Characterization of porcine AIDA-I adhesin and its receptors

A Thesis Submitted to the College
of Graduate Studies and Research in
Partial Fulfillment of the Requirements
for the Degree of Master of Science in
the Department of Veterinary Pathology
University of Saskatchewan
Saskatoon, Saskatchewan

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ABSTRACT

A relatively high percentage of porcine *Escherichia coli* isolates from cases associated with neonatal and post-weaning diarrhea are positive for the gene encoding the adhesin involved in diffuse adherence I (AIDA-I). This gene and its corresponding protein were first identified and characterized in *E. coli* strain 2787 isolated from human infantile diarrhea. Little is known about the role of the AIDA-I protein in pathogenesis of porcine enteric disease caused by AIDA-I positive *E. coli* and the properties of AIDA-I protein expressed by porcine AIDA-I positive *E. coli* isolates and its receptors.

In this study, we demonstrated that AIDA-I adhesin isolated from porcine AIDA-I positive *E. coli* PD20 and PD58 is an acidic protein consisting of five isoforms. It has a molecular weight (100 kDa) similar to the AIDA-I adhesin expressed by human AIDA-I positive *E. coli* strain 2787 and has a relatively high amino acid homology (78-87%) with it. Immunodetection of AIDA-I positive *E. coli* strains using polyclonal anti-AIDA-I antibodies had relatively low sensitivity and specificity, accordingly these tests are unlikely to be used for regular diagnostic detection.

Using affinity chromatography, we isolated from porcine intestinal mucus proteins that bind to purified AIDA-I adhesin. These proteins were separated by one- and two-dimensional electrophoresis and subjected to overlay Western blot with purified AIDA-I adhesin and AIDA-I positive *E. coli* to demonstrate 65 and 120 kDa (p65 and p120) proteins as AIDA-I binding proteins. The identity of p65 was not determined based on LCMS/MS data, whereas p120 was matched to two nuclear proteins (namely, DNA damage binding protein and splicing factor 3b) and one cytoplasmic protein, which is an IgG Fc binding protein. Based on similar amino acid homology, molecular weight,

structural similarity to mucin and reported evidence of being secreted by goblet cells into the intestinal lumen, we think that the IgG Fc binding protein is the most likely candidate to serve as a potential receptor in intestinal mucus for AIDA-I adhesin.

ACKNOWLEDGEMENTS

I deeply understand that this work would not have been possible without guidance from a great group of people during my MSc graduate study. First of all, I would like to thank the members of my advisory committee Drs. Elemir Simko, Dorothy M. Middleton, and Musangu Ngeleka. My primary supervisor, Dr. Simko, has been a great mentor to guide me in numerous ways to finish my MSc program and I thank him for his constant encouragement, patience, support and friendship. I am extremely grateful to be equipped with all the knowledge under his direction after finishing my program, just like a bird to grow up with strong wings and ready to fly high in the future. I believe that his valuable guidance and extremely strong support will benefit the rest of my life. I thank Dr. Middleton and Dr. Ngeleka for their wonderful guidance, teaching, and suggestions. They are very open and helpful at any time when I need. Their support has played a key role to my successes. I would like to extend my thanks to Dr. Andrew Allen and Dr. Marion Jackson, as graduate chairs, for their help with the administrative aspect of my MSc program.

I give my thanks to the Department of Veterinary Pathology for providing me with this great opportunity to study and research at the University of Saskatchewan. I am really thankful to all the members of the department (faculty, stuff to graduate students) for their support during my study. I thank all people in necropsy room, PDS Microbiology Laboratory, and WCVM library staff. I give my special thanks to Brenda Trask for her friendship and help, Jan, Sandy, and Betty in the general office of the department, and Ian Shirley and Jennifer Cowell for their technical support in daily life. I thank Drs John Gordon and Vikram Misra, Department of Veterinary Microbiology, for their ideas and

suggestions during my research. I also thank my colleagues, Farshid Shahriar, Madhu Ravi, and Yanyun Huang for their technical support, encouragement and friendship.

Importantly, I thank all funding agencies, Saskatchewan Agriculture Development Fund (SADF-20020032), Alberta Livestock Industry Development Fund (ALIDF-2003L010R), Sask Pork (2002-09), Saskatchewan Health Research Foundation and Canadian Research Network on Bacterial Pathogens of Swine (CRNBPS) for their support of this research and provision of a personal stipend**.**

Lastly, I would like to thank all my friends who give me support and encouragement in my life. I give my special thanks to Guojian Wei and his family, Enwu Liu and his family. I know it would have been hard to finish my study without my family's great love and support. I thank my parents, Jingzhao Fang and Shujuan Ge, and all other members of my family. Lastly but not least, I deeply thank my wife Lanying Wang and my son Jiarui Fang for their love and patience for all these years.

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LIST OF ABBREVIATIONS

AA	aggregative adherence
AAF	aggregative adherence fimbriae
A/E	attaching and effacing
AEEC	attaching and effacing *E. coli*
eae	attaching and effacing gene
AIDA	adhesin involved in diffuse adherence
CR	coagglutination reagent
CV	column volume
DA	diffuse adherence
DAEC	diffusely adhering *E. coli*
ED-PWD	edema disease and post-weaning diarrhea
EM	electron microscopy
EAEC	enteroaggregative *E. coli*
EAF	EPEC adherence factor
EAST1	enteroaggregative *E. coli* heat-stable enterotoxin
EHEC	enterohemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
ELISA	Enzyme-Linked ImmunoSorbent Assay
E. coli	*Escherichia coli*
FBS	fetal bovine serum

LT	heat labile enterotoxin
ST	heat-stable enterotoxin
IPG	immobilized pH gradient
IEF	isoelectric focusing
pI	isoelectric point
Gp119	119 kDa glycoprotein
LCMS	liquid chromatography mass spectrometry
LA	localized adherence
LEE	locus of enterocyte effacement
MS	mass spectrometry
MW	molecular weight
1D	One-dimensional
ORF	open reading frame
paa	porcine A/E associated gene
PBS	phosphate-buffered saline
PBST	PBS Tween-20
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PWD	post-weaning diarrhea
Stx2e	shiga toxin 2e
SDS	sodium dodecylsulfate
2D	Two-dimensional

1. Literature review

Escherichia coli (*E. coli*) is an important pathogen associated with a variety of human and animal diseases. The majority of *E. coli* are harmless intestinal commensals; however, some are pathogenic and have been regularly recognized as causative agents of these diseases (Gyles and Fairbrother, 2004). Basically, infections due to pathogenic *E. coli* can be divided into three categories based on the clinical syndromes: i) diarrheal disease, ii) sepsis/meningitis, and iii) urinary tract infection (Nataro and Kaper, 1998). Of these, diarrheal disease is the most common and economically important form in humans and animals throughout the world.

Based on pathogenic mechanism, diarrheagenic *E. coli* can be distinguished and divided into six major pathotypes: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adhering *E. coli* (DAEC), which is a newly designated group (Donnenberg and Nataro, 1995; Torres et al., 2005). Among these, ETEC remains the most prevalent cause of porcine diarrhea (Gyles and Alexander, 1994; Hampson, 1994).

1.1. Diarrheagenic *E. coli* in pigs

1.1.1. Enteric diseases of pigs caused by diarrheagenic *E. coli*

Diarrhea caused by *E. coli* is one of the most common diseases in pigs and enteric colibacillosis is a major cause of economic loss in the swine industry because of high

morbidity and mortality (Tubbs and Hill, 1993; Vaillancourt and Tubbs, 1992). Neonatal diarrhea and postweaning diarrhea (PWD) are the two most important enteric diseases of pigs caused by *E. coli*. Neonatal diarrhea usually occurs during the first 3-5 days of life and in PWD diarrhea develops 3-10 days after weaning. The pathogenic serotypes in neonatal diarrhea were first identified in the late 1950s and 1960s (Gyles and Alexander, 1994) and in PWD in 1960s (Hampson, 1994). The major pathotype of *E. coli* in both neonatal diarrhea and PWD is ETEC, while EPEC are also recognized as a cause of PWD (Gyles and Fairbrother, 2004).

ETEC have long been recognized as the main etiological agents in neonatal diarrhea, although some other agents may cause or contribute to diarrhea in newborn pigs (Glock, 1981). Neonatal diarrhea has been investigated by many researchers from different countries. During this process, two important findings led to great progress in the pathogenesis of *E. coli* diarrhea, one being the discovery of enterotoxins produced by *E. coli* strains (Parry and Rooke 1985) and the other, the role of the F4 (K88) antigen in intestinal colonization of *E. coli* through adherence to epithelial cells (Gibbons et al., 1975). A variety of virulence factors have since been identified to be associated with porcine diarrheagenic *E. coli* (Evans et al., 1986; Gyles and Fairbrother, 2004; Nakazawa et al., 1987). Through the use of colostrum-deprived specific pathogen free pigs and gnotobiotic pigs to test the role of virulence factors in porcine diarrhea, it has been found that all diarrheagenic *E. coli* follow the same general scheme to cause diarrhea, namely, colonization of mucosal surface through fimbrial or non-fimbrial adhesins, evasion of host defences, and production of enterotoxin(s) (Gyles and Alexander, 1994; Nataro and Kaper, 1998).

1.1.2. The genus *E. coli*

1.1.2.1. Characteristics of *E. coli*

Briefly, *E. coli* is a member of the family *Enterobacteriaceae*, and is a facultative anaerobe of human and animal colonic flora. *E. coli* grow readily on simple culture media and synthetic media, and utilize carbon and nitrogen sources for their metabolic and energy needs. *E. coli* cultured on lactose-containing MacConkey's medium form large pink colonies, a property which may be used to identify *E. coli* isolates (Gyles and Fairbrother, 2004). Along with bacterial morphology, this characteristic of lactose-fermentation is frequently used to identify organisms as 'coliform', but further biochemical tests are required for definitive identification.

E. coli can be differentiated into various types based on antigenic components within the cell wall. Lipopolysaccharide (O-antigen) is present in the wall of all *E. coli* as Gram-negative organisms, whereas capsular (K) antigens, flagellar (H) antigens and adhesins (fimbriae or pili) are variably present in different types of *E. coli*. A total of 175 O antigens and 53 H antigens in *E. coli* are identified currently (Gyles and Fairbrother, 2004). Fimbrial and non-fimbrial adhesins are very important virulence factors in *E. coli* infections in humans and animals. *E. coli* produce many different types of adhesins and most are expressed specifically in strains linked to particular diseases. The documented role of adhesins is mediation of bacterial adherence to the epithelial cell surface (Evans et al., 1984; Sellwood, 1984; Torres et al., 2005)

1.1.2.2. Characteristics of porcine diarrheagenic *E. coli*

ETEC produce specific fimbrial adhesins and enterotoxins to cause diarrhea. The most common, and the first identified fimbrial adhesin on porcine ETEC, is F4, originally

called K88. F4 is considered a virulence factor specific to porcine ETEC strains (Gyles and Alexander, 1994), and in addition, F5 (K99), F6 (978P), F41, F17, and F18 are found in various porcine ETEC strains (Evans et al., 1986; Gyles and Fairbrother, 2004; Nakazawa et al., 1987). Studies indicate that some ETEC strains isolated from porcine diarrhea cases do not always express these known fimbrial adhesins (Casey et al., 1992; Osek, 1999). A new non-fimbrial adhesin involved in diffuse adherence (AIDA-I) was recently identified on certain porcine ETEC isolates (Ngeleka et al., 2003). The *aidA* gene has very high prevalence in porcine ETEC isolates from neonatal diarrhea and edema disease and post-weaning diarrhea (ED-PWD) (Ha et al., 2003; Mainil et al., 2002; Niewerth et al., 2001; Ha et al., 2004), indicating that AIDA-I may represent an important virulence factor and be involved in the pathogenesis of porcine diarrhea. ETEC produce heat-stable (STa, STb, and EAST1) and heat labile (LT) enterotoxins which interact with intestinal epithelial cells to induce water and electrolyte secretion (Gyles and Fairbrother, 2004).

EPEC also cause PWD in pigs. Originally, EPEC strains were classified based on O:H serotype (Robins-Browne, 1987). Later, EPEC strains were identified to belong to a category of *E. coli* called attaching and effacing *E. coli* (AEEC) which induce an attaching and effacing (A/E) lesion on the intestinal mucosa (Nataro and Kaper, 1998). EPEC do not produce heat labile or heat stable toxins. The virulence factors of EPEC are coded in the locus of enterocyte effacement (LEE) which includes attaching and effacing (*eae*), *espADB* and *sep* genes. *Eae and espADB* genes encode intimin, EspA, EspD, and EspB proteins which induce signal transduction in epithelial cells and are involved in attachment to host cells. In addition, a 27.6 kDa protein encoded by *paa* (porcine A/E

associated) genes may be involved in the initial bacterial attachment in pigs (Gyles and Fairbrother, 2004).

1.1.3. Virulence factors and their role in porcine diarrhea

1.1.3.1. Fimbrial adhesins

Fimbriae are fibrillar proteinaceous structures attached to the bacterial surface that mediate adherence of bacteria to target cells in the gastrointestinal tract. They are composed of myriad copies of a major subunit which provides structure and antigenic specificity, and of several copies of minor subunits which confer binding properties (Gyles and Fairbrother, 2004). *E. coli* adhesins can be sheared from the bacterial surface through homogenization (Korhonen et al., 1980). After precipitation by crystalline ammonium sulfate (de Graaf and Roorda, 1982; Korhonen et al., 1980), the crude proteins can be further purified by sucrose gradient ultracentrifugation or gel filtration chromatography (Korhonen et al., 1980).

The original system of fimbrial nomenclature was based on the structure of capsular antigens, e.g. K88 and K99, which correspond to F4 and F5 in the new F designation. The well-defined fimbrial adhesins of porcine diarrheagenic *E. coli* are F4, F5, F6, F18, F41, and F17.

F4. The first adhesin identified from porcine diarrheagenic *E. coli* was F4. It is coded by the *fae* locus on a plasmid and is divided into three subsets: F4ab, F4ac and F4ad (Guinee and Jansen, 1979; Klemm, 1985). The 'a' factor refers to the conserved antigenic determinants, while the b, c, and d factors refer to the variant-specific antigenic determinants. Purified F4 adhesin has a molecular weight (MW) of 23.5 -27.5 KDa and an isoelectric point (pI) of 4.1-4.2 (Parry and Rooke, 1985). F4 positive ETEC colonize

the entire intestinal tract and attach to the brush border of villus epithelium of the small intestine (Arbuckle, 1970). F4 adhesin is host specific and the ab, ac, and ad variants of F4 recognize different porcine small intestinal receptors (Jin and Zhao, 2000).

F5. F5 are long filamentous structures on the surface of many ETEC and are associated with strains from a wide host range, including calves, lambs (Smith and Linggood, 1972) and also pigs (Morris et al., 1982a; Smyth et al., 1981). Purified F5 consists of two subunits, 22.5 and 29.5 KDa, respectively (Isaacson, 1977). F5 mediates the attachment of ETEC to the intestinal mucosa of the host similar to the attachment of F4 positive *E. coli* (Smith and Linggood, 1972). F5 is not expressed when organisms are cultured at 18ºC (Morris et al., 1980).

F6. F6, coded by the *fas* locus on both chromosome and plasmid, is a 20 KDa protein, with a PI of 3.7 (Parry and Rooke, 1985). F6 positive *E. coli* colonize the lower small intestine in natural infections (Nagy et al., 1977) and cause diarrhea in neonatal but not in older weaned pigs (Morris et al., 1982a). F6 is able to bind glycolipid receptors in the porcine intestinal mucus. These receptors may mediate age-related resistance to F6 positive ETEC (Dean-Nystrom and Samuel, 1994).

F18. F18 is encoded by the *fed* genes located on a plasmid. Two types of F18 have been found: F18ab and F18ac. F18ac is commonly isolated from ETEC strains, while F18ab is mostly associated with STEC strains (Gyles and Fairbrother, 2004). Although the F18 receptor is initially absent at birth, pigs with the F18 receptor genotype develop these receptors by weaning time, thus becoming susceptible to PWD due to F18 positive strains. These strains produce locally extensive small intestinal mucosal colonization in susceptible hosts (Frydendahl et al., 2003).

F41. F41, first demonstrated on a calf F6 mutant strain B41, is mostly co-expressed with F5 (Morris et al., 1982b). Purified F41 has a MW of 29.5 and a PI of 4.6 (de Graaf and Roorda, 1982), and mediates the attachment of *E. coli* to microvilli of calf enterocytes *in vitro* and the microvillous surface from the small intestine of 18h old piglets *in vivo*, leading to induction of diarrhea (Morris et al., 1982b).

F17. F17, a plasmid-encoded, rod-like adhesin on bovine ETEC and necrotoxigenic *E. coli* (NTEC), has four gene variants, namely, a, b, c, and d (Mainil et al., 2000). F17 also mediates adherence of septicemic *E. coli* to the intestinal epithelial cells. Its role in the development of porcine diarrhea is unclear.

1.1.3.2. Non-fimbrial adhesins

A number of non-fimbrial adhesins have been identified in different principal pathotypes of diarrheagenic *E. coli*. The well-defined adhesins include intimin in EPEC and EHEC, Tia and TibA in ETEC, AIDA-I in DAEC and ETEC, each adhesin mediating a unique mechanism of bacterial attachment to eukaryotic cells (Torres et al., 2005).

Intimin. Intimin, a 94 kDa outer membrane protein encoded by the *E. coli eae* gene, functions as an adhesin (Jerse et al., 1991; Jerse and Kaper, 1991). Intimin includes α, β, γ, δ, and ε subtypes (Adu-Bobie et al., 1998; Oswald et al., 2000). Intimin protein binds to its translocated intimin receptor (Tir) on enterocytes which results in bacterial adherence and subsequently the A/E lesion (Luo et al., 2000). A/E lesions are characterized by localized degeneration of the brush border of the luminal surface, formation of a pedestal-like actin structure and loss of microvilli at the attachment site. EPEC and EHEC are characterized by producing this specific histopathological change on human and animal intestinal epithelial cells (Nataro and Kaper, 1998).

Tia and TibA. Tia and TibA, coded by chromosomally borne loci designed *tia* and *tib*, have been cloned from the human ETEC strain H10407 (Elsinghorst and Weitz, 1994; Fleckenstein et al., 1996). Tia is a 25 KDa outer membrane protein, while TibA is a 104 KDa outer membrane glycoprotein (Lindenthal and Elsinghorst, 1999; Mammarappallil and Elsinghorst, 2000). Both Tia and TibA have the ability to bind to human intestinal epithelial cells. When either the *tia or tib* locus is deleted, the ability of ETEC to adhere to epithelial cells is decreased to 25% and 85% respectively (Elsinghorst and Weitz, 1994; Fleckenstein et al., 1996). Polyclonal antibody can block Tia and TibA mediated binding. Tia and TibA may have a functional role in invasion as well as adhesion. Tia is highly homologous with the non-fimbrial adhesin heat-resistant agglutinatinin 1 (Hra-1) identified from the porcine ETEC strain O9: H10:K99 (Lutwyche et al., 1994).

AIDA-I. AIDA-I is a nonfimbrial, 100 kDa bacterial surface protein first identified from the human DAEC isolate 2787(serotype O127:H27) (Benz and Schmidt, 1989, 1992b) and later from porcine ETEC strains associated with ED-PWD (Ngeleka et al., 2003; Niewerth et al., 2001). Two genes (*aidA* and *aah*) direct AIDA-I production. The *aidA* gene encodes a 132 kDa AIDA-I precursor protein and after post-translational modifications by which amino acids are removed from the C-terminal, the precursor matures to a 100 kDa adhesin (α-domain). The *aah* gene encodes a 45 kDa protein which helps modify the AIDA-I adhesin to form the β-domain through the addition of heptose residues at multiple sites. This domain is essential for translocation of the α-domain to the bacterial surface and adherence to HeLa cell (Benz and Schmidt, 2001).

AIDA-I mediates *E. coli* attachment to different human and animal cell types. Immunofluorescent studies have revealed that AIDA-I mediates adherence to human intestinal epithelial cells, monkey fibroblast cells and Chinese hamster ovary cells (Laarmann and Schmidt, 2003). The binding of AIDA-I to HeLa cells is a saturable phenomenon, suggesting that a finite number of receptors exists on the cell surface (Benz and Schmidt, 1992b).

1.1.3.3. Enterotoxins

ETEC produce two types of enterotoxins, namely, heat-stable (ST) and heat-labile (LT). Both ST and LT act on the epithelial cells of intestine, inducing a massive secretion of fluids, resulting in diarrhea.

1.1.3.3.1. Heat-stable enterotoxin (ST).

ST is a poorly antigenic toxin and is resistant to heat treatment at 100ºC for 15 minutes. It can be divided into two subgroups, STa and STb, based on size, molecular structure, and biological activity. The recently defined enteroaggregative *E. coli* heat-stable enterotoxin (EAST1) is related to STa (Gyles and Fairbrother, 2004).

STa is a 2 kDa peptide which is produced by ETEC strains from different species (human, porcine and bovine). It interacts with the intestinal epithelial cell receptor guanylyl cyclase C to stimulate guanylyl cyclase activity, leading to an increased intracellular cGMP level (de Sauvage et al., 1991). The result is reduced absorption of electrolytes and water from the intestine and an increased secretion of fluids (Sears and Kaper, 1996).

STb is a 5 kDa peptide produced mostly by porcine ETEC. Its intestinal receptors and mechanism of pathogenesis are not fully understood. One possible mechanism of

induction of diarrhea is that STb stimulates the release of prostaglandin and 5-hydroxytryptamine into intestinal fluid and induces the secretion of water and electrolytes, thus causing diarrhea. (Gyles and Fairbrother, 2004).

EAST1 is a 4.1 kDa peptide first identified in human EAEC, ETEC and EPEC (Savarino et al., 1993) and later in ETEC from pigs with diarrhea (Yamamoto and Nakazawa, 1997). It has 50% homology with STa and appears to induce diarrhea by the same mechanism as STa (Gyles and Fairbrother, 2004).

1.1.3.3.2. Heat-labile enterotoxin (LT)

LT includes two subgroups of highly antigenic toxins, LTI and LTII, which can be inactivated by heat treatment at 60ºC for 15 min. Based on origin, LTI can be subdivided into LTh-I from human ETEC and LTp-I from porcine ETEC, while LTII is subdivided into LTIIa and LTIIb based on antigenic differences.

LTI is a 88 kDa toxin complex which consists of one 28 kDa A subunit and five 11.5 kDa B subunits. The B subunit recognizes a GM1 ganglioside receptor on the intestinal epithelial cell. After the B subunit binds to its specific receptors, the A subunit is activated, translocates into the cell and activates the adenylate cyclase system, resulting in increased production of cyclic GMP. A high level cyclic GMP in the cell reduces the absorption of electrolytes and water from the intestine and increases secretion of fluids (Nataro and Kaper, 1998). Although LTII has 55-57% homology with LTI, there is no evidence for any role of LTII in human or porcine disease (Gyles and Fairbrother, 2004; Nataro and Kaper, 1998)

1.1.3.4. Detection of *E. coli* adhesins and enterotoxins

The detection of adhesins and enterotoxins in individual *E. coli* isolates is important in order to characterize the specific virotypes involved in diarrheal disease. Immunoassay and genetic methods are commonly used for this purpose.

1.1.3.4.1. Immunoassay

The agglutination test, either as the slide or latex particle agglutination test is a simple, fast and commonly used method to detect *E. coli* adhesins and enterotoxins. Slide agglutination tests have been used to identify F4, F5, F6, and F41 adhesins in *E. coli* isolated from piglets with neonatal diarrhea and PWD (Fairbrother et al., 1988; Nakazawa et al., 1987). The sensitivity of slide agglutination tests is relatively lower than with other techniques, e.g., the detection rate for F41 is only 13.3% when compared with its detection by immunofluorescence (Fairbrother et al., 1988). To increase sensitivity, various monoclonal antibody-based immunoassays have been developed to detect fimbrial adhesins. By coupling monoclonal antibodies to latex particles to form agglutination reagents, a latex particle agglutination test was developed to detect the adhesins F4, F5, F6, and F41 on *E. coli* following culture. The reagents are stable for 20 weeks at 4ºC, thus this test is suitable for use in diagnostic laboratories for examination of *E. coli* strains associated with diarrhea (Thorns et al., 1989).

Enzyme-Linked ImmunoSorbent Assay (ELISA) is a specific and sensitive method to detect fimbrial adhesins. An ELISA for detection of F4 has been developed for use on porcine fecal specimens, and its ability to detect F4 in feces was reported to correlate well with the isolation of F4 positive *E. coli* (Mills et al., 1983). With the use of monoclonal antibodies, the specificity can be increased greatly. Thorns developed a rapid, highly specific (>90%), monoclonal antibody ELISA for simultaneous detection of

bovine coronavirus, rotavirus serogroup A, and *E. coli* F5 antigen in the feces of calves (Thorns et al., 1992), An ELISA for the detection of ETEC STa was found to have 93.9% specificity and 90.9% sensitivity in comparison with the standard suckling mouse assay (Cryan, 1990).

1.1.3.4.2. Genetic methods of detection

Gene probing (DNA hybridization) and polymerase chain reaction (PCR) are the two commonly used genetic-based techniques for the detection of adhesin and toxin genes of *E. coli*.

The basis of gene probe techniques is the DNA double strand. A probe, either DNA or RNA labeled with radioisotope, can hybridize with target DNA if it exists in treated samples. The genes encoding the adhesins F4, F5, F6, F41, AIDA-I, and the enterotoxins LT1, ST1, ST2, Shiga toxin Stx1, Stx2 have been cloned and used to probe *E. coli* isolates from porcine diarrhea (Mainil et al., 2002; Monckton and Hasse, 1988; Woodward and Wray, 1990). DNA hybridization assays are more sensitive and effective than ELISA and slide agglutination for detection of F4 and F5 and of toxins both in bacterial colonies and in fecal swabs (Monckton and Hasse, 1988). Gene probing is also useful in determining the location of the toxin or adhesin genes in plasmids (Mainil et al., 2002).

PCR involves the enzymic amplification of template by using synthetic oligonucleotide probes (primers) which bind to their complementary target. The amplified DNA products are easily detected by gel electrophoresis. PCR has been developed to detect the genes for LT, ST1, Stx1, Stx2, Stx2e, F18, and the AIDA-I gene in *E. coli* from diarrheal disease (Ha et al., 2003; Niewerth et al., 2001; Woodward et al., 1992). PCR has

become a powerful tool to screen for the AIDA-I adhesin gene from *E. coli* isolates in pigs with ED-PWD (Ha et al., 2003).

1.1.4. Pathogenesis of porcine diarrhea due to ETEC and EPEC

1.1.4.1. ETEC

Newborn pigs first come in contact with ETEC through environmental exposure, especially to the mammary glands of the sow and to the farrowing facilities. ETEC gain entry to the gastrointestinal tract of piglets by the oral route and can multiply rapidly in the favorable environment created by the low pH and low activity of digestive enzymes in the stomach and intestine of neonates. When ETEC increase to a sufficient number, colonization and attachment to the intestinal epithelium through interaction of fimbrial or nonfimbrial adhesins with their specific receptors on intestinal epithelial cells or in mucus occurs. These intestinal receptors are discussed later in more detail. After attachment, ETEC produce one or more enterotoxins (STa, STb, EAST1, and LT) which interact with intestinal epithelial cells to induce water and electrolyte secretion into the intestinal lumen. The net effect is watery diarrhea and ionic imbalance, dehydration, metabolic acidosis, and death (Gyles and Fairbrother, 2004).

Although ETEC colonization of intestine is essential for development of PWD, many studies have found that diarrhea cannot always be induced in weaning pigs by dosing with ETEC orally, indicating that predisposing factors may influence the course of the disease. Physiological changes, nutritional deficiency, low environmental temperature and infection by other pathogens all predispose to proliferation of ETEC in the pig and the occurrence of PWD (Hampson, 1994).

1.1.4.2. EPEC

The pathogenic mechanisms by which porcine EPEC cause PWD are not yet known. In human EPEC infection models, pathogenesis involves three-stages: i) localized adherence by which EPEC attach to the microvilli of intestinal epithelial cells via specific adhesins; ii) signal transduction in epithelial cells mediated through the secreted proteins EspA, EspB, EspD and EspF.; and iii) intimate adherence of EPEC, formation of pedestal-like actin structures and loss of epithelial microvilli at the attachment site i.e. the characteristic A/E lesions on intestinal mucosa (Nataro and Kaper, 1998). Diarrhea may be due to the loss of absorptive microvilli in the A/E lesion or increased permeability of tight junctions of epithelial cells. In porcine EPEC infection, similar A/E lesions are found on ileal epithelial cells of weaned pigs, suggesting that porcine and human EPEC have similar pathogenic mechanisms (Gyles and Fairbrother, 2004).

1.1.5. AIDA-I positive *E. coli* infection

1.1.5.1. Humans

EPEC strains are considered the major cause of neonatal diarrhea and infantile gastroenteritis throughout the world. Originally, EPEC strains were studied based on the O:H serotype and the adherence to HEp-2 cells. Later, an EPEC adherence factor (EAF) probe was developed to identify EPEC strains, and the ability to hybridize with this probe became the standard for detecting diarrheagenic *E. coli* strains with localized adherence (LA) phenotype (Baldini et al., 1986). Not all LA *E. coli* strains associated with A/E lesions can be detected by this EAF probe, however, suggesting the existence of some other type of adhesin involved in the attachment of EPEC to the epithelial cells (Knutton et al., 1991). Benz et al. first identified the AIDA-I adhesin from the human EPEC strain 2787 (O127:H27) isolated from a case of infantile diarrhea (Benz and Schmidt, 1989).

AIDA-I positive *E. coli* show diffuse adherence (DA) in the HeLa cell adhesion assay, and, based on this characteristic, AIDA-I positive *E. coli* are now categorized as DAEC (Torres et al., 2005). The 100 kDa AIDA-I protein encoded by 6 kb of plasmid DNA has been localized to the bacterial surface, a finding subsequently confirmed by electromicroscopy (EM) (Benz and Schmidt, 1992b). The 100 kDa protein can be isolated from the bacterial surface by mild heat extraction. Binding of purified 100 kDa AIDA-I protein to HeLa cells is inhibited by anti-AIDA-I serum, providing direct evidence that DA is mediated by the AIDA-I adhesin (Benz and Schmidt, 1992b).

Molecular studies of the AIDA-I mediated DA phenotype have revealed that the mature100 kDa AIDA-I protein is derived from a 132 kDa precursor protein. Two open reading frames (ORF), ORFA (1173bp) and ORFB (3858bp), are necessary for the expression of DA phenotype but only ORFB encodes a 1286 amino acid as a pre-pro-protein (132 kDa) in the bacterial cell. After removal of 49 amino acids and further N-and C-terminal processing, the resultant proteins are the mature 100 kDa AIDA-I adhesin (α-domain) and a membrane-integrated 47.5 protein (β-domain) (Benz and Schmidt, 1992a; Suhr et al., 1996). As indicated earlier, the β-domain directs the translocation of α-domain to the bacterial surface. The AIDA-I protein is further modified by addition of heptose residues to form the functional AIDA-I adhesin. This post-translational modification process is essential for adherence mediated by AIDA-I (Maurer et al., 1997).

Since AIDA-I mediates adherence of *E. coli* to HeLa cells, the receptor for AIDA-I must exist on HeLa cells. By analysing the receptors using ELISA and immunoflorescence microscopy of fixed mammalian cells incubated with purified

AIDA-I, Laarman found that AIDA-I recognized receptors on human intestinal epithelial cells, monkey fibroblast-like cells, and Chinese hamster ovary cells, thus demonstrating that AIDA-I receptors are distributed on a variety of mammalian cells. In addition, the AIDA-I receptor was identified and characterized by immunoprecipitation and SDS-PAGE to be a 119 kDa (gp119) integral membrane glycoprotein (Laarmann and Schmidt, 2003).

AIDA-I adhesin can mediate biofilm formation and autoaggregation of *E. coli* (Pritchard et al., 2004; Sherlock et al., 2004). Biofilms are defined as structured communities of microbial species embedded in a biopolymer matrix on either biotic or abiotic substrata (Xiuping and Pace, 2006). Biofilm formation is a simple developmental process involved in bacterial attachment and colonization, and the bacterial surface structure plays a very important role in this process. After initial aggregation, specific adhesins are produced which interact with the bacterial surface and permit development of a biofilm architecture (Van Houdt and Michiels, 2005). Through use of Gfp-labeled *E. coli* strains, biofilm formation mediated by AIDA-I has been confirmed by confocal scanning laser microscopy (Sherlock et al., 2004). In *in vitro* studies, the AIDA-I adhesin has shown self-association characteristics such as intercellular AIDA-AIDA interaction, resulting in autoaggregation of AIDA-I positive *E. coli*, and interaction with antigen-43 producing *E. coli*. AIDA-I may function as an autoaggregator and efficient initiator of biofilm formation. Specific attachment to a host cell is very important in bacterial pathogenesis. The self-association function of AIDA-I may have some functional association with bacterial survival en route to a mammalian host. In an *in vivo* study,

biofilm formation by AIDA-I positive *E. coli* colonizing the intestinal epithelial cells was observed (Pritchard et al., 2004).

1.1.5.2. Pigs

Although AIDA-I was first characterized in a human *E. coli* isolate responsible for infant diarrhea, a number of reports have since indicated that the AIDA-I adhesin plays a role in porcine diarrhea. In the recent survey of 170 isolates of *E. coli* from clinical cases of neonatal and post-weaning porcine diarrhea, 15 of the 170 isolates were positive for AIDA-I, while only 27 isolates were positive for common attachment factors such as F4, F5, F6 and attaching and effacing factor (EAE) (Ngeleka et al., 2003). When two of these AIDA-I positive isolates were tested, their diarrheagenic ability was confirmed experimentally by the development of diarrhea in colostrum-deprived neonatal pigs infected orally (Ngeleka et al., 2003; Pritchard et al., 2004).

The AIDA-I genes are widely distributed in porcine *E. coli* isolates. The isolation rate of ETEC strains carrying AIDA-I genes is significantly high (63%) in pigs with suspected ED in Belgium (Mainil et al., 2002). The AIDA-I gene was identified in 40.9% (18 out of 44) *E. coli* isolates from pigs with ED-PWD in Germany (Niewerth et al., 2001) and 7.5% (55 out of 604) in Korea (Ha et al., 2003). On the other hand, identification of AIDA-I in human *E. coli* isolates has been very limited: in one study 1.1% (3 out of 262), and in another, 2% of DAEC from human diarrheic cases were AIDA-I positive (Niewerth et al., 2001). The prevalence of AIDA-I *E. coli* in porcine isolates is significantly higher than that in human isolates. These studies indicate that AIDA-I may represent an important virulence determinant in pigs with *E. coli* enteritis.

Several studies have demonstrated that the AIDA-I adhesin is associated with multiple other virulence factors in porcine diarrhea. One study of *E. coli* strains isolated from pigs with ED-PWD indicated a functional and physical link between AIDA-I and F18 and Stx2e, both of which are recognized as major virulence factors associated with ED-PWD; and more than 25% of the isolates were found to be AIDA-I positive (Niewerth et al., 2001). DNA sequence analysis further confirmed the association of AIDA-I with F18 and Stx2e. Among the 174 German porcine verotoxigenic *E. coli* isolates tested, 71 had the virotype Stx2:F18:AIDA-I, 26 were F18:AIDA-I, 2 were Stx2:AIDA-I, and 12 were AIDA-I . The genes of F18 and AIDA-I adhesin are localized on the same plasmid of *E. coli.* (Mainil et al., 2002). In Ha's study, 51% (23 of 45) of *E. coli* isolates that carried AIDA-I genes were Stx2:AIDA-I and 40% (18 of 45) were F18:AIDA-I (Ha et al., 2003). The relationship between AIDA-I and fimbriae F4, F5, F6, F41, and enterotoxins (STa, STb, EAST1 and LT) has also been investigated in Canada and Korea. The prevalence of the AIDA-I:STb virotype in *E. coli* among 170 western Canadian isolates from diarrheic piglets was 20.5%, but no association of AIDA-I with F4, F5, F6, or F41, the fimbriae classically expressed by diarrheagenic *E. coli,* was demonstrated (Ngeleka et al., 2003).

1.1.6. Adherence of diarrheagenic *E. coli*

The adherence of diarrheagenic *E. coli* to the mucosa of the intestinal tract is of prime importance for the development of diarrhea. Attachment mechanisms of diarrheagenic *E. coli* were first investigated *in vitro* using HEp-2 cells. Adhesion to HEp-2 cells remains the gold standard to determine the adhesive pathotype. Essentially, *E. coli* are added to a confluent HEp-2 cell monolayer, and after washing and staining, the

adherence patterns are observed (Scaletsky et al., 1999). Three types of adherence patterns have been described: localized adherence, aggregative adherence and diffuse adherence.

Localized adherence (LA). LA is characterized by bacterial attachment to one or two small areas on the cells (Scaletsky et al., 1984). The LA pattern, typically associated with EPEC causing infantile diarrhea in humans (Echeverria et al., 1987a; Echeverria et al., 1987b), was first shown to be mediated by a 94 kDa plasmid-associated outer membrane protein, and later another 18.5 kDa outer membrane protein was found to be involved in the LA pattern and colonization on mucosal surfaces by EPEC (Vuopio-Varkila and Schoolnik, 1991).

Aggregative adherence (AA). AA is characterized by bacterial attachment in a stacked-brick-like arrangement on the surface of the cells (Nataro et al., 1987). AA is frequently related to persistent diarrhea caused by EAEC (Elliott and Nataro, 1995). Two different plasmid-encoded fimbrial adhesins, termed aggregative adherence fimbriae I (AAF/I) (Nataro et al., 1992),and AAF/II (Nataro et al., 1995), were identified to mediate this attachment.

Diffuse adherence (DA). In DA, bacterial attachment uniformly covers the surface of the cells (Scaletsky et al., 1984). Two adhesins have been identified to be involved in the DA phenotype. F1845 is a fimbrial adhesin which mediates diffuse adherence of wild type strains (Bilge et al., 1989), while the other, AIDA-I, is a non-fimbrial adhesin. Purified AIDA-I can bind to HeLa cells and anti-AIDA-I antibodies can inhibit the adherence of AIDA-I positive DAEC. Most authors recognize DAEC as a new category of diarrheagenic *E. coli,* although the evidence for the association of DAEC strains with diarrheal disease is inconclusive.

The ability to adhere to a specific host cell receptor enables a pathogen to initiate its effect on the host. The mammalian cell surface contains many carbohydrate structures which can serve as receptors to mediate the binding of pathogens. The different adherence patterns of diarrheagenic *E. coli* may represent different molecular attachment mechanisms which utilise different receptor structures. This is a fundamental principle for further study of porcine intestinal receptors for *E. coli in vivo*.

1.2. *E. coli* host cell receptors

The goal of studies on bacterial adhesins and receptors is to understand the interaction between the pathogen and host, and their impact on the disease process. Various methods have been developed to study adhesins and receptors. The first requirement is isolation and characterization of the adhesin, followed by identification at the biochemical level of the specific host receptor at the colonization site.

1.2.1. Intestinal epithelial cell receptors

Intestinal epithelium contains several dynamic cell types that mediate a variety of digestive, transport and secretory functions. The main cell type is the mucosal enterocyte which originates in the crypts and migrates upwards along the villi to be sloughed eventually from the tips. The binding of pathogens to epithelial cells is considered a first step in bacterial colonization of the host mucosal surface and the brush border of the apical border (luminal surface) of the enterocyte is the first cell structure to contact invading pathogenic microorganisms. Most receptors identified by the *E. coli* adhesins are located in the brush border. Also present in the mucosa are mucus-producing goblet cells, which lack a brush border (Moon, 1997). The brush border found on the apical surface of enterocytes is formed by microvilli which possess contractile protein. TEM of isolated

brush border shows a dense core of microfilaments extending to the microvillus membrane. The bundle of microfilaments is composed of a number of polypeptides (Sellwood, 1984).

1.2.1.1. Intestinal epithelial cell receptors for F4 (K88)

The receptors for F4 were the first isolated from porcine intestinal brush border. To date, seven different brush border proteins have been suggested as putative F4 receptors (Jin and Zhao, 2000). Two purified brush border glycoproteins, 240kDa and 210kDa, respectively, were found to bind F4ac *E. coli,* a property which could be completely blocked by purified F4ac adhesin, providing direct evidence that these proteins serve as phenotype-specific F4ac receptor (Erickson et al., 1992). A 74 kDa glycoprotein (GP74) recognized by F4ab and purified from porcine brush border membrane, belongs to the transferrin family. It has identical N-terminal sequence with transferrin and reacts with anti-pig transferrin antibodies (Grange and Mouricout, 1996). In addition, three other brush border glycoproteins (23kDa, 35kDa and 40-70kDa, respectively) have been reported to be F4ab-specific porcine intestinal receptors (Willemsen and de Graaf, 1992). F4ab-specific receptors have also been identified from mouse intestinal brush border. Further glycoproteins (57kDa, 64kDa and 91 kDa) have been identified as F4ab receptors by using an adhesion assay of $^{35}SO_4$-labeled *E. coli* (Laux et al., 1986). In addition to glycoproteins, a glycolipid has also been identified as a receptor for F4ad (Grange et al., 1999). This knowledge permits novel approaches to the prevention of diarrhea due to ETEC F4 by inhibition of the attachment of ETEC to intestine through modification of the attachment sites, i.e. receptors (Jin and Zhao, 2000). By using brush border adhesion test, four porcine phenotypes have been identified and

designated I, II, III, and IV, in respect to genetic inheritance of receptors for F4 variants. Phenotype I, which lacks genes for any of the variants, has no F4 adhesive properties, while phenotype II is adhesive to F4ad, phenotype III adhesive to F4ab and F4ac, and phenotype IV adhesive to all three F4 variants (Bijlsma et al., 1982; Rapacz and Hasler-Rapacz, 1986). The existence of four different adhesive phenotypes associated with three F4 variants may require further study in order to develop prevention approaches.

1.2.1.2. Intestinal epithelial cell receptors for F6 (987P)

Intestinal epithelial cell receptors for F6 (987P) have been identified on intestinal brush border of rabbits and pigs by western blot assay (Dean, 1990; Dean and Isaacson, 1985b). Purified rabbit F6 receptors are low-molecular weight glycoproteins, ranging from 14 kDa to 20 kDa, and have the ability to agglutinate F6 positive *E. coli*. This agglutinating activity can be inhibited by purified F6, amino sugars such as glucosamine, galactosamine and manosamine, and lectin soybean agglutinin such as SBA (*Glycine max* agglutinin) (Dean and Isaacson, 1985b). F6 receptors are distributed in goblet cells along the villous surface as demonstrated by fluorescein-labeled F6 receptor antibody staining. The receptors isolated from rabbits and pigs have a similar antigenic structure (Dean and Isaacson, 1985a). Porcine F6 receptors are 33-40 kDa glycoproteins and have been identified in 1-day-old to 3-4-week-old weaned pigs and in 3-week-old gnotobiotes (Dean, 1990). F6 positive ETEC can cause diarrhea in the 1-day-old piglet and 3-week-old gnotobiotes but not in conventional 3-4-week-old pigs, indicating the existence of intestinal epithelial cell receptors for F6 is not associated with age-related resistance.

1.2.2. Intestinal mucus receptors

1.2.2.1. Intestinal mucus

Mucus, secreted from goblet cells as a viscoelastic gel that adheres to the mucosal surface, consists of glycoproteins, proteins, lipids, water and electrolytes. Glycoproteins, integral structural components of the intestine and the major components of mucus, form the mucus gel layer (mucins) (Deplancke and Gaskins, 2001). Mucous glycoproteins, polymeric structures composed of glycoprotein subunits, form a group of large molecular weight molecules ranging from 250 kDa to 2000 kDa. In pigs, four glycoprotein subunits are reduced by mercaptoethanol and pepsin from native glycoprotein (Allen, 1984). Individual peptides of mucus glycoprotein consist of two major regions, namely, a heavily glycosylated major region and a poorly glycosylated minor region which is susceptible to proteolytic degradation (Neutra and Forstner, 1987). The side chains of mucus glycoprotein are linked with four oligosaccharides, *N*-acetylglycosamine, *N*-acetylgalactosamine, fucose and galactose (Deplancke and Gaskins, 2001).

Lipid is another important component, comprising nearly 40% of the intestinal mucus in rats (Slomiany et al., 1980). Lipids are composed of natural lipids, glycolipids and phospholipids.

Biosynthesis of mucin occurs through the three steps of core peptide and oligosaccharide synthesis, terminal sugar addition, and post-translational modification (Forstner and Forstner, 1994). Synthesized mucins are released from the apical surface of goblet cells through two mechanisms: exocytosis resulting from the acute release of stored mucin granules, and baseline secretion involving the release of newly synthesized mucin granules (Forstner and Forstner, 1994).

1.2.2.2. Functions of mucus

Mucus forms a layer covering the gastro-intestinal (GI) epithelium. When the pathogens invade the GI tract, the first event is interaction with mucus, and binding to it is essential for bacterial adhesion.

Many bacteria have been shown to bind to intestinal mucus. ETEC bind to the mucus of newborn piglets (Blomberg et al., 1993; Metcalfe et al., 1991) and to crude mucus in the mouse small intestine (Laux et al., 1986). *Salmonella typhimurium* interacts with intestinal mucus in the rat and guinea pig (Ensgraber et al., 1992; Vimal et al., 2000). Some probiotic microorganisms show specific adhesion through which the host microbial balance and the gastrointestinal immune system are influenced (Salminen et al., 1998). Some *Entercoccus* strains demonstrate strain-dependent, but host-independent, attachment to human, canine and porcine intestinal mucus (Deplancke and Gaskins, 2001). The intestinal microorganisms interact competitively and selectively with intestinal mucus. This interaction causes aggregation of microorganisms through mucin binding, coating and clumping (Forstner and Forstner, 1994).

Other functions of mucus such as lubrication, diffusion barrier, detoxification, protection and interaction with microfilaments are well documented in standard texts (Forstner and Forstner, 1994).

1.2.2.3. Mucus receptors

Mucus consists of large number of glycoproteins, small proteins, lipids and glycolipids; and there is extensive evidence to suggest that both glycoproteins and glycolipids serve as *E. coli* adhesin receptors in intestinal mucus (Blomberg et al., 1993; Dean-Nystrom and Samuel, 1994; Dean, 1990; Laux et al., 1986), although the exact

biochemical nature of most adhesin-specific intestinal mucus receptors is not fully understood,

Work on porcine intestinal mucus receptors has concentrated largely on the F4 adhesin, and the receptors for its three variants (F4ab, F4ac, F4ad) have been identified in the mucus layer of small intestine (Jin and Zhao, 2000). F4ab receptors, initially isolated from mouse intestinal mucus as 57 and 64 kDa proteins (Laux et al., 1986), have been isolated and identified subsequently from pigs. A 40-42 kDa glycoprotein isolated from 2-7 day old piglets was found to bind F4ab (Metcalfe et al., 1991). A number of porcine F4ac receptors have been isolated by different researchers: two glycoproteins (41 and 26 kDa) in Fang's study (Fang et al., 2000) and three other glycoproteins (80, 60, and 40 kDa) in Jin's study (Jin et al., 2000). Further investigation of the 41 and 26 kDa proteins has demonstrated that the distribution of these receptors differs among pigs, with 80% having the 41 kDa protein receptor and 20% the 26 kDa protein (Fang et al., 2000). Galactosylceramide, a glycolipid, has been identified in 35-day-old porcine mucus and shown to mediate the adherence of F4 *E. coli* and thus be a receptor for F4 (Blomberg et al., 1993).

The F4 receptors in mucus are dependent on the age of the host. F4 receptors in ileal mucus increase 16-fold from birth to 35 days (Conway et al., 1990), decrease again by 47 days and are undetectable in the mucus of 6-month-old pigs (Willemsen and de Graaf, 1992). These findings suggest that the incidence of ETEC infections is inversely proportional to the amount of mucus receptors, so that the low concentration of F4 receptors in the neonatal pig mucus permits bacterial attachment to the underlying

epithelial cells, while the high concentration of F4 receptors in 35-day-old piglet mucus binds ETEC and prevents attachment to the epithelial cells (Conway et al., 1990).

Receptors for F6 in porcine intestinal mucus have been identified by western blot. A low MW (<17 kDa) F6 receptor has been isolated from the intestinal mucus in F6 age-resistant 3-4 week-old pigs, but not from F6 susceptible neonatal and 3-week-old gnotobiotic pigs (Dean, 1990). This suggests that resistance to diarrhea due to F6 positive ETEC results from F6 binding to its intestinal mucus receptor, and indeed F6 ETEC strains are commonly involved in diarrhea in the neonatal piglet, but not in older pigs. This age-related resistance to F6 ETEC is most likely associated with specific glycolipid receptors, lactosylceramide and sulfatide (SFT), found only in the intestinal mucus of older pigs (Dean-Nystrom and Samuel, 1994). These are able to bind with purified F6 and to capture the F6 ETEC, and thus are likely to inhibit bacterial colonization of the epithelium (Dean-Nystrom and Samuel, 1994).

Mucus receptors have been investigated in relation to other bacterial species. A 15 kDa glycoprotein isolated from guinea-pig intestinal mucus aggregates *Salmonella typhimurium* (Ensgraber et al., 1992). Binding of *Salmonella typhimurium* to rat intestinal mucus has been demonstrated, and a 250 kDa glycoprotein receptor isolated by gel filtration and affinity chromatography (Vimal et al., 2000). The binding of bacteria to mucus was confirmed by EM, thereby demonstrating that the components of intestinal mucus play an important role in trapping and colonization of bacteria within the gut.

1.3. Summary

The pathogenesis of *E. coli* diarrhea in pigs remains a field of high research priority because of the economic impact of this disease. Significant advances have been

made in understanding *E. coli* adhesins, toxins, and adherence to epithelial cells. New fimbrial adhesins and non-fimbrial adhesins associated with attachment of diarrheagenic *E. coli* to the intestinal epithelium are still being discovered. Although AIDA-I has been characterized in human diarrheagenic *E. coli*, the porcine AIDA-I along with its role in the pathogenesis of diarrhea in pigs remains unclear. Since the AIDA-I gene has a very high prevalence in porcine ETEC isolates associated with neonatal diarrhea and PWD, there is a definite need for investigation of the role of AIDA-I in the pathogenesis of diarrhea in pigs. In humans, AIDA-I as a non-fimbrial adhesin mediates AIDA-I positive *E. coli* adherence to HeLa cells through a highly specific AIDA-I receptor. In pigs, however, no data are available regarding AIDA-I mediated adhesion in the intestine or on the nature of porcine mucosal components that may act as receptors for the AIDA-I adhesin *in vivo*. Basic requirements for a proper understanding of the role of porcine AIDA-I are characterization of porcine AIDA-I and investigation of the existence of AIDA-I receptors in porcine intestinal mucus.

2. Hypothesis and objectives

2.1. Rationale

AIDA-I positive *E. coli* was first isolated from a case of human infantile diarrhea and subsequently characterized (Benz and Schmidt, 1989, 1992a, b, 1993). A 119 kDa membrane glycoprotein on HeLa cells was reported to serve as a receptor for human AIDA-I (Laarmann and Schmidt, 2003). Subsequently, a relatively high prevalence of the AIDA-I gene in porcine *E. coli* isolates was reported by various researchers from different countries. Our group reported that experimental infection of colostrum-deprived piglets by AIDA-I positive *E. coli* causes diarrhea and that AIDA-I is an important virulence factor associated with biofilm formation and development of diarrhea (Ravi et al., 2007). Intimate stratification of AIDA-I positive *E. coli* and mucus layer within the above mentioned intestinal biofilm suggested potential presence of AIDA-I receptors in porcine mucus.

2.2. Hyopothesis

- **Characterization of porcine AIDA-I**

We hypothesized that AIDA-I expressed by AIDA-I positive *E. coli* isolated from porcine clinical cases of diarrhea is a protein homologous with AIDA-I expressed by AIDA-I positive *E. coli* strain 2787 isolated from human infantile diarrheic feces. In addition, we hypothesized that immuno-blot and coagglutination using polyclonal

anti-AIDA-I antibody will be useful diagnostic tests for detection of AIDA-I positive *E. coli* strains.

- **Characterization of porcine AIDA-I receptors**

Considering the intimate interaction of AIDA-I positive *E. coli* with mucus within intestinal biofilm, we hypothesized that intestinal mucus contains AIDA-I receptors.

2.3. Objectives

2.3.1. Objective 1

- To isolate and characterize AIDA-I protein from porcine AIDA-I positive *E. coli* isolates
- To develop immunodetection tests for porcine AIDA-I positive *E. coli* isolates using polyclonal anti-AIDA-I antibody

2.3.2. Objectives 2

- To determine if porcine intestinal mucus contains AIDA-I receptors and, if so, to isolate and characterize them

3. Characterization and immuno-detection of AIDA-I adhesin isolated from porcine *E. coli*

This chapter contains the complete text of a manuscript published in Veterinary Microbiology (2005) 109, 65-73 by Yuanmu Fang, Musangu Ngeleka, Dorothy M. Middleton and Elemir Simko

3.1. Introduction

Diarrheogenic *Escherichia coli* must be able to attach to intestinal mucosa to cause diarrhea. In our recent study, *E. coli* was isolated from 170 clinical cases of neonatal and post-weaning porcine diarrhea cases. Only 27 of the 170 isolates were positive for common attachment factors such as F4 (n =13), F5 (n =7), F6 fimbriae (n=1) and attaching and effacing factor (EAE) (n=6). In addition to these common attachment pathogenic factors, 15 of the 170 isolates were positive for adhesin involved in diffuse adhesion (AIDA-I) (Ngeleka et al., 2003). The diarrheogenic pathogenicity of two of our AIDA-I positive *E. coli* isolates was tested and confirmed by the development of diarrhea in colostrum-deprived piglets infected orally (Ngeleka et al., 2003; Pritchard et al., 2004). The AIDA-I gene was identified in 40.9% (18 out of 44) of *E. coli* isolated from pigs with edema disease or post-weaning diarrhea in Germany (Niewerth et al., 2001) and in 7.5% (45 out of 604) in Korea (Ha et al., 2003). Out of 174 *E. coli* isolates from piglets with suspected edema disease in Belgium, 110 isolates (63%) were positive for AIDA-I (Mainil et al., 2002). The AIDA-I gene was also identified in 2.3% (23 out of 1002)

of *E. coli* isolated from piglets with pre-weaning diarrhea in Korea (Ha et al., 2004). These reports suggest that prevalence of AIDA-I positive *E. coli* is relatively high in pigs and that it may have an important clinical significance in modern swine industry. However, little is known about relevance of AIDA-I adhesin in pathogenesis of porcine diseases caused by AIDA-I positive *E. coli*.

Benz and Schmidt (1989) were first to identify the AIDA-I adhesin in the *E. coli* strain 2787 isolated from a case of human infantile diarrhea (Benz and Schmidt, 1989). Subsequently, they demonstrated that AIDA-I adhesin, a 100 kDa outer membrane protein, mediates diffuse adherence of this strain to HeLa cells (Benz and Schmidt, 1992a, b). Only 2%, however, of diffusely adherent *E.coli* isolated from human diarrheic feces were positive for AIDA-I adhesin (Jallat et al., 1993). The prevalence of AIDA-I positive *E. coli* in human isolates is relatively low when compared with the prevalence of AIDA-I positive *E. coli* in porcine isolates indicated above. Accordingly, Niewerth et al (2001) suggested that porcine *E. coli* strains appear to be a major reservoir for AIDA-I genes (Niewerth et al., 2001). Little is known about properties of AIDA-I protein expressed by AIDA-I positive *E. coli* isolates from pigs.

In this study, we isolated and characterized biochemically the AIDA-I adhesin from porcine isolates of *E. coli* and investigated if bacterial AIDA-I adhesin could be detected on bacterial surfaces by immuno-dot-blot and coagglutination tests.

3.2. Materials and methods

This research was approved by the University (of Saskatchewan) Committee on Animal Care and Supply (Proposal #20020059), and performed according to guidelines

outlined in 'The Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care'.

3.2.1. Bacteria

Two porcine isolates PD58 and PD20 of AIDA-I positive *E. coli*, both of which were confirmed previously to cause diarrhea in colostrum-deprived neonatal piglets (Ngeleka et al., 2003; Pritchard et al., 2004), were used for isolation of AIDA-I adhesin. Seventeen AIDA-I positive and 19 various AIDA-I negative *E. coli* porcine isolates determined by polymerase chain reaction (PCR) in our previous study (Ngeleka et al., 2003) were used for detection of surface AIDA-I protein by immuno-dot-blot and coagglutination using rabbit polyclonal serum raised against AIDA-I protein isolated from these two porcine AIDA-I positive isolates. The AIDA-I positive *E. coli* strain 2787 originally isolated from human infantile diarrhea (Benz and Schmidt, 1989) was from Dr. J.M. Fairbrother (Faculte de Medecine Veterinaire, Universite de Montreal, Saint-Hyacinthe, Que., Canada)

3.2.2. Isolation of AIDA-I protein

Isolation of AIDA-I protein from two of our porcine isolates (PD58 and PD20) was performed according to previously published method (Stirm et al., 1967) with a few modifications. Briefly, bacteria were cultured in 4L trypticase-soy-broth (BBL™ Trypticase™ Soy Broth, Becton, Dickinson and Company, Sparks, MD, USA) for 36 hr at 37˚C in an incubator shaker and harvested by centrifugation at 6,000 x g for 15 min. Bacterial pellets were washed twice and re-suspended in 100 mL phosphate-buffered saline (PBS) (0.01M sodium phosphate, 0.15M NaCl, pH 7.2). Surface bacterial proteins were detached by heating to 60˚C for 30 min followed by a 10 min friction created by a

homogenizer (Polytron PT3000, Kinematica Ag, Littau, Switzerland) using a 5 mm diameter rotor (Homogenizer Generator PT-DA 3007/2) at 10,000 rpm. Bacteria were removed from suspension by centrifugation at 14,000 x g for 15 min. To prevent bacterial proliferation, 0.05% sodium azide was added to supernatant which was stored at 4˚C for 3 days. Naturally precipitated proteins were removed from suspension by suction filtration through 0.45 μm filter membrane (FP-450 PVDF Membrane Filter, Pall Gelman, Ann Arobor, MI, USA). Precipitated proteins were washed from the filter membrane with 50 mL PBS, sedimented by centrifugation at 2000 x g for 15 min at 4˚C and re-suspended in 50 mL PBS, pH 7.2. The sedimentation and re-suspension procedure was repeated three times. The final step of purification was the concentration of re-suspended proteins using 30 kDa centrifugal concentrators (30K Filtron Brand Macrosep, Pall Gelman, Ann Arobor, MI, USA). The purity of isolated AIDA-I proteins was examined by Coomassie-stained sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels.

3.2.3. Electrophoresis

3.2.3.1. One-dimensional SDS-PAGE

One-dimensional (1D) SDS-PAGE was performed in 12% gels according to the method of Laemmli (Laemmli, 1970) using a small electrophoretic system (Mini-PROTEAN® 3 Cell, Bio-Rad Laboratories, Mississauga, ON, Canada). Samples were prepared with reducing (with 50μL/mL of β-mercaptoethanol) or non-reducing sample buffer and subjected to electrophoretic separation according to the manufacturer's instructions.

3.2.3.2. Two-dimensional SDS-PAGE

Samples were subjected to isoelectric focusing (first dimension) in linear immobilized pH gradient (IPG) strips (Imobiline DryStrip, pH 3-10, 13 cm, Amersham Pharmacia Biotech AB, Uppsala, Sweden) using the IPGphor Isoelectric Focusing System (Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's protocols. Briefly, samples were applied to IPG strips together with 250 μL of rehydration solutions [8M urea, 18 mM DTT, 0.5 % v/v Triton X-100, 0.01% w/v bromophenol blue (Sigma Chemical Co, St Louis, MO, USA) and IPG Buffer 3-10 (Amersham Pharmacia Biotech AB, Uppsala, Sweden)]. Active rehydration was performed at 20°C during 12 h at 30 V，followed by protein separation based on protein charge. Separation conditions were limited to 50 mA per IPG strip with a gradual increase in voltage (500 V for 1 h, followed by 1000 V for 1 h and by 8000 V until total of 17,500 Vh) at a constant temperature of 20°C. Second dimensional SDS PAGE separation of proteins based on molecular weight was performed as previously described (Simko et al., 1999) using a large electrophoretic system (PROTEAN® II Cell, Bio-Rad Laboratories, Mississauga, ON, Canada).

3.2.4. Amino acid sequencing

The isolated AIDA-I protein was separated by 1D SDS-PAGE and visualized by Coomasie Briliant Blue staining. Protein corresponding to the 100 kDa band was excised from a gel and submitted for identification by liquid chromatography mass spectrometry (LCMS/MS) performed by the UVic-Genome BC Proteomics Centre (University of Victoria, Canada) using QSTAR® XL Hybrid LC/MS/MS System (Applied Biosystems, Foster City, CA, USA). The protein sequence data reported in this paper will appear in the Swiss-Port and TrEMBL knowledgebase under the accession number Q03155.

3.2.5. Production of AIDA-I polyclonal antibodies

The isolated AIDA-I protein was subjected to one-dimensional SDS-PAGE and separated proteins in gel were visualized by Coomassie Briliant Blue stain. Narrow strips of polyacrilamide gels containing 100 kDa protein bands were excised, placed in PBS (1:1 weight/volume) and homogenized by tissue grinder. The homogenate was emulsified with an equal volume of either Complete Freund Adjuvant (CFA) or Incomplete Freund Adjuvant (IFA) (Sigma Chemical Co, St Louis, MO, USA). The resultant vaccine contained approximately 0.2 mg/mL of AIDA-I protein. Priming immunization of two rabbits was performed with 1 mL (4x0.25 of CFA) vaccine administered subcutaneously to each rabbit at 4 different sites (0.25 mL per site). Booster immunization with IFA vaccine was performed at day 28 and 42 days after priming immunization and serum was harvested at day 49 after terminal exsanguinations of the rabbits *via* the carotid artery. Serum collected from rabbits before immunization was used as negative control serum.

3.2.6. Western blot

The AIDA-I proteins were detected by chemiluminescence immuno-detection using Bio-Rad's reagents and blotting system (Mini Trans-Blot® Cel, Bio-Rad Laboratories, Mississauga, ON, Canada). Briefly, after equilibration in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol), the samples separated in 1D SDS PAGE gel were electrotransferred to a polyvinylidene difluride (PVDF) membrane for 2 h at 100V. The PVDF membrane with blotted sample was subjected sequentially to the following procedures: blocking of the remaining protein binding sites with blocking buffer (2% non-fat dry milk, 0.1% Tween-20 in PBS); incubation with primary rabbit anti-AIDA-I polyclonal serum (1:4000) for 2 h; five 15-minute washes with PBS Tween-20 (PBST) buffer (0.1% Tween-20 in PBS); incubation with secondary antibodies (1:20,000 Goat

anti-rabbit antibodies, Sigma Chemical Co, St Louis, MO, USA) for 2 h; three 15-minute washes with PBST buffer; and exposure to a substrate solution (Immun-Star Substrate, Bio-Rad Laboratories, Mississauga, ON, Canada). The chemiluminescent signal demonstrating AIDA-I protein was detected by exposure of the PVDF membrane to an X-ray film (Kodak X-Omat AR Film, Eastman Kodak Company, Rochester, NY, USA).

3.2.7. Immuno-dot-blot

Immuno-dot-blotting was used to detect the AIDA-I adhesin on bacterial surfaces of 17 isolates of *E. coli* determined to be positive for AIDA-I gene by PCR in our previous study (Ngeleka et al., 2003). Nineteen isolates of various different non-pathogenic and pathogenic (e.g. F4, F6) *E. coli* negative for the AIDA-I gene (by PCR) were used as negative controls. Briefly, the bacteria were cultured in 5 mL of TSB (BBL™ Trypticase™ Soy Broth, Becton, Dickinson and Company, Sparks) at 37°C for 48 h and harvested by centrifugation at 2000 x g for 15 min. The bacterial pellets were re-suspended and washed twice with PBS by repeating centrifugation procedure. The bacterial concentrations of each bacterial isolate were adjusted to approximately 2 x 10^6 CFUs/mL using a previously reported conversion formula for optical density and CFU for *E. coli* ($CFU/mL=OD_{620}$ x 2.5 x 10^8) (Steinberg and Lehrer, 1997). Duplicates of 50 µL of suspension of each isolate were dot-blotted (Bio-Dot SF Microfiltration Apparatus, Bio-Rad Laboratories, Mississauga, ON, Canada) to a PVDF membrane using a vacuum generated by running tap water. AIDA-I positive *E. coli* blotted on the PVDF membrane were detected by chemiluminescence immuno-detection using Bio-Rad's reagents and the blotting system as described for Western blot. Based on subjective evaluation of the intensity of the detected chemiluminesence, tested bacterial isolates were determined to

have a negative (-), negative with slight positivity (±), or various degrees of positive (+, ++, +++, or ++++) reaction with polyclonal anti-AIDA-I serum. Both negative (-) and negative with slight positivity (±) were considered as negative reactions in this study.

3.2.8. Coagglutination

The same *E. coli* isolates, which were determined to be positive or negative for the AIDA-I gene by PCR and used for above-described immuno-dot-blot, were also used for the coagglutination test. Bacteria were cultured in 10 mL of Todd-Hewitt broth at 37°C for 18 hours. After washing with PBS, bacteria were sedimented by 2000 x g centrifugation and re-suspended in 0.5 mL of PBS containing 0.5% formaldehyde. One drop of bacterial suspension was mixed with one drop of positive or negative monovalent coagglutination reagent (CR) (the positive CR contained rabbit polyclonal anti-AIDA-I serum and the negative CR contained pre-immunization serum from the same rabbit) prepared as described previously (Gottschalk et al., 1993). After 30 s of continuous gentle tilting of slides, the results were recorded semi-quantitatively as negative (-), negative with occasional aggregates (±), or various degrees of positive reaction (+, ++, +++, or ++++). Both negative (-) and negative with occasional aggregates (±) were considered as negative reactions in this study.

3.3. Results

3.3.1. Characterization of AIDA-I protein

An approximately 100kDa protein was isolated (Fig. 3.1, Lane 3 and 4) from a crude suspension of surface proteins of the AIDA-I positive *E. coli* porcine isolates PD58 and PD20 (Fig. 3.1, Lanes 1 and 2) and detected by Western blot using polyclonal anti-AIDA-I serum (Fig. 3.1, Lane 5). Two-dimensional SDS-PAGE revealed that

isolated AIDA-I protein consists of at least 5 isoforms with isoelectric points rangeing from 4.0 to 5.0 (Fig. 3.2). Amino acid sequences determined for various fragments of isolated AIDA-I protein from both isolates (PD58 and PD20) had 86.7 and 78.4%, homology, respectively, with the AIDA-I protein isolated from human *E. coli* 2787 (Benz and Schmidt, 1992a) (Table 3.1). Only two amino acid segments, namely 340-352 and 825-836 of the AIDA-I protein isolated from both PD58 and PD20 were sequenced and in these two segments there was a 100% homology between AIDA-I proteins isolated from two different porcine AIDA-I *E. coli* isolates (Table 3.1). Despite the relatively high amino acid homology between the AIDA-I proteins expressed by porcine and human *E. coli* isolates, the human AIDA-I positive *E. coli* strain 2787 was not detected by immuno-dot-blot (Fig. 3.3) and only weakly detected by Western blot (Fig. 3.4, Lanes 3 and 6) using rabbit polyclonal antiserum raised against AIDA-I protein isolated from porcine AIDA-I positive *E. coli*. To characterize further these detection differences between human and porcine AIDA-I positive *E. coli*, we subjected both the human and the porcine AIDA-I positive isolates to the same culture conditions and isolation procedures of surface proteins (as described in section 3.2). By rough estimation of staining intensity of crude surface proteins separated by SDS-PAGE and visualized by Coomassie Briliant Blue we determined that a higher amount of a 100 kDa protein, previously determined as AIDA-I adhesin, was harvested from the porcine PD20 isolate than from the human *E. coli* strain 2787.

3.3.2. Detection of expression of AIDA-I protein by porcine *E. coli* containing AIDA-I gene

Using rabbit anti-porcine-AIDA-I polyclonal serum, we were able to detect AIDA-I protein on the bacterial surface in 13, 12, and 9 out of 17 PCR-positive AIDA-I *E. coli* isolates by immuno-dot-blot, coagglutination, and both immuno-dot-blot and coagglutination, respectively; whereas, a positive reaction was detected by immuno-dot-blot and coagglutination in two and three isolates, respectively, out of 19 *E. coli* isolates determined to be negative for the AIDA-I gene by PCR (Fig. 3.5 and Table 3.2). Accordingly, if all *E. coli* isolates determined to contain AIDA-I gene by PCR also expressed AIDA-I protein, then the sensitivity of detection of AIDA-I protein on bacterial surfaces by immuno-dot-blot and coagglutination was 76 and 71%, respectively, whereas specificity was 89 and 84%, respectively.

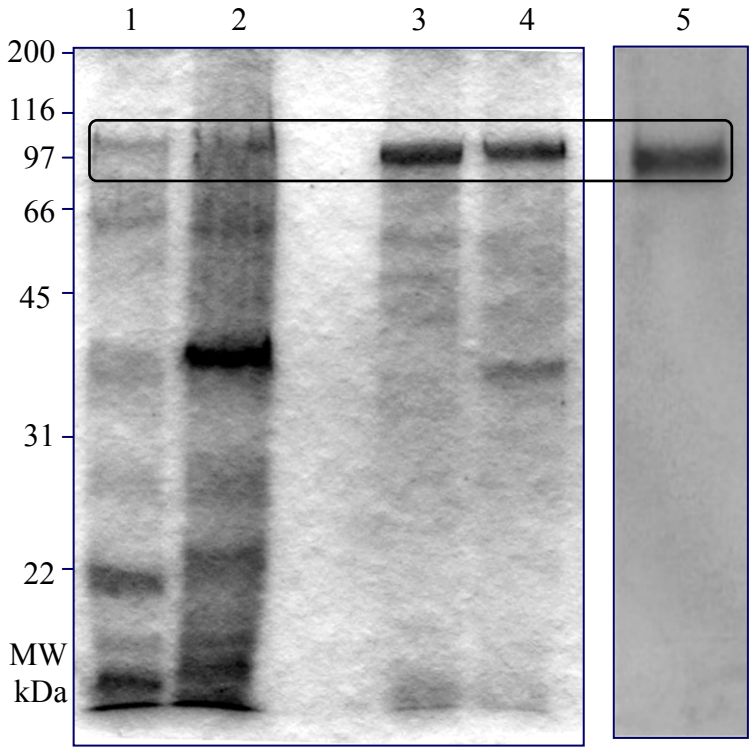


Fig. 3.1. Coomassie-stained SDS-PAGE and Western blot of AIDA-I proteins. Surface bacterial proteins harvested from porcine AIDA-I positive *E. coli* isolates PD58 and PD20 (Lanes 1 and 2, respectively); semi-purified AIDA-I proteins from PD58 and PD20 (Lanes 3 and 4); AIDA-I protein isolated from PD58 and detected by Western blot using rabbit polyclonal antiserum raised against AIDA-I protein isolated from porcine AIDA-I *E. coli* (lane 5). AIDA-I protein bands are encircled.

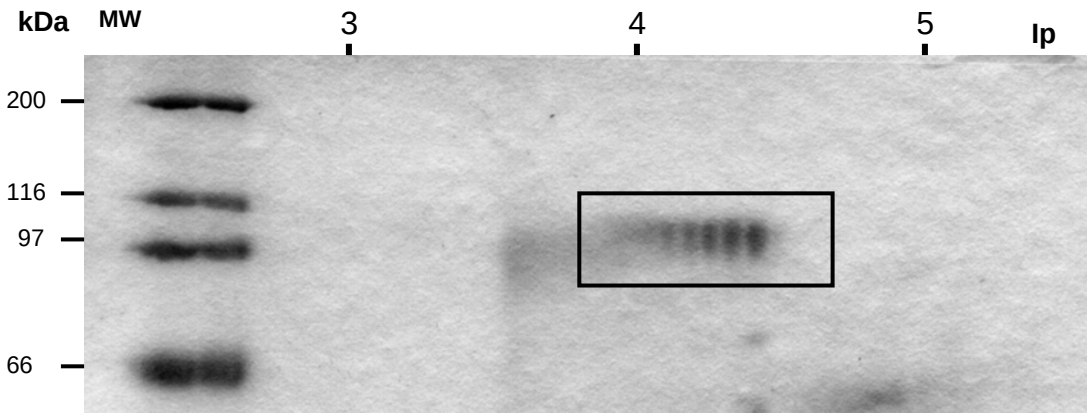
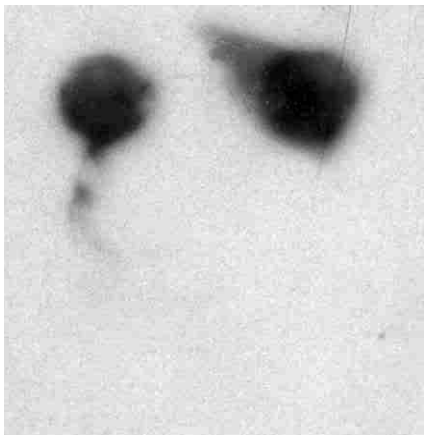


Fig. 3.2. Coomassie-stained AIDA-I protein isolated from porcine AIDA-I *E. coli* and separated on two dimensional SDS PAGE. The isoforms of AIDA-I protein are encircled.



PD20 AIDA-I *E. coli*

AIDA-I *E. coli* strain 2787

Fig. 3.3. Immuno-dot-blot detection of the porcine AIDA-I *E. coli* isolates PD20 and failure of detection of human AIDA-I *E. coli* strain 2787 using rabbit polyclonal antiserum raised against AIDA-I protein isolated from porcine AIDA-I *E. coli.*

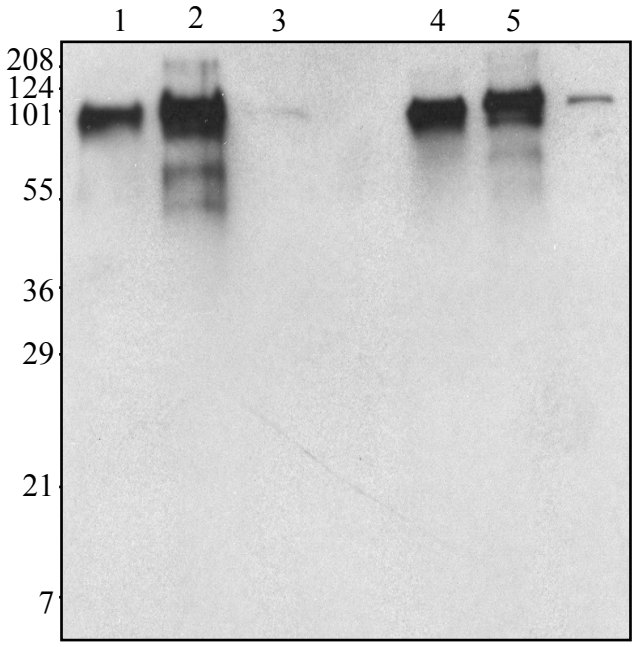


Fig. 3.4. Western blot detection of AIDA-I protein from the porcine isolates PD20 and human AIDA-I *E. coli* strain 2787 using rabbit polyclonal antiserum raised against AIDA-I protein isolated from porcine AIDA-I *E. coli*. Proteins in Lanes 1-3 were separated by reducing SDS-PAGE and proteins in Lanes 4-6 were separated by non-reducing PAGE. Isolated AIDA-I protein from the porcine AIDA-I *E. coli* isolate PD20 (Lanes 1 and 4); surface proteins harvested from the porcine AIDA-I *E. coli* isolate PD20 (Lanes 2 and 5); surface proteins harvested from human AIDA-I *E. coli* strain 2787 (Lanes 3 and 6).

AIDA-1 positive *E. coli* AIDA-1 negative *E. coli*

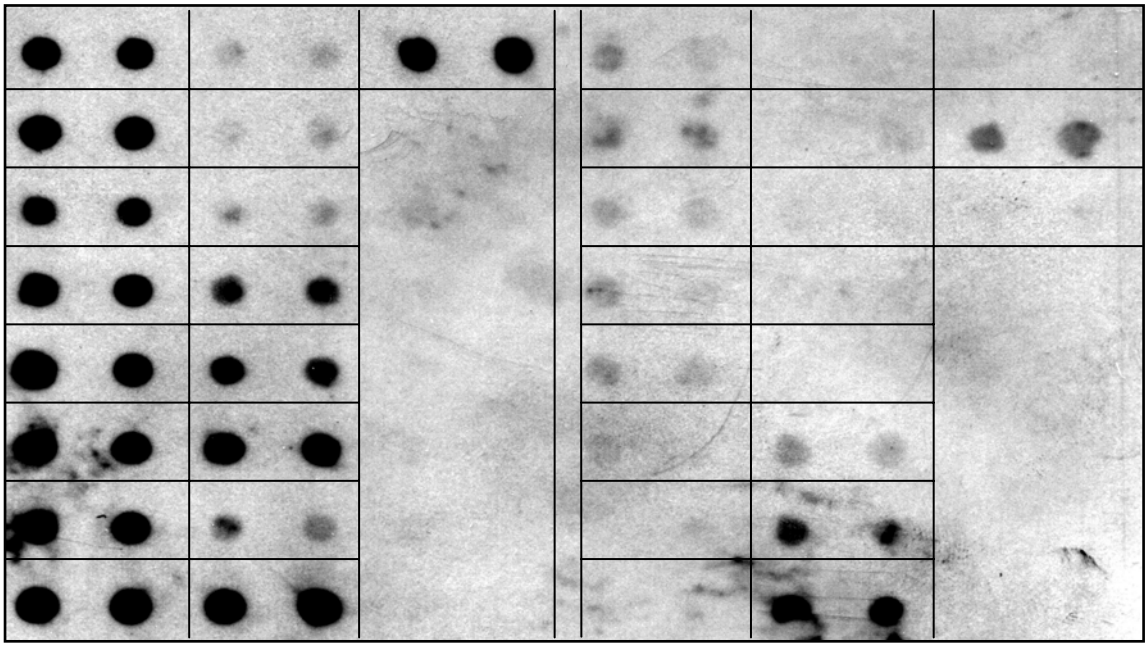


Fig. 3.5. Immuno-dot-blot detection of AIDA-I protein on bacterial surfaces of porcine *E. coli* isolates determined to be positive or negative for AIDA-I gene by PCR. *E. coli* isolates were dot-blotted in duplicates.

Table 3.1

Comparison of amino acid sequences determined by LCMS/MS for fragments of AIDA-I protein isolated from the porcine AIDA-I *E. coli* isolates PD20 and PD58 with AIDA-I protein from the human AIDA-I *E. coli* strain 2787 (Benz and Schmidt, 1992a).

		112					117		338										352		388					393	
Human	…	G	A	T	I	S	T	…	A	I	N	T	L	V	S	D	G	G	Y	Q	H	I	R	…	R	V	L	S	D	G	…
PD 20	…	G	A	T	V	S	T	…	-		N	T	L	V	S	D	G	G	S	Q	H	L	R	…	B	V	L	S	D	G	…
PD 58	…	-	-	-	-	-	-	…	A	L	N	T	L	V	S	D	G	G	S	Q	H	L	R	…	-	-	-	-	-	-	…

		396									405		408						414		473						480		
Human	…	R	G	T	I	L	N	N	S	G	R	…	V	S	N	G	G	V	S	…	A	E	V	Y	S	G	G	K	…
PD 20	…	B	E	T	L	L	N	N	S	G	R	…	V	S	D	G	G	V	S	…	-	-	-	-	-	-	-	-	…
PD58	…																			…	E	V	Y	Y	S	G	G	K	…

		520										530		679					685		691							698		
Human	…	F	A	G	N	V	V	G	T	I	L	N	…	N	S	A	G	R	L	R	…	T	I	N	G	T	T	T	I	…
PD 20	…	F	A	G	N	V	V	G	T	L	L	N	…	-	-	-	-	-	-	-	…	T	V	D	G	S	T	T	L	…
PD58	…	-	-	-	-	-	-	-	-	-	-	-	…	N	S	A	G	M	L	R	…	-	-	-	-	-	-	-	-	…

		797							804		822			825									836		
Human	…	G	S	I	V	I	N	N	S	…	B	L	L	S	A	T	V	N	G	S	L	V	N	N	K	…
PD 20	…	G	S	L	V	S	D	N	S					S	A	T	V	D	G	S	L	V	N	N	K	…
PD58	…	-	-	-	-	-	-	-	-	…	K	L	L	S	A	T	V	D	G	S	L	V	N	N	K	…

		847								855		1177						1183		
Human	…	A	G	N	T	L	T	V	S	N	…	T	V	V	Q	G	A	G	…	
PD 20	…	A	G	N	V	V	T	V	V	N	…	T	V	V	Q	A	A	G	…	
PD58	…	-	-	-	-	-	-	-	-	-	…	-	-	-	-	-	-	-	…	

Shaded amino acids are different in the human and porcine AIDA-I proteins.

Table 3.2
Coagglutination and immuno-dot-blot detection of the AIDA-I protein on bacterial surfaces of porcine *E. coli* isolates determined to be positive or negative for AIDA-I gene by PCR

AIDA-I positive *E.coli*			AIDA-I negative *E.coli*		
Isolate	Coagglutination	Immuno-dot-blot	Isolate	Coagglutination	Immuno-dot-blotblot
PD20	++	++++	D123	±	-
PD22	++	++++	D126	±	-
PD23	+	+++	D127	-	-
PD58	+	++++	D132	+	-
PD74	±	++++	D136	-	-
PD75	±	++++	D140	-	-
PD80	++++	++++	D143	-	-
PD81	++++	++++	D144	-	-
PD100	++	-	D148	-	-
PD101	++	-	D152	-	-
PD102	+	-	D31	-	-
PD108	+	+	D59	-	-
PD124	+++	++	D73	-	-
PD134	+++	++++	D76	±	-
PD153	+	-	D82	+	+
PD80-1	±	++++	D83	++	++
PD80-2	±	++++	D96	-	-
			D99	±	±
			D35	-	-

3.4. Discussion

In this study we demonstrated that AIDA-I adhesin isolated from porcine *E. coli* isolates is an acidic 100kDa protein with a relatively high amino acid sequence homology to the AIDA-I adhesin of human AIDA-I positive *E. coli* strain 2787 (Benz and Schmidt, 1989, 1992a), and that the sensitivity of detection of surface AIDA-I adhesin of PCR-positive AIDA-I *E. coli* by immuno-dot-blot and coagglutination tests was 76 and 71%, respectively, whereas specificity was 89% and 84%, respectively.

Mainil et al (2002) achieved a higher sensitivity (93%) and specificity (90%) of detection of AIDA-I protein in porcine *E. coli* isolates by Western blot using immune serum that was raised against a recombinant *E. coli* K12 (pIB4) expressing AIDA-I protein cloned from the human *E. coli* strain 2787 and that was pre-absorbed with *E. coli* K12 (pBR322) (Mainil et al., 2002). Even though coagglutination detection of AIDA-I positive *E. coli* is a very simple and fast test it is unlikely to be used routinely for diagnostic detection of AIDA-I positive *E. coli* due to the relatively low sensitivity. The specificity of immuno-dot-blot and coagglutination detection of AIDA-I adhesin on the bacterial surface is relatively high and these tests may be potentially useful for identification of false positive reaction by other detection methods.

Given the relatively high degree of homology between the amino acid sequences of certain corresponding segments of porcine and human AIDA-I proteins, we were surprised by a failure to detect human AIDA-I positive *E. coli* strain 2787 by immuno-dot-blot, and by only a weak detection of human AIDA-I protein by Western blot using rabbit polycolonal antiserum raised against AIDA-I protein isolated from porcine AIDA-I positive *E. coli*. The decreased sensitivity of detection could be explained, at least

partially, by an apparent lower AIDA-I protein production by the human AIDA-I *E. coli* strain 2787 when compared to our porcine isolate PD20. Potential immunogenic structural differences between AIDA-I proteins produced by human and porcine *E. coli* strains might also explain failure or weak detection of human AIDA-I *E. coli* strain 2787 by antibodies raised against porcine AIDA-I protein in this study. Since antibodies raised against AIDA-I protein from human *E. coli* were able to detect porcine AIDA-I positive *E. coli* isolates (Mainil et al., 2002), we suspect that low expression intensity of AIDA-I protein, rather than a substantial immunogenic variability, was responsible for failure to detect the human AIDA-I *E. coli* strain 2787 by our immuno-dot-blot test. However, determination of the nucleotide sequence of the AIDA-I gene of porcine *E. coli*, which was not done in this study, would have shown definitively the degree of similarity between AIDA-I genes from porcine and human *E. coli* and identify potential immunogenic differences. In addition, determination of the nucleotide sequence of the AIDA-I gene of various porcine *E. coli* isolates will be necessary to determine if there is a very high homology of the AIDA-I gene among various porcine *E. coli* isolates, as suggested based on our limited comparison of amino acid sequences of AIDA-I proteins isolated from the PD20 and PD58 isoaltes.

We reported previously that AIDA-I gene was present in 9% of *E. coli* isolates from diarrheic pigs in Western Canada. Ha et al (2003, 2004) reported the presence of AIDA-I gene in 2.3% (23 out of 1002) of *E. coli* isolated from piglets with pre-weaning diarrhea and in 7.5% (45 out of 604) of *E. coli* isolated from pigs with post-weaning diarrhea or edema disease in Korea (Ha et al., 2003; Ha et al., 2004). In Germany and Belgium, the prevalence of AIDA-I positive *E. coli* isolated from pigs with edema disease

or post-weaning diarrhea was reported to be 41% and 63%, respectively. The importance of AIDA-I adhesin in pathogenesis of porcine diarrhea is not known and it is being presently investigated in our laboratory. The diarrheogenic pathogenicity of two of our AIDA-I positive isolates was tested and confirmed by oral infection of colostrum-deprived piglets which developed diarrhea (Ngeleka et al., 2003; Pritchard et al., 2004). Preliminary data of our further experiments (Ravi et al., 2007) indicate that AIDA-I positive *E. coli* causes transient non fatal diarrhea in neonatal piglets. We suspect that AIDA-I positive *E. coli* infection may interfere with optimal growth of neonatal and post-weaning piglets and cause economical loses in modern swine industry. However, further studies are needed to substantiate the impact of AIDA-I positive *E. coli* infection and to investigate potential prevention.

3.5. Conclusion

AIDA-I adhesin isolated from porcine AIDA-I positive *E. coli* isolates is an acidic 100 kDa protein consisting of five isoforms. It has a relatively high amino acid homology with the AIDA-I adhesin expressed by the AIDA-I positive *E. coli* strain 2787 isolated from a case of human infantile diarrhea (Benz and Schmidt, 1989). Based on the amino acid sequence of two segments determined for both AIDA-I proteins isolated from our porcine isolates PD20 and PD58, it appears that there is a very high homology among AIDA-I proteins of porcine AIDA-I positive *E. coli* isolates. Our immuno-dot-blot and coagglutination tests are unlikely to be used for diagnostic detection of AIDA-I positive *E. coli* due to relatively low sensitivity; however, they are potentially useful for identification of false positive reactions generated by other diagnostic tests.

4. Isolation and identification of AIDA-I receptors in porcine intestinal mucus

This chapter contains the complete text of a manuscript accepted for publication with minor revisions in Veterinary Microbiology (2007) by Yuanmu Fang, Musangu Ngeleka, Dorothy M. Middleton and Elemir Simko

4.1. Introduction

Bacterial attachment to the intestinal surface is the necessary first step in pathogenesis of diarrhea induced by *Escherichia coli*. In our recent survey, only 27 out of 170 *E. coli* isolates from clinical cases of porcine diarrhea were positive for common attachment virulence factors (i.e. F4, F5, F6, EAE). In addition to these isolates with common attachment factors, a further 15 of the 170 isolates were positive for adhesin involved in diffuse adherence (AIDA-I) (Ngeleka et al., 2003). Previously, we demonstrated high amino acid homology between the AIDA-I adhesin isolated from human and porcine *E. coli* isolates (Fang et al., 2005) and that AIDA-I adhesin is necessary for attachment of porcine AIDA-I positive *E. coli* to HeLa cells and for biofilm formation *in vitro* (Ravi et al., 2007). We demonstrated *in vivo* that porcine AIDA-I positive *E. coli* is associated with biofilm formation and development of diarrhea in colostrum deprived neonatal piglets (Pritchard et al., 2004). Using the following bacterial strains: i) wild type strain: AIDA-I^+/STb^+ *E. coli* isolated from a clinical case of porcine diarrhea, ii) mutant strain: AIDA-I^-/STb^+ *E. coli*, generated by partial deletion of the *aidA* gene from the wild strain, and iii) complemented strain: AIDA-I^+/STb^+ *E. coli*, generated

by reintroducing the full length *aidA* gene into the mutant strain, we also demonstrated that AIDA-I adhesin is an important virulence determinant involved in intestinal colonization with biofilm formation and development of diarrhea in colostrum deprived neonatal piglets (Ravi et al., 2007). Only piglets infected with AIDA-I positive *E. coli* developed diarrhea and intestinal biofilm characterized by distinct mucus and bacterial layers. The bacteria stratified within mucus layers expressed AIDA-I adhesin as demonstrated by immunohistochemistry and immunogold electron microscopy using rabbit polyclonal anti-AIDA-I antibody (Ravi et al., 2007). Based on this intimate interaction between AIDA-I positive *E. coli* and mucus within an intestinal biofilm, we hypothesized that porcine intestinal mucus contains receptor(s) for AIDA-I adhesin. Specific receptors in porcine intestinal mucus have been previously reported for the fimbrial adhesins F4 (Blomberg et al., 1993; Conway et al., 1990; Metcalfe et al., 1991) and F6 (Dean-Nystrom, 1995) of enterotoxigenic *E. coli*. Human AIDA-I adhesin binds a glycoprotein receptor of about 119 kDa on HeLa cells and on a variety of mammalian permanent tissue culture cell types (Laarmann and Schmidt, 2003); however, receptors for AIDA-I adhesin have not been investigated in intestinal mucus. Accordingly, the purpose of this study was to determine the presence of AIDA-I receptors in porcine intestinal mucus using affinity chromatography and *in vitro* adhesion assays.

4.2. Materials and methods

4.2.1. Animals

This research was approved by the University (of Saskatchewan) Committee on Animal Care and Supply (Proposal #20020059), and performed according to guidelines

outlined in 'The Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care'.

4.2.2. Bacteria

Escherichia coli strain PD20 ($AIDA^+$, STb^+) isolated from neonatal pigs with diarrhea was used for isolation of AIDA-I adhesin and for adhesion assays in this study. A mutant *E. coli* strain PD20 ($AIDA^-$, STb^+) generated previously (Ravi et al., 2007) and a clinical isolate of *E. coli* $F4^+$ were used as negative controls.

4.2.3. Isolation and purification of AIDA-I protein

AIDA-I protein was isolated from a porcine clinical isolate of AIDA-I positive *E. coli* and semi-purified as described previously (Fang et al., 2005). Further purification of AIDA-I adhesin was performed by size exclusion chromatography using a HiPrep 16/60 Sephacryl^{TM} S300 High Resolution prepacked gel filtration column (10-1500 kDa fractionation range) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The semi-purified AIDA-I protein was applied to the gel filtration column and eluted by phosphate-buffered saline (PBS) (0.05M sodium phosphate, 0.15M NaCl, pH7.2) at flow rate of 0.5-0.7 mL/min. Optical density (at 280 nm) of eluate was monitored and eluate was collected in 2 mL fractions. The fractions collected between 48 and 52 mL contained purified AIDA-I adhesin and were dialyzed against distilled water for 48 hours and lyophilized. The entire purification procedure was performed at 4°C. To determine purity, AIDA-I protein was applied to SDS-PAGE and gels were stained by silver stain.

4.2.4. Production and affinity chromatography purification of rabbit anti-AIDA-I antibody

Rabbit anti-AIDA-I serum raised against isolated AIDA-I adhesin as described in

a previous study (Fang et al., 2005) was used for purification of anti-AIDA-I antibody using Protein A Sepharose CL-4B affinity matrix (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Briefly, 2 g of Protein A Sepharose CL-4B powder was rehydrated and packed into XK 16/20 column (total matrix volume ~10 mL) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instruction. Affinity chromatography was performed by an ÄKTA purifier 10 chromatography system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at the flow rate of 1 mL/min using PBS (0.01M sodium phosphate, 0.15M NaCl, pH 7.2) as loading and washing (5 column volumes) buffers and a low pH buffer (0.1M citric acid pH 3.0) as elution buffer (3 column volumes). Optical density of the eluate was monitored at 280 nm. The fractions containing eluted antibodies were collected and their low pH was neutralized by 1 M Tris-HCl, pH 9.0 buffer, and then dialyzed against PB buffer (0.01M sodium phosphate, pH 7.2) overnight three times. The protein concentration was determined by colorimetric protein microassay (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories, Ltd. Mississauga, ON) and the final protein concentration was adjusted to 1mg/mL.

Rabbit polyclonal antibodies against whole bacterial cells of AIDA-I positive *E. coli* were also developed for detection of both AIDA-I positive and AIDA-I negative mutant *E. coli* in ELISA assay. Immunization of rabbits with whole bacterial cells was performed according to previously published procedure (Shahriar et al., 2006).

4.2.5. Preparation of mucus

Four colostrum deprived neonatal piglets (Prairie Swine Centre Inc. SK, Canada) were euthanatized, the small intestines were removed, and the small intestinal mucus was collected as previously described (Fang et al., 2000), and stored at -70ºC.

4.2.6. AIDA-I-Sepharose matrix preparation and affinity chromatography

AIDA-I affinity column was prepared by coupling of 50 mg of purified AIDA-I adhesin with 3g of freeze-dried CNBr-activated Sepharose 4B powder (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instruction. A negative control column was similarly generated using 3 g of CNBr-activated Sepharose 4B powder without AIDA-I adhesin. Coupling of AIDA-I adhesin to the Sepharose matrix was confirmed by immuno-dot-blotting as previously described (Shahriar et al., 2006). The AIDA-I-Sepharose and control Sepharose matrices were packed into separate chromatography columns (XK 16/20, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) to be used for isolation of AIDA-I receptors. An ÄKTA purifier 10 chromatography system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was employed with PBS buffer (10mM sodium phosphate, 150mM NaCl, pH 7.2) as the load and wash buffer. Intestinal mucus samples containing 2mg/mL proteins were filtered through 0.45 μm filters (PALL, Gelman Laboratory, MI, USA). Fifteen mL of filtered mucus sample was applied to the AIDA-I-Sepharose column, which was then washed with at least 10 column volumes of PBS buffer until a steady baseline was obtained, as determined by monitoring the OD at 280 nm of the eluate (i.e., <2 mAU). The bound proteins were then eluted from the column with two different elution buffers. The first (low affinity) elution was performed with 3 column volumes of 1M NaCl buffer (in 56mM NaH_2PO_4, 144 mM Na_2HPO_4, pH 7.2) followed by 1 column volume of PBS, while a

second (high affinity) elution was done with 3 column volumes of 0.1M Glycine-HCl (pH 2.5). These two elution fractions were collected and pooled separately, dialyzed for 48 h at 4°C against deionized H_2O, using 1 kDa cut-off cellulose dialysis membranes (Spectra/Por, Spectrum Laboratories Inc, CA, USA), and then lyophilized (Labconco Corp, MO, USA). The same procedure was repeated for the negative control Sepharose column. The entire purification procedure was performed at 4°C.

4.2.7. ELISA assay

ELISA assay was used to determine interaction between isolated AIDA-I receptors and purified AIDA-I adhesin. The protein concentrations of the isolated AIDA-I receptors, crude mucus, and bovine albumin (negative control) were adjusted to 0.11mg/ml. A series of dilutions were prepared for albumin, mucus, and AIDA-I receptors eluted by 1M NaCl from AIDA-I-Sepharose affinity column as described above. 96-well plates were coated with 100 µL of the above-prepared dilutions (2 wells for each dilution), incubated at room temperature for 2 h, washed 3 times with PBST (PBS with 0.05% Tween-20), blocked with PBS-10% fetal calf serum (pH 7.2) for 2 h at room temperature, and washed 3 times with PBST. Fifty µL of purified AIDA-I adhesin (12µg/ml) was added to each well and incubated for 2 h at room temperature. Unbound AIDA-I adhesin was removed from the wells by four sequential washes. Bound AIDA-I adhesin was detected in each well by sequential incubation with primary rabbit polyclonal anti-AIDA-I immunoglobulins (1:4000), secondary goat anti-rabbit IgG conjugated HRP (1:2000, Bio-Rad Laboratories, Ltd. Mississauga, ON), and TMB Peroxidase EIA substrate (Bio-Rad Laboratories, Ltd. Mississauga, ON), and subsequently quantified by a

microplate reader (MPM III, Bio-Rad Laboratories, Ltd. Mississauga, ON) at 450 nm. The data were collected, analyzed and plotted.

Interaction between isolated AIDA-I receptors and AIDA-I positive *E. coli* was also quantified by ELISA assay. The wells were coated with the series of dilutions of isolated AIDA-I receptors and incubated with 100 µL of PBST containing $1x10^5$ CFU/ml of one of the following bacteria: AIDA-I positive *E. coli*, AIDA-I negative mutant *E. coli*, or F4 positive *E. coli*. After three consecutive washes, bound bacteria were detected by sequential incubation with primary rabbit polyclonal anti-F4 serum (1:1000, Dr J.M. Fairbrother, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Que., Canada) (to detect F4 positive *E. coli*) or rabbit polyclonal serum raised against whole bacterial cells of AIDA-I positive *E. coli* (1:8000) (to detect AIDA-I positive and AIDA-I negative mutant *E. coli*), the secondary goat anti-rabbit IgG conjugated HRP (1:2000), and TMB Peroxidase EIA substrate (Bio-Rad Laboratories, Ltd. Mississauga, ON). Quantification of attached bacteria was performed by a microplate reader (MPM III, Bio-Rad Laboratories, Ltd. Mississauga, ON) at 450 nm.

4.2.8. Electrophoresis

4.2.8.1. One-dimensional SDS-PAGE

SDS-PAGE was performed in 12% gels according to a previously published procedure (Laemmli, 1970) using a small electrophoretic system (Mini-PROTEAN® 3 Cell, Bio-Rad Laboratories, Mississauga, ON, Canada) according to manufacturer's instructions.

4.2.8.2. Two-dimentional SDS-PAGE

Isolated AIDA-I receptors were subjected to isoelectric focusing in linear immobilized pH gradient strips (IPG) (Immobiline Dry Strip, pH 3-10, 13 cm, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using an isoelectric focusing system (IPGphor; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). All samples were prepared according to the manufacturer's protocols. Briefly, in each case approximately 2.7 μg (for silver stain detection) of lyophilized protein was diluted in 20 μL of double distilled water, mixed with 230 μL of rehydration solution (8 M urea, 18 mM DTT, 0.5% v/v CHEPS, 0.01% w/v bromophenol blue [Sigma Chemical Co, St Louis, MO], and IPG buffer 3-10 [Amersham Biosciences, Uppsala, Sweden]) and applied to IPG strips placed in strip holders. Active 12-h rehydration at 30 V and 20˚C was followed by isoelectric focusing. Separation conditions were limited to 50 μA per IPG strip with a gradual increase in voltage 1 h 500 V gradient, 1 h 500 V step-on-hold, 1 h 1000 V gradient, 1 h 1000 V step-on-hold, 2 h 8000 V gradient and 8000 V step-on-hold until a total of 14000 V/h was reached at a constant temperature of 20 ˚C. The second dimension fractionation, based on molecular weight, was performed by SDS-PAGE in 12% gels using a large electrophoretic system (PROTEAN II xi Cell, Bio-Rad Laboratories) with a 0.5% w/v agarose stacking matrix and use of a constant current of 24 mA per gel, as described previously (Simko et al., 1999). Separated proteins in one- and two-dimensional SDS PAGE were visualized by Coomassie blue or silver stain according to previously published procedure (Shevchenko et al., 1996) with slight modifications. Briefly, the SDS-PAGE gels were fixed in 50% methanol and 12% acetic acid overnight, washed with 35% ethanol, and sensitized in 0.02% $Na_2S_2O_3$. After washing in distilled water they were

stained in 0.2% $AgNO_3$ and immersed in developing solution (6% Na_2CO_3, 0.05% formaldehyde and 0.0004% $Na_2S_2O_3$). The staining was stopped with 5% acetic acid.

4.2.9. Overlay Western blot

Overlay Western blot was used to confirm interaction between the isolated AIDA-I receptors and purified AIDA-I adhesin or AIDA-I positive *E. coli*. The isolated AIDA-I receptors were separated by one- and two-dimensional SDS-PAGE and electroblotted onto PVDF membranes using small (for 1-D PAGE) or large (for 2-D PAGE) electrophoretic transfer cell apparatus (Bio-Rad Laboratories, Mississauga, ON, Canada) according to the manufacturer's instructions. The PVDF membrane with the blotted sample was subjected sequentially to the following procedures: blocking of any remaining protein binding sites with blocking buffer (2% non-fat dry milk, 0.1% Tween-20 in PBS); incubation with purified AIDA-I adhesin (2.5 μg/ml) or AIDA-I positive *E. coli* ($1x10^5$ CFU/ml; CFU/ml=$OD_{620}x2.5x10^8$) in blocking buffer for 90 minutes at room temperature with gentle shaking; five 15-min washes with PBS Tween-20 (PBST) buffer (0.1% Tween-20 in PBS); incubation with purified primary rabbit polyclonal anti-AIDA-I antibodies (1:5000) for 90 minutes; five 15-min washes with PBST; incubation with secondary goat anti-rabbit IgG conjugated with alkaline phosphatase (1:20,000, Sigma Chemical Co, St Louis, MO, USA) for 90 minutes; three 15-min washes with PBST; and exposure to a substrate solution (Immun-Star Substrate, Bio-Rad Laboratories, Mississauga, ON). Chemiluminescent signal demonstrating AIDA-I receptors was detected by exposure of the PVDF membrane to an X-ray film (Kodak X-Omat AR Film, Eastman Kodak Company, Rochester, NY, USA).

4.2.10. Amino acid sequencing analysis

The AIDA-I binding proteins isolated by affinity chromatography were separated by 1D SDS-PAGE and visualized by Coomassie blue stain. Protein bands with the same molecular weights as those identified by Overlay Western blot of AIDA-I receptors (i.e. 65 and 120 kDa) were excised from the gel and submitted for identification by liquid chromatography mass spectrometry (LCMS/MS) performed at the National Research Council Plant Biotechnology Institution (Saskatoon, SK, Canada). Identity of the proteins that interact with AIDA-I adhesin were determined based on data obtained from LCMS/MS using Mascot 2.1 search engine (Matrix Science Inc. Boston, MA) available through the National Research Council of Canada.

4.3. Results

AIDA-I adhesin isolated by heat extraction (Fig. 4.1, Lane 1) and purified by gel filtration (Fig. 4.1, Lane 3) was coupled to activated sepharose and used for affinity isolation of AIDA-I binding proteins from intestinal mucus (Fig. 4.2). Several mucus proteins were eluted from the AIDA-I-Sepharose matrix by a low affinity elution (1M NaCl) (Fig. 4.3, Lane 1), and subsequently 65 and 120 kDa AIDA-I-binding proteins (p65 and p 120) were eluted by high affinity elution (low pH buffer) (Fig. 4.3, Lane 2). Several proteins ranging form 30 to 60 kDa and from 14 to 25 kDa were eluted from both the AIDA-I-Sepharose and the Sepharose only (negative control) matrices (Fig. 4.3, Lane 1, 2, and 3), accordingly, they were considered to be Sepharose-binding proteins, but not AIDA-I binding proteins.

More intense binding of purified AIDA-I adhesin to the protein fraction eluted by 1M NaCl from AIDA-I-Sepharose matrix than to crude mucus confirmed that affinity chromatography employed for isolation of AIDA-I receptors was effective (Fig. 4.4); and

similarly, the more intense binding to the protein fraction eluted by 1M NaCl from AIDA-I-Sepharose matrix exhibited by AIDA-I positive *E. coli* than by either AIDA-I negative mutant *E. coli* or F4 positive *E. coli* confirmed that the isolated fraction contained proteins interacting specifically with AIDA-I adhesin (Fig. 4.5). The protein fractions isolated by low and high affinity elution from AIDA-I-Sepharose matrix contained several proteins; therefore, Overlay Western Blotting was used to demonstrate that purified AIDA-I adhesin or AIDA-I positive *E. coli* bound exclusively to p65 and p120 separated by 1D or 2D SDS-PAGE and blotted onto PVDF membrane (Fig. 4.6 and 4.7). Based on 2D SDS-PAGE page migration, p120 has 3 isoforms with isoelectric points (pI) ranging from 4.8 to 5.2, and p65 has several isoforms, pI of which ranges from 5.2 to 5.7.

p65 and p120 were excised from 1D SDS-PAGE gels, submitted for LCMS/MS, and peptide fragment mass fingerprints of p120 were matched with Mascot database search to DNA damage binding protein 1 (gi|418316), splicing factor 3b (gi|19527174), and IgG Fc binding protein (gi|4503681) (Table 4.1), whereas peptide fragment mass fingerprints of p65 were matched predominantly to various cytokeratins of stratified squamous epithelial cells (commonly detected as a contaminant protein by LCMS/MS protein identification procedure) and occasionally to cytokeratins of simple epithelia (i.e. cytokeratins 8 and 19) (Table 4.1).

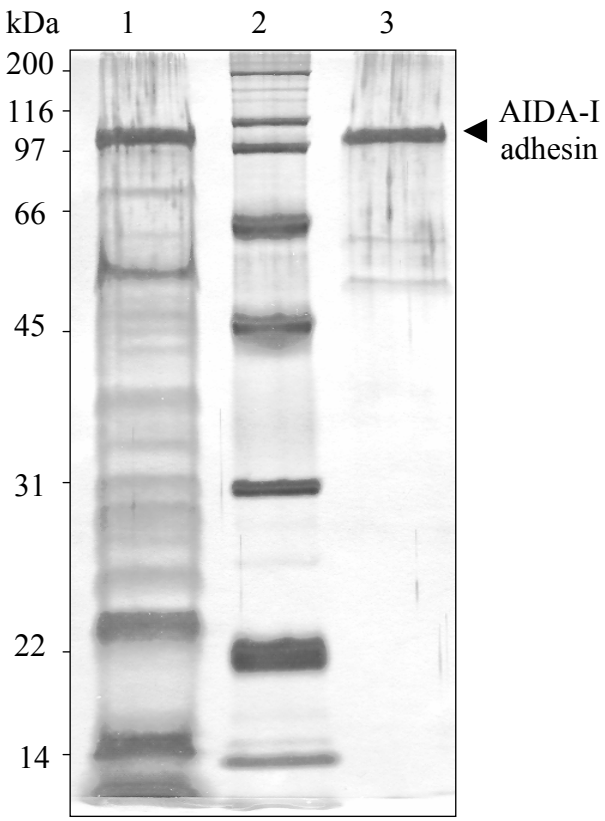


Fig. 4.1. Silver-stained reduced SDS-PAGE of AIDA-I adhesin isolated from porcine isolate of AIDA-I positive *E. coli* by heat extraction (Lane 1) and purified by gel filtration (Lane 3). Molecular weight markers (Lane 2).

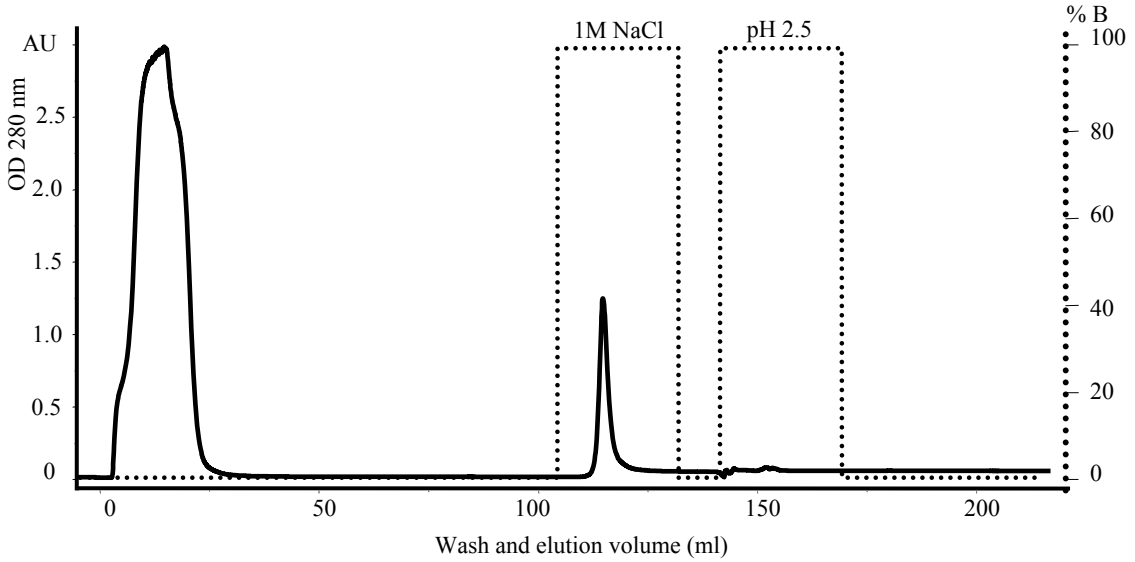


Fig. 4.2. Affinity chromatography isolation of AIDA-I binding proteins from neonatal porcine intestinal mucus using purified AIDA-I adhesin coupled to Sepharose 4B. Intestinal mucus proteins bound to affinity matrix were eluted with low 1M NaCl followed by low pH elution buffers.

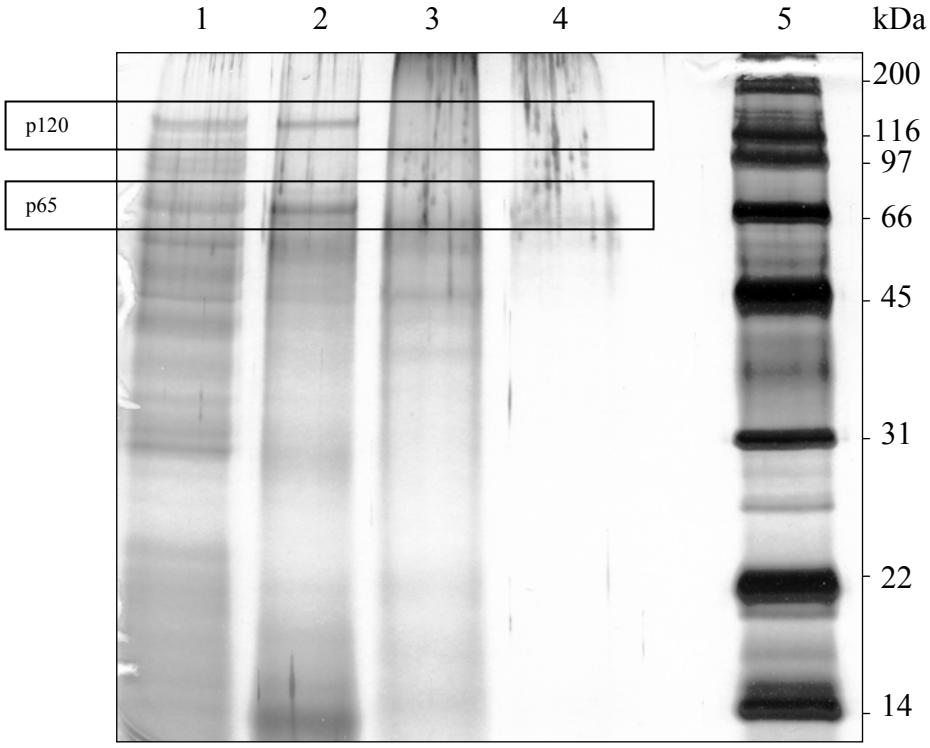


Fig. 4.3. Silver stained reduced SDS-PAGE of AIDA-I binding proteins eluted from AIDA-I-Sepharose matrix with 1M NaCl (Lane 1) followed by pH 2.5 elution buffer (Lane 2), and Sepharose binding proteins eluted from negative control affinity matrix (Sepharose only) with 1M NaCl (Lane 3) followed by pH 2.5 elution buffer (Lane 4). Molecular weight markers (Lane 5).

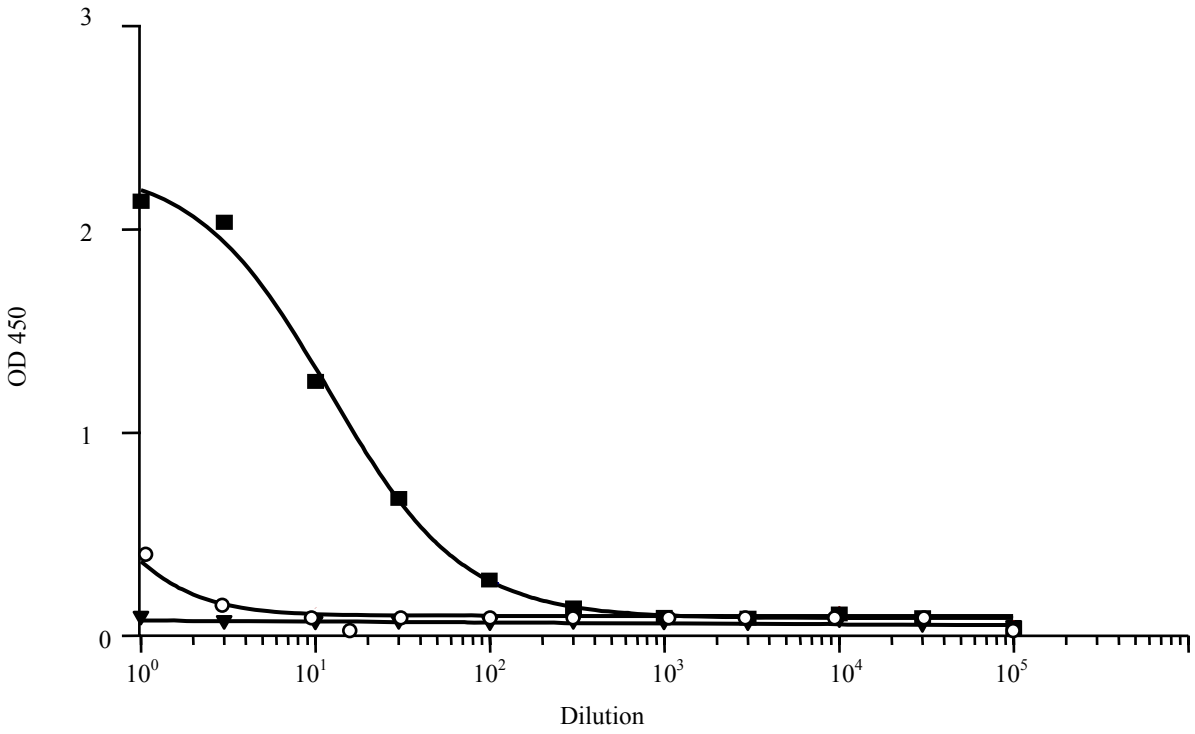


Fig. 4.4. Interaction between purified AIDA-I adhesin and 1) AIDA-I binding proteins eluted by 1M NaCl (■), 2) crude porcine intestinal mucus (○), and 3) albumin (negative control) (▼). AIDA-I adhesin interacted most intensely with AIDA-I binding proteins isolated by affinity chromatography. Quantification of interaction was determined by ELISA assay using polyclonal anti-AIDA-I antibody.

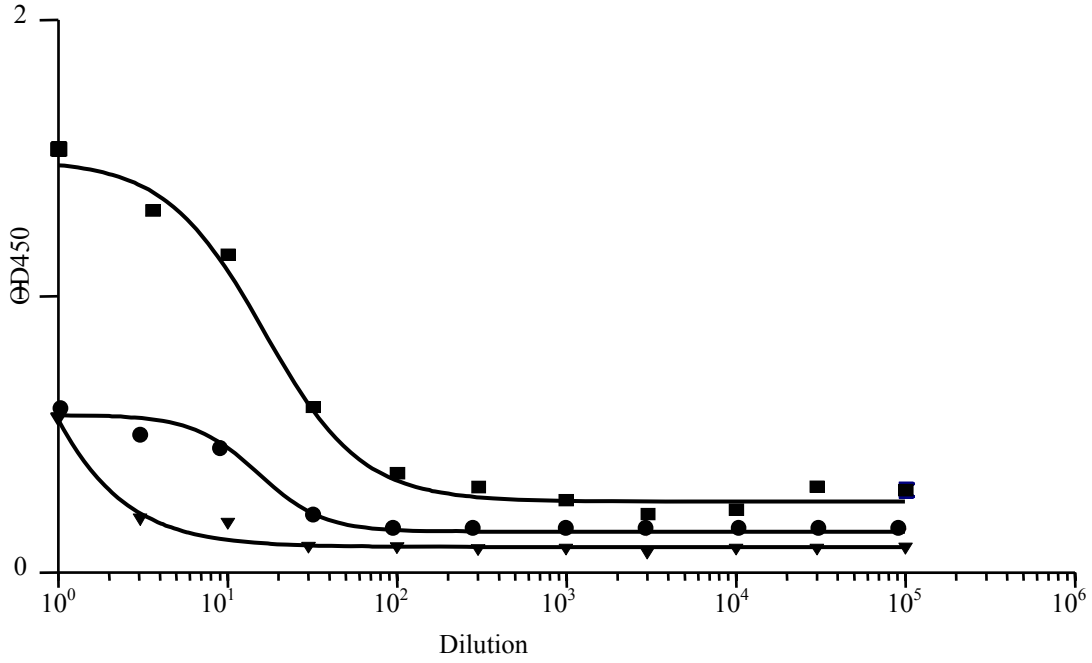


Fig. 4.5. Interaction between AIDA-I binding proteins eluted by 1M NaCl and 1) AIDA-I positive *E. coli* (■), 2) AIDA-I negative mutant *E. coli* (●), F4 positive *E. coli* (▼). AIDA-I binding proteins interacted most intensely with AIDA-I positive *E. coli.* Quantification of interaction was determined by ELISA assay using polyclonal anti-F4 serum for *E. coli* $F4^+$ and AIDA-I antibody polyclonal serum raised against whole AIDA-I positive *E. coli* for detection of both AIDA-I positive and AIDA-I negative mutant *E. coli*.

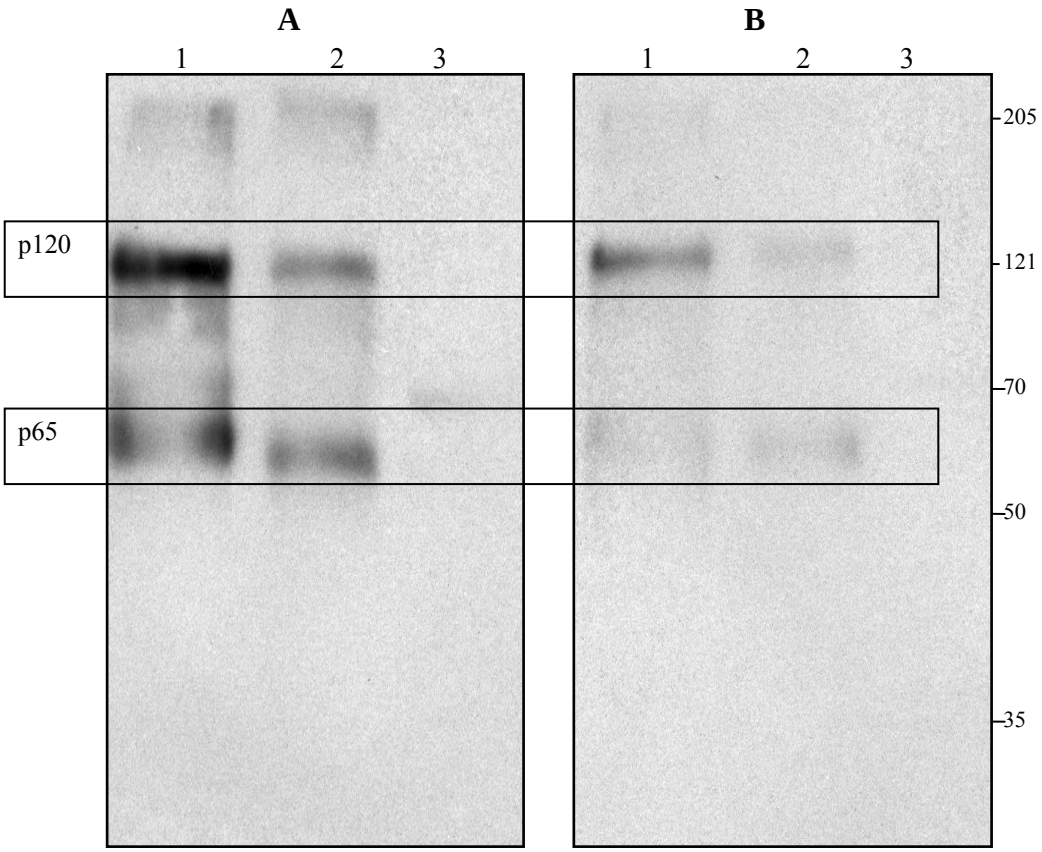


Fig. 4.6. Overlay Western Blot detection of p65 and p120 AIDA-I binding proteins. AIDA-I binding proteins eluted by 1M NaCl (Lane 1A and 1B) and low pH elution buffer (Lane 2A and 2B), and crude intestinal mucus (Lane 3A and 3B) were separated by reduced one-dimensional SDS-PAGE and electroblotted to PVDF membrane. p65 and p120 were detected by purified AIDA-I adhesin (A) and AIDA-I positive *E. coli* (B) as described in Material and Methods.

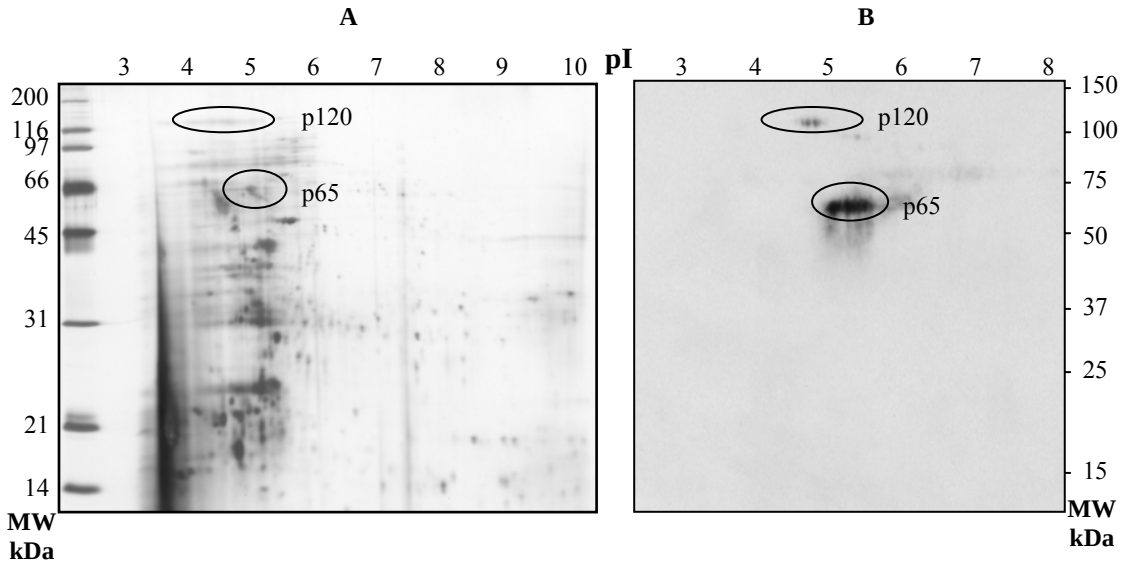


Fig. 4.7. Silver-stained two-dimensional SDS-PAGE of AIDA-I binding proteins eluted by 1M NaCl (A) and Overlay Western Blot detection of p65 and p120 AIDA-I binding proteins using purified AIDA-I adhesin (B) as described in Material and Method

Table 4.1 Matched proteins in MASCOT database search for LCMS/MS peptide fragment mass fingerprints of p120 and p65

AIDA-I binding protein (MW)	Name of matched protein [species] in MASCOT database and NCBI access. #	Total MASCOT protein score	Predicted MW (kDa)	Predicted AA sequence of peptide	MASCOT peptide score	Significance of individual scores	
						Homo-logy	Identity
p120 (120 kDa)	DNA damage binding protein 1 [*Homo sapiens*] gi|418316	287	128	LLASINSTVR	63	>49	>53
				YLAIAPPIIK	41	>48	>53
				IVVFQYSDGK	49	>37	>53
				VTLGTQPTVLR	44	>50	>53
				QSGESIDIITR	59	>46	>53
				EEQMDGTVTLK + Oxidation (M)	15	>28	>53
				IEVQDTSGGTTALRPSASTQALSSSVSSSK	19	>38	>49
	Splicing factor 3b [*Mus musculus* & *Homo sapiens*] gi|19527174 & gi|40254849	176	137	QQEIVVSR	30	>36	>54
				FLAVGLVDNTVR	76	>49	>53
				TVLDPVTGDLSDTR	73	>44	>52
	IgG Fc binding protein [*Homo sapiens*] gi|4503681	83	597*	GSQAVSYTR	42	>39	>52
				CFEGCECDDR	48	>43	>53
	IgG Fc binding protein [Rattus norvegicus] gi|34855405	53	608	GSQAVSYTR	42	>39	>52
				TDSFCPLQCPAHSHYSVCTR	17	>28	>50
p65 (65 kDa)	Cytokeratin 8 [*Mus musculus*] gi|13624315	85	54	YEDEINKR	28	>37	>53
				SLNNKFASFIDK	57	NA	>52
	Keratin 19 [*Gallus gallus*] gi|45384356	81	46	LASYLDK	42	>43	>53
				TTMQNLNDR + Oxidation (M)	40	>43	>53

4.4. Discussion

We have demonstrated that porcine mucus contains 65 and 120 kDa proteins (p65 and p120) that bind with high affinity to AIDA-I adhesin isolated from porcine AIDA-I positive *E. coli* isolate and that AIDA-I positive *E. coli* binds to these proteins with higher affinity than do AIDA-I negative mutant and F4 positive *E. coli*. Based on this we hypothesize that p65 and p120 serve as AIDA-I receptors in porcine intestinal mucus. LCMS/MS peptide fragment mass fingerprints of p120 were matched in Mascot data base to two nuclear proteins, which are unlikely to be AIDA-I receptors, and to an IgG Fc binding protein which appears to be secreted by goblet cells (Kobayashi et al., 1991) and thus may serve as an AIDA-I receptor in intestinal mucus.

Laarmann and Schmidt (2003) demonstrated that AIDA-I adhesin purified from human *E. coli* isolate binds specifically to a surface glycoprotein (approximate molecular weight and pI of 119 kDa and 5.2, respectively) present on HeLa cells. In addition, AIDA-I adhesin was found to bind to a variety of mammalian cell types, suggesting a broad tissue distribution of the receptor moiety (Laarmann and Schmidt, 2003). Based on similarity of molecular weight and isoelectric point, it is possible that our p120 kDa high affinity AIDA-I binding protein identified in porcine mucus is related to the 119 kDa surface glycoprotein identified on HeLa cells by Laarmann and Schmidt (2003); however, since the amino acid sequence of AIDA-I receptor on HeLa cells was not reported, a relationship between 120 kD AIDA-I binding protein in porcine mucus and 119 kDa receptor on HeLa cells was not determined.

We found it challenging to interpret the LCMS/MS results for p65 and p120, even though consistent and reproducible results were obtained from both low affinity (1M

NaCl) and high affinity (low pH) elutions. Identity of p65 was not determined because it was matched predominantly to various cytokeratins of stratified squamous epithelial cells (often encountered as contaminant proteins during LCMS/MS protein identification) and occasionally to cytokeratins of simple epithelia (cytokeratins 8 and 19). While p65 may represent cytokeratins released from disintegrated enterocytes during the mucus collection procedure, it is also possible that the genuine identity of p65 was not determined due to its low concentration and due to interference by contaminant proteins. Alternatively, it could be that p65 protein is a novel protein not previously sequenced and entered into the Mascot database, and its identity therefore could not be determined. On the other hand, p120 was matched to three different proteins despite background contamination by cytokeratin proteins. Two of these are nuclear proteins of similar MW to p120, namely, DNA damage binding protein (128 kDa) and splicing factor 3b (130kDa). It is possible that they also were released from disintegrated enterocytes during the mucus collection procedure and isolated by AIDA-I affinity chromatography. The third protein is an IgG Fc binding protein discovered in the epithelium of human small and large intestines (Kobayashi et al., 1989) and localized specifically in endoplasmic reticulum and secretory granules of goblet cells, which appear to secrete it into the intestinal lumen (Kobayashi et al., 1991). Based on amino acid sequence, it was determined that the IgG Fc binding protein exhibits a mucin-like structure (Harada et al., 1997). IgG Fc binding protein subjected to non-reduced SDS-PAGE consists of >200 kDa and 78 kDa components and in reduced SDS-PAGE, it appears to be 78 kDa (Kobayashi et al., 1989). Further immunoblot analysis using 14 different monoclonal antibodies developed against IgG Fc binding protein revealed that IgG Fc binding protein consists of >200 kDa,

110-140 kDa and 78 kDa in non-reduced SDS-PAGE, whereas in reduced SDS-PAGE monoclonal antibody K17 recognized antigenic components of IgG Fc binding protein at about 124 kDa and 60-70 kDa (Knutton et al., 1991). The same group of researchers observed an apparent increase in synthesis and secretion of IgG Fc binding protein by ulcerated human colon (unpublished data referred to by Harada et al, 1997). This observed induced colonic secretion of IgG Fc binding protein similar to mucus is very interesting. In our previous study, we demonstrated that AIDA-I positive *E. coli,* but not the AIDA-I negative *E. coli* mutant strain, induces colonic mucosal biofilm (intimately stratified layers of mucin and bacteria) and diarrhea in neonatal piglets (Ravi et al., 2007) and in this study we demonstrated that porcine intestinal mucus contains potential AIDA-I receptors one of which is homologous with IgG Fc binding protein secreted by human goblet cells.

Based on amino acid homology, similar molecular weight detected by monoclonal antibody K17 in reduced SDS-PAGE (Knutton et al., 1991), structural similarity to mucin (Harada et al., 1997) and evidence of being secreted by goblet cells into the intestinal lumen (Knutton et al., 1991), we think that IgG Fc binding protein is the most likely candidate to serve as a potential receptor in intestinal mucus for AIDA-I adhesin. Whether AIDA-I adhesin expressed by *E. coli* interacts *in vivo* with IgG Fc binding protein secreted within intestinal mucus is not known, but we believe that our data obtained from *in vitro* experiments justify further investigation to determine the role of IgG Fc binding protein in intestinal colonization by AIDA-I positive *E. coli*. The molecular weight of p65 AIDA-I binding protein reported in this study and the molecular weight of 60-70 kDa of one of antigenic components of IgG Fc binding protein detected by K17 monoclonal antibodies are similar; however, a potential relationship between them was not

determined, due to our inability to identify p65 by LCMS/MS in this study. The biological significance of potential interaction of AIDA-I adhesin with nuclear DNA damage binding protein and splicing factor 3b is questionable, since these proteins are unlikely to be secreted by enterocytes into intestinal lumen.

4.5. Conclusion

We have demonstrated that porcine intestinal mucus contains 65 and 120 kDa proteins (p65 and p120) that bind with high affinity to AIDA-I adhesin isolated from porcine AIDA-I positive *E. coli* isolates and that AIDA-I positive *E. coli* binds to these proteins with higher affinity than do an AIDA-I negative mutant and F4 positive *E. coli*. Based on LCMS/MS data, the identity of p65 was not determined, whereas p120 was matched to two nuclear proteins (namely, DNA damage binding protein and splicing factor 3b) and one cytoplasmic protein, which is an IgG Fc binding protein. Based on similar amino acid homology, molecular weight, structural similarity to mucin and evidence of being secreted by goblet cells into the intestinal lumen, we think that this IgG Fc binding protein is most likely candidate to serve as a potential receptor in intestinal mucus for AIDA-I adhesin. Further studies *in vivo* are needed to determine the role of IgG Fc binding protein in intestinal colonization and pathogenesis of diarrhea induced by AIDA-I positive *E. coli*.

5. General discussion

The aims of the work presented in this thesis were to characterize the adhesin involved in diffuse adherence (AIDA-I) expressed by porcine *E. coli* isolates from clinical cases of diarrhea, and to identify the porcine intestinal receptors for AIDA-I positive *E. coli*. In Chapter 3, characterization of the AIDA-I isolated from porcine AIDA-I positive *E. coli* revealed that it is an acidic 100 kDa protein consisting of five isoforms. Significantly, it has a relatively high amino acid homology (78-87%) with the AIDA-I protein expressed by AIDA-I positive *E. coli* strain 2787 isolated from a case of human infantile diarrhea (Benz and Schmidt, 1989). In Chapter 4, components which can potentially serve as AIDA-I adhesin receptors in porcine intestinal mucus were identified and of these, the most likely candidate is IgG Fc binding protein. Thus our findings demonstrate the presence in porcine AIDA-I positive *E. coli* isolates of an AIDA-I protein with extensive homology with that of a human strain, and, through identification of a candidate receptor protein in mucus, they also contribute significantly to the understanding of the likely mechanism of colonization by AIDA-I positive *E. coli*.

The impetus for this research arose from the observations made at Prairie Diagnostic Services Inc. that AIDA-I positive *E. coli* were being isolated in a significant number of field cases of diarrhea (Ngeleka et al., 2003), consistent with findings in Europe (Mainil et al., 2002; Niewerth et al., 2001) and Korea (Ha et al., 2003; Ha et al., 2004). This work was conducted in parallel with investigations of the role of AIDA-I in

the pathogenesis of diarrhea caused by AIDA-I positive *E. coli* performed also by our group (Pritchard et al., 2004; Ravi et al., 2007).

Significant progress has been made associated with extensive research efforts in the last few decades to determine the important virulence factors associated with diarrheagenic *E. coli* in pigs and other species; yet *E. coli* diarrhea remains an important disease worldwide in animals and humans (Gyles and Fairbrother, 2004; Nataro and Kaper, 1998). Neonatal diarrhea and postweaning diarrhea caused by enterotoxigenic (ETEC) and enteropathogenic (EPEC) *E. coli* continue to be very serious health and economic problems for the modern intensive swine industry (Tubbs and Hill., 1993). ETEC, which are non-invasive pathogens, can cause severe diarrhea through attachment and colonization via fimbrial or nonfimbrial adhesins, and production of enterotoxins which induce water and electrolyte secretion into the intestinal lumen (Gyles and Fairbrother, 2004). Many fimbrial adhesins have been identified and their role explained; however, the role of AIDA-I, a non-fimbrial adhesin, has not yet been fully elucidated, although studies by our group have shown that it is associated with induction of diarrhea in colostrum-deprived neonatal piglets (Ravi et al., 2007). AIDA-I from a human *E. coli* isolate has been characterized (Benz and Schmidt, 1992a, b) but not from isolates derived from pigs. Laarmann and Schmidt demonstrated that AIDA-I adhesin purified from human *E. coli* isolate binds specifically to a surface glycoprotein (approximate molecular weight and pI of 119 kDa and 5.2, respectively) present on HeLa cells (Laarmann and Schmidt, 2003); however, no work on a potential receptor for porcine AIDA-I has been reported. Based on our morphological observations of intimate interaction between

AIDA-I positive *E. coli* and mucus, we hypothesized that the intestinal mucus most likely contains AIDA-I receptors.

In chapter 3, it was shown that porcine AIDA-I is an acidic 100 kDa protein with a significant degree of homology in the amino acid sequences of certain segments to those of human AIDA-I from *E. coli* strain 2787 (Benz and Schmidt, 1989, 1992a), and therefore we suspect that amino acid sequence homology of the entire human and porcine AIDA-I proteins is high. No isoelectric point of human AIDA-I has been reported for comparison with the values of the 5 porcine isoforms identified here. Schmidt's group demonstrated that AIDA-I from human *E. coli* strain (2787) is a 100 kDa protein (Benz and Schmidt, 1989) localized on the surface of bacteria and that it functions as an adhesin to HeLa cells (Benz and Schmidt, 1992b). In our study, AIDA-I protein was isolated from porcine AIDA-I positive *E. coli* strains PD20 and PD58 associated with neonatal diarrhea, and based on a limited comparison, it appears that there is 78-87% amino acid homology between AIDA-I proteins expressed by porcine and human AIDA-I positive *E. coli* isolates. Relatively high amino acid homology points to a likely similarity in immunogenic structure and function, despite our failure to detect the human *E. coli* strain 2787 by immuno-dot-blot and only weak detection of human AIDA-I protein by Western blot using rabbit polyclonal antiserum raised against purified porcine AIDA-I protein. Reciprocity of detection would be expected if homology is high, and this result conflicts with a study in which antibodies raised against AIDA-I protein from human *E. coli* were able to detect porcine AIDA-I positive *E. coli* isolates (Mainil et al., 2002). We suspect, however, that low expression intensity of the AIDA-I protein by human isolate, rather

than a substantial immunogenic variability, was responsible for failure to detect the human AIDA-I *E. coli* strain 2787 by our immuno-dot-blot test.

A reliable method for detection of *E. coli* adhesins constitutes a very useful diagnostic tool to characterize the virotype of *E. coli* pathogens involved in diarrhea disease. Currently, PCR is a commonly used method to investigate clinical *E. coli* isolates and to detect the AIDA-I gene (Ha et al., 2003). We developed alternative tests based on immuno-detection, and compared immuno-dot-blot and coagglutination tests with PCR as the standard. The sensitivity of detection of surface AIDA-I adhesin of PCR-positive AIDA-I *E. coli* by immuno-dot-blot and coagglutination tests was 76 and 71%, respectively, whereas the specificity was 89% and 84%, respectively. While the agglutination test is very simple and fast and it has been used to identify F4, F5, F6, F41 adhesin in *E. coli* isolated from piglets with neonatal and postweaning diarrhea (Fairbrother et al., 1988; Nakazawa et al., 1987), the low sensitivity is the major concern in using this technique. Thus in an attempt to increase the sensitivity, we developed a coagglutination test using monovalent coagglutination reagent as previously described (Gottschalk et al., 1993), but the 71% sensitivity achieved is still too low for routine diagnostic detection of AIDA-I positive *E. coli*. The results achieved suggest that specificity of immuno-detection of AIDA-I adhesin on the bacterial surface is relatively high, especially using immuno-dot-blot; however, due to cost and relatively low sensitivity, it is considered that the use of immuno-dot-blot will be restricted to research purposes for identification of false positive reactions generated by other detection methods. Further investigation and development of an agglutination test using a

monoclonal antibody based latex particle as previously described (Thorns et al., 1989) might lead to improved sensitivity and specificity.

The second aspect of this work, namely the isolation and identification of potential AIDA-I receptors in porcine intestinal mucus, resulted in an advance in the understanding of intestinal colonization by AIDA-I positive *E. coli*. Our group had previously observed through *in vivo* studies that porcine AIDA-I positive *E. coli* colonize the intestinal surface by formation of biofilm composed of stratified layers of bacteria and intestinal mucus (Pritchard et al., 2004) and that AIDA-I is required for this biofilm formation (Ravi et al., 2007). These findings suggested that the intestinal mucus should be investigated for the presence of AIDA-I receptors. As support for this approach, identification of receptors for the fimbrial adhesin F4 and F6 in porcine intestinal mucus has been reported (Blomberg et al., 1993; Conway et al., 1990; Dean-Nystrom, 1995; Metcalfe et al., 1991).

The adhesive properties of AIDA-I-bearing *E. coli* have been investigated both *in vitro* and *in vivo*: human AIDA-I positive *E. coli* adhere to HeLa cells and a variety of mammalian cells (Benz and Schmidt, 1992b; Laarmann and Schmidt, 2003); and expression of AIDA-I mediates diffuse adherence of AIDA-I positive *E. coli* to HeLa cells (Benz and Schmidt, 1992a). Diffusely adherent *E. coli* (DAEC) exhibit diffuse adherence to HeLa cells *in vitro* but reports on their pathogenicity in humans are contradictory (Nataro and Kaper, 1998). An age-dependent susceptibility/resistance might explain these contradictions in results, since several recent epidemiological studies indicate that DAEC are associated with diarrhea in young children but not in infants (Elliott and Nataro, 1995; Nataro and Kaper, 1998). Age susceptibility/resistance is recognized in pigs for F6 positive *E. coli* (Dean, 1990) and inheritance of receptor alleles plays a role in

susceptibility to F4 positive *E. coli* (Bijlsma et al., 1982; Rapacz and Hasler-Rapacz, 1986). Two attachment factors, the fimbria F1845 (Bilge et al., 1989) and the AIDA-I adhesin, mediate diffuse adherence of DAEC to Hep2 cells *in vitro* (Benz and Schmidt, 1989, 1992a); and a 119kDa surface glycoprotein on HeLa cells has been identified as a receptor for AIDA-I (Laarmann and Schmidt, 2003). Little is known, however, about the pathogenesis of DEAC *in vivo*. Thirty three to 75% of human DAEC react positively with a F1845 gene probe; but the specificity of this reaction has not been determined (Elliott and Nataro, 1995; Jallat et al., 1993; Nataro and Kaper, 1998). The AIDA-I gene was present in 2% of DAEC isolates from diarrheic stool specimens of infants, children, and adults in France (Jallat et al., 1993). This prevalence in human *E. coli* isolates is very low in comparison to porcine isolates (Ha et al., 2003; Ha et al., 2004; Mainil et al., 2002; Niewerth et al., 2001).

The investigation of a porcine intestinal crude mucus preparation undertaken in this work indicated that soluble proteins (MW120 kDa and 65 kDa) in this mucus are capable of binding AIDA-I and possibly of acting as AIDA-I receptors. The origin and function of these components *in vivo* are unclear as yet. Although p120 and p65 were isolated from intestinal mucus, potentially they could also represent brush border glycoproteins released into the intestinal lumen from sloughed cells rather than secreted mucus proteins. Adhesin-specific interactions with intestinal mucus may either enhance or inhibit intestinal colonization by enteric *E. coli*: one suggestion is that mucus glycoproteins in the small intestine might be a promoter of colonization by acting as a site for bacterial replication (Sherman and Boedeker, 1987); on the other hand, specific receptors in the intestinal mucus might interfere with the bacterial attachment to epithelial

cells, resulting in more efficient clearance of these bacteria (Drumm et al., 1988). In this study, AIDA-I interacted only with 65 kDa and 120 kDa proteins as demonstrated by Overlay Western blot and AIDA-I binding proteins isolated by affinity chromatography had increased binding capacity with AIDA-I positive *E. coli* compared with AIDA-I negative *E. coli* as demonstrated by ELISA. These results, taken together with *in vivo* biofilm formation within mucus as observed in experimental infection with a wild type AIDA-I positive *E. coli* strain but not with the AIDA-I negative *E. coli* mutant strain (Ravi et al., 2007), suggest that the colonization of AIDA-I positive *E. coli* in the intestine is enhanced by specific interaction of AIDA-I with receptors in the intestinal mucus. Further, re-complementation of the mutant strain with full *aidA* gene restored all the *in vivo* attributes of parent wild strain (Ravi et al., 2007). Additional *in vivo* studies are needed to determine whether for example colostral antibody against AIDA-I adhesin or AIDA-I receptor can prevent AIDA-I mediated adherence of AIDA-I positive *E. coli* and subsequent colonization in neonatal pigs.

In a search of the MASCOT database for LCMS/MS peptide fragment fingerprints of the 65 and 120 kDa proteins isolated from intestinal mucus, a number of matched proteins were identified; however, the cytokeratins matched to p65 were dismissed as likely contaminants and the possible identity of p65 was not determined, while two of the 120kDa proteins were considered unlikely to serve as AIDA-I receptors in mucus due to their nuclear location in the cell. The remaining matched protein, IgG Fc binding protein, warrants serious consideration as the candidate AIDA-I receptor protein in porcine intestinal mucus: taking together its secretion from goblet cells into the intestinal lumen

(Knutton et al., 1991), amino acid homology, close molecular weight (Knutton et al., 1991) and similarity in structure to mucin (Harada et al., 1997), the evidence is persuasive.

In conclusion, this study succeeded firstly in characterizing the AIDA-I protein isolated from porcine AIDA-I positive *E. coli* and demonstrated significant similarities with AIDA-I expressed by the AIDA-I positive *E. coli* strain 2787 isolated from a case of human infantile diarrhea. While the immuno-dot-blot and co-agglutination tests were developed to detect AIDA-I positive *E. coli*, these immunogenic based detection methods are unlikely to be used for diagnostic detection of AIDA-I positive *E. coli* due to relatively low sensitivity; however, they are potentially useful for identification of false positive reactions generated by other diagnostic tests. Secondly, significant progress was made towards identifying an AIDA-I receptor in porcine intestinal mucus. The most likely candidate of the matched proteins corresponding to the p65 and p120 proteins isolated from porcine intestinal mucus is an IgG Fc binding protein which has been identified in human colonic mucosa.

Logical future studies would focus on identifying this protein in the thick mucus layer associated with biofilm formation in experimental porcine AIDA-I positive *E. coli* infection, using monoclonal antibodies in immune based detection methods *in vitro* and in infected intestinal tissue samples *in vivo*. These would include determination of potential age susceptibility should age related expression of the putative receptor protein exist. The potential for active immunization of sows with purified AIDA-I to induce secretion of protective colostral antibody needs to be investigated as a means of reducing the economic impact of diarrhea by AIDA-I positive *E. coli*.

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