Detection and characterization of antimicrobial resistant bacteria from imported reptile and amphibian meat products

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Abstract

Meat from food-producing animals play an important role in the dissemination of antimicrobial resistant bacteria. Active surveillance programs target major agricultural animals but do not include niche food products. In the current investigation, we sought to (1) Identify bacterial pathogens from imported reptile and amphibian meat products and determine the presence of broad spectrum β-lactamase and colistin resistance genes, (2) determine the antimicrobial resistance profiles of *Macrococcus caseolyticus* isolated from imported meat products and (3) develop a culture medium for the selective isolation of *M. caseolyticus*. Fifty-three imported reptile and amphibian meat products were purchased from markets in Vancouver, BC and Saskatoon, SK. We found that 41.5% (22/53) of the products carried antimicrobial resistant bacteria with identifiable extended spectrum β-lactamase (ESBL), AmpC β-lactamase, carbapenemase and mobile colistin resistance genes. Seventy-one isolates from 7 genera of Enterobacteriaceae were recovered (from 41 of the 53 products), with none of the *Salmonella* isolates resistant to any of the antimicrobials tested. One multidrug resistant *E. coli*, isolated from a soft shell turtle, produced the CTX-M-55 enzyme and possessed the *mcr*-1 gene conferring mobile colistin resistance. An NDM-1 carbapenemase-producing *Acinetobacter* spp. was also isolated from a dried turtle carapace. Gram-positive bacteria resembling *Staphylococcus aureus* were isolated from imported meat samples and identified as *Macrococcus caseolyticus*; a commensal bacterium found from animal skin and food products. All isolates were resistant to the β-lactam class of drugs, including meropenem, and possessed the *mec*B gene conferring methicillin resistance. As the ecological distribution of *M. caseolyticus* in nature is largely unknown, we developed a selective culture medium to help facilitate targeted prevalence studies. Of the prepared selective media challenged, colistin nalidixic acid (CNA) blood agar with ampicillin 0.5µg/ml and meropenem 0.5µg/ml worked the best. This

medium facilitated the growth of *M. caseolyticus* while inhibiting the growth of Gram-negative and most Gram-positive bacteria except for *Enterococcus* spp. This is the first study to determine the prevalence of antimicrobial resistance and identify ESBL, AmpC β-lactamase, carbapenemase, methicillin and colistin resistance genes from imported reptile and amphibian meat products. More research is required to evaluate the magnitude of the risk that these products have to public health.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AMP	Ampicillin
AMU	Antimicrobial use
AMR	Antimicrobial resistance
BGA	Brilliant green agar
BPW	Buffered peptone water
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CLSI	Clinical and Laboratory Standards Institute
CLX	Cloxacillin
CNA	Colistin nalidixic acid
COIPARS	Columbian Integrated Program for Antimicrobial Resistance Surveillance
CPO	Carbapenemase-producing organism
DANMAP	Danish Integrated Program for Antimicrobial Resistance Surveillance
ddNTP	Dideoxynucleotide triphosphate
dNTP	Deoxynucleotide triphosphate
ESBL	Extended spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FINRES	Finnish Veterinary Antimicrobial Resistance Monitoring and Consumption of Antimicrobial Agents
IMP	Imipenemase
ITAVARM	Italian Veterinary Antimicrobial Resistance Monitoring
JVARM	Japanese Veterinary Resistance Monitoring System
KPC	*Klebsiella pneumoniae* carbapenemase
LPS	Lipopolysaccharides
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight
MARAN	Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands

MBL	Metallo-β-lactamase
MCR	Mobile colistin resistance
MDR	Multidrug resistance
MHA	Mueller-Hinton agar
MHM	Mueller-Hinton agar + 2μg/ml meropenem
MIA	Medically important antimicrobials
MIC	Minimum inhibitory concentration
MP	Meropenem
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
MSA	Mannitol salt agar
NARMS	National Antimicrobial Resistance Monitoring System
NDM	New Delhi metallo- β-lactamase
NORM-VET	Norwegian Surveillance System for Antimicrobial Drug Resistance
OIE	World Organization for Animal Health
OXA	Oxacillinase
PABA	Para-aminobenzoic acid
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
SCC	Staphylococcal chromosomal cassette
SVARM	Swedish Veterinary Antimicrobial Resistance Monitoring
TSB	Tryptic soy broth
VIM	Verona integron encoded metallo-β-lactamase
VRE	Vancomycin-resistant *Enterococcus*
XLD	Xylose lysine deoxycholate

1 Literature Review

1.1 Antimicrobial resistance

1.1.1 General introduction of antimicrobial resistance

The discovery, commercialization and administration of antibiotics have revolutionized both human and animal medicine. Since their discovery, antibiotics have become the mainstay in preventive and therapeutic treatment of disease. Antibiotics are classically defined as low molecular weight compounds produced by microorganisms which inhibit microbial growth at low concentrations (Giguère et al., 2013). The term antimicrobial encompasses a wide variety of pharmaceutical agents including antibacterial, antifungal, antiparasitic, and antiviral drugs (Leekha et al., 2011). It is defined as any natural, synthetic or semisynthetic compound with the ability to kill or inhibit the growth of microorganisms (OIE, 2011). The terms antibiotic and antimicrobial have been used interchangeably and will for the purpose of this review be used to describe an antibacterial agent.

Antimicrobials have been used to treat bacterial infections for over 70 years. The first natural antibiotic penicillin was discovered by Alexander Fleming in 1928, from a petri dish containing a *Staphylococcus* culture contaminated with a *Penicillium* mold spore and has been used therapeutically since the 1940s (Aminov 2010; Gaynes 2017). Around the same time, Gerhard Domagk introduced the first completely synthetic sulfa drug, sulfonamidochrysoidine (Prontosil), into therapeutic use (Aminov, 2010). Shortly after the introduction of both Penicillin and Prontosil, treatment failures and resistant bacteria were observed (Oldfield and Feng, 2014). Each time a new antimicrobial agent has been developed, emergence of resistance has been detected. Antibiotic resistance is defined as the ability of a microorganism to grow in the presence and resist the effects of a drug designed to kill or inhibit the microorganism (WHO, 2014; CDC, 2018). Antimicrobial

resistance is a natural process that becomes more common as a result of selective pressures applied by antibiotic use.

The emergence and dissemination of antimicrobial resistance is a global public health threat (WHO, 2014). With the decreasing development of new antimicrobial agents, the prospect of a post-antibiotic era of untreatable infections is a real concern (Alanis, 2005). Antimicrobial resistance is associated with increased mortality rates of infected patients and higher economic costs due to longer length of hospital stay, additional diagnostic testing and more extensive treatments (Cosgrove, 2006). The annual economic burden due to increased health care costs is estimated to be approximately $20 billion in the United States and €1.5 billion in Europe (WEF, 2014; WHO, 2014; Blair et al., 2015). The United States Centers for Disease Control and Prevention estimates that more than 2 million infections and approximately 23,000 deaths are caused by antimicrobial resistant organisms annually (CDC, 2018). In Europe it is estimated that 33,000 deaths are attributed each year to multidrug resistant bacterial infections (EU, 2018). A report, reviewing of the global crisis of antimicrobial resistance, commissioned by the UK Prime Minister in 2014, projected that mortality attributable to resistant bacterial infections could reach approximately 10 million by 2050, if resistance rates rose to 100% (O'Neill, 2016).

1.1.2 Mechanisms of antimicrobials

To fully understand how antimicrobial resistance develops in bacteria, it is important to recognize how antimicrobial agents work and where they exert their effects. Five mechanisms of action are briefly described: (1) interference with cell wall synthesis, (2) disruption of cell membrane structure, (3) inhibition of protein synthesis, (4) interference with nucleic acid synthesis, and (5) inhibition of metabolic pathways (Walsh, 2000; McDermott et al., 2003; Tenover, 2006; Giguère et al., 2013).

Interference with cell wall synthesis: Two classes of antibiotics, the β-lactams (e.g. penicillin and cephalosporins) and the glycopeptides (e.g. vancomycin) inhibit bacterial cell wall synthesis (Walsh, 2000; Tenover, 2006). The β-lactam drugs interfere with transpeptidase and transglycosylase enzymes, by binding to penicillin binding proteins (PBPs), required for normal peptidoglycan cross-linking. The glycopeptides sequester the D-alanine termini of the peptidoglycan strands preventing normal cross-linking. Failure to make peptidoglycan cross-links leads to a mechanically weak cell wall, which is susceptible to osmotic lysis (Walsh, 2000).

Disruption of cell membrane structure: This is the mechanism of action of the polymyxins (colistin) and daptomycin. Polymyxins bind to the lipopolysaccharides (LPS) of the outer and inner membranes of the bacteria cell, weakening the integrity of the phospholipid layers (Yu et al., 2015). This increases the membrane permeability resulting in leakage of cell contents and subsequent bacterial cell death. Daptomycin binds to the bacterial cell membrane causing membrane depolarization and cell death (Taylor and Palmer, 2016).

Inhibition of protein synthesis: Ribosomes are the functional units involved in protein synthesis and are structurally distinct in bacterial versus eukaryotic cells (Walsh, 2000). Antibiotics interfere with ribosomal function by binding to the structural subunits resulting in protein synthesis inhibition. The tetracycline and aminoglycoside antibiotics bind to the 30S ribosomal subunit, while macrolides and chloramphenicol bind to the 50S subunit (McManus, 1997; Tenover, 2006).

Interference with nucleic acid synthesis: The quinolones (nalidixic acid) and fluoroquinolones (ciprofloxacin) inhibit nucleic acid synthesis by targeting DNA gyrase and DNA topoisomerase IV, respectively. These enzymes are responsible for relaxing positive DNA supercoils, allowing for the unhindered replication of DNA by polymerase enzymes. Binding of

these enzymes inhibits DNA synthesis. Rifampicin also interferes with nucleic acid synthesis by targeting bacterial DNA-dependent RNA polymerase, thus inhibiting RNA synthesis (McManus, 1997; Walsh, 2000; Tenover, 2006).

Inhibition of metabolic pathways: Trimethoprim and sulfonamides block the folate synthesis pathway and ultimately inhibit DNA synthesis. Sulfonamides compete with para-aminobenzoic acid (PABA) to inhibit the synthesis of dihydrofolic acid by binding to the dihydropteroate synthase enzyme, while trimethoprim directly inhibits the dihydrofolate reductase enzyme preventing the formation of tetrahydrofolic acid (Tenover, 2006). Trimethoprim and sulfonamides have a synergistic effect when given together as they sequentially inhibit the successive steps in the folate synthesis pathway (McManus, 1997).

1.1.3 Mechanisms of acquiring resistance

Resistance can be categorized as intrinsic or acquired. Intrinsic resistance is defined as an innate characteristic of a bacterial species to resist the action of an antimicrobial due to inherent structural or functional properties (Blair et al., 2015). Intrinsic resistance is constitutive to all strains in a bacterial genus or species and is independent of antibiotic selective pressure and the acquisition of novel genetic material (Cox and Wright, 2013). Bacteria can acquire antimicrobial resistance through mutation of chromosomal genes or by acquisition of external genetic determinants of resistance by horizontal transfer (McManus, 1997; Thomas and Nielsen, 2005; Huddleston, 2014). Chromosomal mutation can occur through spontaneous mutation, production of hypermutator strains and adaptive mutagenesis (Watford and Warrington, 2018).

Mutation: Chromosomal mutation is a random event that occurs during DNA replication. Some mutations may confer a fitness advantage such as growth in the presence of antimicrobials,

allowing for the selection of resistance populations of bacteria, while others may be costly to the bacterium, resulting in cell death (Woodford and Ellington, 2007).

Horizontal gene transfer is the movement of genetic material between bacteria. It is the most common means of dissemination of antibiotic resistance genes among bacteria. Mobile genetic elements such as plasmids, transposons, insertion sequences and integrons, containing gene cassettes, are important facilitators of resistance genes, and may contain multiple genes conferring antimicrobial resistance and virulence factors (Thomas and Nielsen, 2005; Arber, 2014; Huddleston, 2014). Classically, the acquisition of resistance genes by horizontal gene transfer occurs by three primary mechanisms: transformation, transduction and conjugation.

Transformation: Is the process whereby bacteria uptake short fragments of DNA and incorporate it into their genome. Briefly, DNA is released into the environment from dead bacterial cells. Competent bacteria take up this free DNA from the environment and through recombination events the acquired DNA is expressed by the recipient cell (Thomas and Nielsen, 2005; Huddleston, 2014).

Transduction: This process occurs when antibiotic resistance genes are transferred by a bacteriophage (a virus which infects bacteria). During the replication process, bacteriophages inadvertently take up bacterial host genomic DNA. Once, the bacteriophage lyses a host cell it is released into the environment. These bacteriophages then infect a new bacterial host and transfer the acquired DNA. This DNA is then incorporated and expressed in the bacterial genome (Arber, 2014; Huddleston, 2014).

Conjugation: Is the transfer of genetic material between bacteria via a pilus or pore. Conjugation requires direct cell to cell contact between the donor and recipient bacterial cells,

creating a mating pair. Plasmids and transposons are the main mobile genetic elements transferred during conjugation, but integrons can also be frequently transferred (Thomas and Nielsen, 2005; Huddleston, 2014).

1.1.4 Mechanisms of antimicrobial resistance

Resistance to antimicrobial agents occur by three main mechanisms – decreased accessibility of antibiotics to its target, inactivation of antibiotics, and modification or alteration of antibiotic targets (McManus, 1997; Blair et al., 2015; Munita and Arias, 2018).

Decreased accessibility of antibiotics: Bacteria can minimize the access of antibiotics into the cell through active efflux pumps and changes to outer membrane permeability, thus conferring resistance. Expression of efflux pumps in the cell membrane reduces the intracellular antimicrobial concentration to below inhibitory levels by actively transporting antibiotics out of the cell (Wright, 2011). Efflux mediated resistance is common for the tetracycline, fluoroquinolone and sulfa drug classes (McManus, 1997). Decreased cell permeability to antibiotics is a recognized mechanism of resistance among Gram-negative bacteria, including *Pseudomonas aeruginosa* to the carbapenems and *E. coli* to the tetracyclines (Wright, 2011; Munita and Arias, 2018). This is achieved by down regulation of porin expression or replacement of non-selective porin channels to more-selective channels (Blair et al., 2015).

Inactivation of antibiotics: Antibiotics can be inactivated by bacterial enzymatic hydrolysis or modification. β-lactamases are enzymes that many Gram-positive and Gram-negative bacteria produce which can degrade β-lactam drugs (McManus, 1997; McDermott et al., 2003; Wright, 2011; Blair et al., 2015; Munita and Arias, 2018). These enzymes hydrolyze the β-lactam ring thus rendering the antibiotics inactive. Antibiotics can also be inactivated by

transferring a chemical group to their vulnerable sites by bacterial enzymes (McManus, 1997; Wright, 2011; Blair et al., 2015). This prevents the antibiotics from binding to its target protein. Several different chemical groups such as adenyl, phosphoryl, or acetyl can be transferred to the antibiotic site by specific enzyme transferases and thus modify the antibiotic (McManus, 1997; Wright, 2011; Blair et al., 2015). Some examples include, aminoglycosides, chloramphenicol, streptogramin, macrolides and rifampicin (McManus, 1997).

Modification or alteration of antibiotic targets: Target site modification can inhibit antibiotic function by preventing binding to the active site (Walsh, 2000). Mutational changes in bacterial structures prevent the antibiotic from binding while retaining cellular functions. β-lactam, aminoglycosides, tetracyclines, fluoroquinolones, and macrolides are some examples of drug classes which can be inhibited by target alteration (McManus, 1997; Blair et al., 2015; Munita and Arias, 2018). In Staphylococci, mutation in the penicillin binding proteins (PBPs) decreases the affinity of β-lactam drugs from binding and disrupting the peptidoglycan layer in the cell wall, thus conferring resistance (McManus, 1997). This is the main mechanism of methicillin resistance in Staphylococci.

1.1.5 Antimicrobial resistance in Gram-negative bacteria

Resistance in Gram-negative bacteria is currently a major issue. In Gram-negative bacteria, broad-spectrum β-lactamase resistance is increasing globally due to the extensive use of antimicrobials in both human and veterinary medicine (Paterson, 2006; Partridge, 2015). Extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae are a serious treat as these enzymes confer resistance to critically important drugs such as 3rd generation cephalosporins, which are used to treat urinary tract infections, bacteremia and invasive gastrointestinal infections caused by Enterobacteriaceae (Pitout, 2010; Rubin and Pitout, 2014; Eng et al., 2015). Multidrug

resistance (MDR) is another major concern for Gram-negative bacteria, as they tend to acquire resistance mechanisms through horizontal transfer of plasmids capable of harbouring resistance genes to multiple classes of drugs (Paterson, 2006; Partridge, 2015; Mathers, 2016; Logan and Weinstein, 2017). This limits the ability of clinicians to effectively treat infections, leading to increased patient mortality (Dicks et al., 2017). The emergence of carbapenem-resistant Enterobacteriaceae including those producing New Delhi metallo-β-lactamase (NDM) and *Klebsiella pneumoniae* carbapenemase (KPC) conferring resistance to last resort drugs, have become a serious threat (Logan and Weinstein, 2017). These resistant bacteria have disseminated around the world where international travel and trade of food is thought to have played a crucial role (Woodford et al., 2014; Hawkey, 2015; Kuenzli, 2016).

1.1.5.1 Broad spectrum β-lactamases

β-lactam antibiotics are an important class of drugs and are considered the first line treatment for many bacterial infections. They bind with PBPs of the cell membrane to inhibit cell wall synthesis. β-lactamase enzymes hydrolyze the β-lactam ring to inactivate these antibiotics (McDermott et al., 2003; Poole, 2004; Paterson, 2006; Tenover, 2006; Wright, 2011). Genes encoding β-lactamase enzymes are either chromosomally mediated or found on mobile genetic elements, including plasmids, transposons and gene cassettes (Poole, 2004). β-lactamases are a broad group of enzymes that have been classified using two schemes: Ambler molecular classification and the Bush-Jacoby-Medeiros functional classification. In the Ambler classification system, β-lactamases are grouped according to protein sequence similarity. There are four classes (A-D) in the Ambler scheme where class A, C, and D are serine β-lactamases and class B are metallo-β-lactamases (Ambler et al., 1991; Bush et al., 1995; Bush, 2013). Conversely, functional similarities of the enzyme substrate and inhibitor profiles are the basis of the Bush-Jacoby-

Medeiros classification (Bush and Jacoby, 2010). Classification of the β-lactamase enzymes are summarized in **Table 1.1**. The most concerning enzymes found in Enterobacteriaceae are the extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC β-lactamases and the carbapenemases.

Table 1.1: Classification of β-lactamase enzymes (adapted from Bush and Jacoby, 2010; Rubin and Pitout, 2014)

Ambler Classification	Buch-Jacoby-Medeiros Group	Active Site	Enzyme	Examples	Spectrum of Resistance	Inhibitors
A	2a, 2b, 2be, 2br, 2ber, 2e, 2f	Serine	Narrow spectrum β-lactamases	TEM-1, 2 and 13, SHV-1	Penicillins, cephalosporins	Clavulanic acid, tazobactam, sulbactam (except 2br)
			Extended spectrum β-lactamases (ESBLs)	TEM (except TEM-1, 2 and 13), SHV (except SHV-1), CTX-M	Penicillins, cephalosporins, monobactams	
			Carbapenemases (KPC type)	KPC, SME, IMI	Penicillins, cephalosporins, monobactams, carbapenems, cephamycins	
B	3a	Zinc-binding thiol group	Carbapenemases (Metallo-β-lactamases)	NDM, VIM, IMP	All β-lactams	EDTA and other metal chelators
C	1	Serine	AmpC (Cephalosporinases)	CMY-2, FOX, ACT, MOX, ACC, DHA	Penicillins, cephalosporins, cephamycins, monobactams	Cloxacillin, oxacillin, boronic acid
D	2df	Serine	Oxacillinase	OXA-48, OXA-181	Penicillins, carbapenems	NaCl

Group 1: Cephalosporinases- higher activity towards cephalosporins than benzylpenicillins, active against cephamycins (cefoxitin), high affinity for aztreonam
Group 2b: Narrow spectrum β-lactamases – hydrolyze penicillins and early cephalosporins (cephaloridine and cephalothin)
Group 2be: Extended spectrum β-lactamases – broad spectrum of activity against penicillins and cephalosporins of subgroup 2b, plus able to hydrolyze one or more oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime and aztreonam)
Group 2df: Oxacillinases – carbapenem hydrolyzing activities, able to hydrolyze cloxacillin or oxacillin
Group 2f: Serine carbapenemases – Able to hydrolyze the carbapenems and the oxyimino-β-lactams
Group 3a: Metallo- β-lactamases – Able to hydrolyze all the β-lactams (NDM) including penicillins, carbapenems and oxyimino-β-lactam

Extended-spectrum β-lactamases (ESBLs): ESBLs are class A enzymes according to Ambler classification. SHV (except SHV-1), TEM (except TEM-1, 2 and 13) and the CTX-Ms are examples of some of the most common ESBLs. The parent enzymes SHV-1 and TEM-1, 2 and 13 have narrow spectrum of activity and are only able to hydrolyze penicillin and first generation cephalosporins. Mutations in these enzymes have broadened their spectrum of activity leading to the designation of SHV and TEM ESBLs (D'Andrea et al., 2013; Rubin and Pitout, 2014). ESBLs are capable of degrading a broad-spectrum of β-lactam antibiotics including, penicillins, cephalosporins, and monobactams but not cephamycins or carbapenems. These enzymes can be inhibited by β-lactamase inhibitors such as clavulanic acid, tazobactam or sulbactam (Lee et al., 2012). The Lahey database is a curated list of clinically important β-lactamase families; there are over 190 SHV and 220 TEM variants described (Bush et al., 2017). The CTX-M enzymes became a public health concern when clinically relevant Enterobacteriaceae acquired them through horizontal gene transfer from *Kluyvera* spp. (Humeniuk et al., 2002; Poirel et al., 2002; Pitout and Laupland, 2008). The name CTX-M describes the preferential hydrolytic activity of this enzyme against cefotaxime than ceftazidime, and the location of München where it was first described (Rubin and Pitout, 2014). There are over 170 CTX-M variants described (Bush et al., 2017). The CTX-M enzymes are classified into five phylogenetic subgroups based on their amino acid sequences and include, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Bonnet, 2004; D'Andrea et al., 2013).

AmpC β-lactamases: This group of enzymes are class C based on Ambler classification. AmpC enzymes are structurally similar to ESBLs but have increased affinity for binding to cephalosporins. These enzymes confer resistance to penicillins, cephalosporins, monobactams and

cephamycins, while cloxacillin, oxacillin and boronic acid are good inhibitors of AmpC enzymes (Jacoby, 2009). Chromosomally mediated AmpC β-lactamases were first detected from *E. coli*, while the plasmid mediated gene was reported from *Enterobacter cloacae* in the 1980s (Philippon et al., 2002). The most common plasmid-mediated AmpC β-lactamase is CMY, which was initially derived from chromosomally encoded AmpC cephalosporinases from *Citrobacter freundii* (Bauernfeind et al., 1996; Wu et al., 1999). Over 130 CMY variants have been identified to date (Bush et al., 2017).

Carbapenemases: Carbapenemases are a group of enzymes capable of hydrolyzing all classes of β-lactam drugs, including the carbapenems. Carbapenems have the broadest spectrum of antibacterial activity of the β-lactam antibiotics and are considered the last resort drugs for treating multidrug resistant Gram-negative infections (Papp-Wallace et al. 2011). Carbapenemases are grouped into two major molecular families: the metallo-β-lactamases and the serine-β-lactamases (Queenan and Bush, 2007). Metallo-β-lactamases (MBLs) differ structurally from the serine-β-lactamases by their requirement for zinc at the active site which facilitates the hydrolysis of β-lactam drugs. MBLs can be inhibited by metal ion chelators, such as EDTA but are uninhibited by clavulanic acid, tazobactam or sulbactam (Queenan and Bush, 2007). MBLs are classified as Ambler class B and functional group 3 (Queenan and Bush, 2007; Bush and Jacoby, 2010). The most common types of MBLs are the Verona integron encoded metallo-β-lactamase (VIM), imipenemase (IMP) and NDM (Nordmann et al., 2011). In fact, NDM is the most common carbapenemase worldwide; it has been identified in more than fifty countries (Logan and Weinstein, 2017). Unlike MBLs, serine β-lactamases contain a serine at their active site and metal ion chelators like EDTA do not inhibit them (Bush, 2013). Serine β-lactamases belong to Ambler class A and D. Some examples include the chromosomally-mediated SME and the plasmid-

mediated KPC. At present, plasmid-mediated KPC is the most common class A carbapenemase found worldwide; it is endemic in an increasing number of countries and are responsible for many major outbreaks (Logan and Weinstein, 2017). Oxacillinases (OXA) with carbapenemase activity are class D enzymes. They have been reported throughout the world, with OXA-48 being the most commonly identified variant (Logan and Weinstein, 2017). The OXA enzymes are a diverse family of β-lactamases with the ability to hydrolyze penicillins at high levels, but the hydrolysis of carbapenems occur only at low levels (Poirel et al., 2012).

1.1.5.2 Colistin resistance

Colistin is one of the last drugs used to combat carbapenem-resistant infections. It is a cationic polypeptide with broad spectrum activity against Gram-negative bacteria (Liu et al., 2016). Colistin was discovered in 1949 and until recently it was considered too toxic for routine use, due to the high incidence of reported nephrotoxicity (Falagas and Kasiakou, 2005; Nation and Li, 2009). Unfortunately, as with all antimicrobials, increased use has resulted in resistance. Resistance is mediated through modification of lipid A by phosphoethannolamine transferase, resulting in decreased binding affinity to the outer membrane and prevention of cell lysis (Nordmann and Poirel, 2016). Resistance to colistin is not a new phenomenon, as chromosomal mutations have been previously described (Falagas et al., 2010). What is problematic is the emergence of the plasmid-mediated colistin resistance gene, *mcr*-1, capable of facilitating horizontal gene transfer and the dissemination of resistance (Liu et al., 2016). The plasmid-mediated nature of these enzymes often results in the acquisition of other resistance genes, leading to multidrug resistance. Recently, a KPC-28 carbapenemase-producing *E. coli* isolate from a hospitalized patient in France exhibited colistin resistance and was found to have acquired a *mcr*-

1 and *bla*$_{\text{OXA-48}}$-bearing plasmid following a three week treatment of colistin (Beyrouthy et al., 2017).

1.1.5.3 Integrons

In Gram-negative bacteria, integrons play an important role in the dissemination of resistance genes between bacteria. Integrons are composed of three distinct structures: an integrase gene (*int*I), a gene cassette receptor site (*att*I) and a promoter (Pc), which allows for the site specific insertion of a gene cassette and the expression of the associated gene (Hall and Collis, 1995). Integrons are not mobile themselves, but the gene cassette within them can be independently mobilized. Gene cassettes are small genetic elements which can be incorporated into an integron or exist as a free, circular piece of DNA (Fluit and Schmitz, 2004). Integrons are categorized into three distinct classes: class 1, class 2 and class 3. Class 1 integrons are most frequently identified from clinical isolates (Bennett, 1999; Fluit and Schmitz, 2004). Integrons are capable of carrying multiple gene cassettes, resulting in multidrug resistance (Rowe-Magnus and Mazel, 2002). The most commonly encoded genes found contained within class 1 integron gene cassettes are streptomycin-spectinomycin, trimethoprim and β-lactam resistance (Deng et al., 2015). These integrons are commonly located on plasmids which can be transferred to other organisms via horizontal gene transfer (Rowe-Magnus and Mazel, 2002; Fluit and Schmitz, 2004).

1.1.6 Antimicrobial resistance in Gram-positive bacteria

Gram-positive bacteria are among the most common bacterial causes of clinical infections (Eades et al., 2017). Although recent global attention has been focused on multidrug resistant Gram-negative organisms, antimicrobial resistant Gram-positive bacteria are also a serious public health concern. Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant

Enterococcus faecium (VRE) are leading causes of healthcare-associated and community-acquired infections (Munita et al., 2015; Eades et al., 2017). In the United States, MRSA and VRE cause approximately 100,000 healthcare-associated infections resulting in more than 12,000 deaths per year (CDC, 2013). Furthermore, MRSA and VRE have the potential for zoonotic transfer between humans and companion animals (Pomba et al., 2016). *Staphylococcus aureus* and *Enterococcus faecium* are commensal bacteria living on the skin and in the intestinal tracts of both humans and animals. The extensive use of antimicrobials in both human and veterinary medicine can select for resistance, while the close contact with companion animals can facilitate transfer (Guardabassi, 2004).

1.1.6.1 Methicillin resistance

Gram-positive bacteria are covered by a thick layer of peptidoglycan, consisting of short glycan chains of N-acetylmuramic acid and β-1-4-N-acetylglucoseamine residues. Pentaglycine cross-bridges are cross-linked in the cytoplasmic membrane through transpeptidation which is catalyzed by the PBP (Stapleton and Taylor, 2002). The β-lactam class of drugs inhibit the transpeptidase activity by acting as an analogue for the terminal D-alanyl bonds of the stem peptides. In methicillin resistant staphylococci, β-lactams have a weak affinity towards PBP2a, an altered form of PBP2. This is due to the structural change to the serine active site resulting in decreased accessibility of β-lactams to its binding site (Stapleton and Taylor, 2002).

In Staphylococcaceae, the *mec* family of genes encoding for the altered penicillin-binding proteins confer methicillin resistance (Peacock and Paterson, 2015). These genes are found in association with the staphylococcal chromosomal cassette (SCC) complex, which is a mobile genetic element responsible for horizontal gene transfer that facilitates integration of the *mec* gene into the chromosome (Stapleton and Taylor, 2002; Peacock and Paterson, 2015; Lee et al., 2018).

There have been four *mec* genes characterized to date and include: *mec*A, *mec*B, *mec*C and *mec*D (Schwendener et al., 2017).

***mec*A**: The *mec*A gene was first discovered in 1986 and is the most common gene expressed in MRSA isolates (Matsuhashi et al., 1986). It is the classical gene conferring methicillin resistance, as it encodes PBP2a which has extremely low affinity for almost all β-lactam drugs, including the penicillins, cephalosporins, monobactams, cephamycins and carbapenems. Thus, in the presence of a β-lactam, the transpeptidase function of peptidoglycan synthesis is retained (Becker et al., 2014).

***mec*B:** The *mec*B gene was first identified in 2009 from *Macrococcus caseolyticus* isolated from a chicken skin swab in Japan (Baba et al., 2009). Then later identified from *Macrococcus canis* isolated from canine skin samples in Switzerland (Cotting et al., 2017). The *mec*B gene can be associated with the SCC*mec* element within the chromosome or found on plasmids (Baba et al., 2009; Tsubakishita et al., 2010; Gómez-Sanz et al., 2015). In February 2016, a *S. aureus* isolate containing a plasmid encoding *mec*B gene was identified from a hospitalized patient during routine MRSA screening (Becker et al., 2018). This is the first description of a *mec*B-mediated methicillin resistance in *Staphylococcus aureus*.

***mec*C:** In 2011, *mec*C was first described from *mec*A-negative bovine and human MRSA isolates in Europe (García-Álvarez et al., 2011). This gene plays a very similar role to *mec*A but the encoded PBP2a has a higher affinity for oxacillin than cefoxitin giving different susceptibility profiles compared with *mec*A encoded MRSA isolates (Lee et al., 2018). This has led to some recommendations from CLSI to use cefoxitin over oxacillin for susceptibility testing (CLSI, 2018).

***mec*D:** Recently, *mec*D was identified from methicillin-resistant *mec*B-negative *M. caseolyticus* from bovine and canine sources in Switzerland (Schwendener et al., 2017). This gene conferred high levels of resistance to all classes of β-lactams including the anti-MRSA cephalosporins and was associated with a novel genomic island, classified as *M. caseolyticus* resistance island, which has characteristics resembling that of SCC*mec* elements (Schwendener et al., 2017). The risk of this novel methicillin resistance determinant in human patients has yet to be determined.

1.2 Detection of antimicrobial resistance

1.2.1 Phenotypic identification of antimicrobial resistance

Phenotypic testing is used routinely in diagnostic laboratories (Markey et al., 2013; Procop, 2017). The principle of phenotypic testing is to compare observable or measurable traits to a known set of criteria. The most common phenotypic techniques used to identify antimicrobial resistant bacterial strains include the use of culture media and antimicrobial susceptibility testing (Jorgensen and Ferraro, 2009; McLain et al., 2016). Underlying resistance mechanisms can often be inferred from antimicrobial susceptibility test results using 'expert rules,' which are based on current clinical microbiological evidence of resistance phenotypes and mechanisms (Leclercq et al., 2013). However, molecular testing is still required to confirm the presence of these mechanisms, as interpretative reading alone cannot determine if multiple resistance mechanisms or if a new mechanism is contributing to the resistance patterns identified (Livermore et al., 2001; Leclercq et al., 2013; McLain et al., 2016).

1.2.1.1 Bacteriological culture media

Bacteriological culture media are designed to support the growth of bacteria. They contain all the elements that most bacteria require for growth and maintenance (Riley, 2013). Culture media can be divided into 5 different categories, including: chemically defined media, basic nutritive media, enrichment broths, differential/indicator media and selective media (Washington, 1996; Markey et al., 2013; Riley, 2013). Broth medium is used for the cultivation of large quantities of organisms, while solid medium is used for isolating bacteria to observe colony characteristics of individual bacterial species (Riley 2013). In diagnostic laboratories one of the most useful media types is selective media.

Selective media: Any agar medium designed for the selective growth of a bacterium or group of bacteria, is classified as a selective medium (Markey et al., 2013). It contains inhibitory substances to prevent growth of unwanted bacterial species while not affecting the bacteria of interest. Many selective media can also be differential (e.g. MacConkey and Mannitol salt agar) when they incorporate dyes or metallic substrates that bacteria utilize, allowing for different bacteria to be recognized based on colony colour (Washington, 1996). Antibiotic-based selective media have been used for the isolation of a variety of resistant bacteria (McLain et al., 2016). This is based on the knowledge that certain bacteria possessing intrinsic or acquired resistance mechanisms to specific antibiotics will grow in the presence of higher concentrations of that antimicrobial agent. Antibiotic selective media are routinely used as a screening tool for specific antimicrobial resistant pathogens (Gazin et al., 2012). Two examples of antibiotic-based selective media are CHROMagar ESBL and Mannitol Salt Agar (MSA) with oxacillin (**Figure 1.1**).

CHROMagar ESBL: CHROMagar ESBL is a chromogenic medium that is both differential and selective for Gram-negative bacteria harbouring ESBLs (CHROMagar, 2016). It

incorporates a chromogenic substrate that once hydrolyzed by a bacterial enzyme causes accumulation of a coloured dye in the bacterial colony, resulting in differentiation of the targeted pathogen (Gazin et al., 2012). The incorporation of a proprietary selective mix allows for the direct detection of presumptive ESBL-producing organisms. One study comparing five media for detecting ESBL-producing Enterobacteriaceae observed that CHROMagar ESBL had a high sensitivity (98.3%) as it was able to detect ESBL-producing strains very consistently, however, this media had a moderate specificity (72.3%) indicating it would frequently detect non-ESBL strains such as those expressing AmpC β-lactamases (Grohs et al., 2013).

Mannitol salt agar with oxacillin: Mannitol salt agar is both a selective and differential growth medium for the detection of *Staphylococcus aureus*. The use of a high salt concentration (7.5 - 10%) inhibits most bacteria, while the incorporation of mannitol and phenol red is used to detect acid production from the fermentation of mannitol (Markey et al., 2013). The addition of oxacillin to MSA allows for the selection of methicillin resistant strains of *S. aureus*. Studies have observed that including oxacillin results in high rates of sensitivity and specificity for the detection of *mec*A-positive MRSA (Kumurya, 2017; Lally et al., 1985; Mathanraj et al., 2009). However, one study demonstrated that the inclusion of cefoxitin in MSA had even greater levels of sensitivity (100%) and specificity (100%) in the detection of *mec*C-positive MRSA strains then with oxacillin, 92% and 96% respectively (Kumurya, 2017). This is due to the higher relative affinity of *mec*C-encoded PBP2a for oxacillin than for cefoxitin, leading to higher levels of resistance to cefoxitin over oxacillin (Kim et al., 2012).

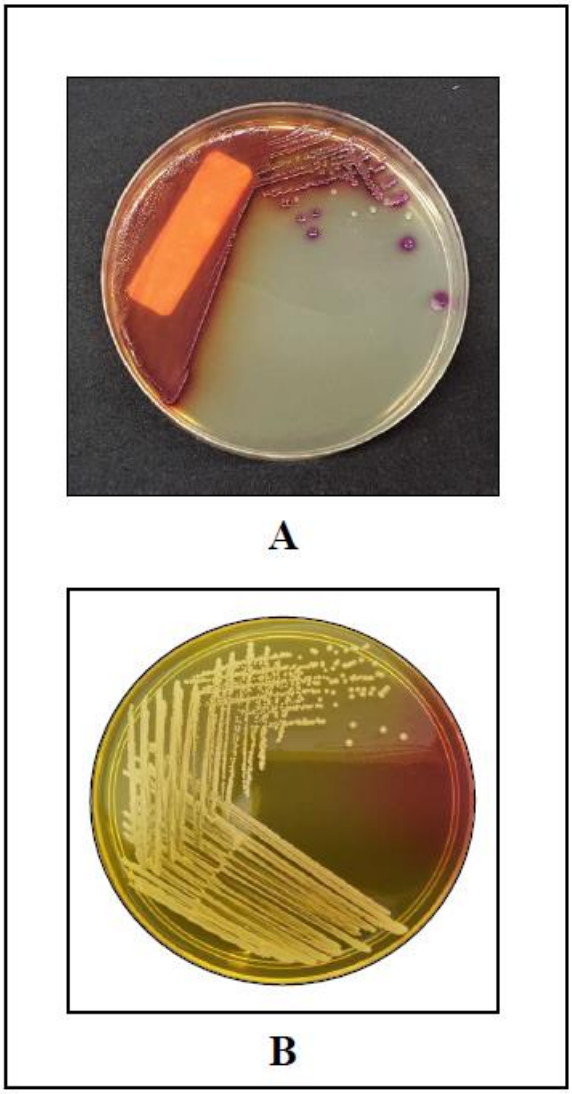

Figure 1.1: Antibiotic-based selective media: (A) CHROMagar ESBL demonstrating the pink colony colour of *E. coli*, and (B) Mannitol Salt Agar with oxacillin showing the yellow pigmented colonies of *S. aureus*.

1.2.1.2 Antimicrobial susceptibility testing

The principle of antimicrobial susceptibility testing is to determine the effectiveness of an antibiotic at inhibiting the growth of a bacterial isolate in a laboratory setting, also known as *in vitro* testing. Antimicrobial susceptibility testing follows a highly standardized protocol to ensure reproducible and reliable results (Balouiri et al., 2016). There are two main international standards committees recognized: The Clinical and Laboratory Standards Institute (CLSI), and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). These committees provide updated protocols for testing, and guideline criteria for interpretation of results. The standardization of antimicrobial susceptibility testing is important for generating accurate results and involves every step of the protocol, including, growth media, antibiotic concentration, inoculum concentration, incubation time and temperature, and the use of control bacterial strains. Failure to adhere to the recommended standards can render the test results meaningless as interpretative criteria for each susceptibility method are based on strict adherence to the standardized protocols (Kiska 1998; Balouiri et al. 2016). There have been numerous methods developed and they are broadly categorized into two main groups: diffusion and dilution based techniques (**Figure 1.2**). Depending on the testing method utilized either a qualitative or quantitative result will be determined.

Diffusion based techniques

Diffusion based techniques include the disk diffusion and gradient strip tests. Both tests use the same standardized protocol. To perform these assays, a fresh bacterial suspension in saline or deionized water is prepared. The inoculum is prepared to a density of a McFarland 0.5 standard, which corresponds to approximately 1.5 x 10^8 CFU/ml. The inoculum is streaked out onto the surface of a 4 mm thick Mueller-Hinton agar (MHA) plate, creating an even lawn of bacteria. After

the plate is inoculated, antibiotic disks or antibiotic strips are placed on the plate and incubated at 35°C for 16-18 hours. Results are interpreted using the CLSI or EUCAST criteria (CLSI, 2018; EUCAST, 2018).

Disk diffusion test: This method was first described by Bauer and colleagues in 1966 and is known as Kirby-Bauer antibiotic testing (Bauer et al., 1966). Antibiotic impregnated discs are placed on agar plates inoculated with the test bacterium. The antimicrobial agent diffuses into the agar radially creating a concentration gradient with high drug concentration immediately surrounding the disk and decreasing outwards (Matuschek et al., 2014). Bacteria grow only up to the maximum concentration they can tolerate resulting in a zone of inhibition where no bacterial growth occurs depending on the susceptibility of the bacterium to an antimicrobial agent (**Figure 1.2A**). The diameter of the inhibitory zone is measured to the nearest millimetre using a ruler or calipers and is interpreted according to CLSI or EUCAST criteria (CLSI, 2018; EUCAST, 2018). The results yielded are qualitative and describe bacterial isolates categorically as susceptible, intermediate or resistant to the antimicrobial agent tested. This technique is the simplest to perform and is the most cost-effective means for screening both human and animal bacterial pathogens in diagnostic laboratories (Jorgensen and Ferraro, 2009).

Gradient strip test: Gradient strips (e.g. Epsilometer or E-test) are commercially available thin inert non-porous plastic reagent strips impregnated with a predefined gradient of an antimicrobial agent (Olsson-Liljequist, 1992; Jorgensen and Ferraro, 2009). The surface of the strip displays a scale delignating the antibiotic concentrations. During the incubation period, an antimicrobial concentration gradient is established with the highest concentration at the top of the strip and decreasing to the lowest concentration at the bottom. Following incubation, the test is read by viewing where bacterial growth occurs at the intersection of the strip and the tear drop

shaped zone of inhibition (**Figure 1.2B**). The results yielded are quantitative, which allows for the determination of the antimicrobial minimum inhibitory concentration (MIC). The MIC is defined as the minimum concentration which completely inhibits the growth of bacteria (Coyle, 2005; Balouiri et al., 2016). This test is a hybrid diffusion-dilution assay. It is simple to perform but errors may occur in reading this test if plates are inoculated poorly or if the gradient strip moves when placed (Brown and Brown, 1991).

Dilution based techniques

Dilution based techniques can be performed using either agar plates or broth media. Both methods consist of making a standard bacterial density prepared from a McFarland 0.5 suspension inoculated into a doubling series of antibiotic concentrations. Results are reported quantitatively as the antimicrobial MIC (CLSI, 2015).

Agar dilution: Mueller-Hinton agar plates are prepared with serial dilutions of antimicrobials and a standard bacterial inoculum of 1.0×10^4 CFU is spotted onto the agar surface (CLSI, 2015). The plate containing the lowest antimicrobial concentration where no growth is observed is the MIC (**Figure 1.2C)**. Although this method is technically simple, it is laborious to perform, thus limiting its routine application in most diagnostic settings.

Broth dilution: Broth dilution consists of a series of antimicrobial concentrations prepared in Mueller-Hinton broth. This technique can be carried out in large tubes (macro broth dilution) or in microtiter plates (micro broth dilution). Broth microdilution is a widely used test in both human and animal diagnostic laboratories. A bacterial inoculum prepared to a density of McFarland 0.5 standard is added to broth media creating a final inoculum of 5.0×10^5 CFU/ml which can be inoculated into commercially prepared 96 well microtiter plates (**Figure 1.2D**). These plates contain numerous serially diluted antimicrobial agents as well as positive and negative controls

(Jorgensen and Ferraro, 2009; CLSI, 2015). The MICs are determined, using a manual (mirror box) or automated viewing device, by observing either a lack of turbidity or lack of a cell pellet in each well.

Automated instrument systems: Fully automated systems, such as the Vitek system, are utilized in large diagnostic laboratories, where bacterial growth curves are used to calculate MICs (Jorgensen and Ferraro, 2009). In brief, bacterial isolates are grown on sheep blood agar and incubated at 37°C for 18-24hrs. A bacterial suspension is prepared to a density of a McFarland 0.5 standard and diluted to a final inoculum concentration of 1.5×10^7 CFU/ml in 2.5 ml of 0.45% sodium chloride. Compact plastic reagent cards that contain microlitre quantities of antibiotics and broth media (45 or 64-well format) are filled, sealed and loaded into the instruments for incubation and reading. MICs are determined by repetitive monitoring of the turbidity (bacterial growth) which is compared to a control well (Ligozzi et al., 2002). A computer software system interprets the results based on established resistant breakpoints by CLSI and reports them as antimicrobial MICs and categorically as susceptible or resistant (Ligozzi et al., 2002; Jorgensen and Ferraro, 2009). The use of automated systems allows for faster test results (4-10 hours), allowing for more timely implementation or changes in antimicrobial therapy (Jorgensen and Ferraro, 2009).

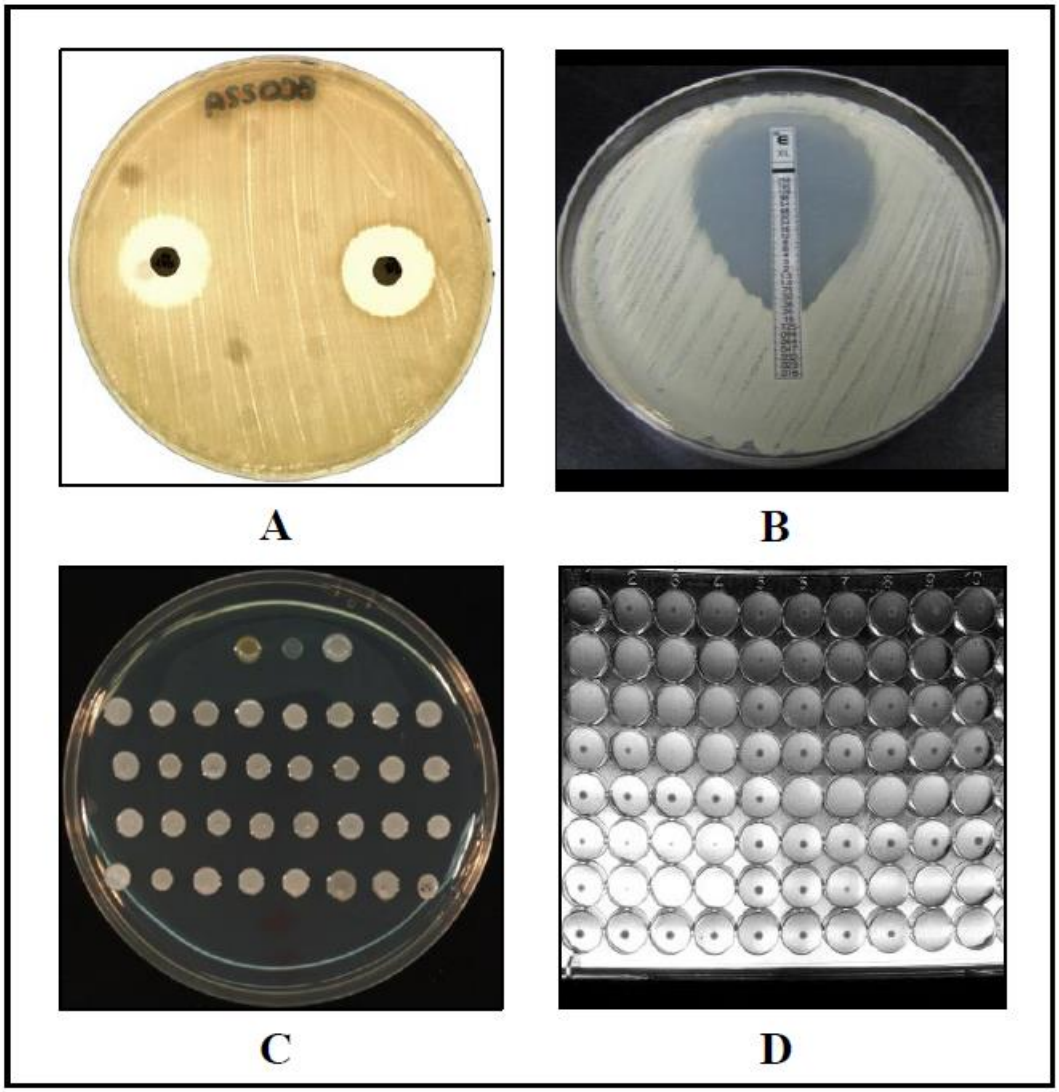

Figure 1.2: Antimicrobial susceptibility testing techniques: (A) Disk diffusion test showing a large zone of inhibition around the antibiotic disks. Susceptible/resistant is determined by measuring the zone of inhibition to the nearest millimetre. (B) Gradient strip test demonstrating the tear drop zone of inhibition. The MIC is determined by measuring the intersection of the zone of inhibition and the strip. (C) Agar dilution with inoculum spotted onto a MHA + antibiotic plate. The MIC is the first plate were no growth is observed. (D) Broth microdilution using a commercially available 96 well plate with cell pellets visible in some of the wells, indicating bacterial growth. The MIC is the first concentration in the serial dilution where no turbidity or cell pellet is observed.

1.2.2 Molecular identification of antimicrobial resistance

Polymerase chain reaction: Polymerase chain reaction (PCR) was first described by Kary Mullis in 1983 (Mullis et al., 1986). It is a technique that exploits the basic biochemistry of DNA replication, allowing for the exponential amplification of specific segments of DNA. The portion of DNA amplified contains diagnostically useful information such as bacterial resistance genes (Mullis and Faloona, 1987). This method produces millions of copies of a target sequence within a short timeframe by using a thermostable DNA polymerase enzyme, known as *Taq* polymerase, to mimic the natural process of DNA replication. It uses existing single-stranded DNA as a template and attaches deoxynucleotide triphosphates (dNTPs) into the replicating strand to make a complimentary copy (Bartlett and Stirling, 2003). Conventional PCR consists of a cycle of three distinct temperature phases repeated 30 to 40 times: denaturation, annealing and extension (Viljoen et al., 2005). This technique can be utilized to identify specific bacteria species and strains, single or multiple resistance genes, integrons, gene cassettes and plasmids.

DNA sequencing: Post-amplification analysis of PCR amplicons by DNA sequencing is commonly used to identify single nucleotide polymorphisms and for identifying specific alleles for strain typing. Amplicon sequencing is based on the dideoxy method also known as sequencing by termination or Sanger sequencing. Traditionally, this method substitutes dideoxynucleotide triphosphates (ddNTP) for deoxynucleotides into a growing strand of DNA during polymerization resulting in the termination of DNA synthesis in a base-specific manner. Four separate sequencing reactions are performed where one radiolabelled-ddNTP analog in addition to the four deoxynucleotides (dATP, dGTP, dCTP and dTTP) are included to terminate at A, G, C and T residues. Each reaction creates specific fragments which could be resolved by gel electrophoresis (Sanger et al., 1977). Improvements to the original Sanger sequencing method increased the speed

and efficiency of DNA sequencing by replacing radiolabelling with fluorometric based detection (allowing for one instead of four reactions) and the use of capillary based electrophoresis (Heather and Chain, 2016).

1.3 Monitoring of antimicrobial use and resistance in animals

1.3.1 Antimicrobial use and resistance in food-producing animals

Antimicrobial agents are widely used in animals. Veterinarians prescribe antibiotics for use in companion animal medicine and in production animal systems including livestock and aquaculture. In animals, antimicrobials are used in four different scenarios, including: therapy (treatment of sick animals due to bacterial infection), prophylaxis (use of antibiotics to prevent bacterial infection in clinically healthy but at risk animals), metaphylaxis (treatment of a group of sick and healthy animals to minimize the spread of infection) and growth promotion (use of antibiotics to increase the muscle tonnage of animals destined for meat production) (Page and Gautier, 2012; Giguère et al., 2013). Generally, antimicrobials are administered to targeted individual animals in the therapeutic and prophylactic treatment of illness, while the administration of antibiotics to the entire group or herd occurs with metaphylactic and growth promotion scenarios (Spicer, 2010; Page and Gautier, 2012).

One major concern is centered around the controversial application of antimicrobials used as growth promoters in animals. There are numerous beneficial effects of using medicated feeds, including, significant increases in production, reduction in infectious diseases and the increased overall well-being of animals (Hao et al., 2014). It is hypothesized that the antibiotics interact with the physical environment of the intestinal tract and associated microflora, leading to positive

effects on growth and health of animals (Lekshmi et al., 2017). However, the administration of low or sub-therapeutic concentrations of antibiotics apply continuous selective pressures on bacteria and has been attributed to the development of antimicrobial resistance in food animals (McDermott et al., 2002). This has led to the ban of antimicrobial growth promoters in many parts of the world (notably Europe), resulting in the increased use of copper and zinc as in-feed alternatives and for pathogen control in swine and poultry production (Wales and Davies, 2015). In Canada, growth promotion claims and related directions for use were removed from medically important antibiotics (MIAs) used in human medicine (classified by the Veterinary Drugs Directorate as category I, II and III antimicrobials), while responsible use statements were included on labels for all in-feed and in-water MIAs (PHAC, 2017). Food animal producers are now required to obtain a prescription for MIAs and their use is for treatment of disease only (PHAC, 2017).

Another major concern is the use of antibiotics, biocides (disinfectants) and heavy metals in agriculture and aquaculture and the development of cross-resistance or the co-selection of resistance to unrelated antimicrobials or antibacterial agents (Seiler and Berendonk, 2012; Wales and Davies, 2015; Romero et al., 2017). This is an indirect selection process due to coupling of resistance mechanisms, which may be mediated by the presence of separate resistance genes, against antibiotics and biocides/heavy metals in the same organism (Chapman, 2003). Multidrug efflux pumps mediating decreased susceptibility towards antibiotics and biocides/heavy metals by their rapid removal out of the bacterial cell is an example of cross-resistance; the genetic linkage of two or more resistance genes located next to each other on a mobile genetic element is an example of co-resistance (Wales and Davies, 2015). In agricultural and aquatic production systems, heavy metals are routinely used as feed additives, organic and inorganic fertilizers,

pesticides and as anti-fouling agents, while quaternary ammonium compounds are used as biocides on farms and in food-processing plants due to its bactericidal activity in the presence of organic matter (Seiler and Berendonk, 2012; Romero et al., 2017). Bacterial communities in these production systems are strongly exposed to the environmental contamination of heavy metals and antibiotics, which increase the likelihood of selection or co-selection of resistance (Seiler and Berendonk, 2012).

1.3.1.1 Livestock

In livestock production, the most commonly used antimicrobial drugs come from six major classes, including, the β-lactams, tetracyclines, aminoglycosides, macrolides, fluoroquinolones and the sulphonamides (McDermott et al., 2002). Antimicrobial use is important in animal production industries, where they are directly used for growth promotion and the treatment and prevention of disease. In the United States, approximately 70% of the nation's annual antimicrobial consumption are used in livestock production, and of the 41 antibiotics approved for use in animals by the United States Food and Drug Administration (FDA), 31 are classified as important, highly important or critically important for human medicine (FDA, 2016; Lekshmi et al., 2017). In Canada, approximately 78% of all antimicrobials distributed/sold were used in production animals, with tetracyclines and β-lactams being the most predominant drug classes (PHAC, 2018). The global trend of antimicrobials used in livestock production is projected to rise to approximately 106,000 tonnes by 2030, up from 63,000 tonnes in 2010, with China, the United States, Brazil, India and Mexico having the largest amounts of antimicrobials consumed (Van Boeckel et al., 2015).

Animal origin food products are possible sources of antimicrobial resistant organisms for transmission into people. A number of published reports have focused on different aspects within

livestock production systems including commercial farms, feedlots, processing plants or packing plants as sources of antimicrobial resistant bacteria (Aslam et al., 2009; Jouini et al., 2009; Sanchez-Maldonado et al., 2017). In each report, meat samples were tested for the presence of antimicrobial resistance in either *E. coli* or *Salmonella* isolates; multi-drug resistant phenotypes and high numbers of resistance genes were identified. One problem with intensive livestock production is the close contact between animals, which may enhance disease transmission and result in the increased need for antimicrobials (Marshall and Levy, 2011). To combat this problem many farms have implemented increased biosecurity measures such as additional vaccinations and improved hygiene protocols. As of yet, there is no evidence that these measures have had any direct impact on decreasing antimicrobial resistance; decreased levels of resistance have been observed in countries that have completely banned the use of specific antibiotics on farms as growth promoters (Hao et al., 2014; Lekshmi et al., 2017). However, the increased use of biocides and heavy metals, in an effort to control antimicrobial use, may select for antibiotic resistance (Wales and Davies, 2015). Slifierz et al. (2015) found that MRSA in nursery pigs was associated with in-feed use of zinc and the frequent disinfection of nursery pens.

1.3.1.2 Aquaculture

The aquaculture industry is the fastest growing food-animal production sector in the world, comprising both marine and inland capture fisheries, and aquaculture operations (FAO, 2018). The world seafood industry produced a total of 171 million tonnes of products in 2016; 151.2 million tonnes of shellfish and finfish were intended for human consumption and the remaining 19.8 million tonnes were used as non-food resources for the production of fishmeal and oil (FAO, 2018). Approximately 89% of all aquaculture production comes from the Asia, followed by Europe (3.7%), Latin America (3.3%), Africa (2.5%) and North America (0.8%); dominance by Asia is

largely attributable to China, comprising 67% of global production (FAO, 2018). Other notable aquaculture-producing countries from Asia (ranked in descending order by production volume) include India, Indonesia, Vietnam and Bangladesh (FAO, 2018)

Globally only a few antimicrobials are approved for use in aquaculture. In Canada, these drugs include, florfenicol, oxytetracycline and combinations of trimethoprim or ormetoprim with sulfadimethoxine or sulfadiazine, for use in lobsters, salmon and trout (Health Canada, 2010). In many countries around the world, tetracyclines are approved for aquaculture use, including Canada, the United States, India, Norway, Indonesia, Japan and China (Tuševljak, 2012). In contrast, the approval of quinolones is less widespread; they are not approved in Canada or the United States but are approved in certain European and Asian countries (Hernández Serrano, 2005).

As the aquaculture industry continues to grow, utilization of antimicrobials may be an important contributor to antimicrobial resistance. In contrast to livestock production systems, antimicrobials are given to treat and prevent specific diseases rather than promote growth (Wall et al., 2016). No antimicrobial agent has been specifically developed for fish or shellfish therapy, therefore, the same agents licensed for use in humans and livestock have been approved for selected use in aquatic animals with established withdrawal times (FAO/OIE/WHO, 2006; Health Canada, 2010; Wall et al., 2016). Aquaculture production systems vary in size and by type of aquatic animals, thus the use of antibiotics in this industry is mainly administered directly to the water where animals live (Hernández Serrano, 2005). As a result, antibiotics are given as medicated food pellets, where the food not consumed by animals together with the drug excreted, eventually reaches the sediment around the pens (Hernández Serrano, 2005). It is estimated that 70-80% of antimicrobials used in aquaculture settle in the surrounding environment applying

selection pressure for the development of resistant bacterial strains in environmental organisms (Samuelsen et al., 1992; Hernández Serrano, 2005).

Resistance genes have been identified in aquatic bacteria, including *Aeromonas* and *Vibrio* spp., and from *E. coli* isolated from farmed fish (Miranda et al., 2013). Muziasari et al. (2014) isolated resistance genes and bacteria resistant to sulphonamides and trimethoprim from the sediment under aquaculture farms, which persisted in the environment during the 6-year observation period. Although resistance in the surrounding environments was less prevalent, these sediments could act as reservoirs for resistance genes to local fish farms and potential transfer to humans through the distribution of food (Muziasari et al., 2014). The association between tetracycline use in aquaculture and the development of resistance has been investigated. In a study by Rhodes et al. (2000), tetracycline resistance genes from *Aeromonas salmonicida* were transferred to *Aeromonas hydrophilia* and *E. coli*, demonstrating the ability of resistance genes from an aquatic pathogen to spread to human pathogens. As resistance to tetracyclines developed there has been increased usage of quinolones in aquaculture systems, resulting in the detection of plasmid-mediated quinolone resistance genes from water samples and farmed fish in several countries, including Egypt and China (Ishida et al., 2010; Jiang et al., 2012; Miranda et al., 2013).

1.3.2 Antimicrobial resistance surveillance programs

Surveillance programs have been created for monitoring trends in resistance among bacteria from animals, food and humans in several countries around the world. Monitoring of antimicrobial use is less well established in most countries, with the exception of Denmark, Sweden and the Netherlands (Grant et al., 2014). Globally, there are fifteen well-established programs including: the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS, 2017), the National Antimicrobial Resistance Monitoring System (NARMS, 2015),

Danish Integrated Program for Antimicrobial Resistance Surveillance (DANMAP, 2017), the Finnish Veterinary Antimicrobial Resistance Monitoring and Consumption of Antimicrobial Agents (FINRES-VET, 2017), the Italian Veterinary Antimicrobial Resistance Monitoring (ITAVARM, 2003), the Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands (MARAN, 2018), the Norwegian Surveillance System for Antimicrobial Drug Resistance (NORM-VET, 2017), the Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM, 2017), the Japanese Veterinary Resistance Monitoring System (JVARM, 2016), the Usage of Antibiotics and Occurrence of Antibiotic Resistance in Bacteria from Humans and Animals in Switzerland (ARCH-VET, 2018), the French Agency for Food, Environmental and Occupational Health & Safety (ANSES, 2014), the German Antimicrobial Resistance Strategy (DART, 2009), the Austrian Resistance Report (AURES, 2017), the United Kingdom Veterinary Antimicrobial Resistance and Sales Surveillance (UK-VARSS, 2017), and the Columbian Integrated Program for Antimicrobial Resistance Surveillance (COIPARS, 2015). Surveillance programs are the most developed in Europe, as they extensively monitor antimicrobial use and resistance in both human and veterinary medicine. A description of global AMR surveillance programs is summarized in **Table 1.2**.

DANMAP is one of the most comprehensive programs that has adopted the 'farm-to-fork' approach for tracking antimicrobial resistance along the food chain and monitors antibiotic consumption in animals and humans (DANMAP, 2017). This program includes surveillance of pet animals, food-producing animals, locally and imported food, and bacterial isolates from humans recovered from diagnostic submissions (DANMAP, 2017). DANMAP collects all the antimicrobial susceptibility data from bacterial isolates to: (1) study associations between antimicrobial consumption and the occurrence of resistance from food animals (on farms, at

slaughter and at processing plants) and retail food products to resistance found in human isolates, and (2) identify and model potential routes of transmission of resistance from animals or food products into humans (Bager, 2000; Grant et al., 2014).

Table 1.2: Summary of global antimicrobial resistance surveillance programs targeting food animal and retail meat bacterial isolates (ITAVARM, 2003; Barlow and Gobius, 2008; Zaidi et al., 2008; Schroeter et al., 2009; ANSES, 2014; NARMS, 2015; Donado-Godoy et al., 2015; CCVO Antimicrobial Use in Animal Agriculture Committee, 2016; JVARM, 2016; AURES, 2017; DANMAP, 2017; FINRES-VET, 2017; Government of Canada, 2017; NORM/NORM-VET, 2017; SVARM, 2017; ARCH-VET, 2018; MARAN, 2018)

Country	Program (Years Active)	AMU Data collection and target animal species*	AMR Animal/Products	Indicator Bacterial Species	Zoonotic Bacterial Species
N. America					
Canada	CIPARS (1997 – Current)	Distribution data – broiler chickens and grower-finisher pigs	Cattle, pig, chicken, turkey	*E. coli*	*Salmonella*, *Campylobacter*
USA	NARMS (1996 – Current)	Sales/distribution data – all food animal species	Cattle, pig, chicken, turkey	*E. coli*, *Enterococcus*	*Salmonella*, *Campylobacter*
Mexico	Integrated food chain surveillance system (Pilot: 2002 – 2005)	Not monitored	Cattle, pig, chicken	Not monitored	*Salmonella*
Europe					
Denmark	DANMAP (1995 – Current)	Veterinary prescription level – most animal species	Cattle, pig, chicken, turkey	*E. coli*, *Enterococcus*	*Salmonella*, *Campylobacter*, MRSA
Finland	FINRES-VET (2002 – Current)	Wholesale data – all animal species	Cattle, pig, chicken, turkey, eggs	*E. coli*	*Salmonella*, *Campylobacter*, MRSA
Netherlands	MARAN (1999 – Current)	Sales/distribution (includes pharmacotherapeutic groups) and farm data – all animal species	Cattle, pigs, chicken, turkey, sheep/lambs, goats, seafood (fish and shrimp)	*E. coli*	*Salmonella*, *Campylobacter*
Norway	NORM-VET (2000 – Current)	Sales data – most animal species	Cattle, pig, chicken, turkey, milk, shellfish and molluscs	*E. coli*, *Enterococcus*	*Salmonella*, *Campylobacter*, MRSA
Sweden	SVARM (2000 – Current)	Sales data -Terrestrial animal species only	Cattle, pig, chicken, turkey, sheep, milk, farmed fish	*E. coli*, *Enterococcus*	*Salmonella*, *Campylobacter*, MRSA

Switzerland	ARCH-VET (2006 – Current)	Sales data – all animal species	Cattle, pig, chicken	*E. coli*, *Enterococcus*	*Salmonella*, *Campylobacter*, MRSA
Italy	ITAVARM (1999 – 2003)	Not monitored	Cattle, pig, chicken, turkey, sheep, molluscs	*E. coli*, *Enterococcus*	*Salmonella*
France	ANSES (1999 – Current)	Laboratory/manufacture data – most animal species	Cattle, pig, chicken, turkey	*E. coli*, *Enterococcus*	*Salmonella*, *Campylobacter*
Germany	DART (2008 – Current)	Not available	Cattle, pig, poultry	*E. coli*, *Enterococcus*	*Salmonella*, *Campylobacter*, MRSA
Austria	AURES (2006 – Current)	Pharmaceutical companies and wholesalers – all animal species	Cattle, pig, poultry	*E. coli*	*Salmonella*, *Campylobacter*
United Kingdom	UK-VARSS (1999 – Current)	Sales/distribution data – all animal species	Pigs	*E. coli*	*Salmonella*
Asia/Pacific					
Australia	Surveillance program for AMR in bacteria of animal origin (Pilot: 2007 – 2009)	Not monitored	Cattle, pig, poultry	*E. coli*, *Enterococcus*	*Salmonella*, *Campylobacter*
Japan	JVARM (1999 – Current)	Manufactures/importers and feed additives – all food animal species	Cattle, pig, chicken	*E. coli*, *Enterococcus*	*Salmonella*, *Campylobacter*
S. America					
Colombia	COIPARS (2007 – Current)	Not available	Poultry	*E. coli*, *Enterococcus*	*Salmonella*, *Campylobacter*

*For target animal species 2 categories are presented: **(1) All animal species** – programs explicitly state that all species are included without listing them, and **(2) Most animal species** – programs list many animal species (or categories of animals) which may include all species

1.3.3 Gaps in antimicrobial resistance surveillance

Most surveillance programs target only major agricultural animal species such as cattle, swine and poultry, while the diversity of other terrestrial and aquatic species consumed globally are ignored. In Canada and the United States, no surveillance data is collected from sheep, veal and aquaculture production systems even though they are consumed by a proportion of the population, routinely given antimicrobials and possess potentially pathogenic bacteria and resistance genes (NARMS, 2015; Government of Canada, 2017; PHAC, 2018). In Scandinavian countries, such as Denmark, Norway, Sweden and the Netherlands, antimicrobial use in farmed fish is monitored and resistance data from seafood products (farmed fish and shellfish) is routinely collected (DANMAP, 2017; NORM/NORM-VET, 2017; SVARM 2017; MARAN 2018). Wildlife and bush meats are also not included in antimicrobial resistance surveillance. These animals act as sentinels for resistant bacteria and resistance genes in the environment (Vittecoq et al., 2016; Arnold et al., 2016). Including these animals and associated products in surveillance programs would allow for monitoring the occurrence of antimicrobial resistance in nature over time.

Another area that is ignored, is the surveillance of imported meat and other food items, including niche products. It has been demonstrated that international travel plays a very important role in the dissemination of antimicrobial resistance genes from regions with high prevalence rates; people can acquire, carry and transmit antimicrobial resistant bacteria or resistance genes back to their home regions where resistance rates may be lower (van der Bij and Pitout, 2012; Arcilla et al., 2014; Kuenzli, 2016). Similarly, the global trade of livestock and food products also have the potential to transmit and disseminate resistant bacteria and/or resistance genes around the world, via the carriage of resistant bacteria in livestock as part of their normal microflora or through

contaminated food products, such as meat and produce (Hawkey, 2015; Hanefeld et al., 2017; George, 2018).

Antimicrobial resistance data collected from food animals and associated products are restricted to specific, zoonotic and potentially pathogenic bacteria and indicator organisms such as *Salmonella*, *Campylobacter* and *E. coli*, while other bacterial species are not included (OIE, 2004; CDC, 2014; Government of Canada, 2017; PHAC, 2018). Other bacteria, besides enteric pathogens, can also carry resistance genes which may be transmitted to gastrointestinal microbiota when consumed (Huddleston, 2014). Thus, surveillance programs may miss potential bacterial reservoirs of resistance genes. Also, the use of indicator organisms like *E. coli* may be an efficient strategy for identifying resistance among bacteria in major agricultural animals (cattle, pig and poultry) where recovery rates are nearly 100%; however, this may not be effective when evaluating other meats or animals with ill defined microbial communities (Ryu et al., 2012; Morrison and Rubin, 2015). When monitoring aquatic food products, the World Organization for Animal Health (OIE) recommends testing for *Vibrio* or *Aeromonas* species, however in those surveillance programs routinely testing seafood, this has not been implemented (OIE, 2004). Overall, these exclusions in antimicrobial surveillance programs constitute major gaps in important information, as national surveillance is an essential resource regarding exposure of humans to resistant bacteria in the food supply.

1.4 Niche products

In the business world, a niche can be defined as a distinct segment within a market, generally a targetable part of a larger market, where the demand for a product or service is unfulfilled (Kotler and Keller, 2012). Niche markets tend to be highly specialized and focus on specific needs of a smaller group of individuals (Shani and Chalasani, 1992). **Figure 1.3** illustrates the layers of a food industry market. Some examples of niche food markets include organic food, vegan, gluten free and specialty coffee.

Within the food industry, a wide variety of food items are sold within a market, such as grocery stores. However, specialty grocery stores exist to cater to the specific needs of the consumer. These specialty groceries are considered sub-markets or niche markets, as they still fit into the larger context of the market but focus on specific consumer needs. A niche product is a specialty item that is not considered mainstream or widely available, in which a subset of the population has a specific demand for it. An example of a niche product within the context of this thesis, is reptile and amphibian meat products, such as soft shell turtle or frog legs, imported into Canada for use in cuisines and herbal preparations not traditionally associated with Western diets or medicine. The sub-market/niche market is specialty grocery stores and the unmet customer demand is the inability of consumers (specific ethnicities) to buy local, traditional ethnic food items. As the population in Canada has become more diverse, there has been an increase in the number of specialty food markets carrying imported niche food items.

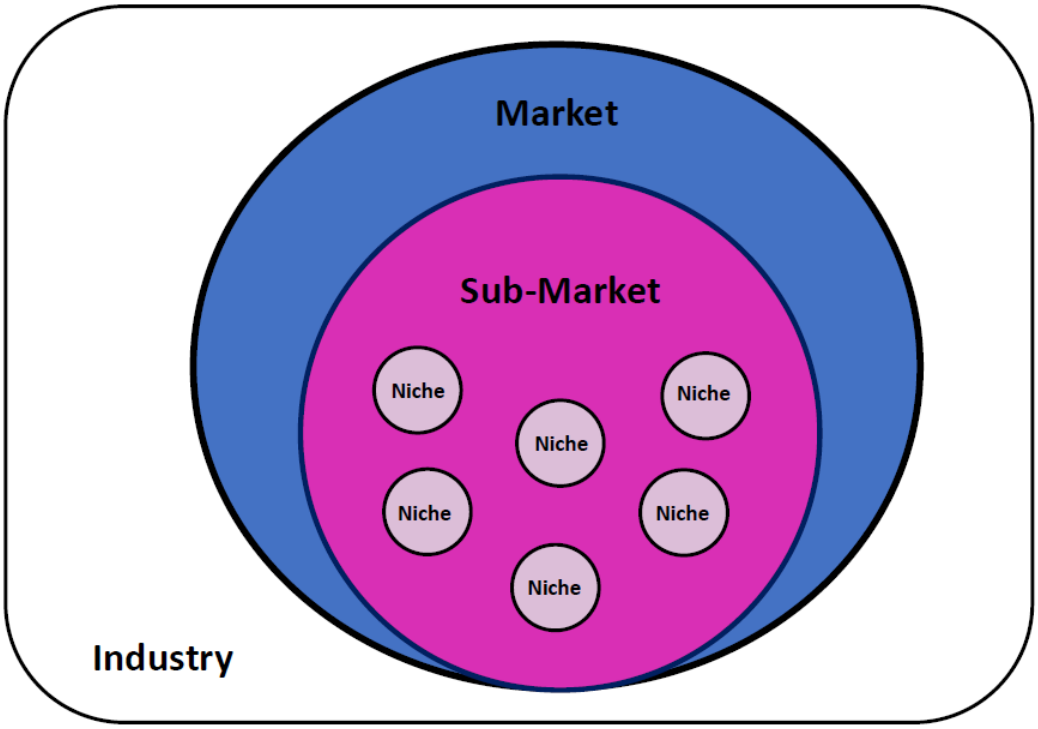

Figure 1.3: Layers of a food industry market. Within the food industry, the market is all retail stores selling food items (groceries, butchers, etc.), the sub-market is specialty groceries and other specialty stores (herbalist shops), and the niche is specialty products (e.g. frog legs, dried snakes).

1.5 Reptile and amphibian derived products

Throughout the world, reptiles and amphibians have been collected or raised for a variety of uses to benefit humans. They serve as invaluable models in medical research for understanding developmental and physiological processes, especially in species capable of limb regeneration (O'Rourke, 2007). Reptiles and amphibians provide potential for new pharmaceuticals, such as analgesics, anti-cancer and antimicrobial drugs (Xiao et al., 2011; Valencia-Aguilar et al., 2013; van Hoek, 2014). Amphibians, more so than reptiles, have historically been used as dissection specimens in high school and secondary educational institutions in Western countries, allowing students to learn basic anatomy (Jensen and Camp, 2003). Reptiles, and also some amphibians, are cultivated for the fashion industry; their skin and hides are processed into leather for the manufacturing of purses, wallets, shoes and bags (Jensen and Camp, 2003; Valencia-Aguilar et al., 2013). Amphibians and reptiles are some of the most common animals involved in the global pet trade; with risks of becoming invasive species, spread of novel pathogens detrimental to local populations and public health risks associated with transmission of disease or direct injuries (Herrel, 2014; Auliya et al., 2016). Reptiles and amphibians serve as important food sources and have been used in traditional medicines and associated with specific cultural beliefs (Klemens and Thorbjarnarson, 1995; Jensen and Camp, 2003; Valencia-Aguilar et al., 2013; Hocking and Babbitt, 2014). The use of reptiles and amphibians for culinary and traditional medicinal purposes will be further described, along with production, consumption and exportation of these products.

1.5.1 Production, consumption and exportation

Reptiles and amphibian derived products are consumed for cultural and medicinal purposes in addition to a nutritive protein source. The extent of consumption of these products are not well known. Most literature tends to focus on the production systems (wild-caught, commercially

farmed or hunted) and uses of these products rather than the distribution within specific populations that consume them (Klemens and Thorbjarnarson, 1995; Jensen and Camp, 2003; Magnino et al., 2009; Valencia-Aguilar et al., 2013; Hocking and Babbitt, 2014; Nijman and Bergin, 2017). Information on exportation is limited to a select number of species (Warkentin et al., 2009; Gratwicke et al., 2010; FAO, 2014).

1.5.1.1 Culinary products

Reptiles and amphibians are used as a food resource in many countries around the world. Although many species of reptiles are ingested by humans, extensive consumption and commercialization of meat is mainly observed in turtles, while meat consumption of snakes, crocodilians and lizards is presumed to be a by-product of commercial harvesting of skin (Klemens and Thorbjarnarson, 1995). In terms of amphibians, certain species of giant salamanders (*Andrias davidianus* and *A. japonicus*) have been used locally in China and Japan for food (Okada et al., 2008; Cunningham et al., 2016). However, the primary form of amphibians consumed by humans are frog legs (Jensen and Camp, 2003; Warkentin et al., 2009).

Turtles: Turtles are an important source of protein especially in Asia; Chinese soft shell turtles (*Pelodiscus sinensis*), are widely cultivated in China, Japan, Korea, Thailand, Taiwan, Vietnam, Malaysia and Indonesia with China being the largest producer and supplying 94% of the export market (Jenkins, 1995; Silpachai, 2001; Magnino et al., 2009; FAO, 2014). Wild sea turtles and tortoises have also been extensively consumed for their meat, eggs and oil, leading to significant declines in marine and inland turtle populations (Merrem et al., 1990; Jenkins, 1995).

Crocodilians: The consumption of crocodilians is not as wide spread as compared with turtles. Crocodile meat is considered a delicacy in regions of the United States, Cuba, South Africa,

Ethiopia, Thailand and Australia (Klemens and Thorbjarnarson, 1995). Farming of American alligator (*Alligator mississippiensis*) occurs in the Southern US (Georgia, Florida, Texas and Louisiana) mostly for their hides, but an established meat market also exists (Magnino et al., 2009; Nickum et al., 2018). In 2014, farmer's in Louisiana sold more than 383 000 m of alligator skin and 445 000 kg of meat valued at $77 million and $7 million respectively; primary export markets include France and Italy for alligator skin and Canada and Hong Kong for the meat (Nickum et al., 2018). Commercial farms in South America produce yacare caimans (*Caiman crocodilus yacare*) for meat and skin, with an estimated value of approximately $900,000 USD per year (Carreira and Sabbag, 2015).

Snakes: The commercial sale of snake meat is not very common except in Southeast Asia, where up to 4000 tonnes are served annually in China (Hoffman and Cawthorn, 2012). Pythons (*Pythonidae*) and Boas (*Boa constrictor*) are commonly hunted as bushmeat in many rural communities in West Africa, while venomous snakes are regarded as a delicacy in South Africa (Jensen, 2017). In the United States, rattlesnakes are hunted and farmed for their skin with the trade of meat as a by-product (Klemens and Thorbjarnarson, 1995; Fitzgerald and Painter, 2000). It is estimated that the whole sale value of rattlesnake skin is approximately $9-$11/linear foot and meat $13-$27/kg, with the overall magnitude of trade estimated to be more than 125 000 snakes/year and greater than 75% of these animals originate from Texas (Fitzgerald and Painter, 2000). Rattlesnake meat is locally available in restaurants and consumed by many indigenous communities (Klemens and Thorbjarnarson, 1995; Fitzgerald and Painter, 2000).

Lizards: Green iguanas (*Iguana iguana*) are found in Central America, Mexico, the Caribbean and Africa, where they are harvested for both their meat and skin (Hoffman and Cawthorn, 2012). In South America, tegus (*Tupinambis rufescens* and *T. merianae*) have been

traditionally hunted and utilized by aboriginal people as a source of fat, protein and leather (Valencia-Aguilar et al., 2013). There have been some attempts to farm tegus in Argentina, however, these giant lizards are only active during the hot summer, making farming very difficult (Saadoun and Cabrera, 2008).

Frogs: Frog legs are considered an international culinary delicacy. Most products originate from Asian countries, with greater than 75% imported into France, Belgium, and the United States, and an annual net worth of around half a billion dollars (Gratwicke et al., 2010). Originally, India was the major supplier of frogs, but concerns of inhumane killing and declining populations lead to an exportation ban in 1987; Indonesia replaced India as the primary exporter of frogs' legs, followed by China (Jensen and Camp, 2003; Kusrini and Alford, 2006; Warkentin et al., 2009; Gratwicke et al., 2010). The majority of frogs from Indonesia are wild-caught and consist of the crab-eating frog (*Fejervarya carncrivora*) and the giant Javan frog (*Limnonectes macrodon*), while frogs from China are mainly farmed and consist of the American bullfrog (*Lithobates catesbeianus*) (Kusrini and Alford, 2006; Warkentin et al., 2009).

1.5.1.2 Medicinal products

Reptile and amphibian meat products are not just a source of protein but are also used for traditional medicinal and religious purposes. In many cultures, particularly in regions with limited access to primary health care, animals have long been used as medicinal sources for healing (Alves et al., 2007, 2009). Reptiles are more commonly utilized in traditional folk medicine, where their role in the prevention and cure of disease has been reported (Alves et al., 2008). Medicinal products from meat, fat, skin, eggs, blood, shell and bones are used as raw materials in the preparation of powdered medicines, oils, and salves, and are sold in rural markets for the treatment of numerous illnesses, such as skin issues, asthma, inflammation, bleeding disorders, rheumatism, arthritis,

cancer and epilepsy (Alves et al., 2007, 2008, 2009; Valencia-Aguilar et al., 2013; Pandey, 2015; Vats and Thomas, 2015; Nijman and Bergin, 2017). In addition to medicinal uses, many parts of these animals are used in rituals, magic spells or as amulets for protection (Alves et al., 2009).

Over the past few years, numerous studies have been conducted to determine the effectiveness of reptile derived substances used in traditional medicine. The body fat from the lizard *T. merianae* is widely used to treat wounds, earaches, swelling, sore throat and bronchitis. Many of these illnesses are associated with bacterial infections, suggesting that reptile fat may contain antibacterial components. Ferreira et al. (2009) demonstrated that this fat does not have significant antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* when used alone. However, the oil was effective for treating edema in the ear by reducing pro-inflammatory mediators (Ferreira et al., 2010). The fat from the *Boa constrictor* is also used to treat a wide variety of ailments such as lung disease, earaches, sore throat, stomach ache, inflammation, erysipelas and boils. In similar tests, the oil extracted from this fat did not demonstrate direct antibacterial activity against *E. coli* and *S. aureus* but did show significant synergistic activity when used in combination with aminoglycosides (Ferreira et al., 2011). Lizards such as *Ameiva ameiva, Tropidurus hispidus* and *T. semitaneniatus*, are used traditionally to treat inflammation, dermatitis, venereal disease, boils, snake bites, sore throat and bacterial pharyngitis and tonsillitis. Due to the small size of these lizards, the whole animal is used in the preparation of a decoction (a form of tea). Santos et al. (2011, 2012) conducted research to test whether these decoctions had effective antibacterial effects against *E. coli, S. aureus* and *P. aeruginosa*. The decoctions alone did not demonstrate any substantial inhibitory effects on the growth of these bacterial strains, but when used in combination with antibiotics they reduced the minimum inhibitory concentrations of the assayed antimicrobials (Santos et al., 2012).

In Western medicine, many drugs are derived from natural sources (Ji et al., 2009). Amphibian skin contains granular glands which produce a wide range of alkaloids, peptides and proteins that protect against infection by bacteria, fungi and predators, with many of these compounds being researched for the development of new pharmaceutical agents (Valencia-Aguilar et al., 2013; Hocking and Babbitt, 2014). In recent decades, more than 200 peptides have been identified and many antimicrobial peptides are being used in the development of new drugs to fight resistant bacteria, such as MRSA, and multidrug resistant infections caused by *Acinetobacter baumannii, Pseudomonas aeruginosa* and Enterobacteriaceae (Xiao et al., 2011; Conlon, 2011). The discovery of the alkaloid epibatidine, isolated from the toxic secretions of the poison dart frog (*Epipedobates tricolor*), demonstrated significant pain suppression, with analgesic potency of 200 times greater than morphine when administered to mice and rats (Daly et al., 2000; Hocking and Babbitt, 2014). Unfortunately, the narrow analgesic to toxic dose range prevented it from moving forward in clinical trials (Daly et al., 2000). Many reptiles produce large quantities of toxins, that tend to be a mixture of peptides, proteins and biomolecules that can produce various effects in humans (King, 2011; Valencia-Aguilar et al., 2013). The study of reptile toxins has contributed to the development of numerous pharmaceuticals used for the treatment of human diseases (King, 2011). The development of captopril, an angiotensin converting enzyme inhibitor for the treatment of hypertension, was synthesized from a peptide found in the venom of the South American lancehead pit viper (*Bothrops jararaca*) (King, 2011; Harvey, 2014). Eptifibatide, a synthetic cyclic heptapeptide derived from a protein found in the venom of the Southeastern pygmy rattlesnake (*Sistrurus miliarius barbouri*), is a drug used to treat acute coronary syndrome by preventing platelet aggregation and clot formation (King, 2011; Harvey, 2014). Finally, exenatide is a synthetic analogue of the protein exendin-4, derived from the saliva of the Gila monster lizard

(*Heloderma suspectum*), used as an anti-diabetic agent for the control of type 2-diabetes (King, 2011; Harvey, 2014).

1.6 Bacterial hazards associated with the consumption of reptiles and amphibians

Like other meats, reptiles and amphibian products have been associated with foodborne pathogens (Magnino et al., 2009). Owing to their popularity as pets, reptiles are recognized as common carriers of *Salmonella* (Minette, 1984). Limited information is available regarding the presence of Salmonellae in reptile meat, other than crocodilians farmed for meat consumption. Studies on crocodile meat have documented *Salmonella enterica* subspecies *enterica*, *salamae*, *arizonae* and *diarizonae*, in both fresh chilled and frozen samples (Manolis et al., 1991; Obwolo, 1993; Madsen, 1993, 1996; van der Walt et al., 1997). Although many of these serotypes are rarely or never associated with human disease, the isolates belonging to subsp. *enterica*, comprises potential human pathogens, such as *S.* Typhimurium and *S.* Enteritidis (Gurakan et al., 2008). From marine turtle meat, *Salmonella* Chester was isolated in Australia and *S*. Typhimurium was isolated in Japan from snapping turtle meat (O'Grady and Krause, 1999; Aguirre et al., 2006).

Cases of human Salmonellosis have been reported following the consumption of raw and cooked meat from soft shell turtles in Japan, and sea turtle meat in Northern Australia (O'Grady and Krause, 1999; Fukushima et al., 2008). Numerous outbreaks of Salmonellosis have occurred following consumption of dried rattlesnake meat used for traditional medicinal purposes in aboriginal communities in the United States (Waterman et al., 1990; Kelly et al., 1995; Magnino et al., 2009). Contamination of frog legs with *Salmonella* is also a well recognized food safety risk and has led the US FDA to issue import alerts for frog legs imported from China, India,

Bangladesh, Indonesia, Philippines, Singapore, Taiwan, Thailand and Vietnam (FDA, 2014). The identification of unapproved drug residues has also led to import alerts for frog legs imported from China, Malaysia and Vietnam (FDA, 2017).

Study Rationale

Animal origin food products are recognized as potential reservoirs of antimicrobial resistant organisms. These organisms may be transmitted to the intestinal microbiota following consumption. For this reason, numerous countries have active surveillance programs targeting resistant bacteria from major agricultural food animal species such as cattle, swine and poultry. These programs focus on specific, zoonotic and potentially pathogenic bacteria and indicator organisms, including *Salmonella*, *Campylobacter* and *E. coli*, while mobile resistance from non-pathogenic bacteria are not included. In Canada, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) collects antimicrobial resistance data from domestically raised food animals and associated products, while other terrestrial and aquatic animal species are ignored, including imported meat. It has been demonstrated that international travel plays an important role in the dissemination of antimicrobial resistance from regions of high to low prevalence rates. Thus, the international trade of food may be a critical player in the transmission and dissemination of resistant bacteria or mobile resistance genes.

Objectives

- To evaluate imported reptile and amphibian meat products for pathogen identification and to determine if broad spectrum β-lactamase and colistin resistance genes are present in bacterial isolates.
- To determine the antimicrobial resistance profiles of *Macrococcus caseolyticus* isolated from imported seafood, reptile and amphibian meat products.
- To develop a bacteriological culture medium for the selective isolation of *Macrococcus caseolyticus*.

2 Identification of ESBL, AmpC β-lactamase, carbapenemase and colistin resistance from imported culinary reptiles and amphibians

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Author contribution:

Conceived and designed the experiments: BJM, JER. Sample collection and preparation: BJM, JER. Performed the experiments: BJM. Analyzed the data: BJM, JER. Wrote the paper: BJM, JER.

2.1 Abstract

Meat from food-producing animals play an important role in the dissemination of antimicrobial resistant bacteria. Although many countries have active surveillance programs targeting resistant organisms in retail beef, pork and poultry, they typically do not capture the diversity of foods available including niche market meats. Therefore, we conducted this investigation to screen imported reptile and amphibian meat products for antimicrobial resistant bacteria. A total of 53 items including whole soft-shelled turtles (n=11), frog legs (n=20), dried geckos (n=12), dried snakes (n=9) and dried turtle carapace (n=1) were purchased from markets in Vancouver and Saskatoon, Canada. Samples were washed in sterile buffered peptone water and rinsates were cultured for *Salmonella, E. coli*, and ESBL and carbapenemase-producing organisms. Antimicrobial susceptibility of isolates was determined by a combination of broth microdilution and agar dilution. Based on phenotypic susceptibility, isolates were screened for ESBLs, carbapenemases and the *mcr*-1 gene by PCR and the identity of amplicons were confirmed by DNA sequencing. *Salmonella*, all pan-susceptible, were grown from 6 dried geckos. *E. coli* were isolated from 19 samples, including 9 isolates from 6 food items which produced CTX-M-type ESBLs. One *E. coli* isolated from a soft shell turtle produced a CTX-M-55 enzyme, was resistant to tetracycline, sulfonamides, chloramphenicol, aminoglycosides and the fluoroquinolones, was also colistin resistant and possessed the *mcr*-1 gene. An NDM-1 producing *Acinetobacter* spp. was also isolated from a dried turtle carapace. Our results suggest that imported reptile and amphibian meats are an underappreciated source of resistant organisms with mobile resistant genes to clinically relevant drugs. The presence of bacteria possessing acquired resistance mechanisms to the last line of defense antimicrobials from these niche market food items represents a potential public health hazard that warrants further investigation.

2.2 Introduction

The food supply is recognized as a potential source of antimicrobial resistant organisms. Recent publications describing the presence of carbapenemase-producing organisms (CPO) and mobile colistin resistance (MCR-1) are particularly alarming (Rubin et al., 2014; Liu et al., 2016). Although many countries have active surveillance programs targeting *E. coli*, *Salmonella* and *Campylobacter* in retail beef, pork and poultry, they typically do not capture the diversity foods available including niche market meats, nor are imported products typically included (Morrison and Rubin, 2015).

Reptile and amphibian derived products are consumed for cultural and medicinal purposes in addition to a nutritive protein source (Klemens and Thorbjarnarson, 1995). The extent of consumption of these products is not well known. Most literature tends to focus on the production systems and uses of these products rather than the distribution within specific populations that consume them. Turtles are an important source of protein especially in Asia; Chinese soft shell turtles (*Pelodiscus sinensis*), are widely cultivated in China, Japan, Korea, Thailand, Taiwan, Vietnam, Malaysia and Indonesia with China being the largest producer and supplying 94% of the export market (Silpachai, 2001; FAO, 2014). Wild sea turtles have also been extensively consumed for their meat, eggs and oil, leading to significant declines in marine turtle populations (Merrem et al., 1990). The commercial sale of snake meat is not very common except in Southeast Asia, but it is consumed in many parts of the world (Magnino et al., 2009). In the United States, rattlesnakes are farmed for meat production and are consumed by many indigenous communities (Kelly et al., 1995). Along with snakes, the consumption of lizard meat is often related to alleged medicinal or cultural benefits derived from their flesh and skin (Alves et al., 2009; Nóbrega Alves et al., 2012). Of the culinary reptiles and amphibians exported internationally, the trade in frog legs is perhaps

the best defined; most products originate from Asian countries, with greater than 75% imported into France, Belgium and the United states (Gratwicke et al., 2010).

Like other meats, reptile and amphibian products have been associated with foodborne pathogens (Magnino et al., 2009). Owing to their popularity as pets, reptiles are well recognized as common carriers of *Salmonella* (Minette, 1984). Cases of Salmonellosis have been associated with the consumption of turtles and rattlesnakes (Waterman et al., 1990; Kelly et al., 1995; Aguirre et al., 2006; Fukushima et al., 2008). Contamination of frog legs with *Salmonella* is also a well recognized food safety risk and has led the US FDA to issue import alerts for frog legs imported from China, India, Bangladesh, Indonesia, Philippines, Singapore, Taiwan, Thailand and Vietnam (FDA, 2014). The identification of unapproved drug residues has also led to import alerts for frog legs imported from China, Malaysia and Vietnam (FDA, 2017a). Despite these potential hazards, few studies have examined the role of these niche market meats in the epidemiology of antimicrobial resistance.

In Canada, a number of recent reports describing CPOs including *Pseudomonas fluorescens* (VIM-2) from a Korean squid, a variety of non-fermenters (OXA-48) from Korean and Chinese seafood products, *Vibrio cholerae* (VCC-1) from Indian black tiger shrimp and a variety of *Enterobacter* spp. (NDM-1, IMI-1 and IMI-2) from Vietnamese clams and shrimp (Rubin et al., 2014; Morrison and Rubin, 2015; Mangat et al., 2016; Janecko et al., 2016). Others have reported the importation of carbapenemase-producing organisms in vegetables (OXA-181) and ornamental fish (OXA-48-like) (Zurfluh et al., 2015; Ceccarelli et al., 2017). There is growing literature describing the presence of CPOs in agricultural animals and retail meats globally (Woodford et al., 2014). However, with the use of carbapenem antibiotics restricted to the treatment of resistant infections in humans and very specific cases in animals, the origin of acquired carbapenemase

genes remains unclear. Similarly, the recognition of the global distribution of MCR-1 (including in people, animals, retail meat and the environment in North and South America, Europe, Asia and Africa) following its initial description in late 2015, highlights the need for a globally focused One-Health approach to understanding the epidemiology of antimicrobial resistance (Schwarz and Johnson, 2016).

Although international travel has been demonstrated to play a role in the global epidemiology of resistant bacteria, relatively little attention has been paid to the role of the international food trade in the dissemination of CPOs (Woodford and Johnson, 2013; Mataseje et al., 2016). The objective of this investigation was therefore to screen niche market reptile and amphibian meat products for antimicrobial resistant bacteria.

2.3 Materials and methods

2.3.1 Study design

This study was conducted to identify potential bacterial pathogens from imported niche reptile and amphibian meat products that are not otherwise investigated by food surveillance programs. International travel has played an important role in the dissemination of antimicrobial resistance around the world, thus, international trade is also likely to be a critical player. Therefore, we evaluated all Gram-negative isolates for extended spectrum β-lactamase, AmpC β-lactamase, carbapenemase and mobile colistin resistance.

2.3.2 Sample collection and processing

A total of 53 reptile and amphibian meat products were purchased from specialty groceries and herbalist shops in Vancouver (n=42) and Saskatoon (n=11), Canada in July 2015. Products included dried gecko (n=12), snake (n=9) and turtle carapace (n=1) and frozen soft shelled turtle

(n=11), and frog legs (n=20) (**Figure 2.1**). Of the 11 soft shelled turtles, one was whole and un-gutted, while the remaining 10 were gutted but contained remnants of intestines where fecal material was collected. No country of origin labeling was present for 22 samples and the remainder were from China (n=28) and Thailand (n=3).

The products were processed using CIPARS retail meat surveillance methodology (PHAC, 2017). For frozen products a 25g sub-sample was dissected and washed in 250ml of buffered peptone water (BPW) in a sterile sample bag. Dried specimens were rehydrated in BPW for 4 hours prior. BPW was then selectively cultured for *Salmonella*, *E. coli,* ESBL-producing Enterobacteriaceae and carbapenem-resistant organisms. Briefly, *Salmonella* was cultured by incubating BPW at 35°C overnight followed by sub-culture to Rappaport-Verssiliadis and tetrathionate broths. Aliquots of each broth were then sub-cultured onto xylose lysine deoxycholate (XLD) and brilliant green agar. Suspect *Salmonella,* black colonies on XLD and pink colonies on brilliant greed agar, were sub-cultured to blood agar and presumptively identified using the triple sugar iron biochemical test (production of hydrogen sulphide). For *E. coli*, equal volumes of BPW rinsate and 2X MacConkey broth were mixed and incubated overnight at 35°C. Broth was then plated onto eosin methylene blue agar. Suspect *E. coli* colonies were sub-cultured to blood agar and presumptively identified using the indole, citrate and urea biochemical tests. Taxa-independent culture for ESBL producers and carbapenem-resistant organisms was achieved by plating BPW aliquots onto CHROMagar ESBL (CHROMagar, Paris, France) and Mueller-Hinton agar + 2µg/ml meropenem (MHM). Any organisms recovered from MHM were Gram stained and any Gram-negatives were identified by phylogenetic analysis of partial 16S sequences using previously published primers (Dorsch and Stackebrandt, 1992). All *Salmonella* isolates were serotyped by the World Organization for Animal Health (OIE) Reference Laboratory for Salmonellosis at the

National Microbiology Laboratory at Guelph, Ontario Canada. Up to 3 isolates per genus type were saved. This was based on colony morphology differences observed on blood agar.

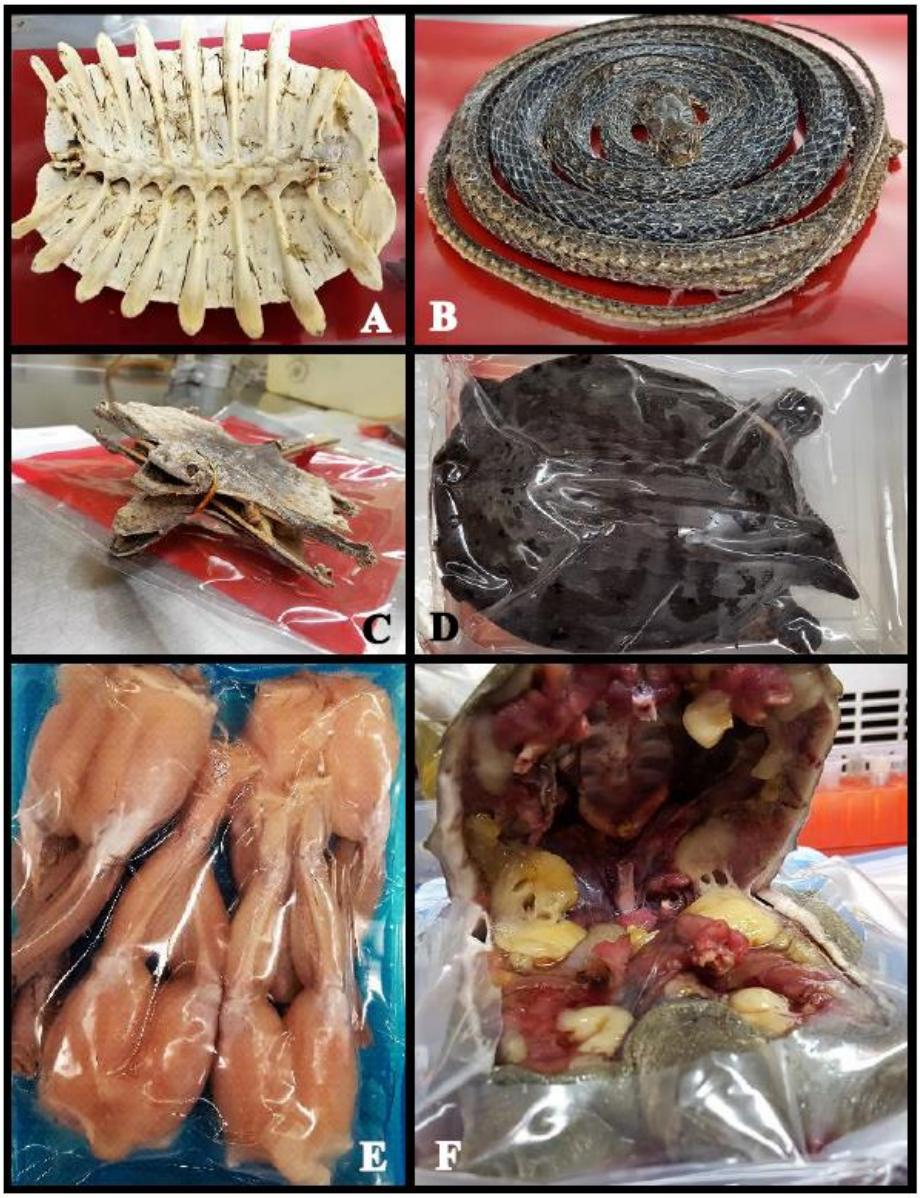

Figure 2.1: Food products collected from retail markets in Vancouver, BC and Saskatoon, SK
A: dried turtle carapace; B: dried snake; C: dried gecko; D: soft shell turtle
in package; E: frog legs in package; F: soft shell turtle opened

Table 2.1: Properties for bacterial isolation and identification. Colonies were selected based on colony morphology on selective media and positive biochemical reactions identified genus of bacteria. Up to three colonies per genus type per sample were saved based on colony morphology differences observed on blood.

	Selective Media	**Colony appearance**	**Biochemical Reactions**	**Identifying Characteristic**
E. coli	Eosin methylene blue (EMB)	Dark metallic green	Tryptophanase production	Indole - positive Citrate - negative Urea - negative
Salmonella	Xylose lysine deoxycholate (XLD)	Black colonies	Gas and hydrogen sulphide production	Triple sugar iron slant (TSI): Red slant with black middle (due to hydrogen sulphide production) and yellow butt lifted off the bottom (due to gas production)
	Brilliant green agar (BGA)	Pink-white colonies with red zones		
ESBL-producing Enterobacteriaceae	CHROMagar ESBL	*E. coli:* Dark pink to red Other Enterobacteriaceae: Metallic blue	Facultative anaerobes	Oxidase negative

2.3.3 Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed by broth microdilution using the Sensititre system (Trek Diagnostics, Cleveland, OH). The CMV3AGNF and GNX3F plate formats were used in this study according to the manufacturer's instructions. In brief, isolates were sub-cultured onto a 5% sheep blood agar plate and incubated overnight at 35°C. To prepare the inoculum, 3 – 4 colonies were suspended in 5ml of demineralized water to a McFarland 0.5 density. Thirty microliters of bacterial suspension was then added to 11ml of cation-adjusted Muller Hinton broth (Trek Diagnostics, OH, USA). Fifty microliters of inoculum was dispensed into the 96 well plate using the Sensititre auto-inoculator. Plates were sealed with adhesive film to prevent evaporation and incubated at 35°C for 18-24 hours. Antimicrobial MICs were interpreted, as susceptible or resistant, according to CLSI guidelines (CLSI, 2018). The antibiotic test ranges used in this study are described in **Table 2.1**. For quality control, *S. aureus* ATCC 29213 and *E. coli* ATCC 25422 were used.

Agar dilution was used to determine colistin susceptibility. Briefly, a series of Mueller-Hinton agar plates containing 0.032-64µg/ml of colistin sulfate were prepared. Isolates were sub-cultured onto 5% sheep blood agar and incubated overnight at 35°C. The inoculum was prepared as above making a McFarland 0.5 density and diluted 1:10. Using a multichannel pipettor, 2µl of each bacterial suspension was spotted onto each agar plate and incubated at 35°C for 18 hours. The presence or absence of growth at each spot was noted. Colistin MIC was interpreted according to the EUCAST guidelines, as there are no MIC breakpoints available using CLSI, and the antibiotic test range used is listed in **Table 2.1**. The clinical breakpoint for colistin resistance is 2µg/ml. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains.

Table 2.2: List of antibiotics used in antibiotic susceptibility testing CMV3AGNF and GNX3F panels.

Antibiotic Class	Antibiotic	Tested Range (µg/ml)
β-lactams	Aztreonam	2 – 16
	Ampicillin	1 – 32
	Cefoxitin	0.5 – 32
	Ceftriaxone	0.25 – 64
	Ceftiofur	0.12 – 8
	Ceftazidime	1 – 16
	Cefepime	2 – 32
	Amoxicillin-clavulanic acid	1/0.5 – 32/16
	Ticarcillin-clavulanic acid	16/2 – 128/2
	Piperacillin-tazobactam	8/4 – 64/4
	Doripenem	0.5 – 4
	Imipenem	1 – 8
	Meropenem	1 – 8
Phenicols	Chloramphenicol	2 – 32
Tetracyclines	Tetracycline	4 – 32
Sulfonamides	Sulfisoxazole	16 – 256
	Trimethoprim-sulfamethoxazole	0.12/2.38 – 4/76
Quinolones	Ciprofloxacin	0.015 – 4
	Levofloxacin	1 – 8
	Nalidixic acid	0.5 – 32
Macrolides	Azithromycin	0.12 – 16
Aminoglycosides	Amikacin	4 – 32
	Gentamicin	0.25 – 16
	Streptomycin	2 – 64
	Tobramycin	1 – 8
Polymyxins	Polymyxin B	0.25 – 4
	Colistin	0.25 – 4

2.3.4 Carbapenemase detection

All organisms grown on MHM were screened for carbapenemase production using the β-CARBA test according to the manufacturer's instructions (Bio-Rad., Montreal, Quebec, Canada). The β-CARBA test is a qualitative colorimetric assay used for the detection of any carbapenemase-producing strains. Briefly, 40µl of reagent R1 and 40µl of R2 are added to a microcentrifuge tube, followed by a 1µl loop of freshly isolated bacterial colony. The mixture was homogenized and incubated at 35°C for 30mins. The test was interpreted within 30 minutes of incubation. Any color change from yellow to orange, red or purple constituted a positive result (**Figure 2.2**).

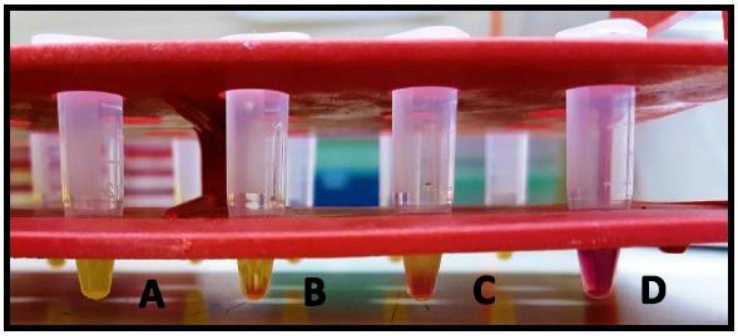

Figure 2.2: The β-CARBA test for detecting carbapenemase production: (A) Negative result showing no color change from yellow; (B) Positive result with some orange-red color at bottom of tube; (C) Positive result with more orange-red color change; and (D) Positive result with complete colour change to pink-red

2.3.5 Bacterial DNA extraction and PCR of targeted gene sequences

Crude DNA extracts, boil preparations, of cultures were made in sterile distilled water. In brief, bacteria were sub-cultured on a 5% blood agar plate and incubated overnight at 35°C. In a sterile 1.5ml microcentrifuge tube, 200µl of ultrapure water was added and several colonies were suspended in it. Tubes were then vortexed and boiled in a heat block for 10 minutes at 100°C. After boiling, tubes were cooled to room temperature, approximately 5 minutes, and centrifuged for 1 minute at 15871 x g. The supernatant was then transferred to a sterile 1.5ml microcentrifuge tube and stored at -20°C until used.

Previously published primers targeting CTX-M-U, CTX-M-G1, CTX-M-G9, TEM, SHV, CMY-2, NDM, IMP, VIM, KPC, OXA-48 and CLR5 (MCR-1) were used to detect ESBL, AmpC, carbapenemase and colistin resistance genes (**Table 2.2**). PCR reactions were carried out in 25µL volumes including 24µL of mastermix and 1µL of bacterial DNA template. In each set of reactions, both positive and negative controls were included. Thermocycler conditions were as follows: initial denaturation at 94°C for 6 mins followed by 35 cycles of denaturation at 94°C for 1 min, annealing at varying temperatures (**Table 2.3**) for 1 min, elongation at 72°C for 1 min and final extension at 72°C for 10 mins. For class I integrons, touchdown PCR was performed with the following conditions: 94°C for 6 mins, 22 cycles of 94°C for 1 min, 78°C -1°C for 1min, 72°C for 1 min, 15 cycles of 94°C for 1 min, 56°C -1°C for 1 min, 72°C for 1 min with final extension at 72°C for 10 mins. Amplicons were resolved by electrophoresis using 1% agarose gel with 0.5µL ethidium bromide at 110 volts for 30 minutes. A DNA gene Ladder was added to the first well for determination of amplicon size. Gels were visualized under UV light using an AlphaImager® HP (Fischer Scientific, Toronto, ON).

Table 2.3: PCR primers used for detecting ESBL, AmpC β-lactamase, carbapenemase, mobile colistin resistance and class I integrons.

Primer Name	Primer Sequence	References
CTX-M-U	5′-ATGTGCAGYACCAGTAARGTKATGGC-3′	(Mulvey et al. 2003)
	5′-TGGGTRAARTARGTSACCAGAAYCAGCGG-3′	
CTX-M-G1	5′-GTTGTTAATTCGTCTCTTCC-3′	(Ghosh 2017)
	5′-AGTTTCCCCATTCCGTTTC-3′	
CTX-M-G9	5′-GACCGTATTGGGAGTTTGAG′	(Hammad et al. 2009)
	5′-ATCTGATCCTTCAACTCAGC-3′	(Gonsalves 2011)
TEM	5′-GCGGAACCCCTATTTG-3′	(Olesen et al. 2004)
	5′-ACCAATGCTTAATCAGTGAG-3′	
SHV	5′-TTATCTCCCTGTTAGCCACC-3′	(Arlet et al. 2006)
	5′-GATTTGCTGATTTCGCTCGG-3′	
	5′-CGGCCCTCACTCAAGGATG-3′ (SHV-F1 sequencing primer)	(Bradford 1999)
	5′-GCGAGTAGTCCACCAGATCC-3′ (SHV-F2 sequencing primer)	(Jung, 2019)
	5′-ATTACCGACCGGCATCTCTC-3′ (SHV-R1 sequencing primer)	(Jung, 2019)
	5′-CTGCTGCAGTGGATGGTG-3′ (SHV-R2 sequencing primer)	
	5′-CGCCGGGTTATTCTTATTTGTCGC-3′ (SHV-UP)	(Perilli et al. 2002)
	5′-TCTTTCCGATGCCGCCGCCAGTCA-3′ (SHV-LO)	
CMY-2	5′-ATGATGAAAAAATCGTTATGCTGC-3′	(Kruger et al. 2004)
	5′-GCTTTTCAAGAATGCGCCAGG-3′	(Hasman et al. 2015)
NDM	5′-GCAGCTTGTCGGCCATGCGGGC-3′	(Peirano et al. 2011)
	5′-GGTCGCGAAGCTGAGCACCGCAT-3′	
KPC	5′-TGTCACTGTATCGCCGTC-3′	(Yigit et al. 2001)
	5′-CTCAGTGCTCTACAGAAAACC-3′	
IMP	5′-GAAGGCGTTTATGTTCATAC-3′	(Pitout et al. 2005)
	5′-GTACGTTTCAAGAGTGATGC-3′	
VIM	5′-GTTTGGTCGCATATCGCAAC-3′	(Pitout et al. 2005)
	5′-AATGCGCAGCACCAGGATAG-3′	

OXA-48	5´-GCGTGGTTAAGGATGAACAC-3´	(Poirel et al. 2011)
	5´-CATCAAGTTCAACCCAACCG-3´	
CLR5 (MCR-1)	5′-CGGTCAGTCCGTTTGTTC-3′	(Liu et al. 2016)
	5′-CTTGGTCGGTCTGTAGGG-3′	
Class I Integron	5´-GGCATCCAAGCAGCAAG-3´	(Pellegrini et al. 2011)
	5´-AAAGCAGACTTGACCTGA-3´	

Table 2.4: Annealing temperatures used for primer binding of ESBL, AmpC β-lactamase, carbapenemase and colistin resistance genes.

Primer Name	Annealing Temperature
CTX-M-U	61.0°C
CTX-M-G1	61.0°C
CTX-M-G9	48.0°C
TEM	58.3°C
SHV	68.0°C
CMY-2	68.0°C
NDM	58.3°C
KPC	54.8°C
IMP	55.1°C
VIM	60.3°C
OXA-48	60.3°C
CLR5 (MCR-1)	56.0°C

2.3.6 PCR amplicon purification and nucleotide sequencing

PCR products were purified using EZ-10 Spin Column PCR purification kit (Bio Basic Canada INC., ON, Canada) and DNA was quantified using a spectrophotometer (NanoDrop^{TM} 1000). Purified DNA with a 1.8 ratio of absorbance at 260nm and 280nm and 2.0 – 2.2 of 260/280 values were considered sufficiently high quality for sequencing. DNA with a concentration between 10 – 50ng/µL was subsequently sequenced by a commercial lab (Macrogen) using amplification primers. DNA sequences were assembled, edited and analyzed using Staden Package software (pregap4 and gap4). Using NCBI BLAST (basic local alignment search tool), nucleotide consensus sequences were compared with a collection database to identify potential resistance genes. The identity of specific resistance gene variants was determined by comparing nucleotide reference sequences listed on the Lahey database (Bush et al., 2017) to the consensus sequences using CLC sequence viewer. Sequences with single nucleotide polymorphisms (SNPs) were further compared at the amino acid level to determine if there was a change in the protein sequence resulting in a new gene variant.

2.4 Results

2.4.1 Bacteria recovery rate

Of the 53 imported reptile and amphibian meat products tested, 71 bacterial isolates from seven genera of Enterobacteriaceae including *Salmonella* (n=6), *Escherichia* (n=48), *Citrobacter* (n=3), *Enterobacter* (n=3), *Klebsiella* (n=6), *Serratia* (n=4) and *Kluyvera* (n=1) were recovered from 41 of the products. Bacteria recovery rate per sample type is summarized in **Table 2.4**. Selective culture for *Salmonella* revealed six samples containing this organism, all dried geckos, and additional characterization revealed five unique serotypes (**Table 2.5**). A total of 48 *E. coli* isolates were recovered from 19 samples, with the largest proportion isolated from soft shell turtles. A great diversity of other Enterobacteriaceae were cultured from CHROMagar ESBL media, with the highest percentage from the frog legs. Finally, three carbapenemase-producing organisms were isolated from soft shell turtles (n=2) and the dried turtle carapace (n=1).

Table 2.5: Bacteria recovery rate per product type of imported culinary reptile and amphibians collected from retail markets in the census metropolitan regions of Vancouver, BC and Saskatoon, SK.

Isolates (n=71)	Reptile and Amphibian Products (n=53)				
	Soft shell turtle (n=11)	**Frog legs (n=20)**	**Dried gecko (n=12)**	**Dried snake (n=9)**	**Dried carapace (n=1)**
Escherichia (n=48)	26 (10/11)	2 (1/20)	11 (5/12)	9 (3/9)	0
Salmonella (n=6)	0	0	6 (6/12)	0	0
Klebsiella (n=6)	0	4 (3/20)	2 (2/12)	0	0
Serratia (n=4)	0	4 (4/20)	0	0	0
Citrobacter (n=3)	1 (1/11)	1 (1/20)	0	1 (1/9)	0
Enterobacter (n=3)	0	2 (2/20)	0	0	1 (1/1)
Kluyvera (n=1)	0	1 (1/20)	0	0	0

Table 2.6: *Salmonella* serotypes identified from imported reptile and amphibian meat products.

Organism	**ID**	**Sample**
Salmonella I:Rough-Or:z6	BR006E1-a	Dried Gecko
Salmonella Poona	BR017E1-a	Dried Gecko
Salmonella Chicago	BR018E2-a	Dried Gecko
Salmonella Poona	BR040E2-a	Dried Gecko
Salmonella Urbana	BR041E2-a	Dried Gecko
Salmonella Weltevreden	BR042E2-a	Dried Gecko

2.4.2 Antimicrobial resistant profiles of *E. coli*

2.4.2.1 MIC distribution of *E. coli* isolates based on drug class

The antimicrobial minimum inhibitory concentrations of the 48 *E. coli* isolates were determined and interpreted based on CLSI guidelines. The MIC distribution of isolates to antibiotics categorized by class are presented in **Table 2.6**. Overall, 52.1% (25/48) of isolates were resistant to ampicillin while ceftiofur and ceftriaxone resistance was observed in 35.4% (17/48) and 33.3% (16/48) of isolates respectively. The frequency of resistance to cefoxitin and amoxicillin-clavulanic acid was lower including 10.4% (5/48) and 6.3% (3/48) of isolates respectively. Resistance to trimethoprim-sulfamethoxazole was observed in 39.6% (19/48) of isolates, while 58.3% (28/48) of isolates were resistant to potentiated sulfisoxazole. Resistance to tetracycline was found in 47.9% (23/48) of isolates, while 45.8% (22/48) of isolates were resistant to chloramphenicol and nalidixic acid. Resistance to colistin was observed in one isolate with a MIC of 16 µg/ml. Within a sample, the susceptibility of isolates recovered were generally homogenous. Resistance profiles for eleven of the most notable isolates is summarized in **Table 2.7**.

Table 2.7: Minimum inhibitory concentration distribution of *E. coli* (n=48) from imported reptile and amphibian meat products.

Drug Class	Name	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	% R Isolates (n=48)	% R Samples (n=19)
β-lactams	AMP								9	10	4			25				52.1 (25)	57.9 (11)
	AUG								9	14	16	6	1	2				6.25 (3)	10.5 (2)
	XNL					5	17	8		2		16						33.3 (16)	42.1 (8)
	FOX								9	10	19	5	2	3				10.4 (5)	21.0 (4)
	AXO					30			1	1		3			13			35.4 (17)	47.4 (9)
Sulfonamides	FIS											6	6	8			28	58.3 (28)	68.4 (13)
	SXT				21	6	2				19							39.6 (19)	47.4 (9)
Quinolones	CIP	20				5	6	1	2	1	13							29.1 (14)	36.8 (7)
	NAL							9	5	6	2	4		22				45.8 (22)	52.6 (10)
Aminoglycosides	GEN					6	6	20	4	2	1	1	8					20.8 (10)	31.6 (6)
	STR								6	4	10	5	2		21			43.8 (21)	63.2 (12)
Tetracyclines	TET									25		1		22				47.9 (23)	52.6 (10)
Phenicol	CHL								2	8	10	6		22				45.8 (22)	52.6 (10)

Ampicillin (AMP), amoxicillin + clavulanic acid 2:1 (AUG), ceftiofur (XNL), cefoxitin (FOX), ceftriaxone (AXO), sulfisoxazole (FIS), trimethoprim + sulfamethoxazole 1:19 (SXT), ciprofloxacin (CIP), nalidixic acid (NAL), gentamicin (GEN), streptomycin (STR), tetracycline (TET) and chloramphenicol (CHL). Cells corresponding to the concentrations tested are in white and resistance breakpoints are denoted by the dark bars. The number of isolates inhibited at each concentration are noted in each cell, isolates not inhibited by the highest concentration of each drug are enumerated in the first concentration above the highest concentration tested.

Table 2.8: Antimicrobial resistance profiles, broad spectrum β-lactamases and other resistance genes identified from *E. coli* isolates (n=11).

ID	Sample	Antimicrobial Resistance Profile*	β-lactamase Genes	Other Genes
BR050B-b	Frog legs	**AMP + XNL + AXO** + **FIS + SXT** + **NAL**+ **GEN** + **TET** + **CHL**	CTX-M-64	
BR027B-c	Soft shelled turtle	**AMP + XNL + AXO** + **FIS + SXT** + **NAL**+ **CHL**	CTX-M-55	
BR028B-c	Soft shelled turtle	**AMP + XNL + AXO** + **FIS + SXT** + **CIP + NAL**+ **TET**	CTX-M-65	
BR028B-d	Soft shelled turtle	**AMP + XNL + AXO** + **TET**	CTX-M-18	
BR029D-a	Soft shelled turtle	**AMP + XNL + AXO + FOX + AUG** + **FIS** + **NAL + CIP** + **GEN** + **TET** + **CHL**	CMY-61, TEM-33	
BR030B-c	Soft shelled turtle	**AMP + XNL + AXO** + **FIS + SXT** + **NAL + CIP** + **GEN** + **TET** + **CHL**	CTX-M-18	
BR030B-d	Soft shelled turtle	**AMP + XNL + AXO + FOX** + **FIS + SXT** + **NAL + CIP** + **GEN** + **TET** + **CHL**	CTX-M-55	*mcr*-1
BR037D-a	Soft shelled turtle	**AMP + XNL + AXO + FOX + AUG** + **FIS + SXT** + **NAL + CIP** + **TET** + **CHL**	CMY-61	
BR051B-b	Soft shelled turtle	**AMP + XNL + AXO** + **FIS + SXT** + **TET** + **CHL**	CTX-M-55	
BR053B-a	Soft shelled turtle	**AMP + XNL + AXO** + **FIS + SXT** + **TET** + **CHL**	CTX-M-55	
BR053B-b	Soft shelled turtle	**AMP + XNL + AXO** + **FIS + SXT** + **NAL + CIP** + **TET** + **CHL**	CTX-M-3	

*A total of six drug classes were tested including (1) β-lactams (Red): Ampicillin (AMP), ceftiofur (XNL), ceftriaxone (AXO), amoxicillin + clavulanic acid (AUG), cefoxitin (FOX), (2) sulfonamides (Blue): Sulfisoxazole (FIS), trimethoprim + sulfamethoxazole (SXT), (3) quinolones (Green): Nalidixic acid (NAL), ciprofloxacin (CIP), (4) aminoglycosides (Black): gentamicin (GEN), (5) tetracyclines (Purple): tetracycline (TET) and (6) phenicols (Brown): chloramphenicol (CHL).

2.4.2.2 Percentage of *E. coli* isolates and samples resistant to different antimicrobial classes

Full susceptibility to all drugs was found in 35.4% (17/48) of isolates from 42.1% (8/19 of samples. Multidrug resistance, defined as resistance to three or more drug classes (Tadesse et al., 2012), was observed in 56.3% (27/48) of isolates. Resistance to all drugs tested was identified in 14.5% (7/48) of isolates from 21.1% (4/19) of samples (**Table 2.8**).

Table 2.9: Percentage of *E. coli* isolates and samples resistant to different antimicrobial drug classes.

Resistance to Antimicrobial Classes*	**Isolates (n=48) N (%)**	**Samples (n=19) N (%)**
Pan-susceptible	17 (35.4%)	8 (42.1%)
1 Antimicrobial class	2 (4.2%)	2 (10.5%)
2 Antimicrobial classes	2 (4.2%)	2 (10.5%)
3 Antimicrobial classes	4 (8.3%)	3 (15.8%)
4 Antimicrobial classes	10 (20.8%)	5 (26.3%)
5 Antimicrobial classes	6 (12.5%)	4 (21.1%)
6 Antimicrobial classes	7 (14.5%)	4 (21.1%)

*Drug classes: β-lactams, aminoglycosides, sulfonamides, quinolones, tetracyclines and phenicols

2.4.3 Antimicrobial resistant profiles of non-*E. coli* Enterobacteriaceae

Antimicrobial susceptibility testing was performed on 23 non-*E. coli* Enterobacteriaceae isolates. The resistance profiles for 17 of the isolates is presented in **Table 2.9**. Of the six *Salmonella* isolates recovered, all were susceptible to all drugs tested. Only one isolate in the *Kluyvera* genus was recovered, *Kluyvera intermedia*, and it was resistant to all 6 antimicrobial classes tested. Of the six *Klebsiella* isolates recovered, all were resistant to ampicillin, ceftiofur, ceftriaxone, tetracycline, trimethoprim-sulfamethoxazole and sulfisoxazole. Resistance to nalidixic acid and gentamicin was observed in 83.3% (5/6) of isolates, while 66.7% (4/6) of isolates were resistant to ciprofloxacin and chloramphenicol. Three isolates of the genus *Citrobacter* were recovered, with 100% (3/3) of isolates resistant to ampicillin and cefoxitin. Resistance to amoxicillin-clavulanic acid, the sulfonamides, ciprofloxacin, nalidixic acid, chloramphenicol and tetracycline was observed in 66.7% (2/3) of isolates. Of the three *Enterobacter* isolates recovered, all were resistant to ampicillin, ceftiofur, cefoxitin, ceftriaxone, trimethoprim-sulfamethoxazole, sulfisoxazole and tetracycline, while 66.7% (2/3) of isolates were resistant to amoxicillin-clavulanic acid, ciprofloxacin, nalidixic acid, gentamicin and chloramphenicol. Lastly, four *Serratia fonticola* isolates were recovered, with 100% (4/4) resistant to ampicillin and 75% (3/4) resistant to ceftriaxone.

Table 2.10: Antimicrobial resistance profiles and β-lactamase genes from non-*E. coli* Enterobacteriaceae isolates (n=17).

ID	Sample	Organism	Antimicrobial Resistance Profile*	β-lactamase Genes
BR004B-c	Dried carapace	*Enterobacter* sp.	**AMP + FOX + AXO + XNL + AUG** + **FIS + SXT** + **TET**	CTX-M-15
BR041B-a	Dried gecko	*Klebsiella pneumoniae*	**AMP + AXO + XNL** + **FIS + SXT** + **NAL** + **GEN** + **TET**	CTX-M-83
BR042B-b	Dried gecko	*Klebsiella pneumoniae*	**AMP + AXO + XNL** + **FIS + SXT** + **GEN** + **TET**	CTX-M-83
BR012B-b	Dried snake	*Citrobacter freundii*	**AMP + FOX + AUG**	CMY-98
BR050B-a	Frog legs	*Citrobacter braakii*	**AMP + FOX** + **FIS + SXT** + **CIP + NAL** + **TET** + **CHL**	TEM-33
BR026B-c	Frog legs	*Enterobacter* sp.	**AMP + FOX + AXO + XNL + AUG** + **FIS + SXT** + **CIP + NAL** + **GEN** + **TET** + **CHL**	SHV-12, TEM-206
BR045B-a	Frog legs	*Enterobacter* sp.	**AMP + FOX + AXO + XNL** + **FIS + SXT** + **CIP + NAL** + **GEN** + **TET** + **CHL**	CTX-M-2, TEM-33
BR019B-a	Frog legs	*Klebsiella oxytoca*	**AMP + AXO + XNL + AUG** + **FIS + SXT** + **CIP + NAL** + **GEN** + **TET** + **CHL**	OXY-2-10
BR019B-b	Frog legs	*Klebsiella oxytoca*	**AMP + AXO + XNL** + **FIS + SXT** + **CIP + NAL** + **TET** + **CHL**	CTX-M- 83
BR025B-a	Frog legs	*Klebsiella oxytoca*	**AMP + AXO + XNL + AUG** + **FIS + SXT** + **CIP + NAL** + **GEN** + **TET** + **CHL**	OXY-2-2
BR048B-c	Frog legs	*Klebsiella oxytoca*	**AMP + AXO + XNL + AUG** + **FIS + SXT** + **CIP + NAL** + **GEN** + **TET** + **CHL**	OXY-2-2
BR021B-c	Frog legs	*Kluyvera intermedia*	**AMP + FOX + AXO + XNL** + **FIS + SXT** + **CIP + NAL** + **GEN** + **TET** + **CHL**	CTX-M-27
BR033B-b	Frog legs	*Serratia fonticola*	**AMP + AXO + AUG** + **CIP + NAL**	FONA-5
BR034B-b	Frog legs	*Serratia fonticola*	**AMP + AXO**	FONA-5
BR047B-c	Frog legs	*Serratia fonticola*	**AMP + AXO + XNL**	FONA-5
BR049B-c	Frog legs	*Serratia fonticola*	**AMP**	FONA-5
BR037B-a	Turtle	*Citrobacter freundii*	**AMP + FOX + AUG** + **FIS + SXT** + **CIP + NAL** + **TET** + **CHL**	CTX-M-1, CMY-83

*A total of six drug classes were tested including (1) β-lactams (Red): Ampicillin (AMP), ceftiofur (XNL), ceftriaxone (AXO), amoxicillin + clavulanic acid (AUG), cefoxitin (FOX), (2) sulfonamides (Blue): Sulfisoxazole (FIS), trimethoprim + sulfamethoxazole (SXT), (3) quinolones (Green): Nalidixic acid (NAL), ciprofloxacin (CIP), (4) aminoglycosides (Black): gentamicin (GEN), (5) tetracyclines (Purple): tetracycline (TET) and (6) phenicols (Brown): chloramphenicol (CHL).

2.4.4 Carbapenemase-producing organisms

The β-CARBA test revealed three carbapenemase-producing organisms among those isolated on MHM. All three isolates were resistant to meropenem, imipenem and doripenem (not shown). Two of the isolates were cultured from soft shell turtles and were identified as a *Stenotrophomonas* sp. and a *Chryseobacterium* sp. The last isolate, an *Acinetobacter* sp. was recovered from the dried turtle carapace. All three isolates were screened for the five most clinically relevant carbapenemase genes including, NDM, KPC, IMP, VIM and OXA-48. Only the *Acinetobacter* sp. possessed NDM-1 and was resistant to the cephalosporins, carbapenems, aztreonam, ciprofloxacin and trimethoprim-sulfamethoxazole and was susceptible to the aminoglycosides, tetracyclines and polymyxins. The other two isolates were negative for the carbapenemase genes tested.

2.4.5 Molecular detection of resistance

2.4.5.1 Broad spectrum β-lactamase and mobile colistin resistance genes

Of the 48 Enterobacteriaceae isolates with antimicrobial resistance phenotypes, up to two isolates of each genus type per sample, 28 isolates from 22 samples, were screened for ESBL and AmpC β-lactamases. A summary of the isolates, sample types and resistance profiles that possessed resistance genes are presented for *E. coli* in **Table 2.7** and the non-*E. coli* Enterobacteriaceae in **Table 2.9**. Of the 28 isolates, 85.7% (24/28) of isolates from 90.9% (20/22) of samples were found to carry ESBL encoding genes, while 14.3% (4/28) of isolates were positive for AmpC encoding genes from 13.6% (3) of samples.

DNA sequencing results revealed of the 24 ESBL-producing isolates, 17 possessed a variety of CTX-M-type enzymes,1 harboured a SHV-12 enzyme and 3 *Klebsiella oxytoca* and 4

Serratia fonticola isolates produced OXY-type and FONA-type ESBLs, respectively. Four isolates were found to harbour CMY-type enzymes which were determined to be CMY-61 (n=2), CMY-83 (n=1) and CMY-98 (n=1). TEM-type β-lactamases were identified in 4 (14.3%) isolates from 4 (18.2%) of samples, which were identified as TEM-33 (n=3) and TEM-206 (n=1). One colistin resistant *E. coli* isolate was found to possess the mobile colistin resistance, MCR-1 enzyme and co-harboured CTX-M-55. Co-location of ESBL and AmpC β-lactamases was observed in one isolate, which harboured the CTX-M-1 and CMY-83 enzymes (**Table 2.10**).

2.4.5.2 Class I integron genes

All 48 Enterobacteriaceae isolates and one *Acinetobacter* sp. were screened for class I integrons and amplicons were sequenced revealing 8 integron positive isolates. Four isolates harboured the *aad*A1 (*E. coli*, n=1) and *aad*A2 (*E. coli*, n=1 and *Enterobacter*, n=2) genes encoding the protein streptomycin 3´-adenylyltransferase, which confers resistance to streptomycin and spectinomycin. Of those that possessed the *aad*A2 gene, the *E. coli* isolate also carried the CTX-M-18 enzyme and one of the *Enterobacter* sp. carried the SHV-12 and TEM-206 enzymes. Three isolates, one CTX-M-15 producing *Enterobacter* sp. and two CTX-M-83 producing *Klebsiella pneumonia*, harboured the *dfr*A1 gene encoding the protein dihydrofolate reductase, which confers resistance to the sulfonamides. Lastly, the NDM-1 producing *Acinetobacter* sp. possessed *fos*C2, which confers resistance to fosfomycin by encoding the protein fosfomycin phosphotransferase.

Table 2.11: Number of ESBL, AmpC β-lactamase and penicillinase-producing Enterobacteriaceae from imported reptile and amphibian meat samples.

Classes of β-lactamase genes tested	Number of samples positive for β-lactamase genes (n=22)	β-lactamase variants
ESBL	20 (90.9%)	CTX-M-1 (1; 4.2%) CTX-M-2 (1; 4.2%) CTX-M-3 (1; 4.2%) CTX-M-15 (1; 4.2%) CTX-M-18 (2; 8.3%) CTX-M-27 (1; 4.2%) CTX-M-55 (4; 16.7%) CTX-M-64 (1; 4.2%) CTX-M-65 (1; 4.2%) CTX-M-83 (3; 12.5%) SHV-12 (1; 4.2%) OXY-2-2 (2; 8.3%) OXY-2-10 (1; 4.2%) FONA-5 (4; 16.7%)
AmpC	3 (13.6%)	CMY-61 (2; 50%) CMY-83 (1; 25%) CMY-98 (1; 25%)
Penicillinase	4 (18.2%)	TEM-33 (3; 75%) TEM-206 (1; 25%)
Both ESBL and AmpC	1 (4.5%)	CTX-M-1 + CMY-83 (1; 100%)
Both ESBL and penicillinase	2 (9.0%)	SHV-12 + TEM-206 (1; 50%) CTX-M-2 + TEM-33 (1; 50%)
Both AmpC and penicillinase	1 (4.5%)	CMY-61 + TEM-33 (1; 100%)

2.5 Discussion

The propensity of reptiles and amphibians to carry pathogenic bacteria, particularly *Salmonella*, is well recognized (Aguirre et al., 2006). Outbreaks associated with pet turtles have led to regulatory changes affecting the ownership of these animals in the United States (Cohen, 1980; Bosch et al., 2015; FDA, 2017b). While zoonotic transmission from exotic pets has been examined, the role of reptile meat in the food supply has not been studied. Interestingly, relatively little attention has been paid to the role of reptile/amphibian derived products in the food supply to the epidemiology of foodborne illness or the dissemination of resistance. In the United States, there are reports of pathogenic *Salmonella* Pomona isolated from alligator meat and a case of recurrent *Salmonella* Arizona infection in a patient who consumed rattlesnake (Cortes et al., 1992; Sakaguchi et al., 2017). Imported reptile and amphibian products have largely escaped study despite the large scale of production; the soft shell turtle production reached 344 800 tonnes in 2014 (FAO, 2014). Interestingly, the role of the international pet trade in the dissemination of resistance was recently highlighted. A *mcr*-1 producing *E. coli* was isolated from Asian grass lizards imported into Germany from Vietnam leading the authors to suggest that the international pet trade may be a vehicle for the dissemination of resistant organisms (Unger et al., 2017).

The emergence of resistance to last line of defense drugs mediated by the carbapenemases and *mcr* family of genes has ignited interest in identifying organisms possessing these genes on food products imported from regions of the world where these resistance mechanisms may be endemic. Since the initial detection of the VIM-2 carbapenemase gene from a Korean squid imported into Canada in 2014, several more studies have detected carbapenemase genes from a variety of Enterobacteriaceae and non-Enterobacteriaceae isolated from imported seafood and vegetables from Southeast Asian countries (Rubin et al., 2014; Morrison and Rubin, 2015; Mangat

et al., 2016; Janecko et al., 2016; Ceccarelli et al., 2017). Also, a few studies have detected the *mcr-1* gene isolated from *E. coli* in imported vegetables and chicken from Thailand, Vietnam and South America (Hasman et al., 2015; Zurfuh et al., 2016). Although the overall prevalence of these resistance genes in imported food remains low, it is worrisome as the consumption of raw or improperly cooked food provide the ideal condition for transmission and spread of resistant bacteria or their respective mobile genetic elements to resident intestinal flora. These studies emphasize the need for further monitoring of antimicrobial resistance and inclusion of imported products into national surveillance programs.

In the current investigation some food safety hazards unique to the products tested compared to foods derived from major agricultural species were identified. We were very surprised to find that one of the soft shell turtles that were included in this investigation was sold un-gutted. The opportunities for cross contamination are substantially greater when whole animals, including intestines with visible fecal material, are dressed and prepared in the kitchen than when other meats are used. It was also observed that the dried products, including the geckos which is where all *Salmonella* isolates were recovered from, were not handled in a manner which was consistent with a high level of meat hygiene. These products were uniformly displayed without packaging in the open air at the retail location and were handled with bare hands.

Reptile and amphibian meat products are not just a source of protein but are also used for traditional medicinal reasons and religious purposes. The meat, fat, skin, blood and bones are used as raw materials in the preparation of powdered medicines, oils, and salves for the treatment and prevention of numerous illnesses, such as sore throat, skin issues, aches, inflammation, burns, asthma, bleeding disorders, rheumatism, cancer and many more (Alves et al., 2008; Vats and Thomas, 2015). Many parts of these animals are used in rituals, magic spells or used as amulets

for protection (Alves et al., 2009). Public health professionals investigating outbreaks should be cognisant of this when designing questionnaires to capture the different types of products that people may have consumed as they are not necessarily considered meat.

In this investigation we have demonstrated that reptile and amphibian derived products are an underappreciated source of resistance. Future studies should seek to identify at what stage of the production cycle (on farm, at slaughter, during processing/packaging or at the retail level) resistant organisms enter the food products. Although the sample size was small, the *Salmonella* serotypes and CTX-M variants identified are not inconsistent with the organisms originating in Asia. Of the *Salmonella* isolated in this study, Urbana and Poona are reptile associated serotypes that have been linked to outbreaks in fresh produce and pet reptiles (Jackson et al., 2013; Walters et al., 2016). *Salmonella* Weltevreden is the most common serotype associated with imported seafood and aquatic production systems and is a frequent and increasing cause of human infection predominating Southeast Asia (Ponce et al., 2008; Makendi et al., 2016). Of the CTX-M enzymes identified, CTX-M-55, -83 and -27 are found most commonly in Asia especially China, but also in North Africa; while CTX-M-15 is the most prominent variant found worldwide (Zhao and Hu, 2013; Tong et al., 2015).

In this study the use of a taxa independent resistance selective culture media allowed for the identification of clinically relevant resistance in bacterial species which are not targeted by resistance surveillance programs. The breadth of non-*Escherichia* or *Salmonella* genera of Enterobacteriaceae possessing ESBLs, and the NDM-1 producing *Acinetobacter* sp. highlight the gaps of traditional surveillance approached. The presumptive location of these broad spectrum β-lactamases on transmissible plasmids in the context of these findings indicates that consideration

of the resistome of the whole bacterial community is required for a holistic understanding of the epidemiology of antimicrobial resistance.

2.6 Acknowledgements

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2.7 Transition statement

The first study was designed to identify bacterial pathogens from imported meat products and to evaluate the prevalence of antimicrobial resistance. A substantial proportion of isolated Gram-negative bacteria from the family Enterobacteriaceae were multidrug resistant and expressed one or more mobile β-lactamase resistance genes. However, resistant Gram-positive bacteria were also found. While isolating potential carbapenem-resistant organisms, there were some bacteria identified with similar colony morphology to *Staphylococcus aureus*. Further characterization revealed these isolates to be *Macrococcus caseolyticus* showing resistance to meropenem. There is not an extensive amount of literature regarding the resistance profile and prevalence of *M. caseolyticus*, but it is known to carry a gene conferring resistance similar to that seen with methicillin-resistant *Staphylococcus aureus* (MRSA). Therefore, we were interested in further investigating its phenotypic antimicrobial susceptibility and developing a culture medium that would selectively isolate this bacterium.

3 Development of a culture medium for the selective isolation of *Macrococcus caseolyticus*

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Conceived and designed the experiments: BJM, JER. Sample collection and preparation: BJM, JER. Performed the experiments: BJM. Analyzed the data: BJM JER. Wrote the paper: BJM, JER.

3.1 Abstract

Macrococcus caseolyticus is a commensal bacterial species that can be isolated from animal skin and food products, but rarely causes human or animal infection. It is closely related to the genus *Staphylococcus* and up until 1998 this bacterium was named *Staphylococcus caseolyticus*. It has acquired plasmid-mediated methicillin resistance which may impact both veterinary and human medicine. To date, there have been very few studies assessing the prevalence of *M. caseolyticus*. Thus, the purpose of this study is to develop a selective culture medium that will facilitate targeted studies and address the question of prevalence more systematically. Nine *M. caseolyticus* isolates cultured from imported meat products containing the *mec*B gene conferring methicillin resistance, were used. Antimicrobial susceptibility testing using broth microdilution was performed. Selective media were challenged using rinsates from animal feces, soil, meat products, and milk samples spiked with two concentrations of *M. caseolyticus* creating final inoculums of 3.0 $x10^6$ CFU/ml and $3.0x10^5$ CFU/ml. The selective medium that worked the best consisted of colistin nalidixic acid (CNA) agar with 5% defibrinated sheep blood with ampicillin 0.5µg/ml and meropenem 0.5µg/ml. This medium inhibited the growth of Gram-negative bacteria and most Gram-positive bacteria except for *Enterococcus* spp., which have intrinsic resistance to numerous drug classes. All nine *M. caseolyticus* isolates grew well on this medium and were recovered from each sample containing a mixture of bacteria. This medium allowed for phenotypic differentiation of *M. caseolyticus* from other Gram-positive bacteria based on colony morphology. Although *Macrococcus caseolyticus* is part of the normal skin microbiota of animals, the potential transmissibility of *mec* encoded β-lactam resistance to other bacteria is a concern. Our results confirm that this medium can selectively isolate *M. caseolyticus* from a variety of samples. This will give researchers a reliable way to conduct further studies on *M. caseolyticus*.

3.2 Introduction

The genus *Macrococcus* are Gram-positive cocci, that are coagulase negative, oxidase and catalase positive, belonging to the family Staphylococcaceae (Mazhar et al., 2018). Macrococci are evolutionarily closely related to the genus *Staphylococcus* but are composed of smaller genomes (Baba et al., 2009). The genus *Macrococcus* was originally classified as *Micrococcus*, then reclassified to *Staphylococcus* and finally in 1998 it received its current designation (Kloos et al., 1998). There are currently eleven species within this genus including, *M. bovicus*, *M. carouselicus*, *M. equipercicus*, *M. epidermidis*, *M. goetzii*, *M. burnensis*, *M. hajekii*, *M. lamae*, *M. bohemicus*, *M. canis* and *M. caseolyticus* (Brawand et al., 2017; Mazhar et al., 2018; Mašlaňová et al., 2018). The ecological distribution of macrococci in nature is not fully known, but they have been isolated from animal skin (ponies, horses, cattle, llamas, sheep, goats, dogs, whales and dolphins), from milk and meat products (Kloos et al., 1998; Mannerova, 2003; Brawand et al., 2017; Mazhar et al., 2018).

M. caseolyticus is a commensal bacterium isolated from animals and food products, but in contrast to staphylococci it is not considered to be a human pathogen, however, this species has been associated with veterinary infections, being isolated from ovine abscesses, bovine mastitis, and canine skin and soft tissue infections (de la Fuente et al., 1992; Gómez-Sanz et al., 2015; Cotting et al., 2017; Schwendener et al., 2017). Methicillin resistance has been described in this species and the basis for this resistance is through the expression of altered PBP2a, encoded by the *mec* gene, conferring resistance to the entire β-lactam class of antimicrobials (Tsubakishita et al., 2010; Cotting et al., 2017; Schwendener et al., 2017). There are currently four *mec* gene homologs (*mec*A-D) described among staphylococci and macrococci (Becker et al., 2014; Schwendener et al., 2017). In the case of methicillin resistant staphylococci, including methicillin resistant *S.*

aureus (MRSA) and methicillin resistant *S. pseudintermedius* (MRSP), the predominant *mec* gene type is *mec*A, with *mec*C found at a very low frequency in MRSA (Peacock and Paterson, 2015). In contrast, *mec*A and *mec*C have not been reported in macrococci, but *mec*B and *mec*D have been found in *M. caseolyticus*, with *mec*B also being reported in *M. canis* (Tsubakishita et al., 2010; Gómez-Sanz et al., 2015; Cotting et al., 2017; Schwendener et al., 2017).

The close relationship between macrococci and staphylococci as commensal bacteria sharing similar ecological niches, raises a concern for the potential transfer of antibiotic resistance, specifically *mec*B and *mec*D-mediated methicillin resistance, to more pathogenic strains (MacFadyen et al., 2018). It has been proposed that the *mec*B gene complex found in *M. caseolyticus* is a primordial form of *mec*A found in methicillin resistant staphylococci (Baba et al., 2009). Recently, evidence for cross-genus transmission of methicillin resistance facilitated by the *mec* genes comes from the discovery of a *mec*B-positive MRSA isolate from a hospitalized patient in Germany (Becker et al., 2018). This is the first description of plasmid-mediated methicillin resistance in staphylococci. *M. caseolyticus* and *M. canis* closely resemble members in the genus *Staphylococcus*, which may lead to potential diagnostic challenges in terms of producing false-positive results in routine MRSA screening and false-negative results from molecular based techniques in identifying *mec*A and *mec*C (Rubin and Chirino-Trejo, 2010; Mazhar et al., 2018; MacFadyen et al., 2018).

Although there is limited information available regarding host preference of *Macrococcus caseolyticus* and its presence in food, the prevalence of this species is still largely unknown. Therefore, the objective of this study was to develop a selective culture medium that will help facilitate targeted studies and address the question of prevalence more systematically.

3.3 Materials and methods

3.3.1 Study design

This study was conducted to develop a selective culture medium for *M. caseolyticus* that will help facilitate targeted studies and address the question of prevalence more systematically. As there have been reports of methicillin resistance in *M. caseolyticus*, we also investigated the antimicrobial susceptibility of these isolates. This was a collaborative study with researchers at the University Hospital of Münster, Germany. Bacterial isolate collection, antimicrobial susceptibility testing and selective culture medium creation was performed at the University of Saskatchewan. Confirmation of *M. caseolyticus* identity using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and molecular characterization of isolates was performed at the University Hospital of Münster.

3.3.2 Bacterial isolates

Nine *Macrococcus caseolyticus* isolates were used in this study. These isolates were cultured from imported seafood (clams, n=2), reptile (soft shell turtle, n=1) and amphibian (frog legs, n=6) meat products. All isolates grew on MHM agar and resembled *Staphylococcus* spp. The isolates were shipped on swabs in Aimes transport medium to Dr. Becker's lab at the University Hospital of Münster for molecular characterization.

3.3.3 Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed on all isolates by broth microdilution using the Sensititre system (Trek Diagnostics, Cleveland, OH). The GPALL1F plate format was used in this study according to the manufacturer's instructions. In brief, isolates were sub-cultured onto a 5% sheep blood agar plate and incubated overnight at 35°C. To prepare the inoculum, 1 –

2 colonies were suspended in 5ml of demineralized water to a 0.5 McFarland density. Thirty microliters of bacterial suspension was then added to 11ml of cation-adjusted Muller Hinton broth (Trek Diagnostics, OH, USA). Fifty microliters of inoculum was dispensed into the 96 well plate using the Sensititre auto-inoculator. Plates were sealed with adhesive film to prevent evaporation and incubated at 35°C for 18-24 hours. Antimicrobial MICs were interpreted, as susceptible or resistant, according to the CLSI guidelines for *Staphylococcus* spp. The clinical antimicrobial breakpoints used in this study are described in **Table 3.1**. For quality control, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were used.

Table 3.1: List of antibiotics with respective CLSI resistance breakpoints used in antibiotic susceptibility testing GPALL1F and CMV4AGNF* panels.

Antibiotic Class	Antibiotic	Tested Range	Resistance Breakpoints**
β-lactams	Ampicillin	0.12 – 8	≥ 0.25
	Penicillin	0.06 – 8	≥ 0.25
	Oxacillin + 2% NaCl	0.25 – 4	≥ 4
	Cefoxitin Screen	8	≥ 8
	Meropenem	0.06 – 4	≥ 4
Glycopeptides	Vancomycin	0.25 - 32	≥ 16
Streptogramin	Quinopristin-dalfopristin	0.5 – 4	≥ 4
Oxazolidinone	Linezolid	1 – 8	≥ 8
Lincosamide	Clindamycin	0.5 – 2	≥ 4
Macrolide	Erythromycin	0.25 – 4	≥ 8
Tetracyclines	Tetracycline	2 – 16	≥ 16
Sulfonamides	Trimethoprim-sulfamethoxazole	0.5/9.5 – 4/76	≥ 4/76
Ansamycins	Rifampin	0.5 – 4	≥ 4
Phenicol	Chloramphenicol	2 – 16	≥ 32
Quinolones	Ciprofloxacin	1 – 2	≥ 4
	Levofloxacin	0.25 – 4	≥ 4
	Moxifloxacin	0.25 – 4	≥ 2
Aminoglycosides	Gentamicin	2 -16	≥ 16

*****The CMV4AGNF panel was used to test meropenem susceptibility

**The resistance breakpoints presented here are those for *Staphylococcus* spp. from the Clinical and Laboratory Standards Institute (CLSI).

3.3.4 Determination of optimal growth conditions

Temperature, pH and salt gradients were performed to determine the optimal growth conditions for *M. caseolyticus*. First the optimal growth temperature was assessed. Each isolate was inoculated onto a 5% sheep blood agar plate and incubated at 5 different temperatures (35°C, 37°C, 39°C, 42°C and 45°C) for 18-20 hours. Growth at different pH and salt concentrations was determined by making 4 different 5% sheep blood agar plates and incorporating 0.5%, 5.0%, 7.5% and 10% NaCl. The pH of a smaller volume of each agar media was then adjusted to 6.0, 6.5, 7.0, 7.5 and 8.0. All isolates were inoculated onto each blood agar plate and incubated overnight at 35°C for 18-20 hours. Each plate was evaluated for growth.

3.3.5 CFU enumeration of *M. caseolyticus*

The concentration of *M. caseolyticus* was enumerated using a spiral plater. In brief, isolates were sub-cultured onto a 5% sheep blood agar plate and incubated overnight at 35°C. To prepare the inoculum, 1 – 2 colonies were suspended in 5ml of saline water to a McFarland 0.5 density. Using a spiral plater (Spiral Biotech Inc., Norwood, MA), a 1:200 dilution of the inoculum was plated on 5% sheep blood agar plates in triplicate and incubated overnight at 35°C. Colony numbers were counted on each plate and the average of the 3 plates was determined (**Figure 3.1**). The concentration of the stock inoculum was back calculated using the following formula: CFU/ml = (number of colonies x dilution factor x 1000) / (volume inoculated onto culture plate). The concentration of a *M. caseolyticus* inoculum at a density of McFarland 0.5 was 1.5×10^7 CFU/ml.

Figure 3.1: *Macrococcus caseolyticus* on 5% sheep blood agar inoculated using a spiral plater (1:200 dilution of McFarland 0.5 standard) for CFU enumeration.

3.3.6 Development of the selective medium

Seven media were selected and prepared to determine a suitable base medium, including: mannitol salt agar, trehalose salt agar, phenol red lactose agar, P agar, chapman stone agar, azide blood agar and colistin nalidixic acid blood agar. All isolates were cultured onto each medium and incubated overnight at 35°C. All plates were assessed for growth. Once a suitable base medium was chosen, the addition of two NaCl concentrations, 0.5% and 5.0%, were incorporated along with two combinations of β-lactam antibiotics (1) 0.5µg/ml meropenem (MP) and 0.5µg/ml ampicillin (AMP) and (2) 0.5µg/ml cloxacillin (CLX) and 1.0µg/ml ampicillin. Thus, a total of 4 selective media were prepared**.** All nine *M. caseolyticus* along with four quality control strains, *E. coli* ATCC 25422, *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212 and *P. aeruginosa* ATCC 27853 were inoculated onto the selective media and incubated overnight at 35°C. All plates were assessed for growth.

3.3.7 Challenge of the selective media

The selective media were challenged by spiking each *M. caseolyticus* isolates into a variety of samples (n=35) including feces (canine and feline), dirt, retail meat products (beef, chicken, pork, fish and shrimp) and mastitic milk samples. Canine (n=3) and feline (n=2) fecal samples were collected fresh on the day of challenge from healthy dog and cats owned by lab members. Dirt samples (n=5) were collected from various locations around Saskatoon, SK. Retail beef (n=3), chicken (n=3), pork (n=2), fish (n=1) and shrimp (n=1) products were purchased from local grocery stores in Saskatoon, SK. Frozen milk samples (n=15) were provided by Prairie Diagnostic Services (PDS) after confirmation of bacterial infection. Briefly, 0.5g of each fecal and dirt sample was homogenized with 5ml of tryptic soy broth (TSB). For the meat samples, 5g of each sample was dissected and rinsed with 50ml of buffered peptone water in a sterile bag; a 5ml aliquot was transferred into a sterile tube and incubated at 35°C for 18 hours. Milk samples were thawed at room temperature and a 5ml aliquot was transferred into a sterile tube. A McFarland 1.0 density of each *M. caseolyticus* isolates, with a known concentration of 3.0×10^7 CFU/ml, was spiked twice into each sample making a 1:10 and 1:100 final dilution. Ten microlitres of each dilution of spiked samples were inoculated onto the CNA blood agar with MP + AMP and CNA blood agar with CLX + AMP selective media and incubated at 35°C for 18 hours. Plates were assessed for growth of *M. caseolyticus*. Any breakthrough growth of bacterial organisms other than *M. caseolyticus* were further investigated. In brief, isolates were Gram stained and phenotypic identification was performed using colony morphology, biochemical tests (catalase, oxidase and coagulase) and antimicrobial susceptibility testing using broth microdilution as described above. PCR and DNA sequencing were performed for molecular identification of the bacterial isolates using primers for the *cpn*60 gene H729 (5'-CGCCAGGGTTTTCCCAGTCACGACGAIIIIGCI

GGIGAYGGIACIACIAC-3') and H730 (5'-AGCGGATAACAATTTCACACAGGAYKIYKIT CICCRAAICCIGGIGCYTT-3') with the underlined regions indicating the M13 sequencing primers (Brousseau et al., 2001).

3.4 Results

3.4.1 Colony morphology of *M. caseolyticus*

Macrococcus caseolyticus grows as non-hemolytic grey to cream 4 – 5 mm colonies on 5% sheep blood agar. Compared with *Staphylococcus pseudintermedius* and *Staphylococcus aureus* there are morphological similarities. *S. pseudintermedius* grows as greyish colonies 1 – 2 mm in diameter and is typically associated with a double zone of hemolysis, while *S. aureus* grows as grey-white to golden colonies 1 – 4 mm in diameter with β-hemolysis on 5% sheep blood agar. **Figure 3.2** shows a side by side comparison of colony similarities of *M. caseolyticus* and *S. pseudintermedius* on 5% sheep blood agar.

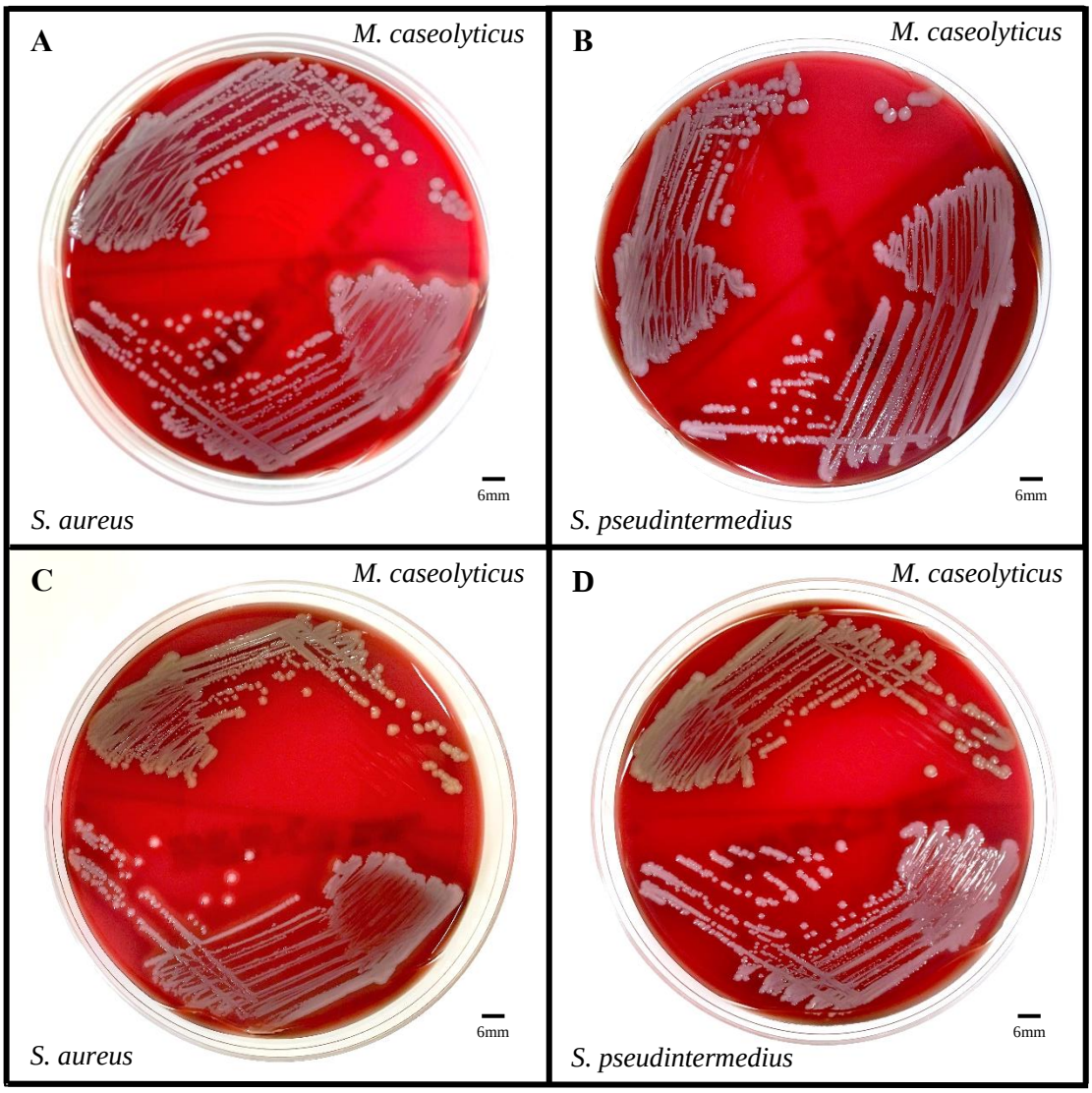

Figure 3.2: Colony morphology comparison between two species of staphylococci and *Macrococcus caseolyticus*. (A) Grey non-hemolytic *M. caseolyticus* colonies (4.0mm) are 2.5x larger than the white-grey hemolytic *S. aureus* colonies (1.6mm). (B) Grey non-hemolytic *M. caseolyticus* colonies (4.0mm) are 2x larger than the white-grey hemolytic S. *pseudintermedius* colonies (2.0mm). (C). Cream pigmented *M. caseolyticus* colonies (3.5mm) are 1.75x larger than the grey *S. aureus* colonies (2.0mm). (D) Cream pigmented *M. caseolyticus* colonies (3.5mm) are 2x larger than the white -grey *S. pseudintermedius* colonies (1.7mm).

3.4.2 Molecular characterization of *M. caseolyticus*

All nine isolates were analyzed with MALDI-TOF and identified as *Macrococcus caseolyticus*, having a score ≥ 1.700 and 2.000 (n=6) indicating probable genus identity and ≥ 2.000 (n=3) indicating secure genus and probable species identification. Isolates were also screened using primers for the 16S rRNA gene and all had 100% sequence identity to *Macrococcus caseolyticus* JCSC5402. All isolates were positive for the *mec*B gene.

3.4.3 Antimicrobial resistance profiles of *M. caseolyticus*

The antimicrobial minimum inhibitory concentrations of all 9 isolates were determined and interpreted based on CLSI guidelines for *Staphylococcus* spp. The MIC distributions of isolates to drugs categorized by class are presented in **Table 3.2**. Overall, there were no pan-susceptible isolates found and 100% (9/9) of isolates were resistant to ampicillin, penicillin, oxacillin, cefoxitin screening test and meropenem. Resistance to tetracycline was frequently observed in 77.8% (7/9) of isolates. Resistance to the quinolones (ciprofloxacin, moxifloxacin and levofloxacin), chloramphenicol and clindamycin was observed in 66.7% (6/9) of isolates. The frequency of resistance to rifampicin, erythromycin, gentamicin, and trimethoprim-sulfamethoxazole was 44.4% (4/9), 55.6% (5/9), 44.4% (4/9) and 33.3% (3/9) of isolates respectively. One isolate was resistant to the antibiotic linezolid. The resistance profiles of isolates are presented in **Table 3.3**.

Table 3.2: Minimum inhibitory concentration distribution of *Macrococcus caseolyticus* (n=9) from imported meat products.

Drug Class	Antibiotic	0.06	0.12	0.25	0.50	1	2	4	8	16	32	%R Isolates (n=9)	%R Samples (n=9)
	Ampicillin							2	7			100 (9)	100 (9)
	Penicillin								9			100 (9)	100 (9)
β-lactams	Oxacillin							9				100 (9)	100 (9)
	Cefoxitin								9			100 (9)	100 (9)
	Meropenem							9				100 (9)	100 (9)
Glycopeptides	Vancomycin			1	8							0 (0)	0 (0)
Streptogramin	Quinopristin-dalfopristin				4	3	2					0 (0)	0 (0)
Oxazolidinone	Linezolid						8		1			11.1 (1)	11.1 (1)
Lincosamide	Clindamycin				3			6				66.7 (6)	66.7 (6)
Macrolide	Erythromycin			3				1	5			55.6 (5)	55.6 (5)
Tetracyclines	Tetracycline						2			7		77.8 (7)	77.8 (7)
Sulfonamides	Trimethoprim-sulfamethoxazole				5	1		3				33.3 (3)	33.3 (3)
Ansamycins	Rifampin				5			4				44.4 (4)	44.4 (4)
Phenicol	Chloramphenicol							3			6	66.7 (6)	66.7 (6)
	Ciprofloxacin					2	1	6				66.7 (6)	66.7 (6)
Quinolones	Levofloxacin			2	1			6				66.7 (6)	66.7 (6)
	Moxifloxacin			2	1			6				66.7 (6)	66.7 (6)
Aminoglycosides	Gentamicin						4		1	4		44.4 (4)	44.4 (4)

Cells corresponding to the antimicrobial concentrations for each drug tested are in white and resistance breakpoints are denoted by the dark bars. The number of isolates inhibited at each concentration are noted in each cell. %R Isolates indicates the number of resistant isolates, while %R Samples indicates the number of samples from which an isolate resistant to each drug was isolated.

Table 3.3: Antimicrobial resistance profiles from *Macrococcus caseolyticus* isolates (n=9).

ID	Sample	Antibiotic Resistance Profile
AM08CR03	Soft shelled turtle	AMP + PEN + OXA + FOX + MP + CLI + ERY + TET + RIF + CIP + LEVO + MXF
AM20CR01	Clams	AMP + PEN + OXA + FOX + MP
AM22CR01	Clams	AMP + PEN + OXA + FOX + MP
14ON049C	Frog legs	AMP + PEN + OXA + FOX + MP + CLI + ERY + TET + CHL + GEN
BR032A-c	Frog legs	AMP + PEN + OXA + FOX + MP + LZD + CLI + ERY + TET + RIF + CIP + LEVO + MXF
BR033A-a	Frog legs	AMP + PEN + OXA + FOX + MP + TET + CHL + CIP + LEVO + MXF + GEN
BR043A-a	Frog legs	AMP + PEN + OXA + FOX + MP + CLI + ERY + TET + SXT + RIF + CHL + CIP + LEVO + MXF + GEN
BR045A-a	Frog legs	AMP + PEN + OXA + FOX + MP + CLI + ERY + TET + SXT + CIP + LEVO + MXF
BR047A-b	Frog legs	AMP + PEN + OXA + FOX + MP + CLI + TET + SXT + RIF + CHL + CIP + LEVO + MXF + GEN

Ampicillin (AMP), penicillin (PEN), oxacillin (OXA), cefoxitin (FOX), meropenem (MP), linezolid (LZD), clindamycin (CLI), erythromycin (ERY), tetracycline (TET), trimethoprim/sulfamethoxazole (SXT), rifampin (RIF), ciprofloxacin (CIP), levofloxacin (LEVO), moxifloxacin (MXF), chloramphenicol (CHL) and gentamicin (GEN)

3.4.4 Optimal growth of *M. caseolyticus*

To determine the conditions that would allow for the optimum growth of *M. caseolyticus*, temperature, pH and salt concentration gradients were performed. Growth and colony diameter were assessed at 24 hours. All isolates were able to grow at temperatures from 35°C to 45°C, at salt concentrations from 0.5% to 10% and at pH 6.5 to 7.5. Colony diameter was maintained at all temperatures. However, it was observed that the incorporation of a higher concentrations of salt (7.5% to 10%) restricted growth as seen by smaller colony diameter and fewer colonies as compared to the addition of lower salt concentrations (0.5% to 5%).

Table 3.4: Summary of temperature optimization gradient for *M. caseolyticus* isolates. A description of growth or no growth is noted for each temperature tested.

Temperature	Growth (Pos/Neg)
35°C	Pos – well defined colonies, colony diameter maintained
37°C	Pos – well defined colonies, colony diameter maintained
39°C	Pos – well defined colonies, colony diameter maintained
42°C	Pos – well defined colonies, colony diameter maintained
45°C	Pos – well defined colonies, colony diameter maintained

*Pos = Growth; Neg = No growth

Table 3.5: Summary of salt concentration and pH gradients for *M. caseolyticus*, including a description of growth or no growth noted at each concentration and pH after incubation at 35°C.

	pH				
Salt Concentration	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**
0.5%	Neg	Pos – well defined colonies	Pos – well defined colonies	Pos – well defined colonies	Neg
5.0%	Neg	Pos – small colonies	Pos – small colonies	Pos – small colonies	Neg
7.5%	Neg	Pos – small colonies	Pos – small colonies	Pos – small colonies	Neg
10.0%	Neg	Pos – Only a few small colonies in first streak	Pos – Some growth in first streak, small colonies	Pos – Only a few small colonies in first streak	Neg

*Pos = Growth; Neg = No growth

3.4.5 Selective media

Of the seven initial selective media prepared and inoculated with *M. caseolyticus*, growth was observed on all media (**Table 3.6**). However, acid production from the fermentation of carbohydrates (mannitol, trehalose and lactose) was not observed by these isolates. Thus, they would not act as a useful differential indicator. Also, the notable clear zone surrounding *S. aureus* colonies grown on CSA was not observed with *M. caseolyticus.* The incorporation of sodium azide to blood agar lowered the growth rate of all *M. caseolyticus* isolates, resulting in small colonies. *M. caseolyticus* grew well on colistin nalidixic acid blood agar. The incorporation of colistin and nalidixic acid inhibits Gram-negative bacteria, acting as a suitable initial base medium that could be built upon. Therefore, colistin nalidixic acid blood agar was chosen as the base medium. Four variations of selective media were prepared including: CNA blood agar with MP + AMP and CNA blood agar with CLX + AMP at two salt concentrations (0.5% and 5%), incubated at 35°C . All *M. caseolyticus* isolates were able to grow on the selective media with 0.5% NaCl. However, not all isolates grew on the selective media with the higher (5%) NaCl concentration. Therefore, only the selective media with 0.5% NaCl were used in the selective media challenge with the incubation temperature of 35°C. Four ATCC quality control isolates were inoculated onto the selective media to determine if the media would inhibit the growth of Gram-negative and other Gram-positive bacteria; *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis* were inhibited (**Table 3.7**).

Table 3.6: Description of seven media prepared and inoculated with *M. caseolyticus*, including the indications for use, unique feature and whether growth was observed.

	Indication	Unique Feature	Growth (pos/neg)
Differential Media			
Mannitol salt agar (MSA)	For the determination of a microorganism to ferment mannitol	Positive fermentation = yellow zone around colonies	Pos – well defined colonies, no fermentation
Trehalose salt agar (TSA)	For the determination of a microorganism to ferment trehalose	Positive fermentation = yellow zone around colonies	Pos – well defined colonies, no fermentation
Phenol red lactose agar (PRL)	For the determination of a microorganism to ferment lactose	Positive fermentation = yellow zone around colonies	Pos – well defined colonies, no fermentation
Selective Media			
P agar (PA)	For cultivation of Staphylococci	No special feature	Pos – well defined colonies
Chapman stone agar (CSA)	For the isolation of Staphylococci	Gelatinase activity = clear zone around colonies	Pos – well defined colonies
Azide blood agar (AzBA)	For the isolation of Streptococci and Staphylococci from mixed bacterial samples	Contains sodium azide to inhibit Gram-negatives	Pos – small colonies
Colistin nalidixic acid blood agar (CNA BA)	Supports the growth of Staphylococci, Streptococci and Enterococci	Contains two antibiotics (colistin and nalidixic acid) to inhibit Gram-negatives	Pos – well defined colonies

Table 3.7: Summary of growth of *M. caseolyticus* and four quality control strains on colistin nalidixic acid (CNA) blood agar with meropenem 0.5µg/ml + ampicillin 0.5µg/ml and CNA blood agar with cloxacillin 0.5µg/ml + ampicillin 1.0µg/ml.

Isolate	Base Medium + Antibiotic Combination	
	CNA BA+ 0.5µg/ml MP + 0.5µg/ml AMP	**CNA BA + 1.0µg/ml AMP + 0.5µg/ml CLX**
14ON049C	Growth	Growth
AM08CR03	Growth	Growth
AM20CRO1	Growth	Growth
AM22CR01	Growth	Growth
BRO32A-c	Growth	Growth
BR033A-a	Growth	Growth
BR043A-a	Growth	Growth
BR045A-a	Growth	Growth
BR047A-b	Growth	Growth
Quality Control Strains		
***E. coli* ATCC 25422**	No Growth	No Growth
***P. aeruginosa* ATCC 27853**	No Growth	No Growth
***S. aureus* ATCC 29213**	No Growth	No Growth
***E. faecalis* ATCC 29212**	No Growth	No Growth

3.4.6 Selective media challenge

To confirm that the selective media could identify *Macrococcus caseolyticus* in a sample with a mixture of bacteria, a challenge study was carried out using fecal, dirt, retail meat and mastitic milk samples with a known concentration of *M. caseolyticus* added. Both selective mediums enabled the growth of each *M. caseolyticus* isolate while inhibiting Gram-negative and most Gram-positive bacteria. Breakthrough growth was observed on both selective media, but a higher occurrence on the CNA blood agar with CLX + AMP. Breakthrough growth isolates were Gram-positive cocci with smaller colony diameters (1.0 to 2.0mm) than that of *M. caseolyticus* (4.0mm). Antimicrobial susceptibility testing using broth microdilution identified higher MICs for ampicillin, oxacillin (cloxacillin) and meropenem then what was added to the media. The isolates were identified as species from the genus *Enterococcus* and *Staphylococcus* (**Table 3.5**).

Table 3.8: Summary of breakthrough growth isolated from colistin nalidixic acid (CNA) blood agar with meropenem 0.5µg/ml + ampicillin 0.5µg/ml and CNA blood agar with cloxacillin 0.5µg/ml + ampicillin 1.0µg/ml with *Macrococcus caseolyticus* included for comparison.

Bacterial Species	**Colony Morphology**	**Minimum Inhibitory Concentration (µg/ml)**		
		Ampicillin	**Oxacillin**	**Meropenem**
Macrococcus caseolyticus	4.0mm, round, cream, non-hemolytic	8.0	≥ 4.0	4.0
Enterococcus faecalis	1.0mm, round, white, hemolytic	1.0	≥ 4.0	4.0
Enterococcus sp.	1.0mm, round, white, hemolytic	2.0	≥ 4.0	≥ 4.0
Enterococcus gilvus	1.0mm, round, white, hemolytic	1.0	≥ 4.0	4.0
Staphylococcus warneri	2.0mm, round, white, non-hemolytic	8.0	≥ 4.0	1.0

3.5 Discussion

Staphylococcus aureus, an important foodborne pathogen, is responsible for food outbreaks and is a causative agent of food poisoning due to the production of heat-stable enterotoxin A (Argudín et al., 2010; Hennekinne et al., 2012; Kadariya et al., 2014). Meat producing animals and food products can become contaminated through infected environmental sources and by food handlers during processing and preparation (Kadariya et al., 2014). *S. aureus* is vulnerable to destruction by heating and sanitization agents, thus the presence is indicative of poor food hygiene or sanitation (Hennekinne et al., 2012). The biggest concern with this organism is the incidence of methicillin resistance (Oniciuc et al., 2017). In this study, bacterial colonies resembling *Staphylococcus aureus,* cultured on MSA and MHM, were isolated from imported seafood, reptile and amphibian meat samples. These isolates were meropenem and oxacillin resistant and identified as methicillin resistant *Macrococcus caseolyticus*, possessing the *mec*B gene.

Most species of *Macrococcus* are sensitive to oxacillin, however, methicillin resistance has been reported in *M. caseolyticus* and *M. canis* (Kloos et al., 1998; Baba et al., 2009; Cotting et al., 2017; Schwendener et al., 2017). Methicillin resistance in *M. caseolyticus* is mediated by the *mec*B and the newly identified *mec*D genes (Tsubakishita et al., 2010; Gómez-Sanz et al., 2015; Cotting et al., 2017; Schwendener et al., 2017). Similar to the *mec*A gene in methicillin resistant staphylococci, the *mec*B gene has been found within the chromosome as part of a mobile Staphylococcal Cassette Chromosome (SCCmec)-like element (Tsubakishita et al., 2010). Most concerning is the identification of plasmid-mediated *mec*B, that has the ability to transfer to more pathogenic bacteria. This was observed by Becker et al. (2018) with the identification of a plasmid-mediated *mec*B-positive MRSA isolate; genetic analysis of the plasmid revealed similarity to a previously described *mec*B containing *M. caseolyticus* plasmid. Also concerning is the *mec*D gene,

most closely related to *mec*B, which encodes resistance to all classes of β-lactams including the anti-MRSA cephalosporins (Schwendener et al., 2017). As of yet, there have been no strains of methicillin resistant staphylococci containing this gene, however, there is a potential for dissemination as it was located on a genomic island containing site-specific integrases with characteristics resembling SCCmec elements (Schwendener et al., 2017).

M. caseolyticus has been found in numerous food animals and associated products. This species was first identified in cow's milk in 1916 and was characterized by its ability to peptonize the milk, thus giving the name *caseolyticus*, meaning casein-dissolving (Mazhar et al., 2018). Since then, methicillin susceptible *M. caseolyticus* have been identified from pigs, goose eggs, sausage and prawns (Wang et al., 2012; Karani et al., 2015; Hansen et al., 2015; Geniş and Tuncer, 2018). While methicillin resistant *M. caseolyticus* have been isolated from bovine skin, mastitic milk, broiler chickens, retail chicken meat, cheese, and bulk milk tanks (Baba et al., 2009; Cicconi-Hogan et al., 2014; Schwendener et al., 2017; Li et al., 2018; MacFadyen et al., 2018).

In this study, a culture medium was developed to selectively isolate *M. caseolyticus*. *M. caseolyticus* has similar colony morphology as staphylococci on blood agar but are larger in size. Thus, the incorporation of 5% defibrinated sheep blood was used in the selective medium for phenotypic identification based on colony morphology. Antimicrobial susceptibility profiles of *M. caseolyticus* isolates were used to determine which antimicrobials to include in the culture medium. The addition of penicillins, carbapenem, polymyxin and quinolone antimicrobials to the medium allowed for the inhibition of Gram-negative bacteria and other Gram-positive bacteria. Therefore, two selective media were prepared: colistin nalidixic acid (CNA) blood agar with meropenem 0.5µg/ml + ampicillin 0.5 µg/ml and CNA blood agar with cloxacillin 0.5 µg/ml + ampicillin 1.0 µg/ml. Both selective media inhibited the growth of *E. coli*, *P. aeruginosa* and *S.*

aureus but not *E. faecalis* control strains, while *M. caseolyticus* grew well on these media and were easily differentiated from *E. faecalis* based on colony morphology.

All nine *M. caseolyticus* isolates grew well on the selective media when spiked into challenge samples. There was considerably more growth with the 1:10 dilution versus the 1:100 dilution; regardless growth occurred at both concentrations. Breakthrough growth was observed on plates inoculated with the meat samples. Isolates were identified as *Enterococcus* spp. and *Staphylococcus warneri*. The colony morphology of the *Enterococcus* spp, were easily differentiated from *M. caseolyticus*, as they were smaller in size and hemolytic. While *Staphylococcus warneri* was only distinguishable based on smaller colony diameter. The *Enterococcus* spp. and *S. warneri* had higher MICs for ampicillin, oxacillin and meropenem which allowed for growth on both selective mediums. This challenge study confirmed that both mediums could be used for the selective isolation of *M. caseolyticus*. However, of the two media, CNA blood agar with MP + AMP had less breakthrough growth, only *Enterococcus* spp. grew, and thus may be more selective than CNA blood agar with CLX + AMP.

3.6 Acknowledgements

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4 General Discussion and Conclusion

The presence of antimicrobial resistant bacteria from imported culinary reptile and amphibian meat products were investigated. Specifically, we examined the occurrence of extended spectrum β-lactamase, AmpC β-lactamase, carbapenemase and mobile colistin resistance in isolated Gram-negative bacteria. Additionally, we investigated the antimicrobial susceptibility of a Gram-positive bacteria, *M. caseolyticus*, that had phenotypic characteristics similar to methicillin resistant *S. aureus*. Finally, we developed a culture media for the selective isolation of *M. caseolyticus*.

We identified seven genera of Enterobacteriaceae from 77% (41/53) of the reptile and amphibian meat samples. *Salmonella* was isolated exclusively from dried geckos (11.3% (6/53) of total samples) and were not resistant to any of the antimicrobials tested. Of the five serotypes identified, Poona and Urbana have been associated with Salmonellosis outbreaks from fresh produce and pet turtles, while Weltevreden has been associated with outbreaks from seafood and aquaculture in Southeast Asia (Ponce et al. 2008; Jackson et al. 2013; Walters et al. 2016; Makendi et al. 2016). *E. coli* was recovered from 35.8% (19/53) of samples, with eleven isolates showing resistant phenotypes; 91% (10/11) were cultured from soft shell turtles while the other was isolated from frog legs. All eleven isolates produced β-lactamase enzymes. Of those, 81.8% (9/11) were ESBLs (CTX-M-type) and 18.2% (2/11) were AmpC (CMY-61) β-lactamases. Two isolates (18.2%) co-produced multiple resistance genes including, one isolate with CMY-61 and a penicillinase (TEM-33) and one isolate with CTX-M-55 and the mobile colistin resistance gene, *mcr*-1. The other five genera of Enterobacteriaceae were isolated from 30.2% (16/53) of the samples and consisted of *Klebsiella* (9.4%), *Serratia* (7.5%), *Citrobacter* (5.7%), *Enterobacter* (5.7%) and *Kluyvera* (1.9%). Seventeen isolates possessed β-lactamase enzymes with 15 (88.2%) ESBLs (CTX-M-type, SHV-type, OXY-type and FONA-type), 1 (5.9%) AmpC (CMY-98) β-

lactamases and 1 (5.9%) penicillinase (TEM-33). Similarly, 3 (17.6%) isolates co-produced two resistance genes including, one *Citrobacter* sp. producing CTX-M-1 and CMY-83, one *Enterobacter* sp. with SHV-12 and TEM-206 and another *Enterobacter* sp. possessing CTX-M-2 and TEM-33. Of the CTX-M-type ESBLs identified in this study, CTX-M-55, -83 and -27 are found in Asia, while CTX-M-15 is the most common variant found throughout the world (Zhao and Hu 2013; Tong et al. 2015). The OXY and FONA-type enzymes are species specific β-lactamases from the organisms *Klebsiella oxytoca* and *Serratia fonticola* respectively, which are biochemically indistinguishable from ESBLs and have thus been placed into subgroup 2be of the Bush-Jacoby-Medeiros functional classification scheme (Bush et al. 1995; Gniadkowski 2001; Blaak et al. 2014). Finally, one carbapenemase-producing Gram-negative non-fermenter, an *Acinetobacter* sp. isolated from dried turtle carapace, possessed the $bla_{\text{NDM-1}}$ gene and exhibited multidrug resistance.

Resistance surveillance programs have been implemented in many countries around the world, where they are designed to detect the emergence of resistance in food (Morrison and Rubin, 2015). However, only major agricultural animal species and meats such as pork, poultry and beef are targeted with the focus on potentially pathogenic indicator organisms such as *E. coli*, *Salmonella*, and *Campylobacter* spp. (OIE, 2004; NARMS, 2015; Morrison and Rubin, 2015; DANMAP, 2017; Government of Canada, 2017). *E. coli* has commonly been used as an indicator organism for antimicrobial resistance for other taxa of the gastrointestinal microbiota, as it has a high recovery rate in bovine, poultry and swine (Franklin et al., 2001; Zhao et al., 2012; Government of Canada, 2017). However, previous studies investigating antimicrobial resistance in imported seafood found *E. coli* recovery rates of less than 10% (Ryu et al. 2012; Wang et al. 2012). Gordon and Cowling (2003), investigated the distribution of *E. coli* in 2300 live non-domesticated

vertebrates including mammals, birds, reptiles, amphibians and fish; observing an *E. coli* recovery rate between 2-33% in reptiles (depending on species), 12% in frogs and 10% in fish. Similarly, in our study, the recovery rate of *E. coli* from reptile products was 34%, with 18.9% from whole un-gutted and gutted soft shell turtle, 9.4% from whole gutted dried gecko and 5.7% from whole gutted dried snake. However, there was only a 1.8% recovery rate of *E. coli* from frog legs which is not surprising as these products were not packaged as whole animals but only as skinned legs. This suggests that *E. coli* may be more adapted to mammalian gastrointestinal tracts and may not be suitable indicator organisms in non-mammalian vertebrates.

In our second study, antimicrobial susceptibility testing was performed on *Macrococcus caseolyticus* isolates, cultured on carbapenem-selective media from imported seafood, reptile and amphibian meat products. All isolates were resistant to the β-lactam class of drugs mediated by *mec*B, the methicillin resistance conferring gene, while 77.8% of isolates were resistant to tetracycline and 66.7% were resistant to the quinolones. *M. caseolyticus* has been identified from animal, meat and dairy sources, but the overall dissemination and prevalence is largely unknown (Mazhar et al., 2018). *M. caseolyticus* is not thought to cause infection in humans but may have a potential as a veterinary pathogen as it has been associated with multiple animal infections (de la Fuente et al., 1992; Gómez-Sanz et al., 2015; Cotting et al., 2017; Schwendener et al., 2017). Of great concern, is the close ecological relationship of *M. caseolyticus* and staphylococci as commensal bacteria and the transmission of plasmid-mediated methicillin resistance (*mec*B gene) to pathogenic *S. aureus* (Becker et al., 2018). This demonstrates a novel risk of methicillin resistance transmission between staphylococci of unknown magnitude that warrants further investigation.

The results from this surveillance investigation revealed the presence of extended spectrum β-lactamase, AmpC β-lactamase, carbapenemase, mobile colistin resistance and methicillin resistance genes from imported meat products. These niche market foods are not routinely targeted by resistance surveillance programs; thus, representing a potential public health hazard that warrants further investigation.

4.1 Limitations of the study

4.1.1 Small sample size of imported reptile and meat products tested

Unfortunately, there was a limited variety of different imported reptile and amphibian meat products and of those available most were from the same distributor. Thus, a smaller sample size was tested in favor of a variety of products from different distributors and regions. Regardless of the sample size, numerous multidrug resistant bacteria with identifiable resistance genes were found. However, a larger sample size would improve the likelihood that the results observed in this study were not due to chance.

4.1.2 Selective culture media

Imported reptile and amphibian meat products were cultured on selective media. This allowed us to systematically identify specific pathogens, such as *Salmonella*, and phenotypically detect ESBL and carbapenemase producers. However, we may have missed other potential foodborne bacterial pathogens by using only selective media. The use of antimicrobials as selective agents may have inhibited susceptible pathogenic bacteria, thus not enabling their identification. Also, many aquatic and environmental bacteria have different nutrient and growth requirements compared to many enteric bacteria, which makes it tremendously difficult to culture them in general and nearly impossible for them to grow on our selective culture media.

4.1.3 Interpretation of antimicrobial susceptibility results from *M. caseolyticus* isolates

Clinical breakpoints have been published for many genera and species of bacteria, however, there are no specific guidelines for *Macrococcus* species. Therefore, the antimicrobial MICs for *M. caseolyticus* was interpreted using *Staphylococcus* genus-specific clinical breakpoints. Although macrococci are closely related to staphylococci they are not 100% identical, and the clinical breakpoints may be different.

4.2 Future directions

This study provides the first description of the presence of antimicrobial resistant bacteria with narrow and broad spectrum β-lactamases, mobile colistin resistance and methicillin resistance, from imported culinary reptile and amphibian meat products. Performing similar studies in other food products not investigated by antimicrobial resistance surveillance programs would help to better understand the epidemiology of antimicrobial resistance and identify other underappreciated sources of resistance. Future studies to identify at what stage of the production cycle (on farm, at slaughter, during processing/packaging or at the retail level) resistant organisms enter food products would be beneficial to inform evidence-based policy to control the spread of antimicrobial resistance. Furthermore, determining the sequence types and genetic relatedness among the different Enterobacteriaceae isolates would help identify transfer patterns of resistance genes. Moreover, it would also be interesting to compare bacterial recovery rates from reptile and amphibian meat products at different points during cooking and medicinal preparation, to help determine food safety hazards as there are no universal internal cooking temperatures to ensure bacterial cell death and many products may be consumed raw. Finally, performing targeted studies utilizing the *Macrococcus* selective culture medium may help to determine the prevalence of methicillin resistant *M. caseolyticus* in animals and food.

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