A broadly effective vaccine against porcine post-weaning diarrhea

by

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B.S., Huazhong Agriculture University, 2012
M.S., Huazhong Agriculture University, 2015

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
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Abstract

Enterotoxigenic *Escherichia coli* are the major causes of porcine post-weaning diarrhea (PWD). Currently, no licensed vaccines for ETEC exist. However, studying ETEC helps to better understand the role of these organelles in biology and pathogenesis, opens a new door to disease diagnosis, prevention and treatment, and enables development of effective vaccines.

In Chapter 2, the study was focused on mapping the immuno-dominant or neutralizing epitopes from the adhesive subunit FedF of F18 fimbriae. Our data showed that seven immune-dominant epitopes were identified from FedF subunit. Epitope fusions induced anti-F18 antibodies in subcutaneously immunized mice. Moreover, antibodies derived from each fusion significantly blocked adherence of a F18-fimbrial *E. coli* bacteria to pig intestinal cell line IPEC-J2. While all seven epitopes exhibited neutralizing activity, results from this study identified FedF epitopes #3 (IPSSSGTLTCQAGT) and #7 (QPDATGSWYD) as the most effective for antibodies against F18 fimbrial adherence and suggested their future application in PWD vaccine development.

In Chapter 3, we further identified B-cell immunodominant epitopes from K88 fimbrial major subunit (also adhesin) FaeG. We found that while all nine FaeG epitope fusions induced antibodies to K88 fimbria, anti-K88 IgG antibodies derived from epitopes #1 (MTGDFNGSVD), #2 (LNDLTNGGTK), #3 (GRTKEAFATP), #4 (ELRKPDGGTN), #5 (PMKNAGGTKVGAVKVN) and #8 (RENMEYTDGT) significantly inhibited adherence of K88-fimbrial bacteria to porcine intestinal cell line IPEC-J2, indicating the ability of these peptides to neutralize EPITOPES of K88 fimbrial major subunit FaeG and suggesting the future application of FaeG epitopes in ETEC vaccine development.

In Chapter 4, a PWD multiepitope fusion antigen (PWD-MEFA) was constructed. Our data showed the expressed fimbriae-toxoid PWD MEFA protein, which was approximately 40 kDa, was verified in Western blot analysis using anti-FaeG, anti-K88epitope-fusion, anti-F18epitope-fusion, anti-CT, anti-STa, and anti-Stx2e antiserum, respectively. Mice SC immunized with PWD MEFA protein developed strong anti-K88, anti-F18, anti-LT and anti-STb IgG antibody responses, and moderate anti-Stx2e and anti-STa IgG responses. Moreover, mouse serum antibodies inhibited adherence of K88- and F18-fimbrial ETEC bacteria and neutralized LT, STa, STb and Stx2e enterotoxicity. Additionally, double mutant LT (dmLT, $LT_{R192G/L211A}$) adjuvant up-immunoregulated PWD MEFA anti-fimbriae and antitoxin antibody responses. These results indicated that this fimbriae-toxoid PWD MEFA induced broadly anti-fimbriae and anti-toxin antibodies, and suggested antigen candidacy for developing an effective vaccine against PWD.

In Chapter 5, we optimized this MEFA to be expressed as a holotoxin-structured and GM1-binding protein in a live host strain to induce mucosal antibodies against ETEC adhesins and toxins. Our data showed that optimized PWD adhesin-toxoid MEFA formed a holotoxin structure and bound to GM1 receptor, and *Salmonella* Ty21a strain, as well as porcine field *E. coli* isolate G58 to produce the new adhesin-toxoid MEFA and secreted the protein outer-membrane. These results suggest that Ty21a or G58 host producing the GM1-binding adhesin-toxoid MEFA can potentially be an effective mucosal vaccine against PWD.

In summary, this study investigated the immunodominate and neutralizing epitopes of F18 fimbrial adhesin subunit FedF and K88 fimbrial adhesin subunit FaeG, and also constructed and optimized a PWD fimbriae-toxoid MEFA inducing broadly effective protection against PWD-associated ETEC infection.

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Chapter 1 - Literature review

1.1 Overview of porcine post-weaning diarrhea

Post-weaning diarrhea (PWD) is common during the first 2 weeks after weaning. PWD-associated symptoms include watery diarrhea and dehydration, which result in acute reduced weight gain and death (Rhouma, Fairbrother, Beaudry, & Letellier, 2017). PWD is a multifactorial disease, which makes prevention difficult **(Fig 1.1)**. Weaned piglets are susceptible to cold or heat stress due to poor regulation of body temperature. Housing temperature plays a critical role in the health of weaned pigs. A difference of over 10 °C in between daytime and nighttime housing temperatures can lead to an approximately 30% increase in the diarrhea rate. The most suitable environment for weaned piglets is 26–28 °C with approximately 70% humidity. Higher humidity increases the incidence of diarrhea. Additionally, good sanitation, especially regular cleaning of bedding and other equipment, can also reduce the risk of PWD effectively (Rhouma et al., 2017).

The structure and function of the digestion system are not fully developed in piglets during the first two weeks after weaning (Heo et al., 2013). The small intestinal villi become shorter than the crypts due to the diet change, which disturbs the absorption of nutrients and can lead to damage by enteric pathogens. Furthermore, weaned piglets have lower digestive enzyme contents and activities, which reduces the absorption of nutrients during the first two weeks after weaning (Parra-Suescun, Agudelo-Trujillo, & Lopez-Herrera, 2015). Simultaneously, a reduction of lactic acid due to the interruption of sow milk intake after weaning increases the pH value in the gastrointestinal (GI) tract, leading to an imbalance of the microbial flora. Intestinal histological changes lead to reduced intestinal function, which not only provides a nutritional

basis for the attachment and proliferation of pathogenic microbes but also increases the GI osmotic pressure, resulting in osmotic diarrhea (Pluske, Turpin, & Kim, 2018). Under such conditions, any improper feeding methods, e.g., a higher proportion of plant proteins, fat or cellulose in the feed, an excess of iron, or a deficiency of zinc, selenium or vitamins, etc., can easily lead to intestinal dysfunction and diarrhea (Heo, Kim, Yoo, & Pluske, 2015; Metzler-Zebeli et al., 2010; Schwab et al., 2015; Sun & Kim, 2017).

The population density of the piglets is another potential factor that contributes to PWD, likely due to the ease of disease transmission (Sun & Kim, 2017). Additionally, the situation of sow farrowing, pregnancy and parturition may also be vital for controlling PWD. However, studies of these factors have been limited to local farm reports and have failed to resolve contradictory observations (Hayakawa, Masuda, Kurosawa, & Tsukahara, 2016). Genetic factors related to the gut receptors of neonatal piglets are also important for infection caused by enteric pathogens, particularly enterotoxigenic *Escherichia coli* (ETEC) (Ogundare, Fasanmi, & Fasina, 2018). For example, the receptors for F4 fimbriae can be isolated from the pig intestine at all life stages, while the receptors for F18 fimbriae can be isolated in pigs commonly after 2-3 weeks of age. Since the success of colonization determines ETEC infection, pigs that do not have fimbria receptors are not susceptible to PWD (Heo, 2013).

The enteric pathogens that cause PWD include rotavirus, ETEC, coccidia, sapovirus and *Cryptosporidium parvum* (Rhouma et al., 2017; Zlotowski et al., 2008). These pathogens either damage intestinal epithelial cells or impair the neonatal immune system, resulting in PWD or enhancing the colonization and infection of ETEC if a mixed infection occurs. For example, ETEC has the ability to produce enterotoxins, such as heat-labile toxin (LT), heat-stable toxin (ST) and Shiga toxin type 2e (Stx2e), to stimulate the secretion of excessive fluid from intestinal

cells and cause diarrhea. During infection, ETEC must first adhere to the intestinal mucosa to release these enterotoxins. Therefore, adhesins, such as fimbriae, are among the main virulence factors in ETEC infection (Dubreuil, Isaacson, & Schifferli, 2016).

1.2 The major pathogen: ETEC

Proliferation of β-hemolytic ETEC in the swine intestine has been considered the major cause of PWD, which is associated with damage to the pig neonatal intestinal epithelium and weakness of the gut barrier function (Rhouma et al., 2017). During weaning, the change in diet, the loss of milk, which supports passive immunity, and the incomplete development of active immunity increase the risk of pathogenic attachment, commonly mediated by binding of fimbrial adhesins to the neonatal mucosal surface (Heo et al., 2013). Contaminated food and water are the most common causes of ETEC infection.

Adhesion of the fimbriae of ETEC bacteria to glycoprotein receptors on the pig small intestinal surface is essential for the release of enterotoxins from ETEC to pig intestinal epithelium cells. Enterotoxins directly result in the secretion of water and electrolytes from intestinal epithelial cells into the intestinal lumen, causing diarrhea and dehydration (Dubreuil et al., 2016). This chapter provides a summary of the fimbriae of porcine ETEC bacteria, such as K88ac fimbriae and F18ac fimbriae, and their roles in ETEC infection in piglets. A review of ETEC toxins associated with PWD, including LT, STa, STb and Stx2e, is also included in this chapter **(Fig 1.2)**.

1.2.1 ETEC fimbriae

Fimbriae or pili on the surface of bacteria are virulence factors with a hair-like structure. Fimbriae are very common in gram-negative bacteria as well as some gram-positive bacteria (Knight & Bouckaert, 2009). Unlike flagella, fimbriae usually do not modulate bacterial movement (except type IV pili, which utilize force generated by adherence during retraction to promote bacterial movement) (Hedlund et al., 2001; Mortezaei et al., 2015). As the major adhesin on the bacterial surface, fimbriae are able to mediate bacterial adherence to host cells or tissues and to avoid clearance by mucus secretion and peristalsis (Ramboarina et al., 2010). Many fimbriae have been characterized, such as chaperone-usher fimbriae (including type 1 fimbriae and P-pili), type IV pili, curli pili and sortase assembled pili (in gram-positive bacteria) (Ramboarina et al., 2010). Fimbriae are considered potential drug targets and vaccine candidates via inhibition of bacterial adhesion (Hallander et al., 2009; Ramboarina et al., 2010).

Porcine ETEC fimbriae adhere to specific receptors on piglet intestinal epithelial cells and module the colonization of bacteria (Luo, Van Nguyen, de la Fe Rodriguez, Devriendt, & Cox, 2015). There are six different fimbrial subtypes, K88 (F4), K99 (F5), 987P (F6), F41 (F7), F17 and F18, in porcine-specific ETEC strains (Dubreuil et al., 2016; Luppi et al., 2016). Other factors, like adhesin involved in diffuse adhesion (AIDA-I), are also related to pig diarrhea disease (Ravi et al., 2007; Zhao, Chen, Xu, Song, & Liu, 2009). K88 (F4) and K99 (F5) are the most common fimbriae among porcine ETEC infections (Luppi et al., 2016; Pereira et al., 2016).

1.2.1.1 K88 fimbriae

K88 (F4) fimbriae are the most frequently detected in neonatal diarrhea and post-weaning diarrhea (Melkebeek, Goddeeris, & Cox, 2013). K88 (F4) fimbriae have three variants, F4ad

(K88ad), F4ac (K88ac), which by far is the most common variant worldwide, and F4ab (K88ab) (Melkebeek et al., 2013; W Zhang, 2014). Conserved determinants such as K88a can be found in all three variants, while variable determinants such as K88b, K88c, and K88d are not shared among the three variants (Dubreuil et al., 2016). The specificity of binding among three variants relates to FaeG, the major structural subunit of K88 (F4) fimbriae, which contains the adhesive domain, whereas the minor tip subunit FaeC is not associated with fimbria-binding activity (Dubreuil et al., 2016). The conformation of the FaeG binding site is heterogeneous among K88 variants (Moonens et al. (2015).

K88 (F4) fimbriae have been studied extensively. Four putative receptors on pig intestinal epithelial cells that are specific to K88 fimbriae have been identified: intestinal mucin-type glycoproteins (IMTGPs), enterocyte membrane-associated transferrin (GP74), intestinal neutral glycosphingolipids (IGLads) and porcine aminopeptidase N (APN) (Table 1.1) (Rampoldi et al., 2011; Xia et al., 2018; Xia, Zou, et al., 2015). The major difference among these four receptors is their carbohydrate composition. Based on their receptors and adherence to three K88 variants, piglets have been grouped into eight phenotypes, which are types A-H (Dubreuil et al., 2016; Nguyen et al., 2017). The relevant young pig phenotypes, host receptors on porcine epithelial cells, K88 fimbriae variants and ETEC infections are listed in Table 1.2. K88ac is considered by some to be the only variant of significance in swine disease (Dubreuil et al., 2016). Thus, recent studies focus on the relationship between K88ac fimbriae and their host receptors on the piglet small intestinal mucosa, as well as the binding domain and binding ability of K88ac fimbriae.

The receptor for *E. coli* K88ac$^+$ fimbriae was extracted from mucus from the piglet small intestine by affinity chromatography and was initially reported to be an 80-kDa glycoprotein

(Jin, Marquardt, and Zhao (2000). Other receptors, 26 and 41 kDa proteins, were also characterized as K88ac fimbrial adhesin-specific receptors in the piglet small intestinal mucosa and concluded to be responsible for nearly all K88ac fimbrial binding to the piglet small intestinal mucosa (Zhou et al. (2013). K88ac$^+$ ETEC could bind efficiently to the intestinal porcine epithelial cell lines IPEC-1 and IPEC-J2 (Verdonck et al., 2008). Amino acids 125-163 of the FaeG subunit have been reported as the variant-specific binding area of K88 (W. Zhang, Fang, & Francis, 2009). Moreover, amino acids 64-107 of the FaeG were shown that associated with the receptor-binding domain (Cao et al., 2013). Live *E. coli* cells have been found that could be efficiently captured and immobilized by antibodies induced by K88ac fimbriae (Das et al., 2013). The tissue-specific mRNA expression of PR-39 in China Jinhua pigs and Landrace pigs was compared during K88ac fimbriae challenge (Gao et al., 2014). The mRNA expression data showed that Jinhua pigs had a stronger response to ETEC K88ac challenge than did Landrace pigs, suggesting that the two breeds have genetic differences in resistance to K88ac$^+$ ETEC infection. Porcine F4ac-binding milk fat globule membranes were demonstrated that consisted of lactadherin, butyrophilin, adipophilin, acyl-CoA synthetase 3, and fatty acid-binding protein 3 and suggested that the interaction of these proteins with F4ac fimbria may obstruct ETEC attachment and colonization (Xia, Zou, et al., 2015). K88 fimbriae were also reported to upregulate IL-1beta, IL-8 and TNF-alpha (Li et al. (2016).

1.2.1.2 F18 fimbriae

F18 is a typical fimbria with one copy of the adhesive subunit FedF at the fimbrial tip, and FedF residues 60-109 are important for attachment (Dubreuil et al., 2016). F18 fimbriae are soft filaments with a length of 1-2 μm under the electron microscope, and they cover the

bacterial surface like hair. The diameter of the fimbriae is approximately 6.7 nm, which can mediate the adhesion of bacteria to the intestinal mucosa. Unlike K88 fimbriae, which cause only porcine diarrhea disease, F18 fimbriae are also associated with porcine edema disease. Based on antigen specificity, two variants have been classified, F18ab and F18ac. The F18ab serotype can be found in ETEC and Shiga-toxin producing *E. coli* (STEC), while F18ac is associated with only ETEC. $F18^+$ ETEC is closely related to diarrhea in weaned pigs, whereas $F18^+$ STEC is associated with swine edema disease.

Two F18 fimbria variants are highly homologous within the FedF subunit, resulting in binding to the same receptors on the surface of the pig gut (Luppi et al., 2016). The C-terminus of FedF is conserved, with no amino acid residue mutations between the two variants. The N-terminus of FedF includes a crystal structure, which supports binding to blood cell molecules, and a polybasic loop, which stabilizes the interaction of F18 with glycosphingolipids on the host intestinal cell surface. However, other evidence suggests that the F18 adhesin does not agglutinate erythrocytes and that its adhesion is not inhibited by mannose (Dubreuil et al., 2016). FedA, with a size of approximately 15.1 kDa, is the major structural subunit of fimbriae. Data from electron microscopy studies suggest that FedA subunits form a symmetrical single-helix structure in a zigzag manner (Hahn et al., 2000).

The FedF subunit of F18ab and F18ac binds to the same receptor molecule on piglet intestinal epithelial cells. The genetic factors related to F18 susceptibility in pigs include the FUT1 alpha (1, 2)-fucosyltransferase gene located on porcine chromosome 6. FUT1 gene encodes a glycosyltransferase which adds fucose residues to F18 glycoprotein receptors for an entire function of fimbrial adherence (Dubreuil et al., 2016). This adhesion can be blocked by antibodies in the milk glycans. Receptors for F18 fimbriae can only be observed in the pigs after

2-3 weeks of age (Luppi et al., 2016), which may suggest the possibility of breeding F18-resistant pigs.

Efforts have been made to prevent F18 ETEC infection. Researchers tried to construct four nanobodies specific for the lectin domain of the F18 adhesive subunit FedF and found that these nanobodies inhibited the attachment of fimbriated pathogens, which could be used as a strategy against $F18^+$ ETEC infection (Moonens et al., 2014). Cranberry extract also was used to inhibit the attachment of $F18^+$ *E. coli* to the pig intestinal surface (Coddens, Loos, Vanrompay, Remon, & Cox, 2017). Researchers from South Korea prepared an inactivated *Salmonella* ghost delivery system to expresses FedF and FedA, as well as the recombinant Stx2eB, as a vaccine candidate against porcine edema disease (Won & John Hwa, 2017; Won, Kim, & Lee, 2017; Won & Lee, 2016, 2018). Preventing swine ED and PWD caused by $F18^+$ *E. coli* has been investigated extensively, especially by using fimbrial antigens. However, no vaccine has been licensed. Unlike K88 fimbriae, which can be used as a protective immunogen for vaccine development, F18 fimbriae, due to their structural characteristics, show lower immunogenicity (Verdonck et al., 2007). Thus, a large number of F18 fimbriae are required to induce protective antibodies against ED and PWD (Verdonck et al., 2002). Recently, studies identified the neutralizing peptide and epitopes, which could be used for subunit vaccine construction (Ti Lu, Seo, Moxley, & Zhang, 2019).

1.2.2 ETEC toxins

Enterotoxins produced by ETEC include heat-labile enterotoxins (LT) and heat-stable enterotoxins (ST). The ETEC LT, STa and STb toxins, alone or more frequently, together, are the virulence determinants that cause watery diarrhea and dehydration in humans and animals.

1.2.2.1 Heat-labile enterotoxins (LT)

LT has two subtypes differentiated by their B subunits: LTI and LTII. LTI includes LTIh (human) and LTIp (porcine) based on their host specificity.

LT is a 1A5B toxin and is homologous to cholera toxin (CT) produced by *Vibrio cholerae*. LT B subunits form a pentamer and bind to GM1 receptors on the host cell surface, which is important for their toxic effect (Dubreuil et al., 2016; W. Zhang et al., 2019). The B subunit may also bond to blood group determinants, as type A blood and A and B glycolipids can enhance its binding to the pig brush border and human erythrocytes (W. Zhang et al., 2019). After binding to GM receptors, the enzymatic A subunit enters the cytoplasm to elevate intracellular cyclic AMP (cAMP) levels, stimulating Protein Kinase A (PKA) activity and causing the secretion of sodium and chloride into the lumen. The LT A subunit can also bind to LPS on the surface of gram-negative bacteria, but this attachment does not inhibit the binding of B subunits to GM receptors (Mudrak & Kuehn, 2010).

The holotoxin of LT enters pig intestinal epithelial cells to initiate fluid secretion and watery diarrhea. After binding to GM1, LT is internalized by receptor-mediated endocytosis, followed by the separation of the A and B subunits in the Golgi and the subsequent release of the A1 subunit into the cell cytosol at the endoplasmic reticulum by proteolytic cleavage (Mudrak & Kuehn, 2010). The A1 subunit will stimulate adenylate cyclase (AC) via the accumulation of cyclic adenosine monophosphate (cAMP) through ADP-ribosylation of the alpha-subunit of a Gs protein, resulting in the activation of the membrane chloride channel cystic fibrosis transmembrane conductance regulator (CFTR) (Moss, 1978). Activation of CFTR enhances

water and electrolyte secretion to the intestinal lumen and inhibits salt reabsorption, resulting in watery diarrhea (W. Zhang et al., 2019) **(Fig 1.3)**.

1.2.2.2 Heat-stable enterotoxins (ST)

Heat stable ST is a small molecule with a unique structure. ST has two variants: STa (type I) and STb (type II). STa is a peptide of 18 or 19 amino acids that is produced by ETEC and associated with diarrhea in humans (hSTa, STaH; STh) and animals (pSTa, STaP, STp) (Dubreuil et al., 2016). The C- terminus of STa has a 13-amino-acid peptide with three disulfide bonds and is highly conserved and essential for enterotoxicity. The disulfide bonds of Sta are needed for its toxic activity, and STaH and STaP have cysteine residues in the same positions to promote their formation (Dubreuil et al., 2016; Loos et al., 2012).

STa binds to guanylate cyclase C (GC-C) receptors on intestinal epithelial cells and activates their intracellular catalytic domain, resulting in increased intracellular cGMP levels to stimulate cGMP-dependent protein kinase II (cGMPKII) for the phosphorylation of CFTR. This process leads to water and electrolyte secretion to the lumen (Dubreuil et al., 2016). The elevation of cGMP levels blocks phosphodiesterase 3 (PDE3) from increasing the cAMP level, likely enhancing water and electrolyte secretion to the lumen and inhibiting salt reabsorption **(Fig 1.3)**.

STb is related to porcine diarrhea, with 48 amino acid residues, including four cysteine residues that form two disulfide bonds, which are important for toxicity (Beausoleil, Labrie, & Dubreuil, 1999; Taillon, Nadeau, Mourez, & Dubreuil, 2008). Two helical areas in residues 10-22 have been found to be amphipathic to promote solubility and also important for the toxicity of

STb (Sukumar et al., 1995). Residues 38-44 are hydrophobic and may be related to STb polymerization (Dubreuil et al., 2016).

STb binds to sulfatides on the pig intestinal epithelium but does not increase cAMP or cGMP levels in the cells (Rousset & Dubreuil, 1999). However, STb can stimulate a toxin-sensitive GTP-binding regulatory protein to increase the secretion of nonchloride anions from gut epithelial cells. STb can also form a pore on the cell membrane and change secretion without inducing cell death (Rousset, Harel, & Dubreuil, 1998) **(Fig 1.3)**.

1.2.2.3 Stx2e

Stx2e is a member of the Shiga toxin family. Shiga toxin is a cytotoxic protein and is the major virulence factor of STEC and EHEC (Melton-Celsa, 2014). The Stx1 (Stx1a) and Stx2 (Stx2a) subtypes are most commonly associated with STEC infections. Stx1a is highly conserved with two variants: Stx1c and Stx1d. This subtype is related to STEC in sheep and shows lower pathogenicity in humans (Pacheco & Sperandio, 2012). Stx2a is more important for human infection. Stx2 has several variants including stx2c, stx2b and stx2d-activatable. Among these variants, the stx2d-activatable toxin is associated with hemolytic-uremic syndrome in STEC infection (Pacheco & Sperandio, 2012). Additionally, stx2e, stx2f and stx2g are related to STEC infection in animals. Among these toxins, Stx2e causes fatal edema disease in swine (Melton-Celsa, 2014). Shiga toxin commonly causes the accumulation of water in animal intestines, as well as kidney damage (Krause, Barth, & Schmidt, 2018).

Shiga toxin 2e (Stx2e) produced by Shiga-toxin producing *Escherichia coli* (STEC) or ETEC (commonly related to F18-expressing ETEC) is the major virulence factor of porcine edema disease (ED) within 1-2 weeks after weaning (Beutin et al., 2008). This disease is also

known as enterotoxemia and leads to a high mortality rate and significant economic losses to the pig industry (Sarrazin, Fritzsche, & Bertschinger, 2000). Stx2e is the 1A5B holotoxin that consists of an enzymatic A subunit (approximately 32 kDa) and five B subunits (approximately 7.7 kDa per subunit) and causes neurological ataxia and local edema. The Stx2e A subunit is cleaved into an A1 domain and an A2 peptide; the A1 peptide is enzymatic, and A2 binds to the B pentamer. A disulfide bridge supports the connection between A1 and A2 (Gyles, 2007). The Stx B pentamer binds to globotriosylceramide (Gb3) or globotetraosylceramide (Gb4), the glycolipid receptor on host cells, and enters the cytoplasm via endocytosis (Melton-Celsa, 2014) **(Fig 1.3)**.

1.3 ETEC vaccine

1.3.1 Vaccines

A vaccine is a biological preparation that prevents disease by enhancing the host immune system (S. A. Plotkin, 2011). Vaccines stimulate active immunity if they comprise weakened, killed, or fragmented disease-causing microorganisms or toxins and passive immunity if they comprise antibodies or lymphocytes that are related to the disease. The routes of vaccination include injection, oral immunization and intranasal administration (S. A. Plotkin, 2011). Edward Jenner, who used the vaccinia virus from cowpox to inoculate a boy and protect against smallpox in 1796, with the first smallpox vaccine made in 1798, is considered the founder of vaccinology (Stewart & Devlin, 2006). Subsequently, a cholera vaccine (1897) and an anthrax vaccine (1904) were designed by Louis Pasteur (S. Plotkin, 2014). This technology was also improved from using live vaccines to live-attenuated or inactivated vaccines from the late 19th century to the middle of the 20th century. Two successful polio vaccines that successfully eradicated polio

disease were created during the late 20th century, with the development of viral tissue culture technology (S. Plotkin, 2014). However, vaccine safety, especially for live-attenuated or inactivated vaccines, has become a major issue for global immunization programs. New techniques were required for vaccinology to develop a safe and cost-effective. In the 21st century, advances in molecular genetics built a new platform for vaccinology (Stern & Markel, 2005). DNA vaccines, recombinant vaccines, plant vaccines, subunit vaccines and novel adjuvants have been developed and applied successfully (Stern & Markel, 2005).

Several new technologies for developing safe vaccines have been widely used in the field. These technologies can shorten the time required for vaccine development by facilitating simple and safe antigen/antibody presentation on subunit vaccines.

Reverse vaccinology is a new technology for screening the genome of a pathogen and identifying immunogenic proteins for vaccine development (Amol M Kanampalliwar, Rajkumar Soni, Amandeep Girdhar, & A Tiwari, 2013). Based on genomic sequencing technology and bioinformatics, reverse vaccinology allows the rapid identification of vaccine candidates without the use of traditional reinfection and reculturing technologies in the laboratory (Amol M Kanampalliwar, Rajkumar Soni, Amandeep Girdhar, & Archana Tiwari, 2013). However, this technology cannot predict the antigenic targets on polysaccharides or lipids, which are essential parts of a successful vaccine (Loomis & Johnson, 2015).

Unlike reverse vaccinology, which isolates the antigenic epitopes from sequencing, structural vaccinology can engineer the protein for epitope maintenance and surface exposure (Loomis & Johnson, 2015). Based on the use of structural biology and bioinformatics, this technology can modify the vaccine candidate for conformational stabilization and better

exposure of epitope scaffolds (Charleston & Graham, 2018). Like reverse vaccinology, structural vaccinology can also be used for only antigenic proteins.

1.3.2 ETEC vaccine development

ETEC vaccine development has been undertaken for several decades, and progress has been made in various areas. However, there are still no effective vaccines for ETEC. Since the process of ETEC-induced diarrhea is the result of the joint action of adhesins and enterotoxins, neither anti-adhesin vaccine candidates nor anti-enterotoxin vaccine candidates can contribute to a comprehensive vaccination. Therefore, a protein adhesion-based toxoid multiepitope fusion antigen (MEFA) strategy represents a new direction for ETEC vaccine research (Table 1.3).

MEFA strategy is a focus of research in our laboratory. We constructed a STb-LT fusion which embedded the mature STb peptide to the LT toxoid backbone to increase the immunogenicity of STb (W. P. Zhang & Francis, 2010). Based on this platform, we continually fused an epitope from B subunit of LT and an STa toxoid epitope into the FaeG major subunit of *E. coli* K88ac fimbriae to induce neutralizing antibodies of anti-LT, anti-STa and anti-K88 (C. Zhang & Zhang, 2010). Considering the GM1-binding function of LT B pentamer, we fused peptides from the K88ac major subunit FaeG, the F18 minor subunit FedF, and the LT toxoid (LT192) A2 and B subunits and constructed a FaeG-FedF-LT192A2:B fusion which elicited protective antibodies specific for K88, F18 and LT in immunized mice and pigs (X. S. Ruan, Liu, Casey, & Zhang, 2011). This monomeric fusion was optimized to a LT-like holotoxin structure expressed by a live attenuated *E. coli* strain and was found that could induce a protective immune response against that adhesin and toxin in pigs (X. Ruan & Zhang, 2013). Moreover, a toxoid MEFA inducing antibodies specific for STa, STb, LT and Stx2e has been

constructed, which could induce protective immunity against ETEC (Rausch et al., 2017). In this study, the low immunogenicity of STa had been an issue for ETEC vaccine development. To solve this problem, we continually constructed a toxoid fusion 3xSTaN12S-dmLT with three STa epitope copies which could induce protective anti-STa antibodies against ETEC diarrhea (Nandre, Duan, Wang, & Zhang, 2017). Those data from early our studies suggested potential application of MEFA strategy in porcine ETEC vaccine development.

Other strategies, like multivalent antigen and nanometer-sized inclusion body (IB) technology, also have been used to develop a broadly protective vaccine against ETEC infection (Jiang et al., 2019; H. H. Zhang et al., 2018). Some groups coated F4 and F18 fimbriae with thiolated Eudragit microspheres (TEMS) as an oral vaccine candidate and found this vaccine candidate could effectively induce an immune response against ETEC adhesion (Lee et al., 2011). Others used spray dried plasma powder (SDPP) containing ETEC fimbrial subunit F4 and LT as a vaccine which improved average daily growth (ADG) and decreased ETEC excretion (Niewold et al., 2007). Vaccine candidates using those strategies have been demonstrated that could broadly and effectively protect piglets against ETEC diarrhea.

F4 fimbriae, as mucosal carrier, has been used as a powerful oral immunogen (Verdonck, De Hauwere, Bouckaert, Goddeeris, & Cox, 2005). Oral immunization with F4 fimbriae could induce a Th17 dominated response with the participation of IL-17B and IL-17F in the immune response (Luo et al., 2015). Purified F4 fimbriae induced IL-6 and IL-8 secretion, which was considered as a potential mucosal adjuvant (Devriendt, Stuyven, Verdonck, Goddeeris, & Cox, 2010). However, industrial application required large quantities of F4 fimbriae. Moreover, the pH of the stomach and digestive enzymes could degrade F4 fimbriae which reduce the protection. To solve those problem, transgenic plants were used to produce large-scale

recombinant proteins against ETEC infection with lower cost. Alfalfa, crop, and tobacco have been used to express FaeG proteins (Joensuu, Kotiaho, et al., 2006; Joensuu, Verdonck, et al., 2006; Kolotilin et al., 2012; Shen et al., 2010). Recombinant plant-produced FaeG proteins could store at room temperature and remained stable longer by using dried plant materials. Recently, some researchers used rice to develop MucoRice-CTB as a vaccine candidate and found that this candidate could protect both suckling and weaned piglets from ETEC diarrhea by inducing both passive and active immunity (Takeyama et al., 2015).

Live attenuated or live wild type avirulent vaccine has also been extensively studied. For example, a $K88^+$ avirulent strain expressing adhesive fimbriae and a nontoxic form of LT was found that could protect piglets from the same fimbrial adhesin- and enterotoxin-expressing ETEC strain (Santiago-Mateo, Zhao, Lin, Zhang, & Francis, 2012). Attenuated *Salmonella* strains have been widely used to secrete multiple fimbriae, like K88ab, K88ac, F41 or K99, from recombinant plasmids and found that those candidates could protect piglets through milk by immunizing sows (Hur & Lee, 2012a, 2012b). Furthermore, ETEC bacterial ghosts (BGs), empty bacterial envelopes containing antigenic comformation, were evaluated in a mouse model recently with high safety and immunogenicity (Ran et al., 2019). Two live oral vaccines, Coliprotec® F4 (Fairbrother et al., 2017) and Coliprotec F4/F18 (Nadeau et al., 2017), have been evaluated in pig challenge models in 2017. Data showed that those vaccines administered once in drinking water to pigs of at least 18 days of age could induce long-term protection against swine ETEC infection. Those vaccine candidates have been marketed in Canada and Europe, but still limited in Unite States.

Current efforts in ETEC vaccine research are focused on induction of protective antitoxin and anticolonization immunity. The adhesin-based protein subunit approach is the most

advanced. Traditional immunization routes, for example, subcutaneous vaccination, induce systemic immunity effectively (L. Zhang, Wang, & Wang, 2015). However, protection is difficult to achieve in the intestinal mucosa, which is the site of origin of bacterial infection (L. Zhang et al., 2015). Based on current vaccinology and vaccine knowledge, it may be a good idea to use a safe probiotic organism to express antigens to stimulate intestinal mucosa immune responses or to produce neutralizing antibodies directly if this probiotic can maintain long-term colonization (Vitetta, Vitetta, & Hall, 2018).

Several vaccine candidates expressing by probiotics show promise for protection against ETEC infection in pigs. For example, *Lactococcus lactis* was used to express FaeG extracellularly and found that this candidate induced immunity against live ETEC challenge (Hu et al., 2009). A recombinant *Lactobacillus casei* strain secreting K99 or K88 or K99-K88-LTB was developed which could induce antibodies and cellular immune responses against K99 and K88 ETEC (L. J. Wen et al., 2012). Other probiotic strain, like *E. coli* Nissle 1917 (EcN) which expressed the K88 fimbrial adhesin on the surface, was found that this recombinant strain induced a humoral immune response (Remer et al., 2009).

1.4 The oral typhoid vaccine strain *Salmonella typhi* Ty21α

The *Salmonella* Ty21α strain is a mutant of *Salmonella* Ty2 strains lacking uridine-diphosphate-galactose (UDP-Gal)-4-epimerase due to chemical inactivation of the galE gene. This strain has been used as the only oral live attenuated vaccine (or Vivotif®) against typhoid fever by the WHO (Organization, 2019). The mutation of galE causes disordered galactose metabolism, leading to the accumulation of galactose in the bacteria. These changes result in bacteriolysis and the loss of bacterial virulence (McKenna, Bygraves, Maiden, & Feavers, 1995;

Robbe-Saule, Coynault, & Norel, 1995). After oral administration, the Ty21α strain proliferates in the jejunum and cecum temporarily, which induces humoral immunity and cellular immunity, including the activation of $CD4^+$ and $CD8^+$ T cells, the response of NK cells and the secretion of IFN-γ (Wahid, Fresnay, Levine, & Sztein, 2015, 2016; Wahid, Simon, Zafar, Levine, & Sztein, 2012) **(Fig 1.4)**. Data from several volunteer studies suggest that Ty21α does not achieve long-term colonization in hosts and has a very low excretion rate in feces (Aebischer et al., 2008). The intestinal tract prevents the adhesion of pathogenic microbes after Ty21a oral immunization via rapid clearance of the challenge strain.

The *Salmonella* Ty21α strain is also effective against non-muscle-invasive bladder cancer (NMIBC) by successfully delivering anticancer molecules deep inside tumors (Domingos-Pereira et al., 2017; Domingos-Pereira et al., 2019). Furthermore, the *Salmonella* Ty21α strain has been used as a vaccine delivery platform to express vaccine antigens against some diseases, such as HIV, anthrax, shigellosis, plague, and human papilloma virus (Amicizia, Arata, Zangrillo, Panatto, & Gasparini, 2017; Baillie et al., 2008; Dharmasena, Osorio, et al., 2016; Fraillery et al., 2007; J. Wen et al., 2012). Since Ty21α is not stable in acidic environments, most research on the Ty21α strain has emphasized effective oral immunization in the context of stomach acidity by using enteric-coated capsules or increasing acid resistance (Dharmasena, Feuille, et al., 2016).

1.5 Purpose of this research

The overall goal of this research is to construct a PWD multiepitope fusion antigen (PWD MEFA) to induce protective immunity against ETEC-associated PWD. This dissertation includes three aims: (1) to map the immunodominant or neutralizing epitopes from the adhesive subunits

of ETEC fimbriae causing swine post-weaning diarrhea via a reverse epitope vaccinology strategy; (2) to construct a PWD monomeric MEFA that induces multiple antifimbria and antitoxin antibodies; and (3) to optimize the PWD MEFA into a holotoxin structure and use the Ty21a strain as the carrier for PWD-MEFA vaccine protein delivery.

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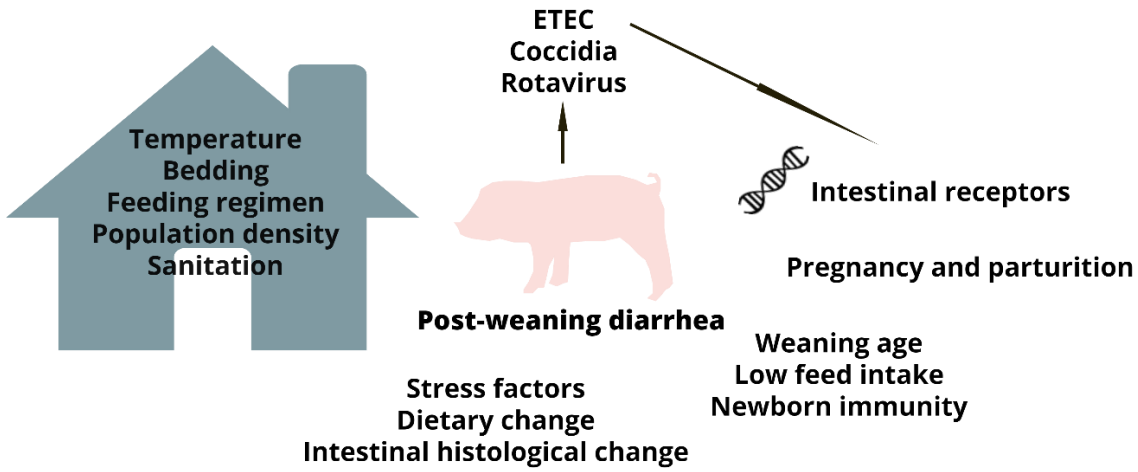

Fig 1.1 Risk factors for post-weaning diarrhea in pigs. As a multifactorial disease, post-weaning diarrhea is one of the most severe diseases and an important cause of death in weaned piglets, leading to losses in the pig industry. This disease results in a lower survival rate for piglets, lower feed returns, slower growth, etc. Furthermore, post-weaning diarrhea makes infection with other pathogens more likely and seriously threatens the growth of the pig industry. This article reviews the causes and prevention of diarrhea in weaned piglets.

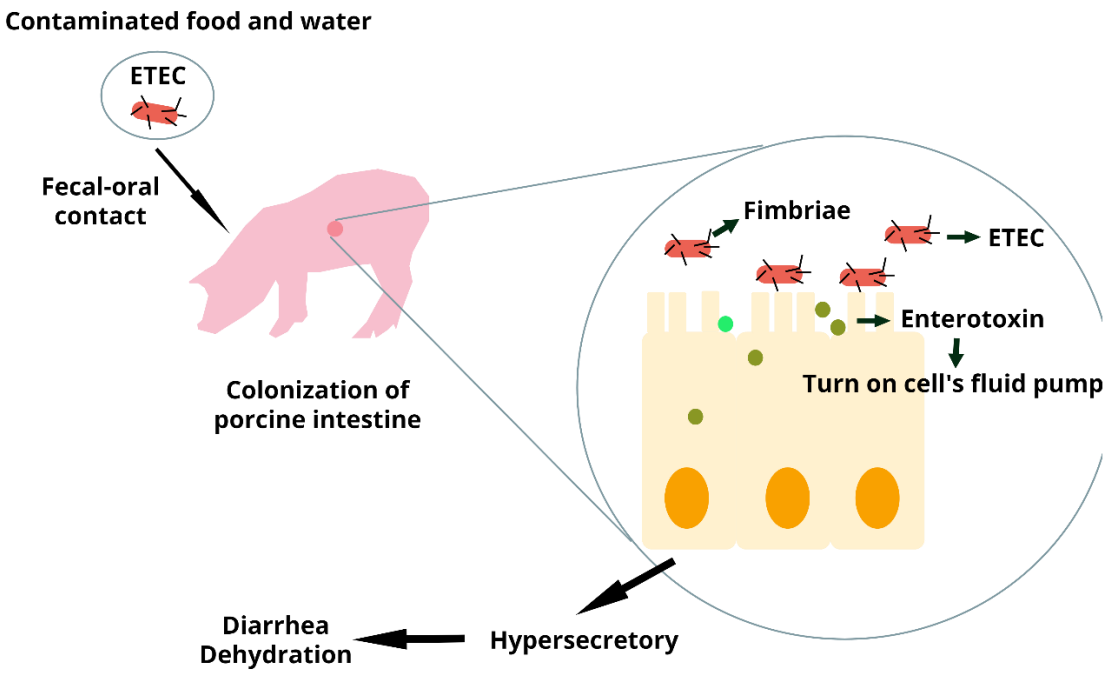

Figure 1.2 The pathogenesis of post-weaning diarrhea in pigs. ETEC bacteria can survive in the environment for at least 6 months if they are protected by feces. Once infection occurs, ETEC colonizes the porcine intestinal surface by fimbrial adhesins, which is essential for the release of enterotoxins from ETEC to intestinal epithelial cells, resulting in the secretion of water and electrolytes into the intestinal lumen, which causes diarrhea and dehydration.

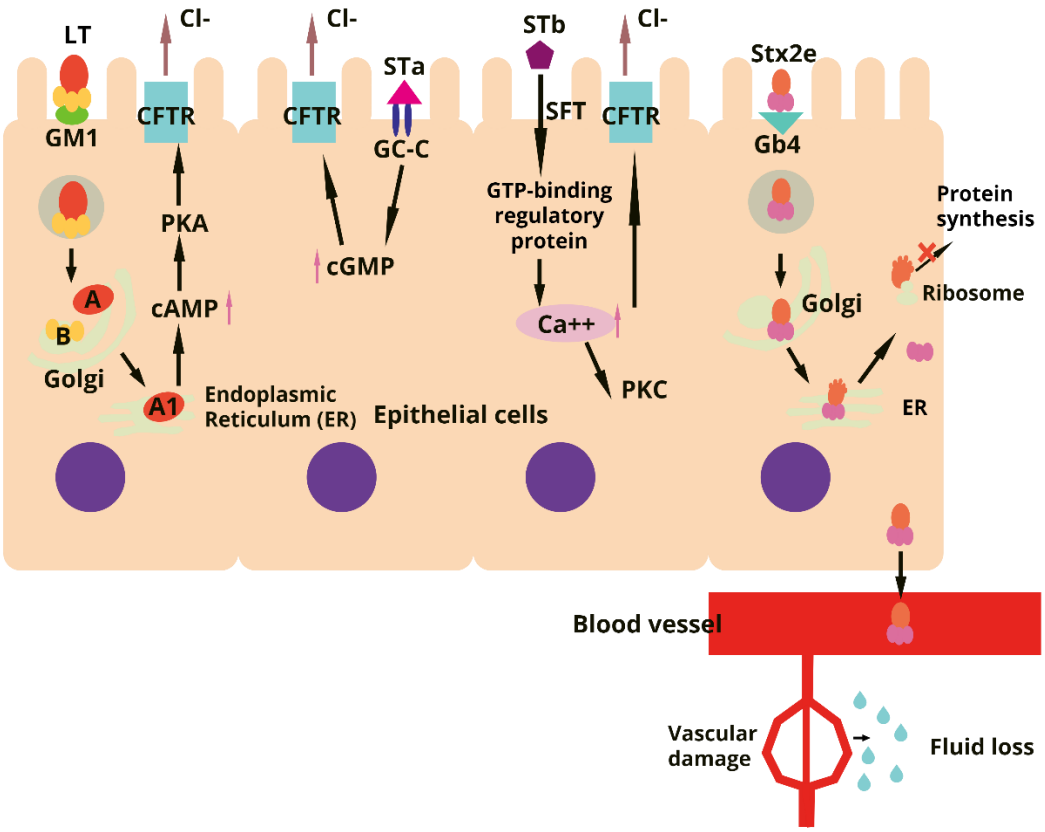

Figure 1.3 Mechanism of ETEC toxins on intestinal epithelial cells. LT binds to GM1 receptor on the intestinal cell and increases the intracellular cAMP level, leading to fluid secretion. STa binds to GC-C receptor on the intestinal cell and increases the intracellular cGMP level. STb binds to sulfatide on the intestinal cell and stimulates GTP-binding regulatory protein. Stx2e binds to Gb4 on the intestinal cell and damages vascular endothelium. CFTR: cystic fibrosis transmembrane regulator; PKA: protein kinase A; PKC: protein kinase C; GM1: Monosialotetrahexosylganglioside; GC-C: guanylate cycles C; SFT: sulfatide; Ca++: calcium ion; Gb4: Globotetraosylceramide.

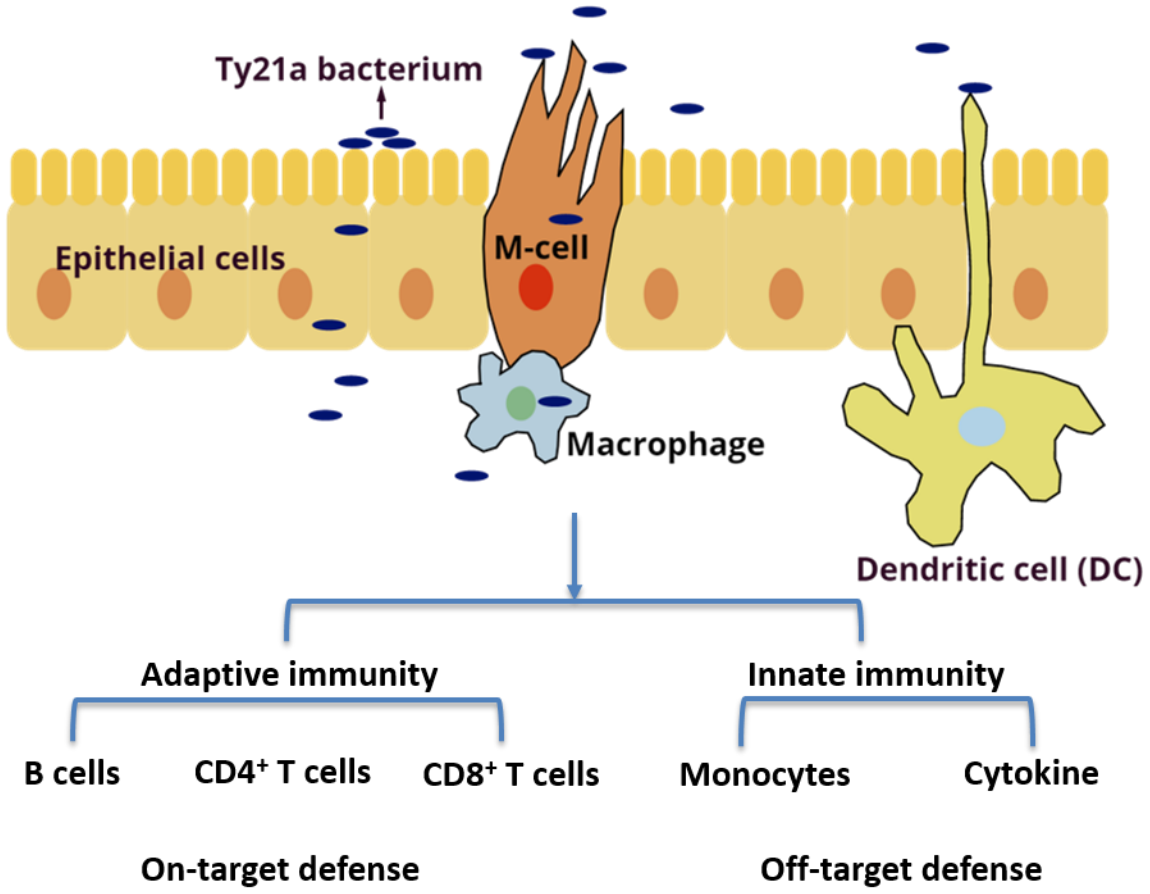

Figure 1.4 Mechanisms of live-attenuated *Salmonella* Ty21a vaccine inducing immunity. After oral immunization, Ty21a strains enter the host by M-cell adhesion, epithelial invasion, and dendritic cells luminal capture. The vaccine antigens are presented to immune cells by antigen-presenting cells, like macrophages or dendritic cells. Moreover, the live oral typhoid vaccine Ty21a induces the 'non-specific' immune response against other common infections, not just *Salmonella*.

Table 1.1 Receptors for F4 (K88) fimbriae.

Fimbriae	Phenotype	Adhesins	Receptor model	Intestinal receptor molecules
F4ab	A B C F H	FaeG(ab)	b	Transferrin *N*-glycan (74 kDa) (GP74)
			bc	IMPTGPs (210–240 kDa)
			bcd	Glycoproteins (45–70 kDa)
			Porcine aminopeptidase N (APN)	
F4ac	A B G H	FaeG(ac)	bc	IMPTGPs
			bcd	Glycoproteins
			Porcine aminopeptidase N (APN)	
F4ad	A C D	FaeG(ad)	d	Neutral glycosphingolipids (IGlads)
			bcd	Glycoproteins
			Porcine aminopeptidase N (APN)	

Table 1.2 Phenotypes of young pigs based on their F4 (K88) receptors.

Phenotype	Host receptor	Recognized fimbrial variant	Related disease
Type A	Intestinal mucin-type glycoproteins (IMTGPs) and intestinal neutral glycosphingolipids (IGLads)	All three K88 fimbrial variants	Typical diarrhea after K88ab$^+$ and K88ac$^+$ infection and no clinical disease after K88ad$^+$ infection
Type B	Intestinal mucin-type glycoproteins (IMTGPs)	K88ab and K88ac	Typical diarrhea after K88ab$^+$ and K88ac$^+$ infection
Type C	Enterocyte membrane-associated transferrin (GP74) and intestinal neutral glycosphingolipids (IGLads)	K88ab and K88ad	Typical diarrhea after K88ab$^+$ infection and no clinical disease after K88ad$^+$ infection
Type D	Intestinal neutral glycosphingolipids (IGLads)	K88ad only	No clinical disease
Type E		No K88 fimbrial *E. coli* variants	
Type F	Enterocyte membrane-associated transferrin (GP74)	K88ab only	Typical diarrhea

Type G	K88ac
Type H	K88ac and K88ad

Table 1.3 Virulence factors of PWD and ED

Type of diarrhea	Virulotypes
Porcine post-weaning diarrhea	LT:STb:EAST1:F4
	LT:STb:STa:EAST1:F4
	STa:STb
	STa:STb:F18
	STa:F18
Porcine edema disease	F18:Stx2e

Chapter 2 - Mapping the neutralizing epitopes of F18 fimbrial adhesin subunit FedF of enterotoxigenic *Escherichia coli* (ETEC)

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Abstract: K88 and F18 fimbrial enterotoxigenic *Escherichia coli* (ETEC) are the major causes of post-weaning diarrhea (PWD) in pigs. A vaccine that induces broad immunity to prevent K88 and F18 fimbrial ETEC bacterial attachment and colonization in pig small intestines and to neutralize enterotoxin enterotoxicity would be effective for PWD. Structure-based multiepitope-fusion-antigen (MEFA) technology using a backbone immunogen to present neutralizing epitopes of representing virulence factors capacitates development of broadly protective ETEC vaccines. Neutralizing epitopes have been identified from K88 fimbrial adhesin (FaeG) and enterotoxins but not F18 fimbrial adhesin. In this study, we in silico identified immunodominant epitopes from F18ac fimbrial subunit FedF which plays a critical role in F18 fimbrial adherence, genetically fused each epitope to a carrier, examined immunogenicity of each epitope fusion, and determined epitope-derived antibodies neutralizing activities against F18 fimbrial adherence. Data showed that seven immune-dominant epitopes were identified from FedF subunit. Fused to heterologous human ETEC adhesin subunit CfaB, epitope fusions induced anti-F18 antibodies in subcutaneously immunized mice. Moreover, antibodies derived from each fusion significantly blocked adherence of a F18-fimbrial *E. coli* bacteria to pig intestinal cell line IPEC-J2. While all seven epitopes exhibited neutralizing activity, results from this study identified FedF epitopes #3 (IPSSSGTLTCQAGT) and #7 (QPDATGSWYD) the most effective for antibodies against F18 fimbrial adherence, and suggested their future application in PWD vaccine development.

2.1 Introduction

Post-weaning diarrhea (PWD) is one of the most important swine diseases (Fairbrother et al., 2005; USDA, 2002). Piglets commonly develop diarrhea 3 to10 days after they are weaned, a clinical condition called PWD. PWD is mainly caused by pathogenic bacteria and viruses including diarrheagenic *Escherichia coli*, coronaviruses [transmissible gastroenteritis (TGE) and porcine epidemic diarrhea virus (PEDV)] and rotaviruses; however, diarrheagenic *E. coli* have a central role in the etiology of PWD (Hampson, 1994). PWD causes weight loss, slow growth and acute death in recently weaned pigs, resulting in economic losses to swine producers in the US and other countries (Haesebrouck et al., 2004; Nagy and Fekete, 1999; Verdonck et al., 2002; Vu-Khac et al., 2007). Diarrhea is also a main reason for using antibiotics on swine farms. Antibiotic exposure is linked to antimicrobial resistance (AMR), casting a major concern for animal and human health (Docic and Bilkei, 2003; Mishra et al., 2012; Torjesen, 2016). However, a ban on the use of food animal growth promoting antibiotics in Scandinavia and Europe spiked PWD outbreaks (Casewell et al., 2003), urgently calling for alternative effective prevention strategies against PWD. Vaccination would be the most economical and likely effective approach to control PWD and an effective means to reduce the use of antibiotics. Though there are products on the market, truly effective PWD vaccines are urgently needed (Fairbrother et al., 2005; Melkebeek et al., 2013; Zhang, 2014).

Of the diarrheagenic *E. coli*, enterotoxigenic *E. coli* (ETEC) is the most common cause of PWD, though the stress of weaning, absence of maternally-derived enteric antibodies, and dietary change are important but indirect factors of clinical PWD (Fairbrother et al., 2005). ETEC strains causing PWD produce fimbriae and enterotoxins. Fimbriae promote initial attachment to host cell receptors, enabling colonization (Smith and Linggood, 1971); colonized

ETEC bacteria deliver enterotoxins to host enterocytes, causing water and electrolyte hypersecretion and diarrhea (Nataro and Kaper, 1998). Thus, fimbriae and enterotoxins are the major virulence determinants of ETEC, and have been targeted in intervention strategies.

ETEC fimbriae and enterotoxins are immunologically heterogeneous (Gaastra and de Graaf, 1982). Fimbriae of ETEC causing PWD include K88 (F4) and F18, and occasionally K99 (F5), 987P (F6) and F41 (F7) (Awad-Masalmeh et al., 1982; Casey and Moon, 1990; Frydendahl, 2002; Moseley et al., 1986; Nagy et al., 1977; Zhang et al., 2007). Enterotoxins produced by ETEC are heat-labile toxin (LT), heat-stable toxin type I (STa), heat-stable toxin type II (STb), Shiga toxin 2e (Stx2e) and enteroaggregative heat-stable toxin type 1 (EAST1) (Frydendahl, 2002; Lee et al., 1983; Moon et al., 1980; Nakazawa et al., 1987; Osek, 1999b; Zhang et al., 2007). Clinical observations and epidemiological studies indicate that a typical ETEC strain expresses one and occasionally two types of fimbriae and one, two or more enterotoxins (Francis, 2002; Frydendahl, 2002; Zhang et al., 2007). Laboratory experimental studies demonstrated that an ETEC strain expressing one type of fimbriae and LT, STb, or STa enterotoxin causes diarrhea in young pigs (Berberov et al., 2004; Erume et al., 2008; Zhang et al., 2006; Zhang et al., 2008). The optimal prevention approach would be to block attachment of different ETEC fimbriae to host receptors and eliminate enterotoxicity of major enterotoxins (LT, STs) to host cells (Walker, 2005; Zhang, 2014; Zhang, 2012).

Blocking attachment of all ETEC fimbriae and neutralizing against enterotoxicity of LT and STs have proven very challenging. However, a recent breakthrough in antigen preparation by using neutralizing epitopes and multiepitope-fusion-antigen (MEFA) technology makes completion of such a task feasible (Duan et al., 2017; Nandre, 2016; Ruan et al., 2014a). Additionally, molecular epidemiological studies showed that the vast majority of ETEC strains

causing PWD express K88 or F18 fimbriae in conjunction with 2 to 3 toxins (Frydendahl, 2002; Zhang et al., 2007). In the US, over 95% of PWD cases are caused by K88 or F18 fimbrial ETEC strains (Francis, 2002; Zhang et al., 2007). Thus, blocking attachment of K88 and F18 fimbriae to enterocyte receptors would be an effective means to prevent ETEC colonization.

Neutralizing epitopes from K88 fimbrial adhesin FaeG (Lu, 2017) and toxins including LT (Huang et al., 2018), STa (Rausch et al., 2017; Ruan et al., 2014b; Zhang et al., 2010), and STb (Rausch et al., 2017) were identified. However, epitopes from F18 adhesive subunit FedF are not mapped and neutralizing epitopes have not been identified. With neutralizing epitopes identified from all ETEC virulence determinants, we should be able to apply the MEFA technology to develop a broadly protective vaccine against PWD. In this study, we in silico identified immunodominant epitopes from F18 FedF subunit, fused individual epitopes to protein carrier CfaB (a structural subunit of heterologous human ETEC fimbria CFA/I), immunized mice with each epitope fusion protein, measured mouse anti-F18 antibody response, and examined epitope-derived antibodies for neutralizing activities against F18 fimbria adherence to determine FedF neutralizing epitopes.

2.2 Methods and Materials

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 2.1. Recombinant CfaB strain 9477 (Ruan, 2015) were used as DNA templates for CfaB gene PCR amplification. CfaB-epitope fusion genes were cloned in vector pET28α (Novagen, Madison, WI) and expressed in *E. coli* BL21-CodonPlus (DE3). F18 fimbrial *E. coli* field isolate 8516 was used in antibody neutralization assays against F18 fimbriae attachment.

F18 FedF epitope in silico prediction and epitope fusion construction.

Immunodominant epitopes from F18 FedF subunit protein was in silico identified with B-cell epitope prediction programs (Larsen, 2006; Saha, 2007). Predicted epitopes were mapped on a FedF protein model generated from Phyre3 (Bennett-Lovsey et al., 2008; Kelley and Sternberg, 2009). The PyMOL Molecular Graphics System (version 2.2; Schrödinger, LLC, New York City, NY, USA) was used to display the location of each epitope on the FedF protein. Nucleotides coding each FedF epitope were embedded into CfaB gene by replacing nucleotides coding 80-86 amino acids of CfaB (a CfaB epitope) using SOE PCR with specifically designed primers (Table 2), as we previously described (Duan and Zhang, 2017; Huang et al., 2018; Lu, 2017). PCR generated CfaB-epitope fusion genes were digested with NheI and EagI restriction enzymes (New England BioLabs, Ipswich, MA), and were cloned into pET28a vector.

CfaB-epitope fusion protein expression and characterization. Recombinant CfaB-epitope (CfaB-FedF-ep) fusion proteins expressed by *E. coli* BL21 (DE3) were extracted with bacterial protein extraction reagent (B-PER; Thermo Fisher Scientific, Rochester, NY) and refolded as we previously described (Huang et al., 2018; Nandre, 2016; Rausch et al., 2017). Refolded fusion proteins were examined in SDS-PAGE with Coomassie blue staining, and were characterized in Western blot or direct ELISAs with anti-F18 mouse antiserum.

Additionally, each CfaB-epitope fusion protein was investigated for blocking F18 fimbriae from reacting with anti-F18 antiserum in competitive ELISAs. Four μg CfaB-epitope fusion protein was incubated with mouse anti-F18 serum dilutions (1:4,000 to 1:32,000) for 30 min at room temperature. Each mixture was added to ELISA plate wells coated with F18 fimbriae (50 ng per well). Incubated at 37^0C for 1h, wells were washed with PBS-0.05% tween-20 (PBST), and incubated with horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG

(1:3000, Sigma, St. Louis, MO). OD600 was measured after exposure to 3,3',5,5'-tetramethylbenidine (TMB; KPL, Gaithersburg, MD) for 30 min at room temperature.

Furthermore, CfaB-epitope fusions were examined as competitive agents to prevent anti-F18 antibodies from inhibiting adherence of F18-fimbrial bacteria to pig intestine cell line IPEC-J2. Since protective anti-F18 antibodies inhibit adherence of F18-fimbiral *E. coli* to host receptors, conformational F18 epitopes react with anti-F18 antibodies (derived from F18 fimbriae) thus abolish antibodies from adherence inhibition. Nearly identical to antibody adherence inhibition assay previously described (Rausch et al., 2017; Ruan, 2013; Ruan et al., 2014a; Ruan et al., 2011), except the addition of CfaB-epitope fusions as the competitive agent, this assay measured interference of CfaB-epitope fusion protein to anti-F18 antibodies in inhibiting F18 fimbriae adherence to target host cells. Ten µg CfaB-epitope fusion protein together with 1.5x106 CFU F18-fimbrial *E. coli* bacteria 8516 were mixed with 25 µl mouse anti-F18 antiserum and incubated at room temperature for 30 min; the mixture was then added pig intestinal epithelial cells of the jejunum (IPEC-J2; 1.5x105 per well) cultured in Dulbecco's modified Eagle medium-F12 (DMEM-F12) medium (ATCC). Incubated in a CO2 incubator for 1 h, IPEC-J2 cells were washed to remove non-adherent *E. coli* bacteria, dislodged with TritonX-100 (0.5%; Sigma), and collected by centrifugation. Collected *E. coli* bacteria were suspended, serially diluted, plated on agar plates, and counted for CFUs after overnight growth at 37^0C.

Mouse immunization with CfaB-epitope fusion protein. Each CfaB-epitope fusion protein was used to subcutaneously immunize mice. A group of five 8-week old female BALB/c mice was each administered with 40 µg CfaB-epitope fusion protein (in 40 µl), adjuvanted with 1 µg dmLT (double mutant LT, $LT_{R192G/L211A}$; provided by PATH). Immunized mice received two boosters of the primary dose at the interval of two weeks. A group of mice without injection

was used as the negative control. Mouse serum samples collected before the primary and two weeks after the final booster were used to prepare serum samples. Mouse serum samples were stored at -80^{0}C until use. Mouse immunization complied with Animal Welfare Act under 1996 National Research Council Guidance and was approved by Kansas State University Institutional Animal Care and Use committee (protocol #3879).

Mouse serum anti-F18 IgG antibody titration. Serum samples from each immunized or control mouse were titrated for anti-F18 IgG antibodies as we previously described (Rausch et al., 2017; Ruan, 2013; Ruan et al., 2011). Briefly, wells of 2HB plates (Fisher Scientific) coated with heat-extracted F18 fimbriae (100 ng per well) were incubated with mouse serum binary dilutions (1:400 to 1:128,000) as primary antibodies and then HRP-conjugated goat-anti-mouse IgG as secondary antibodies (1:3000). OD_{650} measured after exposure to TMB were converted to antibody titers, in a log10 scale.

Mouse serum anti-F18 antibody adherence inhibition and neutralizing epitope identification. Mouse serum samples from the groups immunized with each CfaB-epitope fusion were examined for antibody adherence inhibition using F18-fimbrial *E. coli* bacteria 8516 and IPEC-J2 cells. Briefly, 3x106 (CFUs) bacteria 8516 exposed to 30 µl serum pooled from each immunization group or the control group were added to IPEC-J2 cell (1.5x105 per well) and cultured for 1 h in a CO2 incubator. After washes with PBS to remove non-adherent bacteria, cells were dislodged, lysed, then diluted, and plated on agar plates for 8516 bacteria counting (CFUs) after overnight growth at 37^{0}C. Neutralizing epitopes were identified if mouse serum samples derived from the fusion protein showed significant inhibition against adherence from F18-fimbrial *E. coli* bacteria, compared to the control mouse serum samples.

Data analyses. GraphPad prism software version 7.0 was used for statistical analysis. The ELISA of screening each CfaB-epitope fusion protein by anti-F18 antiserum and competitive ELISA with F18 fimbriae were analyzed by two-way ANOVA. One-way ANOVA was performed to analyze competitive bacteria adherence inhibition assay, mouse serum anti-F18 antibody titration and antibody adherence inhibition assay data. The mean ± standard deviation (SD) was used to express the results. Statistical significance was $p < 0.05$.

2.3 Results

Seven immunodominant epitopes identified from F18 FedF adhesin subunit. A total of seven immunodominant epitopes were in silico identified, at the length ranged from 10 to 14 amino acids (Table 2.3). All seven epitopes are discontinuous, surface-exposed, and located on ß sheets or α-helix extension (**Fig. 2.1**).

CfaB-epitope proteins were expressed and recognized by anti-F18 antiserum. Seven CfaB-epitope fusions were constructed, designed as CfaB-FedF-ep1, CfaB-FedF-ep2, CfaB-FedF-ep3, CfaB-FedF-ep4, CfaB-FedF-ep5, CfaB-FedF-ep6, and CfaB-FedF-ep7 (**Fig. 2.2**). Epitope fusion protein were extracted and refolded (**Fig. 2.3A**), and recognized by anti-F18 mouse antiserum (**Fig. 2.3B**). Additionally, when coated on ELISA plates, each of these seven fusion proteins reacted with anti-F18 antiserum (**Fig. 2.3C**).

Additionally, competitive ELISAs using F18 fimbriae as the coating antigen showed that CfaB-epitope fusion proteins competed with coated F18 fimbriae for binding to anti-F18 antiserum (**Fig. 2.4A**). OD readings were significantly lower in wells with the addition of individual CfaB-epitope fusion proteins or F18 fimbria ($p<0.01$; as the positive control),

confirming FedF epitopes presented in the fusion proteins retained native antigenic conformation.

More importantly, CfaB-epitope fusion proteins bound to anti-F18 antiserum and reduced anti-F18 antibodies from inhibiting the adherence F18-fimbrial *E. coli* bacteria to pig cell line IPEC-J2 (**Fig. 2.4B**). The addition of CfaB-FedF-ep1 or CfaB-FedF-ep3 fusion significantly reduced anti-F18 antiserum neutralizing activity for blocking the adherence of 8516 bacteria to IPEC-J2 cells ($p<0.05$) when compared to other epitope fusions, shown more bacteria adhered to IPEC-J2 cells (**Fig. 2.4B**).

Mice subcutaneously immunized with CfaB-epitope fusions developed antibodies to F18 fimbriae. Mice immunized with CfaB-epitope fusion proteins had anti-F18 IgG antibody titers detected in serum samples at 3.65±0.39 (CfaB-FedF-ep1), 2.42±0.536 (CfaB-FedF-ep2), 3.65±0.148 (CfaB-FedF-ep3), 3.21±0.484 (CfaB-FedF-ep4), 3.68±0.373 (CfaB-FedF-ep5), 3.61±0.308 (CfaB-FedF-ep6), 3.45±0.471 (CfaB-FedF-ep7) (**Fig. 2.5A**). No anti-F18 antibodies detected in the serum of the control mice or from the serum collected prior to the primary immunization. Anti-F18 IgG titers in the group immunized with CfaB-FedF-ep2 were significantly lower than the titers of the groups immunized with the other CfaB-epitope fusions ($p<0.01$).

Mouse serum antibodies derived from CfaB-epitope fusions inhibited in vitro adherence of F18-fimbrial *E. coli* bacteria. Serum samples of the mice immunized with CfaB-epitope fusion proteins exhibited adherence inhibition activities against F18-fimbrial *E. coli* 8516 (**Fig. 2.5B**). Treated with the serum of mice immunized with CfaB-FedF-ep3 or CfaB-FedF-ep7, 8516 had the fewest bacteria adherent to IPEC-J2 cells (15±3.46 %; 16.7±5 %). One-way

ANOVA test indicated that serum antibodies of the mice immunized with CfaB-FedF-ep3 or CfaB-FedF-ep7 were most effective in inhibiting 8516 adherence.

2.4 Discussion

Vaccination with live and subunit vaccines has been explored in the past decades, and remains the most promising for PWD prevention (Fairbrother et al., 2005; Melkebeek et al., 2013). Since ETEC strains expressing K88 or F18 fimbriae cause nearly all PWD cases, F18 and K88 fimbriae have been the main targets in vaccine development. While oral administration of purified K88 fimbriae induced K88-specific antibodies and protected pigs against a homologous challenge (Van den Broeck et al., 1999a, b), administration of purified F18 fimbriae did not induce protective immunity against F18 ETEC challenge in pigs (Verdonck et al., 2007). In similar, live vaccine candidates derived from avirulent *E. coli* field isolates expressing K88 fimbriae induce K88-specific antibodies and protect against colonization by a K88 ETEC challenge strain (Bianchi et al., 1996; Francis and Willgohs, 1991; Fuentes M., 2004; Santiago-Mateo, 2012), but live strains expressing F18 were not effective in the induction of an F18-specific immune response, nor did they protect against challenge with a F18 ETEC strain (Bertschinger et al., 2000; Coddens et al., 2007).

F18 fimbriae, previously known as F107, 2134P and 8813, consist of two antigenic variants: F18ac and F18ab (Imberechts et al., 1997; Nagy et al., 1997), and are associated with PWD and edema disease in young pigs respectively (Amezcua et al., 2002; Frydendahl, 2002; Osek, 1999a; Post KW, 2000). The major structural subunit of F18 is FedA, but the adhesive minor subunit FedF plays a central role in binding to host receptors (Imberechts et al., 1996; Smeds et al., 2001; Smeds et al., 2003), and is highly conserved among F18 strains. A LT-K88-

F18 tripartite antigen that includes FedF peptide (60th-109th aa), as a recombinant protein administered intramuscularly (Ruan et al., 2011) or a holotoxin-structured protein expressed by a live *E. coli* strain when given orally (Ruan, 2013), induced antibodies blocking adherence of K88- and F18-fimbrial ETEC bacteria and protecting pigs against K88-fimbrial ETEC challenge. This tripartite PWD vaccine candidate was not examined for efficacy against F18-fimbrial ETEC challenge due to difficulty in identifying F18 susceptible pigs, and also needs to carry toxin antigens to induce protective antitoxin antibodies for effective protection against PWD.

Incorporating antigenic elements of all virulence determinants into a PWD vaccine product for effective protection against heterogeneous ETEC strains is challenging. Structural based MEFA platform allows a backbone immunogen to present multiple neutralizing epitopes for broad immunity. With neutralizing epitopes from K88 fimbrial adhesin subunit FaeG, toxins including LT, STa and STb are already identified, we need to identify neutralizing epitopes from F18 fimbrial adhesin subunit FedF in order to have a PWD vaccine candidate for immunity against all ETEC virulence determinants. Results from the current study indicated that epitope #3 (IPSSGTLTCQAGT) and epitope #7 (QPDATGSWYD) are the top candidates of F18 adhesin subunit FedF neutralizing epitopes. Imbedding these two FedF epitopes into a MEFA that uses a LT toxoid as backbone and presents neutralizing epitopes of F18, LT, STa and STb, we are a step closer for a broadly protective PWD vaccine, though future challenge studies are needed to confirm whether antibodies derived from these FedF epitopes are protective against F18 fimbrial attachment and colonization.

In conclusion, seven epitopes identified from F18 fimbrial adhesin subunit FedF retained native antigenicity after being fused to heterologous carrier CfaB protein, indicated by each epitope fusion protein recognized by anti-F18 antiserum but also ability to compete with

F18 fimbria for binding to anti-F18 antibodies or to reduce anti-18 antibodies from inhibiting adherence of *E. coli* bacteria expressing F18 fimbriae. Moreover, each CfaB-epitope fusion protein induced antibodies specific to F18 fimbriae in subcutaneously immunized mice. More importantly, derived antibodies showed neutralizing activities against F18 fimbria adherence to pig intestine cell line IPEC-J2. Among seven FedF epitopes that induce neutralizing anti-F18 antibodies, epitope 3 and epitope 7 displayed better in inducing neutralizing anti-F18 antibodies, suggesting their potential application in vaccine development against PWD.

2.5 References

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Table 2.1 *Escherichia coli* strains and plasmids used in the study.

Strains and plasmids	Relevant properties	Reference
Strains		
BL21	*huA2, Δ(argF-lacZ), U169, phoA, glnV44, φ80, Δ(lacZ)M15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17*	GE Healthcare
8516	porcine *E. coli* field isolate, F18	
9477	'CfaB(with signal peptide) + pET28α' in DH5α	(Ruan et al. 2015)
9503	'CfaB (without signal peptide) + pET28α' in BL21	(Ruan et al., 2015)
9668	'CfaB-FedF-ep1 + pET28α' in BL21	This study
9669	'CfaB-FedF-ep2 + pET28α' in BL21	This study
9670	'CfaB-FedF-ep3 + pET28α' in BL21	This study
9671	'CfaB-FedF-ep4 + pET28α' in BL21	This study
9672	'CfaB-FedF-ep5 + pET28α' in BL21	This study
9673	'CfaB-FedF-ep6 + pET28α' in BL21	This study
9674	'CfaB-FedF-ep7 + pET28α' in BL21	This study
Plasmids		
pET28α		Novagen

Table 2.2 Primers used in SOE PCRs to construct CfaB-epitope fusion genes in the study.

Primer	Sequence (5'-3')	Amplified region
CfaB-F	CGGGCTAGCGTAGAGAAAAATATT	upstream of *CfaB* gene, *Nhe*I site underlined
CfaB-R	TTACGGCCGGGATCCCAAAGTCAT	downstream of *CfaB* gene, *Eag*I site underlined
FedF-ep1-F	AGCACTACTCGCACTAGAATTGAT TTTTTTAGTTGCATCGTTTGT	forward and reverse primers to insert FedF epitope 1 into *CfaB* gene
FedF-ep1-R	TCTAGTGCGAGTAGTGCTCAAGTC GATACACCACAGCTTACAGAT	
FedF-ep2-F	AGTGTTTGTCTTCCCTGTGCCAAGT TTTTTAGTTGCATCGTTTGT	forward and reverse primers to insert FedF epitope 2 into *CfaB* gene
FedF-ep2-R	ACAGGGAAGACAAACACTACCCA AATGGATACACCACAGCTTACA	
FedF-ep3-F	GCATGTCAAAGTTCCTGATGAACT AGGAATTTTTTTAGTTGCATC	forward and reverse primers to insert FedF epitope 3 into *CfaB* gene
FedF-ep3-R	TCAGGAACTTTGACATGCCAGGCT GGAACTGATACACCACAGCTT	
FedF-ep4-F	CTGTTGCCCCCACTGAGATTCATTT TTTTTAGTTGCATCGTTTGT	forward and reverse primers to insert FedF epitope 4 into *CfaB* gene
FedF-ep4-R	TCTCAGTGGGGGCAACAGTCACAA GATACACCACAGCTTACAGAT	

FedF-ep5-F	GGAAGAAAGGGGATATGTCTGAG CTTTTTTAGTTGCATC	forward and reverse primers to insert FedF epitope 5 into *CfaB* gene
FedF-ep5-R	ACATATCCCCTTTCTTCCGGTGATG ATACACCACAGCTT	
FedF-ep6-F	TGAAGGCATATCATTTTGGTTGGG TTTTTTAGTTGCATCGTTTGT	forward and reverse primers to insert FedF epitope 6 into *CfaB* gene
FedF-ep6-R	CAAAATGATATGCCTTCATCTAAT GATACACCACAGCTTACAGAT	
FedF-ep7-F	CCACGAGCCTGTTGCATCGGGCTG TTTTTTAGTTGCATCGTTTGT	forward and reverse primers to insert FedF epitope 7 into *CfaB* gene
FedF-ep7-R	GATGCAACAGGCTCGTGGTATGAT GATACACCACAGCTTACAGAT	

Table 2.3 Immunodominant B-cell epitopes in silico identified from F18 fimbrial adhesin subunit FedF subunit.

epitopes	amino acid sequence	position	length (aa)
FedF-ep1	INSSASSAQV	34-43	10
FedF-ep2	LGTGKTNTTQM	48-58	11
FedF-ep3	IPSSSGTLTCQAGT	74-87	14
FedF-ep4	NESQWGQQSQ	115-124	10
FedF-ep5	AQTYPLSSGD	151-160	10
FedF-ep6	PNQNDMPSSN	226-235	10
FedF-ep7	QPDATGSWYD	253-262	10

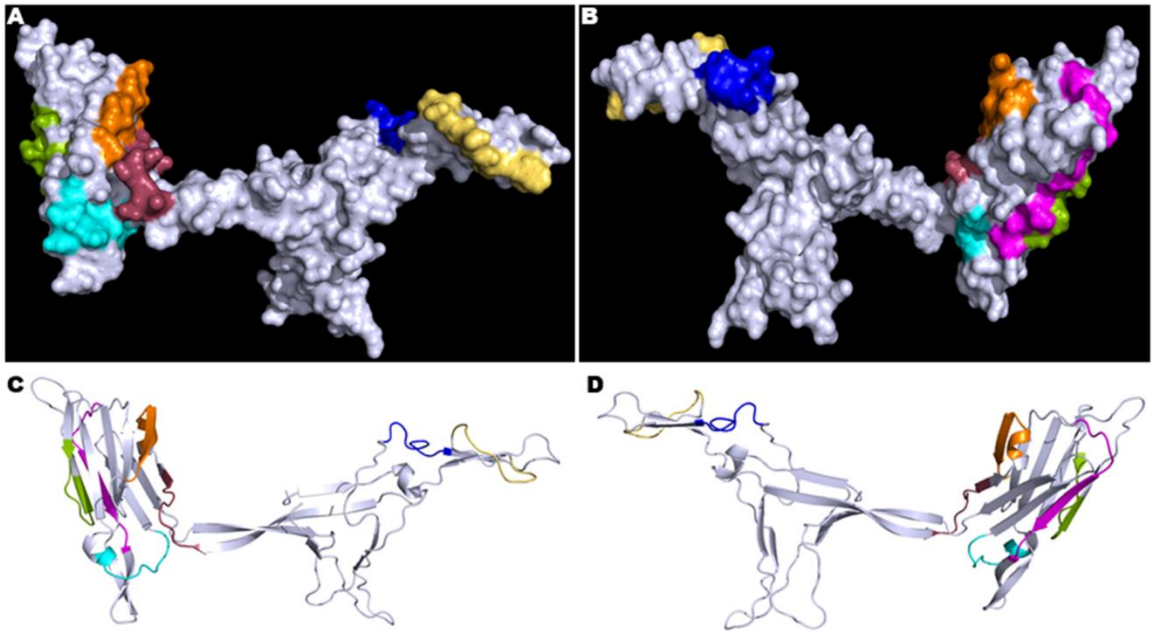

Figure 2.1 F18 fimbrial adhesin subunit FedF protein model. (A, B) and secondary structure (C, D) to show positions of in silico identified epitopes (A & C, front; B & D, back). FedF-ep1 (red), FedF-ep2 (orange), FedF-ep3 (pink), FedF-ep4 (green), FedF-ep5 (cyan), FedF-ep6 (blue), FedF-ep7 (yellow).

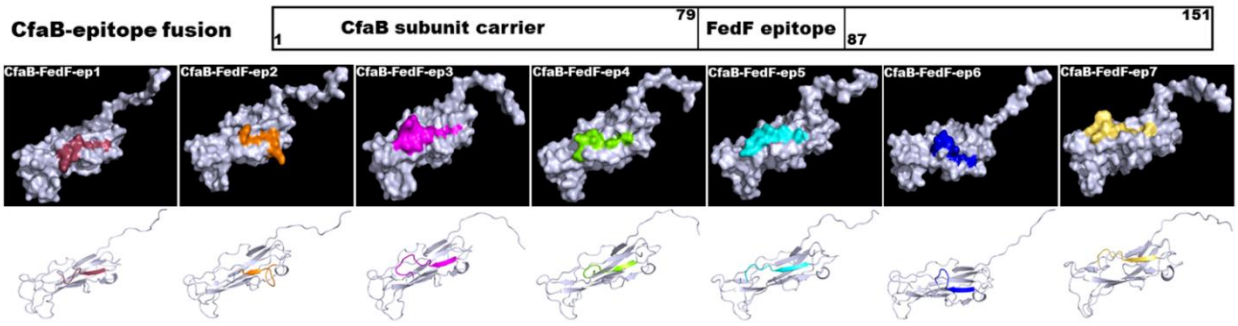

Figure 2.2 CfaB-FedF-epitope fusion genetic structure illustration and fusion protein modeling. Top: fusion gene genetic structure, the carrier protein CfaB peptide epitope (80-86 aa) was replaced with each FedF epitope. Middle: CfaB-epitope fusion protein models (CfaB-FedF-ep1 to CfaB-FedF-ep7). Bottom: CfaB-epitope fusion protein secondary structure. FedF epitopes are shown in different colors.

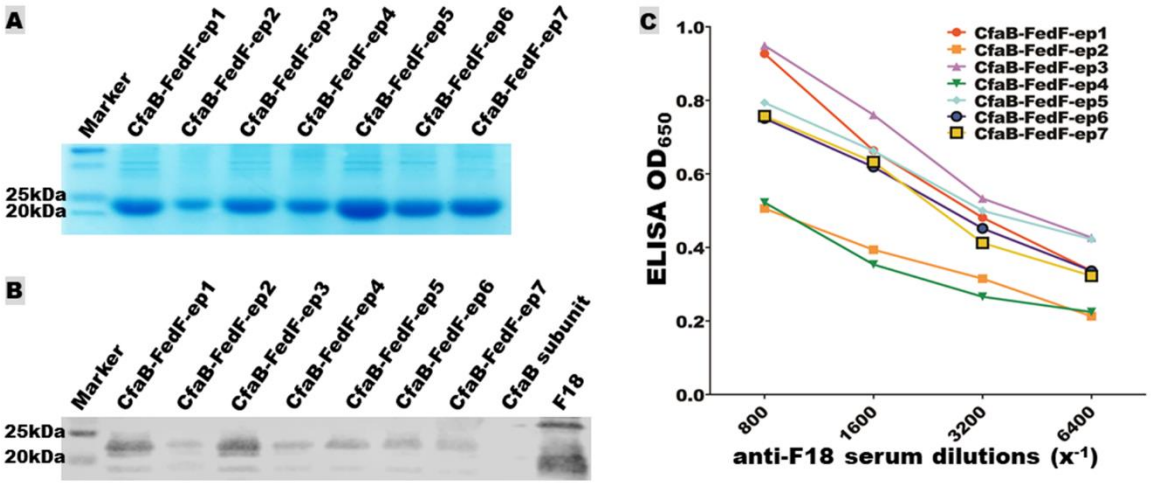

Figure 2.3 CfaB-epitope fusion protein extraction and characterization. (A) SDS-PAGE with Coomassie blue staining to show extracted and refolded CfaB-epitope fusion proteins. (B) Western blot with anti-F18 antiserum to showed recognition of each CfaB-epitope fusion by anti-F18 antiserm, with carrier protein CfaB protein as the negative control and F18 fimbriae as the positive control. (C) ELISAs to show conformational recognition of each CfaB-epitope fusion protein (ELISA coating antigen) by anti-F18 antiserum (at different dilutions).

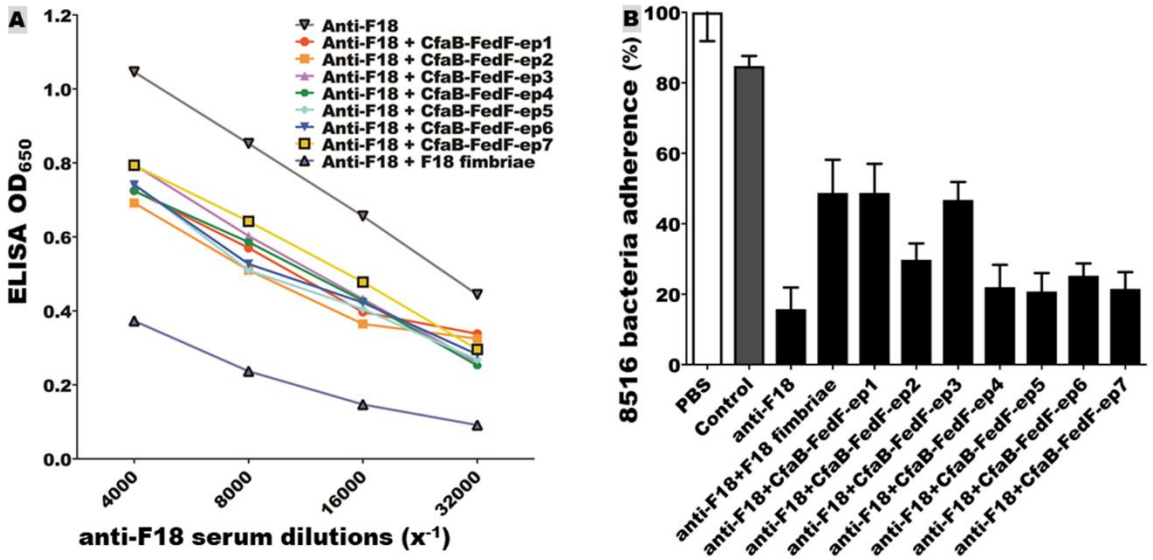

Figure 2.4 Competitive ELISA and bacteria adherence inhibition assay. (A) Competitive ELISAs with F18 fimbriae as the coating antigen, and each CfaB-epitope fusion protein as the competing agent. Anti-F18 antiserum dilutions from 1:4,000 to 1:32,000 were used. (B) antibody adherence inhibition assay using anti-F18 antiserum as the antibodies to inhibit F18-fimbiral *E. coli* strain 8516 binding to F-18 receptor positive pig intestine cell line IPEC-J2, and each CfaB-epitope fusion protein as the agent to compete for anti-F18 antiserum thus to prevent anti-F18 antiserum from blocking the binding between 8516 bacteria to OPEC-J2 cells. PBS, no competing agent and no anti-F18 antiserum; control, control mouse serum only; anti-F18, no competing agent but with anti-F18 antiserum.

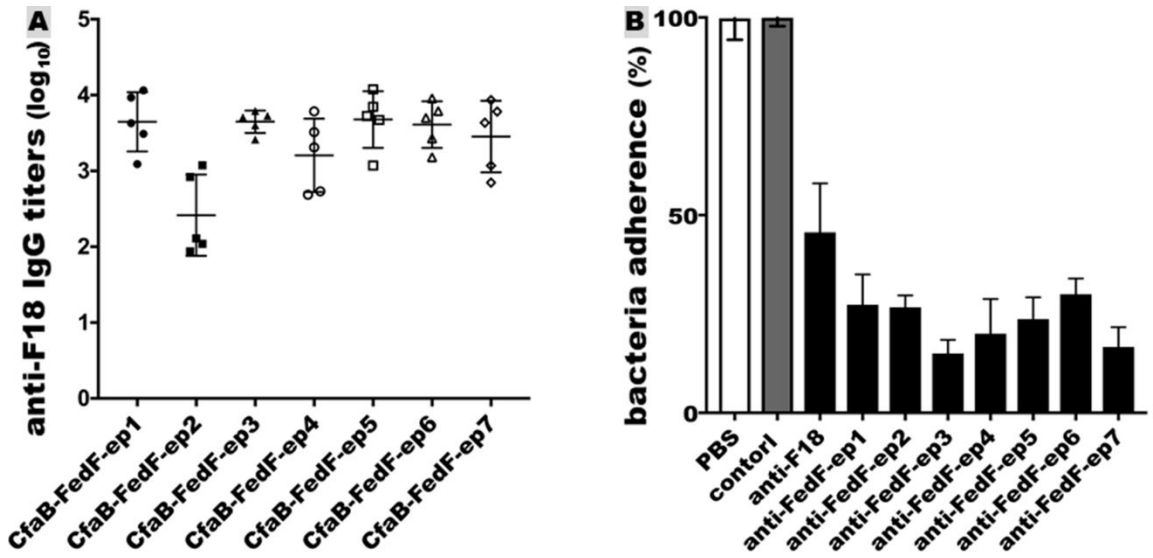

Figure 2.5 Mouse serum anti-F18 antibody titration and antibody adherence inhibition assay. (A) adAnti-F18 IgG titers (in log10) from serum samples of the mice immunized with each CfaB-epitope fusion protein; no anti-F18 IgG titer detected from the negative control mice. (B) mouse serum antibody inhibition assays to show epitope derived antibodies against adherence (in %) of F-18 fimbrial *E. coli* bacteria 8561 to IPEC-J2 cells. PBS, no mouse serum; control, negative control mouse serum; anti-F18, serum samples of mice immunized with F18 fimbriae.

Chapter 3 - Mapping the neutralizing epitopes of enterotoxigenic *Escherichia coli* (ETEC) K88 (F4) fimbrial adhesin and major subunit FaeG

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Abstract: Enterotoxigenic *Escherichia coli* (ETEC) strains that produce immunologically heterogeneous fimbriae and enterotoxins are the primary cause of porcine neonatal diarrhea and post-weaning diarrhea. A multivalent vaccine inducing protective immunity against ideally all ETEC fimbriae and enterotoxins would be effective against diarrhea in young pigs. However, developing a vaccine to broadly protect against various ETEC virulence determinants has been proven challenging. Structure-based multiepitope-fusion-antigen (MEFA) technology to present neutralizing epitopes of various virulence determinants in a backbone immunogen and to mimic epitope native immunogenicity suggests feasibility of developing multivalent vaccines. While neutralizing epitopes of ETEC fimbria F18 and enterotoxins were identified, protective epitopes of K88 (F4) fimbria are to be determined. In this study, we *in silico* predicted B-cell immunodominant epitopes from K88 fimbrial major subunit (also adhesin) FaeG and embedded each epitope in a heterogeneous carrier. We then immunized mice with each epitope fusion protein and examined epitope antigenicity but also neutralizing activities of epitope-induced antibodies. Data showed that while all nine FaeG epitope fusions induced antibodies to K88 fimbria, anti-K88 IgG antibodies derived from epitopes MTGDFNGSVD (ep1), LNDLTNGGTK (ep2), GRTKEAFATP (ep3), ELRKPDGGTN (ep4), PMKNAGGTKVGAVKVN (ep5) and RENMEYTDGT (ep8) significantly inhibited adherence of K88-fimbrial bacteria to porcine intestinal cell line IPEC-J2, indicating these peptides the neutralizing epitopes of K88 fimbrial

major subunit FaeG and suggesting the future application of FaeG epitopes in ETEC vaccine development.

3.1 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) bacteria that express K88 (F4) or F18 fimbria and enterotoxins including heat-labile toxin (LT), heat-stable toxin type I (STa), heat-stable toxin type II (STb) and Shiga toxin type 2e (Stx2e) are a primary cause of diarrhea in pigs (Dubreuil et al., 2016). Porcine neonatal diarrhea is largely prevented by passive protection of maternal antibodies through the immunization of pregnant sows. Post-weaning diarrhea, however, is yet to be effectively controlled. Various preventive approaches including feeding ETEC specific antibody-containing materials, treatment of prebiotics, probiotics or dietary supplementary were attempted but found results inconsistent or unpractical commercially. Vaccination has been considered effective and practical. However, developing effective vaccines for post-weaning diarrhea has encountered challenges. Difficulties include a narrow window for immunization, vaccine and vaccination at a low cost, and more importantly the need of cross protection against heterogeneous ETEC strains. Ideally, piglets are vaccinated when their maternal antibodies drop to levels which sufficiently protect against neonatal diarrhea but do not significantly interfere vaccine antigens to stimulate active immunity, thus piglets develop active immunity against ETEC infection by the time of weaning. Vaccines need to be low-cost and easy for administer. Additionally, since ETEC strains produce immunologically different fimbrial adhesins to attach to specific receptors at pig small intestinal epithelia and deliver various toxins to stimulate water and fluid hyper-secretion in epithelial cells, an effective post-weaning diarrhea vaccine needs to carry antigens from all virulence determinants and to induce broad immunity, thus inhibits

adherence of different ETEC fimbriae and neutralizes enterotoxicity of immunologically distinctive ETEC toxins.

New strategies need to be explored since conventional vaccine technologies encountered difficulties in developing a broadly protective multivalent vaccine against post-weaning diarrhea (Dubreuil et al., 2016). Multiepitope fusion antigen (MEFA), a structure- and epitope-based vaccine technology presents an alternative approach for multivalent vaccine development (Q. Duan et al., 2017). By mimicking epitope native antigenicity and presenting multiple heterologous epitopes in one backbone immunogen, MEFA vaccinology allows a single immunogen (protein) to carry multiple antigenic elements (epitopes or peptides) of various virulence determinants for broad immunogenicity thus development of a broadly immunogenic multivalent vaccine. With this MEFA technology, we have successfully generated ETEC fimbria MEFAs for broadly protective immunity against seven or nine human-ETEC virulence determinants (Q. Duan et al., 2018) and adhesin-toxoid MEFAs for antibodies not only inhibiting adherence of seven human-ETEC adhesins and neutralizing LT and STa enterotoxicity but also protecting against ETEC diarrhea in a pig challenge model . These MEFAs become leading antigens of human ETEC subunit vaccines against children's diarrhea and travelers' diarrhea. MEFA technology has also assisted the construction of a multivalent toxin antigen and the development of a broadly protective anti-toxin vaccine candidate for pig post-weaning diarrhea (Rausch et al., 2017).

Protective epitopes of four toxins (LT, STa, STb and Stx) produced by ETEC strains associated with pig diarrhea were identified (Rausch et al., 2017). However, multivalent toxoid MEFAs do not induce anti-adhesin antibodies to prevent ETEC bacteria adherence to host receptors. Since ETEC causing pig post-weaning diarrhea produce one, two, three or four toxins

and either K88 or F18 fimbria, an effective vaccine ideally needs to induce antitoxin and anti-adhesin antibodies against four ETEC toxins and both K88 and F18 fimbriae. With neutralizing epitopes for F18 fimbrial adhesin subunit FedF are also identified (Lu, Seo, Moxley, & Zhang, 2019), it leaves K88 (F4) fimbria the only virulence determinant without neutralizing epitopes being determined.

In this study, we *in silico* identified immune dominant B-cell epitopes from FaeG, the major structural subunit and also the adhesin of K88ac fimbria, genetically embedded each epitope to a heterologous carry protein, and assessed epitope native topology by examining epitope fusion protein reactivity with anti-K88ac antiserum. We then immunized mice with each epitope fusion protein and measured mouse serum IgG antibodies specific to K88ac fimbria. Furthermore, we examined mouse serum antibodies for inhibition activity against K88ac fimbrial adherence and identified K88ac fimbria neutralizing epitopes.

3.2 Methods and Materials

Bacterial strains and plasmids. Bacterial strains and plasmids for this study are included in Table 3.4. CfaB recombinant strain 9477 was used as the DNA template to PCR amplify *CfaB* gene and the backbone to carry K88 FaeG epitopes for CfaB-epitope fusions. Vector pET28α (Novagen, Madison, WI) was used to clone each CfaB-epitope fusion gene, and *E. coli* BL21-CodonPlus (DE3) was used to express fusion proteins. K88 fimbrial ETEC field isolate 3030-2 (K88/LT/STb/STa) was used in antibody adherence inhibition assays to measure mouse serum antibody neutralization activities.

***In silico* identification of K88ac FaeG epitopes and construction of CfaB-epitope fusions.** K88ac fimbrial subunit FaeG immunodominant epitopes were identified using B-cell

epitope prediction programs. Phyre3 (R. M. Bennett-Lovsey, A. D. Herbert, M. J. E. Sternberg, & L. A. Kelley, 2008; L. A. Kelley & Sternberg, 2009) was used to generate 3-D protein structure of FaeG subunit, and PyMOL Molecular Graphics System (version 2.2; Schrödinger, LLC, New York City, NY, USA) was to map each epitope from the FaeG protein model.

Nucleotides coding each FaeG epitope were embedded into carrier gene *CfaB* by replacing the nucleotides coding a CfaB backbone epitope in a splicing overlap extension (SOE) PCR with primers (Table 3.5), as described previously (Q. D. Duan & Zhang, 2017). After digestion with restriction enzymes NheI and EagI (New England BioLabs, Ipswich, MA), 6xHis-tagged CfaB-epitope fusion genes were individually cloned into pET28a vector.

CfaB-epitope fusion protein expression and characterization. As described previously (Rausch et al., 2017), bacterial protein extraction reagent (B-PER; Thermo Fisher Scientific, Rochester, NY) was used to extract epitope fusion proteins (CfaB-FaeG-ep), and protein refolding buffer (Novogen) was used to refold the extracted fusion proteins. Epitope fusion proteins were then assessed for protein purity and integrity in SDS-PAGE with Coomassie blue staining and with anti-K88 mouse antiserum in Western blot respectively.

Epitope fusion proteins were further characterized in direct and competitive ELISAs to assess FaeG epitope conformation by measuring reactivity between fusion protein and anti-K88 antiserum and. In direct ELISA, each epitope fusion protein (100 ng per well) was used to coat 2HB microtiter plates (Thermo Fisher Scientific), anti-K88 antiserum and horseradish peroxidase-conjugated goat-anti-mouse IgG (1:3000; Sigma, St. Louis, MO) were used as the primary antibodies and the secondary antibodies. In competitive ELISA, K88 fimbriae (100 ng per well) were used to coat 2HB plates, each epitope fusion protein (100 ng per well) to compete the coated K88 fimbriae for reactivity with anti-K88 antiserum (diluted from1:4000 to 1:32000), with HRP-

conjugated goat-anti-mouse IgG was used as the secondary antibodies. OD_{600} values were measured from a plate reader after exposure to 3,3',5,5'-tetramethylbenidine (TMB; KPL, Gaithersburg, MD) for 30 min at room temperature.

Epitope fusion protein mouse immunization. Eight-week old female BALB/c mice (Charles River Laboratories, Wilmington, MA), five mice per group, were subcutaneously immunized with each epitope fusion protein (40 µg) adjuvanted with double mutant heat-labile toxin (dmLT; 1µg). Two boosters at the same dose of the primary were followed at the interval of two weeks. The control group received no injection. Mouse immunization protocol was approved by Kansas State University Institutional Animal Care and Use committee.

Mouse serum anti-K88 antibody titration. Mouse serum samples collected before the primary and two weeks after the final booster were titrated for anti-K88 IgG antibodies. As described previously (Rausch et al., 2017), 2HB plates coated with K88 fimbriae or FaeG protein (100 ng per well) were incubated with mouse serum binary dilutions (1:400 to 1:128000), and then secondary antibody HRP-conjugated goat-anti-mouse IgG (1:3000). Anti-K88 or anti-FaeG IgG antibody titers (log_{10}) were calculated based on OD_{650} values measured after exposure to TMB. Mouse serum IgA antibody response to K88 was not examined.

Mouse serum antibody adherence inhibition against K88 fimbrial ETEC strain. Serum samples pooled from each group immunized with an epitope fusion protein and the control group were examined for antibodies inhibiting adherence of K88ac fimbrial ETEC isolate 3030-2 to IPEC-J2 cells. Briefly, 3030-2 bacteria ($3x10^6$) premixed with mouse serum sample (30 µl) from each group were incubated with 90-95% confluent IPEC-J2 cell ($1.5x10^5$ per well) in a CO_2 incubator. After 1 h incubation, IPEC-J2 cells were washed with PBS to remove non-adherent bacteria, then dislodged with Triton X-100 (0.5%; Sigma). Dislodged cells were collected,

diluted, and plated on agar plates. Bacteria were cultured overnight at 37^0C and were counted for colony forming units (CFUs).

Data analyses. Differences among epitope fusion proteins in reactivities with anti-K88ac antiserum in direct were compared in two-way ANOVA. Differences between each immunization group and the control group for IgG antibody titers, antibody adherence inhibition activities, as well as reactivities with anti-K88ac antiserum in competitive ELISAs were compared in one-way ANOVA. A *p* value less than 0.05 indicated a significant difference.

3.3 Results

Immunodominant epitopes were identified from K88ac fimbrial major subunit FaeG. Nine immunodominant and discontinued epitopes, ranged from 10 to 16 amino acid residues, were identified from FaeG subunit of K88ac fimbria **(Fig. 3.1)**. Protein modeling based on K88ac FaeG sequence showed all epitopes were surface-exposed from the subunit and were located at β sheets or α-helix extensions (**Fig. 3.1**).

Nine epitope fusion proteins were expressed and extracted. With the insertion of each K88 fimbrial FaeG epitope (K88ep1 – K88ep9) into protein carrier CfaB, a 17-kDa major structural subunit of human ETEC fimbria CFA/I, nine epitope fusions (CfaB-epitope) were constructed (**Fig. 3.2**). All 6xHis-tagged epitope fusions of about 22 kDa were expressed and extracted, with a purity of assessed over 95% (**Fig. 3.3**). When linearized at SDS-PAGE and examined by anti-K88ac antiserum in Western blot, however, only fusions of epitope 1, 2, 3, 5, 6, and 9 were recognized (**Fig. 3.3**).

Anti-K88ac antiserum confirmed FaeG epitope conformation. Antigenic conformation of the identified FaeG epitope carried by epitope fusions was verified in direct and competitive

ELISAs using anti-K88ac antiserum. Direct ELISAs showed that fusions of FaeG epitope 1, 2, 3, 4, 5, or epitope 9 showed significantly greater reactivity with anti-K88ac antiserum 1:1600 and 1:3200 antiserum dilutions ($p<0.05$), fusion of epitope 7 showed a moderate reactivity, and fusions of epitope 6 or epitope 8 showed no reactivity (Table 3.1).

Competitive ELISAs with heat-extracted K88ac fimbriae (from ETEC strain 3030-2) as the coating antigen showed significantly lower reactivity with anti-K88 antiserum when fusion proteins of epitope 1, 2, 4, or epitope 5 was used as the competing antigen ($p<0.001$). No reactivity with anti-K88 antiserum was detected when protein carrier CfaB was coated in direct or competitive ELISAs.

Anti-K88ac IgG antibodies were detected in the mice subcutaneously immunized with each epitope fusion protein. All mice in the group immunized with each epitope fusion protein developed anti-K88ac IgG antibodies (Table 3.2). Mice immunized with the fusion protein of epitope 5 developed significantly greater anti-K88ac IgG titers compared to the groups immunized with the fusion of epitope 4, 6, 7, 8 or epitope 9, but not to the groups immunized with fusions of epitope 1, 2 or epitope 3. No anti-K88 IgG was detected from the control group.

Mouse antiserum samples from each immunized group were also confirmed to react with K88 fimbriae or recombinant FaeG protein in ELISA and Western blot assays (**Fig. 3.4**). Data showed that FaeG protein as well as the boiled K88 fimbriae was recognized by antibodies in the serum samples of all immunization groups **(Fig. 3.4B)**. That indicated that epitopes 6, 7, and 8 may locate at the region connecting adjacent FaeG subunits for K88ac fimbriae.

Mouse serum samples from the immunized groups showed inhibition of in vitro adherence of K88 fimbrial ETEC 3030-2. K88ac fimbrial ETEC bacteria 3030-2, after being treated with the serum samples from the groups immunized with fusions of epitope 1, 2, 3, 4, 5, or

epitope 8 showed a significant reduction of adherence to porcine cell line IPEC-J2 ($p<0.01$). No significant adherence reduction was observed from 3030-2 bacteria when treated with the serum from the groups immunized with fusions of epitope 6, 7 or epitope 9 (Table 3.3).

3.4 Discussion

Data from the current study showed that while all *in silico* identified K88ac FaeG epitopes carried by CfaB backbone protein induced anti-K88 IgG antibodies in the subcutaneously immunized mice, only antibodies derived from the fusions of epitope 1, 2, 3, 4, 5 or epitope 8 significantly inhibited the adherence from K88ac fimbrial ETEC bacteria to porcine cell line IPEC-J2. That suggested that while all epitopes are immunodominant, epitopes 1, 2, 3, 4, 5 and 8 represent the neutralizing epitopes of K88ac fimbrial major subunit and adhesin FaeG. It was noted that fusions of epitope 1, 2, 3 or epitope 5 also induced significantly higher anti-K88 IgG antibody titers than the fusions carrying epitope 4, 6, 7, 8 or epitope 9 in the immunized mice. Interestingly, fusion of epitope 4 induced lower anti-K88 IgG titers, but derived antibodies had the second best in inhibiting 3030-2 adherence to IPEC-J2 cells (28.5%; blocking over 70% bacterial adherence). Differed from epitope 4 fusion, fusion of epitope 5 stimulated the highest anti-K88ac IgG titer, and antibodies derived from this fusion protein exhibited the best activity in inhibiting adherence of K88-fimbrial 3030-2 bacteria (blocking over 75% of bacterial adherence). That suggests that epitope 5 and epitope 4 could be the top candidate antigens to be included in a MEFA for developing a multivalent vaccine against ETEC associated diarrhea in young pigs. Indeed, peptides surrounding epitopes 5 and 4 were demonstrated to play a crucial role in K88 fimbria binding to host receptors (Jacobs, Roosendaal, van Breemen, & de Graaf, 1987; Thiry, Clippe, Scarcez, & Petre, 1989). A truncation of epitope 5 or epitope 4 resulted in a total loss of K88

fimbria binding ability to pig small intestinal brush borders (unpublished data). Therefore, antibodies against these two epitopes should prevent K88 fimbriae from attaching to host receptors and colonizing pig small intestines.

We noted that fusions of epitope 1, 2, 3, or 5 which induced greater anti-K88ac IgG titers strongly reacted with anti-K88ac antiserum, demonstrated by Western blot (Fig. 2) and ELISA (Table 3). That likely indicates these epitopes are well exposed on K88ac fimbria. In contrast, fusion of epitope 8 did not react with anti-K88 antiserum, although this fusion induced neutralizing anti-K88 antibodies. That may suggest epitope 8 was poorly exposed on K88 fimbria. The FaeG protein model predicted that epitope 8 is located at one end of the FaeG subunit. It is likely this may be the location FaeG subunits joined together to assemble K88 fimbria. Thus, subunit assembling negatively affects the exposure of epitope 8 on K88ac fimbria, directly resulting in antibodies derived from K88ac fimbria had poor reactivity with epitope 8 carried by the CfaB backbone. Future protein structural studies can further map the location and posture of epitope 8 and provide more details of the structure of FaeG subunit and K88ac fimbria.

With the identification of neutralizing epitopes from K88 fimbria FaeG major subunit and adhesin by this study, we now have all essential antigen elements to construct a MEFA immunogen for the development of a broadly protective vaccine against porcine post-weaning diarrhea in the U.S. This MEFA can use the A subunit of LT toxoid as the backbone. By retaining one or two neutralizing epitopes from the A subunit to induce protective antibodies against LT enterotoxicity, we can replace other LT A subunit epitopes with the neutralizing epitopes of K88 FaeG subunit, F18 FedF subunit, and Stx2e A subunit (Rausch et al., 2017), as well as a STb shorten peptide and STa toxoid (Rausch et al., 2017) for a fimbria-toxin MEFA. We have demonstrated that a replacement of an epitope of the LT A subunit with a foreign epitope abolishes LT enterotoxicity

but does not alter its formation of LT structure. Substitutions of A subunit epitopes of LT toxoid (LT_{R192G} or $LT_{R192G/L211A}$) with the neutralizing fimbria epitopes and toxins Stx2e, STb and STa peptides should eliminate LT enterotoxicity entirely, resulting in a safe but broadly immunogenic MEFA immunogen for vaccines against porcine post-weaning diarrhea.

We need to point out that only K88ac was targeted since K88ac is the predominant variant causing ETEC associated neonatal and post-weaning diarrhea in the U.S. Additional studies will be needed to identify neutralizing epitopes from K88ab variant which primarily cause pig diarrhea in Europe. However, epitopes identified from this study showed homology between K88ac and K88ab variants. Indeed, epitopes 1, 2, 3, 5, 8 and 9 are identical, and epitope 6 differs in one amino acid. Whether antibodies induced by these homologous epitopes also inhibit adherence of K88ab ETEC will need to be verified in future studies. Additionally, perhaps pig anti-K88ac and anti-K88ab antiserum (instead of anti-mouse antiserum) should be used to initially characterize antigenicity of these epitopes. It also needs to point out that the current study identified K88ac FaeG neutralizing epitopes based on cell-based in vitro antibody adherence inhibition assays. Pig immunization and ETEC challenge studies will be needed to further characterize epitopes for antigenicity and to confirm their candidacy as antigens for vaccine development. Nevertheless, data from this study identified potential neutralizing epitopes for antibodies against K88 fimbria; that can move one step closer toward the development of a broadly protective vaccine against porcine post-weaning diarrhea. Additionally, research in identifying neutralizing epitopes and constructing future MEFA from this study may provide helpful information for multivalent vaccine development against other diseases.

3.5 References

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Table 3.1 OD_{650} readings from direct ELISA to measure reactivity between each epitope fusion protein and anti-K88 antiserum.

epitope fusions	Anti-K88 serum dilutions	
	1:1600	1:3200
K88ep1	1.37 ± 0.00	0.96 ± 0.05
K88ep2	1.20 ± 0.11	0.81 ± 0.12
K88ep3	1.07 ± 0.03	0.73 ± 0.10
K88ep4	0.87 ± 0.04	0.52 ± 0.02
K88ep5	1.78 ± 0.23	1.45 ± 0.11
K88ep6	0.15 ± 0.03	0.10 ± 0.04
K88ep7	0.48 ± 0.04	0.39 ± 0.12
K88ep8	0.25 ± 0.03	0.18 ± 0.01
K88ep9	1.31 ± 0.04	1.12 ± 0.08

Table 3.2 Mouse serum anti-K88 IgG antibody titers (log_{10}).

Mouse groups	**IgG titers**	**P values**
ep1 fusion	3.61 ± 0.11	<.001
ep2 fusion	3.53 ± 0.25	<.001
ep3 fusion	3.45 ± 0.26	<.001
ep4 fusion	2.42 ± 0.40	<.001
ep5 fusion	3.89 ± 0.18	<.001
ep6 fusion	2.29 ± 0.30	<.001
ep7 fusion	2.08 ± 0.24	<.001
ep8 fusion	2.86 ± 0.08	<.001
ep9 fusion	2.85 ± 0.21	<.001
control	0 ± 0	

Table 3.3 Mouse serum antibody inhibition against adherence of K88 fimbrial ETEC strain 3030-2 to porcine cell line IPEC-J2.

Mouse group	Mean of attachment (%) ± SD		*P* Value
	Immunized	Control	
K88 fimbriae	9.8 ± 1.7	100 ± 24.2	<0.001
ep1 fusion	41.5 ± 12	100 ± 24.2	<0.001
ep2 fusion	44.3 ± 8.3	100 ± 24.2	<0.001
ep3 fusion	37.2 ± 12	100 ± 24.2	<0.001
ep4 fusion	28.5 ± 12.3	100 ± 24.2	<0.001
ep5 fusion	22.5 ± 5.0	100 ± 24.2	<0.001
ep6 fusion	87.5 ± 11.7	100 ± 24.2	0.88
ep7 fusion	87.2 ± 15.2	100 ± 24.2	0.86
ep8 fusion	66.7 ± 5.4	100 ± 24.2	0.004
ep9 fusion	88.3 ± 23.5	100 ± 24.2	0.92

Table 3.4 *E. coli* strains and plasmids used in this study.

Strains and plasmids	Relevant properties	Reference
Strain		
BL21	*huA2, Δ(argF-lacZ), U169, phoA, glnV44, φ80, Δ(lacZ)M15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17*	GE Healthcare
3030–2	porcine field isolate, K88ac/LT/STb/STa	Zhang et al. 2006
9702	pET28α-FaeG strain in BL21	This study
9477	pET28α-CfaB without signal peptide in DH5α	(4)
9503	pET28α-CfaB without signal peptide in BL21	(4)
9675	pET28α-CfaB-K88-ep1 in BL21	This study
9677	pET28α-CfaB-K88-ep2 in BL21	This study
9678	pET28α-CfaB-K88-ep3 in BL21	This study
9679	pET28α-CfaB-K88-ep4 in BL21	This study
9680	pET28α-CfaB-K88-ep5 in BL21	This study
9681	pET28α-CfaB-K88-ep6 in BL21	This study
9682	pET28α-CfaB-K88-ep7 in BL21	This study
9683	pET28α-CfaB-K88-ep8 in BL21	This study
9676	pET28α-CfaB-K88-ep9 in BL21	This study
Plasmid		
pET28α		Novagen

Table 3.5 PCR primers used to insert K88 FaeG epitope nucleotides into CfaB gene or to amplify FaeG gene.

Primer	Sequence (5'-3')	Amplified region
CfaB-F	CGGGCTAGCGTAGAGAAAAATATT	upstream of CfaB gene, with *Nhe*I site
CfaB-R	TTACGGCCGGGATCCCAAAGTCAT	downstream of CfaB gene, with *Eag*I site
K88ep1-L	CGAACCATTGAAATCACCAGTCATTTTT TAGTTGCATCGTTTGT	Insertion of K88 ep 1 (MTGDFNGSVD)
K88ep1-R	GGTGATTTCAATGGTTCGGTCGATGATA CACCACAGCTTACAGAT	
K88ep2-L	TCCACCATTGGTCAGGTCATTCAATTTT TAGTTGCATCGTTTGT	Insertion of K88 ep2 (LNDLTNGGTK)
K88ep2-R	GACCTGACCAATGGTGGAACCAAAGAT ACACCACAGCTTACAGAT	
K88ep3-L	AGCAAATGCTTCTTTGGTTCGGCCTTTT TAGTTGCATCGTTTGT	Insertion of K88 ep3 (GRTKEAFATP)
K88ep3-R	ACCAAAGAAGCATTTGCTACGCCAGAT ACACCACAGCTTACAGAT	
K88ep4-L	TCCACCATCAGGTTTTCTGAGTTCTTTT TAGTTGCATCGTTTGT	Insertion of K88 ep4 (ELRKPDGGTN)
K88ep4-R	AGAAAACCTGATGGTGGAACTAATGAT ACACCACAGCTTACAGAT	

K88ep5-L	AACTTTAGTGCCCCCTGCATTTTTCATC	
	GGTTTTTTAGTTGCATC	Insertion of K88 ep5
K88ep5-R	GCAGGGGGCACTAAAGTTGGTTCAGTG	(PMKNAGGTKVGAVKVN)
	AAAGTGAATGATACACCA	
K88ep6-L	CGCAGAAGTAACCCCACCTCTCCCTAA	
	TTTTTTAGTTGCATCGTT	Insertion of K88 ep6 (LGRGGVTSADGEL)
K88ep6-R	GGTGGGGTTACTTCTGCGGACGGGGAG	
	CTGGATACACCACAGCTT	
K88ep7-L	CCCAGCCGAGAGTTCAGAACCCCTCGG	
	TTTTTTAGTTGCATCGTT	Insertion of K88 ep7 (PRGSELSAGSA)
K88ep7-R	TCTGAACTCTCGGCTGGGAGTGCCGAT	
	ACACCACAGCTTACAGAT	
K88ep8-L	ATCAGTGTACTCCATGTTTTCCCTTTTTT	
	TAGTTGCATCGTTTGT	Insertion of K88 ep8 (RENMEYTDGT)
K88ep8-R	AACATGGAGTACACTGATGGAACTGAT	
	ACACCACAGCTTACAGAT	
K88ep9-L	GCTGGTAGTTACAGCCTGATTAAATTTT	
	TTAGTTGCATCGTTTGT	Insertion of K88 ep9 (FNQAVTTSTQ)
K88ep9-R	CAGGCTGTAACTACCAGCACTCAGGAT	
	ACACCACAGCTTACAGAT	
FaeG-F	CGGGCTAGCTGGATGACTGGTGATTTC	upstream of FaeG gene, with *Nhe*I site
FaeG-R	TTACGGCCGTTAGTAATAAGTAATTGC	downstream of FaeG gene, with *Eag*I site

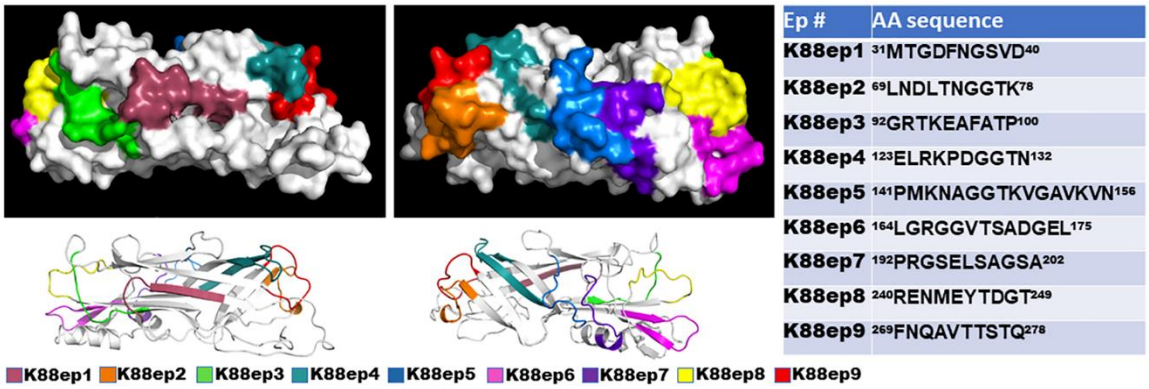

Figure 3.1 K88 fimbrial major structure subunit and adhesin FaeG protein model with *in silico* identified epitopes and epitope amino acid sequences. FaeG-ep1 (brown), FaeG-ep2 (orange), FaeG-ep3 (green), FaeG-ep4 (cyan), FaeG-ep5 (blue), FaeG-ep6 (pink), FaeG-ep7 (ourple), FaeG-ep8 (yellow), FaeG-ep9 (red). Phyre3 and PyMOL Molecular Graphics System (version 2.2) were used to generate 3-D protein structure of FaeG subunit and to map each epitope from the FaeG protein model respectively.

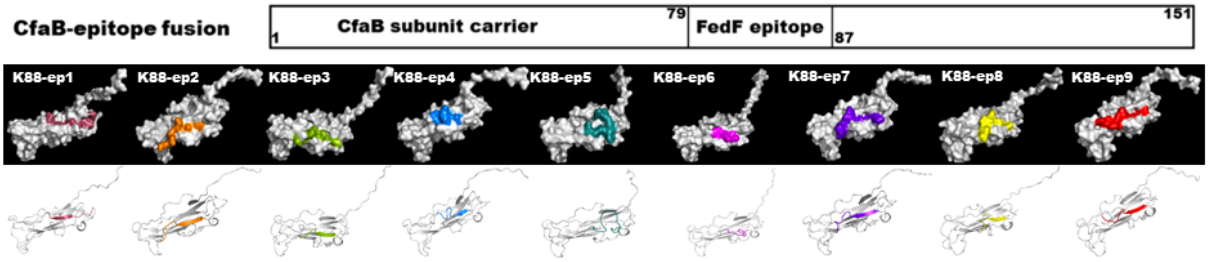

Figure 3.2 CfaB-FaeG-epitope fusion genetic structure illustration and fusion protein modeling. Top: CfaB-epitope fusion protein models (CfaB-FaeG-ep1 to CfaB-FaeG-ep9). Bottom: CfaB-epitope fusion protein secondary structure. FedF epitopes are shown in different colors.

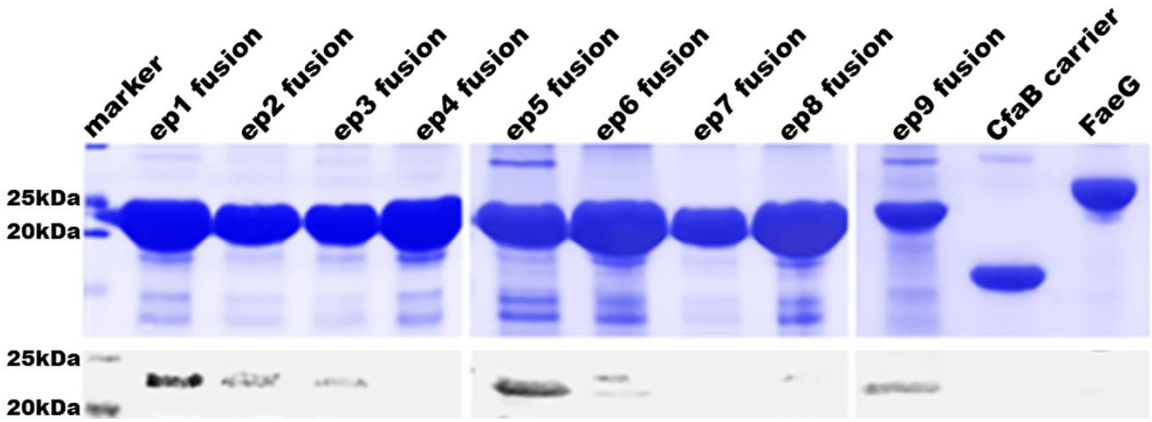

Figure 3.3 CfaB-epitope fusion protein extraction and characterization. Top: extracted and refolded CfaB-epitope fusion proteins from SDS-PAGE Coomassie blue staining. Bottom: reactivity of each epitope fusion protein with anti-K88 antiserum from Western blot.

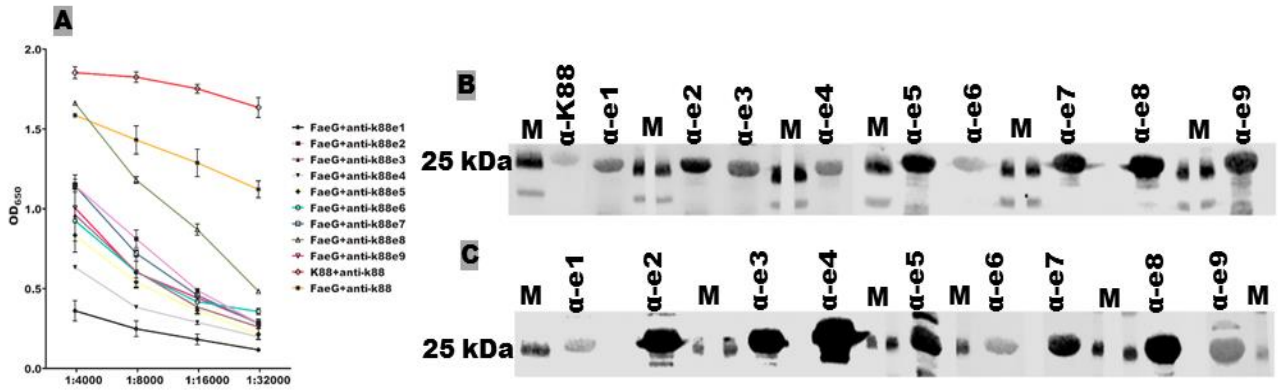

Figure 3.4 Mouse anti- CfaB-K88-epitope antiserum detection of FaeG proteins. (A) ELISA with serially diluted mouse anti-CfaB-K88-epitope antiserum using FaeG recombinant proteins as the ELISA coating antigen. (B) Western blot using mouse anti-CfaB-K88-epitope antiserum to detect denatured K88 fimbriae. (C) Western blot using mouse anti-CfaB-K88-epitope antiserum to detect K88 fimbrial subunit FaeG proteins.

Chapter 4 - Development of a broadly protective multivalent vaccine against porcine post-weaning diarrhea cause by ETEC

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Abstract: Enterotoxigenic *Escherichia coli* strains are the major cause of porcine post-weaning diarrhea (PWD). Currently, there is no effective vaccine for ETEC-associated PWD. Recently, we identified neutralizing epitopes from ETEC virulence factors associated with PWD. In this study, we constructed a multivalent antigen called PWD multiepitope fusion antigen (PWD MEFA) to induce protective immunity against multiple ETEC virulence factors. Neutralizing epitopes of fimbriae K88 and F18, and toxins STa, STb and Stx2e were fused into the A subunit of LT mutant LT_{R192G} using structure-based MEFA technology, a novel structural vaccinology approach. This PWD MEFA protein was characterized in Western blot and ELISAs with anti-K88, -F18, -LT, -STa, and anti-Stx2e antisera. Subsequently, immunogenicity of this MEFA protein was examined in mouse immunization studies. Serum samples of the subcutaneous (SC) immunized mice were titrated for anti-fimbriae and anti-toxin IgG antibody responses. Mouse serum antibody neutralization activities against ETEC fimbrial adherence and enterotoxicity were also measured. Data showed the expressed fimbriae-toxoid PWD MEFA protein, which was approximately 44 kDa, was verified in Western blot using anti-FaeG, anti-K88epitope-fusion, anti-F18epitope-fusion, anti-CT, anti-STa, and anti-Stx2e antiserum, respectively. Mice SC immunized with PWD MEFA protein developed strong anti-K88, anti-F18, anti-LT and anti-STb IgG antibody responses, and moderate anti-Stx2e and anti-STa IgG responses. Moreover, mouse serum antibodies inhibited adherence of K88- and F18-fimbrial ETEC bacteria and neutralized LT, STa, STb and Stx2e enterotoxicity. Additionally, double mutant LT (dmLT, $LT_{R192G/L211A}$) adjuvant up-

immunoregulated PWD MEFA anti-fimbriae and antitoxin antibody responses. These results indicated that this fimbriae-toxoid PWD MEFA induced broadly anti-fimbriae and anti-toxin antibodies, and suggested antigen candidacy for developing an effective vaccine against PWD.

4.1 Introduction

Post-weaning diarrhea (PWD), a disease found in pigs globally, commonly happens during the first two weeks after weaning and results in watery diarrhea, dehydration and acute death, also decreased weight gain in surviving piglets (Fairbrother, Nadeau, & Gyles, 2005; Heo et al., 2013; Rhouma et al., 2017). Epidemiological and clinical studies indicated that enterotoxigenic *E. coli* (ETEC) bacteria are among the most important causes of porcine post-weaning diarrhea (Fairbrother et al., 2005; Rhouma et al., 2017).

ETEC bacteria colonizes pig intestinal surface by fimbrial adhesins. F4 (K88) and F18are the most common fimbriae associated with PWD in piglets (de la Fe Rodriguez et al., 2011; Rhouma et al., 2017; W Zhang, 2014; W. P. Zhang, Zhao, Ruesch, Omot, & Francis, 2007). Both fimbriae consist of a major structural subunit and multiple minor subunits, encoded by multiple genes in a fimbrial gene cassette. F18 fimbriae is a typical fimbria study model with only one adhesive minor subunit, FedF, at the tip of the fimbriae (Do, Byun, & Lee, 2019; Dubreuil et al., 2016; Hahn et al., 2000; Imberechts et al., 1996). FedA is the major structure subunit which has multiple copies surrounding the axis of fimbriae (Barth, Schwanitz, & Bauerfeind, 2011; Hahn et al., 2000). F18 has two variants, which are F18ac and F18ab (Imberechts, Bertschinger, Nagy, Deprez, & Pohl, 1997). F18ac is more commonly associated with PWD while F18ab is related to pig edema disease (Amorim et al., 2000; da Silva, Valadares, Penatti, Brito, & da Silva Leite, 2001; DebRoy, Roberts, Scheuchenzuber, Kariyawasam, & Jayarao, 2009). F18 fimbriae only

pertain to PWD, whereas F4 (K88) fimbriae also relate to neonatal diarrhea in pigs (Dubreuil et al., 2016; Hur, Lee, & Lee, 2011). The major structural subunit of F4 is FaeG which is also the adhesive subunit (Bakker et al., 1991; Bakker, Willemsen, Simons, van Zijderveld, & de Graaf, 1992; Xia, Song, Zou, Yang, & Zhu, 2015). K88 has three variants with K88ac as the most common associated with pig neonatal diarrhea and post-weaning diarrhea. Three K88 variants have highly homologous FaeG but bind to different receptors on pig's intestinal surface (Xia, Zou, et al., 2015; W Zhang, 2014). The adhesion between fimbriae and glycoprotein receptors on the small intestinal surface allows the efficient release of enterotoxins from ETEC to intestinal epithelium cells (Dubreuil et al., 2016). Enterotoxin produced by ETEC includes Heat-labile toxin (LT) and Heat-stable toxin (ST), which can disturb the fluid secretion in neonatal gut epithelial cells (Dubreuil et al., 2016; W Zhang, 2014). LT is a 1A5B toxin which the B pentamar bound to GM1 receptors on pig epithelial cell surfaces (Mudrak & Kuehn, 2010; W Zhang, 2014). After binding, the enzymatic A subunit enters host epithelial cells and toxically elevates cell intracellular cyclic AMP levels, stimulating the protein kinase and causing the secretion of sodium and chloride in the lumen (Beddoe, Paton, Le Nours, Rossjohn, & Paton, 2010; Huang, Duan, & Zhang, 2018; Mudrak & Kuehn, 2010). ST consists of two variants: STa and STb (Dubreuil et al., 2016; Weiglmeier, Rösch, & Berkner, 2010; W Zhang, 2014). STa increases the intracellular cGMP levels, which blocking the absorption of liquid and salt, resulting in diarrhea (Dubreuil et al., 2016; Loos et al., 2012; Weiglmeier et al., 2010). Whereas STb does not increase cAMP or cGMP levels, rather it increases the secretion of non-chloride anions from gut epithelial cells (Dubreuil et al., 2016; Loos et al., 2012).

PWD is a multifactorial disease which remains a challenge for disease control and prevention (Rhouma et al., 2017; W Zhang, 2014). Vaccination is considered the most effective

and practical method against ETEC-associated PWD (Fairbrother et al., 2005; W Zhang, 2014). There are commercial vaccines available to immunize pregnant sows and to protect newborn piglets against ETEC infection through maternal vaccination (Matias, Berzosa, Pastor, Irache, & Gamazo, 2017). However, these vaccines are not effective against PWD due to the shortening of protective antibodies from colostrum and milk, as well as the immature active antibody production of piglets (Matias et al., 2017; Melkebeek et al., 2013). The lack of antibodies in neonatal guts after weaning increases the risk of ETEC infection (Dubreuil et al., 2016; Melkebeek et al., 2013). Furthermore, since ETEC induced diarrhea requires both adhesins and enterotoxins, neither anti-adhesin vaccine candidates nor the anti-enterotoxin vaccine candidates can protect against both virulence factors (Dubreuil et al., 2016; W Zhang, 2014). In many studies on ETEC vaccine development, subunit vaccinology has been a common strategy. A toxoid MEFA based subunit vaccine candidate was demonstrated to protect against most of the toxins related to ETEC infection (Q. Duan et al., 2017; Q. Duan et al., 2018; Rausch et al., 2017). Additionally, a K88-F18-LT genetic fusion protein MEFA was shown as an effective immunogen for a vaccine against porcine F4 and F18 ETEC (X. Ruan & Zhang, 2013; X. S. Ruan et al., 2011). However, neither vaccine candidate included all ETEC fimbriae and toxins for broad protection against PWD.

Therefore, this study aimed to construct a novel fimbriae-toxoid MEFA which carried K88, F18, LT, STa, STb epitopes, as well as one epitope from Shiga toxin 2e (Stx2e), the major virulence factor causing porcine edema disease (ED) (da Silva et al., 2001). The immunogenicity of this vaccine candidate was evaluated in a mouse model for future investigation in piglets.

4.2 Materials and methods

Bacteria and plasmids. Table 4.1 lists all strains and plasmids utilized in this study. All neutralizing epitopes from each virulence factor were embedded into heat-labile toxin (LT) A1 subunit by gene synthases (Genscript, Piscataway, NJ) **(Fig 4.1)**. Monomeric LT-STa recombinant fusion expressed by 8752 strain (Liu et al., 2011) was used for monomeric MEFA construction via SOE PCR. Both A1 part of PWD MEFA and PWD monomeric MEFA were expressed by *E. coli* strain BL21. Porcine ETEC 3030-2 ($K88ac^+$) (W. P. Zhang & Francis, 2010) and porcine *E. coli* field isolate 8516 ($F18^+$) (W. P. Zhang et al., 2007) was used for adherence inhibition assays and also for PCR amplification of FaeG and FedF subunit genes. Vector pET28a (Novagen, Madison, WI) and *E. coli* strain BL21 were used for subunit protein expression. The 9301 and 9302 strains (Rausch et al., 2017) were used to express MBP-STb and MBP-Stx2e, respectively, as the coating antigens for ELISA. The 8020 strain (STb^+) (W. P. Zhang et al., 2007) and 9168 Stx2e-producing *E. coli* strain (W. P. Zhang et al., 2007) were used for cytotoxicity neutralization assays.

Construction of PWD monomeric MEFA. B-cell antigenic epitopes of LT A1 subunit were identified in a previous study (Huang et al., 2018). Neutralizing epitopes were also characterized from FaeG (major subunit of K88) (T Lu & Zhang, 2017), FedF (minor subunit of F18) (Ti Lu, Seo, et al., 2019), STa, STb and Stx2e (Rausch et al., 2017). The 3-D protein modeling was displayed by PyMOL Molecular Graphics System (access: 106826; version 2.2; Schrödinger, LLC, New York City, NY, USA) with PDB transferred from amino acid sequences via Phyre2 online server (R. M. Bennett-Lovsey, A. D. Herbert, M. J. Sternberg, & L. A. Kelley, 2008; L. A. Kelley & Sternberg, 2009; Rigsby & Parker, 2016). Primers PWD-MEFA-A1 nheI-F and PWD-MEFA-B eagI-R (Table 4.2) were designed to amplify the chimera DNA fragment and

cloned it into vector pET-28α (Novagen, Madison, WI), as we previously described (Q. D. Duan & Zhang, 2017; Huang et al., 2018; Ti Lu, Seo, et al., 2019). Plasmid pET28α-STa13-LT192 from 8752 strain was used to amplify monomeric LT A2B DNA fragment and combined with chimera PWD MEFA A1 part by SOE-PCR. Monomeric PWD MEFA fragment was cloned into pET-28α by digesting with NheI and EagI restriction enzymes (New England BioLabs, Ipswich, MA).

Expression and purification of PWD monomeric MEFA. The recombinant plasmids were transferred to into *E. coli* BL21 (DE3) strains, as we previously described (Huang et al., 2018; Ti Lu, Seo, et al., 2019; Rausch et al., 2017). Briefly, a single positive clone was selected and expressed with 30 μM IPTG (Sigma, St. Louis, MO) for 4 hours after culture OD_{600} reached 0.6-0.8. Bacterial pellets were harvested (5000 × g, 10 min, 4 °C), resuspended and lysed in Bacterial protein extraction reagent (B-PER 4 mL/g; Thermo Fisher Scientific, Rochester, NY). 1× IB solubilization buffer (CAPS 50 mM, DTT 1 mM, N-lauroylsarcosine 0.3 %) was added to refold the inclusion bodies after washing. Purified MEFA proteins were assessed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 15 %) stained with Coomassie blue.

Characterization of PWD monomeric MEFA. Antigenicity of purified PWD monomeric MEFA protein was verified by western blot using anti-CT (rabbit, 1:3000; Sigma, St. Louis) or anti-STa (rabbit, 1:2000) or anti-Stx2e (mouse, 1:1500) or anti-FedF (mouse, 1:1500) or anti-FaeG (mouse, 1:1500) sera and IRDye-labeled goat anti-mouse or anti-rabbit IgG antibodies (1:5,000; LI-COR, Lincoln, NE) (Ti Lu, Seo, et al., 2019; Nandre, Ruan, Duan, Sack, & Zhang, 2016). Briefly, A1 part of PWD MEFA and PWD monomeric MEFA protein were loaded onto SDS-PAGE (15 %) separately and transferred to nitrocellulose membranes, blocked with 5% fat-free milk at 4°C overnight. One hour antibody incubations was used at room

temperature, with three times washing with PBST (PBS with 0.05 % Tween-20) between each step. Total proteins of *E. coli* BL21 host strain as the background control were used. LI-COR Odyssey Infrared Gel Imaging System Premium (LI-COR) was used for image analysis.

Mouse immunization. Animal study was approved by the Institutional Animal Care and Use Committee at Kansas State University (IACUC #4056). Eight-week-old BALB/c mouse (10 mice per group) was injected subcutaneously with 40 μg PWD monomeric MEFA protein with 1 μg dmLT (double mutant LT; $LT_{R192G/L211A}$; provided by PATH) adjuvant. Other group with ten mice was immunized with PWD monomeric MEFA protein without dmLT adjuvant. Ten mice injected with PBS were used as negative control. Two boosters at the same dose of the primer will be administered every two weeks. Antiserum samples were harvested from each mouse at day 42 via cardiac puncture after euthanasia. Procedure was shown in **Fig 4.3A**.

Serum antibody measurement. The titrations of antigen-specific IgG antibodies were measured by ELISA (Rausch et al., 2017). Briefly, 96-well Immune 2HB ELISA plates (Fisher Scientific) were coated with 100 ng/well of antigens (including K88, F18, CT, STa, MBP-STx2e and MBP-STb) overnight at 4 °C, blocked with 10 % fat-free milk in PBST. Mouse antisera were added at a 1:400 dilution with 1:1 gradient dilution to each row, and incubated at 37°C for 1 hour. After three washes, the plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000; Sigma) for 1 hour. After color development, enzyme-based colorimeter (650 nm) OD value was determined and a scale of $\log_{10}$ was used for IgG antibody titers (T Lu & Zhang, 2017). Western blot was used to verify the specificity of each antigen-specific IgG antibodies.

Adherence inhibition assay. IPEC-J2 cells (pig jejunum epithelial cell from un-suckled 1-day-old piglets), as well as porcine ETEC 3030-2 ($K88ac^+$) and porcine *E. coli* field isolate

8516 ($F18^+$) were used to verify the antibody neutralizing ability against K88-mediated and F18-mediated bacterial attachment. IPEC-J2 cells were grown as previously described in detail (Ti Lu, Seo, et al., 2019; T Lu & Zhang, 2017). Briefly, IPEC-J2 cells were grown in the 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (1:1; Invitrogen, CA) with 5 % Fetal Bovine Serum (FBS) at 37 ºC with 5 % CO_2 until reaching confluence. Moreover, the cells were re-suspended after 0.25 % Trypsin-EDTA with the added fresh medium and seeded into 48-well cell-plates evenly. Plates were incubated at 37ºC with 5 % CO_2 until reaching 90-95 % confluence per well. The bacterial suspensions were diluted in PBS to a final concentration of 1.5×10^6 CFU/mL. The mixtures with 100 μL of bacterial suspensions (1:10 of cell to bacteria radio) and 30 μL mouse antisera or PBS were incubated at room temperature for 30 minutes at 60 rpm, added to each cell well with 470 μL cell medium without FBS, followed by 1 hour incubation at 37 °C in 5 % CO_2. After three washes, cells were lysed by 500 μL sterile 0.5 % TritonX-100. Dislodged cells and adherent bacteria were removed to sign tubes and re-suspended with 500 μL sterile PBS. The mixtures were diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 100 μL from 10^{-3} or 10^{-4} was speeded on LB agar plates for CFU calculation.

Anti-LT antibody neutralization. Direct cyclic AMP EIA kit (Enzo Life Sciences, Inc., Farmingdale, NY) and human colon carcinoma cell line T84 (ATCC; CCL-248) were used to examine the antibody neutralizing ability against LT toxin. T84 cells were grown as previously described in detail (Huang et al., 2018; Rausch et al., 2017; X. S. Ruan et al., 2011). Briefly, T84 cells were grown in the 75 cm^2 flasks in DMEM and Ham's F12 medium (1:1; Invitrogen, CA) with 5 % FBS at 37 ºC with 5 % CO_2 until reaching confluence. A 24-well cell-plate was used to seed the cells evenly. Plates were incubated at 37 ºC with 5 % CO_2 until reaching 90-95 % confluence per well. The mixtures with 10 ng commercial CT (in PBS, homolog to LT) and 30

μL mouse antisera or PBS were incubated at room temperature for 30 minutes with gentle shaking, added to each cell well with 1000 μL (as the final volume) DMEM/F12 medium (without FBS) with 1 mM IBMX, followed by 3 hours incubation at 37 °C in 5 % CO_2. Cells were lysed by 300 μL HCl (0.1 M) with 0.5 % TritonX-100 and collected (1000 × g, 10 mins). The supernatants were added to cyclic AMP EIA kit for intracellular cAMP levels.

Anti-STa antibody neutralization. Direct cyclic GMP EIA kit (Enzo Life Sciences, Inc., Farmingdale, NY) and T-84 cells were used to examine the antibody neutralizing ability against STa toxin (Rausch et al., 2017; X. Ruan et al., 2014). The mixtures with 2 ng commercial STa toxin and 30 μL mouse antisera or PBS were incubated at room temperature for 30 minutes with gentle shaking, added to each cell well with 1000 μL (as the final volume) DMEM/F12 medium (without FBS) with 1 mM IBMX, followed by 1 hours incubation at 37°C in 5 % CO_2. Cells were lysed by 300 μL HCl (0.1 M) with 0.5 % TritonX-100 and collected (1000 × g, 10 mins). The supernatants were added to cyclic GMP EIA kit for intracellular cGMP levels.

Anti-STb antibody neutralization. The 8020 (STb^+) strain and Vero cells (ATCC; CCL-81) were used to examine the antibody neutralizing ability against STb toxin. Vero cells were grown as previously described in detail (Rausch et al., 2017). Briefly, Vero cells were grown in the 75 cm^2 flasks Eagle's minimum essential medium (EMEM) with 5 % FBS at 37 ºC with 5 % CO_2 until reaching confluence. The cells evenly seeded into 24-well cell-plates. The mixtures with 300 μL bacterial culture filtrates of 8020 strain (50 % cell detachment or CD_{50} to Vero cells) and 150 μL, or 25 μL, or 10 μL of pooled mouse antisera or PBS were added into each well with 700 μL (as the final volume) EMEM medium (without FBS), followed by 1 hours incubation at 37°C in 5 % CO_2. Antibody neutralization titer - the highest dilution for CD_{50} of Vero cells - was examined by microscopy.

Anti-Stx2e antibody neutralization. The 9168 Stx2e-producing *E. coli* and Vero cells were used to examine the antibody neutralizing ability against Stx2e toxin (Rausch et al., 2017). The mixtures with 100 μL bacterial culture filtrates of 9168 Stx2e-producing *E. coli* strain (CD_{50} to Vero cells) and 18.8 μL, 6.3 μL, or 3 μL of pooled mouse antisera or PBS were added into each well with 1 mL (as the final volume) EMEM medium (without FBS), followed by 3 days incubation at 37°C in 5% CO_2. Antibody neutralization titer - the highest dilution for CD_{50} of Vero cells - was examined by microscopy daily.

Statistics. Data management and analysis were performed using GraphPad Prism version 7.0.0 (GraphPad Software, San Diego, California USA). One-way ANOVA was done for antibody titration and neutralization assay data. A p value of <0.05 was considered as statistically significant. The results were expressed by the mean ± standard deviation (SD). All experiments were repeated two times using duplicate samples.

4.3 Results

Expression and detection of PWD monomeric MEFA carried four epitopes of fimbriae and six epitopes of toxins. F18 minor subunit FedF epitopes QPDATGSWYD and IPSSSGTLTCQAGT, and K88 major subunit FaeG epitopes GRTKEAFATP and PMKNAGGTKVGSVKVN were selected as K88 and F18 antigens for this study. These four epitopes, along with STb epitope KKDLCENY, Stx2e epitope QSYVSSLN, two copies of STa toxin epitope CCELCCSPACAGCY were fused into the antigenic sites of LTA subunit by gene synthesis (Genscript, Piscataway, NJ) (**Fig. 4. 1A**). Two LT A subunit neutralizing epitopes DSRPPDEIKRSGG and SPHPYEQEVSA, which were identified in the previous study were

included. Protein 3-D modeling software PyMOL was used to map the locations of epitopes and confirmed that all epitopes were surface exposure (**Fig. 4. 1B-E**). PWD monomeric MEFA (A1 part, A1 domain, and LT B subunit, in a single peptide) of 43 kDa were expressed and purified (**Fig. 4. 2A**). The monomeric structure of PWD-fimbriae-toxoid MEFA reacted with anti-fimbriae and anti-toxin serum from Western blot and direct ELISAs (**Fig. 4. 2A-B**).

Immunogenicity of PWD monomeric MEFA in the mouse model. Eight-weeks-old female BALB/C mice (10 mice per group) SC immunized with PWD monomeric MEFA with or without dmLT developed antigen-specific IgG antibody responses (**Fig. 4. 3**). Anti-fimbriae and anti-toxin IgG responses induced by PWD monomeric MEFA were assessed by measuring Western blot and ELISA. As shown in **Fig. 4. 3B**, PWD monomeric MEFA adjuvanted with dmLT induced antibodies in vivo which detected to each virulence factor protein by Western blot assays. IgG titers (in $\log_{10}$) in the mice immunized with PWD monomeric MEFA were 2.855±0.276 to K88, 2.465±0.301 to F18, 3.137±0.621 to CT, 2.971±0.344 to STb, 2.015±0.171 to Stx2e and 1.350±0.144 to STa (**Fig. 4. 3C**). The difference of each virulence factor IgG response between immunized group and the control group injected with PBS was significant (p<0.001). One immunized mouse did not developed anti-F18 or anti-STa IgG response, and an additional mouse showed no anti-F18 or anti-STa IgG response.

Moreover, dmLT adjuvant could significantly enhance the IgG responses induced by PWD monomeric MEFA. IgG titers (in $\log_{10}$) in the mice immunized with PWD monomeric MEFA and adjuvanted with dmLT were 3.510±0.366 to K88, 2.882±0.381to F18, 3.562±0.370 to CT, 3.269±0.320 to STb, 2.438±0.125 to Stx2e and 1.531±0.263 to STa (**Fig. 4. 3C**). A significant difference was shown between immunized group and the control group injected with PBS with p<0.001. Additionally, the difference of each virulence factor IgG titer between

immunized group with dmLT and immunized group without dmLT was highlighted in **Fig. 4. 3C**.

Anti-PWD monomeric MEFA inhibited adherence of F18$^+$ *E. coli* or K88$^+$ ETEC to porcine cell line IPEC-J2. F18 fimbriae inhibition or K88 fimbriae adherence inhibition was measured in the presence and absence of anti-PWD monomeric MEFA antibodies. Results showed that the antibodies induced by PWD monomeric MEFA significantly inhibited the attachment of F18$^+$ *E. coli* (**Fig. 4. 4A**) or K88$^+$ ETEC (**Fig. 4. 4B**) to porcine cell line IPEC-J2, with $p<0.001$. Anti-PWD monomeric MEFA has a similar ability to inhibit 8516 strain (F18$^+$) adherence to IPEC-J2 compared with anti-F18 sera ($p>0.12$). In contrast, anti-PWD monomeric MEFA induced significant lower ability to inhibit 3030-2 strain (K88$^+$) adherence to IPEC-J2 than that activated by anti-K88 sera ($p<0.005$). No additional adherence inhibition of F18$^+$ *E. coli* or K88$^+$ ETEC to IPEC-J2 was observed in the samples from mice immunized with PWD monomeric MEFA and adjuvanted with dmLT ($p>0.12$).

Anti-PWD monomeric MEFA neutralized STa toxicity *in vitro*. Antibodies from mice immunized with PWD monomeric MEFA and adjuvanted with dmLT neutralized STa toxicity, with the significant reduction of intracellular cGMP in T-84 cells from 9.07±0.396 pmole/ml (cells treated with 2 ng STa and negative mouse sera) to 5.64±0.987 pmole/mL ($p< 0.01$) (**Fig. 4. 4C**). Antibodies from mice immunized with PWD monomeric MEFA without dmLT decreased intracellular cGMP in T-84 cells to 6.48±0.771 pmole/mL ($p=0.06$).

Anti-PWD monomeric MEFA neutralized CT toxicity *in vitro*. Antibodies from mice immunized with PWD monomeric MEFA neutralized LT toxicity, with the significant diminished the intracellular cAMP levels in the T-84 cells from 4.33±0.318 pmole/mL (cells treated with 10 ng CT and negative mouse sera) to 0.825±0.262 pmole/mL ($p=0.02$) (**Fig. 4. 4D**).

No significant increase of LT neutralizing ability was observed in the T-84 cells treated with CT and anti-PWD monomeric MEFA adjuvanted with dmLT (1.07±0.396 pmole/mL) compared with no adjuvant group (p>0.99).

Anti-PWD monomeric MEFA neutralized STb cytotoxicity *in vitro*. Cytotoxicity of STb in 300 μL 8020 strain (STb^+) filtrate was completely neutralized with the anti-PWD monomeric MEFA diluted 1: 7.7 (**Fig. 4. 5D & G**). Diluted 1:100 anti-PWD monomeric MEFA neutralized cytotoxicity of STb in 8020 strain filtrate to 50% Vero cell detachment (CD_{50}) (**Fig. 4. 5F**). Less cell detachment was observed in the Vero cells treated with 8020 strain filtrate and anti-PWD monomeric MEFA adjuvanted with dmLT diluted 1:100 (**Fig. 4. 5I**).

Anti-PWD monomeric MEFA neutralized Stx2e cytotoxicity *in vitro*. Cytotoxicity of Stx2e in 100 μL 9168 Stx2e-producing *E. coli* strain filtrate was completely neutralized with the anti-PWD monomeric MEFA diluted 1:53.2 (**Fig. 4. 6D & G**). Diluted 1:333.3 anti-PWD monomeric MEFA neutralized cytotoxicity of Stx2e in 9168 strain filtrate to 50% Vero cell detachment (CD_{50}) (**Fig. 4. 6F**). Less cell detachment was observed in the Vero cells treated with 9168 strain filtrate and anti-PWD monomeric MEFA adjuvanted with dmLT diluted 1:333.3 (**Fig. 4. 6I**).

4.4 Discussion

Several approaches have been used to develop a better ETEC vaccine. However, lack of effective treatment strategies against ETEC-associated PWD requires a broadly effective ETEC vaccine. In recent years, epitope-based subunit vaccines have turned out to be more promising because they are more immunogenic and more flexible to form fusion with such small size. Previous studies have demonstrated two epitopes as the most immunodominant epitopes of F18

minor subunit FedF as they have the potential to produce to produce F18 neutralizing antibodies (Ti Lu, Seo, et al., 2019). Two epitopes of K88 major subunit FaeG are the most immunodominant epitopes due to their abilities to generate K88 neutralizing antibodies (T Lu & Zhang, 2017). Furthermore, immunogenic epitopes of STb, Stx2e and STa toxin had been embedded into A1 peptide of a monomeric LT mutant LT_{R192G} toxoid to construct a toxoid MEFA which induced neutralizing antibodies against LT, STb, STa and Stx2e in our previous study (Rausch et al., 2017). Neutralizing epitopes of LT A subunit have also been identified (Huang et al., 2018). On this basis, we have developed a monomeric fimbriae-toxiod MEFA comprising immune-dominant epitopes of K88, F18, LT, STa, STb and Stx2e in this study. This fimbriae-toxoid PWD MEFA was evaluated in mice model and could induce broadly anti-fimbriae and anti-toxin antibodies. The results of this study suggested this antigen candidacy could be used for developing an effective vaccine against PWD in the future.

According to our hypothesis, antisera produced by PWD monomeric MEFA were able to recognize K88, F18, STb, Stx2e, STa and LT by western blot and ELISA. The results highlighted anti-PWD monomeric MEFA sera contain a pool of antibodies against specific epitopes of ETEC virulence factors. Likewise, anti-K88, anti-F18, anti-STa, anti-CT and anti-Stx2e were found to recognize PWD monomeric MEFA by ELISA and Western blot, illustrating the existence of antigenic determinants specific to K88, F18, as well as STb, Stx2e, STa and LT. Those results demonstrated that our PWD monomeric MEFA is immunogenic and can induce IgG response, suggesting a significant humoral response. Prior studies that have noted the importance of humoral immune-response in ETEC vaccination (Andersen, Lundgre, Osterud, Volden, & Giercksky, 1985; Evans et al., 1977). Although the titer of STa was found to be lower than those of other virulence factors, which could be explained by its small molecular weight (<2

KDa) (N. E. Aref & Saeed, 2011; N. M. Aref, Nasr, & Osman, 2018; Dubreuil et al., 2016). The poor immunogenic problem of STa was also reported in our previous study (Qiangde Duan & Zhang, 2016; Rausch et al., 2017). Antigenicity of STa may relate to the location and its copy numbers in the MEFA (Rausch et al., 2017). Here we embedded two copies of STa epitope "CCELCCNFACAGCY" including the disulfide bonds into the optimal positions in A1 part of LT toxoid based on the structural vaccinology. Anti-STa titer induced by PWD monomeric MEFA was higher than that induced by our previous toxoid MEFA.

Fimbriae attachment inhibition is one of the major facets to be considered during the vaccine development against ETEC infection (Q. D. Duan, Yao, & Zhu, 2012; W Zhang, 2014). We found anti-PWD monomeric MEFA indicate significant inhibition ability against both K88 and F18 fimbriae attachment, but lower than anti-K88 sera which could be attributed to the polyadhesin structure of K88 fimbriae. Furthermore, enterotoxins which disrupt secretion of water and electrolytes in pig small intestinal epithelial cells is the prime cause of diarrhea in ETEC infection. Thus, toxin neutralization is also an important indicator of ETEC vaccine evaluation (Melkebeek et al., 2013; W Zhang, 2014). Our results established anti-PWD monomeric MEFA sera exhibit significant toxin neutralization ability. Since Stx2e associates with Edema disease, this fimbriae-toxoid MEFA is potential for the protective efficacy of PWD and ED in weaned piglets. We also found that dmLT adjuvant could enhance immunogenicity of PWD monomeric MEFA which is consistent with a lot of previous studies. As a detoxified version, dmLT can enhance mucosal immunity via inducing a cAMP-dependent signal and a B-subunit GM1-binding signal without any gastrointestinal toxicity in host (Leach, Clements, Kaim, & Lundgren, 2012; X. Ruan et al., 2014). Our results suggested that dmLT can be used as the adjuvant for PWD MEFA vaccine in the future.

Several researches are trying to develop recombinant subunit vaccine based on immunogenic capacity of epitopes from each virulence factor to combat ETEC infections, (Holmgren et al., 2013; Luiz et al., 2008; Nazarian et al., 2012). However, the major challenge in the path is that it is difficult to ensure the surface exposure of every epitope. Moreover, there is a potential for the conformational changes of the backbone proteins when embedding multitude foreign epitopes. In this study, we combined the reverse vaccinology with structural vaccinology to design and construct the PWD monomeric MEFA effectively (Cozzi, Scarselli, & Ferlenghi, 2013; Delany, Rappuoli, & Seib, 2013; Dormitzer, Grandi, & Rappuoli, 2012; Q. Duan et al., 2017; Rappuoli, 2001). Here we optimized the platform which improves the traditional proceeding of bacterial vaccine development via bioinformatics and structural proteomics. This platform, while preliminary, offers a possibility to reduce the use of experimental animals during the vaccine development with cost-effectiveness and higher success rate.

In summary, our results suggest that immunization with this PWD monomeric MEFA provides broadly protection against ETEC infection via inducing both anti-adhesin and anti-toxin antibodies. This is the first study to construct a subunit vaccine which includes all the major virulence factors of ETEC related to PWD. Compared to our previous subunit vaccines designed for PWD (Rausch et al., 2017), we propose PWD monomeric MEFA as a better prospective vaccine candidate against ETEC-associated PWD, where PWD monomeric MEFA was found to exhibit broadly protective efficacy in mouse model with the capability of neutralizing LT, STa, STb and Stx2e as well as inhibiting K88 and F18 attachment at the same time. However, being limited to the mouse model, this study lacks direct results which confirm that this fimbria-toxoid MEFA can protect weaned piglets against ETEC-associated PWD. Further work needs to be done in pig challenge model to evaluate the efficacy and safety of this vaccine candidate.

4.5 Reference

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Table 4.1 *Escherichia coli* strains and plasmid used in the study.

Strains and plasmids	Relevant properties	Reference
Strains		
BL21	*huA2, Δ(argF-lacZ), U169, phoA, glnV44, φ80, Δ(lacZ)M15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17*	GE Healthcare
9703	A1 subunit of PWD monomeric MEFA synthesized in pUC57 in DH5α	This study
9715	pET28α-A1 subunit of PWD monomeric MEFA in DH5α	This study
9716	pET28α- PWD monomeric MEFA in DH5α	This study
9718	pET28α-A1 subunit of PWD monomeric MEFA in BL21	This study
9719	pET28α-PWD monomeric MEFA in BL21	This study
8752	pET28α-STa13-LT192 fusion in BL21	(Liu et al., 2011)
3030-2	porcine ETEC challenge strain, K88ac/LT/STb	(W. P. Zhang & Francis, 2010)
8516	porcine *E. coli* field isolate, F18	(W. P. Zhang et al., 2007)
8020	K88/STb (pRAS1 in 1836–2)	(W. P. Zhang et al., 2007)
9168	04-13812 field isolate, F18/Stx2e	(W. P. Zhang et al., 2007)
9301	MBP-STb fusion in pMAL-p5X in DH5α	(Rausch et al., 2017)
9302	MBP-Stx2eA fusion in pMAL-p5X in DH5α	(Rausch et al., 2017)
Plasmids		
pET28α		Novagen

Table 4.2 Primers used in the study.

Primer	Sequence (5'-3')	Amplified region
PWD-MEFA-A1 nheI-F	CGGGCTAGCATGAAAAATATAACTTTC	Upstream of PWD-MEFA A1 subunit gene, with *Nhe*I site
PWD-MEFA-B eagI-R	TTACGGCCGCTAGTTTTCCATACTGAT	Downstream of LTB gene, with *Eag*I site
PWD-MEFA-A1-L	TCATTACAAGTATCACCTGTAATTGTTCTTGAATAATTTTCACAC	Overlapping A1 subunit with A2 subunit
PWD-MEFA-A2-R	AAATTATTCAAGAACAATTACAGGTGATACTTGTAATGAGGAGAC	
PWD-A1only-nheI-F	CGGGCTAGCCCGATGAAAAACATCACCTTTATC	Upstream of PWD-MEFA A1 subunit gene, with *Nhe*I site
PWD-A1only-eagI-R	TTACGGCCGGAAGATGGTACGGCTGTAGTTCTC	Downstream of PWD-MEFA A1 subunit gene, with *Eag*I site

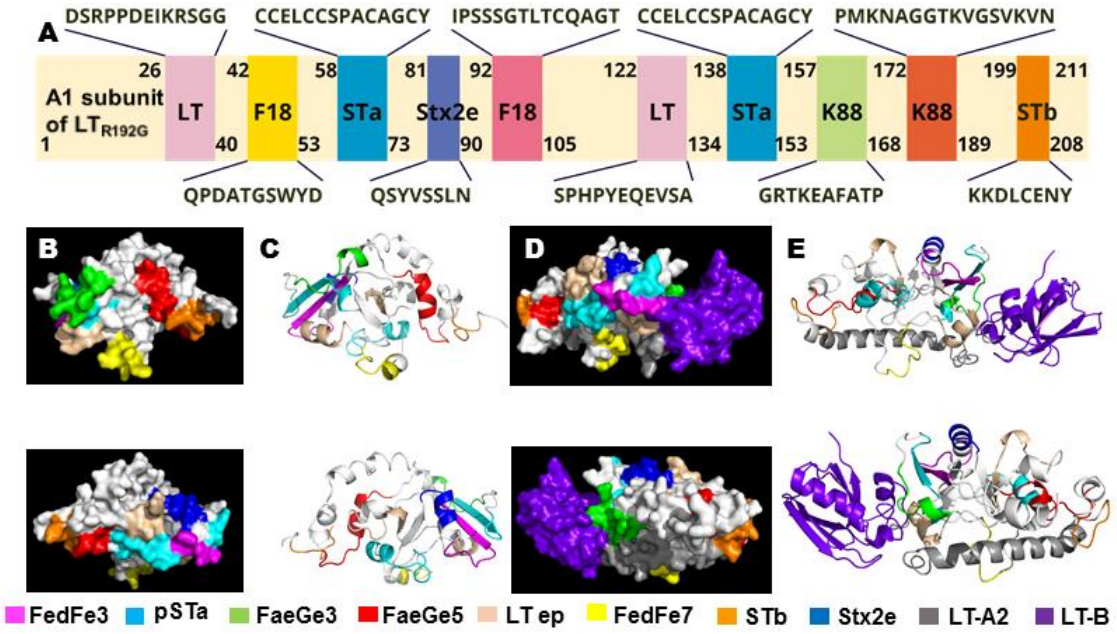

Figure 4.1 Construction of PWD monomeric MEFA. (A) Schematic illustration of the PWD-fimbriae-toxoid MEFA gene. Nucleotides coding two K88 epitopes, two F18 epitopes, Stx2e A subunit epitope, and two copies of part-length STa toxoid STaP12F, STb epitope were synthesized into the A1 subunit of the monomeric LT toxoid LTR192G by GenScript (Piscataway, NJ). (B) (C) (D) (E) Protein computational modeling to show epitopes of A1 subunit of PWD monomeric MEFA and PWD monomeric MEFA by PyMOL. (B) 3D modeling of A1 subunit of PWD monomeric MEFA. (C) Second structure of A1 subunit of PWDmonomeric MEFA. (D) 3D modeling of the monomeric structure of PWD monomeric MEFA. (E) Second structure of PWD monomeric MEFA.

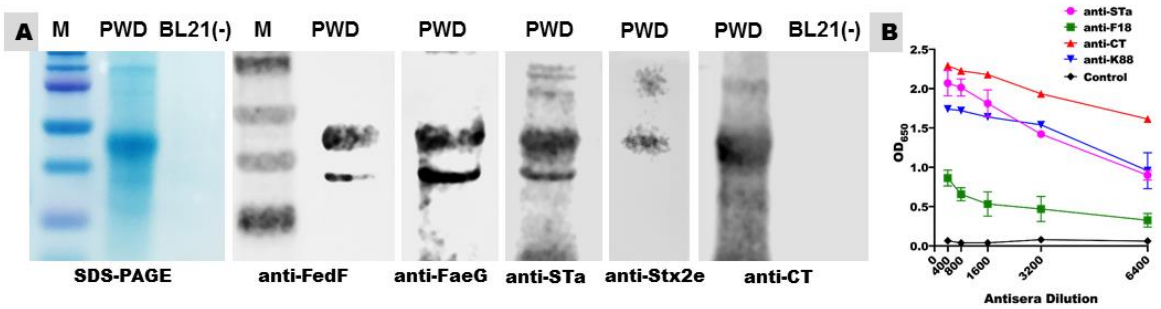

Figure 4.2 Detection of PWD monomeric MEFA. (A) Coomassie blue staining of A1 subunit of PWD monomeric MEFA and PWD monomeric MEFA. (B) (C) (D) (E) (F) Western blot assays to detect A1 subunit of PWD monomeric MEFA (lane 1) and PWD monomeric MEFA (lane 2) with (B) anti-CT (1:3000; Sigma), (C) anti-Stx2e (1:1500) (D) anti-FedF (1:1500) (E) anti-FaeG (1:1500) and (F) rabbit anti-STa (1:2000) antiserum, with IRDye-labeled goat anti-mouse and anti-rabbit IgG (1:5,000; LI-COR) as the secondary antibodies. Protein marker (in kilodaltons; Precision Plus Protein prestained standards; Bio-Rad) and total proteins of *E. coli* BL21 host strain as the background control were used.

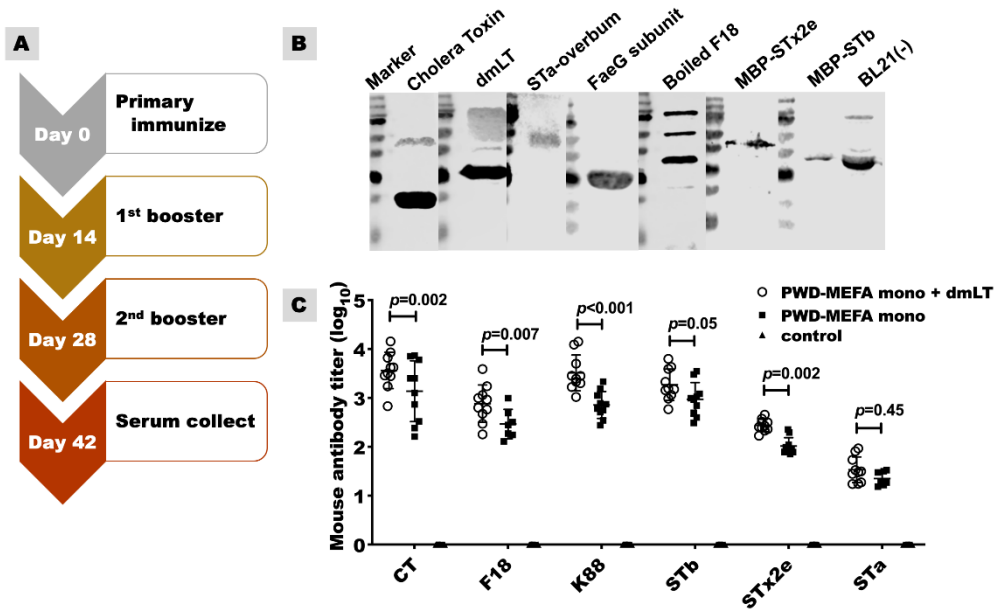

Figure 4.3 Immunogencity of mouse anti- PWD monomeric MEFA antiserum. (A) Timeline of mouse vaccination. (B) Western blot assays to detect STa, FaeG, F18, STx2e and STb with anti-PWD monomeric MEFA with dmLT adjuvant. CT and dmLT were tested with anti-PWD monomeric MEFA without dmLT adjuvant. Total proteins of *E. coli* BL21 host strain was used as the background control. (C) Mouse IgG titers (log_{10}) against each virulence factor of PWD from the group subcutaneously immunized with PWD monomeric MEFA w/o dmLT or the control group immunized with PBS. The mean titer in each group was indicated by bar. Each mouse IgG titer was showed by empty cycle (immunized with PWD monomeric MEFA with dmLT), solid block (immunized with PWD monomeric MEFA without dmLT) and solid triangle (the control mice, *** $p<0.001$ compared to all other immunized groups). No significant difference (ns $p> 0.12$) was found in mouse antiserum detection of STa-overbum protein between mouse groups immunized with PWD monomeric MEFA w/o dmLT.

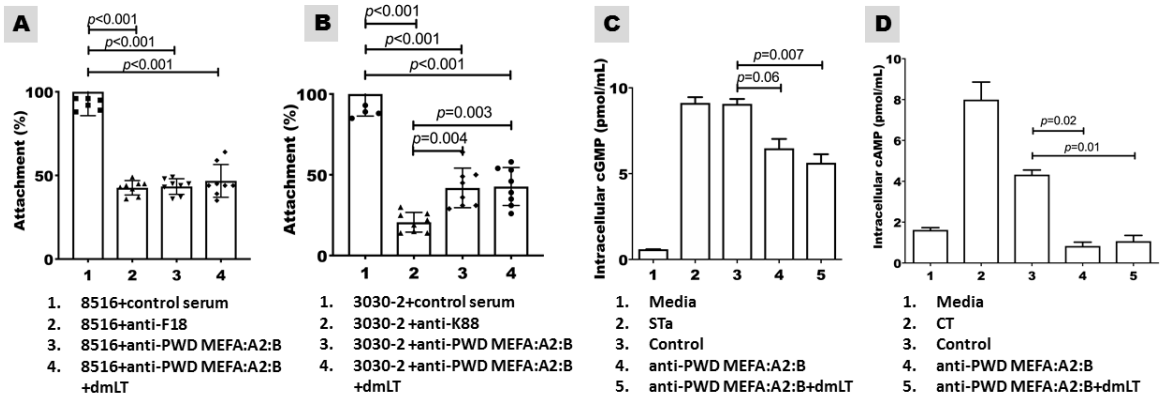

Figure 4.4 Mouse serum antibody in vitro neutralization activity against each virulence factor. (A) Mouse anti-PWD monomeric MEFA w/o dmLT antibody inhibition against adherence of F18 fimbrial ETEC to IPEC-J2 cells (%). The number of adherent bacteria in the control group was considered as 100% (***p<0.001 compared to all other immunized groups). Mouse anti-F18 fimbriae antiserum was used as the positive control (ns p>0.12 compared to mouse groups immunized with PWD monomeric MEFA w/o dmLT). (B) Mouse anti-PWD monomeric MEFA w/o dmLT antibody inhibition against adherence of K88 fimbrial ETEC to IPEC-J2 cells (%). The number of adherent bacteria in the control group was considered as 100% (*p<0.001 compared to all other immunized groups). Mouse anti-K88 fimbriae antiserum was used as the positive control (*p<0.033 compared to mouse groups immunized with PWD monomeric MEFA w/o dmLT). (C) Mouse anti-PWD monomeric MEFA w/o dmLT antibody neutralization activity against STa toxicity. STa (2 ng) were incubated with mouse anti- PWD monomeric MEFA w/o dmLT antiserum and the control serum in T-84 cells. The EIA cGMP kit (Enzo Life Science) was used to measure the T-84 cell intracellular cGMP levels. T-84 cell stimulated cGMP levels by directly added STa and baseline cGMP levels in T-84 cells culture medium (without STa or serum) were also showed. * p<0.033 was found in the cGMP levels in T-84 cells incubated with STa exposed to the mouse control serum compared to that with mouse

anti- PWD monomeric MEFA with dmLT antiserum. No significant difference (ns $p>0.12$) was found in the cGMP levels in T-84 cells incubated with STa exposed to the mouse control serum compared to that without mouse anti-PWD monomeric MEFA with dmLT antiserum. (D) Mouse anti-PWD monomeric MEFA w/o dmLT antibody neutralization activity against LT enterotoxicity. CT (20 ng) were incubated with mouse anti-PWD monomeric MEFA w/o dmLT antiserum and the control serum in T-84 cells. The EIA cAMP kit (Enzo Life Science) was used to measure the T-84 cell intracellular cAMP levels. T-84 cell stimulated cAMP levels by directly added CT and baseline cAMP levels in T-84 cells culture medium (without CT or serum) were also showed. * $p<0.033$ was found in the cAMP levels in T-84 cells incubated with CT exposed to the mouse control serum compared to those with mouse anti- PWD monomeric MEFA w/o dmLT antiserum.

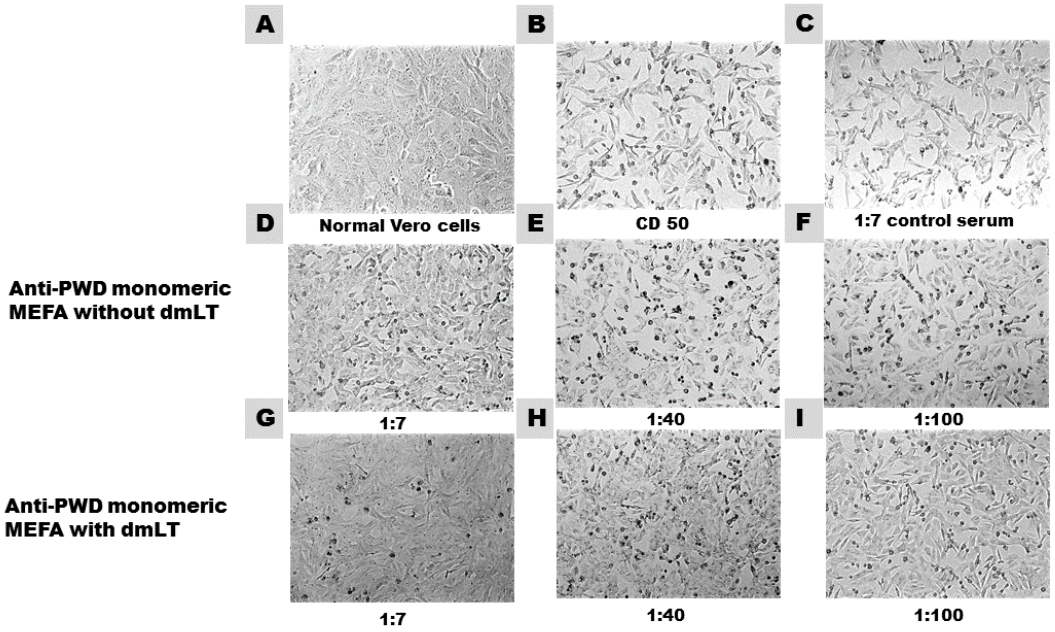

Figure 4.5 Mouse serum antibody in vitro neutralization activity against STb toxin. (A) Normal Vero cells. (B) 50% cell death was showed in vero cells incubated with 300 µL overnight grown culture filtrates from STb$^+$ *E. coli* strain 8020. (C) Vero cells incubated with 300 µL overnight grown culture filtrates from STb$^+$ *E. coli* strain 8020 pre-mixed with 150 µL pooled control serum. (D) Vero cells incubated with 300 µL overnight grown culture filtrates from STb$^+$ *E. coli* strain 8020 pre-mixed with 150 µL pooled anti-PWD monomeric MEFA without dmLT antiserum. (E) Vero cells incubated with 300 µL overnight grown culture filtrates from STb$^+$ *E. coli* strain 8020 pre-mixed with 25 µL pooled anti-PWD monomeric MEFA without dmLT antiserum. (F) Vero cells incubated with 300 µL overnight grown culture filtrates from STb$^+$ *E. coli* strain 8020 pre-mixed with 10 µL pooled anti-PWD monomeric MEFA without dmLT antiserum. (G) Vero cells incubated with 300 µL overnight grown culture filtrates from STb$^+$ *E. coli* strain 8020 pre-mixed with 150 µL pooled anti-PWD monomeric MEFA with dmLT antiserum. (H) Vero cells incubated with 300 µL overnight grown culture filtrates from STb$^+$ *E. coli* strain 8020 pre-mixed with 25 µL pooled anti-PWD monomeric MEFA with dmLT

antiserum. (I) Vero cells incubated with 300 μL overnight grown culture filtrates from STb^+ *E. coli* strain 8020 pre-mixed with 10 μL pooled anti-PWD monomeric MEFA with dmLT antiserum.

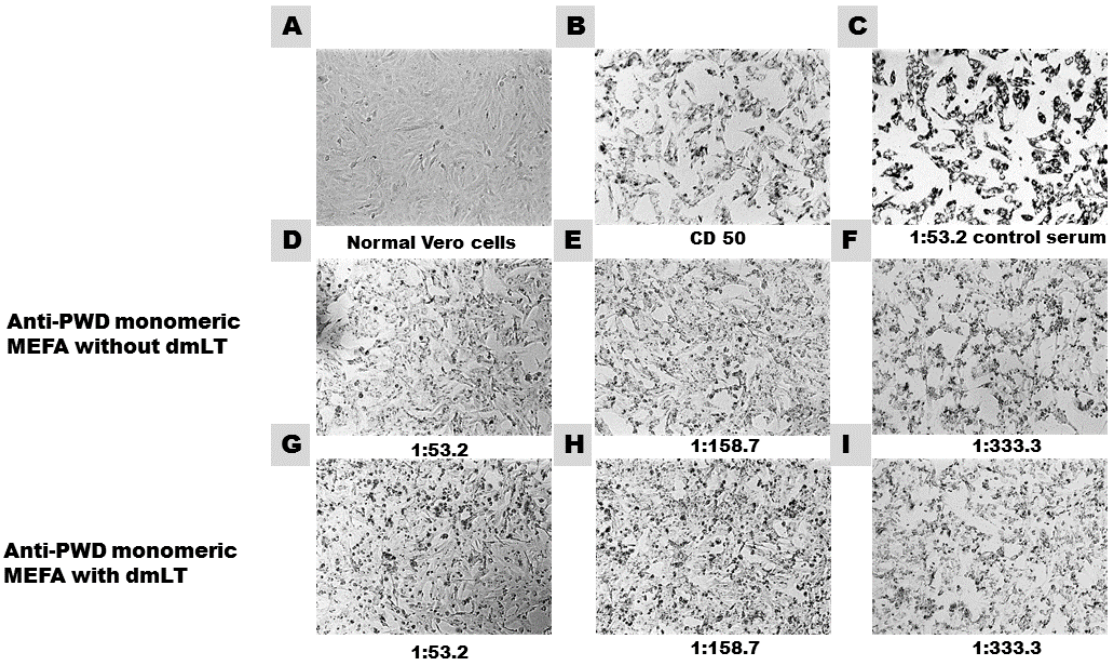

Figure 4.6 Mouse serum antibody in vitro neutralization activity against STx2e toxin. (A) Normal Vero cells. B: 50% cell death was showed in vero cells incubated with 100 µL overnight grown culture filtrates from STx2e$^+$ *E. coli* strain 9168. (C) Vero cells incubated with 100 µL overnight grown culture filtrates from STx2e$^+$ *E. coli* strain 9168 pre-mixed with 18.8 µL pooled control serum. (D) Vero cells incubated with 100 µL overnight grown culture filtrates from STx2e$^+$ *E. coli* strain 9168 pre-mixed with 18.8 µL pooled anti-PWD monomeric MEFA without dmLT antiserum. (E) Vero cells incubated with 100 µL overnight grown culture filtrates from STx2e$^+$ *E. coli* strain 9168 pre-mixed with 6.3 µL pooled anti-PWD monomeric MEFA without dmLT antiserum. (F) Vero cells incubated with 100 µL overnight grown culture filtrates from STx2e$^+$ *E. coli* strain 9168 pre-mixed with 3 µL pooled anti-PWD monomeric MEFA without dmLT antiserum. (G) Vero cells incubated with 100 µL overnight grown culture filtrates from STx2e$^+$ *E. coli* strain 9168 pre-mixed with 18.8 µL pooled anti-PWD monomeric MEFA with dmLT antiserum. (H) Vero cells incubated with 100 µL overnight grown culture filtrates from STx2e$^+$ *E. coli* strain 9168 pre-mixed with 6.3 µL pooled anti-PWD monomeric MEFA with

dmLT antiserum. (I) Vero cells incubated with 100 μL overnight grown culture filtrates from STx2e^{+} *E. coli* strain 9168 pre-mixed with 3 μL pooled anti-PWD monomeric MEFA with dmLT antiserum.

Chapter 5 - Optimizing the immunizing route by using a carrier to deliver the MEFA antigen to local mucosal areas

Ti Lu, Rodney A Moxley, Weiping Zhang

Abstract: Enterotoxigenic *Escherichia coli* (ETEC) is the primary cause of porcine post-weaning diarrhea (PWD). Currently there is no effective vaccine for PWD. A structure- and epitope-based multiepitope fusion antigen (MEFA) to induce broadly neutralizing anti-adhesin (K88 and F18) and antitoxin (LT, STa, STb, Stx2e) antibodies has been constructed. However, this adhesin-toxoid MEFA needs to be extracted as a protein for parenteral immunization. In this study, this MEFA gene was optimized to be expressed as a holotoxin-structured and GM1-binding protein in a live host strain to induce mucosal antibodies against ETEC adhesins and toxins. *Salmonella* Ty21a strain, a mutant of *Salmonella* Ty2 strain lacking Uridine-diphosphate-galactose (UDP-Gal) -4-epimerase by chemically inactivating the galE gene was selected as the host strain to express the optimized holotoxin-structure adhesin-toxoid MEFA. Data showed that optimized PWD adhesin-toxoid MEFA formed a holotoxin structure and bound to GM1 receptor, and Ty21a strain as well as porcine field *E. coli* isolate G58 produce the new adhesin-toxoid MEFA and secreted the protein outer-membrane. These results suggest that Ty21a or G58 host producing the GM1-binding adhesin-toxoid MEFA can potentially be an effective mucosal vaccine against PWD.

5.1 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is considered the major cause of postweaning diarrhea (PWD), which commonly occurs during the first 2 weeks after weaning and exhibits the

clinical symptoms of acute death, diarrhea, and dehydration as well as reduced weight gain in surviving piglets (Rhouma et al., 2017). PWD is a multifactorial disease associated with a reduction in milk immunity and the incomplete development of active immunity in piglets during weaning (W. P. Zhang et al., 2007). When infection occurs, ETEC can adhere to glycoprotein receptors on the pig small intestinal epithelium by fimbrial adhesins on the bacterial surface. Six fimbrial subtypes related to animal diarrhea diseases including K88 (F4), K99 (F5), 987P (F6), F41 (F7) F17 and F18 have been identified. Among them, K88 and F18 are commonly isolated from PWD piglets (Dubreuil et al., 2016). Since K88 receptors can be isolated at all life stages from the pig intestines, while F18 receptors can only be found in pigs after 2-3 weeks of age, K88 fimbriae relate to both neonatal diarrhea (ND) and PWD, but F18 fimbriae only associate with PWD (Heo et al., 2013). After fimbrial attachment, ETEC releases enterotoxins to stimulate the secretion of water and electrolytes into the intestinal lumen, leading to diarrhea and dehydration. Heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) are the main enterotoxins of ETEC. LT is a 1A5B toxin with a B pentamer binding to GM1 receptors on pig intestinal epithelial cells (Huang et al., 2018). The enzymatic A subunit is cleaved into two parts after endocytosis, and the A1 part continually stimulates Protein Kinase A (PKA) by increasing the cAMP level, leading to watery diarrhea. ST comprises STa and STb. STa increases the intracellular cGMP level to activate abnormal secretion of water and electrolytes, leading to diarrheal disease (Qiangde Duan & Zhang, 2016; Q. D. Duan et al., 2012). Unlike LT or STa, STb cannot increase the cAMP or cGMP level, but it does increase the calcium ion concentration to trigger chloride ion channels on gut epithelial cells, resulting in diarrhea (Dubreuil et al., 2016; W. P. Zhang et al., 2007).

PWD is still a global challenge in the pig industry, and there is no effective licensed vaccine. Maternal vaccination against swine ETEC infections has been widely used. A commercial inactivated vaccine or subunit vaccines are commonly given to sows intramuscularly before farrowing (Hur & Lee, 2012b; Matias et al., 2017). Newborn pigs are protected by antibodies in the colostrum and milk during the nursing period. However, this protection will decrease when weaning starts. An oral commercial vaccine against PWD that includes the avirulent live *E. coli* strains O141:K94 (F18ac) and O8:K87 (F4ac) was approved for use in Canada and the European Union recently (Fairbrother et al., 2017). However, since ETEC requires both adhesins and enterotoxins to induce PWD, neither an anti-adhesin nor an anti-enterotoxin vaccine candidate alone can produce broad efficiency. In previous studies, we constructed live *E. coli* vaccine strains expressing fimbriae-toxoid fusions (Rausch et al., 2017; X. S. Ruan et al., 2011; C. Zhang & Zhang, 2010). Moreover, a holotoxin-structured backbone of LT with a complete B pentamer has been shown to enhance local mucosal immunity by successfully binding to GM1 on the pig intestinal surface (X. Ruan & Zhang, 2013).

The *Salmonella typhi* Ty21α strain is a mutant of the *Salmonella typhi* Ty2 strain lacking Uridine-diphosphate-galactose (UDP-Gal)-4-epimerase through chemical inactivation of the galE gene. This strain is part of the only oral live attenuated vaccine (Vivotif®) against typhoid fever used by the World Health Organization (WHO) (Organization, 2019). The mutation in galE disrupts galactose metabolism, leading to the accumulation of galactose in the bacteria, resulting in the loss of bacterial virulence (McKenna et al., 1995). After oral administration, the *Salmonella typhi* Ty21α strain proliferates in the jejunum and cecum to induce humoral immunity and cellular immunity (Wahid et al., 2016). The immunized intestinal tract quickly clears pathogens to prevent salmonellosis. Furthermore, the *Salmonella typhi* Ty21α strain has

been used as a vaccine delivery platform to express vaccine antigens against some diseases, such as HIV infection, anthrax, shigellosis, plague, and human papilloma virus infection (Amicizia et al., 2017; Baillie et al., 2008; Dharmasena, Osorio, et al., 2016; J. Wen et al., 2012).

In our previous study, we constructed a monomeric PWD multiepitope fusion antigen (PWD MEFA) that induced broadly neutralizing anti-adhesin (K88 and F18) and anti-toxin (LT, STa, STb, and Stx2e) antibodies in a mouse model. In this study, we optimized this MEFA to produce a holotoxin-structured protein that could bind to GM1 receptors to induce the mucosal immune response. Furthermore, the *Salmonella typhi* Ty21α strain and porcine field *E. coli* isolate strain G58 were used to deliver the optimized holotoxin-structured adhesin-toxoid MEFA. We compared the secretion of the new adhesin-toxoid MEFA between the *Salmonella typhi* Ty21α strain and porcine field *E. coli* isolate strain G58 and found that both strains could express the GM1-binding adhesin-toxoid MEFA, which has the potential to protect piglets against PWD.

5.2 Materials and methods

Bacteria and plasmids. All strains and plasmids utilized in this study are listed in Table 5.1. The LT recombinant strain 8460 (Liu et al., 2011) was used to construct the holotoxin-structured MEFA by SOE PCR. The vector pBR322 (Promega, Madison, WI) as well as the *E. coli* DH5a strain, porcine *E. coli* isolate strain G58 (X. Ruan & Zhang, 2013) and *Salmonella typhi* Ty21α strain (Vivotif®, PaxVax, Redwood, CA) were used for holotoxin-structured PWD MEFA expression. The porcine ETEC 3030-2 strain ($K88ac^+$) (W. P. Zhang & Francis, 2010) was used for bacterial attachment.

Construction of the holotoxin-structured PWD MEFA. A synthesized heat-labile toxin (LT) A1 subunit including all neutralizing epitopes from each virulence factor was

constructed in a previous study. The primers PWD-MEFA-A1 nheI-F and PWD-MEFA-A1-L were designed to amplify the synthesized DNA fragment. The primers PWD-MEFA-A2 R and PWD-MEFA-B eagI-R were designed to amplify the holotoxin-structured LT A2B DNA fragment from the LT recombinant strain 8460. Two DNA fragments were overlapped and cloned into the pBR322 vector by digestion with NheI and EagI restriction enzymes (X. Ruan & Zhang, 2013). The primers pBR322-check-F and pBR322-check-R were used for DNA sequencing. 3-D protein modeling was displayed by PyMOL Molecular Graphics System (access: 106826; version 2.2; Schrödinger, LLC, New York City, NY, USA) with amino acid sequences transferred from PDB via Phyre2 online server (Lawrence A Kelley, Mezulis, Yates, Wass, & Sternberg, 2015).

Secretion of the PWD holotoxin-structured MEFA. Recombinant plasmids were transferred to the *E. coli* DH5a strain, porcine *E. coli* strain G58 and *Salmonella typhi* Ty21α strain as we previously described (Rausch et al., 2017). Briefly, a single positive clone was selected and cultured overnight in 10 mL Luria broth (LB) medium supplemented with ampicillin (100 μg/mL) at 37°C. The overnight bacterial culture was centrifuged (3000 × g, 30 mins), and the supernatant was concentrated at 4°C for 2 hours. Secretion of the PWD holotoxin-structured MEFA was assessed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 15%) by using a gel stained with Coomassie blue or further detected by Western blotting using anti-CT (rabbit; 1:3000; Sigma-Aldrich, St. Louis, MO) or anti-PWD monomeric MEFA (mouse; 1:2000) and IRDye-labeled goat anti-rabbit IgG or anti-mouse IgG (1:5,000; LI-COR, Lincoln, NE) antibodies.

GM1 ELISA assays. Nunc-Immuno 96-MicroWell ELISA plates (Sigma-Aldrich, St. Louis, MO) were coated with 0.4 μg/well monosialoganglioside GM1 overnight at 4°C and

blocked with 5% BSA in PBST (PBS with 0.05% Tween-20) at 37°C for 1 hour. A single positive clone of the 9740 strain (*E. coli* DH5a + PWD holotoxin-structured MEFA), 9741 strain (*Salmonella typhi* Ty21α + PWD holotoxin-structured MEFA) or 9742 strain (porcine *E. coli* G58 + PWD holotoxin-structured MEFA) was selected and cultured overnight in 10 mL TSB (Tryptic Soy Broth) (Sigma-Aldrich, St. Louis, MO) medium supplemented with ampicillin (100 μg/mL) at 37°C. The overnight bacterial culture was transferred to 10 ml TSB at a 1:10 ratio. When the OD_{600} reached 1.0, the culture was centrifuged (3000 × g, 30 mins), 200 μL supernatant, heat-labile toxin (1 μg/μL) or 1% BSA in PBST was added to the first row, and a 1:1 gradient dilution was used to dilute the molecules into each row below, followed by a 1 hour incubation. After three washes, the plates were incubated with 100 μL/well anti-CT (rabbit, 1:3000; Sigma-Aldrich, St. Louis, MO), anti-STa (rabbit, 1:400), anti-Stx2e (mouse, 1:200), anti-F18 (mouse, 1:1000) and anti-K88 (mouse, 1:1000) antibodies for 1 hour. Afterwards, a horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG (1:3000; Bethyl, Montgomery, TX) or HRP-conjugated goat-anti-rabbit IgG (1:3000; Bethyl, Montgomery, TX) antibody was added to each well as a secondary antibody. The results were analyzed by enzyme-based colorimetry using the OD value at 650 nm, and a $\log_{10}$ scale was used for the IgG antibody titers.

Adherence assays. IPEC-J2 cells (pig jejunum epithelial cells from unsuckled 1-day-old piglets) were cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (1:1; ATCC, Invitrogen, CA) with 5% fetal bovine serum (FBS). The strains 3030-2 ($K88ac^+$), porcine *E. coli* G58 and *Salmonella typhi* Ty21α were cultured overnight in 5 ml TSB medium supplemented with ampicillin (100 μg/mL) at 37°C. The overnight bacterial culture was transferred to 10 ml TSB at a 1:50 ratio for an additional 2 hours. When the OD_{600} reached 0.5-0.6, the bacterial cultures were centrifuged at 3000 × g for 5 mins and resuspended in PBS at a

concentration of 1.5×10^6 CFU/mL. The contents of each well were added to 100 µL diluted bacterial cultures (1:10 ratio of cells to bacteria), followed by a 1 hour incubation at 37°C in 5% CO_2. The cells were lysed in 500 µl sterile 0.5% Triton X-100. Dislodged cells and adherent bacteria were removed to separate tubes and resuspended in 500 µL sterile PBS. The mixtures were diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 100 µl from 10^{-3} or 10^{-4} and spread on TSA (Tryptic Soy Agar) (Sigma-Aldrich, St. Louis, MO) plates for CFU calculations.

Statistics. Data management and analysis were performed using GraphPad Prism software version 7.0. One-way ANOVA was performed with the antibody titration and neutralization assay data. A p-value <0.05 was considered statistically significant. The results were expressed as the mean ± standard deviation (SD). Adherence assays were repeated two times using duplicate samples, while GM1 ELISAs were repeated two times using triplicate samples.

5.3 Results

Expression of the PWD holotoxin-structured MEFA. The synthesized LT A1 part overlapped with the A2 part and B subunit, between which the cistron gene structure was reinserted to reverse an LT-like gene encoding an LT-like holotoxin-structured protein **(Fig 5.1A)**. The protein 3-D modeling software PyMOL was used to map the locations of epitopes and confirmed that all epitopes were surface exposed **(Fig 5.1B-E)**. PWD holotoxin-structured MEFAs of approximately 99.3 kDa were successfully secreted into the supernatant by the *E. coli* DH5a strain **(Fig 5.1F)**. The PWD holotoxin-structured MEFAs reacted with anti-CT and anti-PWD monomeric MEFAs by Western blotting **(Fig 5.1F)**.

Secretion and GM1-binding evaluation of the PWD holotoxin-structured MEFA. A GM1 ELISA showed that the PWD adhesin-toxoid MEFA was successfully optimized to form a holotoxin structure **(Fig 5.2)**. Moreover, the data also indicated that the 9740 strain (*E. coli* DH5a carrying the PWD holotoxin-structured MEFA), the 9741 strain (*Salmonella typhi* Ty21α carrying the PWD holotoxin-structured MEFA) and the 9742 strain (porcine *E. coli* G58 carrying the PWD holotoxin-structured MEFA) could secrete the PWD holotoxin-structured MEFA protein to the outer membrane **(Fig 5.2A)**. At a 1:1600 dilution of bacterial supernatant, all strains carrying the PWD holotoxin-structured MEFA had significantly higher OD_{650} values than the corresponding empty strains ($p<0.001$). The 9741 strain showed a better OD_{650} value than the 9740 or 9742 strain ($p<0.001$), while there was no significant difference between the 9740 and 9742 strains ($p>0.99$).

Detection of each epitope on the PWD holotoxin-structured MEFA. A GM1 ELISA additionally demonstrated that the PWD holotoxin-structured MEFA could react with anti-fimbriae and anti-toxin sera **(Fig 5.2B)**. The 9741 strain showed better reactivity with anti-K88 and anti-F18 sera than the 9742 strain ($p<0.05$), while those two strains had no significant differences in anti-STa, anti-Stx2e or anti-LTA reactions ($p>0.05$).

Abilities of porcine *E. coli* G58 and *Salmonella typhi* Ty21α to adhere to the porcine cell line IPEC-J2. Adherence assays were used to examine whether *E. coli* G58 and *Salmonella typhi* Ty21α had similar attachment activities. The adhered CFUs are reported in Table 5.3. The 3030-2 strain ($18.6\pm6.02 \times 10^3$ CFU) showed better adherence ability than the other strains ($p<0.001$), while the *E. coli* G58 strain ($2.82\pm0.74 \times 10^3$ CFU) and *Salmonella typhi* Ty21α strain ($2.77\pm0.33 \times 10^3$ CFU) showed no significant difference ($p>0.99$).

5.4 Discussion

Researchers have found that traditional injection routes, such as subcutaneous vaccination, may effectively induce systemic immunity (Ti Lu, Seo, et al., 2019). However, it is difficult for protection to reach the intestinal mucosa, which is the gateway of bacterial infection (Pavot, Rochereau, Genin, Verrier, & Paul, 2012). Therefore, an ideal enteric vaccine, first of all, should be able to induce an immune response in the gastrointestinal mucus. In this study, two live PWD MEFA vaccine strains that expressed an LT-like holotoxin-structured MEFA protein were constructed. The PWD holotoxin-structured MEFA was secreted to the outer membrane in the *Salmonella typhi* Ty21α and *E. coli* G58 strain and could bind to the GM1 receptor. These results suggested that these two live vaccine candidates could potentially induce an immune response in the pig gastrointestinal mucosa against PWD.

The *Salmonella typhi* strain Ty21a has been used globally as an oral typhoid vaccine. However, due to the superior immunogenicity of the *Salmonella* antigen, it has rarely been tested as a living carrier for a heterologous antigen (Bumann et al., 2001). Recent studies have shown that the *Salmonella typhi* strain Ty21a can be used as a vector for some pathogens, such as *Helicobacter pylori* (Metzger et al., 2004). These vaccine candidates can induce a comparative immune response in the gastrointestinal mucosa. Moreover, researchers have found that maternal antibodies from immunized sows will not significantly disturb the tissue invasion of the vaccine strain in piglets (Wales & Davies, 2017). Here, the attachment of the *Salmonella typhi* Ty21a strain and the *E. coli* G58 strain to the porcine cell line IPEC-J2 was tested, and the two strains were found to have similar attachment levels. We used the *E. coli* G58 strain as the host strain for PWD vaccine development and determined that this strain could deliver MEFA proteins to the pig intestinal mucosa. Since *Salmonella typhi* Ty21α had an adherence ability similar to that

of *E. coli* G58 when tested with the porcine cell line, *Salmonella typhi* Ty21α could be a host strain for PWD MEFA delivery. This finding has important implications for developing an oral recombinant *Salmonella* vaccine to elicit an efficient swine mucosal response against ETEC infection.

In conclusion, this study constructed two live PWD MEFA vaccine strains expressing an LT-like holotoxin-structured MEFA protein by using the *Salmonella typhi* Ty21α or *E. coli* G58 strain. This PWD holotoxin-structured MEFA could bind to the GM1 receptor with a functional B pentamer. These results suggested that these two live vaccine candidates could potentially induce an immune response in the pig gastrointestinal mucosa against PWD. The data of this study provide new ideas for constructing a recombinant *Salmonella*-based PWD vaccine as well as strong support for porcine vaccination strategies against ETEC-associated PWD. Further studies are required to establish the immunogenicity and safety of these two vaccine candidates in a pig model and optimize the acid-resistance system of the Ty21α host strain to achieve lower costs and industrialized production.

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Table 5.1 *Escherichia coli* strains and plasmids used in the study.

Strains and plasmids	Relevant properties	Reference
Strains		
E. coli BL21	*huA2, Δ(argF-lacZ), U169, phoA, glnV44, φ80, Δ(lacZ)M15, gyrA96, recA1, relA1, endA1, thi-1*, and *hsdR17*	GE Healthcare
S. typhi Ty21α		Vivotif®
E. coli G58	K88ac^{-}LT^{-}STb^{-}	(Francis & Willgohs, 1991)
9703	A1 subunit of PWD MEFA synthesized in pUC57 in DH5α	This study
9740	pBR322 - the holo-structure of the PWD-fimbriae-toxoid MEFA in DH5α	This study
9741	pBR322 - the holo-structure of the PWD-fimbriae-toxoid MEFA in Ty21α	This study
9742	pBR322 - the holo-structure of the PWD-fimbriae-toxoid MEFA in G58	This study
8460	eltAB genes + pBR322 in TOP10, LT recombinant strain	(Liu et al., 2011)
3030-2	porcine ETEC challenge strain, K88ac/LT/STb	(W. P. Zhang & Francis, 2010)
Plasmids		
pBR322		Promega

Table 5.2 Primers used in the study.

Primer	Sequence (5'-3')	Amplified region
PWD-MEFA-A1 nheI-F	CGGGCTAGCATGAAAAATATAACTTTC	Upstream of the PWD-MEFA A1 subunit gene, with an *Nhe*I site
PWD-MEFA-B eagI-R	TTACGGCCGCTAGTTTTCCATACTGAT	Downstream of the LTB gene, with an *Eag*I site
PWD-MEFA-A1-L	ATTACAAGTATCACCTGTGATGGTACGGCTGTAGTTCTCGCA	A1 subunit overlapping with the A2 subunit
PWD-MEFA-A2-R	AACTACAGCCGTACCATCACAGGTGATACTTGTAATGAGGAG	
pBR322-check-F	GTACTGCCGGGCCTCTTG	Upstream of pBR322 from 164 aa
pBR322-check-R	GCCAGCAAGACGTAGCCC	Downstream of pBR322 from 969 aa

Table 5.3 Bacteria adherence assays.

Bacteria	Mean of attachment (CFU) ± SD	*p* value	
		vs Ty21α	vs G58
3030-2	18.6±6.02 ($\times 10^3$)	<0.001	<0.001
Ty21α	2.77±0.33 ($\times 10^3$)	-	>0.99
G58	2.82±0.74 ($\times 10^3$)	>0.99	-

The ETEC bacteria 3030-2 strain and porcine cell line IPEC-J2 were used. The CFUs of ETEC 3030-2, *Salmonella typhi* Ty21α, and *E. coli* G58 bacteria adhered to the IPEC-J2 cells were used to indicate the adhesive ability of each strain. Data are presented as the mean ± SD from three independent experiments. One-way ANOVA was used to calculate the *p* values comparing bacterial attachment among the strains.

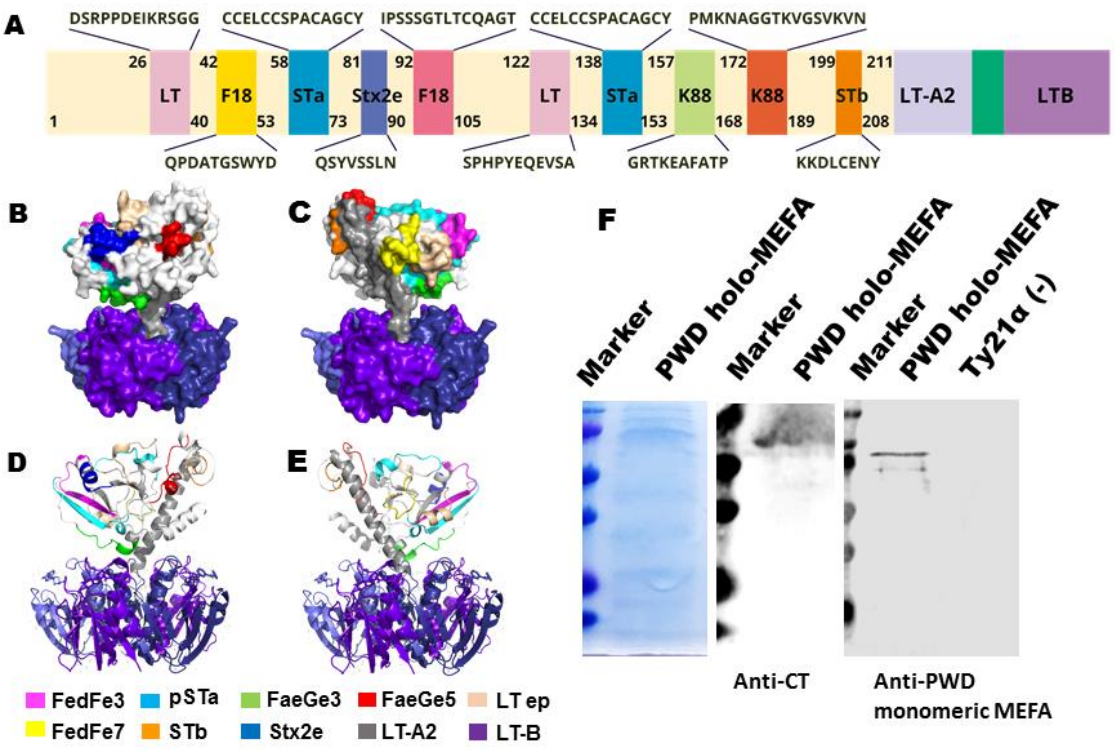

Figure 5.1 Construction of the PWD holotoxin-structured MEFA. (A) Schematic illustration of the PWD holotoxin-structured MEFA gene. The synthesized A1 subunit was fused into the holotoxin-structured LT toxoid LTR192G by GenScript (Piscataway, NJ). (B) (C) (D) (E) Protein computational modeling by PyMOL was used to show the epitopes of the PWD holotoxin-structured MEFA. (A and B) The front (B) and back (C) of the PWD holotoxin-structured MEFA is shown with the 7 fused epitopes indicated in different colors (2 copies of the STa epitope). White, LT A1 domain backbone; gray, LT A2 domain; and purple, LT B subunit. (D and F) A second structure of the PWD holotoxin-structured MEFA with each fused epitope in the same color as that shown in panels A and B is shown. (F) Western blot assays were used to detect the secretion of the PWD-holo-structure MEFA into the supernatant of the 9741 strain culture via anti-PWD MEFA-A1A2:1B (mouse; 1:1000) and anti-CT (rabbit; 1:3000) sera. The supernatant of the Ty21α empty strain was used as the background control.

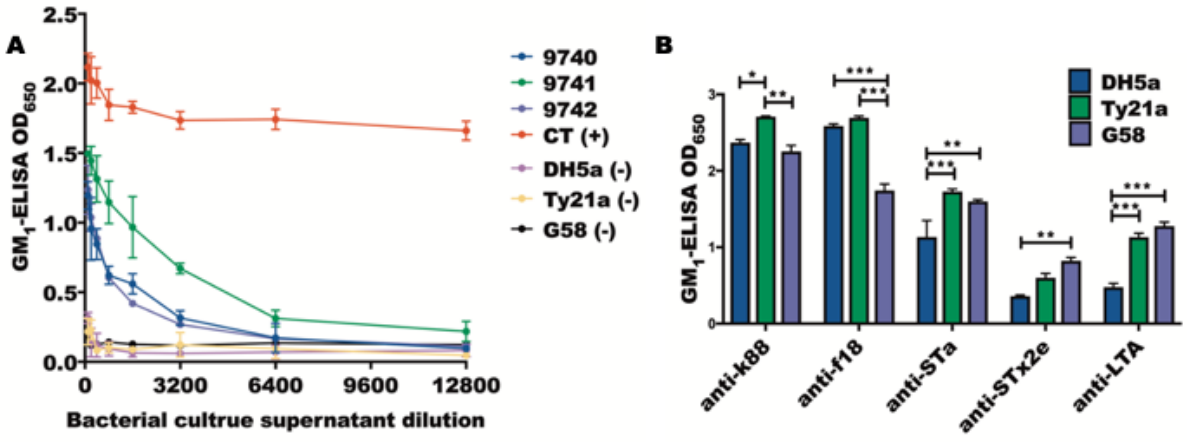

Figure 5.2 Secretion and comparison of the PWD holotoxin-structured MEFA in different bacterial strains. (A) A GM1 ELISA was used to show the outer membrane secretion of the PWD holotoxin-structured MEFA and GM1-binding activity. The *E. coli* DH5α, *Salmonella typhi* Ty21α and *E. coli* G58 strains were used as negative controls. CT (2 μg per well) was added as the positive control. Anti-CT serum (rabbit; 1:3000) was used as the primary antibody. (B) A GM1 ELISA was used to show the exposed epitopes of the PWD holotoxin-structured MEFA. Anti-K88 (mouse; 1:1000), anti-F18 (mouse; 1:1000), anti-STa (rabbit; 1:400), anti-Stx2e (mouse; 1:200) and anti-LTA (mouse; 1:400) sera were used as primary antibodies. HRP-anti-mouse (1:5000) and HRP-anti-rabbit IgG (1:5,000) antibodies were used as the secondary antibodies. ***, $p<0.001$; **, $p<0.002$; *, $p<0.033$; and ns, $p>0.12$.

Chapter 6 - Conclusion

Enterotoxigenic *Escherichia coli* (ETEC) bacteria are the essential cause of post-weaning diarrhea (PWD) in piglets, which causes huge economic losses annually to the swine production. Currently, there are no licensed vaccines for ETEC. Immunodominant and neutralizing epitopes of F18 fimbrial adhesin subunit FedF and K88 fimbrial adhesin subunit FaeG were investigated. Then a PWD fimbriae-toxoid MEFA inducing broadly effective protection against PWD-associated ETEC infection was developed and optimized.

In chapter 2 and 3, we identified seven epitopes from F18 fimbrial adhesin subunit FedF and nine epitopes from K88ac fimbrial adhesin subunit FaeG. Those epitopes could maintain native antigenicity after being fused to heterologous carrier CfaB protein. Each epitope fusion protein was recognized by a special anti-fimbriae serum but also the ability to compete with F18 or K88 fimbria for binding to special anti-fimbriae antibodies or to reduce anti-fimbriae antibodies from inhibiting adherence of *E. coli* bacteria expressing F18 or K88ac fimbriae. Furthermore, each epitope could induced antibodies specific to F18 or K88ac fimbriae in subcutaneously immunized mice. Derived antibodies from all FedF epitope fusions and 6 of 9 FaeG epitope fusions showed neutralizing activities against F18 or K88ac fimbria adherence to pig intestine cell line IPEC-J2. Among those neutralizing epitopes, epitope 3 and epitope 7 of FedF, as well as epitope 3 and epitope 5 of FaeG displayed better in inducing neutralizing anti-F18 or anti-K88 antibodies, suggesting their potential application in vaccine development against PWD.

In chapter 4, we constructed a monomeric PWD multiepitope fusion antigen (PWD-MEFA) which provided broad protection against ETEC infection via inducing both anti-adhesin

and anti-toxin antibodies. Specific neutralizing epitopes of K88, F18, STa, STb, and Stx2e epitopes were embedded into the A subunit of LTR192G using gene synthesis. This is the first study to construct a subunit vaccine which includes all the major virulence factors of ETEC related to PWD. We proposed PWD monomeric MEFA as a better prospective vaccine candidate against ETEC-associated PWD, where PWD monomeric MEFA was found to exhibit broadly protective efficacy in a mouse model with the capability of neutralizing LT, STa, STb, and Stx2e as well as inhibiting K88 and F18 attachment at the same time. However, being limited to the mouse model, this study lacks direct results which confirm that this fimbria-toxoid MEFA can protect weaned piglets against ETEC-associated PWD. Further work needs to be done in the pig challenge model to evaluate the efficacy and safety of this vaccine candidate.

In chapter 5, we continually optimized the monomeric PWD MEFA to an LT-like holotoxin-structured protein that could bind to GM1 receptor with a functional B pentamer. This PWD holotoxin-structured MEFA was expressed in *Salmonella* Ty21α strain or *E. coli* G58 strain as the oral live vaccine candidates. We found that these two live vaccine candidates could potentially induce immune response on pig gastrointestinal mucus against PWD. Data of this study provide new ideas for constructing a recombinant *Salmonella*-based PWD vaccine, as well as strong support for porcine vaccination strategies against ETEC-associated PWD. Further works are required to establish the immunogenicity and safety of these two vaccine candidates in pig models and optimize the acid-resistant system of Ty21α host strain for lower cost and industrialized production.

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