The binding mechanism of Enterotoxigenic *Escherichia coli* (ETEC) to swine intestinal epithelia

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the requirements of the degree of Master of Science

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Colibacillosis (diarrhea in piglets caused by *Escherichia coli*) is the leading killer to piglets. Pathogenic *E. coli* responsible for this onset is referred as diarrheagenic *E. coli*. In six defined groups of diarrheagenic *E. coli*, enterotoxigenic *E. coli* (ETEC) is an important cause of post weaning diarrhea (PWD) in pigs. ETEC was first recognized by their ability to secrete two defined groups of enterotoxins: heat-stable toxin (STs) and heat-labile enterotoxin (LTs). F4 and F18 fimbriae, the most prevalent adhesins are commonly expressed on porcine ETEC to facilitate bacterial colonization. In addition to enterotoxins and fimbrial adhesins, functions of many other colonization factors are constantly identified.

In 2009, EtpA, an exoprotein, was identified in human ETEC and confirmed to interact with a conserved region of flagella. This interaction appears critical for ETEC adherence to human intestinal epithelia. Our current study demonstrated that EtpA gene was also present in some wildtype porcine ETECs. However, a subsequent correlation analysis showed ETEC adherence ability cannot be solely
attributed to a single pathogenic feature, such as the expression of enterotoxins, fimbrial adhesins or EtpA protein. It appears that pathogenesis of ETEC is more complicated than previously thought. To study the pathogenesis of ETEC adherence, this project firstly focused on the recognition between cellular glycoconjugates and bacterial adhesins that accounts for the initiation of bacterial adherence. The removal of cellular sialic acid from porcine intestinal epithelia confers a favoured environment for ETEC to enhance binding. Monosaccharides were subsequently utilized as competitive inhibitors in order to determine the recognition sites on ETEC adhesin. Our result suggests that subterminal N-acetylgalactosamine (GalNAc) seems responsible for the recognition of F4ac fimbriae. Also, in all ETECs responsive to monosaccharide treatments, 100% of Neu5Ac saturated ETECs, as well as 80% of Mannose saturated ETECs showed enhanced binding ability to desialylated IPEC-J2 cell monolayers. An earlier proposed model of conformation change in bacterial adhesin was adopted to explain this phenomenon. It is suggested that there is an allosteric effect on ETEC adhesin after first binding where an essential recognition of free Neu5Ac initiates bacterial adherence, and certain sugar, such as fucose or mannose can possibly synergistically function with Neu5Ac on adhesin and contribute to better binding.
La colibacillose (diarrhée causée par *Escherichia coli* chez le porcelet) constitue la principale cause de décès chez le porcelet. La souche pathogène d’*E.coli* responsable de ce phénomène est connue sous le nom d’*E.coli* diarrheagénique. Parmi les six différents groupes identifiés d’*E.coli* diarrheagéniques, la variété *E. coli* entérotoxigénique (ETEC) est la cause majeure des diarrhées survenant après le sevrage chez le porc. ETEC a initialement été identifiée pour sa capacité à sécréter 2 groupes distincts d’entérotoxines : l’entérotoxine ST (*heat-stable*) et l’entérotoxine LT (*heat-labile*). Il a été démontré que les formes F4 et F18 des fimbriae, adhésines les plus communes, sont communément exprimées dans les ETEC de porcs facilitant ainsi la colonisation bactérienne. En plus des entérotoxines et des adhésines, de nombreux autres facteurs de colonisation sont constamment identifiés.

En 2009, EtpA, une exoprotéine, a été identifiée dans des ETECs chez l’humain et son interaction avec la région conservée des flagelles a été confirmée.
Cette interaction est primordiale pour l’adhérence des ETECs sur l’épithélium intestinal humain. Notre étude a montré que le gène EtpA est aussi présent dans les ETECs « *wildtype* » chez le porc. Une analyse de corrélation a révélé que la capacité d’adhérence des ETECs ne peut pas être uniquement attribuée à un seul trait pathogénique tel que l’expression d’entérotoxines, d’adhésines ou d’EptA. Il semble que la pathogénicité des ETECs est très complexe. Dans le but d’étudier les mécanismes d’adhérence des ETECs, ce projet s’est d’abord concentré sur la reconnaissance entre les glycoprotéines des cellules et les adhésines des bactéries, étape cruciale pour l’adhérence bactérienne. Dépriver les cellules de l’épithélium intestinal porcin d’acide sialique a ainsi permis d’augmenter la liaison des ETECs. Différents monosaccharides ont ensuite été utilisés en tant qu’inhibiteurs compétitifs dans le but de déterminer les sites que reconnaissent les adhésines des ETECs. Nos résultats suggèrent que le résidu subterminal N-acétylgalactosamine (GalNAc) semble être responsable de la reconnaissance de la forme F4ac des fimbriae. De plus, chez toutes les ETECs répondant aux traitements avec les monosaccharides, 100% des ETECs saturées en Neu5Ac ainsi que 80% des ETECs saturés en Mannose ont montré une augmentation de la capacité de liaison sur les monocouches cellulaires IPEC-J2 dépouvrues d’acide sialique. Un modèle
de changement de conformation des adhésines bactériennes a déjà été proposé et a été accepté pour expliquer ce phénomène. Il a été suggéré qu’un effet allostérique se produirait : après la liaison sur les adhésines des ETECs, où la reconnaissance des sites Neu5Ac libres est essentielle pour initier l’adhérence bactérienne, certains sucres, comme le fucose ou le mannose pourraient agir avec Neu5Ac sur les adhésines et contribuer ainsi à une meilleure liaison.
I am heartily thankful to my supervisor, Dr. Xin Zhao, whose encouragement, guidance and supports from the initial to the final level enabled me to accomplish this thesis. This thesis would not also have been done without many financial assistances from Rotary Club of Montreal, CDPQ (centre de développement du porc du Québec), and FAES (Faculty of Agriculture and Environmental sciences).

I would also like to thank many beloved laboratory colleagues who always selflessly share their knowledge and hands in the past two years. Special thanks to Baurhoo Bushansingh and Neerusha Baurhoo who bear me in mind at all times and keep me accompanied unconditionally. Lastly, the deepest gratitude is given to my parents who sacrificed many of their own dreams in expense of mine and never lost faith on me.

I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.
INTRODUCTION

Diarrhea is the frequent passing of loose or watery stools in humans and animals. It is a symptom of infections caused by bacterial, viral and parasitic organisms. In human, it means 3 or more passages of loose or liquid stools per day and it is the second leading cause of death in children under five years old, and is responsible for killing 1.5 million children every year (WHO, 2009). Diarrhea can last several days, and can deprive the body of water and salts that are necessary for survival. Most people die from diarrhea due to severe dehydration and fluid loss. In pigs, post-weaning diarrhea is responsible for major economic losses due to mortality, morbidity, decreased growth rate, and cost of medication. Before weaning, piglets are continuously exposed to immunoglobulin IgA from sow’s milk that confers passive immune protection. After weaning, external IgA is no longer available and therefore piglets are susceptible to infection. Among all pathogens, rotavirus and *Escherichia coli* (*E. coli*) are the two most common causes of diarrhea.

*E. coli* is the type of species of the genus Escherichia, which contains mostly motile gram-negative bacilli within the family Enterobacteriacea, commonly found in
the gut of humans and warm-blooded animals. Most strains of \textit{E. coli} are harmless and confined to the intestinal lumen. These commensal \textit{E.coli} strains rarely cause diseases except in immune-compromised hosts or where normal gastrointestinal barrier are breached. Other than commensal \textit{E.coli} strains, there are several highly adapted pathogenic \textit{E.coli} strains with acquired specific virulence attributes, which confer an increased ability to cause a spectrum of diseases. A group of pathogenic \textit{E.coli} that causes diarrheal diseases is referred as diarrheagenic \textit{E.coli} which includes enterotoxigenic \textit{E. coli} (ETEC), enteropathogenic \textit{E. coli} (EPEC), enterohemorrhagic \textit{E. coli} (EHEC), enteroinvasive \textit{E. coli} (EIEC), enteroaggregative \textit{E. coli} (EAEC), diffusely adherent \textit{E. coli} (DAEC) and perhaps others that are not yet well characterized.

EHEC, EPEC and ETEC are three major studied categories of diarrheagenic \textit{E. coli} that could be differentiated from each other on the basis of pathogenic features. In general, EHEC can produce toxins, known as verotoxins or Shiga-like toxins which cause bloody diarrhea (haemorrhagic colitis) and haemolytic uremic syndrome (HUS). It was firstly recognized as a public health problem in 1982, following an outbreak in the United States of America. \textit{E. coli} O157:H7 is the most important EHEC in relation to public health and recognized as a cause of hemorrhagic colitis.
Its pathogenic mechanism has been well investigated and the information related to
*E. coli* O157:H7 has led to development of the control and prevention methods for
EHEC. Preventive detection of *E. coli* O157:H7 has become an indicator for food
contamination in some countries (Hughes et al. 2008). Histopathology of EPEC
infections, known as “attaching and effacing” (A/E) lesions, shows that the bacteria
attach intimately to intestinal epithelial cells, causing striking cytoskeletal changes,
effacing the microvilli of the intestines. EPEC has been mostly linked to infant
diarrhea in the developing world and was the first pathotype of *E. coli* to be
described. Numerous advances in the understanding of the pathogenesis of EPEC
have been made since 1979 (Nataro and Kaper 1998, Kaper, 2004).

ETEC colonizes the surface of the small intestinal mucosa and is characterized
by elaborating at least one member of two defined groups of enterotoxins: LT and
ST, that are protein toxins released by microorganisms in the intestine. This
organism causes watery diarrhea which is an important cause of childhood diarrhea
and is also the main cause of diarrhea in travellers to developing countries. Its
wide-spectrum of infection also appears in warm blood animals. In pigs,
ETEC-induced outbreaks mostly occur in early-weaned piglets and these strains
express fimbrial intestinal adherence factors, such as F4 and F18, which are not
found in human ETEC strains, suggesting existence of different pathogenic mechanisms in these two hosts. In comparison with EPEC and EHEC, the pathogenic mechanisms of ETEC are less clear.

Bacterial adhesion is a primary step of bacterial colonization. It is mediated by a various type of adherence factors, such as fimbriae, enterotoxins and virulence proteins. These adherence factors, also called adhesins, allow bacterial pathogens or many non-pathogens to adhere with a high degree of specificity to tissues. They are specified proteins located on the surface of bacteria that bind to the glycoconjugates on host cell surface. The mechanisms for adhesion of ETEC to hosts appear still elusive. For example, fimbrial adhesins are not constitutively expressed but are stringently regulated on the molecular level (Hacker 1992). In addition, participation of enterotoxins in ETEC adherence has also complicated the studies toward ETEC pathogenesis. Moreover, recent research has unveiled that certain specialized proteins, other than defined bacterial adhesins, play rather determinant roles in bacterial colonization. For instance, a newly established exoprotein from ETEC, EtpA, was able to interact with a highly conserved region of flagellin and these interactions were critical for adherence and intestinal colonization (Roy et al. 2009). Apparently, many questions about the pathogenesis
of ETEC remain unclear. More research is needed before we can develop effective protective measures against ETEC.

Therefore, the objective of this project was to investigate how ETEC colonize porcine intestinal epithelia, in perspectives of the participation of carbohydrates on epithelial cell surface and the secreted protein, EtpA, from ETEC. The in-vitro experiments were designed to correlate levels of bacterial binding with biological features, such as serotypes, pathotypes and EtpA expression of ETECs. It was hoped that the results from this project might shed some lights on mechanism of ETEC binding to swine intestinal epithelial cells.

LITERATURE REVIEW

The meat industry is the largest sector in Canadian food processing industries, representing 10% of Canada's agri-food exports with annual sales of over $21.3 billion. As for pork production, the Canadian industry produces 14-15 million pigs a year and $2.77 billion of pork were exported to over 130 countries in 2010. The
productivity is directly related to the swine health which could be affected by
genetics, nutrition supplement, farm management and disease control (Canadian
Meat Council).

2-1 *Escherichia coli* in Pig Intestine

The intestine is an extremely complex organ and represents the body’s largest
surface area. The intestinal epithelium takes parts in the digestion and uptake of
nutrients. Its huge surface is constantly exposed to intestinal microflora and other
contents and therefore provides the most important entry for foreign antigens.

Bacteria are the main type of microorganisms present in the mammalian intestine,
although protozoa and fungi are also found. The population and diversity of bacteria
differ in different parts of the gastro-intestinal tract. The stomach and small intestine
have relatively low bacterial densities (10^3–10^6 organisms per gram or ml of luminal
contents in mice, consisting mainly of acid-tolerant lactobacilli and streptococci).

The distal portion of the small intestine, the ileum, is a transition zone with higher
bacterial densities (10^8 per gram) and species diversity, but the most dense
colonization is in the colon \((10^{10} - 10^{12} \text{ per gram})\), which hosts more than 400 bacterial species (Smith and Gorbach 1995; Oswald 2006).

*Escherichia coli* are the type of species of the genus *Escherichia*, which contain mostly motile gram-negative bacilli within the family *Enterobacteriacea*. They are predominant anaerobes of colonic flora and usually remain harmless. They are confined in the intestinal lumen except in an immuno-compromised host or when the gastrointestinal barrier is violated. By contrast, pathogenic *E. coli* strains are those with acquired specific virulence attributes, which confer an increased ability to cause a spectrum of diseases.

Prior to identification of specific virulence factors, serotypic analysis is a predominant means of categorizing pathogenic strains of *E. coli*. *E. coli* are serotyped in terms of their O (lipopolysaccharide, LPS), H (flagellar), and K (capsular) surface antigen profiles. These serotypes themselves do not necessarily confer the virulence. Instead, the serotypes and serogroups, a group of bacteria containing a common antigen, sometimes including more than one serotype, species, or genus, serve as readily identifiable markers that correlate with specific
virulent clones (Whittam et al. 1993). Empirically, a specific combination of O and H antigens defines the serotype of an isolate (Table 1).

<table>
<thead>
<tr>
<th>Category</th>
<th>Serogroup</th>
<th>Associated H antigen(s)</th>
<th>Category</th>
<th>Serogroup</th>
<th>Associated H antigen(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETEC</td>
<td>O6</td>
<td>H16, H9, H27, H11, NM, H42, NM, H7, H11, H12, H7, H28, H10, H20, NM</td>
<td>EHEC</td>
<td>O26</td>
<td>H11, H32, NM, H7, H8 NM, H21, H14, H7</td>
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<td></td>
<td>O8</td>
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<td>O11</td>
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<td>O115ab,</td>
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<td>O15</td>
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<tr>
<td></td>
<td>O159</td>
<td></td>
<td></td>
<td>EPEC</td>
<td>H6, NM, H34, NM, H2, H12, NM, H6, NM, H21, H27, NM, H6, NM, H2, H12, H6</td>
</tr>
<tr>
<td></td>
<td>O173</td>
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<td>O55</td>
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<td>O86</td>
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<td>O111</td>
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<td>O119</td>
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<td>O125ac</td>
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<td>O126</td>
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<td>O128</td>
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<td>O142</td>
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<td></td>
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<td></td>
<td></td>
<td>EIEC</td>
<td>O28ac, O29, O112ac, O124, O136, O143, O144, O152, O159, O164, O167</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>EAEC</td>
<td>O3, O15, O44, O86, O77, O111, O127, O9*</td>
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</table>

* O antigen un-typable by conventional methods. Reference: (Whittam et al. 1993)
Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains: (i) urinary tract infection, (ii) sepsis/meningitis, and (iii) enteric/diarrheal disease (Nataro and Kaper 1998; Prescott et al. 2005). There are at least six well characterized classes that can cause intestinal diseases: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroaggressive *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and Diffusely adherent *E. coli* (DAEC). Most of the pathogenic *E. coli* remains extracellular, but enteroinvasive *E. coli* (EIEC) is a true intracellular pathogen that is capable of invading and replicating within epithelial cells and macrophages. Other *E. coli* stains might be internalized by epithelial cells at low levels, but do not seem to replicate intracellularly (Kaper et al. 2004). Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC) and Enteroaggressive *E. coli* (ETEC) are three main categories that can cause diseases in animals therefore much research over the last decades has been focused on the studies of their pathogenesis.
Like most other pathogens, pathogenic *E. coli* utilizes similar steps in their infection. After being transmitted to an appropriate environment, pathogenic bacteria must be able to attach to host cells and tissues. Further colonization indicates the establishment of a site for microbial reproduction. Meanwhile, a successful competition for nutrients with host’s commensal microbiota must have happened while specialized structures allow them to compete for surface attachment. To achieve this, diarrheagenic *E. coli* in animals utilizes many shared virulence factors that are also present in human strains, such as enterotoxins, capsule and lipopolysaccharide (LPS). These virulence factors are molecules expressed or secreted from pathogens that enable them to achieve infectious advantage. Many common *E. coli* virulence factors are tabulated in Table 2. In addition to shared virulence factors, animal strains contain certain unique colonization factors. For example, porcine diarrheagenic *E. coli* strains possess specific fimbrial antigens that enhance their intestinal colonizing ability and allow adherence to the small intestinal mucosa (Levine et al. 1984). This versatility of colonization of *E. coli* is conferred mainly by two genetic configurations: virulence-related plasmids and chromosomal pathogenicity islands (PAI). It has been known that all categories of diarrheagenic *E. coli* carry at least one virulence-related property upon a plasmid. In
addition, some virulence genes of EPEC and EHEC organized as a cluster on chromosome are referred as a pathogenicity island (PAI) (Nataro and Kaper 1998).

Detailed features of various _E. coli_ are further discussed below.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Pathotype</th>
<th>Activity/effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paa</td>
<td>EPEC, EHEC</td>
<td>Adhesin</td>
</tr>
<tr>
<td>ToxB</td>
<td>EHEC</td>
<td>Adhesin</td>
</tr>
<tr>
<td>Efa-I/LifA</td>
<td>EHEC</td>
<td>Adhesin</td>
</tr>
<tr>
<td>Long polar fimbriae (LPF)</td>
<td>EHEC, EPEC</td>
<td>Adhesin</td>
</tr>
<tr>
<td>Saa</td>
<td>EHEC</td>
<td>Adhesin</td>
</tr>
<tr>
<td>OmpA</td>
<td>MNEC, EHEC</td>
<td>Adhesin</td>
</tr>
<tr>
<td>F1C fimbriae</td>
<td>UPEC</td>
<td>Adhesin</td>
</tr>
<tr>
<td>S fimbriae</td>
<td>UPEC, MNEC</td>
<td>Adhesin</td>
</tr>
<tr>
<td>Type-1 fimbriae</td>
<td>All</td>
<td>UPEC adhesin: binds to uroplakin</td>
</tr>
<tr>
<td>Curli</td>
<td>Various</td>
<td>Adhesin: binds to fibronectin</td>
</tr>
<tr>
<td>Aggregative adherence fimbriae</td>
<td>EAEC</td>
<td>Adhesin; &gt;4 subtypes</td>
</tr>
<tr>
<td>P (Pap) fimbriae</td>
<td>UPEC</td>
<td>Adhesin; induces cytokine expression</td>
</tr>
<tr>
<td>Dr adhesins</td>
<td>DAEC, UPEC</td>
<td>Adhesin, binds to decay-accelerating factor (DAF), activates PI-3-kinase, induces MICA; &gt;10 Dr adhesins described</td>
</tr>
<tr>
<td>Intimin</td>
<td>EPEC, EHEC</td>
<td>Adhesin, induces TH1 response; 10 variants described</td>
</tr>
<tr>
<td>AslA</td>
<td>MNEC</td>
<td>Promotes invasion</td>
</tr>
<tr>
<td>IbeA, B, C</td>
<td>MNEC</td>
<td>Promotes invasion</td>
</tr>
<tr>
<td>Aerobactin</td>
<td>EIEC</td>
<td>Iron acquisition, siderophore</td>
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<tr>
<td>Yersiniabactin</td>
<td>Various</td>
<td>Iron acquisition, siderophore</td>
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<tr>
<td>IreA</td>
<td>UPEC</td>
<td>Iron acquisition, siderophore receptor</td>
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<tr>
<td>Chu (Shu)</td>
<td>EIEC, UPEC, MNEC</td>
<td>Iron acquisition, haem transport</td>
</tr>
<tr>
<td>IroN</td>
<td>UPEC</td>
<td>Iron acquisition, siderophore receptor</td>
</tr>
<tr>
<td>IcsA (VirG)</td>
<td>EIEC</td>
<td>Nucleation of actin filaments</td>
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<tr>
<td>Bundle-forming pilus (BFP)</td>
<td>EPEC</td>
<td>Type IV pilus</td>
</tr>
<tr>
<td>K antigen capsules</td>
<td>MNEC</td>
<td>Antinphagocytic: &gt;80 K types</td>
</tr>
<tr>
<td>Dispersin</td>
<td>EAEC</td>
<td>Promotes colonization; aids mucous penetration</td>
</tr>
<tr>
<td>Flagellin</td>
<td>All</td>
<td>Motility; induces cytokine expression through TLR5; &gt;50 flagella (H) serotypes</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>All</td>
<td>Induces cytokine expression through TLR4; &gt;180 O types</td>
</tr>
</tbody>
</table>

CFA, colonization factor antigen; CS, coli surface antigen; MICA, MHC class I chain-related gene A; PCF, putative colonization factor; PI-3-kinase, phosphatidylinositol 3-kinase; TLR, Toll-like receptor

Modified from reference: (Kaper et al. 2004)
Enteropathogenic *E. coli* (EPEC) are primarily related to infantile diarrhea in developing countries and produce a characteristic histopathology known as attaching and effacing (AE) lesion. AE lesion is characterized by the effacement of microvilli and intimate attachment between a bacterium and epithelial cell membrane. Another hallmark is the cytoskeletal changes, including accumulation of polymerized actins right beneath the adherent bacteria (Nataro and Kaper 1998). The ability to induce this AE histopathology is encoded by genes on a 35-kb pathogenicity island (PAI) called the locus of enterocyte effacement (LEE). A 94-kDa outer membrane protein called intimin encoded by this genetic locus is responsible for mediating intimate attachment of EPEC to epithelial cells. Intimin serves not only as a ligand for epithelial cell adhesion but also stimulate mucosal immune response and intestinal crypt hyperplasia (Higgins et al. 1999).

Apart from the membrane protein, bacteria can also take advantage of protein secretion pathways to modulate the infection. A few secretion pathways of
prokaryotes are now well studied: the Sec-Dependent pathway, Type I (ABC) protein secretion pathway, Type II and Type III protein secretion pathways. Several gram-negative pathogens have type III protein secretion pathway and use it to secrete virulence factors. Therefore, Type III secretion system and effector proteins within the open reading frames of LEE PAI play roles in the bacterial attachment. One of these effector proteins, designated as Tir, is secreted and inserted into the host-cell membrane, where it functions as a receptor for the intimin outer-membrane protein(Kenny et al. 1997). Outside the LEE PAI, additional EPEC virulence factors have also been described. Lymphostain (LifA) inhibits lymphocyte activation and EAF (EPEC adherence factors) plasmid encode a type IV pilus that is known as bundle forming pilus (BFP), which mediates interbacterial lateral adhesion and possibly adherence to epithelial cell. Several investigators have shown that EPEC are capable of entering a variety of epithelial cell lines, however they do not multiply intracellularly or escape from phagocytic vacuole and thus do not appear to be specifically adapted for intracellular survival (Donnenberg et al. 1989; Francis et al. 1991).
Symptoms of the diseases caused by EHEC include abdominal cramps and diarrhea that may in some cases progress to bloody diarrhea (haemorrhagic colitis) in human. In a small proportion of patients (particularly young children and the elderly), the infection may lead to a life-threatening disease, such as haemolytic uremic syndrome (HUS).

The percentage of EHEC infections which progress to HUS varies between sporadic cases (3%-7%) and those associated with outbreaks (20% or more).

Enterohemorrhagic *E. coli* (EHEC) is a closely related pathotype to EPEC which also produce AE lesion with the excretion of potent cytotoxin that broadens the spectrum of illness, including bloody diarrhea and haemolytic uremic syndrome (HUS). EHEC strains of O157:H7 serotypes are the most important EHEC pathogens in North America, the United Kingdom and Japan, but several other serotypes, particularly those of O26 and O111 serogroups can also cause diseases in many countries (Kaper et al. 2004).
The key virulence factor for EHEC is Shiga-like toxin (Stx), which is also known as verocytotoxin (VT). Stx consists of five identical B subunits that are responsible for binding the holotoxin to the glycolipid globotriaosylceramide (Gb3) on the target cell surface, and a single A subunit is translocated to the cytoplasm where it subsequently cleaves ribosomal RNA and ceases protein synthesis in host cells (Kaper and Alison 1998). Stx is produced in the colon by *E. coli* and travels to the kidney by bloodstream, where it damages renal epithelial cells and stimulates the renal inflammation that induces local cytokine and chemokine production. Stx also induces apoptosis in intestinal epithelial cells (Jones et al. 2000).

**2-2-3 ENTEROTOXIGENIC E. COLI (ETEC)**

Enterotoxigenic *E. coli* (ETEC) are a major cause of travellers’ diarrhea that produces heat-stable toxin (STs) and heat-labile enterotoxin (LTs) as well as a variety of intestinal colonization factors (Kaper 2005). Adhesins of human and porcine ETEC strains are encoded by plasmids, as are many other known ETEC virulence factors.
factors and enterotoxin productions (Johnson and Nolan 2009).

LTs and STs are two subgroups of enterotoxins secreted by ETEC: LT is used synonymously with LT-I in differentiation to a second type of LT, LT-II, primarily found in animal ETEC isolates. LTs consist of a single A subunit and five identical B subunits which is structurally and functionally related to cholera enterotoxin (CT) expressed by vibrio cholerae. The B subunits of LT-I mediate binding of holotoxin to the cell surface GM1 gangliosides and weakly to GD1b (Fukuta et al. 1988), while the A subunit is responsible for the enzyme activity of toxin. LT-I transfers an ADP-ribosyl moiety from NAD to the α-subunit of the stimulatory G-protein on the basolateral membrane which leads to the activation of adenylate cyclase and increases levels of intracellular cAMP. Subsequent activation of cAMP dependent kinases phosphorylate chloride channel in apical epithelial cell membrane, thus increase secretion of Cl⁻ and water efflux from secretory crypt cells to intestinal lumen and therefore cause diarrhea (Sears and Kaper 1996). LT-II increase intracellular cAMP level by a similar mechanism to that involved with LT-I toxicity, however, LT-II uses GD1 as its receptor rather than GM1 (Fairbrother et al. 2007).
STs are small, single peptide toxins that include two unrelated classes: STa and STb, which differ in both structure and mechanisms of action. Only toxin of STa class has been associated with human diseases (Nataro and Kaper 1998). The main receptor for STa is a membrane-spanning guanylate cyclase. Stimulation of guanylate cyclase activity leads to increased intracellular cGMP and consequently increases secretion. STb toxin is primarily associated with ETEC strains isolated from pigs although some human strains have been reported to express STb. Studies have also identified sulfatide as the receptor for STb toxins (Francis 2002; Dubreuil et al. 2007). Unlike the chloride ion secretion elicited by STa, STb stimulate the secretion of bicarbonate from intestinal cells, and can elevate cytosolic Ca2+ concentrations and stimulate the secretion of serotonin, all of which could lead to increased ion secretion (Dubreuil 1997).

ETEC colonizes the surface of the small bowel mucosa; in human, the colonization is mediated by one or more proteinaceous fimbrial or fibrillar colonization factors (CFs), which are known as CFA (colonization factor antigen), CS (coli surface antigen) or PCF (putative colonization factor) followed by a number for distinguishing purpose (Kaper et al. 2004). However, ETEC are also an important cause of diarrheal disease in animals. Other than certain fimbriae
mutually expressed in human and animal strains, like type1 fimbriae, porcine ETEC strains express unique fimbrial intestinal colonization factors, such as F4 (K88), F5 (K99) and F18, which are not found in human ETEC strains.

**2-3 COLONIZATION OF ENTEROTOXIGENIC ESCHERICHIA COLI (ETEC) TO PORCINE INTESTINAL EPITHELIA.**

Adhesion is an essential and very first step in microbial infection which requires the establishment of the pathogen at the appropriate portal of entry. Microorganisms have usually developed tissue adherence mechanisms and some abilities to overcome or withstand the constant pressure of the host defences at the surface. Bacterial adherence or attachment to a eukaryotic cell or tissue surface requires the participation of two factors: a receptor and a ligand. The binding sites of receptors so far defined are usually specific carbohydrate or peptide residues on the eukaryotic cell surface. The bacterial adhesin is typically a macromolecular component of the bacterial cell surface which interacts with the host cell receptor. Adhesins and receptors usually interact in a complementary and specific fashion with specificity comparable to enzyme-substrate relationships or antigen-antibody
Porcine ETEC strains adhere to small intestinal epithelia via specific adhesive factors that include certain defined secreted proteins and, in majority, filamentous proteins on the surface of bacteria. For instances, a gene for afimbrial adhesin
called AIDA has been commonly detected from weaned pigs with PWD and edema disease (Ngeleka et al. 2003; Daniel 2010). However, filamentous bacterial fimbriae designated F4 and F18 are types that are widely detected and in charge of bacterial adhesion. Genes for F4 and F18 were identified in 92.7% of all porcine ETEC (Frydendahl 2002). F4 fimbriae are associated with diarrhea of weaning pigs as well as in weaned pigs. It presents in ETEC strains of all the main PWD serogroups, but with O149 strain in particular (Tzipori 1985). In contrast, F18 fimbriae are typically associated with diarrhea of weaned pigs; moreover, it has been connected with edema disease, an abnormal accumulation of fluid beneath the skin or in one or more cavities of the body (Bertschinger et al. 1990; Fairbrother et al. 2005). Other fimbrial structures, such as F5, F6 and F41, are also associated with porcine ETEC strains but are relatively rare. One study conducted in Denmark reported that F4 and F18 were detected in 96(44.7%) and 86 isolates(39.3%) respectively out of 219 isolates (Frydendahl 2002). These results were further confirmed by another study which detected the prevalence of fimbrial adhesins and toxin gene detected in 215 Escherichia coli isolates from pig with post-weaning diarrhea in China (Chen et al. 2004). (Table 4)
The K88 (F4) antigen was first described in 1961, the first colonization factor of the porcine ETEC types to be discovered. F4+ ETEC strains are the most common etiological agents of neonatal and post-weaning diarrhea in pigs (Garcillan-Barcia et al. 2009). F4 are flexible fimbriae that occur as F4ab, F4ac and F4ad variants, the “a” antigenic region is conserved and a second antigenic region is variable and designated “b”, “c” and “d”. F4ac is the dominant type, worldwide. For example, Alexa et al. (2001) showed that F4ac variant was present in 98% of 237 F4 positive porcine ETEC isolates while F4ab and F4ad were present only in 0.8% and 1.3%. A study on F4 fimbriae binding specificity suggested
that three F4 (K88) variants bound preferentially to purified glycosphingolipids (GSL) containing a β-linked N-acetylhexosamine (HexNAc), either GlcNAc or N-acetylgalactosamine, in the terminal position or, in the penultimate position with galactose in the terminal position (Grange et al. 2002). However, the fidelity of bacterial carbohydrate binding specificity to immobilized purified glycosphingolipids was questionable, since Grange et al. (2002) have suggested that the conformation change of a receptor may alter the binding specificity as a consequence. In the study of Grange et al (2002), sequential treatments of glycosphingolipid, porcine serum transferrin (pSTf), with neuraminidase followed by β-galactosidase and then HexNAcase increased the F4ab adhesin-binding activity of pSTf in comparison to that of the untreated control. They proposed that the removal of the terminal three residues on each antenna causes a conformational change in the remaining oligosaccharides, which makes it a better receptor for F4ab.

F4 fimbriae are encoded by 10 different genes (faeABCDEFGH) which are on a large non-conjugative plasmid (Huisman et al. 1993). It includes regulatory genes (faeAB), a major subunit (faeG), minor subunits (faeCFHIJ), usher and chaperones (faeDE). The operon is temperature-regulated and is expressed at 37°C, but not
18°C, a feature consistent with a need for these fimbriae when the bacteria are in
the intestine of host but not in the environment.

2-3-2 K99 PLASMID (F5 FIMBRIAE)

Neonatal diarrhea in piglets is caused primarily by ETEC strains possessing F5
fimbriae. The F5 antigen is encoded by a ~78 kb conjugative plasmid (Smith and
Linggood 1971). The F5 antigen is expressed as mannose resistant fimbriae of ~9
nm in diameter. F5 fimbriae mediate bacterial binding to the small intestinal
glycolipid ganglioside of newborn pigs. The trait is transmissible and these plasmids
have been found to be highly conserved among K99+ isolates (Johnson et al.
2006).

The F5 operon (fanABCDEFGH) includes positive regulators, a major pilin
subunit, an usher, a chaperon and pilus assembling and elongation genes
(Roosendaal and De Graaf 1989; Simons et al. 1990). Expression of F5 is
temperature dependent, activated at body temperature and inhibited at lower
temperature (van der Woude et al. 1990; Johnson and Nolan 2009).
F6 fimbriae are also found in porcine ETEC strains that cause neonatal diarrhea in pigs, with F6 acting to mediate adhesion to intestinal cells (Dean et al. 1989). The operon encoding F6 fimbriae can be located on plasmids or on the bacterial chromosome (Casey et al. 1993). The F6 fimbrial gene cluster contains eight genes, fasABCDEFGH, and the plasmids containing F6 operon range in size from 35 to 40 MDa (Schifferli et al. 1990).

F18 fimbriae are long flexible appendages that show a characteristic zigzag pattern (Nagy et al. 1997) and occur as two antigenic variants, F18ab and F18ac (Salajka et al. 1992). Typically, F18+ isolates also possess other plasmids encoding STa and STb. Five genes have been identified as members of the operon that encode F18 fimbriae: fedA (major pilus subunit), fedB (usher), fedC (chaperone), fedE (adhesin) (Smeds et al. 2001; Smeds et al. 2003). They appear to be located
on a 200-kb transmissible plasmid, whereas STa and STb were encoded on a separate plasmid.

2-3-5 ETPA (ETEC TWO-PARTNER SECRETION A PROTEIN)

Gram-negative bacteria contain multiple secretion pathways that facilitate the translocation of proteins across the outer membrane. For example, a collection of large virulence exoproteins, Ca2+-dependent cytolsin, an iron acquisition protein and several adhesins, are secreted by a two-partner secretion (TPS) pathway. The hallmark of this TPS pathway is the presence of N-proximal module called the secretion domain in the exoproteins (TpsA) and the channel-forming β-barrel transporter protein (TpsB). The genes for a cognate exoprotein and a transporter protein are usually organized in an operon. Specific secretion signals are present in a highly conserved region of the secretion domain of TpsAs. TpsBs probably serve as specific receptors of the TpsA secretion signals and as channels for the translocation of the exoproteins across the outer membrane (Dubuisson et al. 2001).
An identification of an ETEC two partner secretion locus (*etpBAC*) on the pCS1 virulence plasmid of prototype strain H10407 confirms the existence of a large 170-kD glycoprotein, EtpA, exhibiting a linear peptide sequence and predicted structural homologies with known high-molecular weight adhesins produced by other two partner secretion locus (Fleckenstein et al. 2006; Roy et al. 2009).

The actual function of EtpA was demonstrated to interact with highly conserved regions of the flagellin, FliC. These interactions are critical for adherence and intestinal colonization (Roy et al. 2009). Despite FliC are mostly buried in the flagellar shaft, they are at least transiently exposed at the tips of the flagella where they capture EtpA adhesin molecules for the presentation to eukaryotic receptors. Previous studies also reported that EtpA was able to optimize H10407 colonization in the murine intestine, and mice vaccinated with a truncated 110kD, non-glycosylated version of EtpA were protected against subsequent intestinal colonization with ETEC H10407 (Roy et al. 2008).
AIDA is a bacterial afimbrial adhesin associated with diarrheagenic *E.coli* strains. It was originally detected in a human isolate and reported as an autotransporter protein that is active in its glycosylated form on the bacterial surface (Fairbrother et al. 2005). AIDA mediates adherence to a variety of surfaces and promotes bacteria to bacteria adherence because of its capacity of autoaggregate and forming biofilms. AIDA is present in a subgroup of porcine ETEC negative for classical fimbrial antigens (F4, F5, F6, F18 and F41). The genes encoded for AIDA are located on a large plasmid; an in-frame deletion mutation in the aidA gene of an AIDA-positive ETEC resulted in a loss of ability to colonize the intestine and induce disease (Daniel 2010).

2-3-7 PAA

Porcine Attaching and Effacing-associated (Paa) protein was firstly identified in porcine Enteropathogenic *E. coli* as a gene whose inactivation leads to a loss of AE activity. Paa is also present in EHEC O157:H7 and ETEC O149:H10 serotypes and its genes are localized to a virulence region on a plasmid, which also encodes both
drug resistance and enterotoxin STa. However, the role of Paa in pathogenesis of porcine ETEC is unknown.

To sum up, ETEC are a diverse group of pathogens that have in common the ability to colonize the small intestine, where they produce and deliver plasmid-encoded heat-labile (LT) and/or heat-stable (ST) enterotoxins. In piglets, fimbriae F4 and F18 are mostly detected colonization factors in diarrheagenic ETEC and are believed to play an important role in the pathogenesis. Interestingly, many strains do not always produce recognizable colonization factors due to stringent controls on gene expression in response to environments. Therefore, other non-fimbrial adhesins of ETEC could play roles in adhesion. In addition to many virulence factors expressed by bacteria, host factors could certainly influence ETEC infection. Innate and acquired host defences and the genetic background of the host all influence the clinical presentation of ETEC infection following ingestion of a sufficient inoculum.
2.2 INNATE IMMUNE DEFENCE OF GUT EPITHELIUM IN PIGS

The innate defence system plays a crucial role in maintenance of the intestinal integrity and protection of hosts against a vast amount of potential microbial pathogens (Muller et al. 2005). The innate defence system takes the first hit and rapidly eliminates many infections. In contrast, the adaptive immunity specific to pathogens usually requires days to take place. Intestinal epithelial cells (IEC) have developed a variety of mechanisms for advanced defence (Pitman and Blumberg 2000).

The intestine is the most important route of entry for foreign antigens. The gastrointestinal tract is lined by a continuous monolayer of epithelial cells. A primary function of these intestinal epithelial cells in innate defence is to act as a physical barrier, separating the contents of luminal space from the interior milieu (Gewirtz et al. 2002). Besides, a complete protection requires a mucus layer lining above intestinal epithelium. Mucin glycoproteins (glycocalyx) form a viscoelastic gel which appears as a continuous protective barrier on the colonic mucosal surface for maintenance of gut health (Kindon et al. 1995). In addition, antibacterial peptide
synthesis and cytokine/chemokine network are also regarded as important parts of the innate immune response. Finally, the interactive process between IEC and underlying immune system could also mediate subsequently acute inflammation and other adaptive immune responses.

2.2.1 BARRIER FUNCTION OF EPITHELIAL CELL

Epithelial integrity is critical in maintaining a physical but selective barrier between external and internal environment. This barrier function is maintained by well-organized intercellular structures including tight junctions, adherence junctions and desmosomes surrounding the apical region of epithelial cells (Gumbiner 1996).

Tight junctions of epithelial cells consist of a belt like structure in the apical region of lateral plasma membrane. They hold cells together and provide a barrier function. Cell and cell adhesion at this junction is mediated by the interaction of multiple membrane-spanning proteins claudin and occluding, proteins protruding from the plasma membrane of adjacent IEC. Other proteins, such as Zonula
Occludens (ZO-1) and ZO-2 and cingulin are also involved in the regulation of tight junctions (Oswald 2006).

Adherence junctions occur usually basal in comparison with tight junctions. Adherence junctions are defined as cell junctions whose cytoplasmic face is linked to the actin cytoskeleton. They can appear as bands encircling the cell (zonula adherens) or as spots of attachment to the extracellular matrix (adhesion plaques). Adherence junctions consist of interaction mediated by the homophylic adhesion of single membrane spanning proteins E-cadherin and desmosomes (Oswald 2006; Dann and Eckmann 2007).

### 2.2.2 MUCUS LAYER PROTECTION

The mucus layer is composed of glycoprotein mucin associated with other proteins and lipid. It forms a continuous gel into which a bicarbonate rich fluid is secreted to maintain a neutralizing pH at the epithelial surface. Mucin is subdivided into two subgroups: the membrane anchoring (gel-forming) and the secreted mucins (non gel-forming) groups (Desseyn et al. 2000). They are secreted by Goblet cells through baseline secretion and active exocytosis. The tight adherence
of mucin to the apical surfaces of the epithelium is due to formation of a specific complex between mucin oligosaccharides and a mucin binding protein on the epithelial cells (Oswald 2006). Mucins are a distinct group of highly glycosylated glycoproteins that contain numerous glycans which are predominantly O-linked to the abundant Ser or Thr residues present in all mucins.

Mucins are involved in gut physiology since they form a selective diffusion barrier permeable to small molecules of nutrients but not to macromolecules. Their protection is against an acidic environment and protease from endogenous or bacterial origin (Montagne et al. 2004). Immunoglobulin A (IgA) is transported to the mucus and contributes to the prevention of epithelial colonization by inhibiting binding of bacterial surface structure to the epithelium (Fagarasan and Honjo 2003).

Pig mucins have been characterized at the genomic and biochemical levels. Many dietary factors including fibre, protein and anti-nutritional factors have been shown to influence secretion of mucins in pig intestine. Other than dietary factors, enterotoxins are also demonstrated to disrupt the integrity of intestinal mucins, and thus enhance enteric bacterial colonization and pathogenicity. For example, heat-labile enterotoxin from ETEC can help overcome the innate mucosal barrier by a toxin-mediated fluid secretion. This disruptive breach gives pathogens access to
the enterocyte, leading to further colonization of enterotoxigenic *Escherichia coli* (Glenn et al. 2009).

### 2.2.3 Secretion of Defensins and Antimicrobial Peptides

Another mechanism of mucosal defence is secretion of antimicrobial properties which disrupt the integrity of microbial membranes through their net positive charges and their ability to fold into amphipathic structures. Two main families of antimicrobial peptides can be distinguished: the defensins and the cathelicidins (Oswald 2006).

Paneth cells are a type of specialized epithelial cells residing at the base of the crypts and fulfil the crucial role in innate immunity (Porter et al. 2002). Mucosal epithelial cells and Paneth cells produce a variety of antimicrobial peptides (defensins, cathelicidins, cryptdin-related sequence peptides, bactericidal/permeability-increasing protein, chemokine CCL20) and bacteriolytic enzymes (lysozyme, group IIA phospholipase A2) that protect mucosal surfaces (Muller et al. 2005). Some of the released antimicrobial peptides are able to attract antigen-presenting cells and lymphocytes, thus Paneth cells serve a role in warning
the adaptive immune system of severe and persistent infection (Yang et al. 2002).

The pattern of antimicrobial factors produced by enterocytes differs from that of Paneth cells, as described below (Muller et al. 2005).

<table>
<thead>
<tr>
<th>Antimicrobial molecule</th>
<th>Producing intestinal cells</th>
<th>Basis of antimicrobial activity</th>
<th>Additional activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Defensins HD-5, HD-6</td>
<td>Paneth cells</td>
<td>pore formation*</td>
<td></td>
</tr>
<tr>
<td>β-Defensins HBD1, HBD3, HBD4</td>
<td>Intestinal epithelial cells</td>
<td>pore formation*</td>
<td>chemotactic for leukocytes</td>
</tr>
<tr>
<td>Cathelicidin intestinal LL-37/hCAP-18</td>
<td>epithelial cells</td>
<td>pore formation*</td>
<td>chemotactic for leukocytes; stimulates epithelial wound; healing; angiogenic; neutralizes LPS; opsonic</td>
</tr>
<tr>
<td>Bactericidal permeability-increasing protein (BPI)</td>
<td>intestinal epithelial cells</td>
<td>disruption of outer and inner membranes of Gram-negative bacteria</td>
<td></td>
</tr>
<tr>
<td>Chemokine (MIP-3α) CCL20</td>
<td>intestinal epithelial cells</td>
<td>?</td>
<td>chemotactic for dendritic and T cell subsets</td>
</tr>
<tr>
<td>Group IIA phospholipase A2 (PLA2)</td>
<td>Paneth cells</td>
<td>degrades bacterial lipids</td>
<td>?</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Paneth cells</td>
<td>degrades bacterial peptidoglycan</td>
<td>modulates the inflammatory ability of peptidoglycan</td>
</tr>
</tbody>
</table>

Reference: (Muller, Autenrieth et al. 2005)
*Additional antimicrobial activities, such as activation of bacterial autolysins, are possible

2.2.4 ADAPTIVE IMMUNITY

The synergistic protection amongst innate immune defence and adaptive immunity originates from the active production of chemokines and cytokines which are responsible for the recruitment of specialized immunocytes. Traditionally, production of cytokines is mediated by cells belonging to immune system (lymphocytes, macrophage and dendritic cells). However, intestinal epithelial cells (IEC), not considered as a part of immune system, can also produce cytokines and
chemokines (Oswald 2006). Several constitutively secreted cytokines such as TGF-α, IL-1, IL10, IL15 and IL18 play crucial roles in the basal influx of immune cells into mucosa, in epithelial cell growth and homeostasis. Other cytokine including IL-1α or β, IL-6, TNF-α, CCL-20 are expressed in response to microbial infection. IEC can not only secrete chemokines but also respond to chemokines synthesized by underlying specialized cells belonging to intestinal immune system. For example, IEC could respond to IL-1 via their IL-1 receptors and thus amplify the effects against inflammatory reaction. Intestinal immune system is comprised of several specialized cell types. One major component, gut associated tissue (GALT), is a loosely organized non-encapsulated cluster of lymph tissues along the intestinal tract. An afferent part of GALT is Peyer’s patches responsible for sampling luminal antigens to ensure their uptake by antigen-presenting cells. Specialized M cells accomplish the translocation of sampled antigens and microorganisms via transcytosis to the subepithelial dome, an area populated with professional antigen-presenting cells. Upon activation and migration, cells of GALT would create an effector part consisting IgA-producing plasma cells and mature T cells which are diffusely scattered around the intestinal mucosa (Artis 2008).
In addition, specialized intestinal dendritic cells (DCs) located in the lamina propria of the small intestine express tight junction proteins and can extend their dendrites between epithelial cell tight junctions to directly sample luminal environment. Intestinal DCs are known to efficiently acquire antigens from the lumen and express a wide range of pattern-recognition receptors, including Toll-like receptors (TLRs) that recognize microbial components. Apart from the expression of TLRs, IECs also express intracellular nucleotide-binding oligomerization domain (NOD) like receptors (NLRs). In general, ligation of TLRs and NLRs results in the activation of innate immune responses, including the expression of pro-inflammatory cytokines, chemokines and antimicrobial peptides (Medzhitov 2007).

3.1 EFFECTS OF CARBOHYDRATES, NEURAMINIDASE AND EXOPROTEIN, ETPA, ON PORCINE ETEC ADHERENCE

3.1.1 INTRODUCTION
The surface of many cells, both eukaryotes and prokaryotes, is decorated with glycoconjugates that play important roles in a wide range of biological processes, including cell-cell and small molecule-cell recognition. Glycoconjugate is a general classification for carbohydrates covalently linked with other chemical species. Cellular sialic acid is an important component of glycoconjugate that is mostly found on glycoproteins and gangliosides. A large family of neuraminic acid derivatives are acidic, negative charged monosaccharides, common in higher animals and microorganisms. The largest structural diversity of sialic acid happens at carbon 5 (Figure. 4), and this is also a site for its involvement in a variety of biological functions. Up to date, the most abundant and best studied sialic acid is N-acetylneuraminic acid (Neu5Ac). The term sialic acid (Sia) has been therefore used in literature to mean this particular chemical.

Sia is mainly a terminal component of glycoprotein and gangliosides (Figure. 5) and is mostly found glycosidically linked to either 3- or 6- hydroxyl group of galactose(Gal) residues or to the 6-hydroxyl group of N-acetylglucosamine(GlcNAc) and N-acetylgalactosamine (GalNAc) residues (Lehmann et al. 2006; Severi et al. 2007).
Their negative charges confer electronic repulsion forces that influence the conformation of gangliosides and contribute to the supermolecular structure in cell membranes, thus affecting their functions (Schauer 2000). Physiological roles of cellular Sia could be viewed from its dual roles, either masking recognition sites or, in contrast, representing a biological target allowing recognition by Sia specific binding proteins, collectively called lectins. The pathophysiological role of cellular Sia to bacterial colonization is rather ambiguous. It has been reported that a large number of bacteria like Streptococcus and Helicobacter species attach to cell via recognizing Sia. For example, a Sia-specific adhesin SabA on H. pylori draws special attention due to its involvement in facilitating binding to oral and gastric mucins (Schauer 2009). In contrast, Sakarya et al (2010) have reported that Staphylococcus aureus has better adherence to desialylated pharynx epithelial cell monolayer comparing to normal
cell monolayers, which implies a masking feature of cellular Sia on the terminal position of glycoconjugates to bacterial adherence. Alternatively cellular Sia could have no effects on bacterial adherence. In the same study, the adherence of *M. catharralis* is not affected after nuraminidase, a glycoside hydrolase to Sia, is applied. Expression of Sia is found age-dependent in pigs. The expression of membrane NeuAcα2,6 moieties is high in new born pigs, declined slightly during sucking and is very low in weaned animals. Conversely, the expression of membrane NeuAcα2, 3 moieties is low at birth but higher in sucking and weaned animals (King et al. 1995). Thus, it seems that cellular Sia is likely involved in bacterial adherence in pigs. However, the exact biological function of Sia is still unclear.

The complementary interaction between glycoconjugates on cell membrane and adhesins on bacteria are selective and pivotal to initiate ETEC colonization to porcine intestine. Many studies have been done on bacterial adhesins of porcine ETEC. F4 and F18 fimbriae positive ETEC strains have been reported responsible for PWD, however many current studies have suggested that ETEC infectious mechanism is more complicated than previously appreciated. An earlier described exoprotein, EtpA, from ETEC H10407, consolidated this notion.
There are growing concerns about the increasing pool of drug resistance genes in bacteria in the intestine of animals. To date, the prevention and medication of infected piglets from porcine ETEC rely mainly on the use of antibiotic treatment. However, the potential emergence of antimicrobial resistance has required us to develop non-antibiotic preventive measures. Also, the mounting pressures of selection from overuse of antibiotics, to a certain extent, accelerate the emergence of zoonotic virulent strains amongst pig and human that has again emphasized the importance of seeking alternatives to antibiotics. So far, there have been a number of initiatives to evaluate vaccines, prebiotics, probiotics, bacteriophage and management procedures in order to minimize the use of antibiotics (Fairbrother et al. 2005).

3.1.2 HYPOTHESIS AND OBJECTIVE

Our first objective was to determine the biological role of cellular Sia on porcine intestinal epithelia by removing Sia through addition of neuraminidase from Clostridium perfringens (C. welchii). The enzyme hydrolyzes α(2→3), α(2→6), and α(2→8)-glycosidic linked terminal sialic residues from various glycomolecules.
Secondly, different monosaccharides were provided as competitive inhibitors to saturate the binding sites of ETEC adhesins in order to unveil the minimum recognition sugar on the epithelial receptors required to initiate subsequent colonization.

Finally, the potential correlation between types of adhesins or virulence factors of ETEC, such as types of fimbriae, enterotoxins, and exoprotein, EtpA, to the capacity of colonization was determined. We hoped that the results from this study could possibly shed light on the potential colonization mechanisms of ETEC to the pig epithelial cells.
3.1.3 MATERIALS AND METHODS

Reagents

Neuraminidase (NANase) type V extracted from *Clostridium perfringens (C. welchii)* with a specific activity 1.2U mg⁻¹ (Sigma, Canada) was reconstituted in 50 mmol l⁻¹ Na acetate (pH 5.5) immediately prior to use.

Bacteria strains and cell line

The porcine jejunum epithelial cell line (IPEC-J2) was freshly thawed for every use and routinely cultured at 37°C under a humidified (v/v) 5% CO₂ atmosphere, in D-MEM/F-12 (1:1) (Gibco) containing 5% fetal bovine serum (Sigma) without antibiotic. Twenty four wild strains of Enterotoxigenic *Escherichia coli* (Table. 7) causing pig diarrhea were obtained from University of Montreal (Québec, CA) and cultured on trypticase soy agar (Fluka). The bacterial growth curve was constructed beforehand. The bacterial concentration was monitored spectrophotometrically at optical density A₆₀₀ nm, accordingly.
Bacterial Adherence Assay

IPEC-J2 cell were seeded in black sided, clear bottom, 96-well, tissue culture plates at a density of \(1 \times 10^4\) cell cm\(^{-2}\) and were grown to confluence. Required amount of bacteria (MOI 20:1 or 50:1) were incubated with 100 \(\mu g\) ml\(^{-1}\) fluorescein-5-isothiocyanate (FITC) in PBS for 30 min at room temperature. FITC-labelled bacteria were used subsequently to infect IPEC-J2 monolayer for 1 hr in triplicates. Non-adherent bacteria were removed by gentle washing with PBS. The fluorescent intensity of each well representing FITC-labelled ETEC and background fluorescence was assessed by multi-wells plate reader (DTX 880, Beckman coulter) with excitation and emission filters set at 485 and 535 nm, respectively. The percentage of the binding was calculated by the following equation:

\[
\text{Percent bound} = \frac{(I_{\text{exp.}} - I_{\text{neg.}})}{(I_{\text{pos.}} - I_{\text{neg.}})} \times 100
\]

Where \(I_{\text{exp.}}\) indicates the fluorescence intensity measured in wells containing cultured cells and FITC-labelled bacteria. \(I_{\text{neg.}}\) represents the background fluorescence agitated from cell monolayer. \(I_{\text{pos.}}\) simply stands for fluorescence
intensity of wells incubated with IPEC-J2 cells and FITC-labelled bacteria in the absence of any treatment.

**Cell viability assay to determine Nuraminidase shock**

The cytotoxicity of enzyme NANase treatment was quantified by the neutral red uptake (Olano Martin et al. 2003). The culture plates of 96 wells were co-incubated with 200μl of 0.1% (w/v) neutral red solution for 90 min at 37°C under a 5% CO₂ atmosphere. Procedures with neutral red were all carried out under the light protection by foil. Cells were subsequently washed; the retained dye was then extracted from the viable cells through addition of 100ml absolute alcohol/ 0.1M citrate buffer (pH 4.2 ) (v/v 1:1) to each well. The plate was agitated for 20 min at room temperature and the absorbance was read at 550 nm using an ELX808 ultra-microplate reader.

The percentage of IPEC-J2 cell death was calculated from the equation by the comparison with control wells that did not endure enzyme treatment.

\[
\% \text{ survival} = \frac{\text{mean absorbance of treated cells}}{\text{mean absorbance of control}} \times 100
\]
Neuraminidase effects on bacterial adherence to IPEC-J2 cell

Confluent monolayer IPEC-J2 cells were washed three times with calcium-free PBS. Afterwards, they were treated with 100mU ml-1 of NANase in 50 mmole l⁻¹ sodium acetate (pH5.5) buffer containing 5 mmole l⁻¹ CaCl₂, or medium alone, and were cultivated for 3h at 37°C, 5% CO₂ humid atmosphere. Cells were then washed three times with PBS and used in the adherence assay as described earlier.

Effects of penultimate monosaccharides beneath sialic acid of porcine epithelial cells on ETEC adherence

Effects of monosaccharides representing potential residues on surface glycoconjugates of cells were assessed by adding exogenous monosaccharides into the medium. N-Acetyl-D-glucosamine(GlcNAc), D(+)−Glucose(Glu), D−(−)-Galactose(Gal), N-Acetyl-D-galactosamine(GalNAc), D(+)-Fucose(Fuc), D(+)-mannose(Man) (Sigma-Aldrich Chemical Co), were prepare as 50 mM solutions in PBS, whereas Sialic acid (N-Acetylneuraminic acid, NANA) was prepared in 10 mmole L⁻¹ Tris-HCl buffered to pH 6.9. Thirty µl E. coli isolate
suspension were pre-incubated with an identical volume of monosaccharide solutions, making a final 25 mM concentration, for 30 min. After three washes with PBS, bacteria pellets were then resuspended in Opti-MEM(Invitrogen) to 1 ml and presented to IPEC-J2 treated with NANase or media alone for 1 hr as the above described adherence assay. The assay was performed to elucidate the effects of monosaccharides on bacterial adherence.

**Preparation of cDNA**

Twenty five isolates of Enterotoxigenic *Escherichia coli* (Table 7) kindly provided from University of Montreal (Québec, CA) were cultured overnight until the log-phase growth in trypticase soy broth (Fluka). Bacteria were lysed in a lysis buffer containing 2-mercaptoethanol and the lysate was then homogenized by sonication. Supernatants containing total RNA were recovered in Spin Cartridge and collection tubes (Ambion. Inc). On-Column DNAase I treatment (PureLink TM) removed DNA contaminants from deteriorating the quality of isolation. RNA quality and concentration were subsequently quantified by Nano-drop 2000 spectrophotometer (Thermo Scientific) at wavelength A268/280. The ratio should
be between 1.85 - 2.10. Reverse-transcription (1 µg RNA) was carried out following manufacturer’s instruction (BioRad). In brief, RNA was reverse transcribed with 1 µg/µl random hexamer primers at a reaction protocol was as below: 5 min at 25°C for primer annealing; 30 min at 42°C for reverse transcription; 5 min at 85°C to inactivate the reaction; Hold at 4°C.

**Polymerase Chain reaction**

PCR was used to detect the presence of the gene encoding EtpA and to characterize F4 antigenic variants. The primer pairs used for amplification were etpa.7f: (TCAGAACAGCCAGAACATGG) etpa.6r: (CCACCGTCCAGTACAATGTT) which amplified 660bp fragments (Table. 6). cDNA templates from various *E. coli* strains were prepared as described above. PCR was carried out in a 25 µl reaction volume containing 5X PCR buffer (5 µl), 25 mM MgCl2 (1.5 ml), dNTPs (10 mM, 0.5 µl), each primer (0.5 µl, 20 pmol), DNA template (0.5 µl), water (16.3 µl) and *Taq* polymerase (0.2 µl) (BioRad, Canada). A PCR protocol for EtpA was as follows: 94°C for 30 s, 53.1°C for 30 s and 68°C for 45 s for 35 cycles, followed by 68°C for 7
min. An expression negative control was included by choosing non-ETEC *E. coli* strains. The reference strain (H10407) was used as a positive control.

As for F4 variants (F4ab, F4ac and F4ad) characterization, primer pairs used in this study were AM005: sense (GGTGATTCAATGGTTCGGTC) which anneals to a conservative region in all three F4 variants; while MF007 antisense, (TGCAGCACCAGAAACAGTCGTCGT) specifically anneals to F4ab; MF008 antisense, (CCCAGCCGACGATTACAGAACCCT) anneals to F4ac and MF009 antisense, (TGCAGAATTTCGTGAACATTTCGTGCG) anneals to F4ad variants.

DNA amplification was performed using less than 1µg cDNA, 90 ng oligonucleotide primers, 0.2 mM each of dATP, dGTP, dCTP, and dTTP, 10 mM Tris HCl (pH 8.8); 1.5 mM MgCl2; 50 mM KCl; one unit of *Taq* polymerase (BioRad, Canada), and added distilled water to final volume of 50 µl. The PCR protocol included a denaturing step at 94°C for 30 s, followed by annealing of the primers at 56°C for 30 s, with an extension step at 72°C for 1 min. The 35 cycles of these three-step procedures were performed in a thermal cycler, followed by a 10-min extension at 72°C.
The amplified products were visualized by standard gel electrophoresis in 1% agarose gels (Type I, Sigma) in Tris–acetate–EDTA (TAE) buffer. The gels were analyzed under UV light (300 nm) and photographed with a Kodak camera system.

**RealTime PCR**

Gene expressions of EtpA and GAPDH, in seven ETEC isolates were evaluated using a comparative real-time PCR. Each 20-µl reaction contained 2 µl of reverse-transcribed cDNA, 10 µl of Platinum® SYBR® Green qPCR SuperMix-UDG, 500 nM of each primer, and nuclease-free water. The sequences for primers of EtpA and GAPDH are given in Table 6. Primers were designed to contain minimal internal structures (i.e., hairpins and primer-dimer formations, as identified by the software) and avoid the highly repeated region in EtpA operon. The specificity and efficiency of each primer was determined by melt curve analysis. Amplification and detection were carried out on Bio-Rad CFX 384TM-Real Time System with an initial temperature of 50 °C for 2 min. Activation Platinum®Taq DNA polymerase was achieved at a 95 °C temperature for 2 min, followed by 39 amplification cycles at 95 °C for 15 s; 59.1 °C for 48s;. Subsequent melt curve analysis involved heating
the products to 95 °C for 1 min, followed by cooling to 55 °C and slowly heating to 95 °C while monitoring fluorescence. Fluorescence data were collected at the end of each cycle. No template control (NTC) and no-reverse transcriptase (NRTC) controls were included for each assay and no Ct values were consistently obtained for all negative controls after 39 cycles of PCR (data not shown). Relative mRNA levels were determined according to a separate tube comparative critical threshold (2−ΔΔCT) real-time PCR while laboratory EHEC strain H10407 functions as a reference sample (Biosystems User Bulletin No. 2). The housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Carey et al. 2009) was used to normalize input amounts of RNA and the level of EtpA gene was determined.

<table>
<thead>
<tr>
<th>Table 6. oligonucleotides used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligonucleotide</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td><strong>Conventional PCR</strong></td>
</tr>
<tr>
<td>etoa.7f</td>
</tr>
<tr>
<td>etoa.8r</td>
</tr>
<tr>
<td>AM005</td>
</tr>
<tr>
<td>AM007</td>
</tr>
<tr>
<td>AM008</td>
</tr>
<tr>
<td>AM009</td>
</tr>
<tr>
<td><strong>Real-time qPCR</strong></td>
</tr>
<tr>
<td>GAPDH.f</td>
</tr>
<tr>
<td>GAPDF.r</td>
</tr>
<tr>
<td>etoA10.f</td>
</tr>
<tr>
<td>etoA10.r</td>
</tr>
</tbody>
</table>

a reference (Choi and Chae 1999)
Statistical Analysis

Data were analysed as multi-way ANOVA, using the General Linear Models (GLM) procedure of SAS (SAS Institute, 2003). Effects of each trait were tested using Bonferroni’s Multiple Comparison test, using a 5% probability level for accepting or rejecting hypothesis.
TABLE 7. WILD TYPE ETEC ISOLATES AND PATHOTYPE CHARACTERIZED

<table>
<thead>
<tr>
<th>Group</th>
<th># designation</th>
<th># ECL</th>
<th>Other Designation</th>
<th>Pathotype</th>
<th>Serotype</th>
<th>Isolation</th>
<th>Diagnostic</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4+</td>
<td>Isolation 2</td>
<td>8559</td>
<td>1999-1184-3-1</td>
<td>LT:Sta:Tb:East1:Paa:F4</td>
<td>O149</td>
<td>feces</td>
<td>unknown</td>
</tr>
<tr>
<td>F4+</td>
<td>Isolation 6</td>
<td>1307</td>
<td>F305-2</td>
<td>LT:Stb:East1:F4</td>
<td>O149</td>
<td>feces</td>
<td>diarrhea</td>
</tr>
<tr>
<td>F18+</td>
<td>Isolation 17</td>
<td>14637</td>
<td>2007-9288</td>
<td>Stx2:East1:Aida:F18</td>
<td>0139</td>
<td>ileum</td>
<td>oedema</td>
</tr>
<tr>
<td>F18+</td>
<td>Isolation 18</td>
<td>2124</td>
<td>2004-175-5449</td>
<td>Stx2:East1:Aida:F18</td>
<td>0139</td>
<td>feces</td>
<td>oedema</td>
</tr>
<tr>
<td>F18+</td>
<td>Isolation 19</td>
<td>14724</td>
<td>2007-55-969</td>
<td>Stx2:East1:Aida:F18</td>
<td>0139</td>
<td>ileum</td>
<td>oedema</td>
</tr>
<tr>
<td>Others</td>
<td>Isolation 21</td>
<td>12370</td>
<td>2004-175-7556</td>
<td>Sta:Stb</td>
<td>O139</td>
<td>ileum</td>
<td>unknown</td>
</tr>
<tr>
<td>Others</td>
<td>Isolation 22</td>
<td>12444</td>
<td>2004-C896</td>
<td>Sta:Stb</td>
<td>O139</td>
<td>ileum</td>
<td>diarrhea, pneumonia</td>
</tr>
<tr>
<td>Others</td>
<td>Isolation 23</td>
<td>3448</td>
<td>2004-C896</td>
<td>Sta:Stb</td>
<td>O139</td>
<td>ileum</td>
<td>diarrhea</td>
</tr>
<tr>
<td>Others</td>
<td>Isolation 24</td>
<td>12809</td>
<td>F18D4-1</td>
<td>Sta:Stb</td>
<td>O139</td>
<td>ileum</td>
<td>diarrhea</td>
</tr>
</tbody>
</table>

Bacteria were categorized into groups by types of fimbriae expressed, pathotypes, serotypes, diagnostic syndromes and locations in the host where they were isolated. Twenty four isolates were kindly provided by University of Montreal.

Twenty-four wild type ETEC isolates were tabulated in terms of types of fimbriae expressed, pathotypes, serotypes, diagnostic syndromes and locations in the host where isolated. As for adhesin identification, fourteen isolates express F4 fimbriae while another five isolates express F18 fimbriae and none of either fimbria is detected in the rest five isolates. All isolates carry at least one enterotoxin gene.
(LT or/and ST) and many virulence factors were identified as shown in Table 7.

Result from bacteria serotyping indicates that fourteen O149 serotyped isolates consistently express F4 fimbriae.
FIGURE 6. EFFECTS OF NURAMINIDASE TREATMENT ON IPEC-J2 CELL MONOLAYERS (WRIGHT-GIEMMSA STAINING)

Potential cytotoxicity of nuraminidase treatment was visualized by Giemmsa-Wright staining. Morphological changes of cell monolayer were pictured by inverted microscope, magnification 200X.

Neuraminidase from Clostridium perfringens hydrolyzes α(2→3), α(2→6), and α(2→8)-glycosidic linkages of terminal sialic residues. It has optimal cleavage function at 50 mmol L⁻¹ Sodium acetate (pH 5.5), which is lower than normal
physiological pH range (7.35-7.45). In order to assess the potential cytotoxicity of
nuraminidase and the damage caused from lower pH, cell morphology change was
observed and cell viability was measured. Results from the Wright-Giemmsa
staining indicated that cell monolayers did not exhibit apparent morphological
change or detachment after four hours of Sia cleavage reaction (Figure. 6).

FIGURE 7. ASSESSMENT OF CELL VIABILITY TO NURAMINIDASE
TREATMENTS BY NEUTRAL RED UPTAKE

Potential cytotoxicity of nuraminidase treatment was measured by Neutral red uptake. Experiments
were carried out in triplicate. There were no differences among different sampling points (P > 0.05)

As for cell viability, the experiment with trypan-blue viable cell counting was first
performed. Cell monolayers after nuraminidase treatment for four hours were
severely lysed and the integrity of cell membrane was not maintained. We suspected that the detachment step by the addition of trypsin to nusraminidase treated cells could possibly contribute to this destructive phenomenon.

Alternatively, cell viability was assessed by measuring neutral red uptake. The neutral red uptake assay provides a quantitative estimation of the number of viable cells in a culture. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. The uptake of neutral red depends on the cell’s capability to maintain pH gradient through production of ATP. Inside the lysosomes, there is a proton pump to maintain a lower pH inside the lysosomes. Therefore, neutral red uptake can not only be applied to assess cytotoxicity but suggests a lost function of maintenance of physiological pH in cells. Although there were no differences among different time points, however, as shown in Figure. 7, cell reached its lowest uptake of neutral red at the first hour which indicates a pH shock as NANase started reaction during the first hour of treatment. Cell monolayers then began to recover from environmental shock and to retrieve the capability of pH maintenance as observed after the one hour. Cell monolayers reached its maximum dye uptake at the third hour. We therefore adopted three
hours as the optimal NANase treatment time on porcine intestinal epithelium for subsequent experiments.

**FIGURE 8. SYNERGISTIC EFFECT OF ETEC INCUBATION AND NURAMINIDASE TREATMENT (WRIGHT-GIEMMMSA STAINING)**

Potential cytotoxicity of nuraminidase treatment and the addition of bacteria were visualized by Giemmsa-Wright staining. Morphological changes of cell monolayer were pictured by inverted microscope, magnification 200X.
To assess the cytotoxicity induced by ETEC infection after NANase treatment, untreated cell monolayers and those with three hours NANase treatment were both infected by bacteria for one hour and two hours (Sakarya et al. 2010). The results showed that two hours of ETEC incubation (Figure 8a) to porcine intestinal epithelium was cytotoxic to affect cell monolayer integrity. A three hours NANase treatment to IPEC-J2 cells prior to two-hour bacterial infection (Figure 8b) excessively destroyed cell integrity and caused cell death, despite the fact that IPEC-J2 cell monolayer was sustainable up to four hours of NANase treatment (Figure 6). While bacterial incubation time was reduced to one hour, no difference of cell monolayers was observed between one-hour ETEC incubation alone without NANase treatment and three hour NANase treatment followed by ETEC incubation (Figure 8c, d). Thus, this condition was used in subsequent studies.
To evaluate the capacities of porcine ETEC adherence to IPEC-J2 cell monolayers, the experiment was initially carried out by counting bacterial CFU. The results did not show great consistency within repeats. Thus, an alternative method using fluorescein-5-isothiocyanate (FITC) was adopted.

Bacteria were labelled by FITC in PBS (pH 9.0) for 30 minutes. After incubation with cell monolayers, adhered FITC-labelled bacteria after washing were quantified by excitation and emission wavelength set at 485 and 535 nm.

Human ETEC, H10407, and *E. coli* J5 strain were also used as references to examine bacterial binding specificity. *E. coli* J5 strain related to mastitis in cows had
the least binding capacity to IPEC-J2 monolayer. Meanwhile, the clinic human strain H10407 showed a moderate binding to IPEC-J2 cell monolayers.

The binding of ETECs to IPEC-J2 monolayer was shown in Figure 9. Isolate 13 and isolate 16 had the highest binding in all porcine ETEC isolates. Although certain isolates, such as isolate 9 and isolate 11, did not appear to be highly adhesive, in general, ETEC isolates from diarrheic piglets had greater binding capacity than H10407 and *E. coli* strain J5.
TABLE 8. EFFECTS OF NEURAMINIDASE ON BACTERIAL ADHERENCE TO IPEC-J2 CELL (M.O.I 50:1)

<table>
<thead>
<tr>
<th>E. coli isolates</th>
<th>Fluorescence Intensity of bacteria adherent to Monolayers before NA/Nase treatment</th>
<th>Monolayers after NA/Nase treatment</th>
<th>% Adherent Increase</th>
<th>E. coli isolates</th>
<th>Fluorescence Intensity of bacteria adherent to Monolayers before NA/Nase treatment</th>
<th>Monolayers after NA/Nase treatment</th>
<th>% Adherent Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>isolate 1</td>
<td>1265.00±19.74</td>
<td>1461.00±148.07</td>
<td>113</td>
<td>isolate 14</td>
<td>1031.50±128.69</td>
<td>1472.00±288.72</td>
<td>143</td>
</tr>
<tr>
<td>isolate 2</td>
<td>1257.00±36.30</td>
<td>1575.00±227.05</td>
<td>125</td>
<td>isolate 15</td>
<td>2178.03±202.86</td>
<td>1489.4±102.88</td>
<td>686</td>
</tr>
<tr>
<td>isolate 3</td>
<td>1303.50±53.03</td>
<td>2668.50±375.33</td>
<td>204</td>
<td>isolate 16</td>
<td>1248.50±301.08</td>
<td>2207.09±315.89</td>
<td>182</td>
</tr>
<tr>
<td>isolate 4</td>
<td>1075.00±157.96</td>
<td>1442.50±190.08</td>
<td>160</td>
<td>isolate 17</td>
<td>1114.00±312.80</td>
<td>3630.00±463.96</td>
<td>347</td>
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<tr>
<td>isolate 5</td>
<td>1078.00±115.17</td>
<td>1406.50±156.30</td>
<td>129</td>
<td>isolate 18</td>
<td>1396.50±63.23</td>
<td>4447.00±636.99</td>
<td>318</td>
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<tr>
<td>isolate 6</td>
<td>1100.50±14.61</td>
<td>1219.50±1.65</td>
<td>163</td>
<td>isolate 19</td>
<td>1200.20±176.04</td>
<td>2484.00±519.50</td>
<td>208</td>
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<tr>
<td>isolate 7</td>
<td>966.50±51.00</td>
<td>1570.00±286.28</td>
<td>162</td>
<td>isolate 20</td>
<td>1088.50±106.94</td>
<td>1396.00±32.91</td>
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<tr>
<td>isolate 8</td>
<td>1665.50±152.62</td>
<td>7865.00±740.07</td>
<td>510</td>
<td>isolate 21</td>
<td>1054.00±105.30</td>
<td>1911.50±162.62</td>
<td>181</td>
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<tr>
<td>isolate 9</td>
<td>1750.00±102.01</td>
<td>6840.50±740.07</td>
<td>362</td>
<td>isolate 22</td>
<td>5438.00±171.11</td>
<td>6768.50±204.22</td>
<td>1245</td>
</tr>
<tr>
<td>isolate 10</td>
<td>1275.00±144.78</td>
<td>2467.00±25.32</td>
<td>194</td>
<td>isolate 23</td>
<td>1929.50±54.29</td>
<td>2817.00±250.77</td>
<td>1313</td>
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<tr>
<td>isolate 11</td>
<td>1181.50±93.78</td>
<td>1487.00±200.72</td>
<td>126</td>
<td>isolate 24</td>
<td>993.00±129.33</td>
<td>2226.50±245.14</td>
<td>224</td>
</tr>
<tr>
<td>isolate 12</td>
<td>1253.50±179.33</td>
<td>2504.50±18.10</td>
<td>200</td>
<td>H10407</td>
<td>1143.50±133.27</td>
<td>3157.00±406.03</td>
<td>276</td>
</tr>
<tr>
<td>isolate 13</td>
<td>1352.50±256.02</td>
<td>4120.00±113.52</td>
<td>325</td>
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</tbody>
</table>

Effects of NA/Nase on ETEC adherence to desialyalted IPEC-J2 cell monolayers. FITC labelled bacteria were quantified at 485/535 wavelength. Each isolates were repeated three times. Adherent increase is presented in percentage compared to the respective control of untreated group.

One objective of this research was to determine the physiological role of Sia on glycoconjugates of porcine intestinal epithelia cells in ETEC adhesion. The result revealed that, at M.O.I. 50:1, all porcine ETECs appeared to increase adhesion to desialyalted IPEC-J2 cell monolayers. Certain isolates, such as isolate 22 and isolate 23, show a significant increase of binding, with over 10 times of enhancement. Human strain H10407 also increases its binding by 276%. In general, results on Table 8 indicate that ETECs performed better adhesion to porcine intestinal epithelium while terminal Sia was removed from cell surface.
To determine the participations of subterminal monosaccharides on glycoconjugates of porcine intestinal epithelial cells in bacterial adherence, seven different mono-sugars were applied to saturate bacteria before the binding assay. The subsequent binding ability comparing to PBS control were examined and tabulated in Table 9. The cytotoxic effect of ETEC adhesion on cell monolayers at M.O.I 50:1 was apparent in presence of certain sugar treatments. Therefore, infectious dose of bacteria was reduced to M.O.I. 20:1. Binding abilities of nineteen ETEC isolates to the epithelial cells were successfully quantified, while binding

### Table 9. Effects of Monosaccharides on ETEC Adherence to Desialylated Porcine Intestinal Epithelium in Comparison to PBS Control

<table>
<thead>
<tr>
<th>Bacteria saturated with</th>
<th>Number (percentage) of F4+ bacteria show responsive</th>
<th>Number (percentage) of F18+ bacteria show responsive</th>
<th>Number (percentage) of F4- and F18- bacteria show responsive</th>
<th>Total number (percentage) of porcine ETEC shows responsive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Increase</td>
<td>Decrease</td>
<td>Increase</td>
<td>Decrease</td>
</tr>
<tr>
<td>Glucose</td>
<td>2 (20%)</td>
<td>3 (30%)</td>
<td>5 (50%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Galactose</td>
<td>1 (10%)</td>
<td>2 (20%)</td>
<td>7 (70%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Fucose</td>
<td>3 (30%)</td>
<td>4 (40%)</td>
<td>3 (30%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Mannose</td>
<td>4 (40%)</td>
<td>2 (20%)</td>
<td>4 (40%)</td>
<td>2 (40%)</td>
</tr>
<tr>
<td>NANA</td>
<td>7 (70%)</td>
<td>0 (0%)</td>
<td>3 (30%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>3 (30%)</td>
<td>3 (30%)</td>
<td>4 (40%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>0 (0%)</td>
<td>3 (30%)</td>
<td>7 (70%)</td>
<td>1 (20%)</td>
</tr>
</tbody>
</table>

*10 F4+ isolates of ETECs were studied in this trial. 5 F18+ isolates of ETECs were studied in this trial. 4 isolates of ETECs neither expressing F4 nor F18 were studied in this trial.
abilities of five isolates (isolates 1, 2, 11, 12 and 20) were not quantified due to severe cytotoxic effects.

Bacteria showing significant changes of binding in response to sugar saturation, either increasing or decreasing, were defined responsive. In contrast, non-responsive group represents bacteria had no significant binding difference in response to sugar pre-treatment. Adherences of F4 expressing ETECs were relatively susceptible to the treatment of monosaccharides saturation. In contrast, F18 expressing ETECs were nearly non-responsive to all sugar saturations (Table 9).

In responsive bacteria, bacteria pretreated with NANA consistently increased their binding ability to desialylated cell monolayers. Similarly, 80 % of bacteria saturated with Mannose showed increasing binding ability. Moreover, it appears that there might be a fimbriae-dependent inhibition by GalNAc. Bacteria expressing F4 fimbriae seemed to have lower adherences to desialylated cell monolayers if they were pretreated with N-acetyl-Galactosamine.
TABLE 10. DIFFERENTIATION OF ETEC IN TERMS OF BINDING ABILITY.

<table>
<thead>
<tr>
<th>Porcine ETECs</th>
<th>High Adhesive ETEC</th>
<th>Low Adhesive ETEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>isolate 1</td>
<td>isolate 5</td>
<td>isolate 7</td>
</tr>
<tr>
<td>isolate 2</td>
<td>isolate 6</td>
<td>isolate 9</td>
</tr>
<tr>
<td>isolate 3</td>
<td>isolate 8</td>
<td>isolate 11</td>
</tr>
<tr>
<td>isolate 4</td>
<td>isolate 12</td>
<td>isolate 23</td>
</tr>
<tr>
<td>isolate 13</td>
<td>isolate 10</td>
<td>isolate 24</td>
</tr>
<tr>
<td>isolate 14</td>
<td>isolate 15</td>
<td></td>
</tr>
<tr>
<td>isolate 18</td>
<td>isolate 20</td>
<td></td>
</tr>
<tr>
<td>isolate 22</td>
<td>isolate 16</td>
<td></td>
</tr>
</tbody>
</table>

In Figure 9, ETEC showed various binding capacity to porcine intestinal epithelia. Among all, high adhesive ETECs are of most research interests due to their potential high pathogenicity. The adherence ability of human ETEC H10407 was regarded as a reference to evaluate binding capacity of ETEC to porcine intestinal epithelia. This value was arbitrarily used to categorize porcine ETECs into two subgroups with respects to binding capacity (Table 10).
TABLE 11. BINDING PATTERNS OF HIGH ADHESIVE ETEC IN RESPONSE TO SUGAR TREATMENTS

<table>
<thead>
<tr>
<th>Monosaccharide Saturation</th>
<th>Isolate 3</th>
<th>Isolate 4</th>
<th>Isolate 5</th>
<th>Isolate 6</th>
<th>Isolate 8</th>
<th>Isolate 10</th>
<th>Isolate 13</th>
<th>Isolate 14</th>
<th>Isolate 15</th>
<th>Isolate 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.467</td>
<td>2.000</td>
<td>0.535</td>
<td></td>
<td></td>
<td>2.203</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>0.560</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.511</td>
<td></td>
<td></td>
<td></td>
<td>2.824</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.737</td>
<td>0.267</td>
<td>1.570</td>
<td>5.816</td>
<td></td>
<td>0.693</td>
<td></td>
<td></td>
<td></td>
<td>2.208</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.589</td>
<td>2.094</td>
<td>1.551</td>
<td></td>
<td></td>
<td>0.707</td>
<td>1.899</td>
<td>3.394</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NANA</td>
<td>4.823</td>
<td>5.786</td>
<td>2.593</td>
<td>6.625</td>
<td>2.583</td>
<td>2.317</td>
<td></td>
<td></td>
<td></td>
<td>5.671</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>0.339</td>
<td>1.692</td>
<td>0.074</td>
<td>0.690</td>
<td>1.579</td>
<td>2.271</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactosamine</td>
<td>0.343</td>
<td>0.495</td>
<td>0.706</td>
<td></td>
<td></td>
<td>0.585</td>
<td></td>
<td></td>
<td></td>
<td>0.585</td>
</tr>
</tbody>
</table>

Adherence abilities of ten high adhesive and responsive ETECs were normalized into scale of ratio by respective PBS controls. Each value represents the mean value of three experiments. Only significant changes of bacterial binding (at $P<0.05$) in response to monosaccharides saturation were tabulated. Values larger than 1 indicate increasing binding ability. In contrast, values less than 1 indicate decreasing binding ability.

It had been noticed that there seemed to be certain patterns of ETECs adhesion in the presence of certain sugars. This study was especially interested in the binding patterns of high adhesive ETECs. To unveil the binding patterns, adhesion ability of individual bacteria was first normalized by respective PBS controls and present in scale of ratio.

Although there are seventeen high adhesive isolates as indicated in Table 10, the binding abilities of some isolates are not responsive to sugar saturation. Therefore Table 11 showed the binding patterns of ten high adhesive and responsive ETEC isolates in response to monosaccharide pretreatments. Values larger than 1 indicate monosaccharide treatment increase bacterial binding ability, in contrast,
values less than 1 indicate binding ability was abolished. In ten highly adhesive ETECs, bacteria pretreated with NANA consistently showed enhanced binding ability. Similar binding patterns were observed in bacteria pretreated with Fucose or Mannose.

**FIGURE 10. IDENTIFICATION OF F4 FIMBRIAE VARIANTS.**

![Image of gel electrophoresis results]

Fourteen F4+ isolated were sub-serotyped by PCR. Three pairs of primers for F4ab, F4ac and F4ad were employed. The PCR results were loaded respectively in the gel (above to bottom), and confirmed by electrophoresis image.

F4 are flexible fimbriae that occur as F4ab, F4ac and F4ad variants. Each F4 variant shows different binding affinity to ganglioside receptors (Van Den Broeck et al. 2000). To study the participation of F4 fimbriae in bacterial adherence, it was
necessary to identify the variants of fimbriae used in this study. Three pairs of primers specifically amplifying each variant were utilized as suggested by Choi and Chae (1999). Fourteen F4 positive isolates of ETECs in this study were all identified as F4ac serotype as shown in Figure 10.

FIGURE 11. IDENTIFICATION OF ETPA EXPRESSION IN PORCINE ETEC.

Expression of EtpA gene in porcine ETECs by PCR. P4 and J5 are negative controls; H (H10407) represents a positive control; NC is a control lacking of template.

EtpA was reported to optimize H10407 colonization via the association with conserved region of flagella. This protein was first identified in human prototype ETEC strain H10407 by Fleckenstein et al (2009). No report has been published on the presence of this gene in porcine ETEC. We were the first to confirm the
existence of EtpA gene in certain porcine ETEC (Figure 11). H10407 was used as a positive control to evaluate the specificity of primer designed. Six porcine ETECs (isolate 5, 7, 11, 12, 20 and 22) were found to express EtpA out of twenty-four ETEC isolates tested in this study.

**TABLE 12. CALCULATION OF POTENTIAL CORRELATION BETWEEN BACTERIAL BINDING ABILITY AND BIOLOGICAL FEATURES**

<table>
<thead>
<tr>
<th>Trait</th>
<th>DF</th>
<th>Contrast SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fimbriae</td>
<td>2</td>
<td>20639.43508</td>
<td>10319.71754</td>
<td>1.03</td>
<td>0.3815</td>
</tr>
<tr>
<td>LT</td>
<td>1</td>
<td>8442.40120</td>
<td>8442.40120</td>
<td>0.84</td>
<td>0.3736</td>
</tr>
<tr>
<td>STa</td>
<td>1</td>
<td>339.98660</td>
<td>339.98660</td>
<td>0.03</td>
<td>0.8563</td>
</tr>
<tr>
<td>STb</td>
<td>1</td>
<td>16335.51087</td>
<td>16335.51087</td>
<td>1.63</td>
<td>0.2218</td>
</tr>
<tr>
<td>East1</td>
<td>1</td>
<td>13322.50870</td>
<td>13322.50870</td>
<td>1.33</td>
<td>0.2675</td>
</tr>
<tr>
<td>Paa</td>
<td>1</td>
<td>13703.35838</td>
<td>13703.35838</td>
<td>1.37</td>
<td>0.2611</td>
</tr>
<tr>
<td>Aida</td>
<td>1</td>
<td>4764.00256</td>
<td>4764.00256</td>
<td>0.48</td>
<td>0.5012</td>
</tr>
<tr>
<td>Stx2</td>
<td>1</td>
<td>21325.39369</td>
<td>21325.39369</td>
<td>2.13</td>
<td>0.1661</td>
</tr>
<tr>
<td>Source of Isolation</td>
<td>1</td>
<td>4740.70442</td>
<td>4740.70442</td>
<td>0.47</td>
<td>0.5022</td>
</tr>
<tr>
<td>EtpA</td>
<td>1</td>
<td>10686.97155</td>
<td>10686.97155</td>
<td>1.86</td>
<td>0.3283</td>
</tr>
</tbody>
</table>

Data were analysed in a multi-way model, using the General Linear Models (GLM) procedure of SAS. Effects of each trait were tested using Bonferroni’s Multiple Comparison test, with 1% probability for acceptance.

In earlier results, we categorized porcine ETECs into two groups depending on their binding abilities. Although many colonization factors of porcine ETEC have been identified, no particular marker has been confirmed to indicate ETEC adhesion ability to porcine intestine epithelium. To look for potential candidate, we correlated
ETEC binding ability with a broad spectrum of bacteria features (Table 12). In this analysis, parameters such as bacterial fimbriae (F4ac, F18 or neither of both), pathotypes (enterotoxins and virulence proteins as listed in Table 7), location where bacteria were isolated as well as the expression of EtpA protein, were evaluated whether any of these parameters could explain the variation of porcine ETEC adherence to intestinal epithelium. The result indicated that the variation of bacterial adherence ability cannot be solely ascribed to any individual parameter analysed in the model at P<0.05 confidence.
EFFECTS OF MULTIPLE SUGARS TREATMENT ON ETEC ADHERENCE TO PORCINE INTESTINAL EPITHELIA

Figure 12. Effects of saccharide pretreatments of ETEC isolate 14 on adherence to porcine intestinal epithelium.

Figure 13. Effects of saccharide pretreatments of ETEC isolate 14 on adherence to porcine intestinal epithelium.

Figure 12 and 13 were conducted to evaluate our hypothesis of allosteric effect of ETEC adhesin when Sia was recognized. Isolates10 and isolate14 were randomly chosen for this experiment. To saturate binding adhesin on ETEC
isolate10, four sugars, NANA, Fucose, Mannose and N-Acetyl-Glucosamine, were used based on earlier results. Similarly, NANA, Fucose, Glucose and N-acetyl-Glucosamine, were chosen for saturation of ETEC isolate14. Saturations with two sugars were manipulated to imitate the binding where disaccharide recognition might be required. Isolate 10 and isolate14 saturated with Sia both performed consistently higher adherence ability than PBS control either when Sia was provided alone or with other sugars. Surprisingly, further enhancement of bacterial adherence was observed when isolate 14 was pretreated with Sia and Fucose.
3.1.5 DISCUSSION:

**EtpA expression in porcine ETEC**

A novel virulence protein, EtpA, has attracted our attention as it is a potent candidate for vaccination against the colonization of ETEC H10407 (Roy et al. 2008). The role of EtpA protein in ETEC pathogenesis has been carefully evaluated. First, mice vaccinated with a truncated 110kD, non-glycosylated version of EtpA has been proved to provide protection against subsequent intestinal colonization with ETEC H10407 (Roy et al. 2008). In this study, EtpA is confirmed to be expressed in certain porcine ETEC isolates, further studies are needed to determine whether porcine ETEC utilize EtpA in the same way as established in human H10407 strain. Thus, EtpA can possibly become a common target protein for vaccination against ETEC and simultaneously benefits human and pig. The other aspect is the potential emergence of a virulent zoonotic strain. Roy et al (2009) reported that there was an EtpA dependent colonization in H10407. The association of EtpA protein with flagella accounts for bacterial binding ability as an etpa mutant nearly lost its adherence. Thus, it is supposed that an additional
acquisition of flagella gene in EtpA expressing porcine ETECs may lead to infectious zoonotic strains that possess distinctively novel virulence factors of animal strains but are capable of transmitting to human via the aid of EtpA protein.

**Correlation of ETEC binding ability with biological parameters**

Although statistical analysis cannot not correlate porcine ETEC binding ability to a particular biological parameter, involvement of these proteins including enterotoxins, virulence factors and EtpA, in the adhesion cannot be completely ruled out at this stage. Adhesion of bacteria to host may depend on binding of a unique bacterial adhesin to a unique terminal sugar or carbohydrate motifs on cell surface glycoconjugates, however most frequently, bacteria adhesion relies on simultaneous interactions between multiple adhesins with different receptors (An and Friedman 2000). Enterotoxins and virulence proteins are found to possess different binding specificities other than fimbriae. For example, LT-I recognizes cell surface GM1 gangliosides and weakly to GD1b (Fukuta et al. 1988); and LT-II uses GD1 as its receptor rather than GM1 (Fairbrother et al. 2007).
Certain non-specific forces also determine the strength of bacterial binding (Ono et al. 1989). For instance, the exact distance between the specific epitope of the moiety and the hydrophobic ceramide moiety is critical for F5 fimbriae to achieve the best recognition by ETEC. It seems that many factors contribute to the pathogenesis of ETEC, and thus complicate our study on finding a determinant factor for ETEC adhesion.

Many earlier studies have elucidated the receptor of F4 fimbriae is a β-linked Galactose (Mouricout 1991; Blomberg et al. 1993; Van Den Broeck et al. 2000). However, Grange et al. (2002) suggested that HexNAc is the minimal recognition sequence for F4 adhesin-binding activity instead and the presence of a terminal galactose residue is not essentially required for recognition but enhances F4 adhesin binding. The discrepancies among earlier reports could be due to an over simplified experimental design. In fact, most studies to evaluate the binding specificity of ETEC adhesins were carried out by using purified glycan or receptor. The conformation of purified gangliosides and glycoprotein may not remain identical as in vivo after preparation procedures and thus affect the results.
Roles of Sialic acid and penultimate saccharides on ETEC adhesion

To elucidate the pathogenesis of ETEC, our research was focused on the interactions between bacterial adhesins and cellular glycoconjugates. This project started with determining the involvement of Sia on porcine intestinal epithelial cells in the binding of ETEC by using a non-transformed porcine jejunum epithelial cell line (IPEC-J2) as an in-vitro model. This cell line has been shown to secrete glycocalyx bound mucins and its functional integrity has made it a relevant model system for porcine intestinal pathogen-host cell interactions studies (Schierack et al. 2006).

Sialic acid on the eukaryotic cell surface is an important determinant responsible for cell's interaction with other cells and invading microbes. In term of cell-cell interaction, cellular Sia could act as anti-recognition agent, in this way, it marks cells being “self” and prevent cells from sequestration. There have been many researches supporting this observation. For example, the uptake of desialylated glycoproteins by hepatocytes was firstly reported in 1971 and lately, Schauer et al (2009) demonstrated that desialylation of erythrocytes and lymphocytes leads to sequestration from circulation by liver and spleen
macrophages through galectin, a family of lectin which binds to beta-galactosides. As such, it is believed that the exposure of Gal residue on erythrocytes and lymphocytes and trapping by Gal recognizing receptor on macrophages account for this phenomenon (Wiederschain 2009). Electronegative nature of Sia together with its bulky, hydrophilic chemical structure could also possibly contribute to this masking effect. They influence the conformation of, for example, gangliosides and contribute to the supramolecular structures in cell membranes, thus influencing their functions.

In contrast to masking, Sia also participate in many cellular recognition processes as a ligand/receptor with specific proteins such as lectins. Sialic-acid-binding Immunoglobin like lectins (Siglecs) are thought to promote cell-cell interaction and mediate the function of cells in the innate and adaptive immunity via glycan recognition (Crocker et al. 2007).

Cellular sialic acid mostly comes to attention due to its pathophysiological role on bacterial adherence. For example, Sia-specific adhesin, SabA, on *H. pylori* and hemagglutinin (HA) of influenza are both proved to recognize sialic acid, thus suggesting that Sia can function as a ligand/receptor for pathogen recognition (Severi et al. 2007; Schauer 2009). In contrast, removal of cellular Sia from
terminal position of glycoconjugates gives a better condition for *S. aureus*
attachment. This indicates an masking function of cellular Sia on bacterial
adherence (Sakarya et al. 2010). It is hard to describe a general role of Sia as the
environment to which it is bound may influence the biological effects.

This project utilized neuraminidase to remove the terminal Sia on the surface of
porcine intestinal epithelium (IPEC-J2) to study the adhesion of porcine ETEC.
Neuraminidase from *Clostridium perfringens* was applied to hydrolyzes α(2→3),
α(2→6), and α(2→8)-glycosidic linkages of terminal sialic residues. The subsequent
desialylated epithelium was demonstrated to enhance ETEC adherence as shown
in Table 8. These studies imply that sialic acid expressed on the porcine intestinal
epithelium may affect ETEC adherence via specific (sub-terminal monosaccharides
as a receptor) or/and non-specific (surface electric charge change, conformational
change) mechanisms.

To illustrate the mechanism of how sialic acid is involved in ETEC binding to
porcine intestinal epithelium, we first hypothesised Sia acts as an anti-recognition
agent by shielding recognition sites, such as penultimate monosaccharide of glycan
chains on gangliosides or glycoproteins. Seven monosaccharides, GlcNAc, GalNAc,
Glu, Gal, Fuc, Man and NANA, commonly found as components of gangliosides are
were utilized as competitive inhibitors to saturate the recognition sites of binding adhesins on bacteria (Yu et al. 2007). Table 9 summarized monosaccharide pre-treatment ETEC adherence to desialylated cells comparing to respective PBS controls.

Monosaccharides were initially regarded as competitive inhibitors in order to determine the sub-terminal monosaccharide which was presumed to be responsible for the binding of bacterial adherence. Our result suggested that N-acetyl-Galactosamine may be the receptor for F4 fimbriae recognition as F4 ETEC saturated with GalNAc diminished their adherence (Table 9). This observation is supported by Grange et al. (2002) who reported that F4 fimbriae bound preferentially to a β-linked N-acetyltetrasamine, such as N-acetyl-Galactosamine.

In contrast, all responsive ETECs showed greater adhesion to NANA treatment. It seems that binding of porcine ETEC to desialylated cell monolayers could be enhanced in response to the pre-treatment of NANA. Likewise, 80% of responsive ETECs had higher binding performance while pretreated with Mannose.

The observation that NANA was capable of improving bacterial adherence seems contradictory to our previous discussion. Table 8 showed that the removal of
Sia increased bacterial adherence and suggested that Sia might be a masking agent. However, Table 9 showed that binding of free Sia to ETEC enhanced bacterial adherence instead. These two seemingly contradictory observations suggest that our hypothesis of Sia being a masking agent might not be correct.

Other hypothesis assuming the participation of Sia in ETEC adherence via non-specific mechanism was examined. Two possible models are proposed to explain the contradicting roles of Sia in ETEC adherence to cell monolayers:

(i) Sia’s electronegative nature together with its bulky, hydrophilic chemical structure could possibly contribute to bacterial binding.

(ii) After recognition, conformation change of the bacterial Sia-binding lectin influences bacterial adherence.

The negative charges of Sia confer electronic repulsion forces that influence the conformation of gangliosides and contribute to the supermolecular structure in cell membranes, thus affecting their functions (Schauer 2000). Therefore, microbes may have better attachment to desialylated cell monolayer due to the abolishment of electronic repulsion force on cell surface. However, other than NANA, 80% of Mannose saturated bacteria also improved binding (Table 9). It seems that
negative-charge-carrying Sia is not the only monosaccharide capable of enhancing ETEC adherence. Therefore electronic repulsion forces from Sia cannot well explain our results.

Finally, the last model suggests there might be a conformation change of the bacterial lectin after Sia is recognized. It is believed that this conformation change contribute to an environment facilitating better attachments and favouring bacterial binding. This explanation seems more likely as certain monosaccharides in our studies, for examples, NANA, Fucose and Mannose, consistently enhance bacterial adherence in most responsive porcine ETECs (Table 9 and Table 11).

An allosteric influence of Sia was first proposed in 1990. As being a masking agent to hinder the recognition of Gal residue, Sia was postulated to bind to receptor and thus exerted modulating effects on galactose-mediated binding (Lee, et al. 1990). In addition to allosteric effect, sialic acid specific bacterial lectin has been confirmed on ETEC (Lindahl et al. 1988), therefore we hypothesised that Sia exerts a cooperative allosteric effect on the attachment of porcine ETEC to cell monolayers. In this sense, the conformation of bacterial Sia-binding lectin changes after association with free Sia, and this structural change possibly facilitates the
subsequent recognition of bacterial ligand toward subterminal oligosaccharides on the desialylated cell monolayers (Ono et al. 1989).

Binding potency of terminal sialic acid residue on glycoprotein dramatically decreases with the increasing number of branch on the oligosaccharides (Lee et al. 1990). Thus it is possible that immobilized Sia on the epithelial monolayer are not as recognizable by bacterial adhesin as free sialic acid used to saturate ETEC in the study. This observation reasonably explains why porcine ETEC has less adhering potency to cell monolayers than to desialylated ones.

Grange et al. (2002) suggested that there is a minimal recognition sequence for microbial recognition. In their study, HexNAc is proposed to be the minimal recognition sequence for F4 adhesin-binding activity while the presence of a terminal galactose residue is not essentially required for recognition but enhances F4 adhesin binding. Our results support this model as Sia appears to be the minimal recognition sequence that facilitate bacterial adherence. The highest binding appears when Sia and Fucose were applied to isolate14 and Sia and Mannose to isolate10 (Figure 12 and 13). Fucose and Mannose alone have already been
noticed to increase ETEC binding ability in Table 11, therefore it is not surprising that there are synergistic effects of binding when Sia is simultaneously added.
The expression of EtpA in certain porcine ETEC has been confirmed, although the exact role of this protein in pathogenesis of porcine ETEC remains unclear. It is essential to determine the expression of flagella in porcine ETECs in order to predict whether EtpA also associates with flagella for subsequent colonization. Molecular cloning of recombinant EtpA would help better understand the effects of EtpA on porcine ETEC pathogenesis.

The in-vitro porcine intestinal epithelial monolayer was used in this project to study porcine ETEC adherence. It is noticed that the pathogenesis of ETEC is complicated than previously reported as ETEC adherence to host cells is a cumulative process which requires many specific and non-specific interactions among host and bacteria.

Further study to determine the role of sialic acid in ETEC adherence to swine intestinal epithelia suggests that Sia might be a minimal recognition sequence for bacterial attachment. The binding of Sia to bacterial lectin on ETEC modulates the conformation change and thus influences ETEC adherence to porcine epithelia. Fucose and mannose are found not essentially required for binding but enhance
bacterial binding ability. Although our results can be reasonably explained by the allosteric model adopted from Lee et al. (1990). Further research is still needed to validate this model.

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