Pathogenesis and clinical significance of AIDA-I-positive *E. coli* in diarrhea of pigs

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ABSTRACT

Escherichia coli remains a significant cause of diarrhea worldwide and in recent years a relatively high number of *E. coli* carrying gene for AIDA-I (**a**dhesin **i**nvolved in **d**iffuse **a**dherence) has been isolated from cases of neonatal and post-weaning diarrhea in pigs. AIDA-I adhesin and its gene *aidA* were first identified and characterized in *E. coli* isolated from a human case of infantile diarrhea. Recent studies have demonstrated a significant degree of homology between the AIDA-I adhesin isolated from porcine neonatal diarrheagenic *E. coli* isolates and that from a human *E. coli* isolate; however, the role of AIDA-I adhesin in the pathogenesis of diarrhea and the clinical significance of the AIDA-I *E. coli* virotype are unknown in humans or in animals.

First, in order to evaluate the role of AIDA-I adhesin, colostrum deprived newborn pigs were infected with: i) a wild strain PD20 (AIDA-I^+/STb^+) *E. coli*; ii) a mutant strain PD20M (AIDA-I^-/STb^+), generated by partial deletion of the *aidA* gene from the wild strain, iii) a complemented strain PD20C (AIDA-I^+/STb^+), generated by reintroducing the full length *aidA* gene into PD20M strain, and iv) a nonpathogenic *E. coli* strain PD71 used as negative control. Pigs infected with wild type (PD20) and complemented (PD20C) strains developed diarrhea between 15-19 h and 27-31 h after oral inoculation, respectively, in contrast to pigs infected with strains PD20M or PD71 that did not developed diarrhea. Intestinal colonization was evaluated by histology, imunohistochemistry (IHC), transmission electron microscopy (TEM), including immunogold electron microscopy (IGEM), and showed heavy bacterial colonization with biofilm formation in the large intestine with AIDA-I^+ strains (PD20 and PD20C), but not in pigs infected with AIDA-I^- strains (PD20M and PD71). *In vitro* assays

showed marked diffuse adherence to HeLa cells, enhanced bacterial autoaggregation and significant biofilm formation by AIDA-I$^+$ strains, when compared to AIDA-I$^-$ strains.

Second, 110 F4 negative *E. coli* isolates from problematic cases of diarrhea in pigs were subjected to multiplex polymerase chain reaction (M-PCR) for detection of the genes encoding the virulence factors F4, F5, F6, F18, F41, AIDA-I, EAE, STa, STb, LT, EAST1 and Stx2e. In this study, the prevalence of *aidA* gene among the 110 isolates was 8.2%, and the *aidA* gene was shown to be associated most commonly with EAST1 and STb genes. The genes for the F4, F5, F6 and F41 fimbriae were absent in all the AIDA-I$^+$ *E. coli* isolates.

The clinical significance of the AIDA-I$^+$ *E. coli* was studied using clinical data available for 35 of the 110 *E. coli* isolates, originating from 18 cases of diarrhea. Among these 18 diarrhea cases, 3 cases (5 isolates) were found to have AIDA-I$^+$ *E. coli* and these were significantly associated with diarrhea cases of post-weaning age group. Enterotoxigenic *E. coli* strains were isolated from the majority (72.5%) of 18 diarrhea cases and a high proportion (23.1%) of these ETEC cases carried AIDA-I$^+$ *E. coli*.

In conclusion, AIDA-I adhesin appears to be a significant virulence factor for intestinal colonization and induction of biofilm formation. Further, experimental studies and clinical data suggest that the AIDA-I/STb virotype may be important in the pathogenesis of pre-weaning and post-weaning diarrhea in pigs. Our results suggest that AIDA-I may play a significant role in the development of diarrhea in pigs.

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AAF	aggregative adherence fimbria
AIDA-I	adhesin involved in diffuse adherence
A/E	attaching and effacing
Ag43	antigen 43
Amp	ampicillin
Amp^r	ampicillin resistant
BFP	bundle forming pili
bp	base pairs
Caco-2	human Caucasian colon adenocarcinoma
cAMP	cyclic adenosine monophosphate
CFU	colony forming units
CFA	colonization factor antigen
cGMP	cyclic guanosine monophosphate
Cl	chloride ion
CNF	cytotoxic necrotizing factor
CT	cholera toxin
DA	diffuse adherence
DAEC	diffusely adherent *Escherichia coli*
DNA	deoxyribonucleic acid
DNTP	Deoxynucleotide-triphosphate
EAE	*Escherichia coli* attaching and effacing
EAF	EPEC adherence factor
EAST1	enteroaggrgative stable toxin
E. coli	*Escherichia coli*
ED	edema disease
EHEC	enterohaemorrhagic *Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
ENS	enteric nervous system
EPEC	enteropathogenic *Escherichia coli*
EPS	extracellular polymeric substance
ETEC	enterotoxigenic *Escherichia coli*
Gm	gentamycin
Gm^r	gentamycin resistant
GuSCN	guanidinium thiocyanate
HeLa	**He**nrietta **La**cks cell line (a cell line derived from cervical cancer cells)
Hep-2	**h**uman **ep**idermoid cell line (a carcinoma cell line)
h	hour
IGEM	immuno-gold electron microscopy
IHC	immunohistochemistry
IgA	immunoglobulin A
IgG	immunoglobulin G
kb	kilo base pairs
kDa	kilo-Dalton

Km	kanamycin
Km^r	kanamycin resistant
LA	localized adherence
LEE	locus of enterocyte effacement
LT	heat-labile toxin
ME	mercapto-ethanol
MRHA	mannose resistant haemagglutinin
MW	molecular weight
M-PCR	multiplex polymerase chain reaction
Na	sodium ion
nm	nano-meter
NTEC	necrotoxigenic *Escherichia coli*
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.1% Tween
PCR	polymerase chain reaction
PGE2	prostaglandin E2
PI	post-inoculation
pM	picomoles
PWD	post-weaning diarrhea
rRNA	ribosomal RNA
ST	heat-stable toxin
STEC	shiga toxin producing *Escherichia coli*
Str	streptomycin
Str^r	streptomycin resistant
Stx	shiga like toxin
TEM	transmission electron microscopy
TSB	tryptic soy broth
VT	vero-toxin
VTEC	verotoxigenic *Escherichia coli*

1 - Literature review

1.1 - Introduction

Infectious diarrhea is one of the most common and important disease in farm animals because of its high morbidity and mortality. Diarrhea in swine represents 10.8% of pre-weaning mortality in some geographic locations of the world (152). Among bacterial causes of diarrhea, *E. coli* is the most common and economically important (10, 66). Among different groups of diarrheagenic *E. coli*, enterotoxigenic *E. coli* (ETEC) are the most commonly reported etiological agents of neonatal and post-weaning diarrhea in pigs (31, 140, 145, 146).

Non-pathogenic *E. coli* and other microorganisms typically colonize the colon immediately after birth, and these organisms and the host derive mutual benefit (38). When the barriers of gastrointestinal tract are violated or in cases of debilitation and immunosupression of the host, even non-pathogenic *E. coli* strains can cause infection. Pathogenic *E. coli* infections are limited to mucosal surfaces or disseminated throughout the body. In general, there are three distinct clinical syndromes caused by pathogenic *E. coli* strains: (i) enteritis/diarrhea, (ii) septicemia/meningitis, and (iii) urinary tract infection (109).

1.2 - Enterotoxigenic *E. coli* (ETEC) infection in pigs

1.2.1 - History and importance

Neonatal diarrhea is one of the economically important diseases in

contemporary swine industry. Pigs are most susceptible to ETEC induced diarrhea during pre-weaning and immediately after weaning. Pre-weaning diarrhea is most commonly reported in 0-4 day old pigs and is caused predominantly by ETEC strains (106).

ETEC specifically colonize the small intestine by using fimbrial adhesins and produce one or more enterotoxins to cause diarrhea. The most commonly detected fimbrial adhesins among ETEC strains of pigs include F4, F5, F6, F41 and F18 (57). There are reports that some strains of *E. coli* isolated from porcine diarrhea cases carry genes for one or more toxins, but not for classical fimbrial adhesins (F4, F5, F6, F41 and F18) (17, 18, 122). Casey et al. (1992) reproduced diarrhea experimentally in pigs with two such strains that were negative for F4, F5, F6 and F41, and demonstrated that both strains expressed a common surface antigen that was thought to be a novel adhesin (13). Other studies described that some *E. coli* strains isolated from neonatal and post-weaning diarrhea cases of pigs were negative for classical fimbriae (F4, F5, F6, F41) and a relatively high proportion of them (7-25%) were positive for *aidA* gene which encode **a**dhesin **i**nvolved in **d**iffuse **a**dherence (AIDA-I), a non-fimbrial adhesin (59, 113, 115). These studies led to the speculation that AIDA-I may be regarded as an important virulence factor and may be involved in the pathogenesis of diarrhea in pigs.

Bacterial pathogens employ a variety of virulence factors to cause infection and disease in animals. Expression of virulence factors is dependent upon complex regulatory circuits. Several invasion themes are common to diverse microorganisms, and enable them to evade host immune defenses, which is a key to the success of a pathogen. Maintenance of virulence factors on mobile genetic elements called plasmids

and pathogenicity islands facilitate constant evolution of new strains of pathogens. Understanding the common themes of virulence mechanisms in bacterial pathogenicity is critical to the development of preventive strategies.

1.2.2 - Predisposing factors and modes of transmission

Pigs in the neonatal and immediate post-weaning period are primarily infected by ETEC. Their immediate environment can potentially harbor enterotoxigenic *E. coli*, usually by fecal contamination of farrowing crate and skin of the dam particularly at parturition, thus facilitating transmission to piglets soon after birth. Newborn pigs acquire infection by ingestion of large numbers of environmental *E. coli* carrying virulence factors. In one study in Vietnam, samples collected from porcine feces, feed and water from irrigation system were positive for *Stx* and *eae* genes determined by polymerase chain reaction (PCR) (78). Nevertheless, neonates are protected against these environmental pathogens by colostral and lactogenic maternal immunity. Ingestion of colostrum within the first few hours of life provide neonates with specific antibodies and nonspecific antimicrobial factors that protect them against various infections during the first weeks of life (65). Breakdown of colostral and lactogenic immunity can occur if dams are not exposed to pathogens, including ETEC, present in the current environment or if they develop mastitis and agalactia. Another example of failure of colostral and lactogenic protection against ETEC includes genetically resistant dams that do not express receptors for F4 fimbrial adhesins, and accordingly, lack colostral and lactogenic anti-F4 immunoglobulins to protect their offspring that express receptors for F4 fimbriae. Up to 50% of pigs are reported to lack receptors for F4 adhesin (2, 50, 57, 58). Neonatal pigs have the ability to absorb colostral antibodies

within the first 24-48 hours of life, after which gut closure for absorption of macromolecules takes place (12). The longer the interval between birth and the first ingestion of colostrum, the lower the amount of protective antibody absorbed, resulting in inadequate protection against systemic infections.

Poor hygiene and moisture in the farrowing area plays an important role in the survival and multiplication of *E. coli*. Proper design of the farrowing crate is important because it affects fecal contamination by the sow. The incidence of diarrhea in piglets is higher with concrete floors than with perforated floors. Other important factors in *E. coli* infection include hypothermia, reduced gastric acidity in neonates and post-weaning stress. In newborn pigs the environment of stomach and duodenum is less acidic resulting in lower production of digestive enzymes, thus favoring rapid multiplication of bacteria such as *E. coli*. In the post-weaning period, pigs experience variety of stressors such as loss of maternal contact, movement to new pens, mixing with new pen mates, over crowding, waning maternal antibody and changes in feed. Diarrhea in post-weaning pigs usually starts at 3-5 days after weaning and lasts for about a week, resulting in weight loss, emaciation or mortality (44, 58).

1.2.3 - Clinical signs and pathology

The severity of ETEC infection usually depends on the virulence factors expressed by the specific *E. coli*, and the age and immune status of the piglets. In severe cases of diarrhea, affected animals develop dehydration and metabolic acidosis and die due to hypovolemic shock. Profuse watery diarrhea can develop in single neonatal pig or in a whole litter. In acute cases, affected animals are dull, listless and decline to suckle. The color of the feces varies from whitish yellow to green, and the consistency

varies from watery to mucoid and sometimes bloody. Lesions at necropsy may include the stomach full of milk curd, and dilatation of small intestine due to fluid accumulation. Histological lesions in the mucosa vary with the ETEC strain involved and mostly are minimal. In the case of F4-positive *E. coli*, most commonly a layer of *E. coli* is adhered to the villus enterocytes of jejunum and ileum, especially on the tips of villi. Less frequently, histological lesions with ETEC may include vascular congestion and increase in neutrophils and macrophages in lamina propria and microvascular thrombosis. Some degree of villus atrophy is attributed to STb. In severe cases, fibrin micro-thrombi may be observed in lamina propria of stomach, small intestine and colon, accompanied with villus necrosis (44, 58).

1.2.4 - General features of *Escherichia coli*

Escherichia coli, a member of the *Enterobacteriaceae* family, is a gram-negative, facultative anaerobic, rod-shaped (1.1 - 1.5 X 2 - 6 μm), motile organism commonly found as part of the commensal microflora in the intestinal tract of humans and animals. *E. coli* is identified by a positive indole reaction, and lack of production of urease, citrate and hydrogen sulphide. It can be readily grown on a simple bacteriological medium such as MacConkey agar giving characteristic pink colonies. It is the dominant organism recovered from aerobic culture of feces. Isolates producing F4 or F18 fimbriae and certain isolates producing F6 fimbriae are also hemolytic. An international serotyping system has been developed based on the polysaccharide portion (O) of cell wall lipopolysaccharide (types: O1-O175), capsular (K) polysaccharide (types: K1-K103), and flagellar (H) protein (types: H1-H53). Pathogenic *E. coli* are best identified based on their possession of virulence factors such as enterotoxins,

cytotoxins, colonization pili and adhesins (57, 99, 109). With this system, the enteric *E. coli* pathogenic for livestock are divided into three broad classes: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC). Among these, ETEC still remains the most prevalent cause of diarrhea in farm animals (45).

1.2.5 - Virulence factors expressed by enterotoxigenic *E. coli* (ETEC)

Detection of virulence factors with any specific pathogenic *E. coli* is important for identification, characterization and understanding of the mechanism of pathogenesis of that particular type of *E. coli* (85, 99, 109). ETEC strains express variety of virulence factors, such as fimbrial adhesins, non-fimbrial adhesins, and enterotoxins. Detection of genes encoding these virulence factors helps in identification and classification of *E. coli*.

1.2.5.1 – Fimbrial adhesins

Fimbriae or pili are the arm-like surface structures that mediate the attachment of bacteria to host tissues (144). Each fimbrial unit consists of hundreds of antigenically specific major and minor structural subunits with specific binding properties. F4, F5, F6, F17, F18 and F41 are most commonly recognized fimbrial adhesins of porcine ETEC isolates.

F4 (K88): F4 fimbria is encoded by *fae* locus on a plasmid. Adherence of F4 is specific to pigs, and F4-positive *E. coli* infections occur commonly in both neonatal and post-weaning pigs. F4 mediated adherence and colonization are mostly confined to the small intestine. Three variants of F4, namely ab, ac, and ad, are reported (157). The mucin type glycoprotein of the intestinal brush border may be a biological receptor for F4 (57);

however, it has been reported that nearly 50% of pigs lack receptors for the F4 adhesin. The allele for the F4 receptor is dominant and the genetic resistance associated with the absence of receptor is inherited in a Mendelian fashion. If the sow, but not the boar, is genetically resistant (homozygous recessive, ss), it delivers piglets susceptible to F4-positive *E. coli* infections, in the absence of parenteral immunization (57, 134).

F5 (K99): F5 fimbria is encoded by *fan* locus on a plasmid. It is most commonly recognized in ETEC isolates from pigs, cattle and sheep. F5 mediated attachment and colonization are mostly confined to the lower part of the small intestine, particularly in young animals (57).

F6 (987P): The *fas* locus encoding F6 fimbria is found both on chromosome and plasmid. F6 mediated colonization is mostly confined to the lower small intestine of neonatal pigs (57). F6 is a heteropolymer made of FasA, FasF and FasG subunits (132). Age related resistance to F6 mediated attachment is due to the appearance of new receptors in the mucus of post-weaning pigs, which inhibit F6 mediated attachment by competing with membrane anchored receptors (30).

F17: This fimbrial adhesin is identified mostly in bovine necrotoxigenic *E. coli* strains, which are also positive for cytotoxic necrotizing factor (CNF) 1 and CNF 2. The CNF1 and CNF2 genes are located on the chromosome and a plasmid, respectively. Their role in the development of diarrhea is not fully understood (57).

F18: The *fed* locus encoding F18 fimbria is present on a plasmid. Two F18 variants, ab and ac, have been recognized. F18ab has been associated with Stx2e positive strains and is poorly expressed *in vitro* and *in vivo*. F18ac is most commonly associated with ETEC strains and is expressed efficiently both *in vitro* and *in vivo*. Newborn pigs lack

receptors for F18; therefore, they are resistant to F18 mediated colonization. However, pigs of weaning age are more susceptible due to increased expression of F18 receptors (57), hence F18-positive *E. coli* is most common in weaned pigs (16, 32, 92, 122)

F41: It is encoded by genes present on the chromosome, and is mostly co-expressed with F5, although some strains express it alone. Bovine and porcine strains of ETEC may be positive for F41, which mediates colonization in the lower small intestine of pigs, calves and lambs resulting in diarrhea (57).

1.2.5.2 - Identification of fimbrial adhesins

Identification of fimbrial antigens on strains isolated from diarrhea feces or intestinal contents offers presumptive diagnosis of *E. coli* as the causative agent (65). Following the isolation of *E. coli*, fimbrial virulence factors are identified by routine slide agglutination test using polyvalent or monospecific sera or monoclonal antibodies (49, 159). Further, more reliable procedures for detection of fimbrial antigen have been developed including enzyme immuno-assay (98) and fluorescent antibody test performed on intestinal impression smears (49). Colony hybridization assays (91, 100, 104) and PCR assays (1, 121, 151, 164) that reliably detect genes for fimbriae and enterotoxins have also been developed. These methods have enhanced the reliability of diagnosing ETEC and eliminated tedious bioassays.

1.2.5.3 – Non-fimbrial adhesins

So far, 4 non-fimbrial adhesins that appear to mediate attachment to host epithelial cells have been recognized: a 57-kDa adhesion-mediating protein (176), the CF16K molecule (71), the Tib system (105, 139) and the AIDA-I system (5).

Escherichia coli 73-1 (serotype O73:H33) and 5-2 (serotype O89:H-) isolated from human diarrhea cases possess characteristics of a novel hemagglutinin associated with diffuse adherence to tissue culture cells (HeLa and HEp-2) and coverslips (plastic and glass). This adherence is mediated by a 110-kb plasmid designated pEDA1, and correlates with D-mannose-resistant hemagglutinin (MRHA) detected with bovine, sheep, or human erythrocytes. The MRHA region is duplicated on pEDA1 and mediates the production of the 57-kDa outer membrane protein. These strains have no detectable pili and produced marked adherence to mucus and colonic epithelium in humans (176).

Some diffusely adherent *E. coli* (DAEC) strains isolated from human diarrhea cases produced two major surface proteins of 16 and 29-kDa. The 16-kDa protein (CF16K) is involved in adhesion to Caco-2 and HEp-2 cells *in vitro*, and is unrelated to the Dr Family of adhesins recognizing the Dr Blood group antigen as receptor. Pretreatment of bacteria with anti-CF16K serum, or of Caco-2 cells with purified CF16K, greatly decreased the adherence to both cell types by *E. coli* CF1085 strain producing the CF16K protein. The CF16K adhesin was found in 9.5% of the 335 *E. coli* strains from human diarrhea cases studied by colony immunoblot assays using anti-CF16K serum (71).

TibA is a potent bacterial adhesin associated with a number of ETEC strains isolated from human diarrhea cases. It mediates bacterial attachment to a variety of human cells and also promotes invasion of these cells. This adhesin is a surface-displayed glycoprotein belonging to a family of bacterial autotransporter proteins. The *tib* locus is chromosomally encoded and the *tibA* gene directs the synthesis of a 104-kDa outer membrane protein (105, 139).

AIDA-I adhesin, first characterized from *E. coli* O126:H27 strain isolated from human infantile diarrhea, produces diffuse adherence to a variety of mammalian cells *in vitro*. AIDA-I belongs to the autotransporter family and its gene, *aidA,* is located in a plasmid which encodes a 100-kDa mature outer membrane glycoprotein derived from 132-kDa protein precursor protein (5, 7, 149). Interestingly, the *aidA* gene is associated with the Stx2 toxin in some *E. coli* strains from porcine edema disease (59, 113, 115) and the DNA sequences encoding AIDA-I and F18 fimbriae are reported to be located in the same plasmid (92).

1.2.5.4 – Enterotoxins

ETEC strains produce two major classes of enterotoxins: a heat-labile toxin (LT) and a heat-stable toxin (ST), neither of which produce distinct morphological lesions in the intestinal mucosa (57).

Heat-labile toxins (LT): LT is a large, oligomeric toxin closely related to cholera toxin (CT), and is inactivated at 60˚ C for 15 min. The genes encoding LT (*elt or etx*) reside on a plasmid which may also harbor genes encoding ST and/or colonization factor antigen (CFA). LT can be divided into two subgroups, LTI and LTII, which do not cross-react immunologically.

Two variants of LTI, which exhibit partial antigenic cross-reactivity, have been recognized: LTp-I, produced by porcine ETEC strains; and LTh-I, produced by human ETEC strains. LTI is a 86-kDa complex toxin consisting of a biologically active subunit A, and five identical subunits B of 11.5-kDa, which can bind to GM1 ganglioside receptors on enterocytes (53). LT can also bind to other receptors such as GD1b, asialo GM1, GM2, galactoproteins and galactose containing glycolipids. The subunit A

consists of an A1 fragment containing the active site and an A2 fragment which links A1 to the B subunit. After binding of B subunit to the specific receptor on the host cell membrane, the A1 fragment translocates into the cell and activates the adenylate cyclase system, stimulating the production of cyclic AMP (cAMP) which in turn activates the cAMP dependent protein kinase leading to excessive phosphorylation of chloride channels located in the apical cell membrane. The net result is an increased secretion and decreased absorption of Cl^-, Na^+ ions and water in the lumen, resulting in an osmotic type of diarrhea. Other possible diarrheagenic mechanisms include: stimulation of prostaglandin E and the enteric nervous system (ENS), which regulates intestinal motility, secretion and a proinflamatory cytokine response (109).

Two antigenic variants, LTIIa and LTIIb, were recognized in LTII with the same A subunit as LTI but with different B subunits. LTII increases cellular cAMP levels by mechanisms similar to those of LTI, but LTII binds to a GD1 receptor, unlike LTI. There is no evidence that LTII is associated with human or animal disease, but ETEC with the genes for LTII have been isolated from humans, cows, buffalo, and pigs (57, 109).

Heat-stable (ST) toxins: There are two classes of heat-stable toxins, STa (STI) and STb (STII), which differ structurally and functionally. These are small, monomeric toxins resistant to heat treatment at 100° C for 15 min. The genes encoding both these toxins are present on plasmids (57, 109).

Two toxin variants of STa, each of 2-kDa peptides, produced by ETEC have been recognized: STaP (STIa) associated with porcine, bovine and human strains of ETEC, and STaH (STIb) produced by human ETEC strains. STa binds to the guanylyl

cyclase C (GC-C) intestinal epithelial receptor to stimulate guanylyl cyclase activity, which in turn stimulates the production of cGMP. The resulting increase in cGMP levels leads to the stimulation of chloride secretion and/or inhibition of sodium chloride absorption, resulting in net increase in intestinal fluid secretion (133).

STb, a 5-kDa peptide, is primarily present in porcine strains, although some of the human ETEC strains are also positive. Unlike STa and LT toxins, STb less frequently induces morphological damage to the intestinal epithelium by destruction of villus epithelial cells and partial villus atrophy (109). The receptor for STb is unknown, but a recent study showed that STb binds to a 25-kDa protein in murine intestinal cell membranes. STb does not stimulate intracellular cAMP or cGMP levels, but increases the intracellular calcium levels and the secretion of bicarbonate from intestinal epithelial cells (133). Other mechanisms by which STb induces diarrhea include secretion of PGE2 and serotonin, suggesting possible ENS involvement in the secretory response to this toxin.

Enteroaggregative heat-stable toxin 1 (EAST1): This toxin, first identified in human isolates of enteroaggregative *E. coli* (EAEC), is a 4.1-kDa peptide sharing 50% homology with STa. In one study, it was reported that 38% of non pathogenic *E. coli* strains express EAST1 (129). Subsequently, the *astA* gene encoding EAST1 has been associated with various virotypes found in human ETEC and EPEC, and also in ETEC strains from pigs and calves with diarrhea. It has been proposed that the mechanism of action of EAST1 is similar to STa in increasing cGMP; however, the exact role of EAST1 in the development of diarrhea is still unclear (57, 109)

1.2.5.5 – Detection of toxins

Initially, the most common and practical ways of detecting enterotoxins included biologic and immunologic assays (65). Subsequently, DNA colony hybridization, DNA probes and PCR assays have been used more frequently for the detection of ST and LT along with other virulence factors of ETEC strains (1, 33, 59, 91, 92, 104, 113, 115, 121, 151).

1.2.6 - Virulence factors of other diarrheagenic non-ETEC *E. coli*

Various strains of non-enterotoxigenic *E. coli* are characterized from neonatal farm animal diarrhea cases similar to those of diarrhea syndromes described in humans. These strains are grouped under EPEC and EHEC. Unlike ETEC, EPEC and EHEC usually do not produce ST and LT toxins (65). Although both EHEC and EPEC produce attaching and effacing (A/E) lesions, EHEC can be differentiated from EPEC by its production of shiga toxin (Stx) (109).

EAE: Strains of the EPEC and EHEC groups attach to intestinal epithelial cells and cause effacement of the microvilli and intimately attach to enterocyte surface resulting in characteristic A/E lesion visualized by histopathology. Ultrastructurally, the A/E lesion is characterized by marked cytoskeletal changes resulting in accumulation of polymerized actin (pedestal-like structure) underneath the adherent bacteria. The gene for A/E lesion formation is encoded by the *eae* gene within a pathogenicity island called the locus of enterocyte effacement (LEE) (109, 155).

Adhesins: Some EPEC strains express surface type 1 fimbria (54) and bundle-forming pilus (BFP) (19) that mediate their attachment to intestinal epithelial cells. A few other EPEC strains isolated from human diarrhea cases produced two different patterns:

localized adherence (LA) and diffuse adherence (DA), which are mediated by a plasmid encoded adherence factor (73, 130, 131).

Cytotoxins: EHEC strains produce potent toxins, verotoxin I (VTI) and II (VTII), that can damage and kill host epithelial cells. EHEC causes two disease syndromes in people, namely, hemorrhagic colitis and hemorrhagic uremic syndrome (HUS) (37). Verotoxins derived their name from their toxic effect on Vero cell lines. VTI is structurally and immunologically related to toxin produced by *Shigella dysenteriae* type 1, therefore also called shiga like toxin 1 (Stx1). VTII toxin is immunologically related to Stx1, and VTII can also be called as shiga-like toxin 2 (Stx2) (72). Stx2e toxin is produced by *E. coli* strains associated with edema disease which causes illness and death in pigs, generally during the first 2 weeks after weaning (68, 107). These toxins are encoded by bacteriophages related to the classic lamda (λ) phage. Each toxin is composed of a single A subunit non-covalently associated with a pentamer composed of identical B subunits. The B subunits bind specifically to globotrioacyl ceramide and related glycolipids on host cells. The subunit A is taken up by endocytosis and transported to the endoplasmic reticulum. The toxin targets the 28S rRNA, causing depurination at a specific adenine residue, followed by cessation of protein synthesis and death of infected cells by apoptosis. Receptors for Stx are found on endothelial cells. Renal microvascular endothelial cells appear to be particularly sensitive to the toxin. It is presumed that Stx enters the systemic circulation after translocation across the intestinal epithelium and damages endothelial cells, leading to activation of the coagulation cascade, formation of microthrombi, intravascular hemolysis, and ischemia (37).

1.2.7 – Immunohistological studies of *E. coli* colonization and pathogenesis

Immunohistochemistry has been used by many investigators to study the colonization of different groups of diarrheagenic *E. coli* expressing various adhesins (28, 94, 178). Only limited colonization studies have been done with AIDA-I$^+$ *E. coli* (124) and none to date have employed immunohistological technique.

Strains of *Escherichia coli* expressing the K1 capsular polysaccharide colonize the large intestine of newborn infants. An immunohistochemical study of the nature of the colonization by wild strains expressing K1, and K1 defective mutants, showed that the wild strains efficiently colonize the large intestine and the mutants have distinct colonization defects (94).

The invasive ability of F4 positive *E. coli* O18 isolated from porcine neonatal diarrhea studied by streptavidin-biotin immunohistochemistry using polyclonal *E. coli* O18 antiserum revealed the presence of bacilli in the cytoplasm of enterocytes and in the lamina propria (167).

Immunohistochemistry was used on formalin fixed, paraffin embedded intestinal tissues to demonstrate the FasG, which is a 987P (F6) fimbrial adhesin subunit, mediated interactions with the brush border molecules (Histone H1) of intestinal epithelial cells from neonatal pigs. The histone H1 molecules stabilize fimbrial interaction by simultaneously binding to the membrane and to F6 (178).

Colonization by EHEC strain O157:H7 producing A/E lesions was studied extensively in various animal hosts using immunohistochemistry (20, 21, 29, 81, 127, 148, 168). Calves less than 36 h old experimentally inoculated with EHEC O157:H7 developed diarrhea and enterocolitis with characteristic attaching and effacing (A/E)

lesions in both the large and small intestines. The A/E bacteria detected by hematoxylin and eosin (H&E) staining were confirmed by horse radish peroxidase (HRPO) staining with goat anti-O157:H7 antibodies (29).

Cytotoxic necrotizing factor 2 produced by necrotoxigenic *E. coli* (NTEC) caused intestinal and extraintestinal lesions in experimentally infected colostrum-restricted newborn calves. The NTEC bacteria in the intestinal lesions were detected by DNA hybridization, and further confirmed by immunohistochemistry using polyclonal anti-O78 and monoclonal anti-F17b antisera (156).

1.2.8 - Pathogenesis of diarrhea caused by ETEC

The gastrointestinal tract of newborn animals is sterile at birth and rapidly becomes colonized with bacteria from the dam and environment. *E. coli* colonizes the intestine immediately after birth of animals, and remains a part of intestinal microflora for the entire life (39). The numbers of *E.* coli are small in the upper small intestine but this increases progressively more distally to reach a maximum in the large intestine. Strong intestinal peristalsis, villus pumping, the flow of ingesta and mucus secretion serve to propel the bacteria through the intestine and thereby regulate their establishment (102). Most of *E. coli* found in the normal flora are nonpathogenic with the exception of shiga toxin producing *E. coli* (O157) found in normal flora of cattle and other ruminants (57). Attachment and colonization of the mucosal surface is considered to be the initial step in the pathogenesis of any intestinal bacterial infection. Once attached, the bacteria multiply to a significant number, and if successful in evading the natural host defenses, cause damage to the host. Despite intestinal peristalsis, ETEC are able to colonize intestinal mucosa and produce enterotoxins

resulting in net increase in secretion of water and electrolytes with little or no change to epithelial morphology, while also competing for nutrients with the normal flora of the gut (101, 109). Although all *E. coli* including non-pathogenic strains have fimbriae, the diarrheagenic *E. coli* express specific fimbrial or non fimbrial adhesins that enhance colonizing ability by facilitating adherence to small intestine where normally colonization does not occur (85, 88, 109, 162). Differences in fimbriae and host mucosal receptors account for variations in ETEC infections by age (neonatal or older pigs), site (small or large intestine), and the host itself (species or genotype within species) (101). Pathogenicity of various *E. coli* is associated with virulence genes encoded by plasmids, bacteriophages and pathogenicity islands (PAI). Plasmid encoded genes include enterotoxins, fimbriae or pili; shiga toxin genes are phage encoded, and PAI encode the *eae* genes of EPEC and EHEC (57). ETEC adhered to intestinal mucosa and cause diarrhea through the action of enterotoxins LT, ST or both (109). These toxins affect the efflux of water and electrolyte secretion in small intestine by the mechanisms explained above (1.2.5.4), which leads to diarrhea if the large intestine is unable to absorb the excess fluid.

1.2.9 - Enteroaggregative *E. coli* (EAEC)

In humans, some strains of *E. coli* were described that did not secrete ST and LT toxins, but instead adhered to epithelial cells with a characteristic stacked brick pattern, thus termed enteroaggregative *E. coli* (EAEC) (109). In developing countries these EAEC are regarded as the major etiological agents of persistent diarrhea in children (77). An important characteristic of EAEC is the ability to adhere to both small and large intestinal mucosa in a thick biofilm of aggregated bacteria (108, 154, 162).

Mucosal biofilm formation may be important in the pathogenesis of EAEC by promoting persistent colonization that forming a barrier for penetration of antibiotics (118).

Using electron microscopy, Knutton et al. (1992) studied 44 EAEC strains isolated from infants with diarrhea in India and the United Kingdom to determine their ability to adhere to human intestinal mucosa *in vitro*, and found that none of these strains adhered to human duodenal mucosa and six strains tested did not adhere to ileal mucosa. All the 44 strains adhered to human colonic mucosa as localized aggregates, adhesion of which was mediated by fimbrial structures. Accordingly, this study suggested that EAEC strains were large bowel pathogens and that colonization of the colon was mediated by a fimbrial adhesion (77).

Hicks et al. (1995) used organ cultures of small and large intestinal mucosa from children to examine the interactions of five EAEC strains. The study showed a variable pattern of adhesion by each strain: 2 strains adhered to all intestinal levels, 1 to jejunum and ileum, 1 to ileum only, and 1 to ileum and colon. All 5 strains showed aggregative or stacked-brick pattern of adherence on HEp-2 cells. Electron microscopy of infected small intestinal mucosa showed bacterial association with a thick mucus layer above an intact enterocyte brush border and absence of a mucus layer in non-infected controls (63). Pritchard et al. (2004) orally inoculated colostrum deprived neonatal pigs with two *Escherichia coli* strains, PD58 and PD149 of the AIDA-I/STb/EAST1 virotype, isolated from porcine diarrhea cases, to study the colonization patterns by light and transmission electron microscopy. PD58 colonized the upper colon through the formation of a

biofilm like colonization with layers of bacteria embedded within glycocalyx. The same isolate also produced diffuse aggregative pattern on Hep2 cells *in vitro* (124).

Gnotobiotic, colostrum deprived neonatal pigs as well as conventional pigs have been used as animal models for studying the pathogenesis of different groups of diarrheagenic *E. coli,* for their relevance to acute onset of infantile diarrhea (55, 119, 124, 153, 154).

1.2.10 - AIDA-I positive *E. coli* infections

1.2.10.1 - Humans

EPEC strains were considered the chief bacterial agents causing neonatal and infantile gastroenteritis throughout the world (86). Initial studies demonstrated that human EPEC strains isolated from human infantile diarrhea, belonged to serotype O126 (E2348/69), and that they adhered to Hep-2 cells. The genes for the adhesin, termed as EPEC adherence factor (EAF), were located on plasmids. Infants infected with this EPEC strain mounted serum IgA and IgG responses to a 94-kDa plasmid-associated outer membrane protein of strain E2348/69 (87). Reports by a number of authors have indicated that strains of *E. coli* belonging to the most common EPEC serotypes express an adhesion factor which is involved in the adherence to epithelial cells (14, 25, 64, 76). Further, some EPEC showed two distinct patterns of adherence in adhesion assays using HeLa/HEp-2 cell culture systems: (i) localized adherence (LA) with focal attachment to the cell surface to form micro-colonies and (ii) diffuse adherence (DA) with bacteria adhered over the whole cell surface (110, 111, 131). In one study in France, out of 335 DAEC isolated from sporadic cases of diarrhea in humans, 13 (3.9%) were positive for AIDA-I (71). Benz et al. (1989) isolated EPEC strain 2787 (O126:H27) from infantile

diarrhea case that showed DA to HeLa cells. Subsequently, they demonstrated that DA was mediated by a 100-kDa protein encoded by 11-kb bacterial DNA. By insertion of this 11-kb DNA into a recombinant clone (pIB6), they confirmed that DA was in fact mediated by the 100-kDa protein named AIDA-I (5). The antiserum raised against recombinant clone (pIB4 with 9.2-kb DNA insert) inhibited the adherence by *E. coli* 2787 and recombinant clone pIB6 to HeLa cells (6). Further studies revealed that the DA phenotype is mediated by a 100-kb plasmid bearing 6.0-kb DNA fragment denoted as *aidA* gene which encodes a 140-kDa precursor molecule and a 50-kDa degradation molecule as detected by Western blotting with antiserum to heat extracted AIDA-I protein. The expression of mature100-kDa protein occurred in the 2787 wild strain, recombinant DA positive strains, but not in mutant DA negative strains. Immunogold electron microscopy revealed that the 100-kDa AIDA-I adhesin distributed on the bacterial cell surface is not part of a filamentous pilus-like structure (9). Further analysis of plasmid derived 6.0-kb DNA fragment revealed two open reading frames ORF A (1173-kb) and ORF B (3858-kb) denoted as *aidA* gene encoding 45-kDa and 132-kDa proteins, respectively. The 132-kDa protein [1286 amino acids (aa)] is an AIDA-I precursor protein and it is processed by removal of 49-amino acid signal sequence. The resultant protein is located in the periplasm and further undergoes C- and N-terminal processing by autocatalytic action resulting in translocator (439aa) and passenger (798aa) domains respectively. This N- and C-terminal processing of AIDA-I precursor 132-kDa protein generates the three distinct domains, which are characteristic for autotransporter systems (4,7).

The autotransporters, a family of proteins secreted by Gram-negative bacteria, possess three functional domains: the amino-terminal leader sequence, the secreted mature protein (passenger domain) and a carboxy-terminal (beta-) (transporter domain) that forms a beta-barrel pore to allow the secretion of the passenger protein. In many Gram-negative organisms, autotransporters proteins have been considered as important or putative virulence factors (62).

AIDA-I adhesin was classified under the autotransporter protein family by possessing an overall unifying structure comprising three functional domains: N-terminal pre-peptide, the alpha domain and the C-terminal beta-domain (62, 84, 95, 96). The beta-domain integrates with the outer membrane and is responsible for translocation of the mature alpha-domain (AIDA-I) (4, 7, 9, 79, 84, 149). Deletion of open reading frame (ORF) A gene (denoted as *aah*) resulted in expression of a non functional adhesin indicating that the AIDA-I adhesin is post-translationally modified by glycosylation which is mediated by the *aah* gene product, a 45-kDa autotransporter adhesin heptosyl-transferase (aah), a step essential for its adherence function (8). Further investigations revealed that the AIDA-I adhesin recognizes 119-kDa (gp119) integral N-glycosylated membrane protein receptor on HeLa cells and causes specific attachment of bacteria to HeLa cells (82), and that AIDA-I adhesin plays a novel role in autoaggregation and biofilm formation (138).

Although the molecular characterization of AIDA-I and its *in vitro* functions have been investigated, the exact mechanism involved *in vivo* in the pathogenesis of infantile diarrhea caused by AIDA-I^{+} *E. coli* in humans is still not clear.

1.2.10.2 - Pigs

The AIDA operon was detected by multiplex PCR in 25% of *E. coli* isolates from pigs with edema disease and post-weaning diarrhea (ED-PWD). Co-expression of AIDA-I with F18 and Stx2e was suspected to have functional linkage (115). The same study also reported that defects in *orfB* (*aidA* gene) were associated with absence of disease. Mainil et al. (2002) demonstrated that F18 and AIDA-I adhesin are localized on the same plasmid in verotoxigenic *E. coli* (VTEC) and non-VTEC strains (92). In Korea, the *aidA* gene was present in 7.5% of *E. coli* isolates from PWD or ED cases (59). A Canadian study showed that 8.8% of *E. coli* isolates carried gene for AIDA-I from diarrheic piglets, and further that these AIDA-I positive isolates induced diarrhea following experimental infection of newborn pigs (112, 113, 124). Pritchard and co-workers demonstrated the stacked brick pattern of aggregation characteristic of EAEC on Hep-2 cell adhesion assay using PD149 *E. coli* strain (AIDA-I/STb/EAST1) isolated from porcine diarrhea suggesting these strains are hybrids of ETEC, EAEC and DAEC (124). In addition, it was demonstrated that the AIDA-I adhesin from porcine diarrheagenic isolates is an acidic protein with similar molecular weight (100-kDa) and amino acid homology (78-87%) to the AIDA-I adhesin expressed by human AIDA-I^{+} *E. coli* strain 2787 (46).

1.2.10.3 - Other animals

Interestingly, *E. coli* isolates from cattle, horse, sheep and rabbit do not seem to carry genes for the AIDA operon, indicating that AIDA has a certain host specific function in pigs (115). However, more isolates need to be tested in these species to asses the prevalence of AIDA-I adhesin. Ngeleka et al. (2005) reported that

approximately 1% of *E. coli* isolates from calves were AIDA-I positive, however, these isolates simultaneously expressed F17 fimbriae and EAST1, suggesting that the latter factors may have played a role in pathogenesis of diarrhea in these calves (114)

1.2.11 - AIDA-I association with other virulence factors

In light of the significant association of AIDA-I with shiga-like toxin and F18 genes among *E. coli* isolated from ED-PWD of pigs (115), Mainil and co-workers also found that out of 174 verotoxigenic porcine *E. coli* isolates, 71 were AIDA-I/Stx2/F18, 26: AIDA-I/F18, 12: AIDA-I only, and 2 were AIDA-I/Stx2 (92). In Korea, Ha et al. (2003) demonstrated that 45 (7.5%) isolates were positive for *aidA* gene out of 604 *E. coli* strains isolated from pigs with post-weaning diarrhea or edema disease. The study also found that 87% of enterotoxigenic *E. coli* strains carried *aidA* gene and its association with other virulence factors as follows: AIDA-I/F18/Stx2e/STa (4 isolates), AIDA-I/F18/Stx2e/STa/STb (4 isolates) and AIDA-I/EAST1 (3 isolates) (59). In Canada, Ngeleka et al. (2003) investigated various virulence factors among 170 *E. coli* isolated from diarrhea cases of piglets, and reported the following prevalence of AIDA-I virotype: 4.1% AIDA-I/STb/EAST1, 2.3% AIDA-I/F18, 1.2% AIDA-I alone and 0.6% each for AIDA-I/STb and AIDA-I/EAE/EASTI. The same study also found that AIDA-I was not associated with fimbriae F4, F5, F6 and F41 commonly expressed by diarrheagenic *E. coli* (113).

Although AIDA-I adhesin was first reported and characterized from *E. coli* isolated from a human infantile diarrhea case, a relatively high percentage of porcine *E. coli* isolates carry the *aidA* gene; however, in both species its specific role in the pathogenesis of AIDA-I$^+$ *E. coli* diarrhea remains unknown. More studies are necessary

to fully understand the specific role of AIDA-I adhesin in the pathogenesis of diarrhea in both humans and pigs.

1.3 - Bacterial biofilm formation

1.3.1 - Introduction

Bacteria live in two basic states, either as planktonic or as sessile cells. Planktonic cells are important for proliferation and spreading into new territories, whereas sessile cells focus on their persistence. Studies showed that the sessile cells grow in communities called biofilms which are present in all natural and host environments (22, 26). “Biofilms are defined as structured communities of microbial species embedded in a biopolymer matrix on either biotic or abiotic substrata” (173). Biotic and abiotic surfaces include animal or plant and mineral or metal, respectively. Biofilm formation is considered as a simple developmental process involving differential gene expression. Bacterial colonization and biofilm formation has been recognized in lung, intestine and on medical implants. The beneficial effects of biofilms are used in industrial processes e.g. water and wastewater treatment, remediation of contaminated soil and groundwater, microbial leaching. On the other hand, bacterial biofilms creates challenges in the treatment of chronic biofilm related infections due to ineffective antibiotic therapy and poor clearance by host humoral and cellular defense mechanisms. The bacterial attachment to a surface is the first and necessary step in biofilm formation. Attachment is a complex process, which is regulated by various factors such as growth medium, substratum and host cell surface. Biofilm associated bacteria are differentiated from their mutants by generation of extracellular polymeric

substance (EPS) matrix, reduced growth rates and up- and down-regulation of specific genes (22, 34-36, 40, 42).

Microbial biofilms have gained greater importance recently because of their recognized role in certain chronic infectious diseases (e.g. dental caries, periodontitis, otitis media, cystic fibrosis pneumonia), and in a variety of device-related infections (e.g. dental implants, orthopedic implants, intravascular catheters). A better understanding of biofilm formation is necessary to formulate effective control strategies and thus to improve patient care.

1.3.2 - Structure and function

Several studies using tools such as the transmission/scanning electron microscopy and more recently, confocal laser scanning microscopy have enabled researchers to understand that biofilms are structured, homogeneous deposits of bacteria and accumulated slime. Biofilms consists of complex communities of bacterial cells enclosed in a polymer matrix or glycocalyx channels through which transport of water, nutrients and cell-to-cell signaling takes place (22, 26, 135, 141).

Biofilm formation by *Pseudomonas aeroginosa* has been extensively studied in humans affected by cystic fibrosis. The current models of biofilm formation and structure are derived from *Pseudomonas* Spp. organism (22, 158). In humans and animals studies of lungs infected with *Pseudomonas pseudomallei,* it was shown by transmission electron microscopy that the bacterial cells were surrounded by radially arranged fibres that constitute well defined glycocalyx structure (exopolysaccharide) produced by the bacteria (150, 165).

Biofilm formation involves five distinct phases (158): (i) initial attachment of planktonic bacteria that come in contact with the solid surface through motility or by fluid stream; (ii) transition from reversible attachment to irreversible attachment by production of bacterial extracellular matrix and/or by specific adhesins which interact with the surface; (iii) early development of biofilm architecture; (iv) development of microcolonies into mature biofilm with the help of extracellular polymeric matrix which serve as adhesive matrix, and forms water channels and pores to supply the nutrients; and (v) dispersion from biofilm state to planktonic state.

Biofilm phenotype can be described in terms of the genes expressed by biofilm-associated cells (35, 36, 158). Although there is an evidence for biofilm formation as the basis of several infectious diseases, the exact mechanisms by which biofilms elicit disease are poorly understood.

1.3.2 - *E. coli* biofilm formation

Escherichia coli is the best-known organism and most abundant species present in normal aerobic intestinal flora of humans and animals and responsible for neonatal diarrhea, urinary tract infections and bacteremia. Biofilm formation on in-dwelling catheters in human patients leads to an increased incidence of urinary infections (5-10%) (169). Biofilm formation by *Escherichia coli* has been associated with complex interaction between different bacterial surface molecules and structures. These surface determinants, when expressed, play a role in the biofilm formation. Specific surface determinants that play a role in various stages of biofilm formation by *E. coli* are summarized in the following table (158):

Surface determinants involved in *E. coli* biofilm formation

Determinant	Stage of biofilm formation
1. Flagella and motility	Surface contact and reversible attachment
2. Type 1 fimbriae, Curli PGA polysaccharide (EPS)	Irreversible attachment
3. Motility Curli Antigen 43 (autotransporter protein) Colanic acid (EPS) PGA polysaccharide (EPS)	Microcolony formation and early development of biofilm architecture
4. Colanic acid (EPS) Curli, Conjugative pili	Maturation
5. Flagella and motility	Dispersal

Certain *E. coli* express adhesive phenotypes such as Ag43, AIDA-I and TibA, all of which belong to an autotransporter subfamily. Autotransporter proteins are self-recognizing adhesins which contain information for their expression and receptor recognition (62). Antigen 43 is prominent surface protein which protrudes 10 nm beyond the outer membrane and is the product of *agn43* gene (also called *flu*) (75). Antigen 43 mediates autoaggregation (studied through settling kinetics of cells through Ag43-Ag43 interactions), thus promoting biofilm formation based on visualization under phase contrast microscopy and quantification on polystyrene microtitre plates (23, 74).

Tib A is homologous with AIDA-I and Ag43. A mature, glycosylated TibA is associated with epithelial adherence, autoaggregation through TibA-TibA interaction and enhancement of biofilm formation (139).

Curli are proteinaceous filamentous appendages consisting of major (CsgA) and minor (CsgB) subunits that exhibit adherence properties involved in biofilm formation of several *E. coli* strains. The genes for curli production are organized in the operons *csgBAC* and *csgDEFG*, but only the function of some of these genes is fully recognized. Bacterial phenotype overexpressing gene *csgA* forms biofilm, whereas the knockout mutant did not exhibit adherence characteristic for biofilm formation (163).

Motility of pathogens is considered to favor the colonization of the host surfaces by promotion of the initial host-pathogen contact. Motility of *E. coli* strains is mediated by multiple peritrichous flagellae. In addition to the host-pathogen contact, flagellae may be involved in the dispersal of the bacteria from the biofilm formed on the host surfaces (70).

Adhesion to host tissue by majority of pathogenic *E. coli* strains has been mediated by fimbriae (48). Type 1 fimbriae are the most common surface adhesins found in both commensal and pathogenic *E. coli* isolates as well as in other *Enterobacteriaceae*. Type 1 fimbria appeared to be necessary for early biofilm formation by their involvement in initial stable cell-to-surface attachment. The *fim* gene cluster encodes the structural components of the fimbrial organelle as well as the fimbrial biosynthesis machinery (158). The involvement of various other types of fimbriae in biofilm formation has been studied to a lesser extent. Different types fimbriae produced by *E. coli* strains were classified on the basis of their adhesive, antigenic or physical properties, or on the basis of similarities in the primary amino acid sequence of their major protein subunits (90). The initial adhesion by enteroaggregative *E. coli* was reported to be mediated by aggregative adherence fimbriae namely, AAF/I

and AAF/II (137), and autoaggregation is mediated by type 4 fimbriae or bundle-forming pili (BFP) (11). AAF, type 4 fimbriae were considered to promote biofilm formation.

Most *E. coli* strains as well as other species of the *Enterobacteriaceae* produce colanic acid (M antigen), which is an extracellular polysaccharide. The genes required for the synthesis of colanic acid are grouped in the *wca* cluster, formerly called *cps* (147). Confocal scanning laser microscopy revealed that a colanic acid-defective biofilm consisted of tightly packed cells, but lacked both the depth and complex architecture of a wild-type biofilm (24, 47). These results suggest that colanic acid plays a role in the third and fourth developmental stage of *E. coli* biofilm formation and it may be speculated that RcsC, which upregulates genes involved in polysaccharide production, but represses genes encoding fimbriae, curli and Ag 43, is involved in development into a mature biofilm (47).

The plasmid-encoded gene for conjugative pili was demonstrated to induce planktonic bacteria to form biofilms. Since medically relevant plasmid-bearing strains are more likely to form a biofilm, it was suggested that the conjugation and biofilm formation may enhance horizontal spread of virulence factors (52). Recently, it was confirmed that TraA, the major filament protein of the F-pilus, is essential for the development of highly organized, mature *E. coli* K-12 biofilms (125). The presence of TraA pili could support biofilm maturation even in the absence of flagellae, type 1 fimbriae or Ag43, thus providing further confirmation that the latter are surface appendages that promote the initial adsorption of bacteria to the solid surface, but are dispensable for later stages of biofilm development.

In general, biofilm formation by bacteria is a complex regulatory process that depends on strain dependent production of surface determinants and coordinated expression of multiple types of cell surface structures, which in turn influenced by various environmental conditions and challenges within the host. Understanding the role of individual bacterial determinant will help us in understanding mechanism of biofilm formation *in vivo*.

1.3.2.1 - Role of AIDA-I in *E. coli* biofilm formation

ADIA-I$^+$ diarrheagenic porcine *E. coli* isolates colonized the upper colon through the aggregative adherence and biofilm type of colonization (124). *In vitro* studies using human AIDA-I$^+$ *E. coli* strain showed that AIDA-I adhesin possesses self association characteristics (i.e. AIDA-AIDA interactions), which mediate bacterial autoaggregation and AIDA-I interaction with Ag 43 producing bacteria. In the same study, AIDA-I expression by *E. coli* was found to enhance biofilm formation on abiotic surfaces (138).

1.4 - Anatomy and histology of intestinal tract in piglets

The small intestine is the longest portion of gastrointestinal tract and it is divided into three regions namely: duodenum, jejunum (longest portion) and ileum. There is no clear visible boundary between these regions, but histologically they differ. The wall of small intestine consists of four layers: mucosa, submucoa, lamina muscularis and serosa. Finger-like projections of mucosa called villi increase the luminal surface area and thus the efficiency of digestion and absorption. The mucosa is lined by simple columnar epithelial cells called enterocytes, covering the underlying lamina propria that contains aggregates of leukocytes and foci of lymphoid nodules.

The major functions of small intestine include digestion and absorption of nutrients. The large intestine consists of caecum, colon and rectum. The caecum is a blind cylindrical sac that connects at its base to the distal ileum and to proximal colon. The mucosa of large intestine is similar to small intestine except that it lacks villi. The main functions of large intestine are the absorption of water, electrolytes and certain nutrients produced by bacterial fermentation and it also harbors large numbers of commensal organisms (177).

Although most nutrients are absorbed by small intestine, absorption of nutrients can also occurs in the large intestine, especially in the proximal portion of spiral colon in newborn pigs, in which the rudimentary microvilli are found, but they gradually disappear during the first few days of life (175). The enterocytes in large intestine are similar to those in small intestine through possessing long microvilli at the apical border, the ability to absorb aminoacids and glucose, and digestive enzyme activities located at the brush border. It has been suggested that, in the newborn, colon complements the immature small intestine by having additional function of digestion and absorption (174).

The mucosal enterocytes are the main cells is involved in intestinal digestion and absorption. The mucus-producing goblet cells scattered among the enterocytes are increasingly found distally. The microvilli found on the apical surface of enterocytes are thin, uniform, finger-like structures clearly visible under electron microscope and histologically these give a striated appearance called striated border or brush border membrane. These microvilli tremendously increase the absorptive surface area of an enterocyte. Digestive enzymes are anchored to the brush border membrane and

constitute part of glycocalyx (177). Glycocalyx is a thin extracellular matrix layer secreted by prokaryotic and eukaryotic cells, made up of mucins secreted by various epithelial cells of eukaryotes (enterocytes in case of intestine). The various glycoproteins present in mucins interact with bacterial adhesins; however the traditional aqueous fixatives are unable to preserve the intact mucus coat to demonstrate this interaction (83, 142). In contrast, non-aqueous fixation using 1% solution of osmium tetraoxide dissolved in perfluorocarbon was reported to retain the mucus structure of the intestinal glycocalyx (142).

1.5 – Summary

This brief review highlights the importance of *E. coli* infections in pigs and various virulence factors and related genes involved in colonization and toxin production employed by pathogenic *E. coli*. Despite numerous studies on various *E. coli* virulence factors, the role of AIDA-I expressed by diarrheagenic *E. coli* is not clear either in humans or in animals. Considering the relatively high prevalence of AIDA-I positive *E. coli* isolates from clinical cases of porcine diarrhea, the fact that relatively high number of porcine ETEC strains were positive for *aidA* gene in the absence of other classical fimbrial adhesins, and AIDA-I association with other enterotoxins, there is a need for better understanding of the role of AIDA-I adhesin in the pathogenesis of diarrhea in pigs.

The next chapter addresses the hypothesis and objectives of the research presented in this thesis, which focused on investigation of pathogenesis and clinical significance of the AIDA-I adhesin in diarrhea of pigs caused by AIDA-I^{+} *E. coli*.

2 - Research hypothesis and objectives

In this study, the role of the AIDA-I adhesin in the pathogenesis of coliform diarrhea in pigs, the association of AIDA-I with other known virulence factors of diarrheagenic *E. coli*, and its clinical significance in cases of porcine diarrhea are investigated.

2.1 – Objectives and Hypothesis

Objective 1: Determination of significance of AIDA-I adhesin in colonization and pathogenesis of diarrhea.

Rationale: AIDA-I adhesin was demonstrated to mediate *in vitro* diffuse adherence to HeLa or Hep2 cells exhibited by AIDA-I^+ *E. coli* isolated from human infantile diarrhea (6).

Hypothesis: AIDA-I adhesin expressed by AIDA-I^+ *E. coli* isolated from porcine diarrhea case mediates *in vitro* DA to HeLa cells and is essential for intestinal colonization and development of diarrhea in experimentally infected pigs.

The significance of AIDA-I adhesin in colonization and pathogenesis of diarrhea was determined by:

(i) experimental infection of colostrum-deprived newborn pigs with AIDA-I positive wild type PD20 and complemented PD20C strains, and the AIDA-I negative mutant PD20M strain; and

(ii) *in vitro* assessment of the role of AIDA-I adhesin in aggregation and biofilm formation.

Objective 2: Determination of prevalence and clinical significance of AIDA-I virotype in F4-negative *E. coli* isolates from clinical cases of diarrhea.

Rationale: Relatively high (7-25%) percentage of *E. coli* isolates from clinical cases of pre–weaning and post–weaning diarrhea were positive for *aidA* gene and negative for classical fimbriae (F4, F5, F6, F41) (59, 60, 92, 113, 115). The clinical significance of this is not known.

Hypothesis: AIDA-I is significantly associated with clinical cases of porcine diarrhea among porcine diarrheagenic *E. coli* isolates, in the absence of the most common virulence factor, F4 fimbria.

Prevalence and clinical significance of AIDA-I virotype in F4-negative *E. coli* isolates from clinical cases of diarrhea were determined by:

i) screening for *aidA* gene in all 110 F4-negative *E. coli* strains isolated from cases of diarrhea in pigs from the prairie provinces of Western Canada;

ii) analyzing the data obtained through survey questionnaires from swine practitioners; and

iii) screening for common virulence factors of *E. coli* isolates from cases with completed questionnaires.

3 - Contribution of AIDA-I to the pathogenicity of a porcine diarrheagenic *E. coli* and intestinal colonization through biofilm formation in pigs

This chapter contains the complete text of the manuscipt submitted for publication to Veterinary Microbiology (2006), by Madhu Ravi, Musangu Ngeleka, Sang-Hyun Kim, Carlton Gyles, Frédéric Berthiaume, Michael Mourez, Dorothy Middleton and Elemir Simko. Compared to the submitted manuscript, the materials and methods have been expanded to include greater detail of experimental design.

3.1 - Introduction

Escherichia coli remains one of the leading causes of diarrhea in pigs worldwide. Among the different groups of diarrheagenic *E. coli* in pigs, enterotoxigenic *E. coli* (ETEC) is still the most prevalent (57); however, other groups such as *E. coli* attaching and effacing (EAE) and porcine Shiga toxin-producing *E. coli* (STEC) are also frequently isolated from pigs with diarrhea (57, 113). Few studies have recently described an emerging group of enterotoxigenic *E. coli* that are negative for classical fimbrial antigens (F4, F5, F6, F41), but express the **a**dhesin **i**nvolved in **d**iffuse **a**dherence (AIDA-I) (113). AIDA-I has been previously described in *E. coli* isolated from children with diarrhea as well as from asymptomatic children (4, 71). This adhesin has also been reported in *E. coli* isolates from weaned pigs with edema disease or with

post-weaning diarrhea, mostly in association with F18 fimbriae (115). Recent studies suggest that the AIDA-I gene is widely distributed in *E. coli* isolated from diarrheic pigs (59, 92, 113, 115). In addition, Fang and co-workers (2005) recently demonstrated that AIDA-I protein from porcine *E. coli* isolates is highly homologous (78% identity) with AIDA-I extracted from human *E. coli* strain 2787 (46).

Porcine or human AIDA-I is a 100-kDa autotransporter protein (9, 46) distributed on the bacterial outer membrane, and appears to be responsible for bacterial attachment to enterocytes leading to colonization (113, 124), and for bacterial auto-aggregation leading to biofilm formation *in vitro* (138). However, the mechanism by which AIDA-I-positive *E. coli* induce diarrhea in both animals and humans has not been determined.

In other studies, Ngeleka and co-workers observed that AIDA-I-positive *E. coli* was isolated mainly from diarrheic, but not from non-diarrheic pigs, and induced diarrhea in experimentally infected newborn pigs (113,124). Furthermore, the porcine AIDA-I^{+} *E. coli* used in that study colonized the colon of pigs through a biofilm-like colonization, which differs from usual ETEC or EPEC colonization patterns. Thus, AIDA-I plays a significant role in development of diarrhea disease in pigs, through colonization with biofilm formation. The objectives of the present study were two fold:

i) to assess the association of AIDA-I with pathogenicity of AIDA-I positive wild type PD20 and complemented PD20C strains, and AIDA-I negative mutant PD20M strain in colostrum-deprived newborn pigs by experimental infection,

ii) and to further characterize the porcine *E. coli* AIDA-I mediated adhesion pattern *in vitro*.

3.2 - Materials and methods

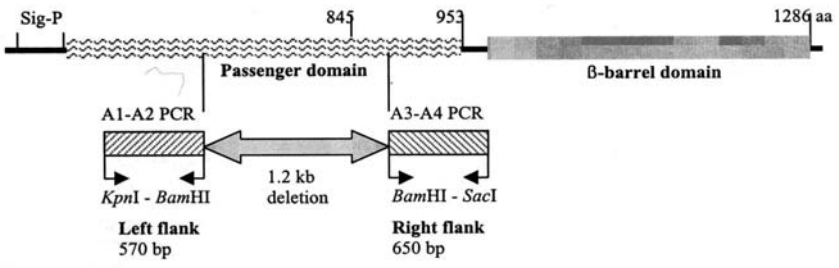
3.2.1 - Bacterial strains, plasmids, and culture conditions

E. coli strain PD20 of serogroup O143, (AIDA-I^{+}, STb^{+}) was isolated from newborn pigs with diarrhea. Strain PD71 is a porcine non-pathogenic *E. coli* that was used as negative control (113). Strain PD20^{Str} is a spontaneous streptomycin-resistant derivative of PD20, and was used as recipient for the mating experiment with *E. coli* strain SM10λ*pir* (*thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^{r}). A recombinant plasmid pT*aidA* was used to complement the AIDA-I-negative mutant PD20M (AIDA-I^{-}, STb^{+}). Bacteria were cultured in Tryptic Soy Broth (TSB) or Luria Bertani (LB) medium (Beckton, Dickinson and company, MD, USA) containing appropriate antimicrobial agents, when necessary, at 37°C for 18 h. Ampicillin (Amp), streptomycin (Str), kanamycin (Km), and gentamicin (Gm) were used at 100 µg/ml, 100 µg/ml, 30 µg/ml and 20 µg/ml, respectively.

3.2.2 - Generation of AIDA-I-negative mutant

A Δ*aidA*::*aacC1* mutation was created in PD20^{Str} by an allelic exchange technique (41). Briefly, an internal deletion of a 1.2 kb DNA fragment within the reading frame of *aidA* gene was obtained by joining two PCR products amplified from the PD20 genomic DNA (Fig. 3.1).

Fig. 3.1 – Schematic illustration of strategy for creation of Δaida-I::aacC1 mutation



Two sets of primers were used to amplify the *aidA* left flank and right flank DNA, respectively:

AidA1 forward (5' GTGTCAGGTACCACAGTGAATG 3') and

AidA2 reverse (5' ATTTGGGATCCAGATACCTCG 3');

AidA3 forward (5' TAGTTGCCGGATCCTATGATTAC 3') and

AidA4 reverse (5' CCATGTGAGCTCATTCAGGTT 3').

The primer sequences were based on the prototype *aidA* gene (GenBank accession no. X65022). The left and right flank DNA fragments obtained from PCR were digested with *Kpn*I-*Bam*HI and *Bam*HI-*Sac*I, respectively. These products were purified and ligated into a single fragment at the *Bam*HI site and cloned into a pBlueScript vector digested with *Kpn*I-*Sac*I. The resulting recombinant plasmid (pAIDA-ID) carrying Δ*aidA* DNA was further manipulated for insertion of a non-polar Gm-cassette. Plasmid pAIDA-ID digested with *Bam*HI was ligated with the *aacC1* gene fragment derived from pUCGM (GenBank accession no. U04610) digested with *Bam*HI so that the Gm-cassette was inserted in the middle region of the Δ*aidA* DNA carried in pAIDA-ID. The resulting plasmid, named pAIDA::Gm, was digested with *Kpn*I and *Sac*I and purified for subcloning into suicide vector pRE107 (*ori*R6K, RP4 *mob*, *sac*B, Amp^r). The resulting suicide plasmid (pRE7AIDA::Gm) carrying the Δ*aidA*::*aacC1* allele was introduced into *E. coli* SM10 by electroporation, then transferred to *E. coli* $PD20^{Str}$ by conjugation. Exconjugants were selected on LB plates containing Str and Amp at 37°C for 18 h. The resistant colonies (Str^r + Amp^r) were spread onto LB agar plates containing 7% sucrose and Gm, and incubated at 30°C for 18 h, in order to promote a double crossover event. A few desired colonies (Str^r/Gm^r/Amp^r) were

purified and confirmed for Δ*aidA*::*aacC1* mutation by PCR. The expected size reduction of the AidA1-AidA4 amplicon of the mutant was confirmed on 1% agarose-gel electrophoresis with the wild type control (Fig. 3.2A). The desired mutant (PD20M) was further confirmed by slide agglutination test using O143 antiserum.

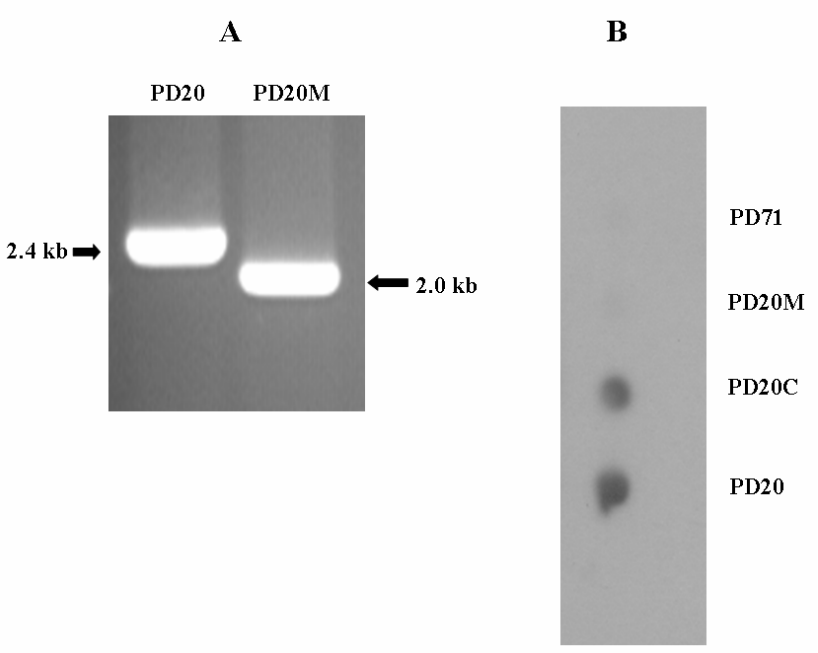


Fig. 3.2 - The PCR reaction with AidA1 and AidA4 primers shows the desired size reduction in PD20M (Δ*aidA*::*aacC1*; 2.0 kb), compared to the wild type strain PD20 (2.4 kb) (A). Using anti-AIDA-I serum, the immunodot assay shows the presence of AIDA-I protein in the wild type strain PD20 and the complemented mutant strain PD20C, but not in the AIDA-I$^-$ mutant PD20M or the negative control strain PD71 (B)

3.2.3 - Construction of pT*aidA* to generate strain PD20C

The *aidA* gene was amplified from the genome of *E. coli* strain PD20 by PCR using primers;

Nco_Aid (5' TAATAACCATGGGTAAGGCCTACAGTATCATATGG 3') and

Xba_Aid (5' GGATAAAATACAGCTTCTGATAATCTAGATAA 3')

to generate a 3.8 kb fragment. This fragment was purified from the gel and ligated in the *Nco*I and *Xba*I restriction sites of the pTrc99A expression vector (Pharmacia Biotech) under the control of the IPTG-inducible p*trc* promoter. The recombinant plasmid (pT*aidA*) was transformed into the mutant strain PD20M (Δ*aidA::aacC1*) by electroporation to generate the AIDA-I-positive complemented strain PD20C. The complementation was confirmed by immunodot with anti-AIDA-I serum as previously described (46) for probing expression of AIDA-I adhesin (Fig. 3.2B).

3.2.4 - Experimental infection of newborn pigs

The AIDA-I-negative mutant (PD20M) was evaluated for pathogencity by gastric inoculation of colostrum-deprived newborn pigs kept in a controlled environment, as described previously (124). Briefly, conventionally derived, colostrum-deprived, newborn pigs were obtained from a commercial high-health swine operation. Piglets, snatched-farrowed from several gilts and sows, were lightly sprayed with an iodine and alcohol preparation (10% Isospray, PVL), the umbilical cord clamped with an autoclaved navel clip and the piglets placed under a heat lamp in a disinfected (Virkon, Vetoquinol) plastic tub until transported to Animal Care Unit at the Western College of Veterinary Medicine. The piglets were weighed and identified with permanent ink numbers on the back, and randomly assigned to each test group in individual environmentally controlled containment rooms. The details of experimental infection carried out in 3 different experiments are as follows: in the first, 3 groups containing 2, 3 and 2 piglets were infected with PD20 wild, PD20M and PD71 strains, respectively; in the 2nd experiment, 3 groups containing 4, 4 and 2 piglets were infected with PD20 wild, PD20M and PD71 strains, respectively; and 3rd experiment was carried

out once PD20C became available, with 3 groups containing 2, 2 and 6 piglets that were infected with PD20 wild, PD20M and PD20C strains, respectively. Three hours after inoculation piglets were bottle-fed with a commercial pig milk replacer (Wet Nurse™, PMT Group, SK). Feeding and clinical observations were done at 4 h intervals over each 24 h period. The data for each group was pooled from these three experiments (Table 3.1); however in the last experiment the data related to PD20M infected group were deleted since that group was cross-contaminated with PD20 wild strain. The animals were handled according to the guidelines outlined in *The Guide to the Care and Use of Experimental Animals of the Canadian Council of Animal Care*.

Piglets from each group were euthanized at 24 and 48 h post-inoculation or at the time of detection of diarrhea. Following gross examination, intestinal samples were collected from terminal ileum, cecum and proximal spiral colon for evaluation by histology and immunohistochemistry, and from colon for electron microscopy. A segment of the ileum was cultured on blood and MacConkey agar plates (Becton and Dickinson) and the infecting *E. coli* organism confirmed by PCR and immunodot as previously described (46).

3.2.5 - Histology and immunohistochemistry (IHC)

The intestinal tissues were fixed in 10% neutral-buffered formalin, processed and embedded in paraffin wax using standard procedures. Four-micron sections mounted on glass slides were stained with haematoxylin and eosin (H&E) and Brown and Brenn (B&B) modified Gram stain for histology and bacterial examination, respectively. IHC was performed on paired tissue sections as previously described (61), using rabbit polyclonal anti AIDA-I (1:500) for the detection of the AIDA-I^+ strains

(PD20 and PD20C) or anti-O143 (1:4000) sera for the detection of the PD20M mutant strain along with its parent wild type strain PD20 and the complemented strain PD20C. Strain PD71 was used as a negative control for both AIDA-I and O-143 antiserum. Rabbit pre-immune serum was used as the primary antibody negative control.

3.2.6 - Transmission (TEM) and Immunogold (IGEM) electron microscopy

Tissues for TEM were fixed using perfluorocarbon and osmium, and embedded in epon as previously described (124). Ultrathin sections (70-90 nm thickness) of colon from pigs infected with the wild type strain PD20, the mutant PD20M or the complemented strain PD20C were selected based on IHC staining and semi-thin sections to confirm colonization, and placed on copper grids (124). Expression of AIDA-I protein by strains PD20, PD20M and PD20C *in vivo* was evaluated by a post-embedding IGEM technique of Polak and Van Noorden (123) with few modifications. Briefly, the perflourocarbon fixed, ultrathin sections of colon were placed on nickel grids that were floated on 4% goat blocker solution [4% goat serum and 0.1% Tween in Phosphate Buffer Solution (PBS), pH 7.4] for 30 min. The grids were drained and incubated in 1:50 dilution of rabbit anti-AIDA-I IgG and goat blocker solution at room temperature for 1 h. Sections of colon from pigs infected with strain PD20 were incubated with pre-immune rabbit serum and used as negative control. Grids were then rinsed twice in PBS containing 0.1% (v/v) Tween (PBST), 5 times in PBS and 3 times in PBS containing 0.1% ovalbumin. Subsequently, the grids were incubated in 1:100 dilution of goat anti-rabbit IgG conjugated with 10 nm gold particles (British Biocell International, UK) in goat blocker solution for 1 h. The grids were then rinsed twice in PBST, 3 times in PBS and 4 times in distilled water. All rinses were done under gentle

agitation on a magnetic stirrer for 5 min. Finally, the grids were dried and contrasted with uranyl acetate. TEM was carried out using a Philips 410LS electron microscope.

3.2.7- *In vitro* assays

3.2.7.1 - Bacterial adhesion to HeLa cells

Adhesion of bacteria to HeLa cells was assessed as described previously (5) with few modifications. Briefly, the four test strains (PD20, PD20M, PD20C and PD71) were grown statically at 37°C in TSB (Becton, Dickinson and Company, MD, USA) for 48 h and the bacterial concentration adjusted to 10^8 CFU/ml, as previously described (46). Preliminary studies showed that adherence of the wild type strain PD20 was greatest using bacterial culture incubated for 48 h compared to 24 h culture (unpublished data). HeLa cells were grown in an eight-chambered polystyrene tissue culture plate (BD Falcon, MA, USA) and fixed on ice with PBS containing 1% (v/v) formalin for 30 min. The wells were inoculated in duplicate with 500 µl of TSB containing 10^8 CFU/ml of bacteria and incubated at 37°C for 3 h. Bacterial adherence was evaluated by light microscopy (124). Adhesion of bacteria to HeLa cells was carried out in two independent experiments.

3.2.7.2 - Bacterial auto-aggregation

This assay was performed by investigating the kinetics of settling of the four different test strains as previously described (74). The absorbency of the supernatant bacterial culture collected at 3, 6, 18 h intervals was measured at an optical density (OD) of 600 nm. Bacterial auto-aggregation was carried out in two independent experiments.

3.2.7.3 - Biofim formation

Production of biofilm was evaluated as described previously (74) with few modifications. The bacterial concentration of the four test strains was adjusted to 10^6 cfu/ml in fresh TSB medium and 200 µl of this culture was incubated at 37°C for 48 h on polystyrene 96 well microtitre plate (BD Falcon, NJ, USA) in triplicate in three independent experiments. Biofilm formation was analyzed by measuring the absorbency of bacterial staining with 0.1% crystal violet, at an optical density of 595 nm on an ELISA reader. In order to normalize distribution, we transformed the collected data into logarithmic values and analyzed these statistically by ANOVA for comparison of paired groups with the Scheffe test using STATA 8.2 (Stata corp. LP, TX, USA).

In addition, phase-contrast microscopy for biofilm evaluation was performed as described previously (23) with slight modifications. The test strains were grown in 3 ml TSB medium at 37°C for 48 h in a polystyrene culture dish (Corning, NY, USA). After incubation, the micro dish was rinsed and the attached bacteria were examined by phase-contrast microscope (Zeiss Axiovert 135) with the images captured using an Eclipse software version 6.0.

3.3 - RESULTS

3.3.1 - Generation of AIDA-I-negative mutant PD20M and its complemented strain PD20C

An allelic exchange procedure was employed for generating the Δ*aidA*::*aacC1* mutant PD20M, as described in the materials and methods. The desired mutation introduced was verified by PCR and immunodot (Fig. 3.2A, 3.2B). The PCR reaction with AidA1 and AidA4 primers shows the desired size reduction in PD20M

(Δ*aidA*::*aacC1*; 2.0 kb), compared with wild type (2.4 kb). An approximately 400 bp size reduction is expected in the Δ*aidA*::*aacC1* allele, compared with wild type, because ~ 800 bp of the *aacC1 Bam*HI fragment was inserted in the deleted region of the *aidA* gene lacking an internal 1.2 kb DNA within the reading frame. This PD20M was then transformed with pT*aidA*, giving the complemented strain PD20C. Both the mutant (PD20M) and the complemented (PD20C) strains were confirmed as derivatives of wild type strain PD20 by slide agglutination test, using O143 antiserum. Restoration of AIDA-I expression on the bacterial surface in PD20C was confirmed by immunodot (Fig. 3.2B).

3.3.2 - Experimental infection of newborn pigs

3.3.2.1 - Clinical observations and bacteriology

All pigs inoculated with the wild type strain PD20 or the complemented strain PD20C developed a pasty diarrhea between 15-19 h or 27-31 h after inoculation, respectively. In contrast, neither the AIDA-I$^-$ mutant strain PD20M nor the non-pathogenic strain PD71 induced diarrhea up to 48 h after inoculation (Table 3.1). Bacterial cultures from the ileum of infected pigs were confirmed as infecting strains by PCR, immunodot using anti-AIDA-I serum or by slide agglutination using anti-O143 serum (results not shown). All rectal swabs taken from pigs prior to experimental infection were negative on aerobic and anaerobic bacterial cultures, suggesting no bacterial colonization of the intestine before inoculation of these pigs.

Table 3.1 - Pathogenicity of AIDA-I$^-$ mutant (PD20M) and AIDA-I$^+$ complemented strain (PD20C) in newborn colostrum-deprived pigs.

E. coli strain	Characteristics	Number of pigs**		
		Inoculated	Affected	Detection of dirahhea (hr PI)*
PD20	AIDA-I$^+$, STb$^+$	8	8	15-19
PD20M	ΔAIDA-I$^-$, STb$^+$	7	0	None
PD20C	pT*aidA* (AIDA-I$^+$, STb$^+$)	6	6	27-31
PD71	Negative control	4	0	None

* PI: Post inoculation
**The number represents total from 3 experiments

3.3.2.2 – Histology

On H&E stained sections, there were no changes in the intestinal mucosal morphology of the infected pigs by the end of the experimental period at 48 h post-inoculation. AIDA-I$^+$ strains, however, were intimately associated with the mucus barrier layer and the apical border of the colonic and cecal epithelium whereas the AIDA-I$^-$ mutant and negative control PD71 had a more haphazard distribution, and less mucus was present. On B&B stained sections, large numbers of Gram-negative bacteria were seen in close contact, with the cecal and colonic epithelium of pigs inoculated with AIDA-I$^+$ strains, but not the AIDA-I$^-$ strain, at 24 and 48 after inoculation (results not shown), whereas ileal colonization was negligible with all strains.

3.3.2.3 – Immunohistochemistry

Using anti-AIDA-I serum, sections of cecum and colon from pigs inoculated with AIDA-I^+ strains PD20 (Fig. 3.3A) or PD20C (Fig. 3.3E) show positive staining of the bacterial population and confirmed heavy colonization, whereas only very weak bacterial staining and sparse colonization were detected in pigs infected with the AIDA-I^- mutant PD20M (Fig. 3.3C) and no staining with the negative control strain PD71 (not shown). Using anti-O143 serum, we have observed a light colonization of the large intestine of pigs inoculated with the mutant strain PD20M (Fig. 3.3D) when compared to that of strains PD20 or PD20C (Fig. 3.3B & 3.3F, respectively).

3.3.2.4 - Transmission electron microscopy and Immunogold electron microscopy

On ultrathin sections of colon from piglets inoculated with the AIDA-I^+ strains PD20 (Fig. 3.4A) or PD20C (Fig. 3.4C), the majority of bacteria were stratified within the thick mucus layer immediately overlying the enterocyte microvillus border while some bacteria were in contact with the colonic microvilli. In contrast, no spatial organization or layering of bacteria within mucus occurred in piglets inoculated with the AIDA-I^- mutant strain PD20M (Fig 3.4B).

On immunogold labeling, many gold particles were present on the bacterial surface of the AIDA-I^+ strains PD20 (Fig. 3.5A) and PD20C (Fig. 3.5B) whereas labeling of PD20M (Fig. 3.5C) was minimal compared to both its parent strain (PD20) and its complemented strain (PD20C). No labeling was seen in the negative control sections of colon from pigs infected with strain PD20, stained with pre-immune rabbit serum (Fig. 3.5D).

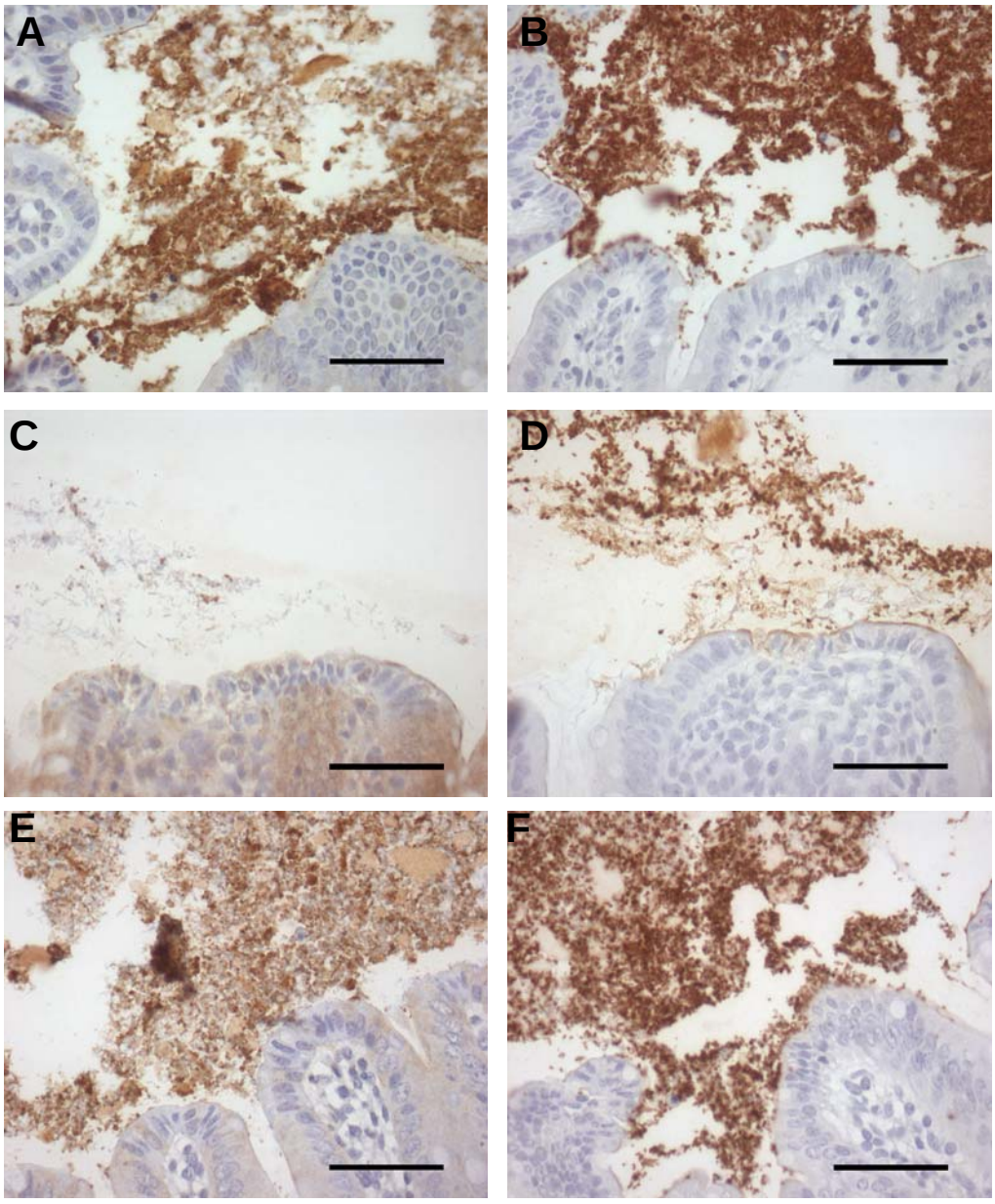


Fig. 3.3 - Immunohistochemical staining of sections of colon shows heavy bacterial colonization and bacteria intimately associated with the mucosa of pigs infected with the wild type strain PD20 (A, B) and the complemented strain PD20C (E, F) using AIDA-I antiserum (1:500) and O143 antiserum (1:4000) respectively). In contrast, specific staining is absent and markedly lesser colonization is seen in the colon of pigs infected with the AIDA-I$^-$ mutant PD20M when stained with AIDA-I (C) or with O143 antiserum (D) respectively. Bars = 50 μm.

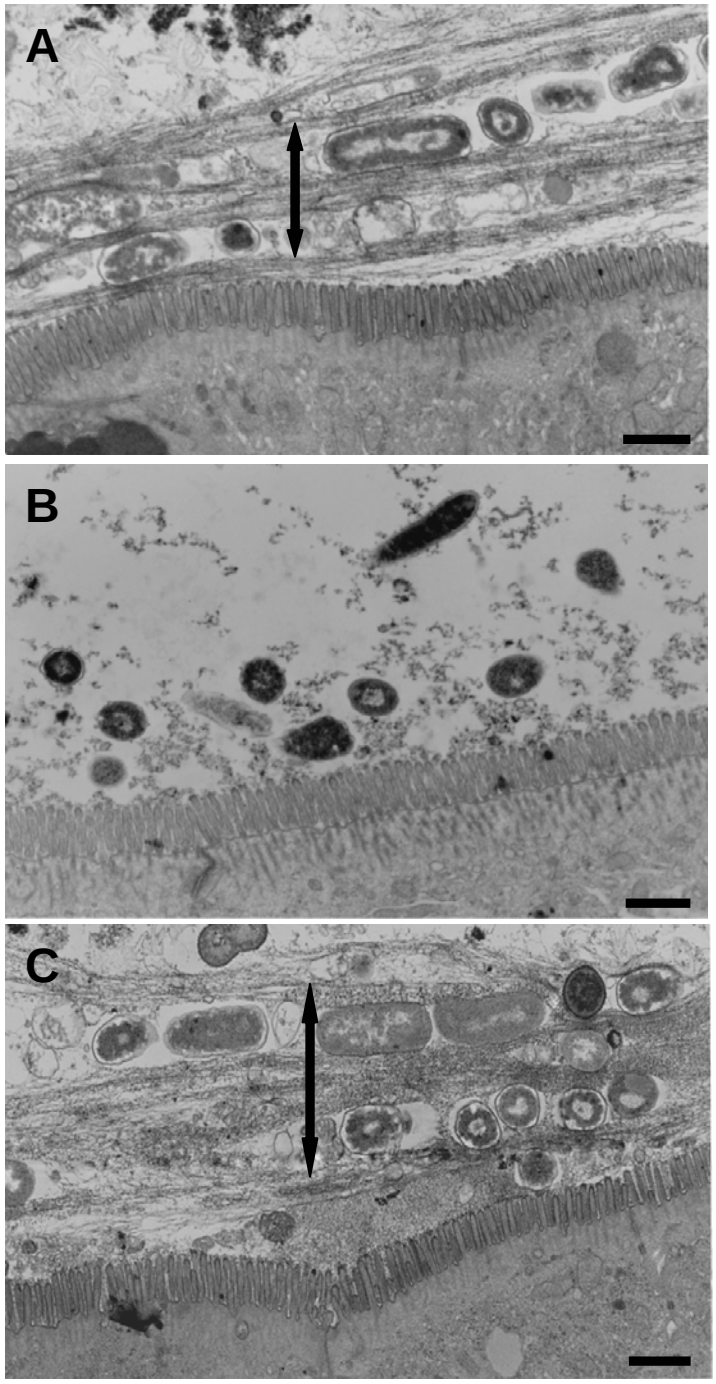


Fig. 3.4 - Transmission electron micrographs of perfluorocarbon fixed, epon-embedded colonic epithelium show bacteria stratified within mucus layers, forming a biofilm (arrows) in pigs infected with AIDA-I^{+} wild type PD20 (A) or the complemented strain PD20C (C). In contrast, with the AIDA-I^{-} mutant strain PD20M (B), no biofilm was formed and bacteria were irregularly distributed above the colonic enterocytes. Bars = 1 μm.

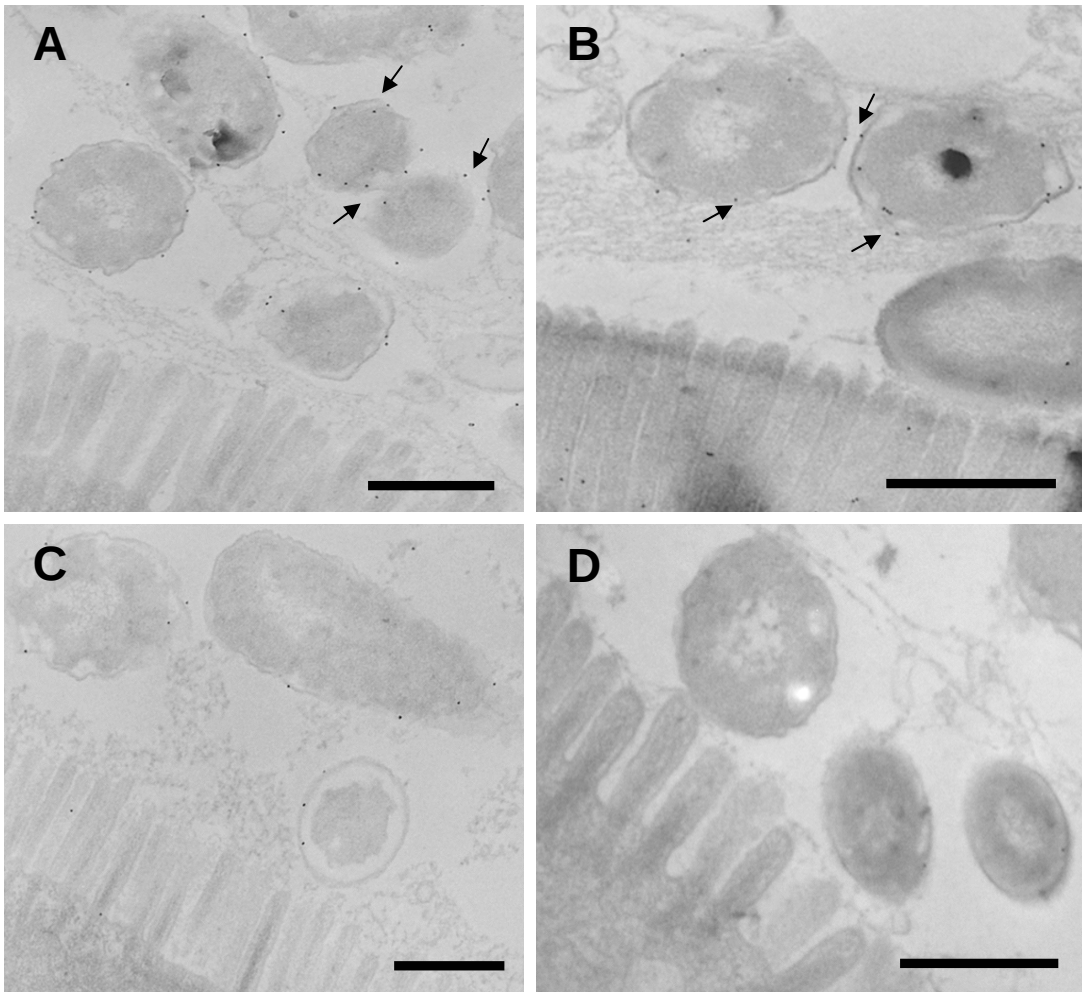


Fig. 3.5 - Immunogold EM of perfluorocarbon fixed, epon embedded colonic epithelium using anti-AIDA-I IgG antibody (1:50) shows gold labeling (10 nm particles) of the cell membrane (arrows) of the wild type strain PD20 (A), the complemented strain PD20C (B) and minimal labeling of the mutant strain PD20M (C). Rabbit preimmune serum (1:50) was used as a negative control to stain colonic tissues of pigs infected with strain PD20 (D). Bars = 0.5 μm.

3.3.2.5 - *In vitro* adherence, bacterial auto-aggregation and biofilm formation.

3.3.2.5.1 – Adherence assay

Using HeLa cells to test adherence, results similar to those described previously (5) were observed. Both AIDA-I$^+$ strains (PD20 and PD20C) diffusely adhered to HeLa cells (Fig. 3.6A and B, respectively), also multifocal aggregative and stacked brick-like pattern (insets of Fig. 3.6A and B), but this was not demonstrated by either the AIDA-I$^-$ mutant PD20M (Fig. 3.6C) or the negative control strain PD71 (Fig. 3.6D).

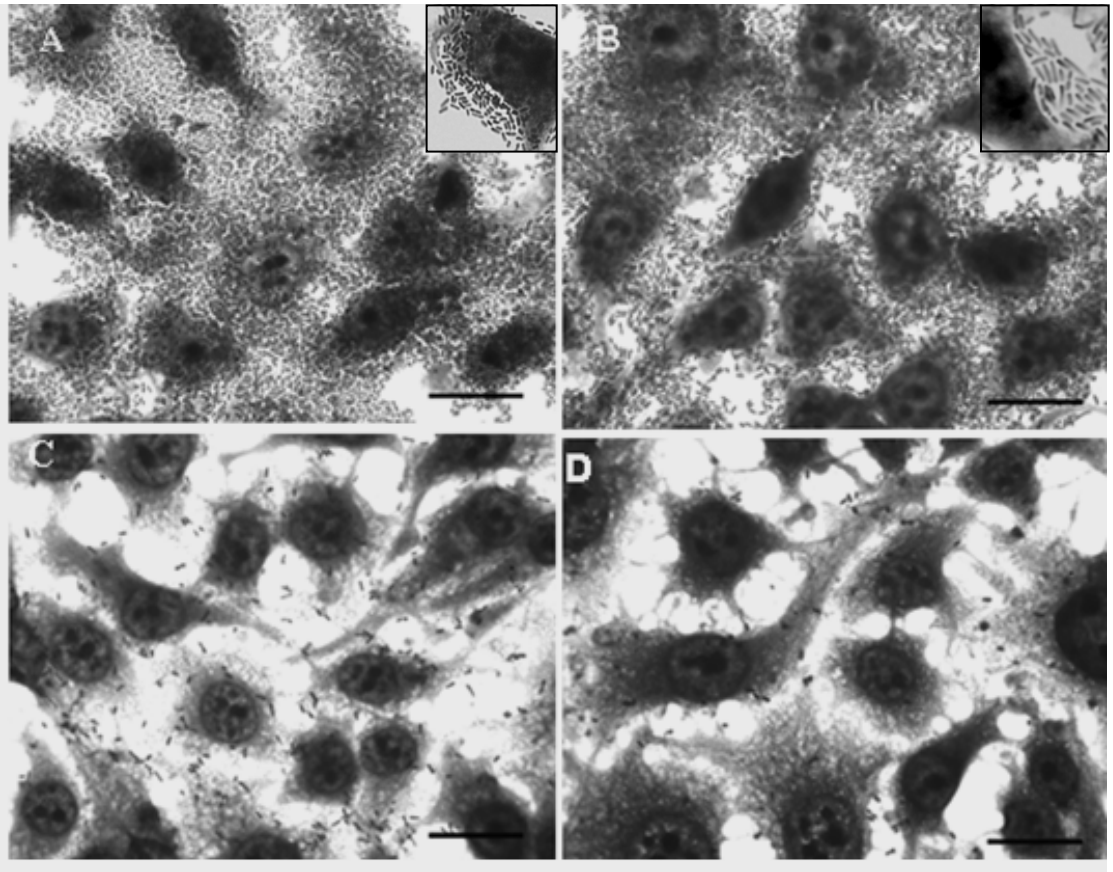


Fig. 3.6 - AIDA-I mediated attachment to HeLa cells is demonstrated by diffuse adherence by, the AIDA-I$^+$ wild type PD20 (A) and the complemented strain PD20C (B) strains. Insets (A and B) show stacked brick pattern of bacterial arrnagement on HeLa cell by PD20 and PD20C strains. In contrast, very little adherence to HeLa cells occurred with the mutant strain PD20M (C) and the negative control strain PD71 (D). Bars = 25 μm.

3.3.2.5.2. Aggregation assay

In addition, the bacterial settling kinetics assessing auto-aggregation showed that both AIDA-I^+ strains settled faster than the AIDA-I^- mutant and the negative control strains, consistent with *in vitro* aggregation by these positive strains (Table 3.2).

Table 3.2 - Auto-aggregation assay was performed in TSB medium and bacterial settling kinetics at 0, 3, 6, 18 hr intervals were assessed by evaluation of absorbance of cultures at an optical density of 600 nm.

	OD_{600} of culture*			
E. coli strain	0 hr	3 hr	6 hr	18 hr
PD71	0.355 ± 0.0	0.254 ± 0.01	0.239 ± 0.031	0.195 ± 0.089
PD20M	0.355 ± 0.0	0.232 ± 0.009	0.218 ± 0.016	0.167 ± 0.059
PD20C	0.355 ± 0.0	0.210 ± 0.045	0.180 ± 0.008	0.092 ± 0.022
PD20	0.355 ± 0.0	0.173 ± 0.058	0.150 ± 0.028	0.065 ± 0.058

*Results indicate the median ± range of the data obtained from two independent experiments.

3.3.2.5.3 –Biofilm assay

In vitro Biofilm formation was significantly greater ($p < 0.05$) for both the AIDA-I^+ strains (PD20 and PD20C) than for the AIDA-I^- mutant (PD20M) and the negative control strain, PD71 (Table 3.2).

Table 3.3 - *In vitro* biofilm* formation by AIDA-I^+ *E. coli* strains (PD20 and PD20C) and AIDA-I^- strains (PD20M and PD71).

E. coli strain	Characteristics	Mean biofilm formation (OD)**
PD20	AIDA-I^+, STb^+	0.452 ± 0.044^a
PD20M	ΔAIDA-I^-, STb^+	0.223 ± 0.057^b
PD20C	pT*aidA* (AIDA-I^+, STb^+)	0.414 ± 0.204^a
PD71	Negative control	0.154 ± 0.148^b

*Bacterial biofilms were stained by crystal violet and quantified by optical absorbance at 595 nm. The data represent mean OD with standard deviation from 3 independent experiments.
**Means ± standard deviation with a superscript not in common are significantly different ($p<0.05$).

Aggregation and biofilm formation on a polystyrene surface, demonstrated by phase microscopy, was noted with AIDA-I$^+$ strains (Fig. 3.7A and B, respectively), but not with the AIDA-I$^-$ mutant and the negative control strains (Fig. 3.7C and D, respectively).

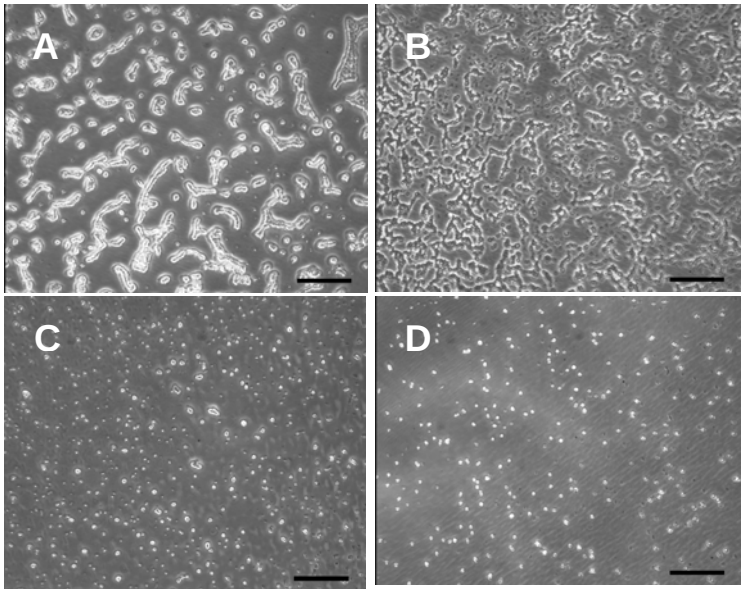


Fig. 3.7 - Phase contrast microscopy of *E. coli* strains grown in TSB on polystyrene dishes showing bacterial aggregation and biofilm formation by the AIDA-I$^+$ strains, wild type strain PD20 (A) and the complemented mutant PD20C (B). In contrast, neither bacterial aggregation nor biofilm formation was observed with the AIDA-I$^-$ mutant PD20M (C) or the negative control PD71 strains (D). Bars = 50 μm.

3.4 - Discussion

We have demonstrated that the wild type *E. coli* strain PD20 (AIDA-I$^+$, STb$^+$) showed diffuse aggregative adherence to HeLa cells, auto-aggregation and biofilm formation *in vitro*, and colonized the large intestine of infected pigs with biofilm formation and induced diarrhea in these animals, in contrast to its isogenic mutant strain PD20M (AIDA-I$^-$, STb$^+$). When this mutant strain was complemented with the full length *aidA* gene, the resulting strain PD20C (AIDA-I$^+$, STb$^+$) regained all these *in vitro* and *in vivo* attributes, including the capacity to induce diarrhea in neonatal pigs. These results suggest that the AIDA-I protein expressed by porcine diarrheagenic *E. coli* is an important virulence determinant in the pathogenesis of diarrhea in pigs.

AIDA-I, a non-fimbrial, 100 kDa autotransporter outer membrane protein encoded by the *aidA* gene belongs to a family of autotransporters in which the translocation mechanism and adhesive properties are located towards the C and N-terminus portions of precursor molecule, respectively. Its capacity to bind to eukaryotic cells is mediated by the protein glycosylation conferred by the autotransporter adhesin heptosyltransferase (*aah*) gene (8, 149). Recent in *vitro* studies on AIDA-I$^+$ *E. coli* isolated from diarrhea in humans suggest that the protein plays a role as an adhesin with high affinity to a variety of eukaryotic cells. In addition, this protein is capable of inducing bacterial aggregation mostly via intercellular self-recognition and is a highly efficient initiator of biofilm formation (138). In the past few years, AIDA-I protein has been associated with diarrheagenic *E. coli* in neonatal and post-weaned pigs (92, 113, 115); however, the specific role of the AIDA-I adhesin in induction of diarrhea in either pigs or humans has not been determined.

In general, bacterial attachment to enterocytes, with subsequent colonization of the intestinal epithelium, is regarded as the initial and crucial step in the pathogenesis of enteric diseases. This feature is one of the strategies used by diarrheagenic bacteria to overcome gastro-intestinal host defences such as the mucus barrier that prevents enteropathogens from coming into close contact with enterocytes, or the peristaltic cleansing mechanism (171). Pritchard et al. (2004) recently reported that porcine AIDA-I$^+$ *E. coli* strains induced aggregation and colonized the proximal spiral colon through biofilm-like formation in the experimentally infected pigs. In the same study using an AIDA-I$^+$ PD149 porcine *E. coli* isolate produced stacked brick-like pattern of adherence on Hep-2 cells *in vitro*. This same pattern was also demonstrated apart from diffuse adherence with HeLa cells in the present study, suggesting porcine AIDA-I$^+$ *E. coli* have mixed characteristics of ETEC, EAEC and DAEC (124).

In the present study, we clearly demonstrate that porcine AIDA-I is expressed as a surface protein (Fig. 3.5A) promoting bacterial auto-aggregation, biofilm formation and induction of diarrhea in pigs by the porcine AIDA-I$^+$ strain PD20, in contrast to the AIDA-I$^-$ isogenic mutant PD20M. Complementation of this mutant with the full length *aidA* gene restored these properties and resulted in clinical signs of diarrhea in experimentally infected colostrum deprived piglets between 27-31 h, in comparison to induction of diarrhea between 15-19 h post-inoculation by the wild type strain PD20. There was no apparent difference in the *in vitro* growth rate between the wild type and complemented strains, and the reason for later onset of diarrhea in groups infected with the complemented strain needs further investigation.

Although the mutant strain PD20M was confirmed negative for AIDA-I by immunodot, weak staining and low labeling of this strain was observed on immunohistochemistry and immunogold EM respectively. It is possible that non-specific binding of polyclonal antibodies occurred with the low dilutions of AIDA-I antiserum (1:500) and anti AIDA-I IgG (1:50) used respectively, compared to high dilution (1:4000) of serum used for the immunodot analysis.

In both previous (124) and present studies, we have observed that AIDA-I$^+$ bacteria, including the complemented strain PD20C preferentially colonize the large intestine of pigs in large numbers. Electron microscopy of colon from pigs infected with AIDA-I$^+$ strains shows that the bacteria are distributed in a stratified pattern within the mucus immediately overlying the enterocytes and that some bacteria come into close contact with the enterocytes. The mucus layer above the microvillus border of the intestinal tract has been reported to contain domains of glycoprotein mucins that are able to interact with bacterial adhesins (56, 83). The mucosa of the cecum and colon is rich in goblet cells and it is possible that porcine AIDA-I$^+$ *E. coli* recognize a receptor within the mucus, explaining the heavy colonization of the large intestine rather than the small intestine, which was demonstrated by histology and immunohistochemistry. These observations and other preliminary results (Fang et al., unpublished data) suggest that AIDA-I$^+$ bacteria attach to receptors in the mucus within which the organisms become enmeshed, thereby facilitating auto-aggregation, and proliferation with biofilm formation in the large intestine of neonatal pigs. Subsequently, the biofilm allows AIDA-I$^+$ *E. coli* to persistently colonize the large intestine leading to impaired water and nutrient absorption, thus resulting in the development of pasty diarrhea in the

infected pigs. This is especially likely in newborn pigs where the colon provides additional capacity to the immature small intestine for nutrient digestion and absorption (174).

It is important to note that the wild type AIDA-I$^+$ strain PD20 used in this study is also positive for enterotoxin STb, suggesting that it may be a member of the ETEC group. Interestingly, however, strain PD20 did not induce watery diarrhea characteristic of ETEC that typically colonize the small intestine, and neither did the AIDA-I$^-$, STb$^+$ mutant strain PD20M, although these bacteria were established in the intestinal lumen. Therefore, the significance of STb enterotoxin in diarrheagenic AIDA-I$^+$ *E. coli* is not clear and needs more investigation. The inability of the mutant PD20M to attach to HeLa cells, aggregate and induce biofilm formation as well as its lack to induce diarrhea in pigs, may be associated by lack of expression of functionally mature AIDA-I adhesin induced by the partial deletion of related gene, in this mutant strain.

Fang et al. (2005), reported recently that porcine and human AIDA-I adhesin have similar molecular weight and share approximately 78% homology (46). The present *in vitro* experiments provide additional functional similarities between porcine and human AIDA-I and are in agreement with those of Sherlock et al. (2004) (138). Specifically, the AIDA-I$^+$ strain PD20 and its complemented strain PD20C were capable of auto-aggregation (Table 3.2) and distinct biofilm formation (Table 3.3), whereas the AIDA-I$^-$ mutant was not. Therefore, AIDA-I is likely an essential virulence attribute which may assist diarrheagenic AIDA-I$^+$ bacteria to cause persistent and chronic infection, and perhaps also to resist antimicrobial agents (22), all strategies useful for survival in a hostile environment, such as the gastro-intestinal tract of pigs.

Additionally, until clearly demonstrated otherwise, there needs to be awareness of the potential, based on structural and functional similarities between human and porcine AIDA-I, for cross infection between these two host species by AIDA-I^{+} *E. coli*.

In conclusion, we have demonstrated that AIDA-I adhesin expressed by a porcine diarrheagenic *E. coli* is an important virulence determinant involved in colonization of large intestine with biofilm formation and development of diarrhea in neonatal pigs. The adhesin is also associated with *in vitro* cell adhesion, auto-aggregation and biofilm formation.

4 - Prevalence and clinical significance of AIDA-I associated virotypes among non-F4 (K88) *E. coli* isolates from problematic cases of diarrhea in pigs

4.1 - Introduction

Escherichia coli remains a significant etiological agent of bacterial diarrhea in pigs. Diarrheagenic *E. coli* express various fimbrial adhesins to attach to the intestinal mucosa resulting in bacterial proliferation, colonization and induction of diarrhea as a result of enterotoxin production (109). The classification of diarrheagenic *E. coli* into various groups is based on distinct epidemiological, clinical features and specific virulence determinants. The following classes among diarrheagenic *E. coli* have been identified in humans: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (65, 85, 109). Among these, ETEC strains are isolated most commonly from cases of bacterial diarrhea in farm animals, especially in suckling and post-weaning pigs (31, 140, 145, 146). Diarrheagenic ETEC strains differ from non-pathogenic strains by carrying genes that express various virulence determinants used for attachment such as fimbriae (F4, F5, F6, F18 or F41) and enterotoxins (heat-stable toxin [ST] and heat-labile toxin [LT]) for induction of diarrhea (31). Among the fimbrial adhesins, $F4^+$ ETEC strains predominate worldwide and cause profuse watery diarrhea in pigs (18, 80, 117, 122, 166). Other diarrheagenic but non-ETEC strains in pigs include shiga-toxin producing *E. coli* (STEC), a subset of EHEC, harboring *stx2v* genes encoding Stx2e, a variant of the

shiga-like toxin (Stx2) causing edema disease (107). Some EPEC strains isolated from humans and pigs cause a characteristic attaching and effacement (A/E) lesion and the genes encoding the proteins involved in the development of A/E lesion are located in a pathogenicity island called locus of enterocyte effacement (LEE) (37, 93, 109, 116, 120). An EAEC group with a characteristic stacked aggregative adherence (AA) to HEp-2 cells *in vitro* and *in vivo* has been described in humans and pigs (67, 124, 136, 153). EAST1 was first recognized in EAEC of human origin. Later studies demonstrated that *E. coli* isolates from diarrheic and non-diarrheic farm animals also widely carry the *astA* gene encoding EAST1, but its role in development of diarrhea in animals is still unknown (161). In recent years, another group of *E. coli,* designated as DAEC, isolated from human infantile diarrhea case has been shown to adhere diffusely to HeLa and Hep2 cells and to be negative for LA or A/E attributes (71, 160). Strains of DAEC express non-fimbrial adhesins such as, CF16K (71), a 53-kDa adhesin (176), TibA (139) and AIDA-I (4). AIDA-I, a non fimbrial adhesin, belongs to the autotransporter protein family, first detected in human infantile diarrrheagenic *E. coli* (4-7, 9). Recently, the gene for AIDA-I was also detected among *E. coli* isolates from pre-weaning and post-weaning diarrhea cases of pigs (59, 92, 112, 115). Fang et al. (2005) recently characterized AIDA-I adhesin from *E. coli* strains isolated from pigs with diarrhea (46), and we have demonstrated that AIDA-I mediates aggregation and intestinal colonization through biofilm formation (submitted for publication-chapter 3).

Reports that some of the porcine diarrheagenic *E. coli* isolates were positive for various enterotoxins but negative for classical fimbria (F4, F5, F6, F18 and F41), led to speculation that as yet unknown adhesins might exist in diarrheagenic *E. coli* (16, 18,

33, 113, 122). Further studies have shown that a relatively high percentage (7-25%) of porcine ETEC strains are positive for *aidA* gene encoding AIDA-I adhesin and negative for genes encoding the classical fimbriae: F4, F5, F6, and F41 (59, 92, 113, 115) lending support for the existence of non-fimbrial adhesins among these non-fimbrial diarrheagenic *E. coli*. Little information is yet available on the prevalence and clinical significance of AIDA-I factor and its associated virotypes in diarheagenic *E. coli* cases in pigs.

In light of existence of i) the various non-fimbrial adhesins which can mediate the bacterial attachment and colonization resulting in the development of *E. coli* diarrhea in human and animals (4, 113, 124, 139), and ii) of speculation on the existence of possible reservoir for AIDA-I system in pigs (115), the present study was undertaken to asses the prevalence and clinical significance of AIDA-I associated virotypes among F4-negative *E. coli* strains isolated from problematic cases of diarrhea in pigs from commercial swine herds of western Canadian prairie provinces.

The objective of the present study was achieved by determining:

i) prevalence of AIDA-I$^+$ *E. coli* through screening for *aidA* gene among 110 F4-negative *E. coli* strains isolated from cases of diarrhea in pigs from the prairie provinces of Western Canada;

ii) clinical significance of AIDA-I associated virotype in *E. coli* by analyzing the data obtained through survey questionnaires from swine practitioners;

iii) association of AIDA-I adhesin with other virulence factors by testing of related genes among *E. coli* isolates from cases for which the questionnaire were completed.

4.2 - Materials and Methods

4.2.1 - Collection of clinical data

A total of 110 *E. coli,* isolated from problematic cases of F4-negative *E. coli* diarrhea in pigs which tested negative for F4 fimbria by standard slide agglutination test, were used in the present study. These cases originated from commercial swine herds located in the Western Canadian prairie provinces: Saskatchewan, Manitoba and Alberta, and were submitted to Gallant Custom Laboratories, Ontario, and Prairie Diagnostic Services Inc., Saskatoon, Saskatchewan between 1999-2005. The clinical data for these isolates were obtained by contacting the practitioners and asking them to fill out a questionnaire. The information obtained for each isolate through the questionnaires included: description of the clinical problem associated with the sample (diarrhea/poor-doer), and clinical problem of diarrhea at the farm level (persistent/recurrent/sporadic/at single-point of time); type of diagnostic investigations (necropsy, bacteriology, virology, parasitology, PCR, typing of pathogen) requested by the practitioner and the most beneficial approach (antibiotics/autogenous vaccines/others) to manage the said clinical condition; type of farming operation; type of sample (pooled from more than one pig or individual pig); age of the pig(s) sampled, morbidity and mortality associated with the clinical condition; type of diarrhea (profuse watery/semi-solid/pasty/bloody/mucoid) observed, and duration of diarrhea in those pigs that recovered.

4.2.2 - Bacterial strains and culture conditions

All the 110 *E. coli* strains isolated from problematic cases of diarrhea in pigs and the reference control *E. coli* strains (Table 4.1) for multiplex PCR were cultured on sheep blood agar plates at 37°C for 18h.

Table 4.1 - Reference *E. coli* strains used as controls in this study

Bacterial strain	Serotype	Target DNA	Source
P81-603A	O9:K-	STa, STb, F6	Dr. J. M. Fairbrother, University of Montreal
P88-1199	O139:K82	F18, AIDA-I	
P97-2554B	O149:K91	LT, F4, EAST1	
B44S	O9:K30	F5	
STJ348	O157:H7	EAE, VT2	
BD142	un known	F41	

4.2.3 - PCR primers. The PCR primers were chosen for each of the target gene from published sequences (113). The EC1 primers, specific for universal *E. coli* gene (*uspA*), were used as previously described (15) as the internal control for specific detection of *E. coli*. The sets of primers (Table 4.2) selected had the similar melting temperature and the resultant products were distinguishable by their size on agarose gels. Primers for EC1, F4, F5, F6, F18, F41, EAE, LT, STa, STb, EAST1, Stx2e and AIDA-I will amplify DNA fragments of sizes 884, 790, 431, 601, 275, 450, 171, 327, 510, 125, 377, 333 and 585 base pairs, respectively.

Table 4.2 - Characteristics of oligonucleotide sequences used in PCR and size of the amplified fragment (bp)

Target Gene	Primer	Oligonucleotide sequence (5' → 3')	Amplicon size (bp)	Reference
Fimbrial adhesins:				
fae	K88-F(F4)	ATCGGTGGTAGTATCACTGC	601	(122)
	K88-R (F4)	AACCTGCGACGTCAACAAGA		
fan	K99-F (F5)	TGGGACTACCAATGCTTCTG	450	(122)
	K99-R (F5)	TATCCACCATTAGACGGAGC		
fas	F6-F(987P)	TCTGCTCTTAAAGCTACTGG	333	(113)
	F6-R(987P)	AACTCCACCGTTTGTATCAG		
fedA	F18-F	GTGAAAAGACTAGTGTTTATTTC	510	(69)
	F18-R	CTTGTAAGTAACCGCGTAAGC		
F41	F41-F	GAGGGACTTTCATCTTTTAG	31	(122)
	F41-R	AGTCCATTCCATTTATAGGC		
Toxins:				
estI	STa-F	TCCCCTCTTTTAGTCAGTCAACTG	171	(113)
	STa-R	GCACAGGCAGGATTACAACAAAGT		
estII	STb-F	GCAATAAGGTTGAGGTGAT	368	(89)
	STb-R	GCCTGCAGTGAGAAATGGAC		
eltI	LT-F	TTACGGCGTTACTATCCTCTCTA	275	(51)
	LT-R	GGTCTCGGTCAGATATGTGATTC		
astA	East1-F	TCGGATGCCATCAACACAGT	125	(128)
	East1-R	GTCGCGAGTGACGGCTTTGTAG		
Stx2v	Stx2-F	CTATATCTGCGCCGGGTCTG	327	(172)
	Stx2-R	AGACGAAGATGGTCAAAACG		
Non-fimbrial Adhesins:				
eaeA	EAE-F	CATTATGGAACGGCAGAGGT	790	(3)
	EAE-R	ATCTTCTGCGTACTGCGTTCA		
aidA	Aida1-F	ACAGTATCATATGGAGCCA	585	(4)
	Aida1-R	TGTGCGCCAGAACTATTA		
***E. coli* universal gene:**				
uspA	EC1-F	CCGATACGCTGCCAATCAGT	884	(15)
	EC1-R	ACGCAGACCGTAAGGGGCCAGAT		

4.2.4 - Bacterial DNA extraction:

Escherichia coli DNA was extracted from 110 isolates using a standard phenol extraction technique (126). Briefly, four to six colonies were added to 400µl of lysis buffer [4 M GuSCN, 25 mM sodium citrate (pH 8.0), 0.5% sarcosyl, 0.1 M βME], vortexed for 10 s. Following addition of 200 µl of phenol-chloroform mixture (1:1 v/v, pH 8.0), the whole mixture was vortexed for 30 s and incubated for 10 min at -20˚C. The aqueous phase was removed into a clean tube after centrifuging at 15,000 x g for 5 min at -4˚C. 200 µl of phenol-chloroform mixture was added the aqueous phase, and the resulting mixture vortexed for 30 s. The aqueous phase was again removed into a clean tube after centrifuging at 15000 x g for 5 min at -4˚C, then twice volume of salt saturated 95% ethanol added to the aqueous phase, and the whole mixture was incubated over night at -20˚C. DNA was recovered by centrifuging at 15000 x g for 15 min at -4˚C. The DNA was washed with 80% ethanol to remove the excess salt, dried under vacuum for 5-10 min and then the DNA pellet was dissolved in 40 µl of sterile purified water. Bacterial strains used as controls during extraction include: AIDA-I^+ *E. coli* (PD20) as positive control, AIDA-I^- *E. coli* (PD71) and *Pseudomonas aeroginosa* were used as negative controls.

4.2.5 - PCR detection of *aidA* gene

Multiplex PCR (M-PCR) was performed initially to screen all the 110 *E. coli* strains for *uspA* and *aidA* genes using a set of primers EC1 and AIDA-I, respectively, in a total volume of 50 µl reaction mixture containing the following components: 2 µl of bacterial DNA, 30 µl of water, 5 µl of 10x PCR buffer, 4 µl of 25 mM $MgCl_2$, 0.5 µl of

25 mM deoxyribonucleotide triphosphate (dNTP), 2 µl of each primer (20 pM) and 0.5 µl of Taq polymerase (5 U/µl). EC1 was used as *E. coli* positive internal control as previously described (121). Other PCR positive controls included DNA extracted from *E. coli* containing gene of interest. Water were used as negative PCR reaction controls, respectively.

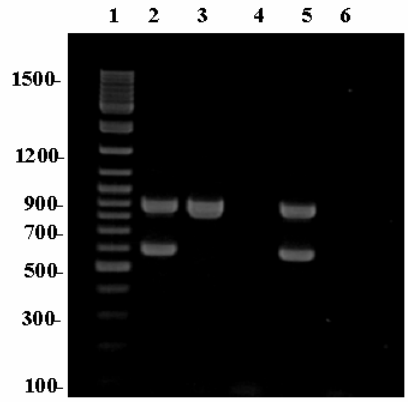


Fig. 4.1 – Agarose gel electrophoresis of PCR-amplified *E. coli* control DNA products. lane 1, 100 base pair DNA ladder; lane 2, AIDA-I$^+$ *E. coli* [uspA (884bp) and aidA (585)]; lane 3, AIDA-I negative PD71 *E. coli* [uspA (884bp)]; lane 4, *Pseudomonas aeroginosa* (negative control); lane 5, AIDA-I positive reference control DNA [uspA (884bp), AIDA-I (585bp)]; lane 6, negative control (water). (Lanes 2, 3 and 4 are extraction controls, and 5 and 6 are PCR controls).

4.2.6 - PCR detection of fimbrial/non-fimbrial adhesins and toxin genes

The first step in this study was PCR detection using DNA from reference *E. coli* strains using a set of primers corresponding to different genes in six different multiplex PCR reactions (Fig. 4.2). This method was successfully applied to screen for fimbrial/non-fimbrial adhesins, toxins and *uspA* gene markers (Table 4.2) among 35 non-F4$^+$ *E. coli* strains associated with clinical information, and in an additional 4 AIDA-I$^+$ *E. coli* isolates. Briefly, a panel of primers (Table 4.3) in a total volume of 50 µl containing the following components: 2 µl of bacterial DNA, 26 µl of water, 5 µl of 10x PCR buffer, 4 µl of 25 mM $MgCl_2$, 0.5 µl of 25 mM dNTP, 2 µl of each primer (20

pM) and 0.5 µl of Taq polymerase (5 U/µl). EC1 primer used to detect universal gene (*uspA*) of *E. coli* (121), and the DNA extracted from reference *E. coli* control strains and water were used as positive and negative PCR reaction controls, respectively.

PCR amplification was performed in four steps: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s; annealing at 60 °C for 30 s; extension at 72 °C for 30 s and the final extension for 10 min at 72 °C.

10 µl of the PCR reaction product was mixed with 3 µl of loading buffer and the mixture then applied and separated on 1.25% agarose gel by electrophoresis. The amplified and separated DNA was stained with 0.1% ethidium bromide and visualized under UV light exposure.

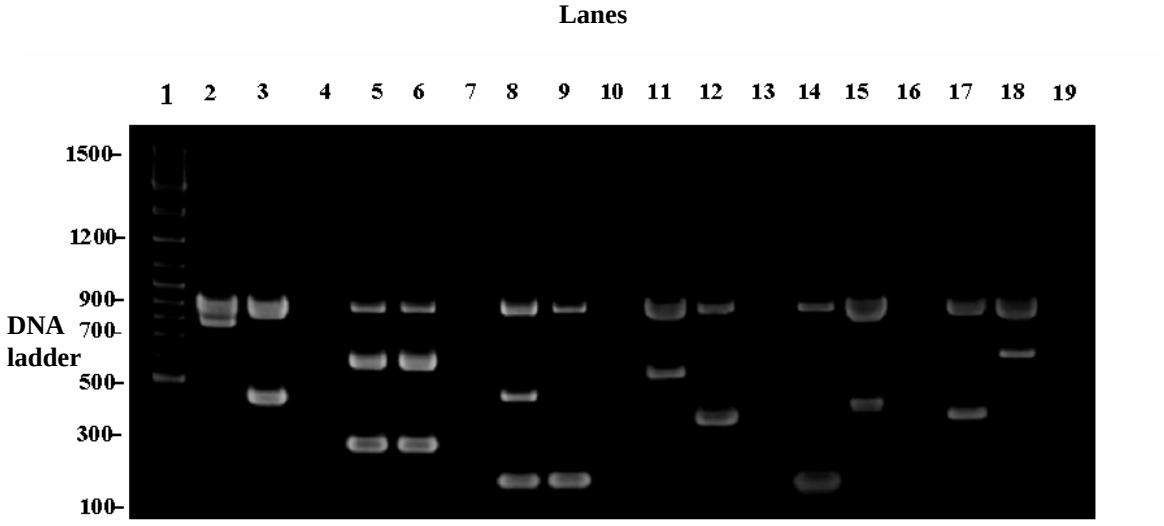


Figure 4.2 - Agarose gel electrophoresis of multiplex PCR-amplified *E.coli* reference control DNA products. lane 1, 100-base pair DNA ladder; lane 2, uspA (884bp), EAE (790bp); lane 3, uspA (884bp), F41 (431bp); lane 5 and 6, uspA(884bp), F4 (601bp), LT (275bp); lane 8, uspA (884bp), F5 (450bp), STa (171bp); lane 9, uspA (884bp) STa (171bp); lane 11, uspA(884bp), F18 (510bp); lane 12, uspA (884bp), Stx2e (327bp); lane 14, uspA (884bp), EAST1 (125bp); lane 15, uspA (884bp), STb (368bp); lane 17, uspA (884bp), F6 (333bp); lane 18, uspA (884bp), AIDA-I (585bp). Lanes 4, 7, 10, 13, 16, 19 Water used as negative control.

Table 4. 3 - Primer combinations used in each panel of multiplex PCR

Panel	Primer combination
1.	EC1 + EAE + F41
2.	EC1 + F4 + LT
3.	EC1 + F5 + STa
4.	EC1 + VT2 + F18
5.	EC1 + EAST1 + STb
6.	EC1 + F6 + AIDA

4.2.7 - Data management and statistical analysis

Clinical and PCR data were stored in a data base (Microsoft Access® 2000). The clinical data were analyzed by comparing the only AIDA-I^+ and AIDA-I^- cases without any other pathogens identified, through chi-square analysis with Epi-info 3.3.2 (27).

4.3 - Results

4.3.1 - Detection of *aidA* gene

The primer specific for AIDA-I yielded a PCR product of expected size in positive controls (Fig. 4.1) and in 9 out of 110 isolates (8.2%), while the primer specific for EC1 (positive internal control) yielded a PCR product of expected size in all the 110 *E. coli* field isolates. The 9 AIDA-I^+ isolates (5 associated with clinical data, and 4 without clinical data) were further tested by M-PCR for the expression of other virulence factors, which are summarized in the table 4.4.

Table 4.4 - Association of AIDA-I with other virulence factors among 9 AIDA-I^+ *E. coli*

Adhesin	STb/EAST1	F18/STb/EAST1	EAST1/F18	Total
AIDA-I	7	1	1	9

4.3.2 - Detection of fimbrial/non-fimbrial adhesins and toxin genes

Thirty-nine *E. coli* isolates (35 out of 110 isolates from cases for which clinical data were available and 4 additional AIDA-I^{+} isolates for which clinical information unavailable) were tested by M-PCR for various virulence factors. The results are summarized in the tables 4.5 and 4.4.

Table 4.5 - Mutual relationship between fimbrial and non-fimbrial adhesins with toxin genes detected by multiplex PCR in 35 *E.coli* strains isolated from pigs.

Adhesin	No. of strains	EAST1 STb	EAST1	EAST1 STb STa Stx2e	EAST1 STa Stx2e	STa	STb	Stx2e	Toxin negative
F4	0	0	0	0	0	0	0	0	0
F5	0	0	0	0	0	0	0	0	0
F6	1	0	0	0	0	1	0	0	0
F18	1	0	0	1	0	0	0	0	0
F41	0	0	0	0	0	0	0	0	0
AIDA-I	5	5	0	0	0	0	0	0	0
EAE	2	0	1	0	0	0	0	0	1
Adhesin negative	26	1	8	0	1	0	1	1	14
Total	35								

Table 4.6 - Clinical information and various virulence factors associated with 35 *E. coli* isolates of 18 diarrhea cases and co-pathogens

Case #	Sample submitted	Isolate #	Adhesins							Toxins					Age (days)	Diarrhea	Co-infection	Morbidity (%)	Mortality (%)
			F4	F5	F6	F18	F41	EAE	AIDA-I	STa	STb	LT	Stx2e	EAST1					
1	ind	1	-	-	-	-	-	-	-	-	-	-	-	-	21-60	l, s	mixed *E. coli*	0-5	5-10
		2	-	-	-	+	-	-	-	+	+	-	+	+					
		3	-	-	-	-	-	-	+	-	+	-	-	+					
2	ind	4	-	-	-	-	-	-	-	-	-	-	-	-	21-60	l, sp	$F4^+$ *E. coli*	5-10	1-25
3	ind	5	-	-	-	-	-	+	-	-	-	-	-	-	21-60	l, s	*Salmonella typhimurium*	0-5	5-10
4	ind	6	-	-	-	-	-	+	-	-	-	-	-	+	0-7	l, ps	*Clostridium perfringens*	25-50	1-5
		7	-	-	+	-	-	-	-	+	-	-	-	-					
5	ind	8	-	-	-	-	-	-	-	-	-	-	-	-	0-7	l, ps	-	5-10	1-5
6	ind	9	-	-	-	-	-	-	-	-	+	-	-	+	8-14	pw, ps	-	unknown	unknown
7	ind	10	-	-	-	-	-	-	-	-	-	-	-	-	0-7	pw, p, ps	*Clostridium perfringens*	10-25	1-5
8	ind	11	-	-	-	-	-	-	-	-	-	-	-	-	0-7	pw, r	-	25-50	1-5
9	ind	12	-	-	-	-	-	-	-	-	-	-	-	+	0-7	pw, sp	-	0-5	10-25
10	pool	13	-	-	-	-	-	-	+	-	+	-	-	+	21-60	pw, sp	*Salmonella typhimurium*, $F4^+$ *E. coli* mixed *E. coli*	5-10	50-75
		14	-	-	-	-	-	-	-	-	-	-	-	+					
		15	-	-	-	-	-	-	+	-	+	-	-	+					
		16	-	-	-	-	-	-	+	-	+	-	-	+					

(continued)

Table 4.6 (continued)

Case #	Sample submitted	Isolate #	Adhesins							Toxins					Age (days)	Diarrhea	Co-infection	Morbidity	Mortality
			F4	F5	F6	F18	F41	EAE	AIDA-I	STa	STb	LT	Stx2e	EAST1					
11	ind	17	-	-	-	-	-	-	-	-	-	-	-	-	0-7	pw, ps	*Clostridium perfringens*	10-25	1-5
		18	-	-	-	-	-	-	-	-	-	-	-	+					
12	ind	19	-	-	-	-	-	-	-	-	-	-	-	+	0-7	pw, ps	$F4^+$ *E. coli*	25-50	5-10
		20	-	-	-	-	-	-	-	-	-	-	-	+					
		21	-	-	-	-	-	-	-	-	-	-	-	+					
13	pool	22	-	-	-	-	-	-	+	-	+	-	-	+	21-60	l, sp	-	10-25	10-25
14	ind	23	-	-	-	-	-	-	-	-	-	-	-	-	0-7	l, r	mixed *E. coli*	5-10	1-5
		24	-	-	-	-	-	-	-	-	-	-	-	-					
		25	-	-	-	-	-	-	-	-	-	-	-	+					
		26	-	-	-	-	-	-	-	-	+	-	-	-					
		27	-	-	-	-	-	-	-	-	-	-	-	-					
15	ind	28	-	-	-	-	-	-	-	-	-	-	-	-	0-7	l, r	mixed *E. coli*	5-10	1-5
		29	-	-	-	-	-	-	-	-	-	-	-	-					
		30	-	-	-	-	-	-	-	-	-	-	-	+					
		31	-	-	-	-	-	-	-	-	-	-	+	+					
16	ind	32	-	-	-	-	-	-	-	-	-	-	-	-	8-14	l, r	mixed *E. coli*	5-10	unknown
		33	-	-	-	-	-	-	-	-	-	-	-	-					
17	ind	34	-	-	-	-	-	-	-	-	-	-	-	-	8-14	pw, l, ps	-	10-25	1-5
18	ind	35	-	-	-	-	-	-	-	-	-	+	-	-	8-14	pw, l, ps	-	10-25	1-5

ind=individual sample; pool=pooled sample; pw= profuse watery; l=loose; p=pasty; ps=persistent; r=recurrent; s=sporadic; sp=single point of time

4.3.3 - Assessment of clinical significance

Clinical information was available for 35 isolates (5 AIDA-I$^+$ and 30 AIDA-I$^-$) representing 18 diarrhea cases (Table 4.6). At the farm level, the clinical problem of diarrhea among these 18 cases was persistent (50%), recurrent (22.2%), at single point of time (16.6%) or sporadic (11.1%). These cases represented three different age (in days) groups: 0-7 d (n=9), 8-14 d (n=4) and 21-60 d (n=5), in farrow-finisher swine operations. There were 5 AIDA-I$^+$ *E. coli* isolates representing 3 (16.7%) clinical cases. All the AIDA-I$^+$ *E. coli* belonged to the 21-60 days old group while 87% of the AIDA-I$^-$ *E. coli* were from the pigs younger than 14 days old (p=0.009) (Table 4.7). No other clinical findings were significantly associated with the presence/absence of AIDA-I$^+$ *E. coli*.

Table 4.7 - Association of AIDA-I$^+$ *E. coli* with age among F4$^-$ *E. coli* diarrhea cases of pigs.

Age group (days)	AIDA-I$^+$ cases (%)	AIDA-I$^-$ cases (%)	p value
0-7	0	60	0.009
8-14	0	27	
15-20	0	0	
>21	100	13	

4.4 – Discussion

Among 110 F4-negative *E. coli* isolated from diarrhea cases, *aidA* gene was detected in 9 (8.2%) isolates. The clinical data was available for 35 isolates (30 AIDA-I^- and 5 AIDA-I^+ isolates) representing 18 clinical cases (15 AIDA-I^- and 3 AIDA-I^+ cases). All the AIDA-I^+ cases were associated with clinical cases of diarrhea in pigs 21-60 d of age, while the majority AIDA-I^- cases were associated with diarrhea in piglets during the first two weeks of life. The *aidA* gene was most commonly associated with the genes for STb and EAST1 (7 out of 9). Earlier, Ngeleka et al. (2003) also found AIDA-I association with STb and EAST1 among AIDA-I^+ *E. coli* isolates from diarrhea cases submitted for diagnosis and suggested that EAST1 is probably not an important marker in the diarrhea of pigs (113). These results suggest that the AIDA-I+STb virotype appears to be important in strains of porcine diarrheagenic *E. coli*.

The major concerns in the present study on the clinical significance of AIDA-I associated virotype were the bias introduced by a relatively low (32%) response to questionnaires by practitioners and the reliability of the clinical information obtained. The clinical information requested from the practioners however was very basic and due to lack of information on the clinical significance of AIDA-I^+ *E. coli* infections either in humans or in animals, it was still considered useful to analyze the clinical data for these 35 isolates. Despite the small number of cases (n=18), higher prevalence of AIDA-I^+ *E. coli* isolates from diarrhea cases in post-weaning versus pre-weaning cases was found in the present study, which is consistent with findings of Ha et al. (2003) (59). Overall prevalence of AIDA-I^+ *E. coli* isolates from diarrheic pigs in this study is lower than the prevalence (25-63%) previously reported in Germany (115) and Belgium (92) even

though these studies included also isolates from edema disease in addition to post-weaning diarrhea. On the other hand 8.2% prevalence of AIDA-I^+ isolate in this study is comparable with those reported previously in Canada (113) and Korea (59).

The absence of AIDA-I^+ *E. coli* in the pre-weaning age group found in this study in contrast to the previous reports of AIDA-I^+ *E. coli* isolates from pre-weaning diarrhea in piglets (60, 113, 115) could be due to the low number (n=25) of *E. coli* tested from the pre-weaning age group, and lack of clinical information for the other 4 AIDA-I^+ *E. coli* isolates.

The percentage (23.1%) of AIDA-I^+ *E. coli* among ETEC cases in the present study was consistent within the range (7-25%) as previously reported (59, 92, 113, 115). The lack of significant difference between AIDA-I^+ and AIDA-I^- cases with regard to other clinical data in the present study may be due to the small number of cases studied and.

The absence of genes for F5, F6 and F41 in AIDA-I^+ *E. coli* isolates in the present study is consistent with previous reports (59, 113). These findings suggest that AIDA-I, a non-fimbrial adhesin, is an important virulence factor of diarrheagenic *E. coli* in pigs lacking classical fimbriae.

Although there are reports of isolation of *E. coli* carrying the gene for EAST1 alone from cases of pre-weaning (18) and post-weaning (17) diarrhea of pigs, the relationship between the presence of EAST1 and disease has not been determined (161). In previous experimental studies, an *E. coli* isolate (PD71) carrying the gene for EAST1 alone did not produce diarrhea in colostrum deprived newborn pigs (113, 124). In the present study, except in one case in which bacteriology was the only diagnostic

procedure undertaken, those diarrhea cases associated with *E. coli* isolates expressing only EAST1 were also infected with other bacterial or protozoal pathogens or co-infected with *E. coli* isolates carrying other virulence factors. Accordingly, it is difficult to interpret the role of EAST1$^+$ *E. coli* in the pathogenesis of diarrhea. However, the association of EAST1 with AIDA-I$^+$ *E. coli* in the present and previous reports (59, 60, 115) warrants further studies to find out the EAST1 role in intestinal fluid secretion in the absence of other virulence factors.

Earlier reports found that fimbrial adhesins (F4, F5, F6, F41) are less frequently detected in *E. coli* isolated from cases of post-weaning diarrhea in pigs (65, 103, 122). In this study, from F4-negative *E. coli* isolates for which clinical data were available, only one isolate tested positive for F6 fimbria and none were positive for F5 and F41. Although the very low prevalence of F6 and absence of F5 and F41 could be due to low number of isolates investigated (this aspect of study was limited to only to those isolates for which clinical data were available), our results corroborate those from previous studies.

ETEC strains are often the sole cause of diarrheal disease in pigs less than a week age but mixed infections with other viral, bacterial and protozoal agents are common in older pigs (106). In our study, we also found mixed bacterial/protozoal infections among these diarrhea cases, which might explain why these became problematic cases.

The significance of mortality could not be analyzed due to co-infection with bacterial agents in all but for one of AIDA-I$^+$ cases for which the clinical information was available (Table 4.6). Based on the information from the questionnaires, the

veterinarians in the field were able to manage the majority of these problematic cases through antibiotic treatment combined with improvement in sanitation and environment. Since no single method or chemical completely eliminates biofilm-forming microorganisms, use of combination of methods (chemical and physical intervention) may have been beneficial in managing these problematic cases.

The current study identified *E. coli* isolates with genes for one or more toxins but without any virulence genes for known fimbrial/non-fimbrial adhesins. If no other pathogen was responsible for diarrhea in these cases, the absence of adhesins from *E. coli* isolated from diarrheic cases may be explained by loss of plasmids harboring the relavant adhesin genes during culture conditions (143) or more likely, that these strains may have unknown virulence determinants. This warrants further investigation.

In conclusion, based on the small sample size used, we found that all AIDA-I$^+$ isolates were associated with post-weaning diarrhea cases, and that 8.2% of F4-negative *E. coli* isolates from porcine diarrhea cases were positive for *aidA* gene, which in turn found to be commonly associated with the gene for STb. Accordingly, AIDA-I+STb may be an important virotype in the pathogenesis of porcine post-weaning diarrhea caused by AIDA-I$^+$ *E. coli*.

5 - General Discussion

Infectious diarrhea caused by *E. coli* is the most common and important disease of pre-weaning and post-weaning pigs worldwide, causing economic losses due to high morbidity and mortality (65, 152). Pathogenic *E. coli* express various fimbrial and non-fimbrial adhesins that allow the bacterium to attach to host and colonize the intestine. These bacteria elaborate various toxins (LT, ST and Stx) which alter the net intestinal secretion and absorption rate leading to diarrhea (109).

During the last few years there has been an increase in the number of *E. coli* isolates from enteric colibacillosis of pigs which have been negative for classical fimbriae (F4, F5, F6 and F41), but positive for the *aidA* gene encoding AIDA-I adhesin (112). AIDA-I, a non-fimbrial adhesin expressed by diarrheagenic *E. coli*, belongs to the autotransporter protein family. AIDA-I adhesin was first identified and characterized as a 100-kDa protein from *E. coli* (2787), isolated from human infantile diarrhea case and involved in the diffuse adherence phenotype demonstrated on various mammalian cells (4-7, 9). Further experimental studies of colostrum-deprived newborn pigs infected with AIDA-I$^+$ *E. coli* strains, resulted in induction of diarrhea in these pigs (113, 124). In a study by Prichard et al., histological examination at different levels of the small and large intestines in experimental AIDA-I$^+$ *E. coli* infection showed extensive bacterial colonization in large intestine, especially in the colon, rather than in the small intestine as anticipated, and electron microscopy of the colonic mucosa

showed bacteria stratified in the mucus layer suggestive of biofilm formation. Interestingly, in the same study, an AIDA-I$^+$ PD149 *E. coli* isolated from porcine diarrhea produced a stacked brick pattern of adherence on Hep-2 cells *in-vitro* suggesting the porcine AIDA-I$^+$ *E. coli* strains have mixed characteristics of ETEC, EAEC and DAEC (124). *In vitro* studies using human strains of AIDA-I$^+$ *E. coli* showed that AIDA-I adhesin recognizes an integral membrane glycoprotein as receptor on HeLa cells (82), and that AIDA-I expression dramatically enhanced biofilm formation on abiotic surfaces (115). Little was known, however, about the role of AIDA-I in the pathogenesis of diarrhea in either humans or pigs.

The research presented in this thesis investigated the role of AIDA-I adhesin in the pathogenesis of *E. coli* diarrhea in pigs in light of the relatively high number (7-25%) of AIDA-I$^+$ *E. coli* isolated from porcine diarrhea cases compared to human isolates (3.9%). This research had two broad objectives: 1) investigation of the contribution of AIDA-I to the pathogenicity of a porcine diarrheagenic *E. coli* and 2) determination of the prevalence and clinical significance of AIDA-I associated virotypes. The first objective was achieved by: (i) experimental infection of colostrum-deprived newborn pigs with AIDA-I positive wild type PD20, a complemented PD20C strains, and an AIDA-I negative mutant PD20M strain; and (ii) *in vitro* assessment of the role of AIDA-I adhesin in aggregation and biofilm formation. The second objective was achieved by determination of: i) prevalence of AIDA-I among F4-negative *E. coli* strains isolated from cases of diarrhea in pigs from the prairie provinces of Western Canada; ii) clinical significance of AIDA-I associated *E. coli* virotype by analyzing the data obtained through survey questionnaires from swine practitioners; and iii)

association of AIDA-I adhesin with other virulence factors of *E. coli* isolates from cases for which the questionnaires were completed.

In earlier pathogenesis studies, experimental infection of pigs using AIDA-I^+ *E. coli* strains and non pathogenic *E. coli* strains as control, pigs infected with AIDA-I^+ strains developed diarrhea, and extensive colonization by AIDA-I^+ *E. coli* occurred in the large intestine (113, 124). To assess the specific role of AIDA-I adhesin in the pathogenesis of diarrhea caused by AIDA-I^+ *E. coli*, further similar studies were done using genetically modified strains through manipulation of the *aidA* gene encoding AIDA-I adhesin. An AIDA-I^- mutant (PD20M) was derived by partial deletion of *aidA* gene from the wild strain (PD20). The full length *aidA* gene was reintroduced into mutant PD20M strain resulted in the AIDA-I^+ complemented strain (PD20C).

In the present research, the contribution of AIDA-I to the pathogenicity of AIDA-I^+ *E. coli* was evaluated by infecting the colostrum deprived newborn pigs as reported earlier (124), with PD20, PD20M, PD20C *E. coli* strains, and a non-pathogenic *E. coli* strain, PD71strain as negative control. The PD20 strain, but not PD20M, induced diarrhea in pigs between 15-19 h PI. Complementation of PD20M with full length *aidA* gene restored virulence function in PD20M as shown by induction of diarrhea in pigs infected with PD20C between 27-31 h PI. AIDA-I^+ *E. coli* strains, PD20 and PD20C colonized in large numbers, preferentially in the large intestine, especially in the caecum and proximal spiral colon as determined by histology, and by immunohistochemistry using an anti-AIDA-I antibody. The relatively weaker positive immunostaining of PD20C strain compared to PD20 could be due to lower AIDA-I expression by PD20C caused by stress that may occur due to gene manipulation.

Techniques such as microarray analysis or quantitative real-time PCR could have been used to investigate the level of *aidA* gene expression among PD20M and PD20C strains to substantiate the above stated supposition. Electron microscopy of the colon from pigs infected with AIDA-I$^+$ bacteria including PD20C showed the bacteria were distributed in a stratified pattern within the mucus immediately overlying the enterocytes and these bacteria were confirmed as AIDA-I$^+$ *E. coli* by immunogold EM using anti-AIDA-I IgG antibody. The results of histology and electron microscopy in the present study are in agreement with other studies on AIDA-I positive field isolates (124). Bacterial adhesins are able to interact with domains of glycoprotein mucins which can act as receptors. In the present study AIDA-I$^+$ *E. coli*, in contrast to its AIDA-I$^-$ mutant, may have recognized receptors within the mucus of large intestine facilitating their attachment, auto-aggregation and biofilm formation, which allowed their persistent colonization. This might have impaired water and nutrient absorption and potentially resulted in pasty diarrhea in the infected pigs. Even though the mechanism of diarrhea observed in piglets infected with AIDA-I$^+$ *E. coli* was not determined, these results suggest that AIDA-I adhesin expressed by porcine AIDA-I$^+$ *E. coli* played an important role in the pathogenesis of diarrhea.

AIDA-I, a 100-kDa non-fimbrial adhesin, belongs to the autotransporter protein family, in which the translocation mechanism and adhesive properties are located towards the C- and N-terminus portion of the precursor molecule, respectively. Glycosylation of AIDA-I adhesin is essential for its binding to eukaryotic cells (8). In addition, this adhesin is capable of aggregation by self-recognition and it is efficient initiator of biofilm formation *in vitro*. The AIDA-I adhesin isolated from porcine

diarrheagenic PD20 *E. coli* have similar molecular weight and share 78% amino acid homology with AIDA-I isolated from human diarrheagenic *E. coli* (46). The current *in vitro* findings with PD20 also suggested that AIDA-I adhesin from procine AIDA-I^+ *E. coli* has functional homology with AIDA-I adhesin of human AIDA-I^+ *E. coli*. The lack of aggregation, adhesion to HeLa cells and significant reduction in biofilm formation *in vitro* by PD20M in contrast to its parent (PD20) or complemented (PD20C) strains was considered to be lack of expression of functionally mature AIDA-I adhesin caused by partial deletion of *aidA* gene. The *in vitro* results in the present study provide additional functional similarities between the porcine and human AIDA-I adhesin and are in agreement with those of Sherbrook et al. (138) and Pritchard et. al (124). In the present study, it was clearly demonstrated by immunogold EM that AIDA-I adhesin was located on the surface of AIDA-I^+ *E. coli*. Although the mutant strain was confirmed negative for AIDA-I adhesin by immunodot and Western blotting, the weak staining on immunohistochemistry and low labeling on immunogold EM observed with mutant strain may be due to possible non-specific binding of polyclonal antibodies to bacterial surface membrane associated with the low dilutions of antiserum used. In addition, unequivocal difference in bacterial attachment to HeLa cells, auto-aggregation and biofilm formation *in vitro* between AIDA-I^+ and AIDA-I^- strains demonstrated that the functionality of AIDA-I adhesin was annihilated completely by partial deletion of *aidA* gene in PD20M, even though it was weakly stained with IHC and immunogold with polyclonal anti-AIDA-I antibodies.

The F4 fimbria is the most important and most common attaching virulence factor in *E. coli* isolated from pre-weaning and post-weaning diarrhea in pigs. In the

present study, 110 *E. coli* isolates, negative for F4 fimbrial antigens by slide agglutination test were selected to determine the prevalence and clinical significance of AIDA-I adhesin. Survey questionnaires were sent out to swine veterinarians to obtain specific clinical information for the 110 *E. coli* isolates. All 110 isolates were screened by multiplex PCR for the *aidA* gene encoding AIDA-I adhesin along with the *E. coli* specific universal *uspA* gene as an internal positive control. Nine isolates (8.2%) were positive for *aidA* gene and all the 110 isolates for *uspA* gene.

A major concern in the present study was a poor response by the practitioners to the survey questionnaires and reliability of the clinical information obtained from them. Only 35/110 (32%) questionnaires were returned and they accounted for 18 diarrhea cases. It was still considered however useful to analyze the clinical data for these 35 isolates because the clinical information requested was simple and there is lack of information on the clinical significance of AIDA-I$^+$ *E. coli* infections either in humans or in animals. Multiplex PCR was designed to detect two virulence genes simultaneously along with *E. coli* specific *uspA* gene, and used to screen for virulence genes present in all the 35 isolates (30 AIDA$^-$ and 5 AIDA$^+$ isolates) for which clinical data were obtained. In addition, we also screened four other AIDA-I$^+$ isolates for which clinical data were not obtained. It was found that the *aidA* gene was most commonly (7/9) associated with the genes encoding EAST1, STb and none of the AIDA-I isolates harbored genes for F4, F5, F6 and F41 fimbriae. AIDA-I was the main adhesin detected in this study among 35 non-F4 *E. coli* isolates and all the AIDA-I$^+$ *E. coli* isolates were associated with post-weaning diarrhea cases. This is consistent with previous reports (59, 115). It would be interesting, therefore, to examine the role of AIDA-I in the

pathogenesis of post-weaning diarrhea in pigs, in addition to its role in the pathogenesis of diarrhea in neonatal pigs demonstrated by our experimental infection. The significance of mortality among AIDA-I$^+$ and AIDA-I$^-$ *E. coli* cases could not be analyzed due to association of most of these cases with co-infections with other bacterial/parasitic agents. However, contribution of persistent infection with biofilm formation to mortality by AIDA-I$^+$ *E. coli* is not known and it warrants further investigation. The lack of significance between AIDA-I$^+$ and AIDA-I$^-$ virotypes with regard to other clinical data in the present study could be due to the small number of cases analyzed and multiple infections among some of these cases. Further investigation is required to clarify the clinical significance of AIDA-I$^+$ *E. coli* in porcine diarrhea.

Despite the small number of clinical cases analyzed, a significant association of AIDA-I$^+$ *E. coli* with post-weaning diarrhea cases was noticed. The absence of AIDA-I$^+$ *E. coli* in pre-weaning age group in the present study may be due to lack of clinical information about the remaining four AIDA-I$^+$ *E. coli* isolates. Relatively low prevalence or absence of F5, F6, F18 and F41 positive isolates in the present study could be due to low number of isolates investigated. Further studies can be done to identify the virulence genes in the remaining 75 *E. coli* isolates, and obtain the clinical information associated with them.

The diarrhea problem in all 18 cases with clinical follow-up was reported to be either persistent, sporadic or at single point of time. The majority of these diarrhea cases were managed by treating the animals with antibiotics along with sanitation.

In Chapter 3, biofilm formation by AIDA-I$^+$ *E. coli* was demonstrated on abiotic surface and biotic colonic mucosal surface. Biofilm formation by pathogenic bacteria

can be a strategy to escape host defense mechanism and a hostile host-environment especially the intestines. Biofilm formation by pathogenic bacteria has been recognized in various chronic bacterial infections in humans and treating these infections can be a challenge due to their persistence and resistance to antibiotics. It was also suggested that horizontal transfer of genetic material may occur rapidly in a biofilm, making it fertile environment for emergence of new pathogens through acquisition of the antibiotic resistance, virulence factors, and environmental survival capabilities (170). Researchers have identified specific determinants that play a role in various stages of biofilm formation by *E. coli* (158). Biofilm formation by *Escherichia coli* has been associated with complex interaction between different bacterial surface molecules and structures. These specific determinants when expressed play a role in the biofilm formation. In the present study, it was also proposed that biofilm formation allows AIDA-I^+ *E. coli,* in contrast to AIDA-I^- mutant *E. coli* strain, to persistently colonize the large intestine leading to impaired water and nutrient absorption, thus resulting in the development of pasty diarrhea in the infected pigs. From the field clinical data, it was found that the AIDA-I^+ cases had profuse watery to loose/semi-solid feces, which is different from pasty diarrhea produced by AIDA-I^+ strains under experimental conditions. This could be due to the fact that these problematic cases of post-weaning diarrhea were found to have mixed bacterial infections with other bacterial/protozoal agents or *E. coli* strains carrying different genes for adhesins and/or toxins.

Various strains of *E. coli* harboring different genes for virulence factors in a single diarrhea case can be potentially explained by coordinated involvement of these strains in biofilm formation. It has been recognized that multispecies biofilm

development depends on the bacterial species involved, the surface composition, and the sequence of attachment (97, 170). In an *in vitro* study, using Hep-2 cells where an *E. coli* biofilm was first formed, followed by challenge with *Salmonella enterica* serovar *typhimurium*, resulted in significant biofilm formation by that Salmonella species (43). In the present study, a case with persistent diarrhea and high mortality was associated with both AIDA-I$^+$ *E. coli* F4-positive *E. coli* and *Salmonella typhimurium*. This might suggest that multispecies biofilm formation *in vivo* warrants further investigation.

Studying the prevalence of virulence genes that are normally expressed as virulence factors by pathogenic bacteria will help us in understanding the common themes of virulence mechanism in bacterial pathogenicity. The findings from the present study suggest that AIDA-I/STb is an important virotype involved in the pathogenesis of diarrhea.

As determined earlier, intestinal attachment and colonization through fimbrial/non-fimbrial adhesins are the initial steps in the pathogenesis of diarrhea caused by diarrheagenic *E. coli*. This research has demonstrated the role of AIDA-I adhesin in diffuse adherence to HeLa cells, autoaggregation and biofilm formation *in vitro*. In addition, AIDA-I adhesin is necessary for *in vivo* colonization in the large intestine with biofilm formation and for induction of diarrhea by PD20 (AIDA-I$^+$/STb$^+$) wild *E. coli* strain, in contrast to its PD20M (AIDA-I$^-$/STb$^+$) mutant strain. When the PD20M was complemented with full length *aidA* gene, it regained all the *in vitro* and *in vivo* attributes of the parent wild strain, including the capacity to induce diarrhea in newborn pigs.

The prevalence study showed that 8.2% percentage of F4 negative *E. coli* isolated from problematic diarrhea cases carry the gene for AIDA-I adhesin and that AIDA-I$^+$ *E. coli* is significantly associated with post-weaning diarrhea cases.

In conclusion, we have shown that AIDA-I, a non-fimbrial adhesin, is an essential virulence determinant expressed by *E. coli*. This information may be useful in understanding the pathogenesis AIDA-I$^+$ *E. coli* diarrhea in pigs and for further development of preventive strategies.

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