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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.

1. Introduction

Post-weaning diarrhea (PWD) is one of the most important swine diseases (Fairbrother et al., 2005; USDA, 2002). Piglets commonly develop diarrhea 3–10 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.

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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), 987 P (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, 2008). The optimal prevention approach would be to block attachment of different ETEC fimbriae to host receptors and to eliminate enterotoxicity of major enterotoxins (LT, STs) to host cells (Walker, 2005; Zhang, 2014; Zhang and Sack, 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 et al., 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 2–3 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 and Zhang, 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. Methods and materials

2.1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. Recombinant CfaB strain 9477 (Ruan et al., 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 adherence inhibition assay.

2.2. 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 et al., 2006; Saha and Raghava, 2007). Predicted epitopes were mapped on a FedF protein model generated from Phyre3 (Bennett-Lovsey et al., 2008; Kelley and Sternberg, 2009). 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 and Zhang, 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.

Table 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

2.3. 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 et al., 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°C for 1 h, 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). OD_{600} 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-2. 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 and Zhang, 2013 Ruan et al., 2014a, 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 ug CfaB-epitope fusion protein together with 1.5×10^6 CFU F18-fimbrial *E. coli* bacteria 8516 were mixed with 25 μl mouse anti-F18 antiserum and incubated at room temperature for

Table 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	AGCACTACTCGCACTAGAATTGATTTTTTTAGTTGCATCGTTTGT	forward and reverse primers to insert FedF epitope 1 into *CfaB* gene
FedF-ep1-R	TCTAGTGCGAGTAGTGCTCAAGTCGATACACCACAGCTTACAGAT	
FedF-ep2-F	AGTGTTTGTCTTCCCTGTGCCAAGTTTTTAGTTGCATCGTTTGT	forward and reverse primers to insert FedF epitope 2 into *CfaB* gene
FedF-ep2-R	ACAGGGAAGACAAACACTACCCAAATGGATACACCACAGCTTACA	
FedF-ep3-F	GCATGTCAAAGTTCCTGATGAACTAGGAATTTTTTTAGTTGCATC	forward and reverse primers to insert FedF epitope 3 into *CfaB* gene
FedF-ep3-R	TCAGGAACTTTGACATGCCAGGCTGGAACTGATACACCACAGCTT	
FedF-ep4-F	CTGTTGCCCCCACTGAGATTCATTTTTTTTAGTTGCATCGTTTGT	forward and reverse primers to insert FedF epitope 4 into *CfaB* gene
FedF-ep4-R	TCTCAGTGGGGGCAACAGTCACAAGATACACCACAGCTTACAGAT	
FedF-ep5-F	GGAAGAAAGGGGATATGTCTGAGCTTTTTAGTTGCATC	forward and reverse primers to insert FedF epitope 5 into *CfaB* gene
FedF-ep5-R	ACATATCCCCTTTCTTCCGGTGATGATACACCACAGCTT	
FedF-ep6-F	TGAAGGCATATCATTTTGGTTGGGTTTTTTAGTTGCATCGTTTGT	forward and reverse primers to insert FedF epitope 6 into *CfaB* gene
FedF-ep6-R	CAAAATGATATGCCTTCATCTAATGATACACCACAGCTTACAGAT	
FedF-ep7-F	CCACGAGCCTGTTGCATCGGGCTGTTTTTAGTTGCATCGTTTGT	forward and reverse primers to insert FedF epitope 7 into *CfaB* gene
FedF-ep7-R	GATGCAACAGGCTCGTGGTATGATGATACACCACAGCTTACAGAT	

30 min; the mixture was then added pig intestinal epithelial cells of jejunum (IPEC-J2, 1.5×10^5 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 Triton X-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^{0}C.

2.4. 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 25 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).

2.5. 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 and Zhang, 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 (1:3000) as secondary antibodies. OD_{650} measured after exposure to TMB were converted to antibody titers, in a $\log_{10}$ scale.

2.6. 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, 3×10^6 (CFUs) bacteria 8516 exposed to 30 µl serum pooled from each immunization group or the control group were added to IPEC-J2 cell (1.5×10^5 per well) and cultured for 1 h in a CO_2 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.

2.7. Data analyses

Data were analyzed statistically using GraphPad prism software version 7.0 (La Jolla, CA). ANOVA (Two-way) was used to compare data of competitive ELISA of screening each CfaB-epitope fusion protein competing against F18 fimbriae for reactivity with anti-F18 antiserum, whereas ANOVA (One-way) was performed to analyze Cfab-epitope fusions in competitive bacteria adherence inhibition assay, mouse serum anti-F18 IgG antibody titration and antibody adherence inhibition assay data. The mean ± standard deviation (SD) was used to express the results; a p value of < 0.05 indicated differences significant.

3. Results

3.1. 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 3). All seven epitopes are discontinuous, surface-exposed, and located on ß sheets or α-helix extension (Fig. 1).

3.2. 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-

Table 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

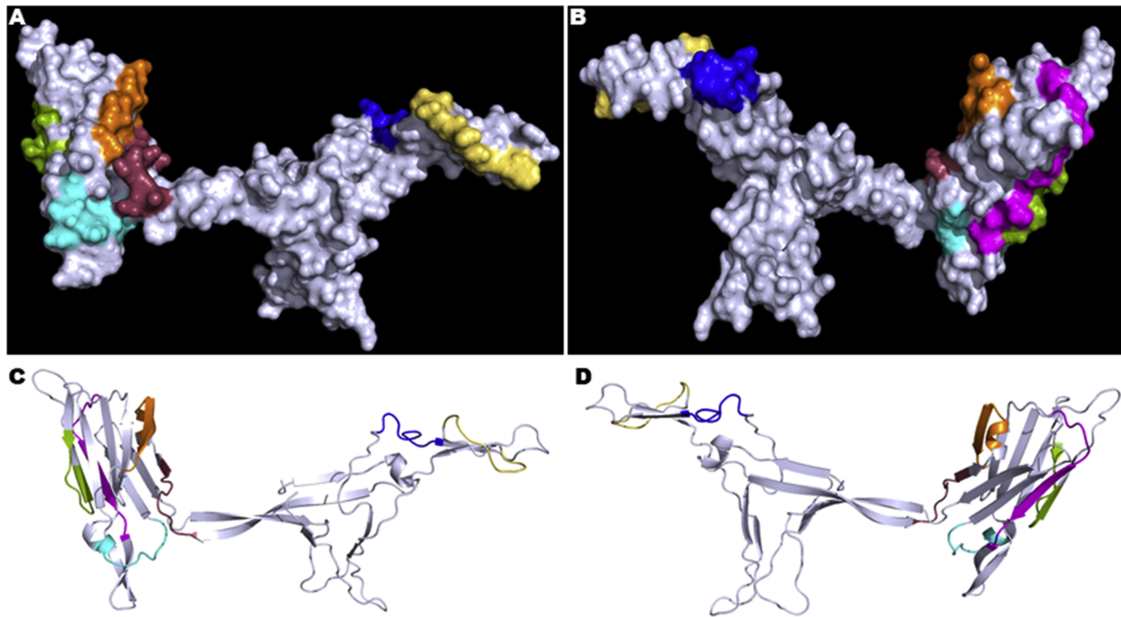

Fig. 1. F18 fimbrial adhesin subunit FedF protein model (A, B) and secondary structure (C, D) to show positions of *in silico* identified epitopes (A & C, front; B & D, back). FedF-ep1 (red), FedF-ep2 (orange), FedF-ep3 (pink), FedF-ep4 (green), FedF-ep5 (cyan), FedF-ep6 (blue), FedF-ep7 (yellow).

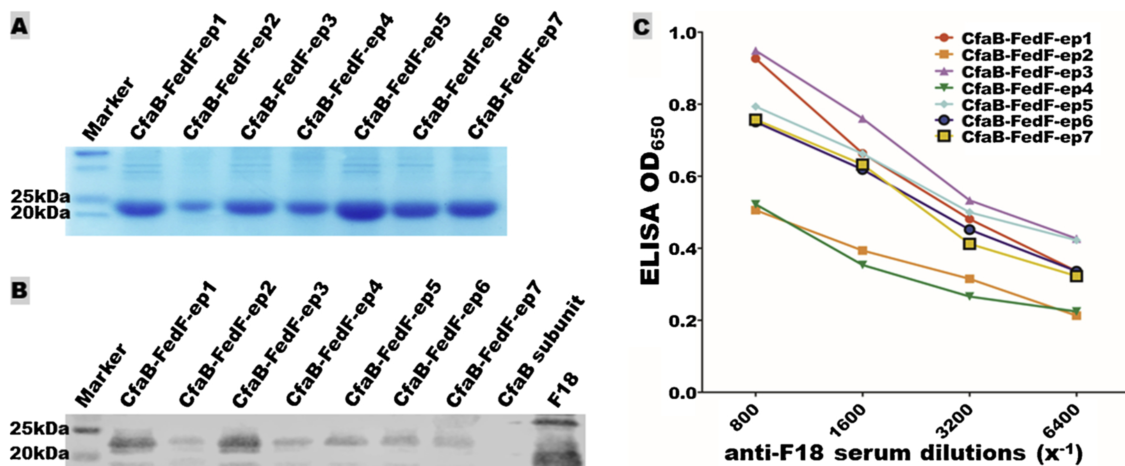

Fig. 2. CfaB-epitope fusion protein extraction and characterization. A: SDS-PAGE with Coomassie blue staining to show extracted and refolded CfaB-epitope fusion proteins. B: Western blot with anti-F18 antiserum to showed recognition of each CfaB-epitope fusion by anti-F18 antiserm, with carrier protein 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).

ep5, CfaB-FedF-ep6, and CfaB-FedF-ep7 (Fig. S1). Epitope fusion protein were extracted and refolded (Fig. 2A), and recognized by anti-F18 mouse antiserum (Fig. 2B). Additionally, when coated on ELISA plates, each of these seven fusion proteins reacted with anti-F18 antiserum (Fig. 2C).

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. 3A). 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. 3B). 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. 3B).

3.3. 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), and 3.45 ± 0.471 (CfaB-FedF-ep7) (Fig. 4A). 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 ($p < 0.01$).

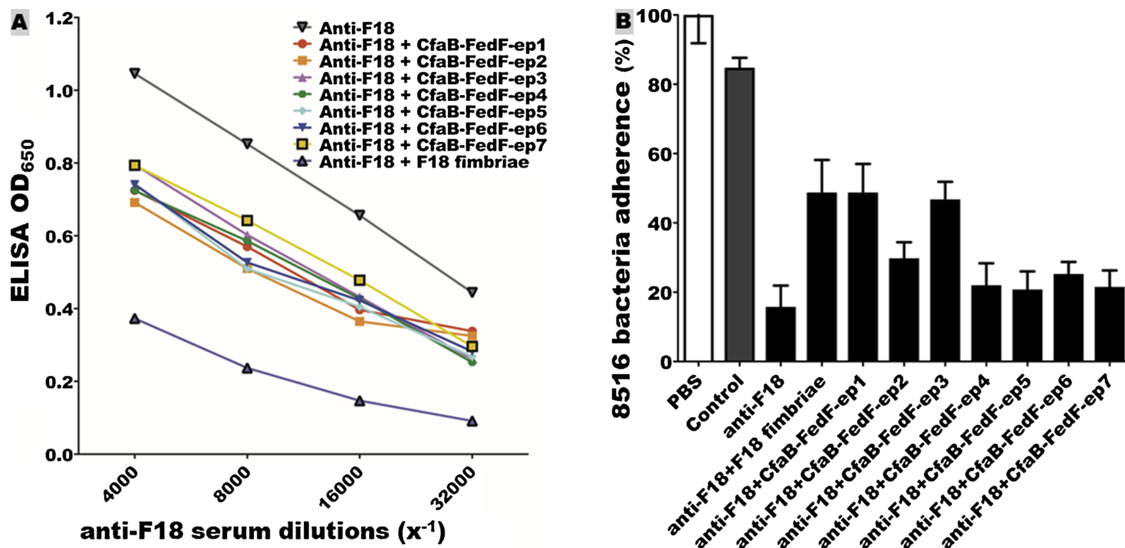

Fig. 3. Competitive ELISA and bacteria adherence inhibition assay to show CfaB-epitope fusion proteins blocking binding of anti-F18 antiserum with F18 fimbriae or F18-fimbrial *E. coli* bacteria 8516. A: competitive ELISAs with F18 fimbriae as the coating antigen, and each CfaB-epitope fusion protein 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.

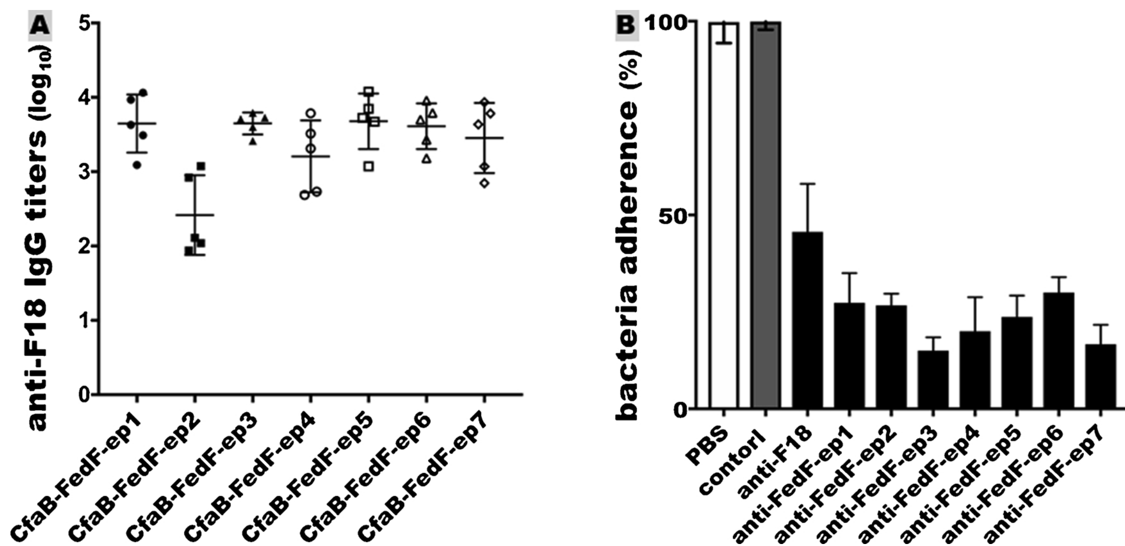

Fig. 4. Mouse serum anti-F18 antibody titration and antibody adherence inhibition assay. A: anti-F18 IgG titers (in $\log_{10}$) from serum samples of the mice immunized with each CfaB-epitope fusion protein; no anti-F18 IgG titer detected from the negative control mice. B: mouse serum antibody inhibition 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.

3.4. 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. 4B). Treated with the serum of mice immunized with CfaB-FedF-ep3 (15 ± 3.5, %) or CfaB-FedF-ep7 (16.6 ± 5, %), 8516 had the fewest bacteria adherent to IPEC-J2 cells. 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.

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). Similarly, live vaccine candidates derived from avirulent *E. coli* field isolates expressing K88 fimbriae induced K88-specific antibodies and protected against colonization by a K88 ETEC challenge strain (Bianchi et al., 1996; Francis and Willgohs, 1991; Fuentes et al., 2004 Santiago-Mateo et al., 2012). However, live strains expressing F18 were not effective in the induction of an F18-specific

immune response, nor protective against challenge with an F18 ETEC strain (Bertschinger et al., 2000; Coddens et al., 2007).

F18 fimbriae, previously known as F107, 2134 P 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 et al., 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, 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 and Zhang, 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.

5. Conclusion

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.

Conflict of interest statement

Authors declare no conflict of interests from this study.

Declaration of interest

Drs. Zhang and Moxley are developing a broadly protective vaccine against PWD.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.02.015.

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