**)**; Correlation analysis results are represented in **Table A.6.** for Rabbit 4E; **Table A.7.** for Rabbit 4G; **Table A.8.** for Rabbit 4H; PCA results are represented in **Figures A.5.10. - A.5.12.** for Rabbit 4E; **Figures A.6.10. - A.6.11.** for Rabbit 4G; **Figures A.7.10. - A.7.12.** for Rabbit 4H.

c.2) cecum fluorescence maps **(Figures A.12.1. to A.12.9.** for Rabbit 4E; **Figures A.13.1. to A.13.9.** for Rabbit 4G; and **Figures A.14.1. to A.14.9.** for Rabbit 4H**)**; Correlation analysis results are represented in **Table A.13.** for Rabbit 4E; **Table A.14.** for Rabbit 4G; **Table A.15.** for Rabbit 4H; PCA results are represented in **Figures A.12.10. - A.12.12.** for Rabbit 4E; **Figures A.13.10. - A.13.12.** for Rabbit 4G; **Figures A.14.0. - A.14.12.** for Rabbit 4H.

Figures A.1.1. to A.1.8. Rabbit 1 G Untreated infected control – Jejunum sample. Fluorescence maps.

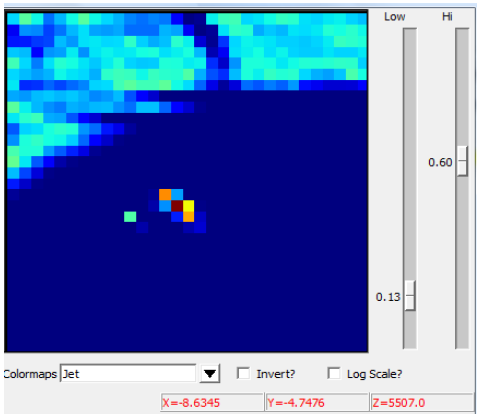


Figure A.1.1. Calcium k α

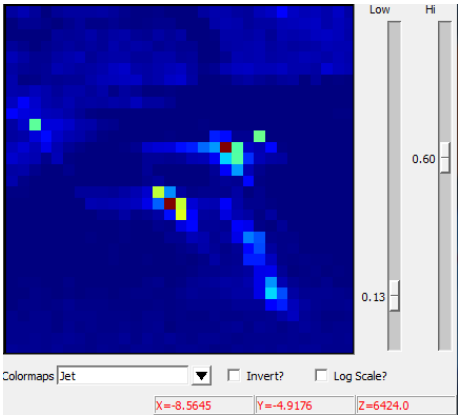


Figure A.1.2. Iron k α

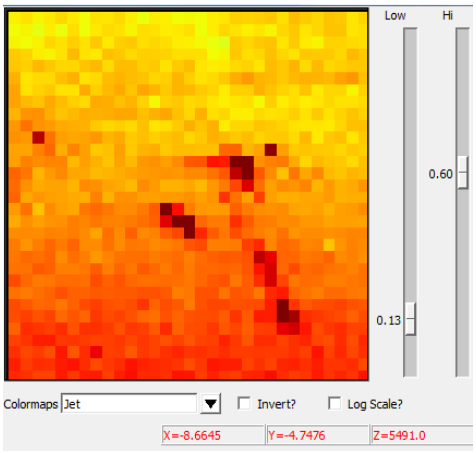


Figure A.1.3. Iron K β

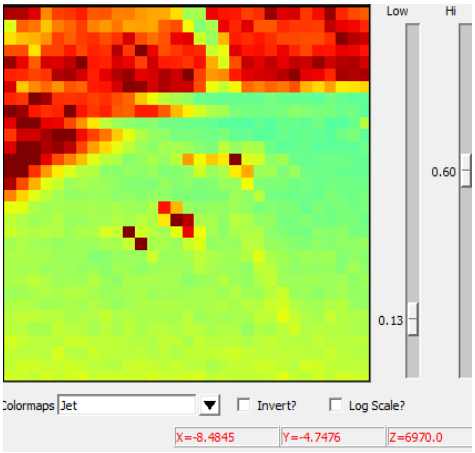


Figure A.1.4. Zinc k α

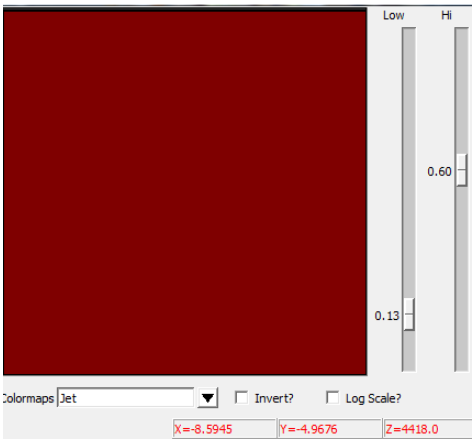


Figure A.1.5. Gallium k α

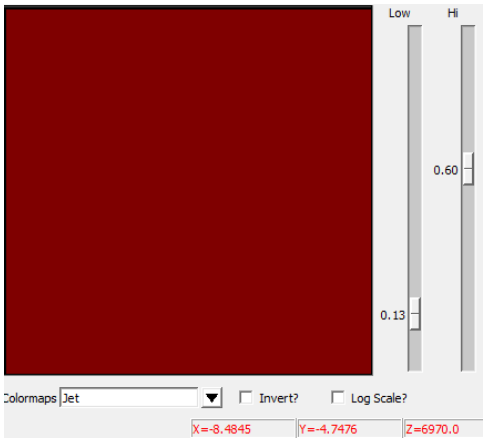


Figure A.1.6. Zinc k β

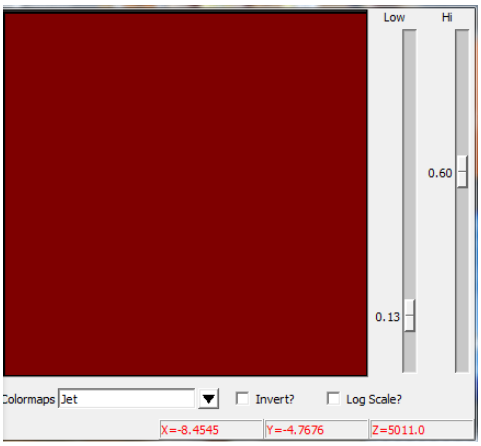


Figure A.1.7. Background scatter lower end

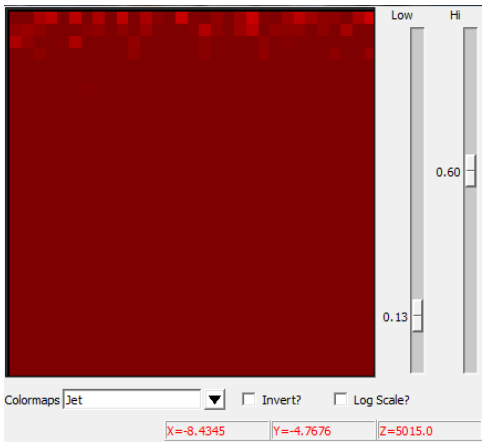


Figure A.1.8. Background scatter higher end

Figures A.1.1. to A.1.8. Fluorescence maps. In this sample analysis, potential anatomical landmarks are grossly estimated from maps of **Fe k α, Ca k α** and **Zn k α**. Zinc and Iron are somehow opposite in appearance. Unfortunately the map location obtained is not matching what was chosen at the beginning of the analysis.

Table A.2. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.41	-10.01999	57262.1463
Cu k α	0.92	1.664063	1152.8842
Fe k α	5.96^{-6}	-0.0129017	6601.3
Fe k β	0.53	0.7974821	1230.82
Zn k α	0.24	-1.70464073	15551.79
Zn k β	0.85	1.189159	1793.97
Background low	0.83	0.96172	39.769
Background high	0.95	19.287085	-2462.5179

Table A.2. Correlation: R^2 values are provided in the first column whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. No gallium was detected in the untreated infected control, as expected. However, regardless of its known absence, background scatter energy, both low and high correlated to inexistent data with high r^2 values, showing a doubtful ability for the detector to record the right fluorescence signal.

Figure A.1.9. PCA: Component 1.

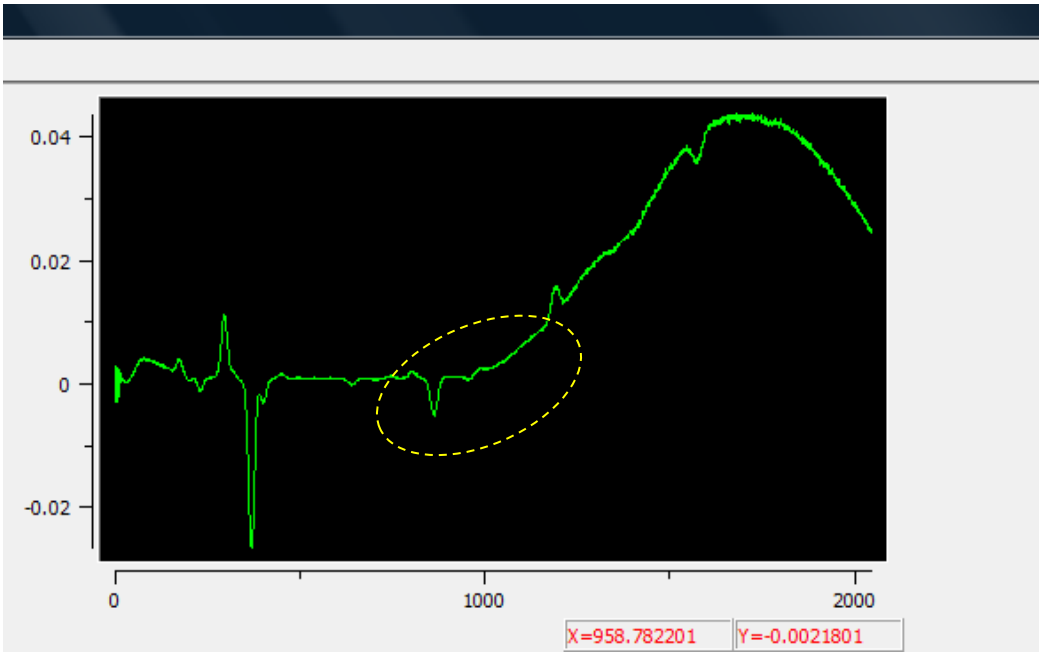
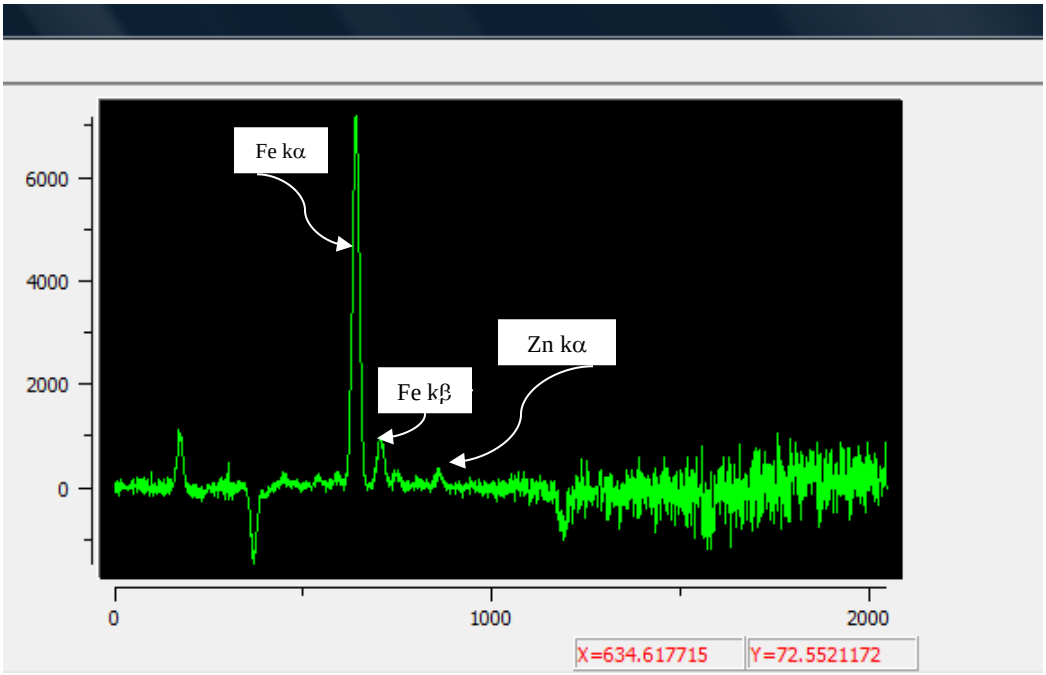


Figure A.1.10. PCA: Component 4.



Figures A.1.9 and A.1.10 - PCA Analysis. In component 1, no detectable Ga k α peak is visible in the selected ROI (yellow interrupted line), as expected. On the other hand, in component 4, the peaks for **Fe k α, Fe k β** and **Zn k α** are well discernible (labels).

Figures A.2.1. to A.2.8. Rabbit 2C - Uninfected treated control – Jejunum sample. Fluorescence maps.

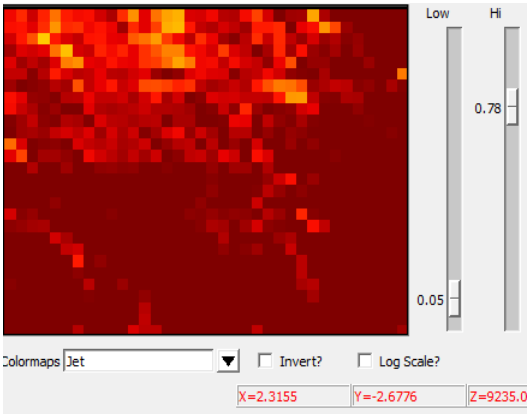


Figure A.2.1 Calcium k α

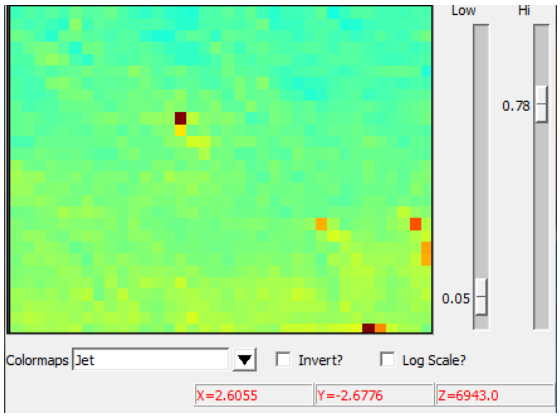


Figure A.2.2.Iron k α

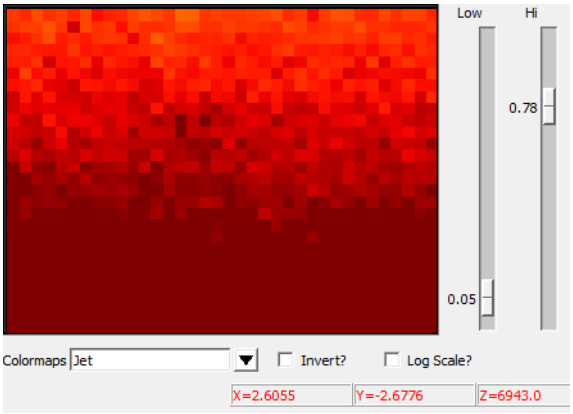


Figure A.2.3. Iron k β

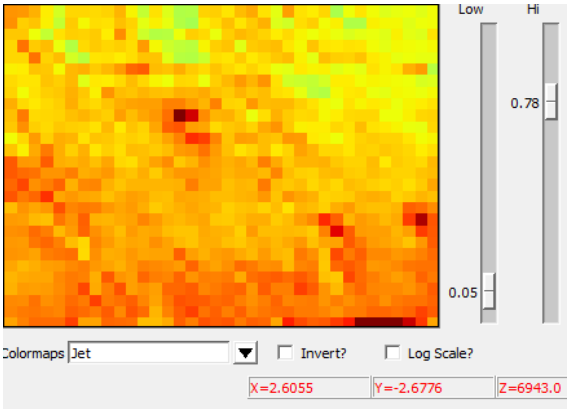


Figure A.2.4 Zinc k α

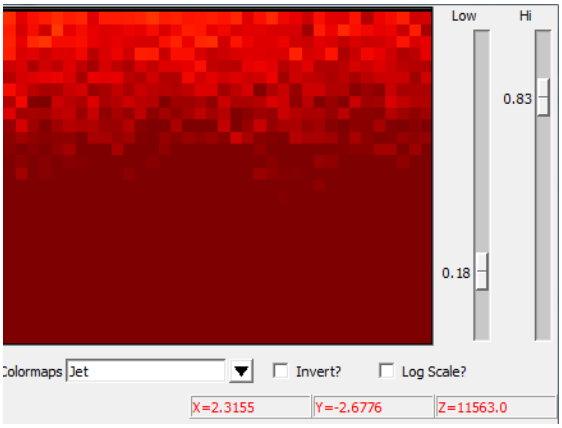


Figure A.2.5. Gallium k α

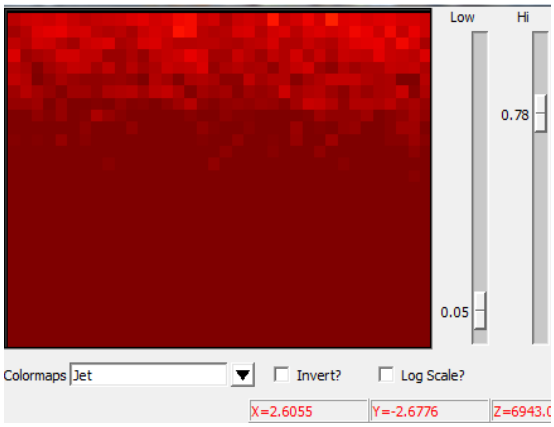


Figure A.2.6. Zinc k β

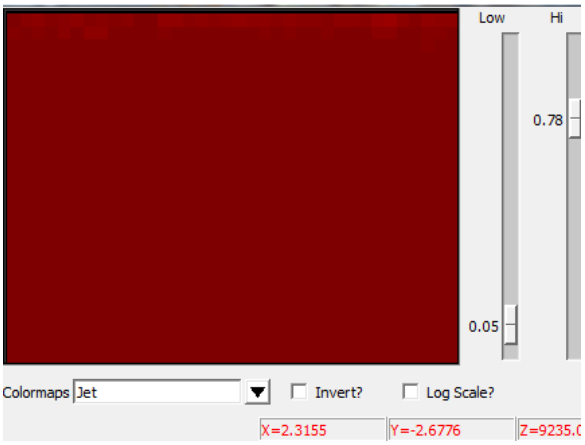


Figure A2.7.Background scatter higher end

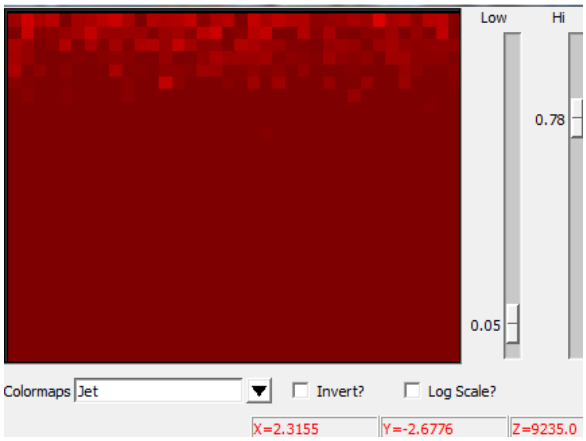


Figure A.2.8. Background scatter lower end

Figures A.2.1. to A.2.8. Fluorescence maps: In this sample analysis, potential anatomical landmarks are grossly estimated from maps **Fe k α**, **Ca k α** and **Zn k α**. Zinc and iron are somehow opposite, unfortunately the map location obtained is not matching what was chosen at the beginning of the analysis.

Table A.3. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.46	1.27533	1325.08345
Cu k α	0.92	1.79918	377.9566
Fe k α	0.42	1.30568	672.935
Fe k β	0.87	1.036957	76.629399
Zn k α	0.45	1.502523	1953.608648
Zn k β	0.90	1.565903	202.8178
Background low	0.90	0.946659	-37.492276
Background high	0.94	18.170999	-3.387846

Table A.3. Correlation: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Although not much was detected on the fluorescence maps in the uninfected treated control, Cu k α, Fe k β, Zn k β and background scatter energy, both low and high, correlated to very feeble data, with high r^2 values (≥ 90%), showing a doubtful ability for the detector to record the right fluorescence signal for gallium.

Figure A.2.9. PCA: Component 1

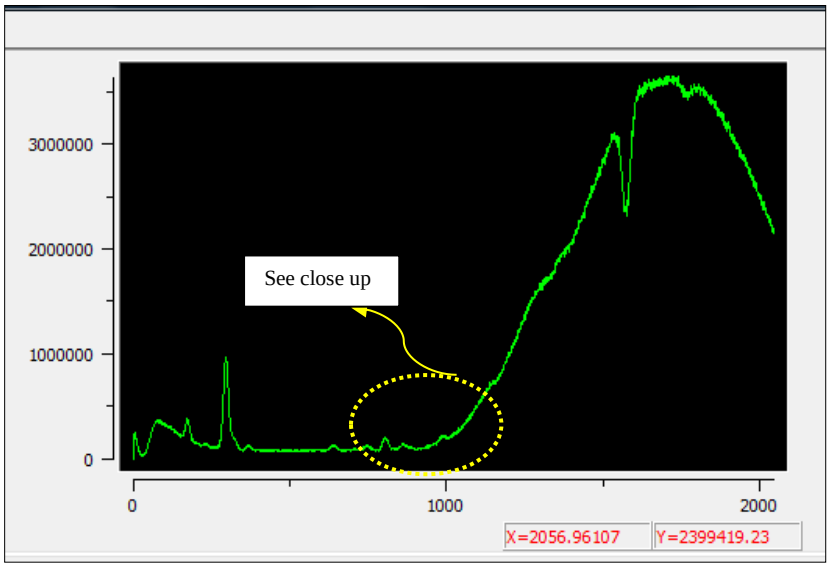


Figure A.2.10. PCA: Component 1, close-up image.

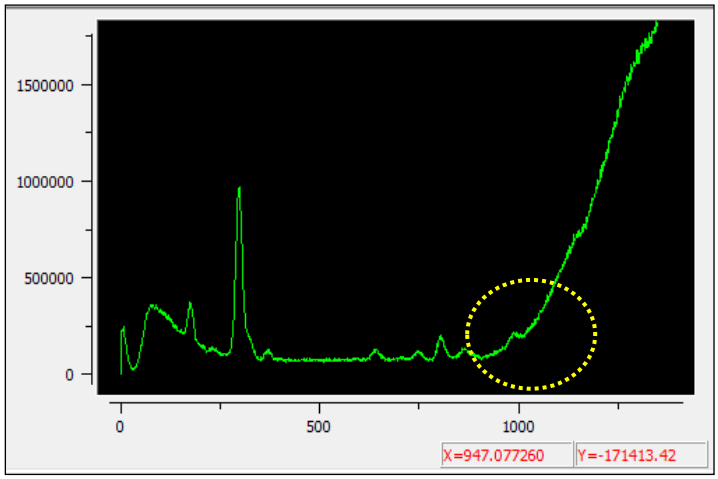
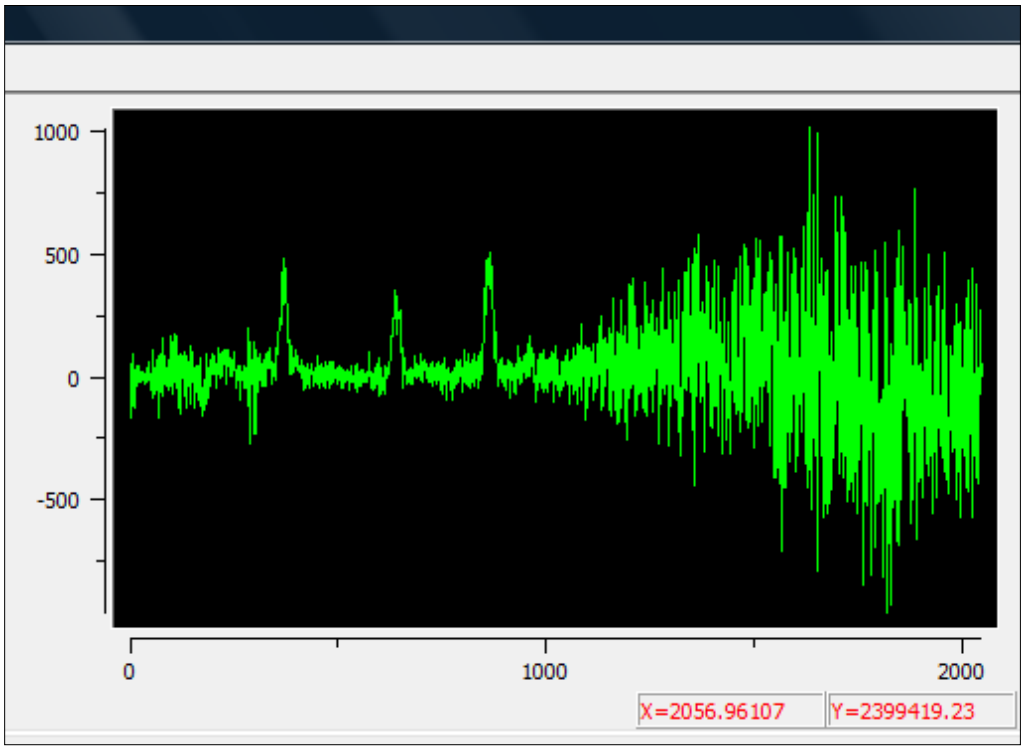


Figure A.2.11. PCA: Component 3



PCA analysis: In Component 1. No detectable **Ga k α** peak is visible on scatter shoulder, whereas possibly a **Zn k β** peak on the said shoulder could be visible. In Component 3: the scatter generated noise is too big and it masks any peak that is not major, such as for **Fe k α** and **Ca k α**.

Figures A.3.1. to A.3.9. Rabbit 2D - Uninfected treated control – Jejunum sample. Fluorescence maps.

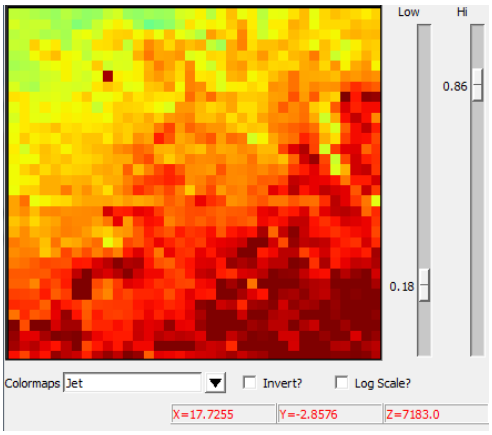


Figure A.3.1 Calcium k α

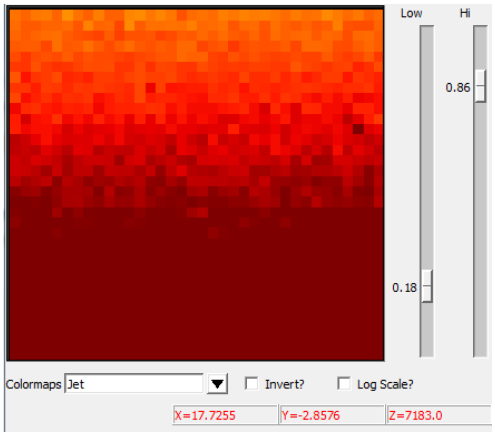


Figure A.3.2. Copper k α

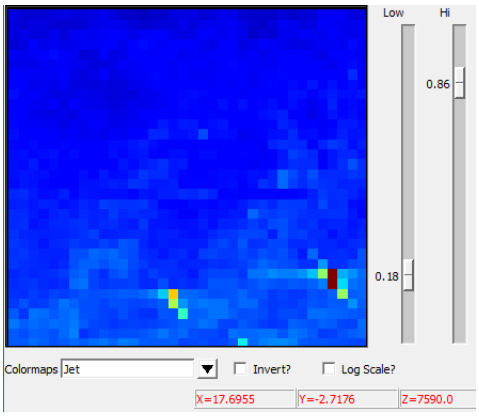


Figure A.3.3 Iron k α

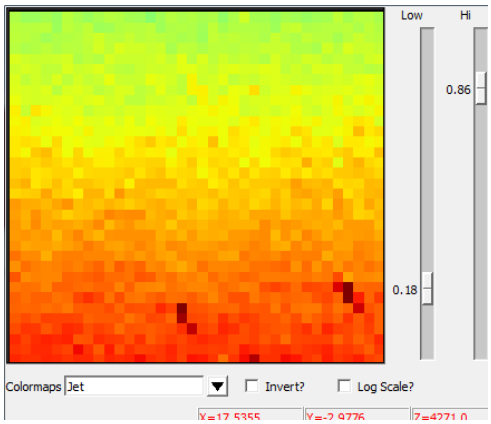


Figure A.3.4. Iron k β

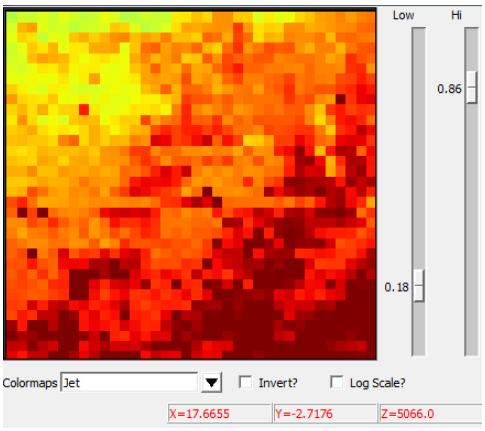


Figure A.3.5.Zinc k α

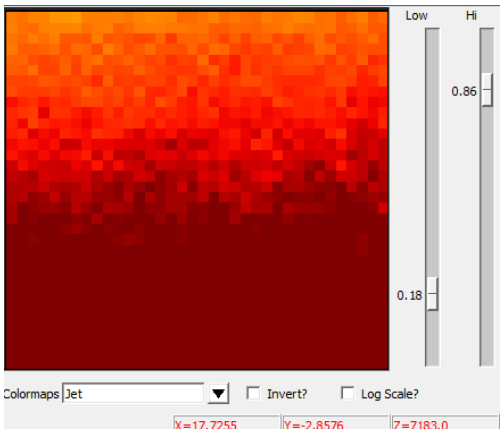


Figure A.3.6. Zinc k β

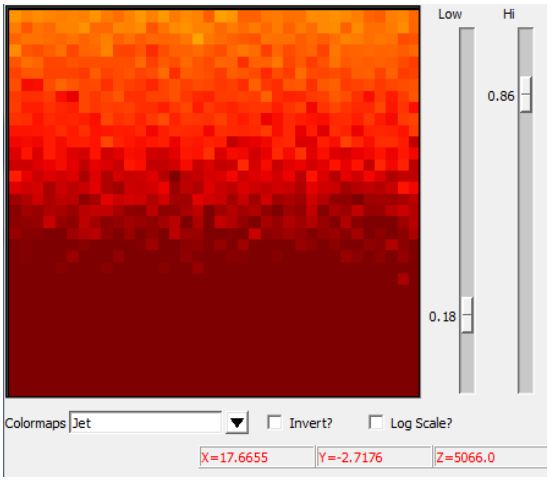


Figure A.3.7. Gallium k α

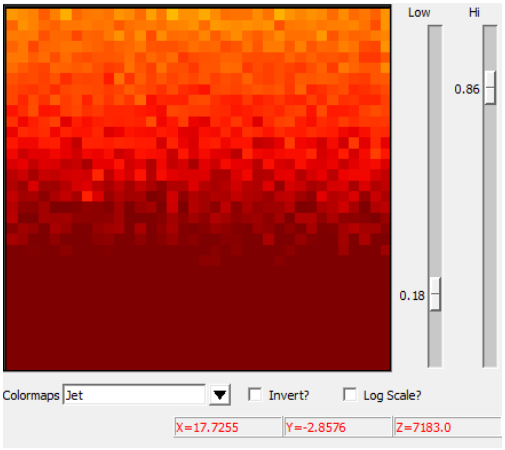


Figure A.3.8 Background scatter lower end

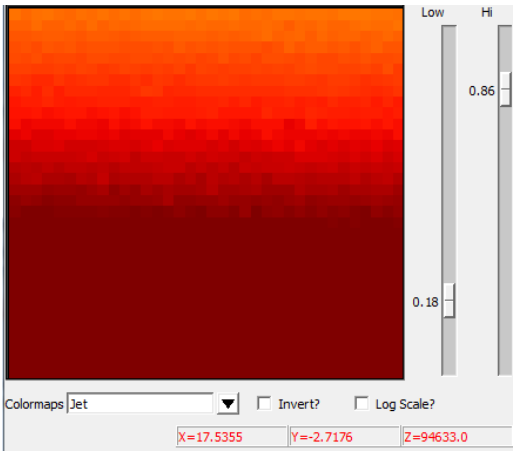


Figure A.3.9.Background scatter higher end

Figures A.3.1 – A.3.9. Fluorescence maps: In this sample analysis, potential anatomical landmarks are difficult to be grossly estimated from maps of **Fe k α, Ca k α** and **Zn k α**. Furthermore based on the coordinates selected and the ones recorded (motor and camera discrepancies), the map location obtained is not matching the chosen area for the analysis.

Table A.4. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.65	2.084988	-480.581647
Cu k α	0.95	1.77639	454.75752
Fe k α	0.42	1.54166	-523. 6469
Fe k β	0.90	1.056127	24.891482
Zn k α	0.58	1.675958	740.06465
Zn k β	0.94	1.54404	261.222698
Background low	0.93	0.910397	169.836561
Background high	0.96	18.019053	1651.6919

Table A.4. Correlation: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Although not much was detected on the fluorescence maps in the uninfected treated control, Cu k α, Fe k β, Zn k β and background scatter energy, both low and high, correlated well to very feeble data, with high r^2 values (> 90%), showing a doubtful ability for the detector to record the right fluorescence signal for gallium.

Figure A.3.10. PCA: Component 1

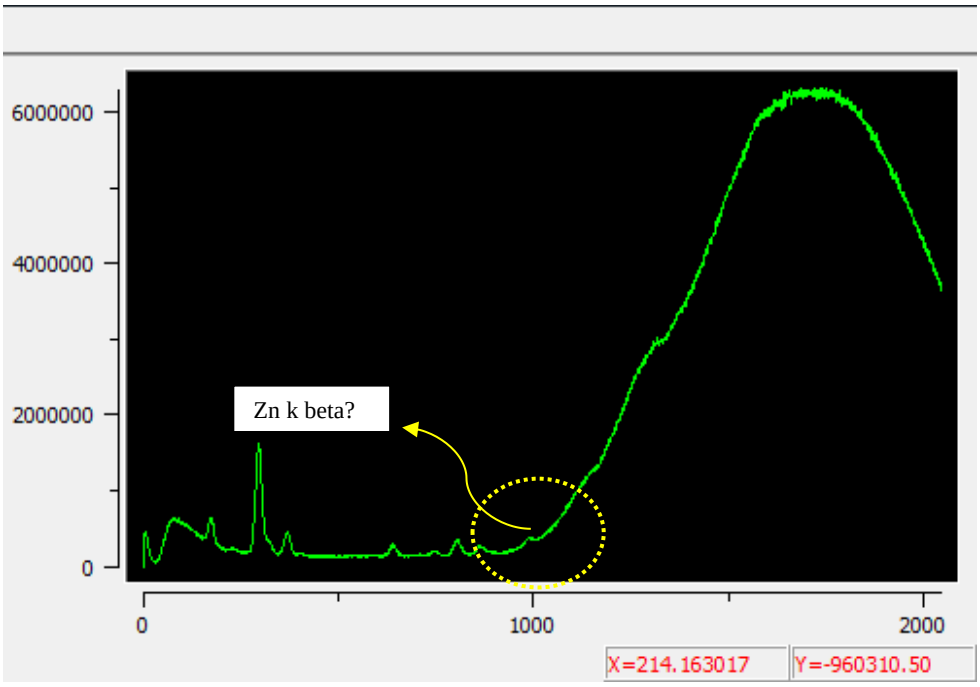
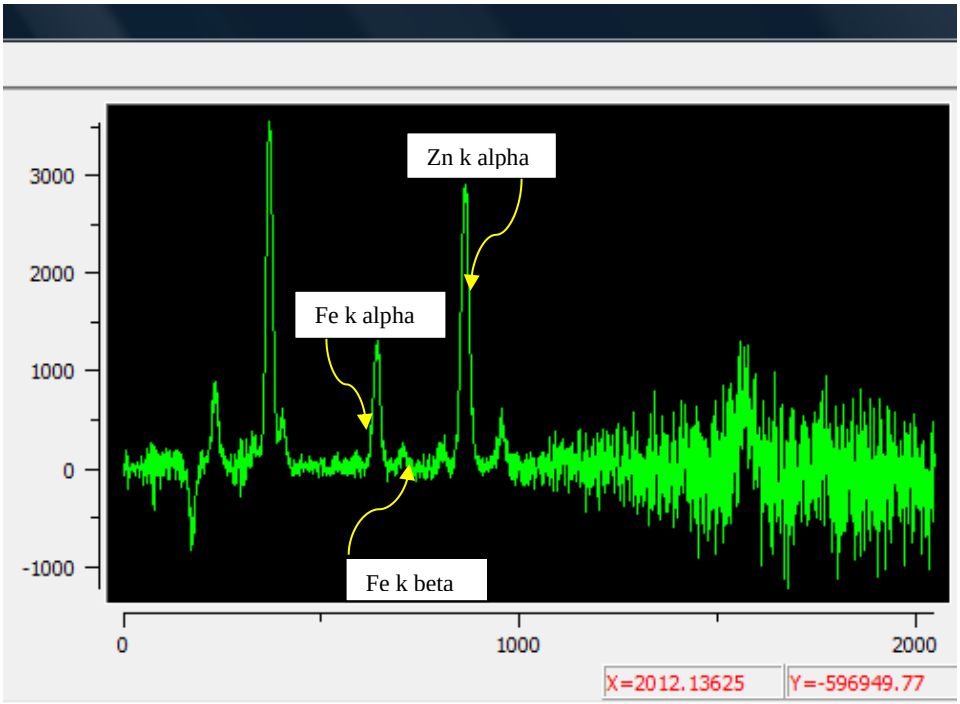


Figure A.3.11.PCA: Component 3



PCA analysis: No appearance of a Gallium peak is noted on PCA components 1 and 3. Component 4 and higher are too scattered/noisy to be considered analytically helpful.

Figures A.4.1. to A.4.9. Rabbit 2F - Uninfected treated control – Jejunum sample. Fluorescence maps.

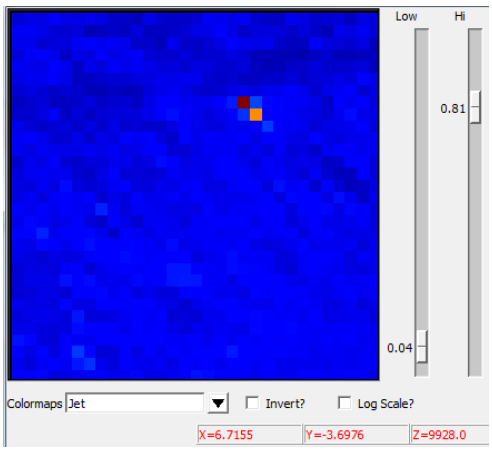


Figure A.4.1. Calcium k α

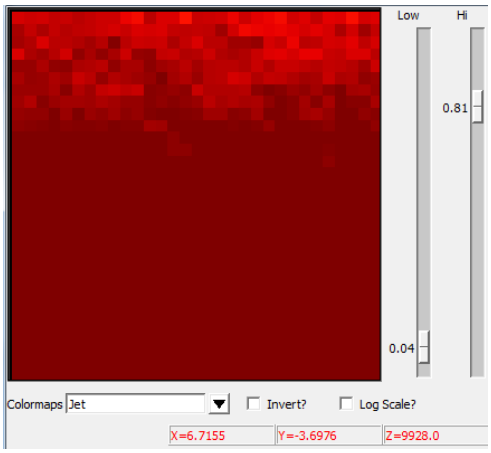


Figure A.4.2. Copper k α

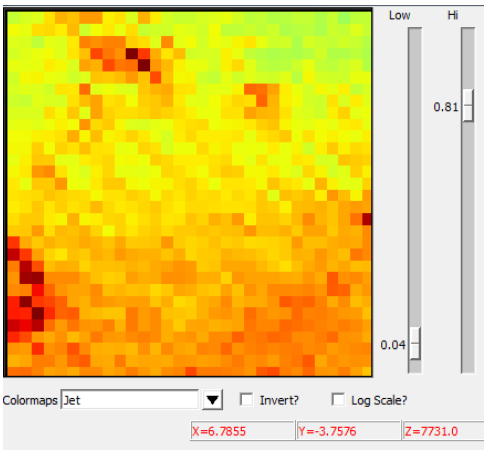
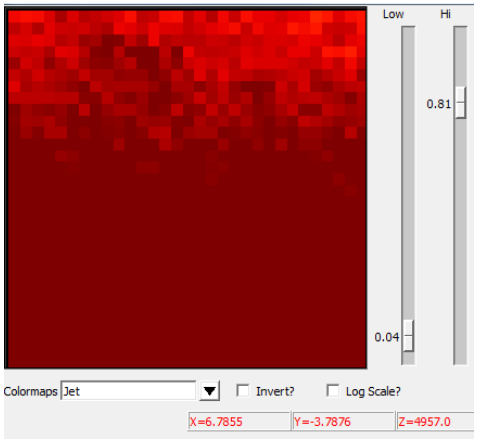


Figure A.4.3. Iron k α



FigureA.4.4. Iron k β

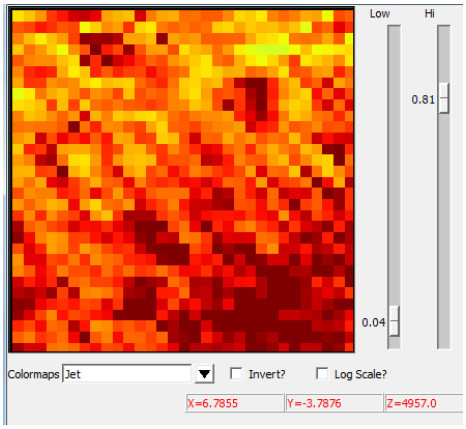


Figure A.4.5. Zinc k α

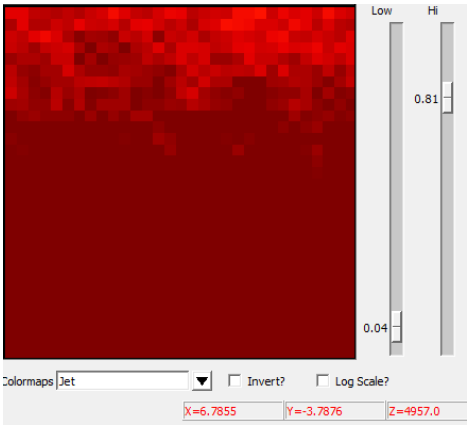


Figure A.4.6. Zinc k β

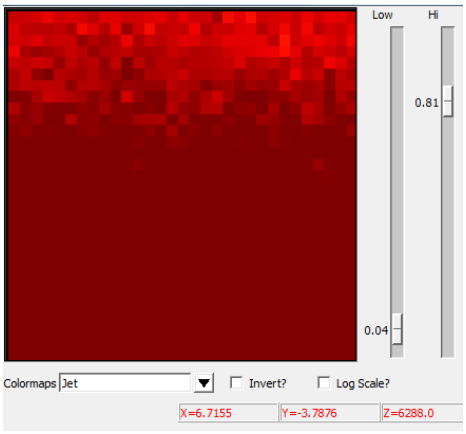


Figure A.4.7. Gallium k α

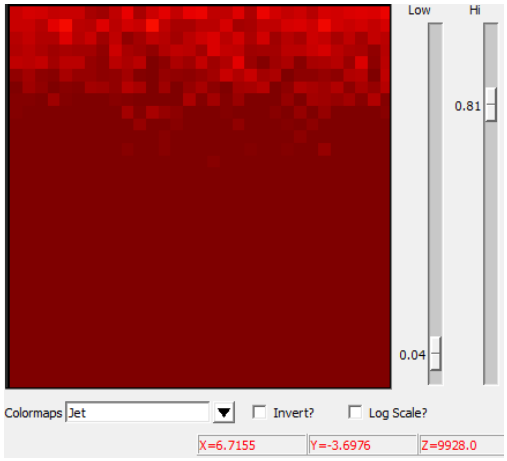


Figure A.4.8.Background scatter lower end

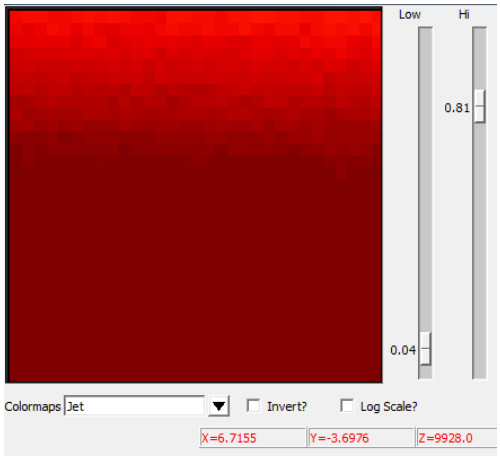


Figure A.4.9. Background scatter higher end

Figures A.4.1 – A.4.9. Fluorescence maps: Again the high resemblance of maps of **Fe k β, Zn k β, Ga k α** and **Cu k α** with the background scatter of both lower and higher ends, is fairly obvious. Very mild anatomical landmarks are somewhat discernible in **Zn and Fe k α**'s maps, although likely are not detailed enough to make a precise reference to the anatomical site analysed.

Table A.5. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.03	1.234004	2775.176
Cu k α	0.92	1.863117	430.60602
Fe k α	0.46	1.107286	1208.72575
Fe k β	0.89	0.957053	418.277862
Zn k α	0.38	1.762516	740.8549
Zn k β	0.92	1.555237	248.324067
Background low	0.91	0.876281	299.300
Background high	0.94	19.17968	-3906.857094

Table A.5. Correlation: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Although not much was detected on the fluorescence maps in the uninfected treated control, Cu k α, Fe k β, Zn k β and background scatter energy, both low and high, correlated well to very feeble data high r^2 values (> 89%), showing a doubtful ability for the detector to record the right fluorescence signal for gallium.

Figure A.4.10. PCA: Component 1

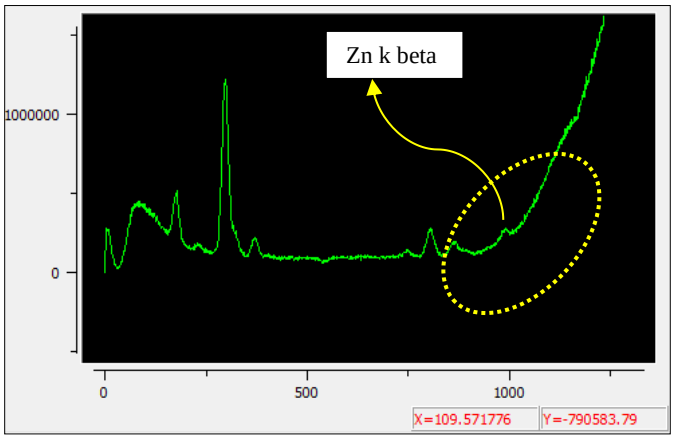


Figure A.4.10. PCA analysis: shows no Gallium peak on the component 1 analysis, and only **Fe k α** and **Zn k α** peaks in component 3, besides scatter signal.

Figures A.5.1. to A.5.9. Rabbit 4E - *Lawsonia intracellularis* infected and treated – Jejunum sample. Fluorescence maps.

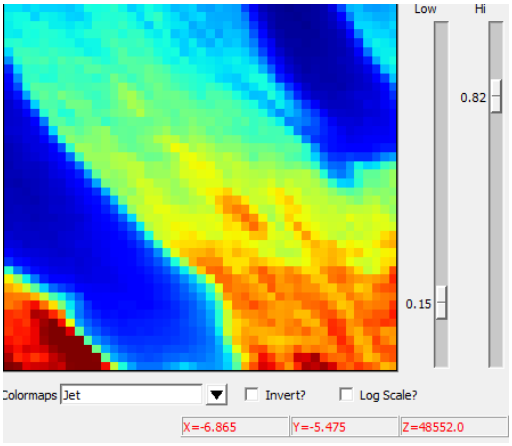


Figure A.5.1 Calcium k α

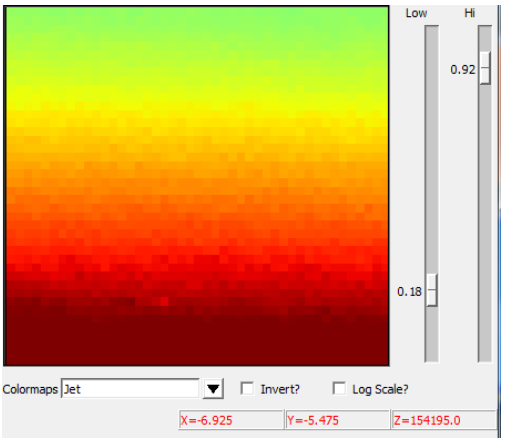


Figure A.5.2. Copper k α

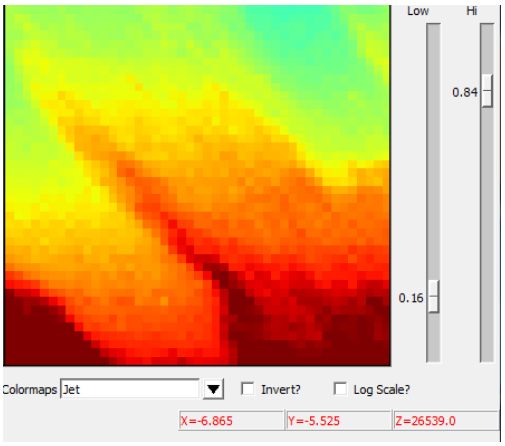


Figure A.5.3. Iron k α

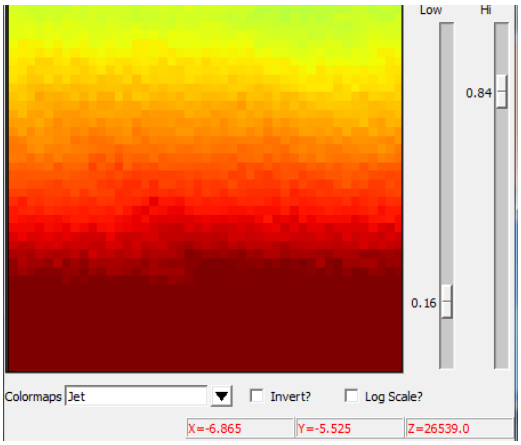


Figure A.5.4. Iron k β

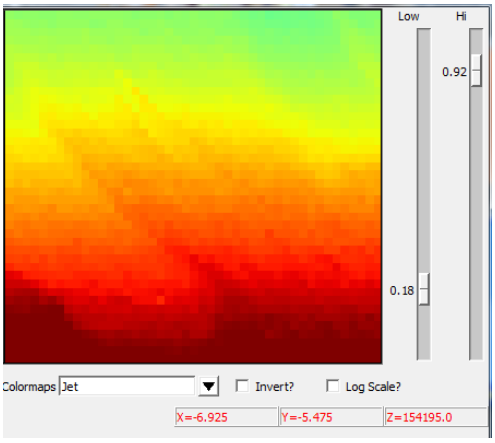


Figure A.5.5. Zinc k α

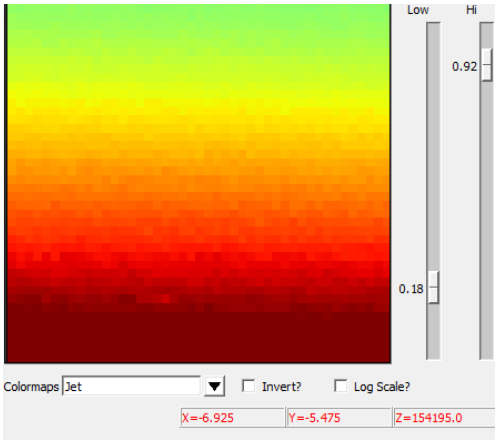


Figure A.5.6. Zinc k β

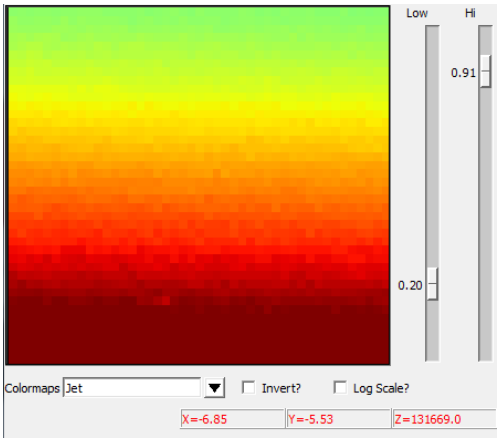


Figure A.5.7. Gallium k α

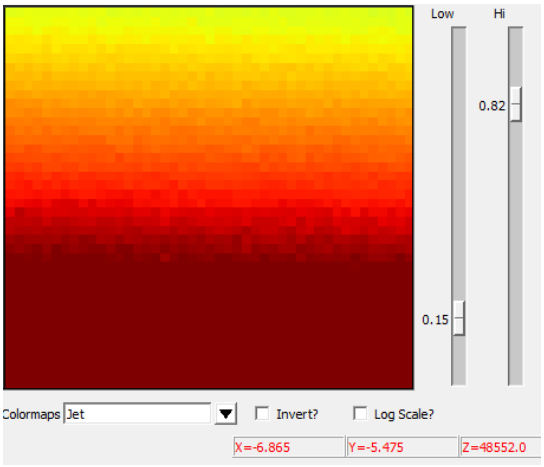


Figure A.5.8.Background scatter lower end

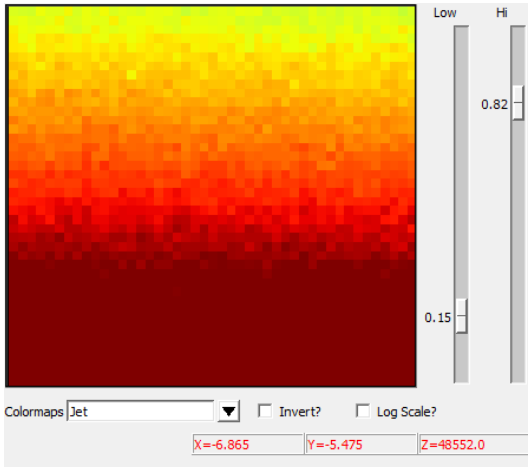


Figure A.5.9. Background scatter higher end

Figures A.5.1. – A.5.9. Fluorescence maps: An appearance of anatomical structure is recognizable only in the **Fe k α** and **Ca k α** maps. **Zn k α** has only a very vague appearance of tissutal structure. However none of these maps definition will be sufficient to relate this analysis to a specific location on the sample. No structure at all visible on the other maps.

Table A.6. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.18	0.241365	-1272.561
Cu k α	0.99	0.998	466.48059
Fe k α	0.83	0.2018	2031.715
Fe k β	0.99	0.25725	394.121
Zn k α	0.98	1.01663	4001.6974
Zn k β	0.99	1.237822	295.51616
Background low	0.99	0.06956	9.67816
Background high	0.99	0.71011	-270.7562

Table A.6. Correlation: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Although not much was detected on the fluorescence maps in the infected treated control, Cu k α, Fe k β, Zn k α, Zn k β and background scatter energy, both low and high, correlated well to very feeble data high r^2 values (> 98%), showing a doubtful ability for the detector to record the right fluorescence signal for gallium.

Figure A.5.10. PCA: Component 1.

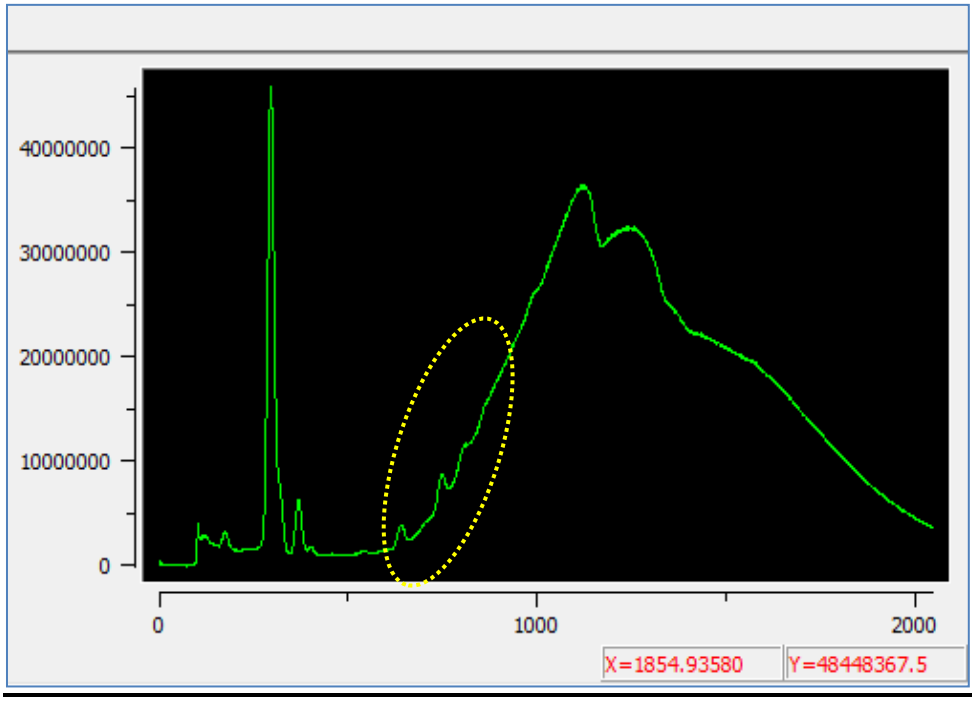


Figure A.5.11.PCA: Component 3.

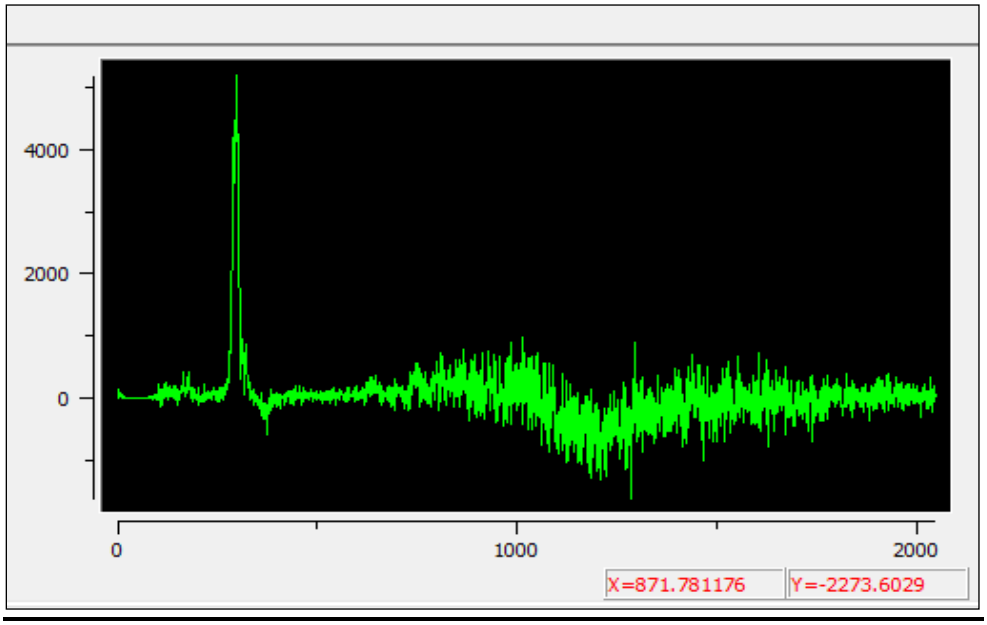
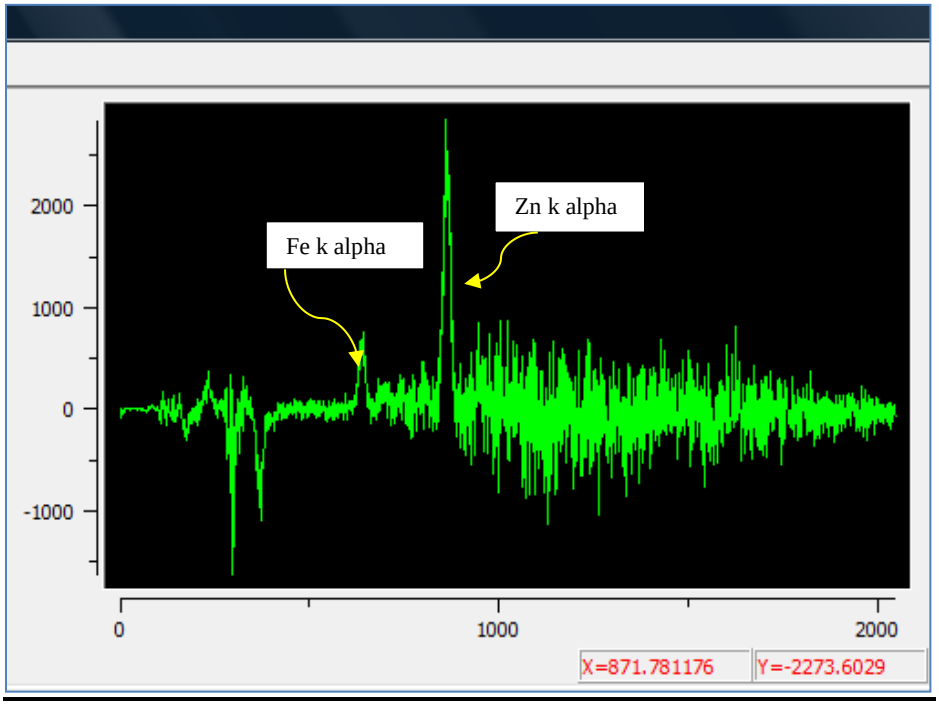


Figure A.5.12.PCA: Component 4.



PCA analysis: No detection of **Ga k α, Zn k β, Fe k β,** or **Cu k α**. The only peaks discernible in Component 4 are **Fe** and **Zn k α**'s. The other elemental peaks are masked by the scatter signal.

Figures A.6.1. to A.6.9. Rabbit 4G - *Lawsonia intracellularis* infected and treated – Jejunum sample. Fluorescence maps.

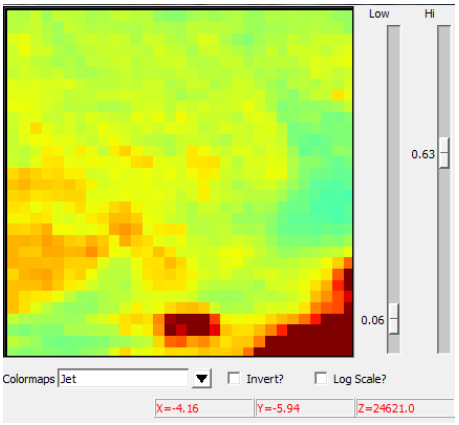


Figure A.6.1. Calcium k α

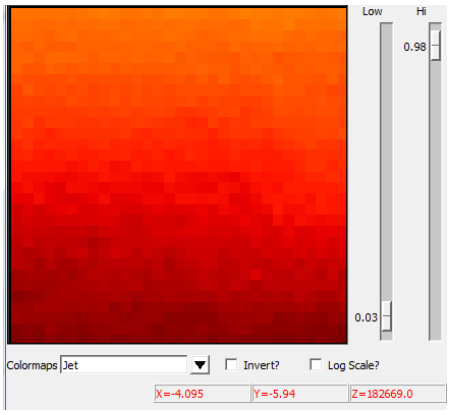


Figure A.6.2. Copper k α

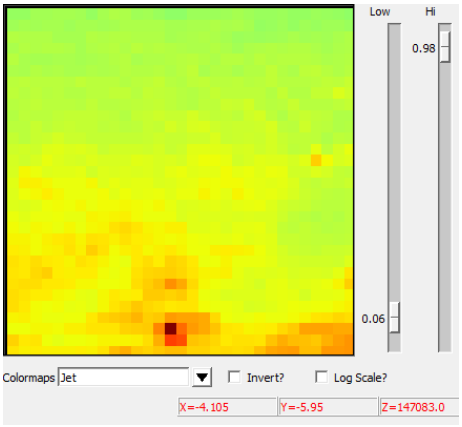


Figure A.6.3 Iron k α

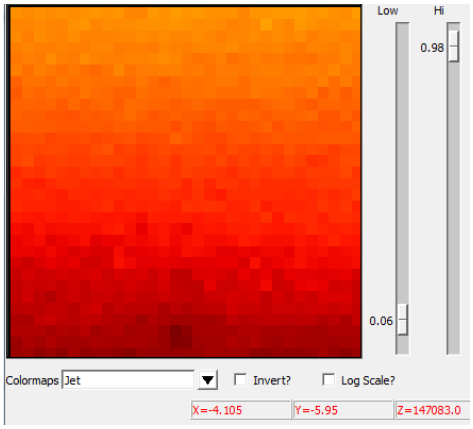


Figure A.6.4. Iron k β

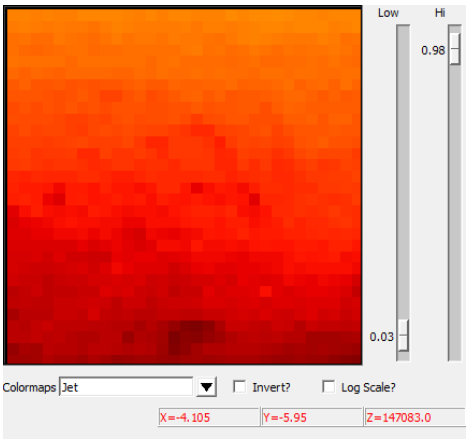


Figure A.6.5 Zinc k α

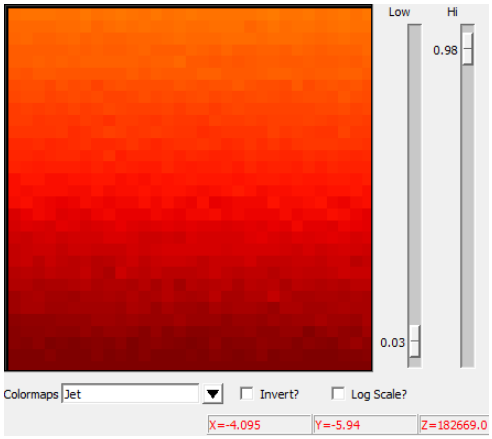


Figure A.6.6 Zinc k β

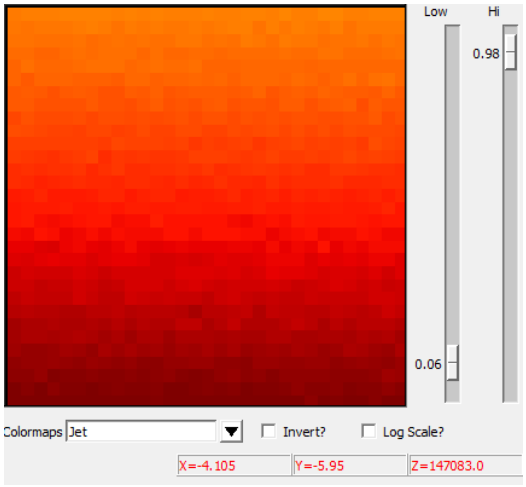


Figure A.6.7. Gallium k α

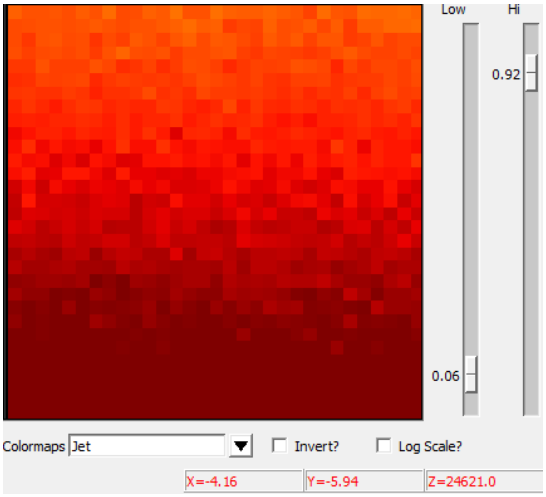


Figure A.6.8.Background scatter lower end

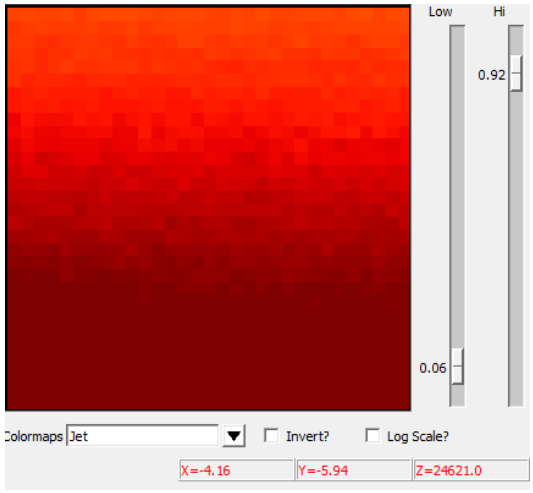


Figure A.6.9. Background scatter higher end

Figures A.6.1 –A.6.9. Fluorescence maps: In most elemental fluorescence maps of this set of analyses, an anatomical structure is not discernible at all. Mainly some hint of structure is recognizable in **Fe k α** and **Ca k α** and barely perceptible in **Zn k α.**

Table A.7. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.15	0.105256	-29.978003
Cu k α	0.98	0.674341	5989.751348
Fe k α	0.71	0.1818	4902.0901
Fe k β	0.98	0.261759	222.908
Zn k α	0.96	0.954722	13852.306
Zn k β	0.99	1.20796	3482.339118
Background low	0.97	0.07157	-135.109988
Background high	0.99	0.689794	2346.2549

Table A.7. Correlation: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Although not much structure was detected on the fluorescence maps in the infected treated control, Cu k α, Fe k β, Zn k α, Zn k β and background scatter energy, both low and high, correlated too well to very feeble data, with high r^2 values (> 96%), showing a doubtful ability for the detector to record the right fluorescence signal for gallium. Also, to note is that data elements usually not at all correlated, a high R^2 is shown (71% for Fe k α). The doubt is that something emitting similar fluorescence signal, regardless of the element (mostly overwhelming or null) was mapped instead of tissue. Could that be glass/quartz of paraffin?

Figure A.6.10. PCA: Component 1

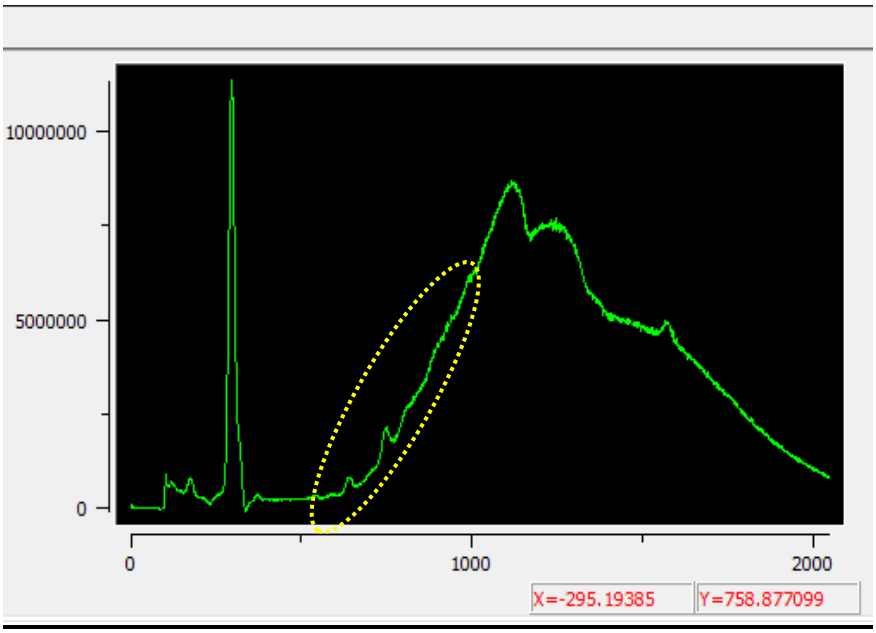
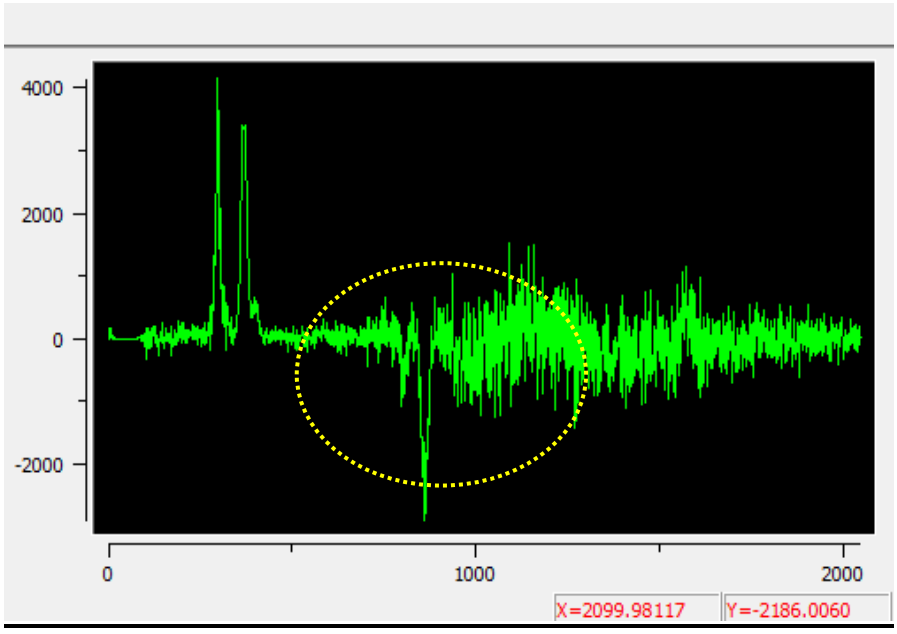


Figure A.6.11. PCA: Component 3



Figures A.6.10. - A.6.11. PCA Analysis: No elemental peak is noticeable (likely masked by the scatter signal shoulder), for **Ga k α, Zn k α, Zn k β, Fe k β,** and **Cu k α**. May be the scatter wave is too long to distinguish any specific metal characteristics, even in case that we did not map glass or paraffin, but legitimate tissue.

Figures A.7.1. to A.7.9. Rabbit 4H - *Lawsonia intracellularis* infected and treated – Jejunum sample. Fluorescence maps.

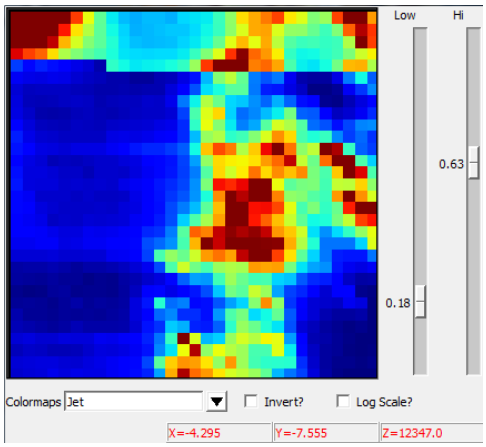


Figure A.7.1. Calcium k α

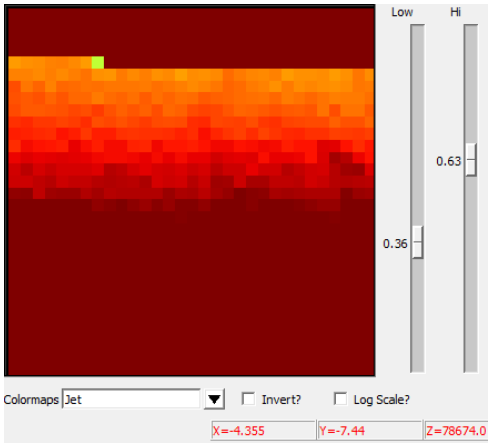


Figure A.7.2 Copper k α

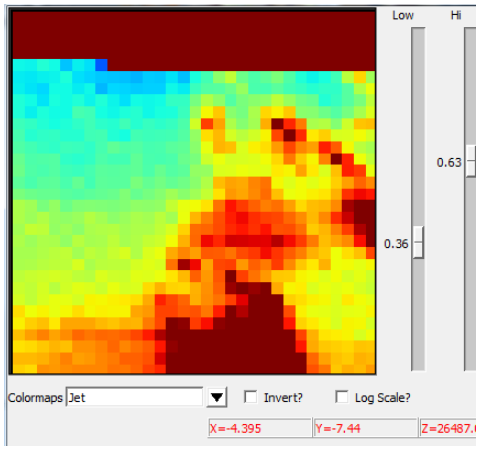


Figure A.7.3. Iron k α

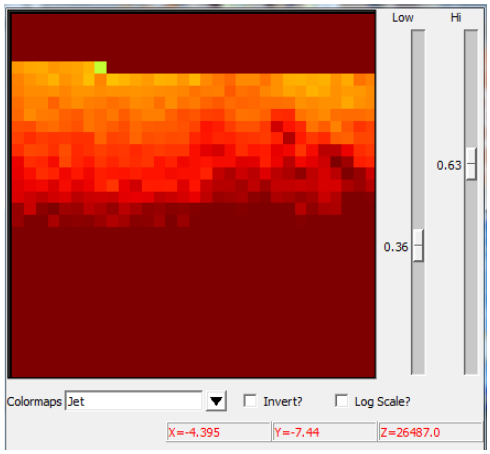


Figure A.7.4. Iron k β

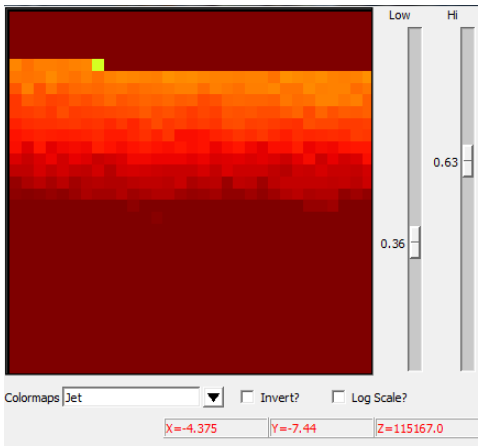


Figure A.7.5. Zinc k α

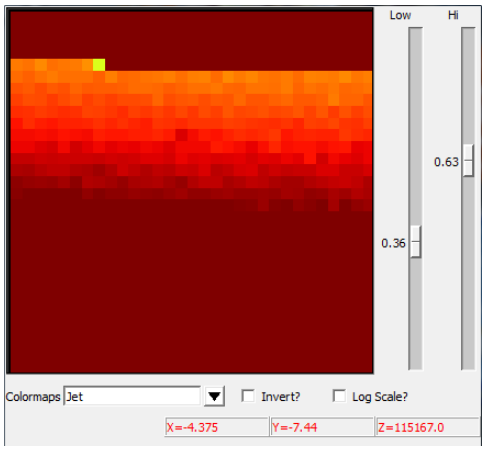


Figure A.7.6. Zinc k β

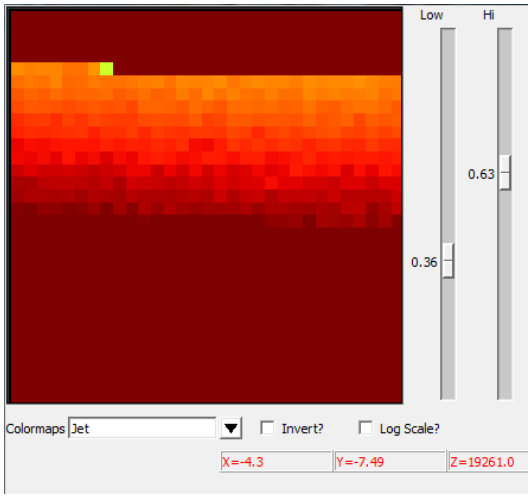
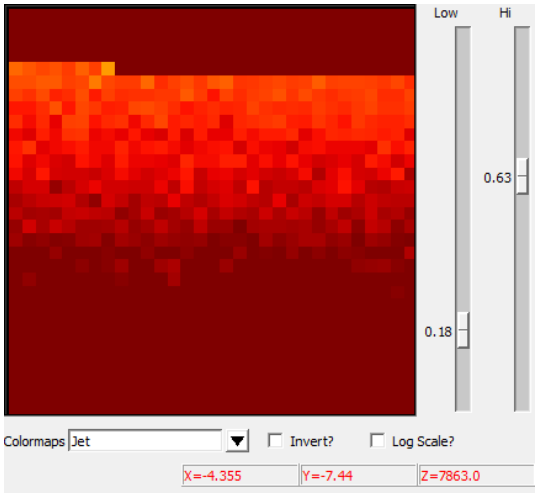


Figure A.7.7. Gallium k α



FigureA.7.8. Background scatter lower end

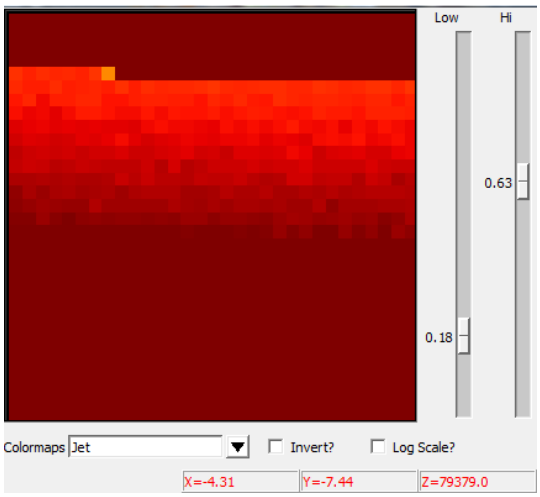


Figure A.7.9. Background scatter higher end

Figures A.7.1. – A.7. 9. Fluorescence maps: Anatomical landmarks are not obvious, once again, except for **Fe k α** and **Ca k α** (slightly more defined). In addition, the energy injection within the mapping time (upper quarter) is not helpful in defining the areas of interest, due to the marked contrast in energy intensity. Once again **Ga k α** looks much alike **Zn k α, Zn k β, Fe k β, Cu k α,** and both ends of the background scatter signal shoulder.

Table A.8. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.12	0.16083	2850.319
Cu k α	0.99	0.708873	-356.2897
Fe k α	0.75	0.20963	-609.8286
Fe k β	0.99	0.264915	-193.0506
Zn k α	0.99	1.031291	-468.92256
Zn k β	0.999	1.2333	-158.160162
Background low	0.99	0.07122	-72.7349
Background high	0.999	0.716905	-152.0740

Table A.8. Correlation: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Although not much structure was detected on the fluorescence maps in the infected treated control, Cu k α, Fe k β, Zn k α, Zn k β and background scatter energy, both low and high, correlated too well to very feeble data, with high r^2 values (≥ 99%), showing a doubtful ability for the detector to record the right fluorescence signal for gallium. Also, to note is that data elements usually not at all correlated, a high R^2 is shown (75% for Fe k α). The doubt is that something emitting similar fluorescence signal, regardless of the element (mostly overwhelming or null) was mapped instead of tissue. Could that be glass/quartz of paraffin?

Figure A.7.10. PCA: Component 1

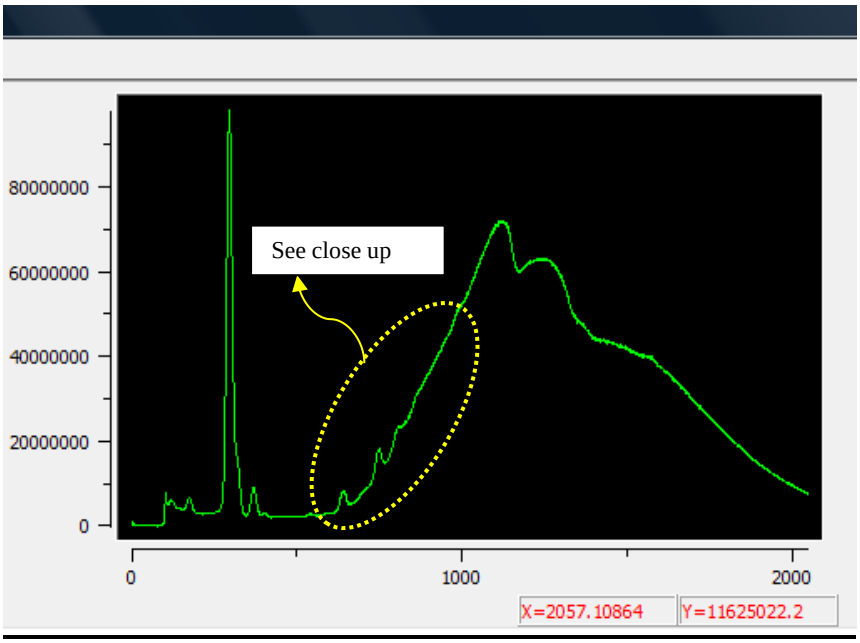


Figure A.7.11. PCA: Component 1: close-up

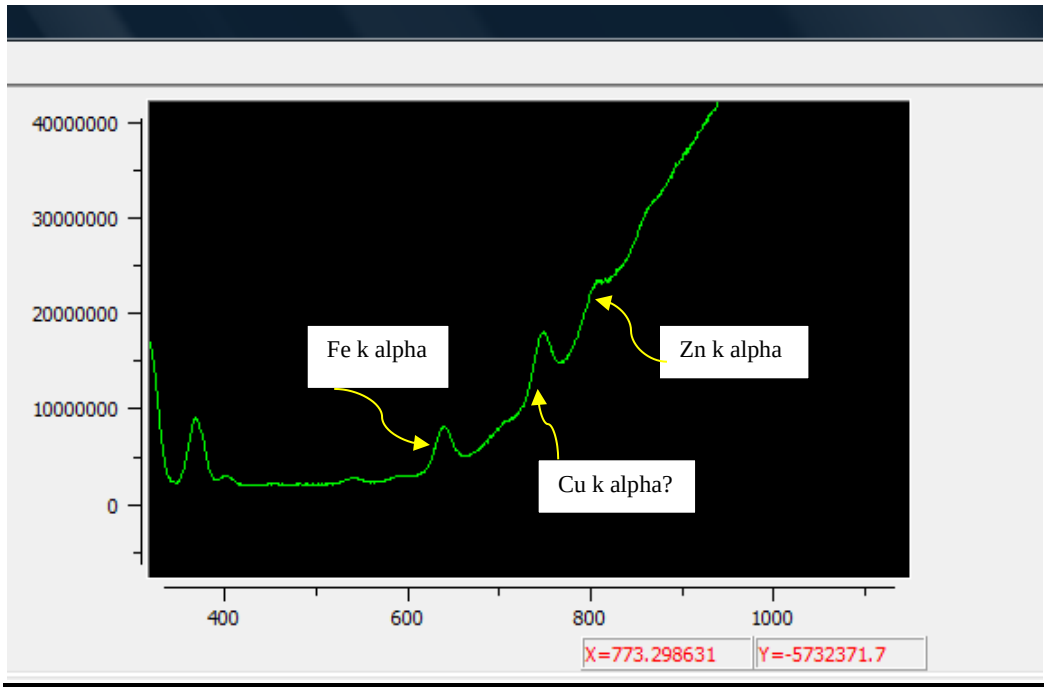
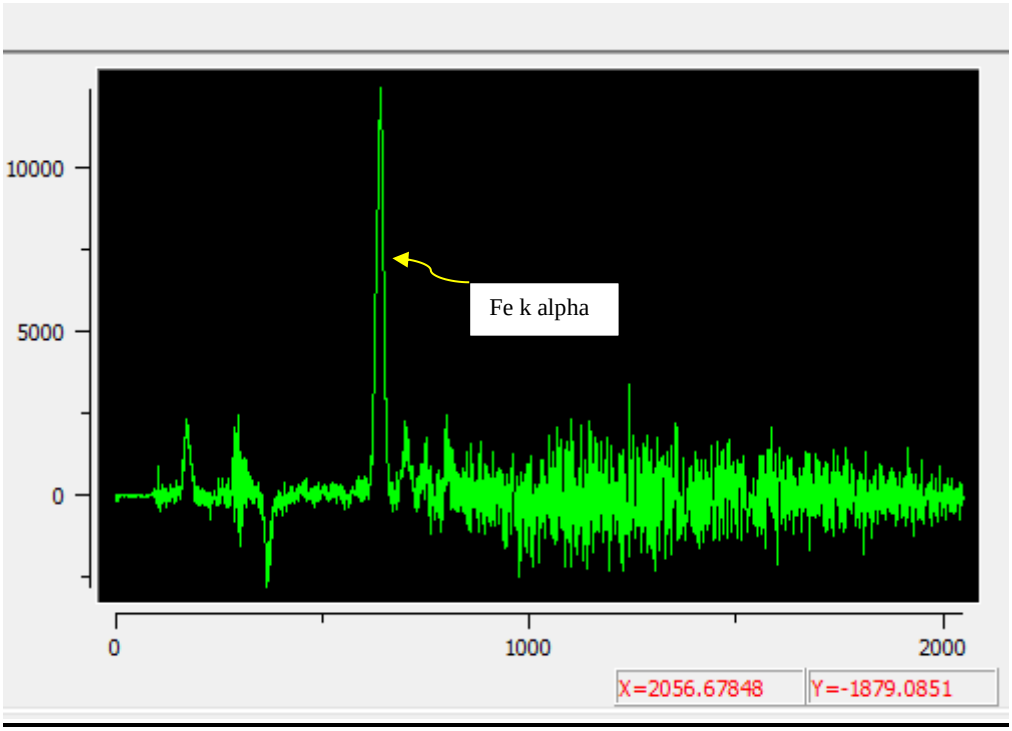


Figure A.7.12. PCA: Component 3.



PCA Analysis: No elemental peak is noticeable, as likely it is masked by the very large scatter signal shoulder, for **Ga k α, Zn k α, Zn k β, Fe k β**. The only ones recognizable are **Fe k α** and **Ca k α** and potentially **Cu k α**, but it is dubious.

Figures A.8.1. to A.8.9. Rabbit 1G - *Lawsonia intracellularis* infected and untreated – Cecum sample. Fluorescence maps.

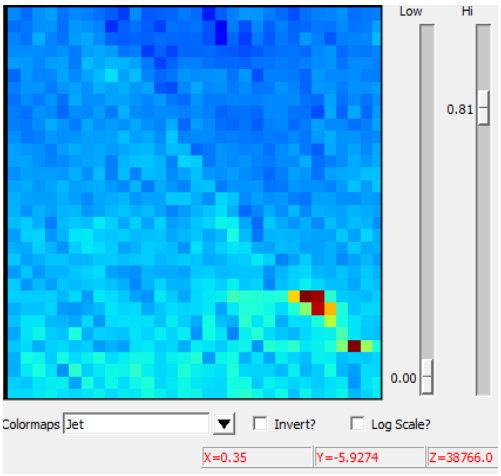


Figure A.8.1 Calcium k α

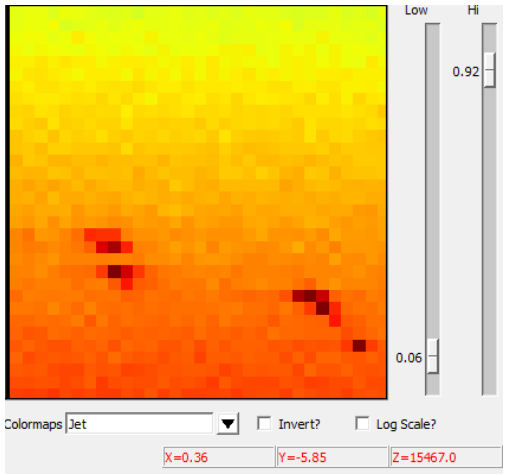


Figure A.8.2. Copper k α

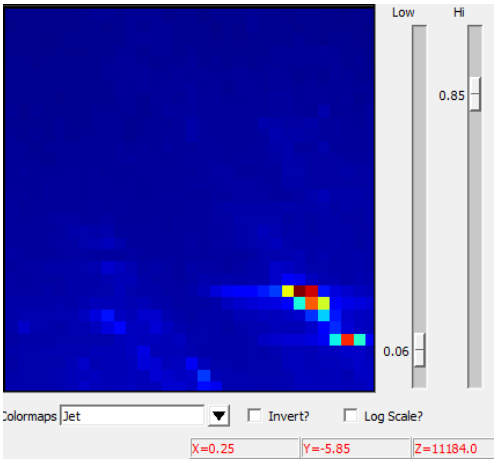


Figure A.8.3. Iron k α

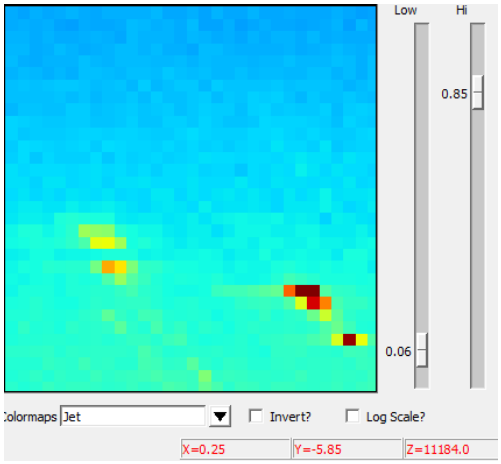


Figure A.8.4. Iron k β

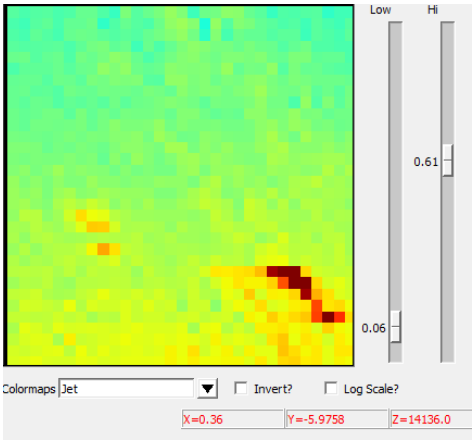


Figure A.8.5.Zinc k α

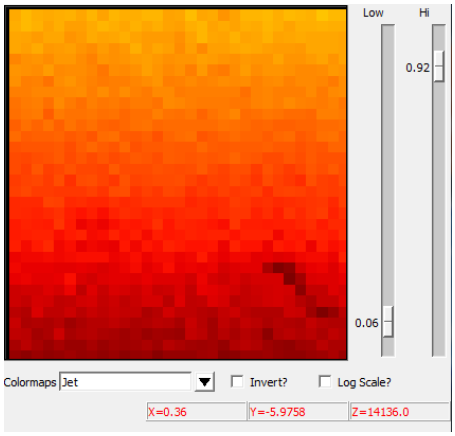


Figure A.8.6. Zinc k β

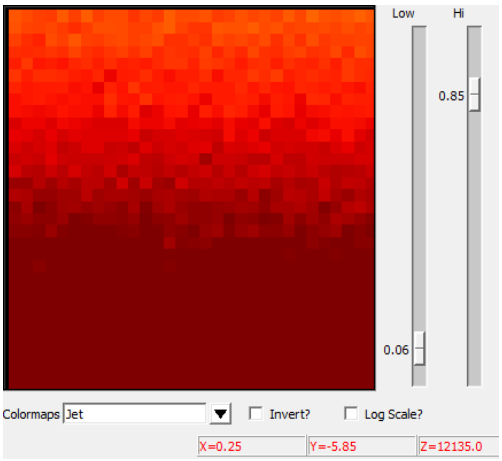


Figure A.8.7. Gallium k α

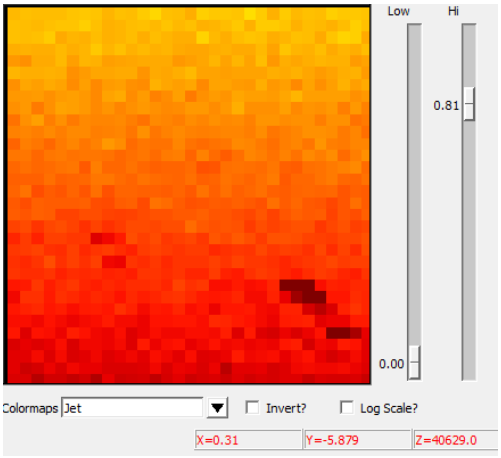


Figure A.8.8. Background scatter lower end

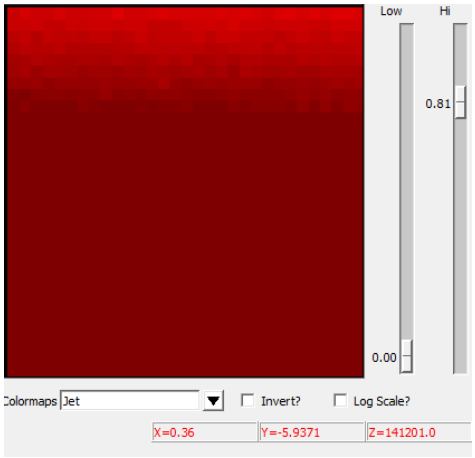


Figure A.8.9 Background scatter higher end

Figures A.8.1 – A.8.9. Fluorescence maps: Some anatomical structure is recognizable on the lower right corner of each fluorescence map. However, it is different than the area originally chosen for analysis, as it should have occupied the whole map space. As suspected during collection of data, the sample stage skip-stepped with respect to the detector, and the micro-camera did not record such a change.

Table A.9. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.41	5.940566	-27119.64
Cu k α	0.90	1.2750	218.5209
Fe k α	0.12	2.436354	-15303.298
Fe k β	0.48	0.930019	-2122.4678
Zn k α	0.50	1.6434	-2772.764
Zn k β	0.97	1.770	74.57269
Background low	0.92	0.64	-55.7519
Background high	0.97	12.015	6844.8416

Table A.9. Correlation: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Although not gallium was present (untreated subject), Cu k α, Zn k β and background scatter energy, both low and high, correlated well to inexistent data, with high r^2 values ($\geq$ 90%), showing a doubtful ability for the detector to record the right fluorescence signal for gallium. In this case a low correlation with Fe k β is noted as well (48%).

Figure A.8.10. PCA: Component 1

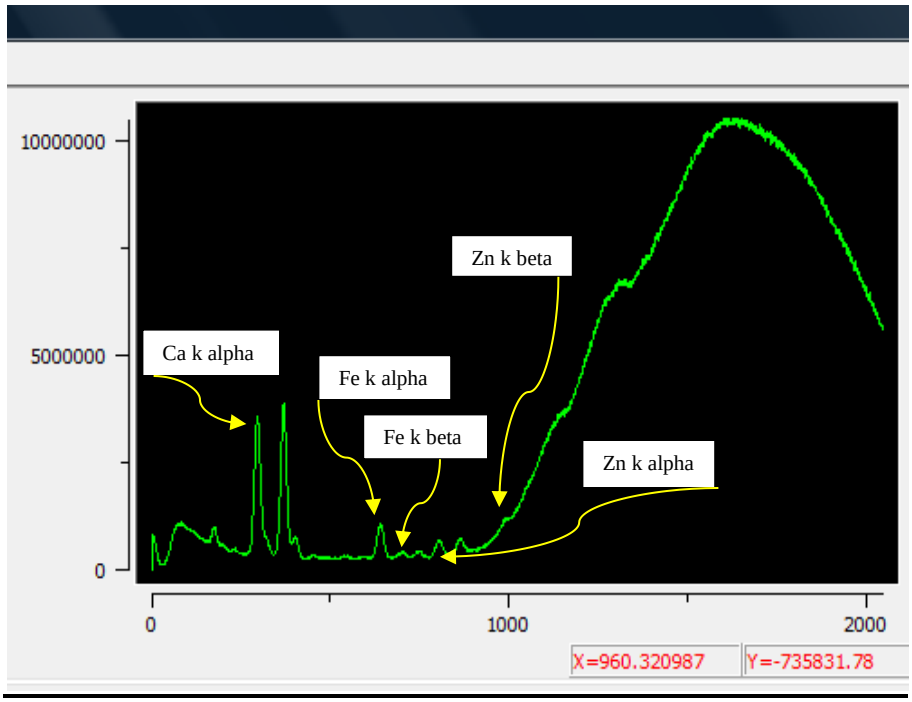


Figure A.8.11. PCA: Component 2.

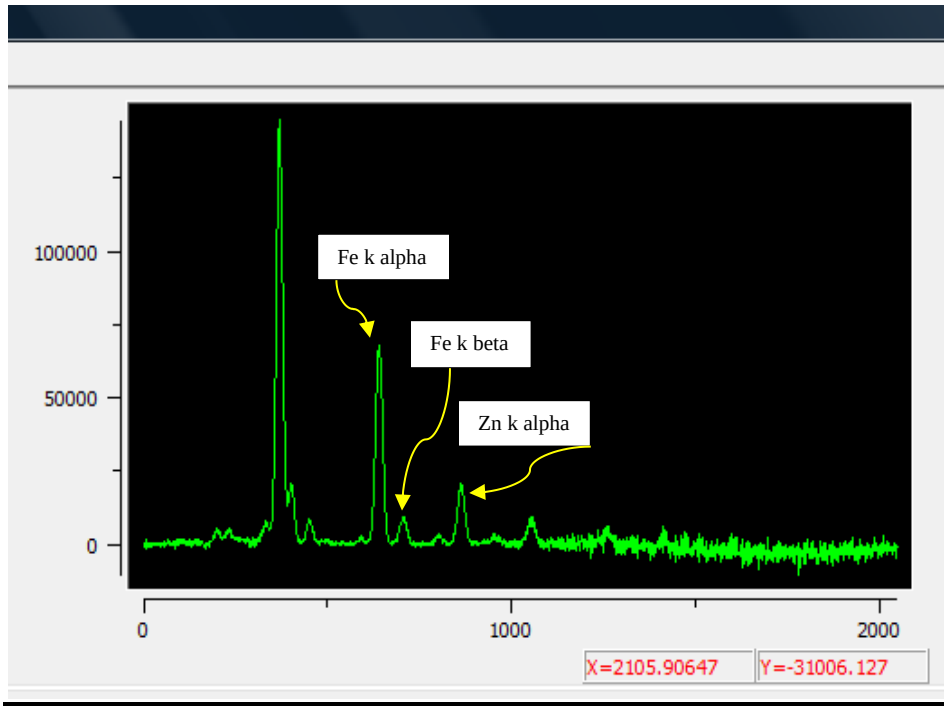
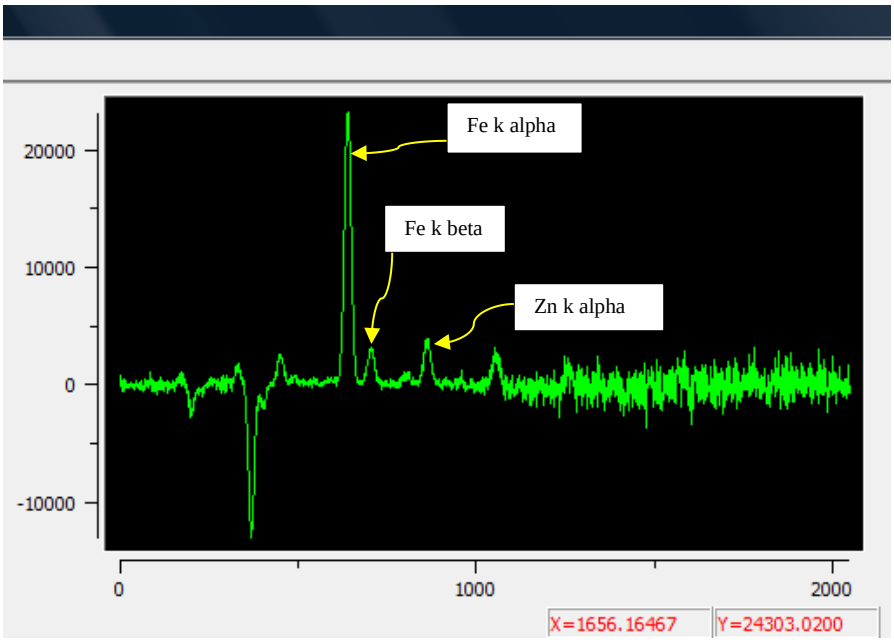


Figure A.8.12. PCA: Component 3.



PCA Analysis: On component 1 all the peaks of interest are discernible: **Ca k α, Cu k α, Fe k α, Fe k β, Zn k α, Zn k β** (although this last one is pretty obviously buried in the background scatter peak's shoulder). As expected there is no gallium. Both components 2 and 3 report the named peaks as well.

Figures A.9.1. to A.9.9. Rabbit 2C - Uninfected treated control – Cecum sample. Fluorescence maps.

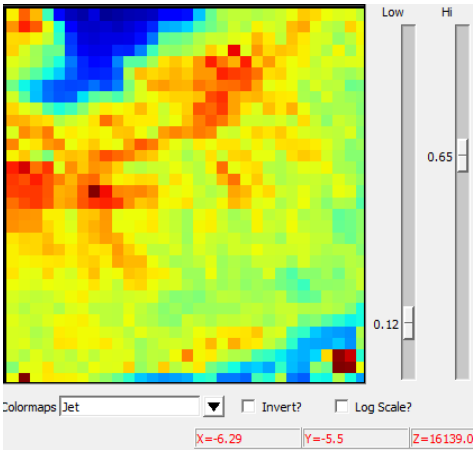


Figure A.9.1. Calcium k α

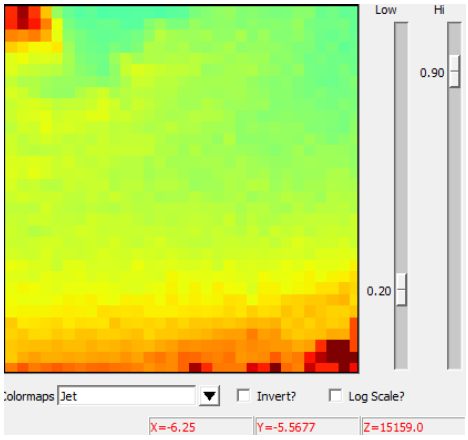


Figure A.9.2. Copper k α

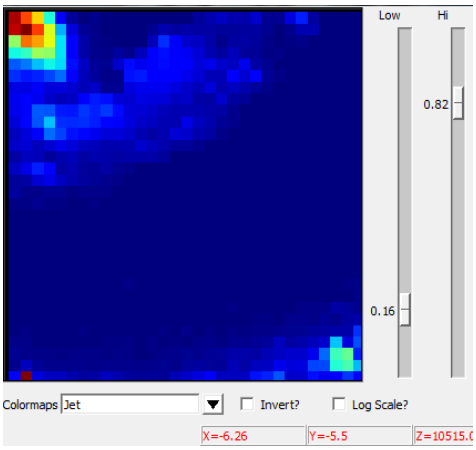


Figure A. 9. 3. Iron k α

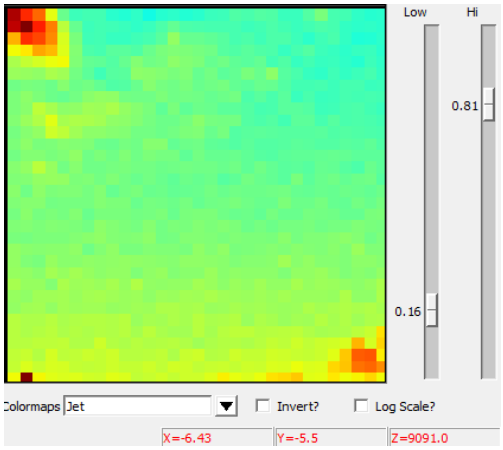


Figure A.9.4. Iron k β

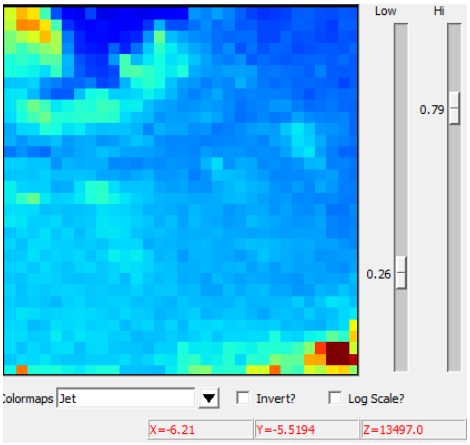


Figure A.9.5. Zinc k α

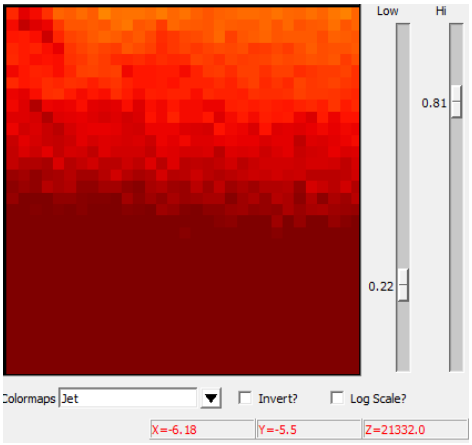


Figure A.9.6. Zinc k β

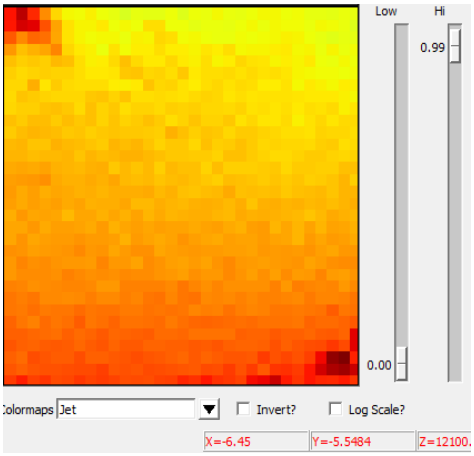


Figure A.9.7. Gallium k α

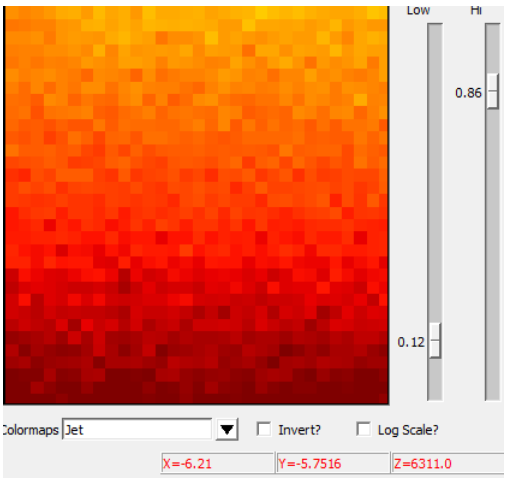


Figure A.9.8.Background scatter lower end

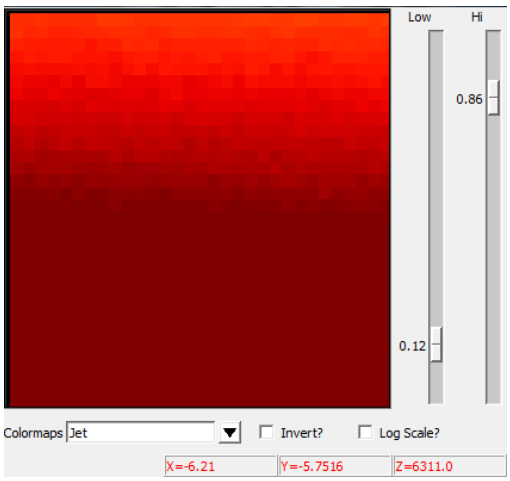


Figure A.9.9. Background scatter higher end

Fluorescence maps: several element channels report marked details in opposite corners (top left and bottom right), including **Fe k α, Fe k β, Ga k α, Ca k α, Cu k α** and **Zn k α**. However it is to be verified what is really imaged in the Gallium channel is gallium and not an association of detection of **Cu k α, Zn k β** and **Ga k α** or more simply **Cu k α** and **Zn k β** only. Also, although placeability of this map could be feasible, the sample stage sidestepped as we obviously mapped a large gap in the sample (rather than sample itself) having relevant materials only at the edge of the map. Such objective was not desirable and likely the mapping of this sample will not be useful for future fruition.

Table A.10. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.005	0.40582	-21370.02
Cu k α	0.87	1.53372	-1765.60116
Fe k α	0.07	1.38103	-3864.133
Fe k β	0.67	0.6729	417.916199
Zn k α	0.46	1.319236	-853.301
Zn k β	0.85	1.495795	2454.2
Background low	0.77	0.528798	977.7682
Background high	0.75	10.1648	22749.539

Table A.10. Correlation: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Although gallium detected feebly, Cu k α, Zn k β and background scatter energy, both low and high, correlated well to weak data, with medium-high r^2 values (≥ 75%), showing a doubtful ability for the detector to record the right fluorescence signal for gallium.

Figure A.9.10. PCA: Component 1

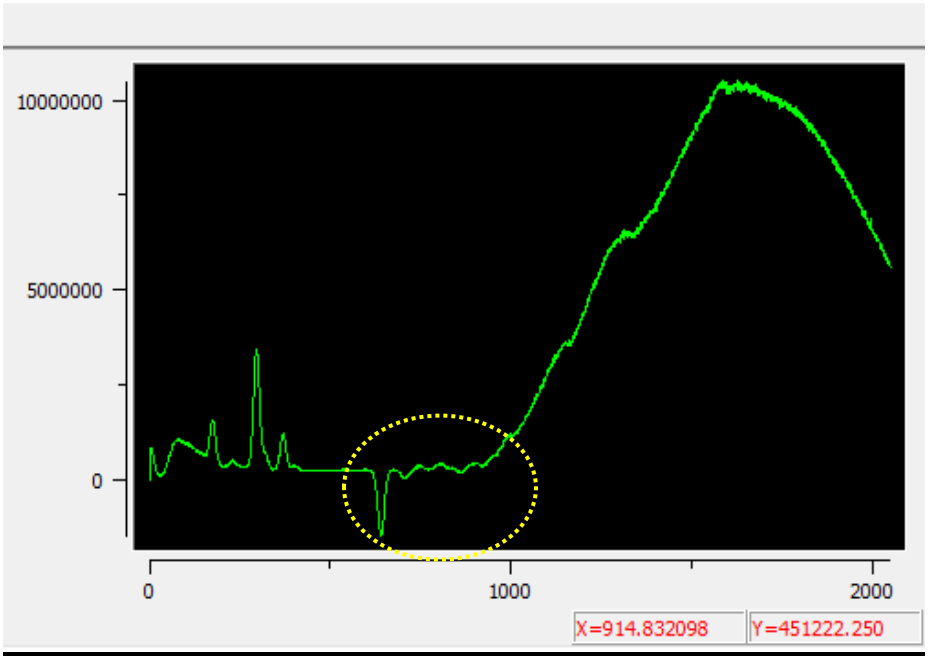


Figure A.9.11. PCA: Component 1, close-up view.

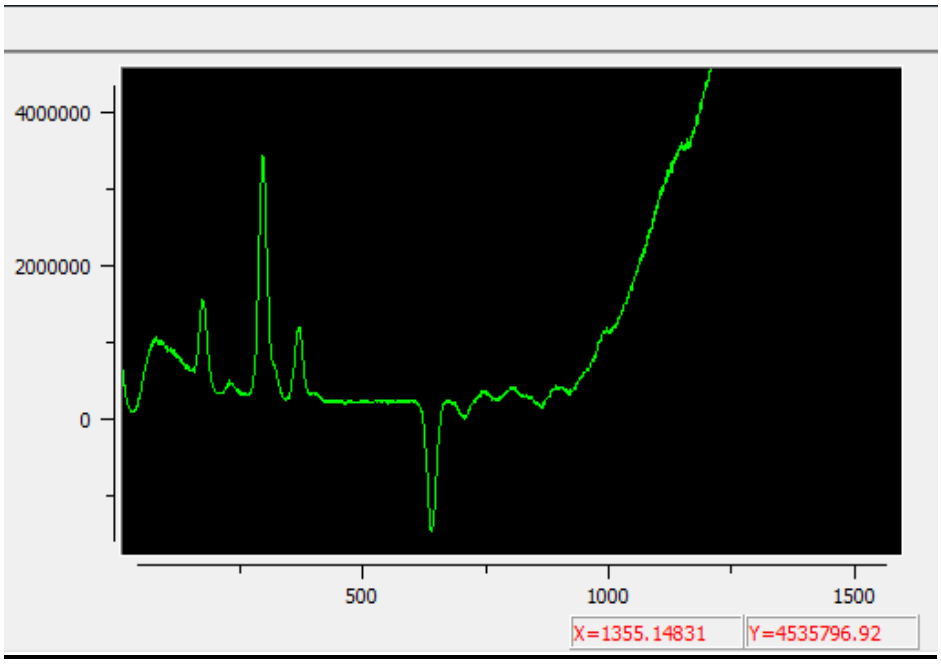


Figure A.9.12. PCA: Component 2.

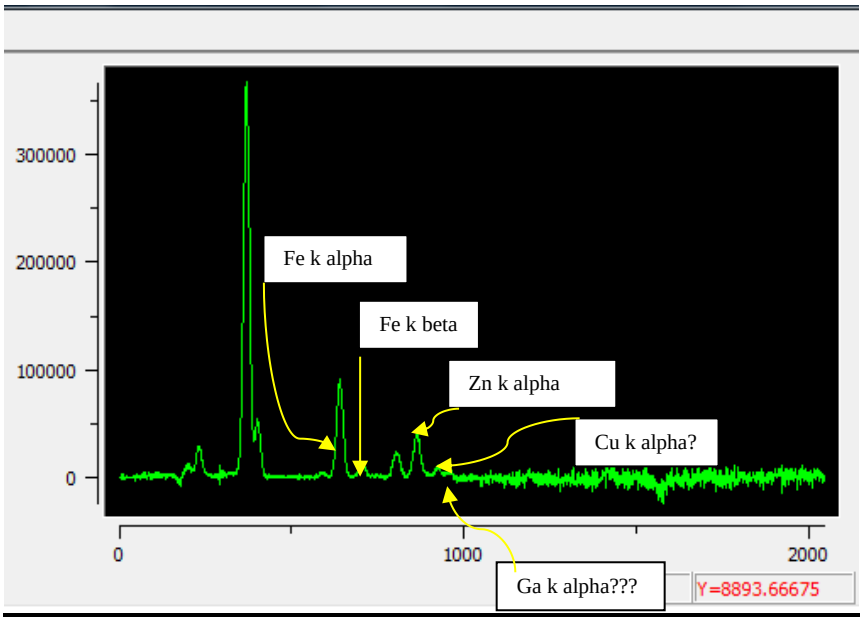
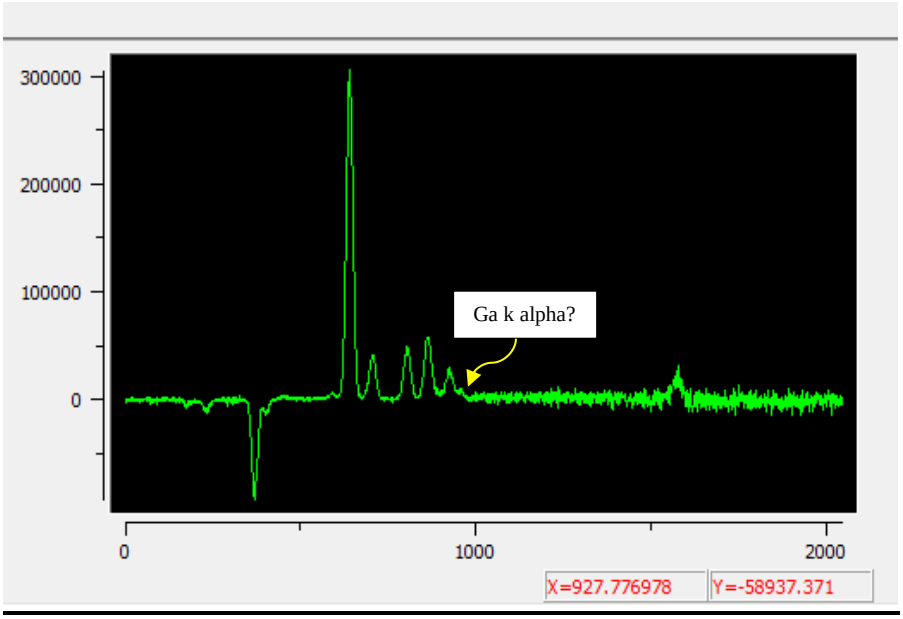


Figure A.9.13. PCA: Component 3.



PCA Analysis: On component 1 the possible location of **Zn k β** peak and **Ga k α** are somewhat buried in the higher end background scatter shoulder. However, in Component 2 and 3 **Ga k α** is visible after **Cu k α** and **Zn k α,** as a small peak (9170 – 9220 eV, ROI limits at 9100 – 9400). Although a peak is present, I'm still skeptical about its significance because of the lack of XAS to confirm it and the inability to determine if I'm mapping intestinal lumen content or intestinal wall, which was ultimately what I was aiming to analyze.

Addendum: Further fluorescence maps for Rabbit 2C

In the addendum, there is a further analysis conducted to compare gallium and zinc findings to ascertain if the peak detected was truly Gallium. Unfortunately the incapability of conducting X-ray absorption spectroscopy (XAS) at VESPERS is detrimental to our results, because we could not prove beyond reasonable doubt that the peak detected is Gallium (Figures A.9.14 to A.9.16).

Figures A.9.14. to A.9.16. Addendum.

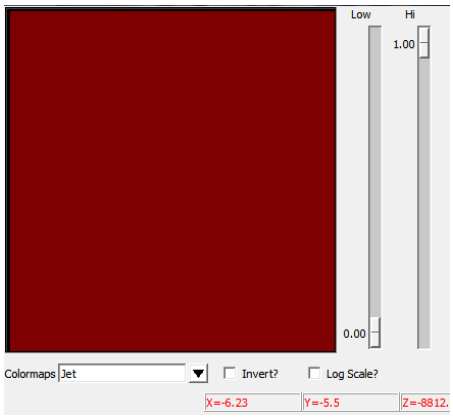


Figure A.9.14 Gallium k α minus Background scatter (higher end)

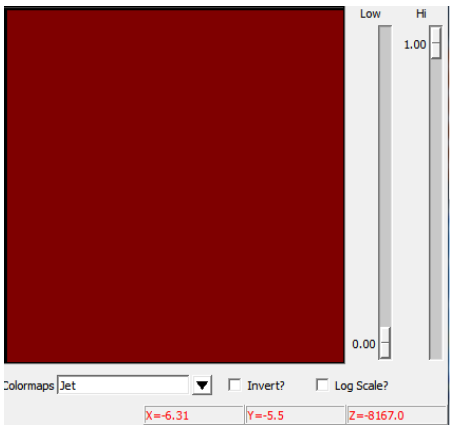


Figure A.9.15 Gallium k α minus Copper k α

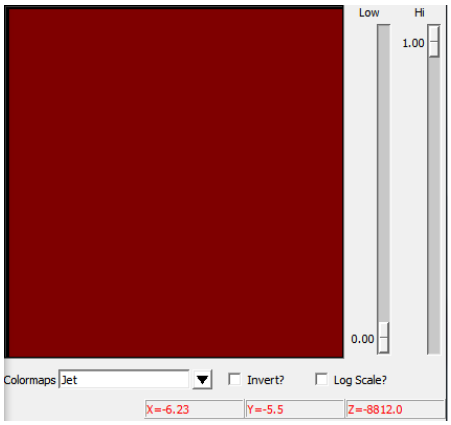


Figure A.9.16. Gallium k α minus Zinc k β

Figures A.9.14. - A.9.16. Fluorescence maps subtractions. The images were obtained subtracting mathematically from the values of each step in the Gallium fluorescence map the values from each step of fluorescence maps obtained from higher-end back ground scatter, Copper k α and Zinc k β channels, whose ROI's have similar or adjacent voltage to Gallium. The results were a complete "null value" and confirmed the suspicion that chances of having really detected gallium were very small, and more likely the findings were only pertinent to detections of elements from overlapping ROIs.

Figures A.10.1. to A.10.9. Rabbit 2D - uninfected and treated – Cecum sample. Fluorescence maps.

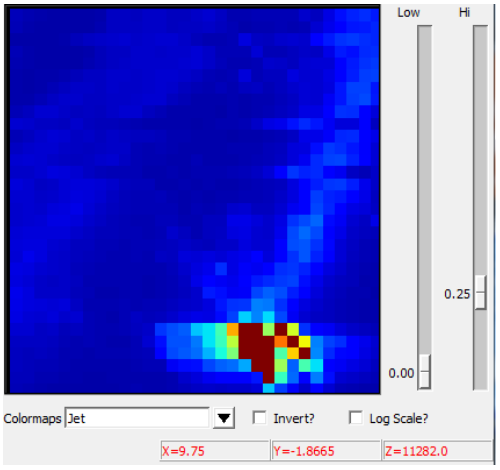


Figure A.10.1. Calcium k α

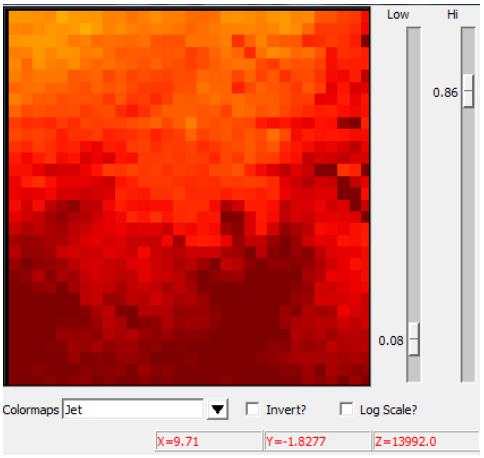


Figure A.10.2. Copper k α

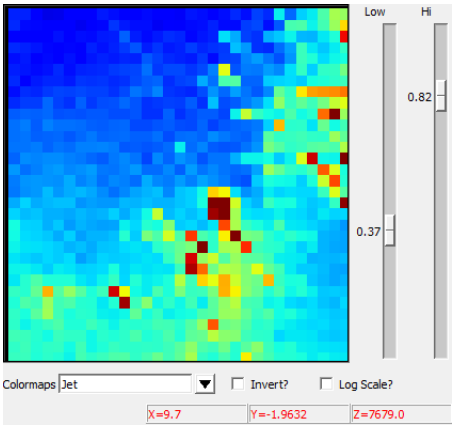


Figure A.10.3. Iron k α

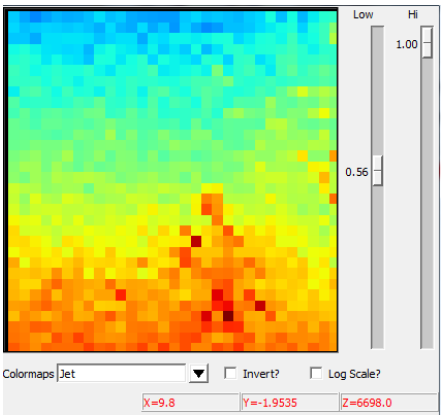


Figure A.10.4. Iron k β

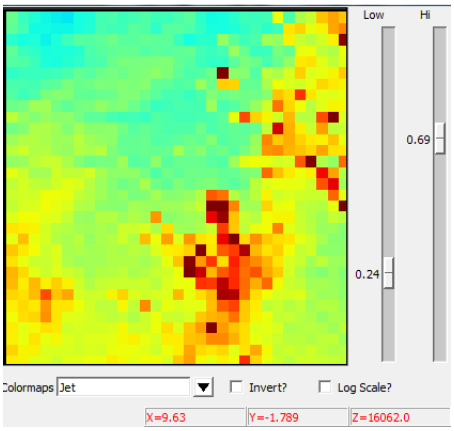


Figure A.10.5 Zinc k α

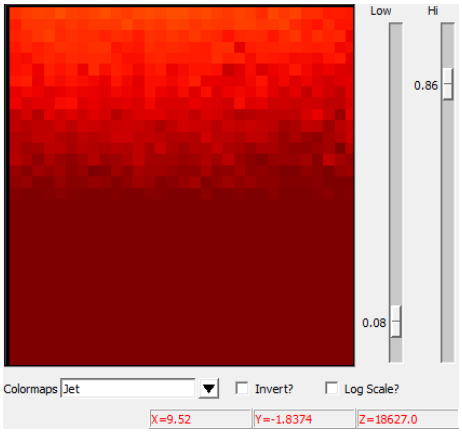


Figure A.10.6 Zinc k β

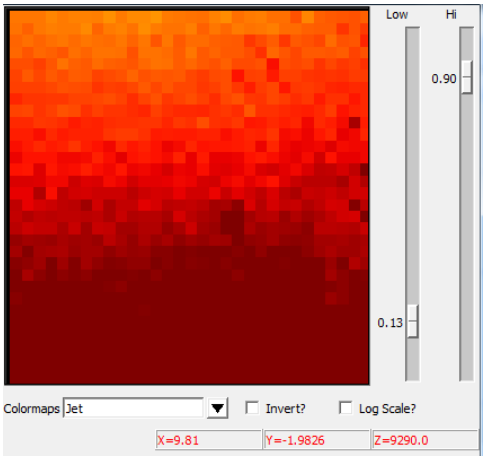


Figure A.10.7. Gallium k α

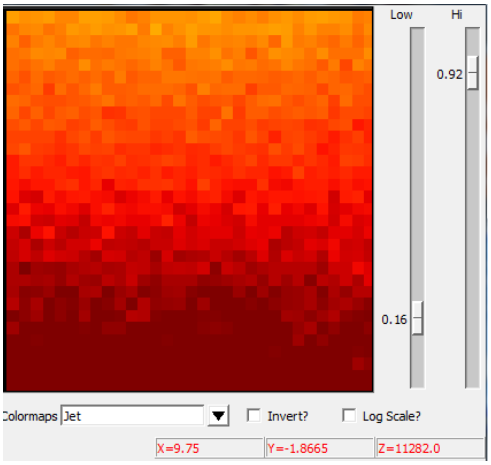


Figure A.10.8. Background scatter lower end

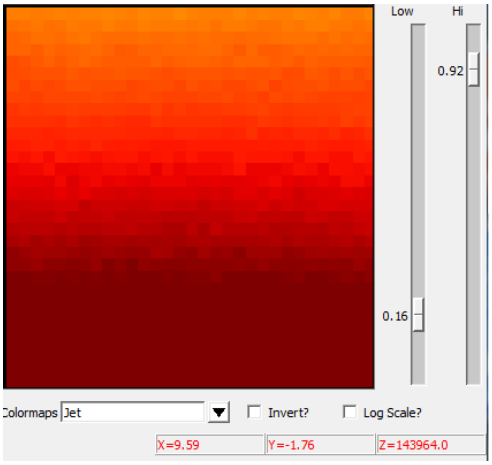


Figure A.10.9. Background scatter higher end

Figures A.10.1. –A.10.2. Fluorescence maps: Some anatomical landmark is barely perceptible from elemental channels of **Ca k α, Cu k α, Fe k α, Fe k β, Zn k α.** No high concentration spot of gallium was detected in what should be animal tissue. Moreover background scatter (high and low end), **Ga k α** and **Zn k β** look very much alike each other, with no distinction in the appearance of fluorescence maps.

Table A.11. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.05	15.3593	-131305.137
Cu k α	0.82	1.14237	1816.1535
Fe k α	0.34	0.7907	667.30825
Fe k β	0.93	0.65109	387.9919
Zn k α	0.35	0.8473	2663.52858
Zn k β	0.96	1.6918	455.889
Background low	0.94	0.579212	464.9330
Background high	0.95	11.7734	6307.2438

Table A.11. Correlation: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Although gallium detected feebly, Cu k α, Fe k β Zn k β, and back ground scatter energy, both low and high, correlated well to weak data, with medium-high r^2 values (≥ 82%), showing a doubtful ability for the detector to record the right fluorescence signal for gallium.

Figure A.10.10 PCA: Component 1

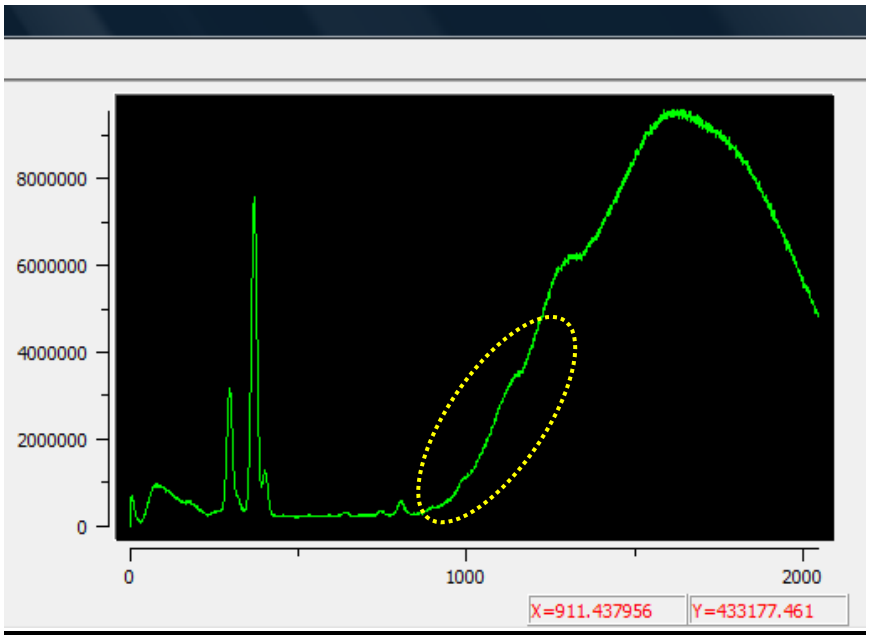


Figure A.10.11 PCA: Component 1, close-up.

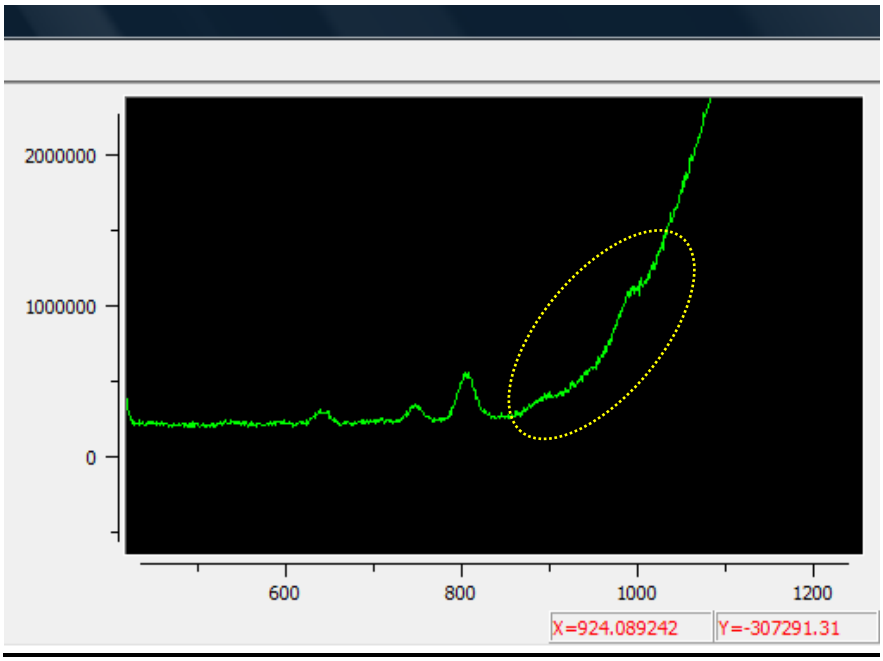


Figure A.10.12 PCA: Component 2.

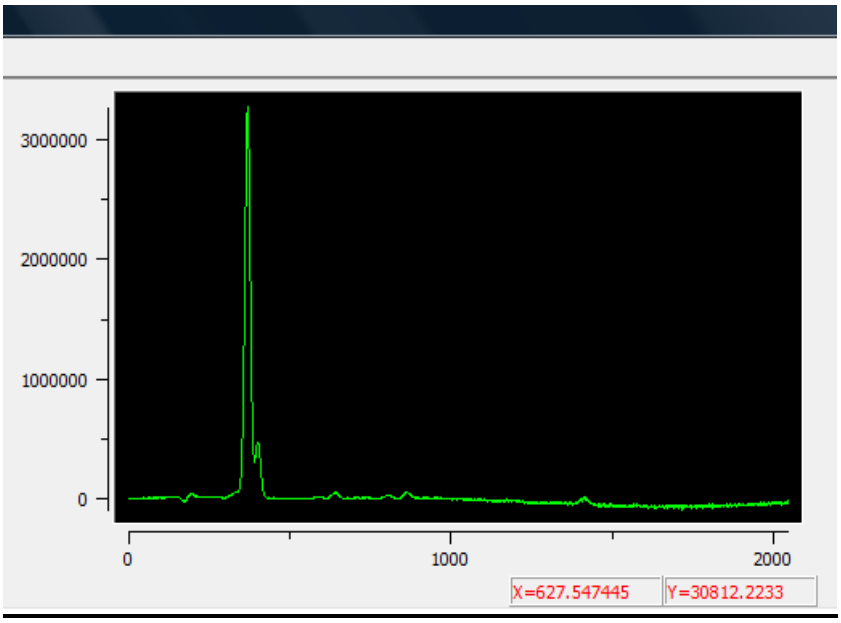
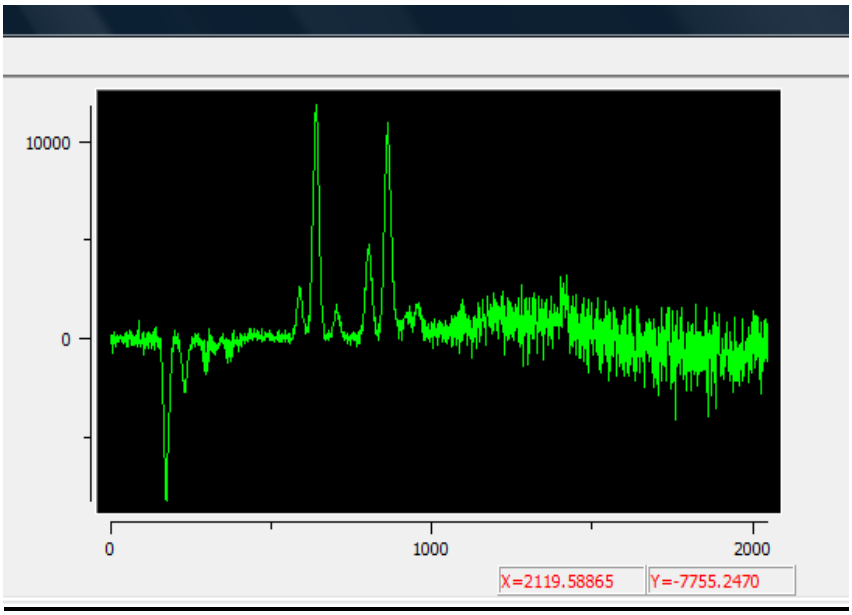


Figure A.10.13. PCA: Component 3.



PCA Analysis: On component 1 the possible locations of **Zn k β** peak and **Ga k α** respectively are annulled/buried in the higher end background scatter shoulder. Also, no **Fe k α** nor **Fe k β** are recognizable. In component 2 a very small **Fe k α** is detectable, and Component 3 is very noisy, although iron and zinc peaks are probably recognizable.

Figures A.11.1. to A.11.9. Rabbit 2F - uninfected and treated – Cecum sample. Fluorescence maps.

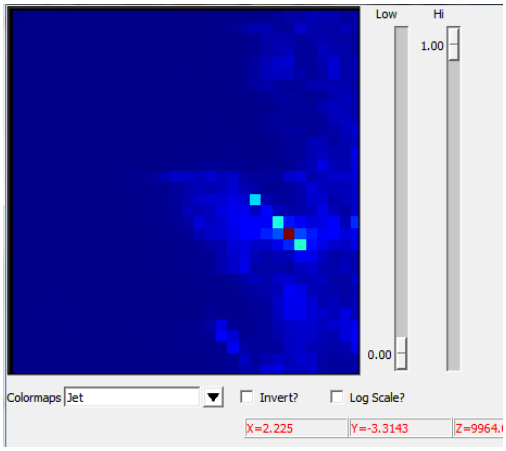


Figure A.11.1. Calcium k α

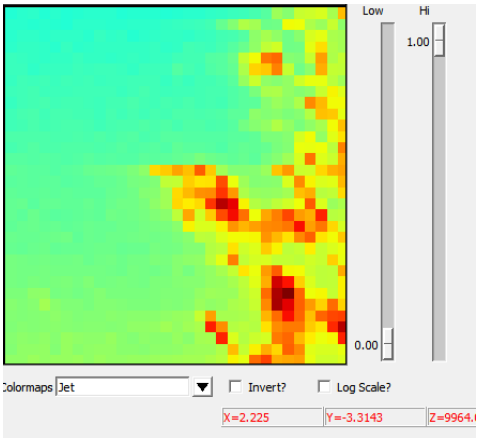


Figure A.11.2 Copper k α

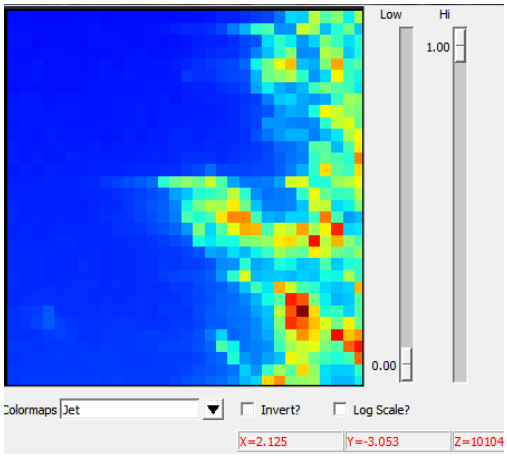


Figure A.11.3. Iron k α

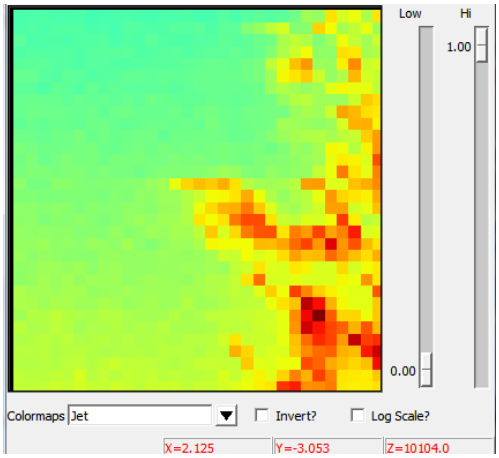


Figure A.11.4. Iron k β

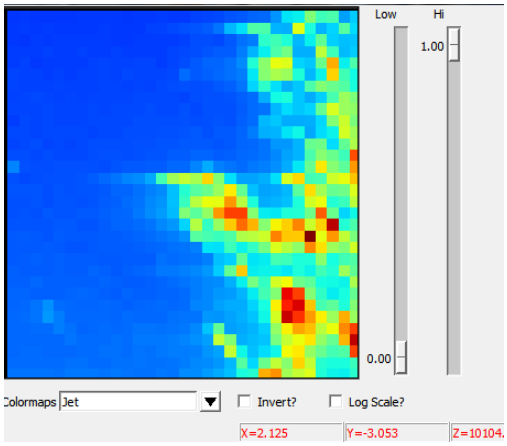


Figure A.11.5. Zinc k α

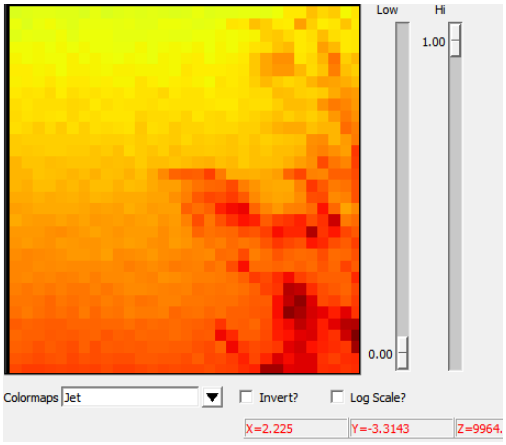


Figure A.11.6. Zinc k α

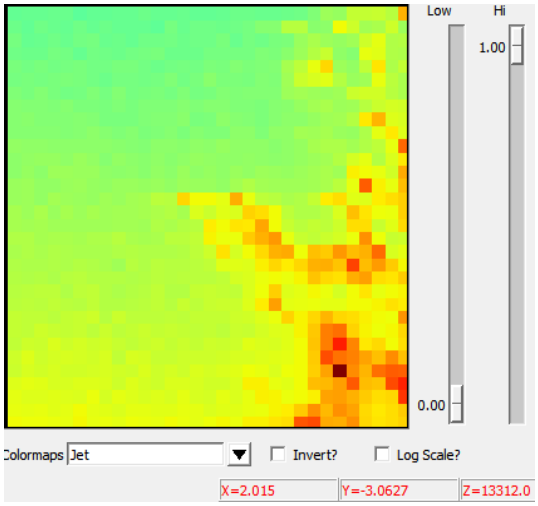


Figure A.11.7. Gallium k α

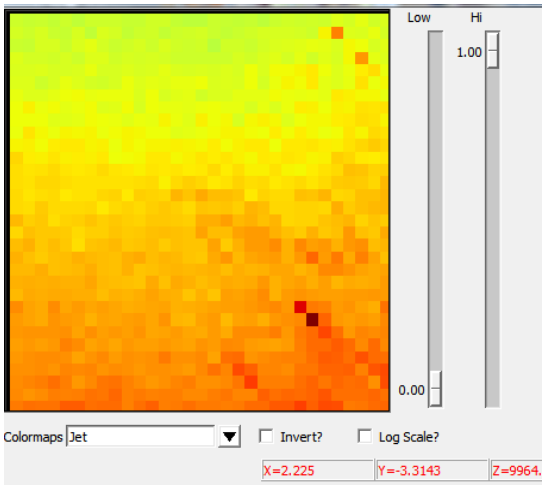


Figure A.11.8 Background scatter lower end

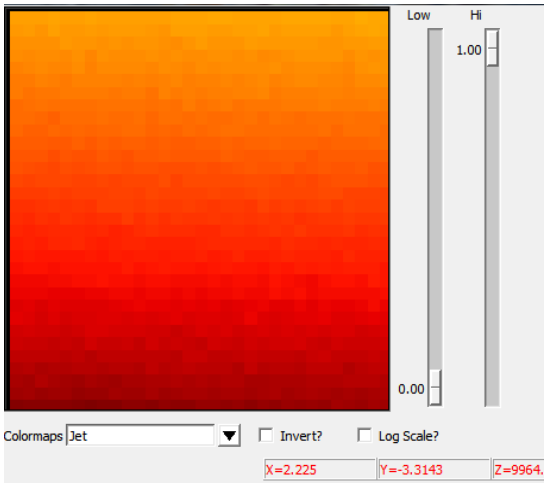


Figure A.11.9 Background scatter higher end

Figures A.11.1. – A.11.9 Fluorescence maps: For the first and only time in this experiment, anatomical structures are easily recognizable in every elemental channel fluorescence map. Also the gallium fluorescence map is astoundingly beautiful in comparison to all the previous ones, as it is comparable for details to iron, zinc and calcium.

Table A.12. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.24	11.797	-116006.39
Cu k α	0.83	2.0846	-8406.6979
Fe k α	0.65	4.8248	-41944.269
Fe k β	0.90	0.92303	-2502.6956
Zn k α	0.68	4.6218	-37653.329
Zn k β	0.88	1.29924	4707.781
Background low	0.66	0.3352	3002.996
Background high	0.43	5.1284	77729.65

Table A.12. Correlation: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Gallium was detected considerably well and differently than usual feebly, Cu k α, Fe k β, Zn k were variable between medium and medium high r^2. Back ground scatter energy, both low and high, were not as well correlated well to Gallium data, differently than previously noted. However, the higher correlations seem to be with those elements that, when subtracted, give a zero result and a null fluorescence map.

Rabbit 2 F - Addendum: Fluorescence maps, elemental subtraction.

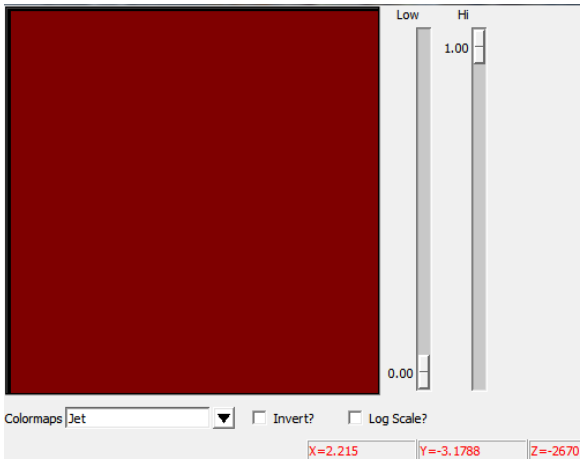


Figure A.11.10 Gallium k α minus Background scatter (high)

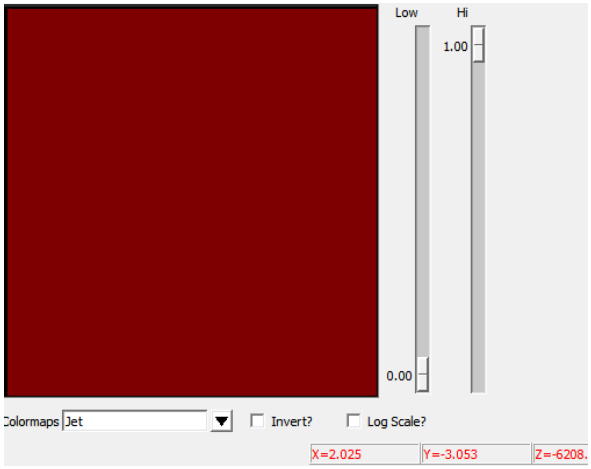


Figure A.11.11 Gallium k α minus Copper k α

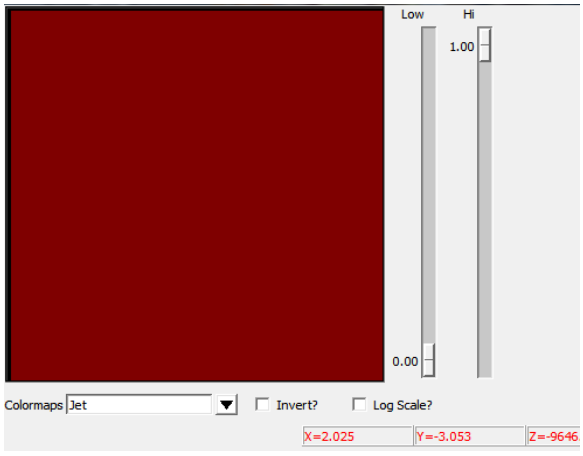


Figure A.11.12 Gallium k α minus Zinc k β

Figure A.11.13. PCA: Component 1.

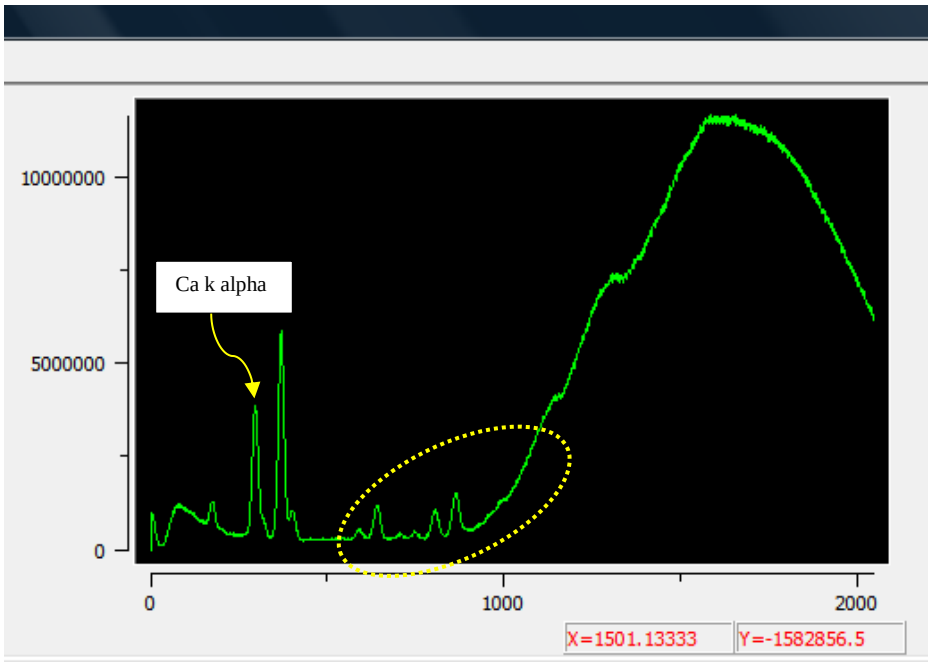


Figure A.11.14. PCA: Component 1, close-up view.

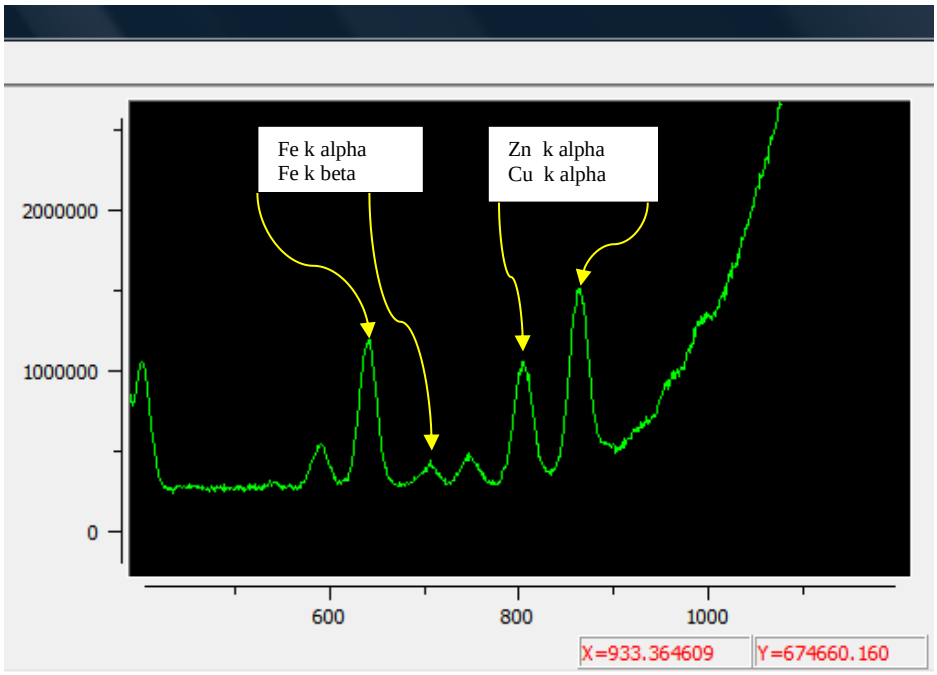


Figure A.11.15. PCA: Component 2

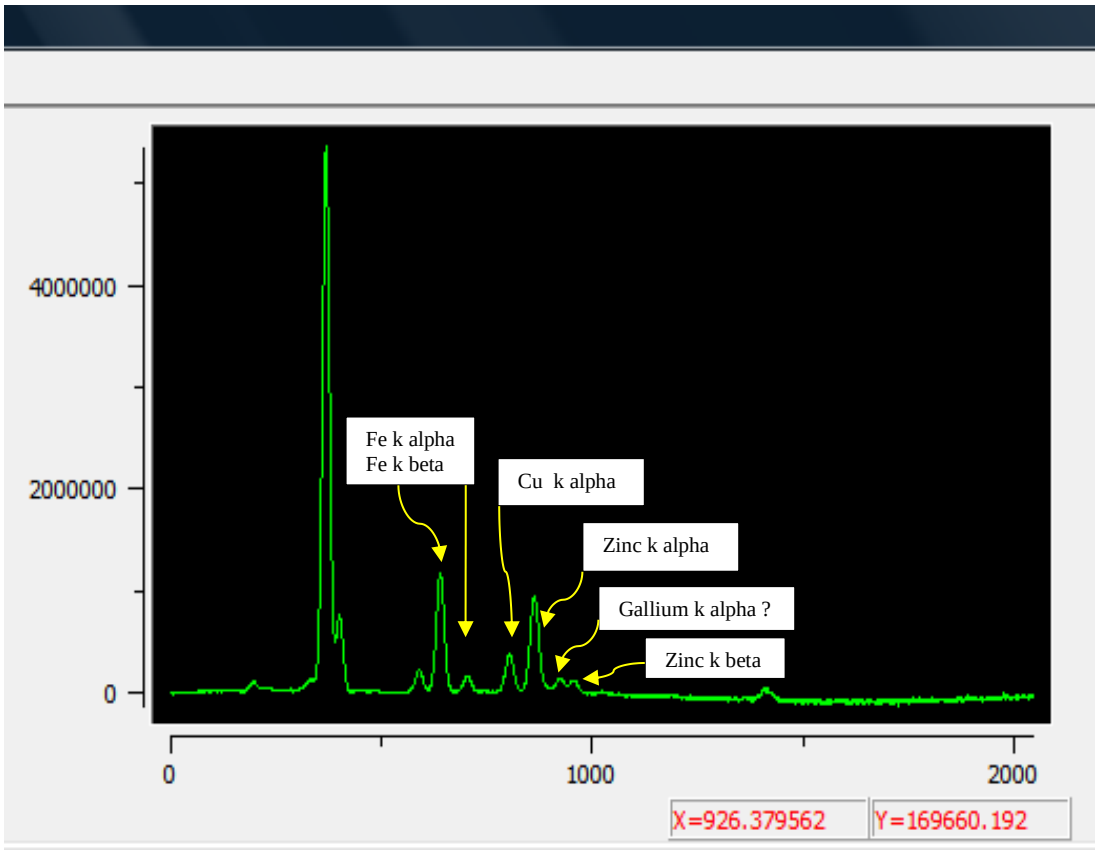
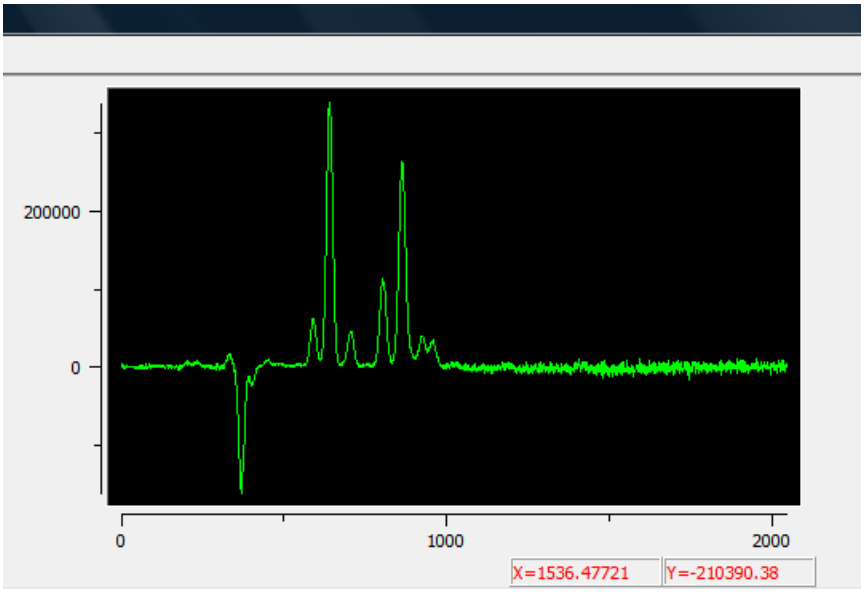


Figure A.11.16. PCA: Component 3.



Figures A.11.13 –A.11.16. PCA analysis: On component 1 **Ga k α** and **Zn k β** are buried in the higher end scatter shoulder, whereas **Fe k α, Fe k β, Cu k α, Zn k α** and **Ca k α** are clearly visible. On components 2 and 3 **Ga k α** and **Zn k β** peaks appear distinct and adjacent, not included in the large amount of background scatter. However, a simple mathematical map subtraction reports a null result when we subtract from a gallium fluorescence map's set of values the set of values deriving from fluorescence maps of **Zn k β, Cu k α** and higher end background scatter signal, as shown in the addendum – Rabbit 2F).

Figures A.12.1. to A.12.9. Rabbit 4E - *Lawsonia intracellularis* infected and treated – Cecum sample. Fluorescence maps.

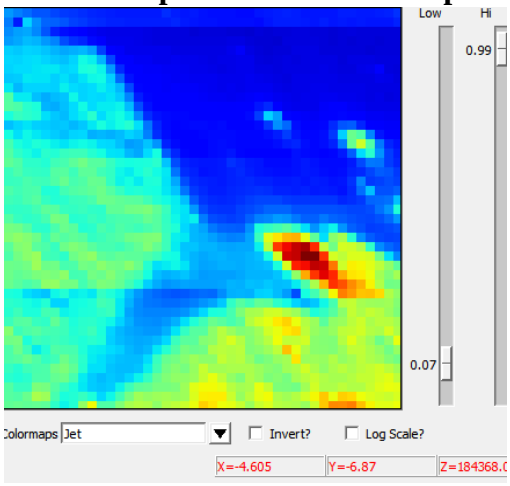


Figure A.12.1. Calcium k α

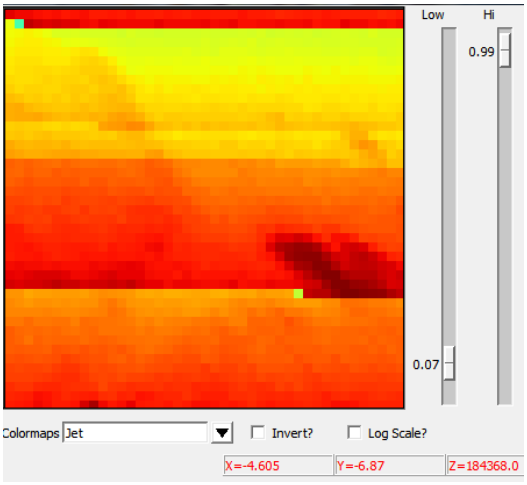


Figure A.12.2. Copper k α

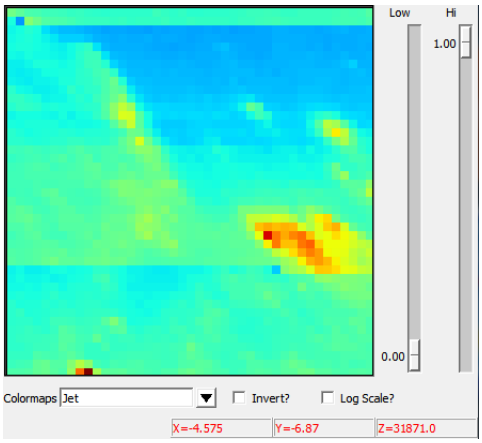


Figure A.12.3. Iron k α

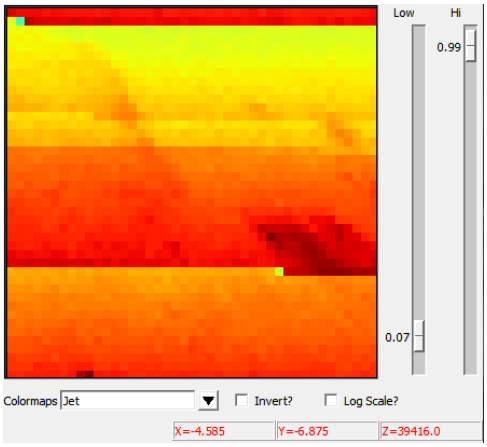


Figure A.12.4. Iron k β

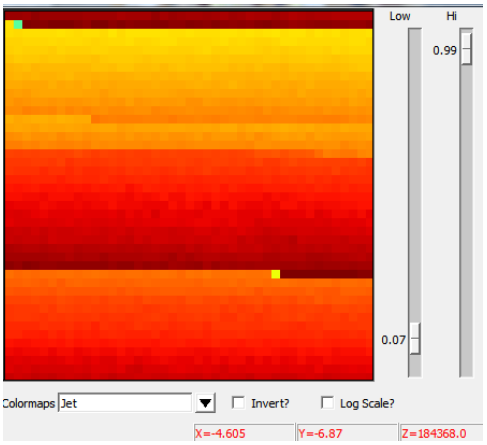


Figure A.12.5. Zinc k α

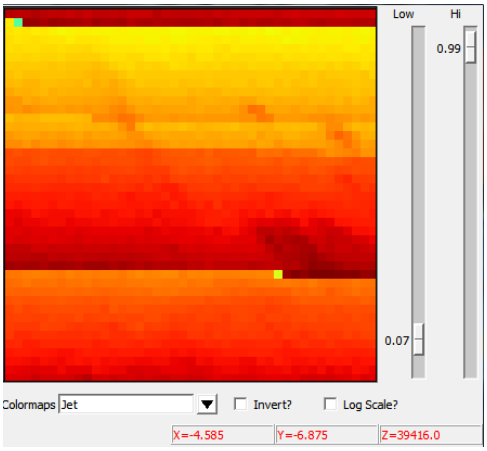


Figure A.12.6. Zinc k β

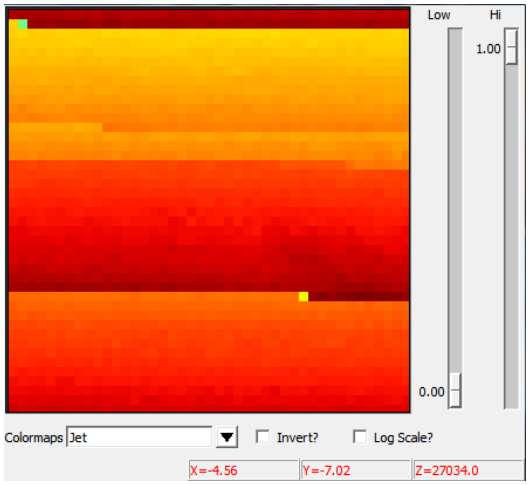


Figure A.12.7. Gallium k α

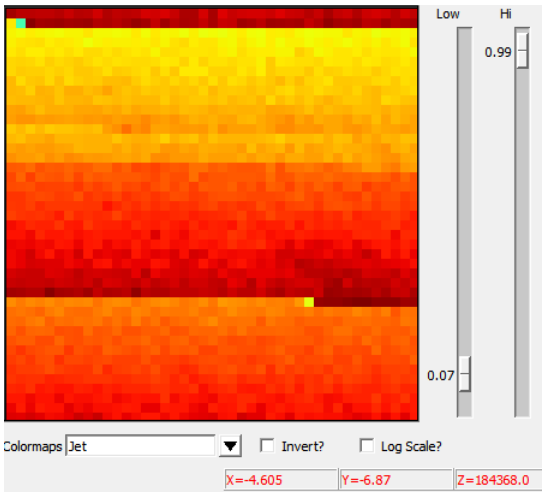


Figure A.12.8.Background scatter lower end

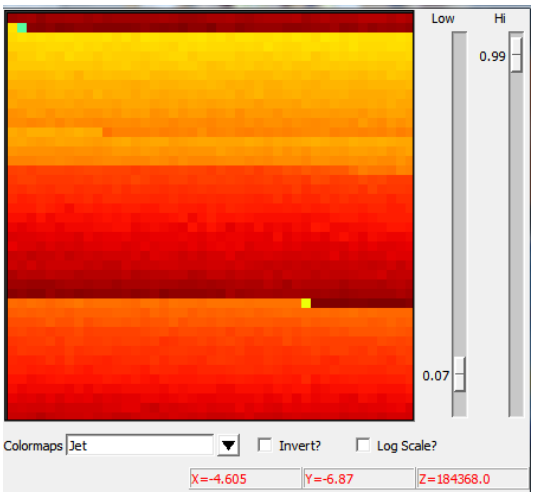


Figure A.12.9. Background scatter higher end

Figures A.12.1 – A.12.9. Fluorescence maps: Maps show some recognizable anatomical structure for **Fe k** α and **Ca k α.** However these same structure references are barely recognizable in **Zn k α** and **Cu k α**, likely because of the side- stepping of the stage motors and the beam itself, whose flux kept being interrupted during this sampling.

Table A.13. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.29	0.37847	-29779.81
Cu k α	0.96	0.775013	-6892.461179
Fe k α	0.47	0.2934	-9809.2031
Fe k β	0.97	0.272347	-1534.3678
Zn k α	0.99	1.04944	-3017.10304
Zn k β	0.99	1.223121	966.86041
Background low	0.98	0.07092	-175.20539
Background high	0.99	0.6975	499.83406

Table A.13. Correlation: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Gallium was detected feebly, if at all, with Cu k α, Fe k β, Zn k, as well as the background scatter energy (low and high ends) showing high r^2 (≥ 96%). Back ground scatter energy, both low and high, were not as well correlated well to Gallium data, differently than previously noted. However, the higher correlations seem to be with those elements that, when subtracted, give a zero result and a null fluorescence map.

Figure A.12.10. PCA: Component 1

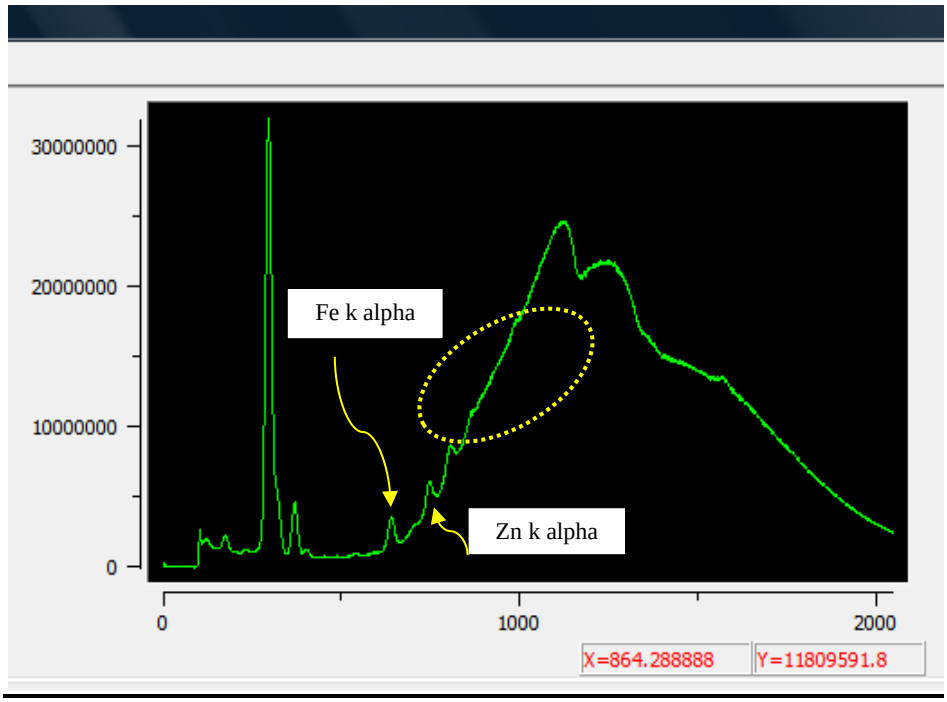


Figure A.12.11.PCA: Component 2.

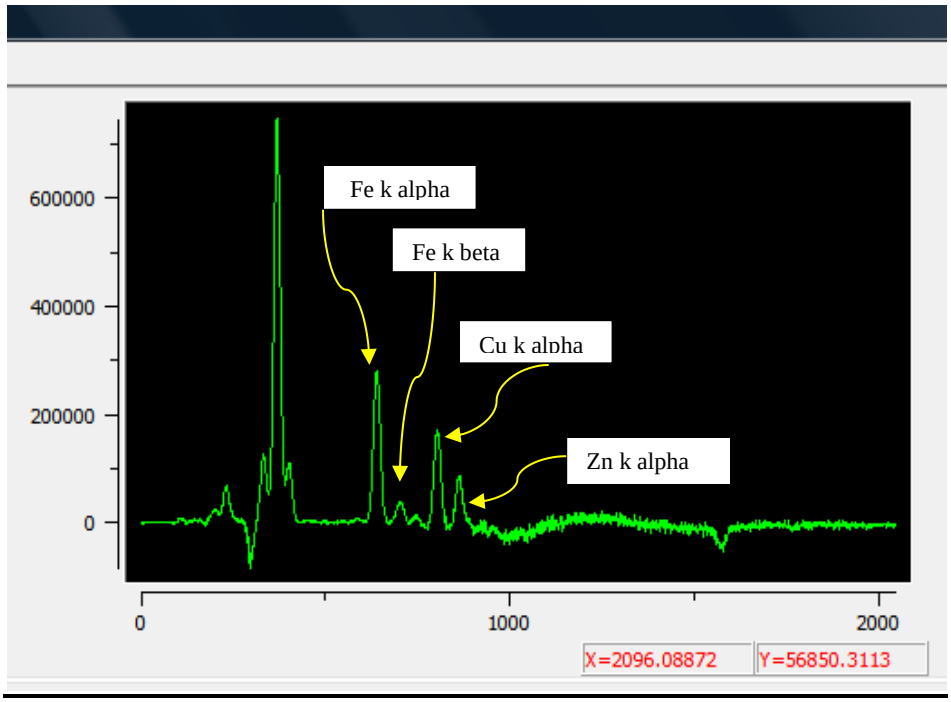
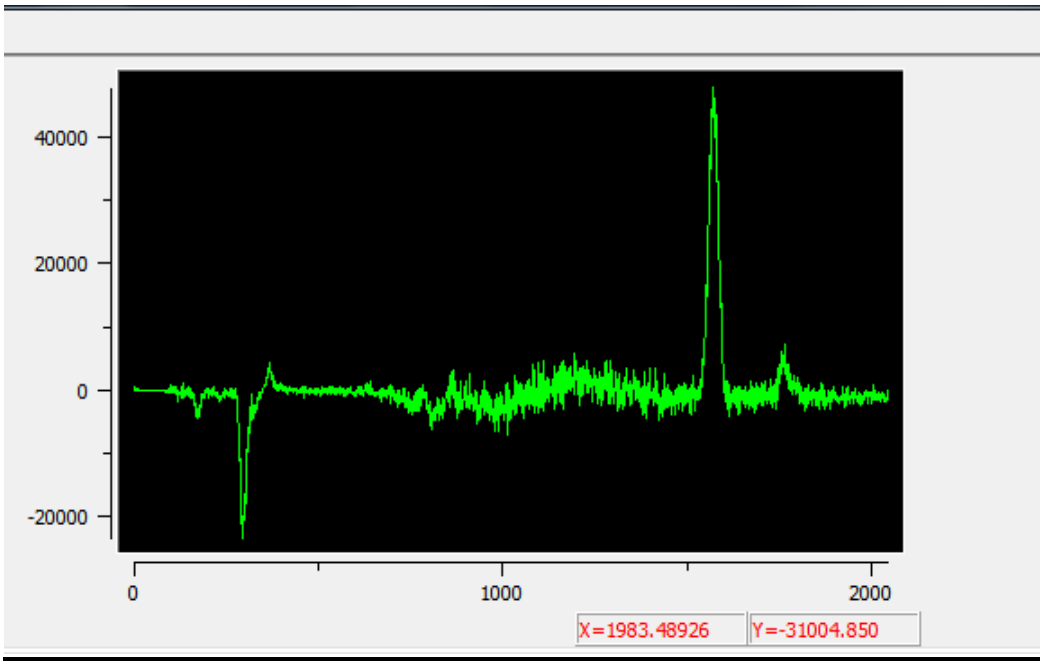


Figure A.12.12. PCA: Component 3



Figures A.12.10 – A.12.12. PCA analysis: High scatter signal "noise" in component 3 is likely not consistent with any specific peak of interest (**Ga k α** or **Zn k β**). The only ones recognizable are mainly in component 2, as they are not very clear in component 1, are **Fe k α** and **Ca k α**, **Zn k α**, and potentially **Cu k α**, but they should be confirmed because the scatter signal shoulder is very large and rises very much before the 10000 eV threshold.

Figures A.13.1. to A.13.9. Rabbit 4G - *Lawsonia intracellularis* infected and treated – Cecum sample. Fluorescence maps.

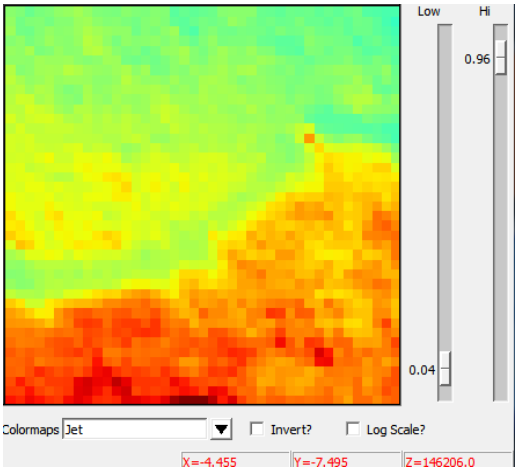


Figure A.13.1.Calcium k α

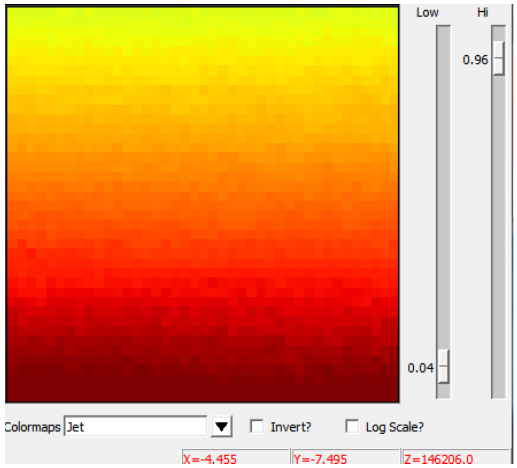


Figure A.13.2 Copper k α

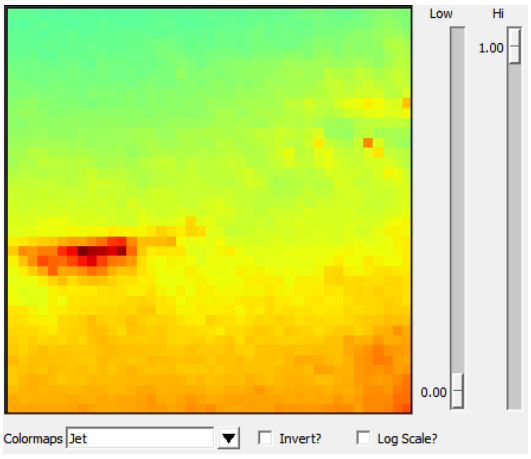


Figure A.13.3 Iron k α

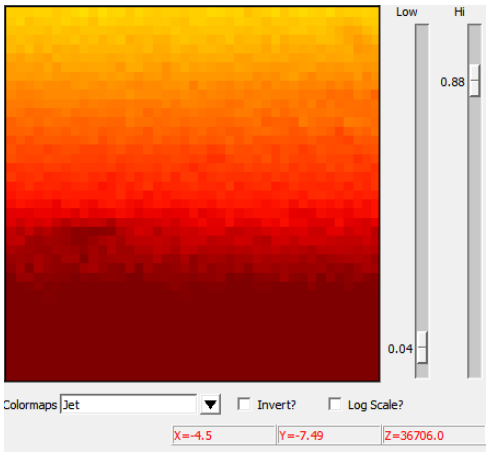


Figure A.13.4 Iron k β

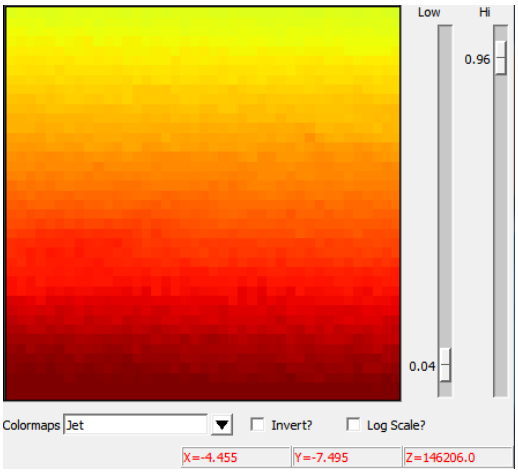


Figure A.13.5. Zinc k α

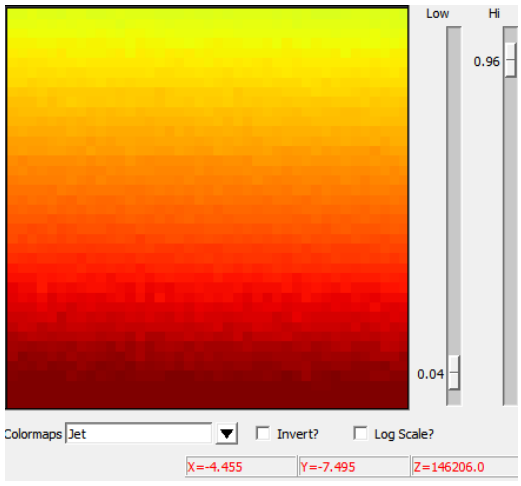


Figure A.13.6. Zinc k β

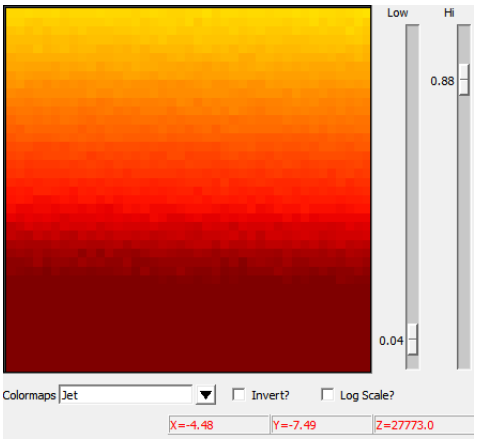


Figure A.13.7. Gallium k α

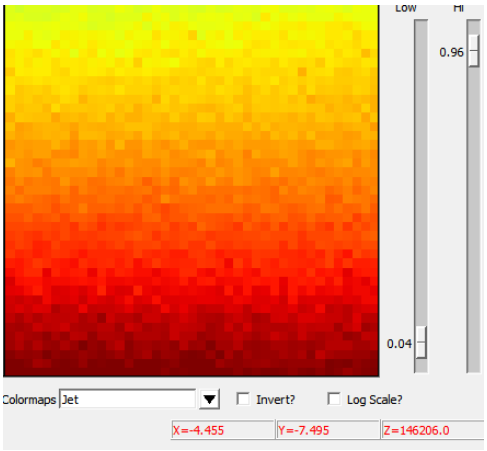


Figure A.13.8.Background scatter lower end

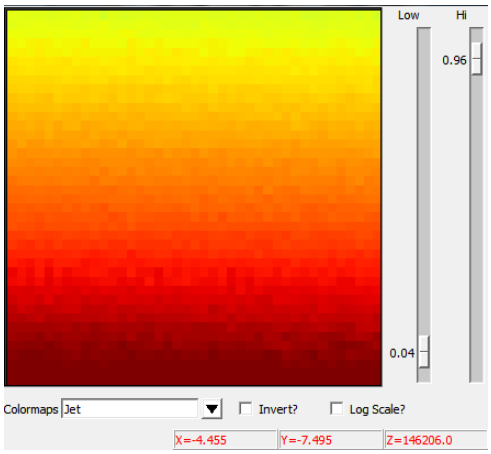


Figure A.13.9. Background scatter higher end

Figures A.13.1 – A.13.9. Fluorescence maps: As noted previously in this experiment, fluorescence maps show some recognizable anatomical landmark in the **Fe k α** and **Ca k α** channels. Nothing at all is distinguishable in the channels set for other elements' fluorescence, as most of them look like background scatter signal.

Table A.14. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.81	0.104722	-1014.5405
Cu k α	0.99	0.6990	819.5353
Fe k α	0.80	0.17668	2690.395
Fe k β	0.99	0.25133	941.71066
Zn k α	0.99	1.01494	1936.8852
Zn k β	0.999	1.2329	39.009286
Background low	0.98	0.0668	420.88045
Background high	0.99	0.70696	85.52276

Table A.14. Correlations: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Although not much structure was detected on the fluorescence maps in the infected treated rabbit, Cu k α, Fe k β, Zn k α, Zn k β and background scatter energy, both low and high, correlated too well to very feeble data, with high r^2 values (> 98%), showing a doubtful ability for the detector to record the right fluorescence signal for gallium, particularly in the case of **Fe k α** and **Ca k α**. With this knowledge, and the PCA results, we can speculate that the background scatter signal was too large to allow appropriate detection of other elements' fluorescence. Also, to note is that data elements usually not at all correlated, a high R^2 is shown (80% for Fe k α). The doubt is that something emitting similar fluorescence signal, regardless of the element (mostly overwhelming or null) was mapped instead of tissue.

Figure A.13.10. PCA: Component 1.

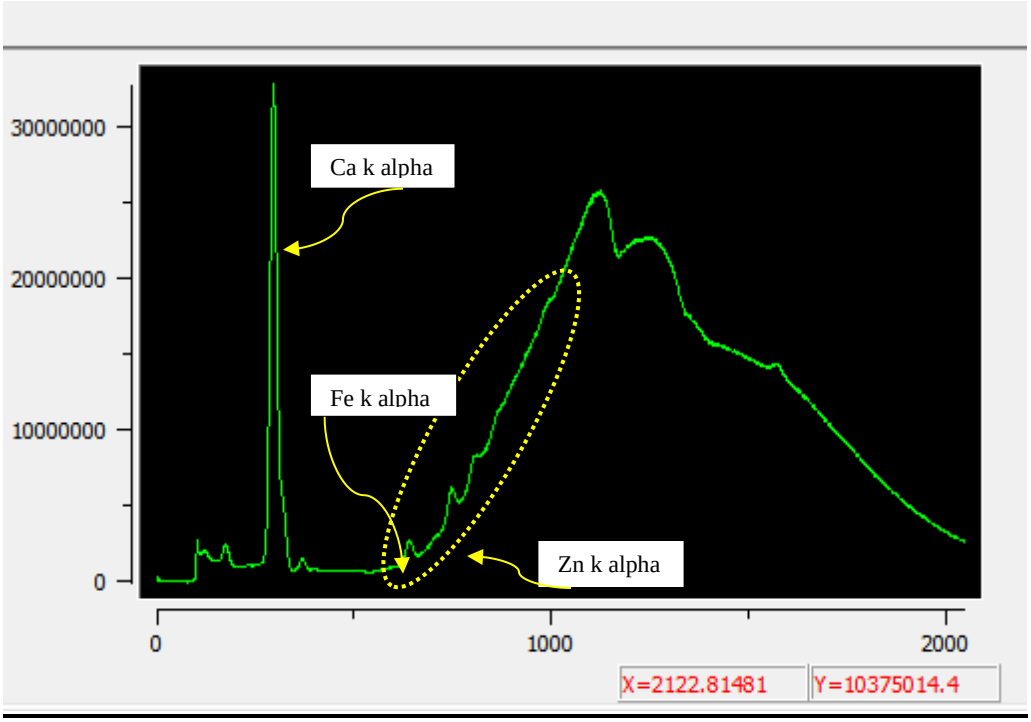


Figure A.13.11. PCA: Component 2.

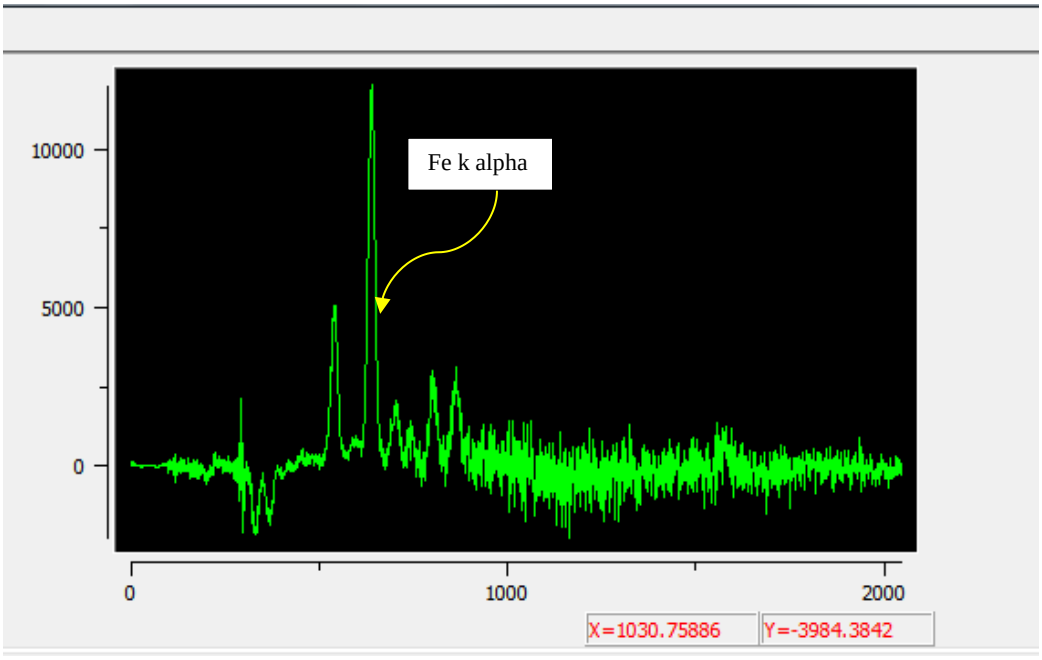
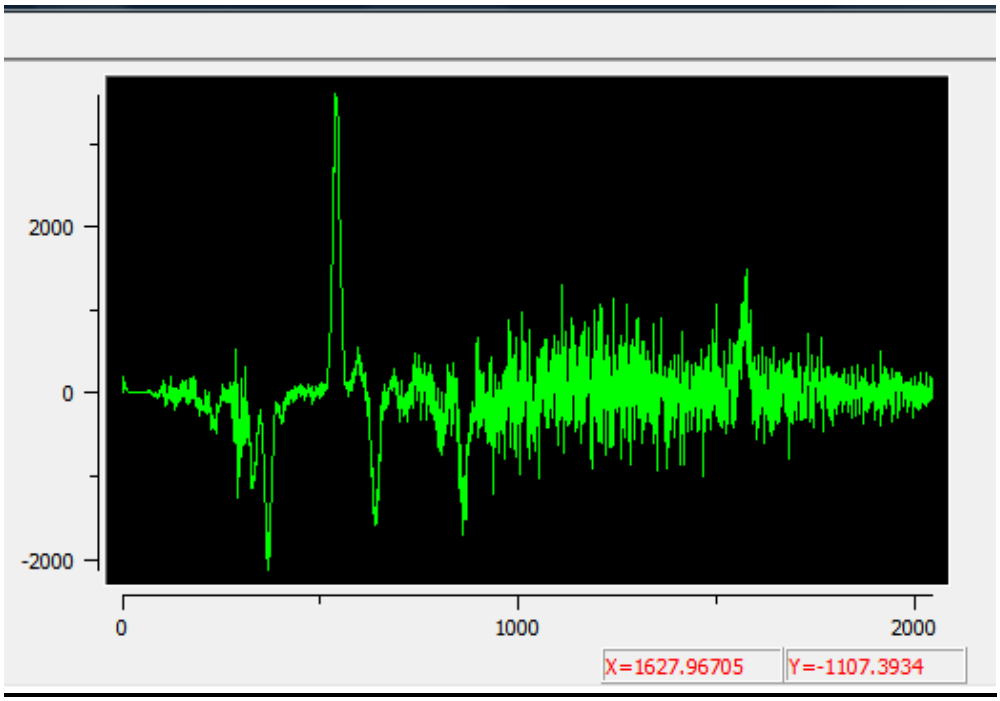


Figure A.13.12. PCA: Component 3.



Figures A.13.10. – A.13.12. PCA analysis. Component 1 shows a very large background shoulder which likely cancels (in other words, it absorbs it in full) **Zn k β** and likely (if any was detected) **Ga k α**. Components 2 and 3 show **Fe k α** peaks, but do not show anything else of interest. Larger components are just increasing levels of scatter signal.

Figures A.14.1. to A.14.9. Rabbit 4H - *Lawsonia intracellularis* infected and treated – Cecum sample. Fluorescence maps.

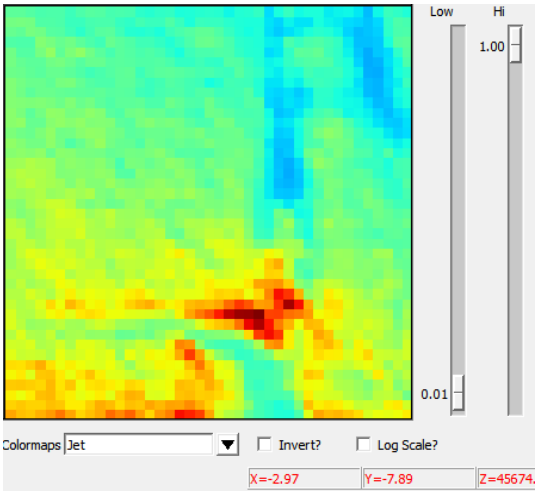


Figure A.14.1. Calcium k α

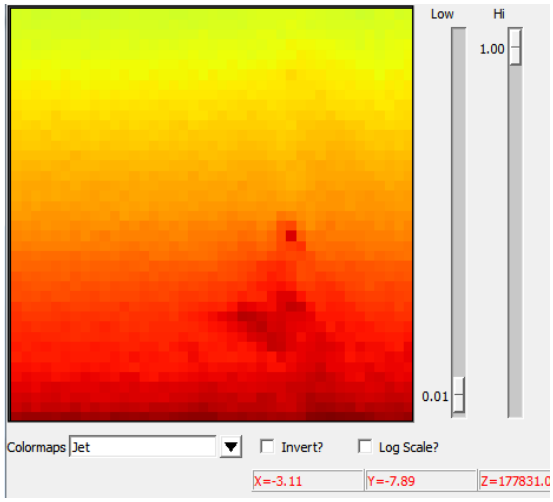


Figure A.14.2. Copper k α

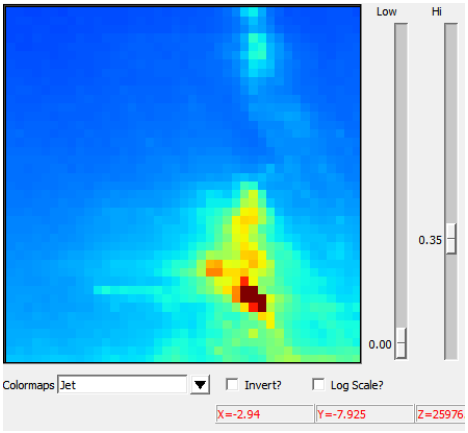


Figure A.14.3. Iron k α

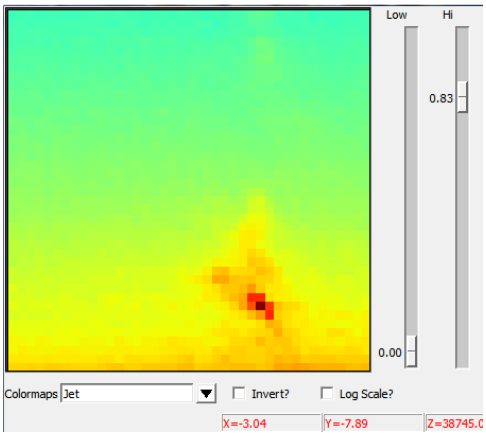


Figure A.14.4 Iron k β

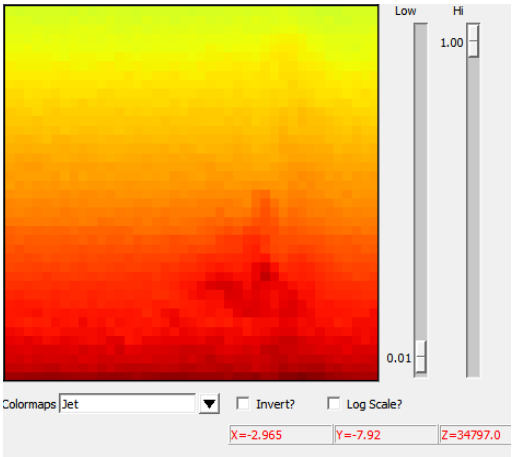


Figure A.14.5. Zinc k α

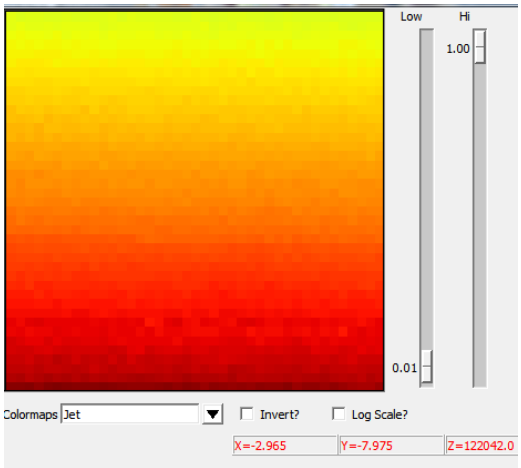


Figure A.14.6. Zinc k β

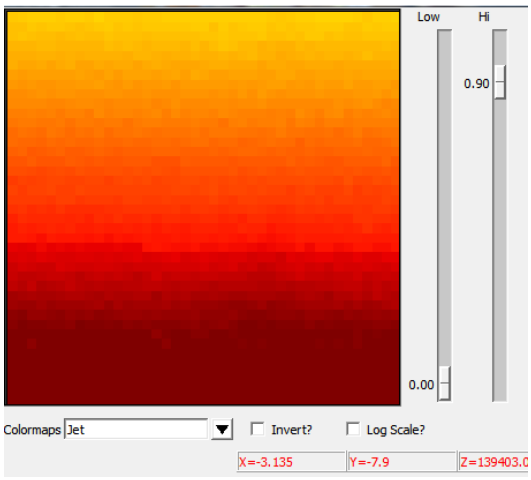


Figure A.14.7. Gallium k α

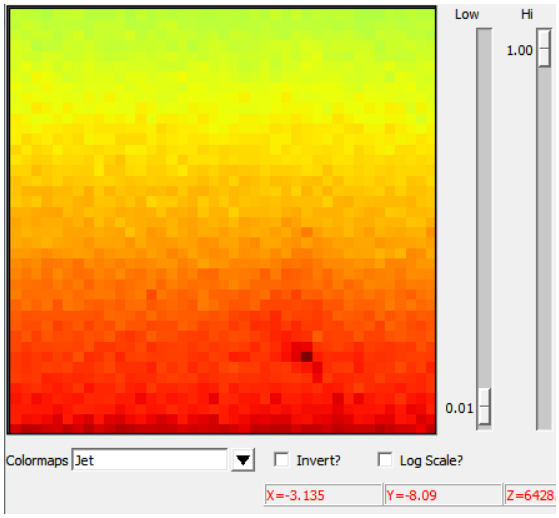


Figure A.14.8 Background scatter lower end

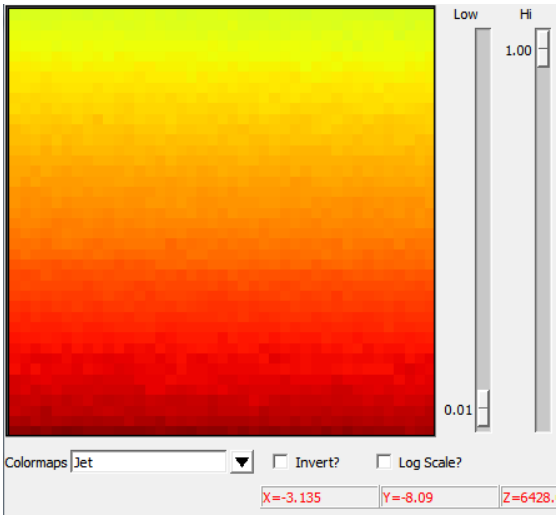


Figure A.14.9 Background scatter higher end

Figures A.14.1 – A.14.9. Fluorescence maps: the appearance of the elemental fluorescence detected is, once again, showing lack of anatomical specific landmarks, although this time some structure is discernible in **Fe k α** and **Ca k α,** and, yet much more approximate, in **Fe k β, Zn k α** and **Cu k α**.

Table A.15. Correlations (R^2) between elements: comparisons with Gallium.

	Ga k α	Slope	Intercept
Ga k α	1	1	0
Ca k α	0.55	0.36219	-6761.6759
Cu k α	0.98	0.741828	-2526.419
Fe k α	0.28	0.41387	-20105.942
Fe k β	0.91	0.29568	-3222.855
Zn k α	0.99	1.060981	-1633.829
Zn k β	0.998	1.2192	1444.299
Background low	0.98	0.07313	-225.57183
Background high	0.99	0.73662	-939.4531

Table A.15. Correlations: R^2 values are provided in the first column, whereas slope and intercept of the correlation line are provided in the 2nd and 3rd columns. Although not much structure was detected on the fluorescence maps in the infected treated rabbit, Cu k α, Fe k β, Zn k α, Zn k β and background scatter energy, both low and high, correlated too well to very feeble data, with high r^2 values (> 91%), showing a doubtful ability for the detector to record the right fluorescence signal for gallium.

Figure A.14.10. PCA: Component 1.

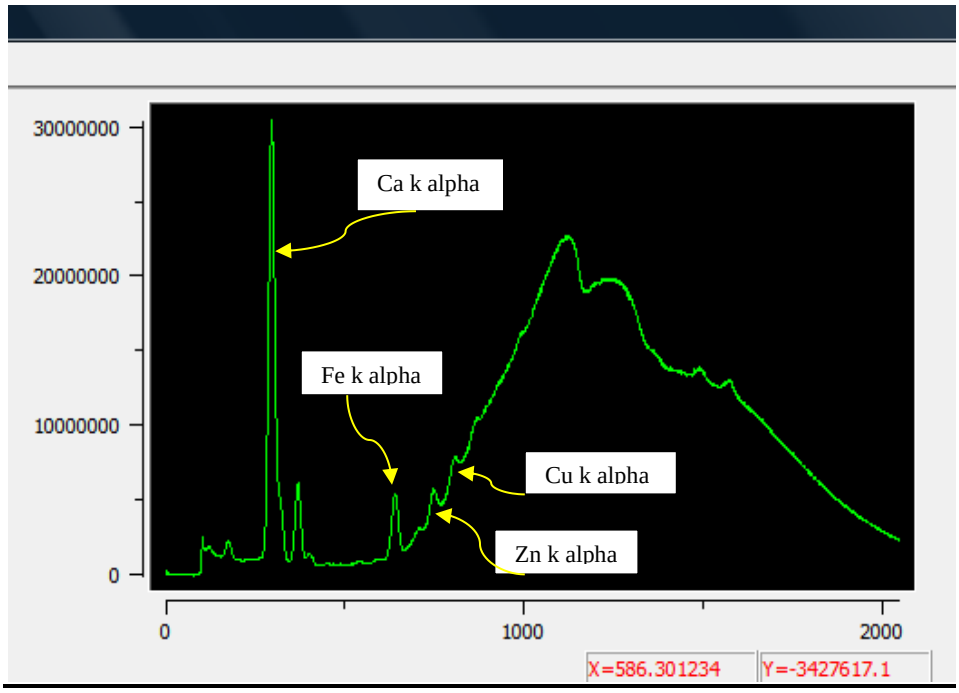


Figure A.14.11. PCA: Component 2.

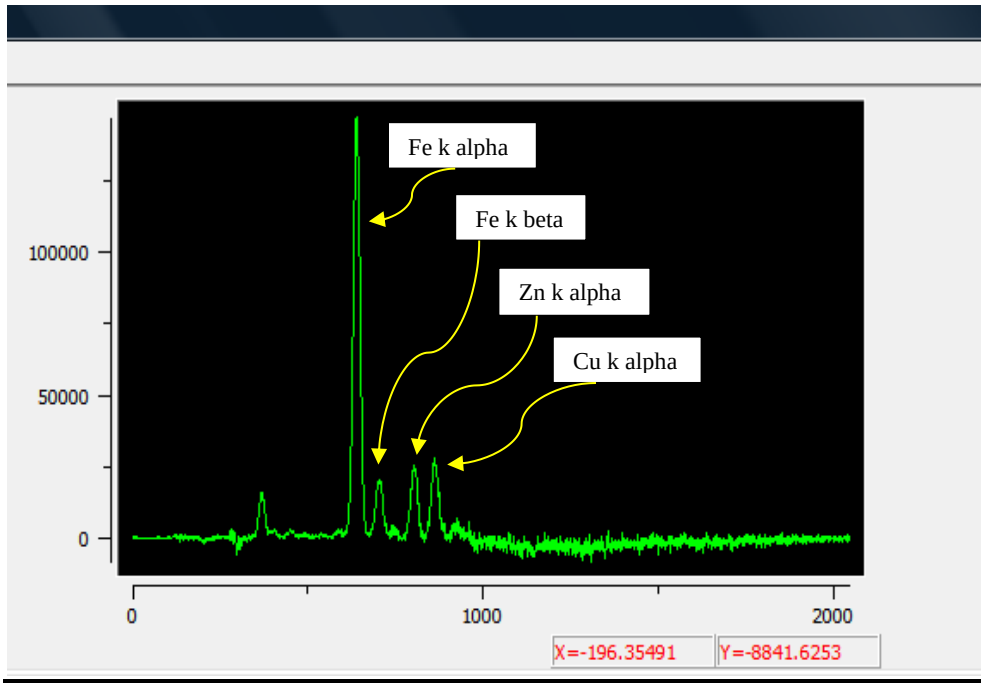
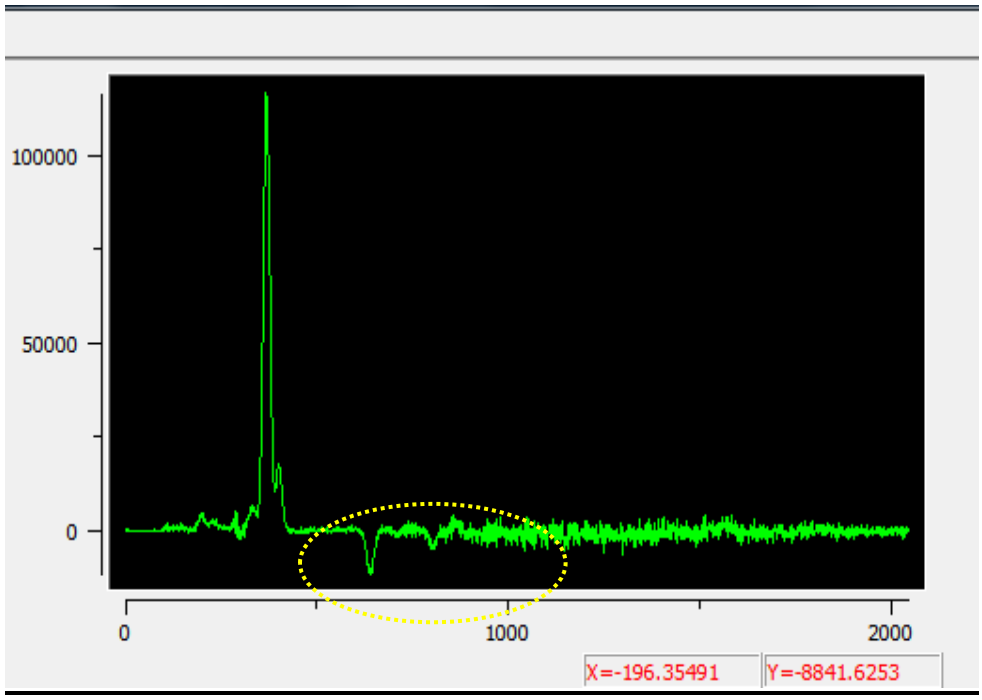


Figure A.14.12. PCA: Component 3



Figures A.14.10- A.14.12. PCA analysis: In component 1 **Fe k α, Ca k α, Zn k α** and **Cu k α,** whereas all the other elemental fluorescence peaks are not noticeable because absorbed entirely by the large background scatter signal shoulder. In component 2 **Fe k α, Fe k β, Zn k α** and **Cu k α** are visible, whereas in component 3 the same elements are overshadowed again with background scatter noise.