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Identifying Immuno-Dominant and Neutralizing Epitopes from K88 Fimbriae of Enterotoxigenic *Escherichia coli* (ETEC)

T. Lu[1] *and W. Zhang*[1]

Summary

Enterotoxigenic *Escherichia coli* (ETEC) bacteria are the primary cause of diarrheal disease, especially porcine post-weaning diarrhea (PWD). Post-weaning diarrhea is one of the most common diseases in piglets 3 to 10 days after weaning and causes the loss of millions of dollars annually to United States swine industry and other countries.[2] These ETEC bacteria produce two types of virulence factors: 1) fimbriae adhesins, which promote bacterial attachment and colonization in pig small intestine; and 2) enterotoxins that disrupt fluid homeostasis and cause fluid hype-secretion and watery diarrhea. The F4 (K88) is the most important fimbria in ETEC bacteria causing PWD. An effective vaccine against PWD would have to induce antibody responses against the K88 fimbriae.[3] In this study, we *in silico* identified epitopes from the K88 fimbriae of ETEC that are associated with pig neonatal diarrhea and PWD. We genetically fused each epitope to non-homologous human ETEC CFA/I adhesin subunit CfaB to present each K88 fimbrial major subunit FaeG epitope, and examined each fusion protein with anti-K88 antiserum to identify immunodominant epitopes. Furthermore, each epitope fusion was used to immunize mice. Mouse serum samples were titrated for IgG antibody response specific to K88 fimbriae. Mouse serum samples were further examined for antibody neutralization activity against adherence of K88 fimbriae using porcine cell lines IPEC-J2 and porcine ETEC wildtype strain 3030-2. To verify whether epitope conformation alteration could lead to the loss in reacting to anti-K88 serum or in inducing anti-K88 antibody responses, we expressed the FaeG subunit protein and used FaeG protein as the coating antigen in ELISAs and Western Blot to examine antibodies derived from each epitope fusion.

Data from this study showed that K88 FaeG epitopes reacted differently to anti-K88 antiserum. Epitopes MTGDFNGSVD, LNDLTNGGTK, GRTKEAFATP, and PMKNAGGTKVGAVKVN showed strong reactivity to anti-K88 fimbria antise-

[1] Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University.

[2] Dubreuil, J., R. Isaacson, D. Schifferli. 2016. Animal Enterotoxigenic *Escherichia coli*. EcoSal Plus 2016; doi:10.1128/ecosalplus.ESP-0006-2016.

[3] Melkebeek, V., B.M. Goddeeris, E. Cox. 2013. ETEC vaccination in pigs. Veterinary Immunology and Immunopathology. Vol. 152, Iss. 1–2, P. 37-42, ISSN 0165-2427, https://doi.org/10.1016/j.vetimm.2012.09.024.

rum. Epitope ELRKPDGGTN-induced antibodies were shown strongly neutralizing against adherence of K88+ ETEC strain 3030-2 but exhibited a low titer, while epitope FNQAVTTSTQ had a high titer but weak neutralizing ability. Epitopes LGRGGVT-SADGEL, PRGSELSAGSA, and RENMEYTDGT were less immunogenic and their derived antibodies showed weak neutralizing activity against K88 fimbria adherence. Interestingly, all mouse serum samples showed strong responses to the FaeG subunit and the denatured K88 fimbriae (not always to the whole K88 fimbriae), indicating some epitopes are located at the adjacent regions of FaeG subunits in assembling to K88 fimbriae.

These results indicated epitopes MTGDFNGSVD, LNDLTNGGTK, GRT-KEAFATP, and PMKNAGGTKVGAVKVN are immuno-dominant and induced neutralizing antibodies. This study suggests these epitopes are potential antigens for developing precision vaccines against ETEC associated with PWD.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) bacteria are the primary cause of swine post-weaning diarrhea (PWD). Post-weaning diarrhea is one of the most common diseases in piglets 3 to 10 days after weaning and causes the loss of millions of dollars annually to United States swine industry and other countries.[4] These ETEC bacteria produce two types of virulence factors: 1) fimbriae adhesins, which promote bacterial attachment and colonization in pig small intestine; and 2) enterotoxins that disrupt fluid homeostasis and cause fluid hype-secretion and watery diarrhea.[2]

The F4 (K88) is the most important fimbria in ETEC bacteria causing PWD.[2] An effective vaccine against PWD would have to induce antibody responses against the K88 fimbriae.[5] A recent study demonstrated that an adhesion-toxoid multiepitope fusion antigen (MEFA) induced protective antibodies against K88 and heat-labile toxin (LT) and suggested that this adhesin-toxoid MEFA can be an antigen for a vaccine against PWD.[6] However, this adhesin-toxoid MEFA does not carry antigens to induce antibodies against other enterotoxins, including heat-stable toxin II (STb). A more recent study showed that toxoid MEFA LT-STa-STb-Stx2e induced protective antibodies against all four ETEC toxins, but this toxoid MEFA did not carry ETEC fimbria antigens.[7] A broadly protective PWD vaccine likely needs to carry all the toxin antigens

[4] Dubreuil, J., R. Isaacson, D. Schifferli. 2016. Animal Enterotoxigenic *Escherichia coli*. EcoSal Plus 2016; doi:10.1128/ecosalplus.ESP-0006-2016.

[5] Melkebeek, V., B.M. Goddeeris, E. Cox. 2013. ETEC vaccination in pigs. Veterinary Immunology and Immunopathology. Vol. 152, Iss. 1–2, P. 37-42, ISSN 0165-2427, https://doi.org/10.1016/j.vetimm.2012.09.024.

[6] Ruan, X., M. Liu, T.A. Casey, W. Zhang. 2011. A Tripartite Fusion, FaeG-FedF-LT_{192}A2:B, of Enterotoxigenic *Escherichia coli* (ETEC) Elicits Antibodies That Neutralize Cholera Toxin, Inhibit Adherence of K88 (F4) and F18 Fimbriae, and Protect Pigs against K88ac/Heat-Labile Toxin Infection. Clinical and Vaccine Immunology: CVI, 18(10), 1593–1599. http://doi.org/10.1128/CVI.05120-11.

[7] Rausch, D., X. Ruan, R. Nandre, Q. Duan, E. Hashish, T.A. Casey, W. Zhang. 2017. Antibodies derived from a toxoid MEFA (multiepitope fusion antigen) show neutralizing activities against heat-labile toxin (LT), heat-stable toxins (STa, STb), and Shiga toxin 2e (Stx2e) of porcine enterotoxigenic *Escherichia coli* (ETEC). Veterinary Microbiology, Vol. 202, P. 79-89, ISSN 0378-1135. https://doi.org/10.1016/j.vetmic.2016.02.002.

and the fimbria antigens.[8] To include antigenic elements from these ETEC toxins and fimbriae into a vaccine product, we applied structure-based vaccinology to develop an epitope-based PWD vaccine, by identifying neutralizing epitopes from each ETEC toxin and fimbria and then packing them into a single antigen component.

Procedures

Bacteria Strain, Plasmid and Cell Line

The *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* strain 9477 was used as DNA templates to PCR amplify the CfaB gene,[9] and *E. coli* strain 9503 for the expression of CfaB protein. Porcine ETEC field strain 3030-2 (K88/LT/STb/STa) was utilized for FaeG subunit gene amplification. Vector pET28α was used to clone the CfaB-K88-epitope fusions and the FaeG subunit. Fusion proteins CfaB-K88-epitope and FaeG subunit protein were expressed by *E. coli* strains BL21 and DH5α in Lysogeny broth (LB) supplemented with kanamycin (30 μg/mL). Porcine intestinal cell line IPEC-J2 was used to examine antibody neutralizing activity against the adherence of K88 fimbriae.

Epitope Identification and Epitope Fusion Protein Preparation

Computer software was applied to *in silico* identify epitopes from FaeG major subunit of K88 fimbria. Each FaeG epitope was genetically fused to non-homologous human ETEC CFA/I adhesin subunit CfaB using splicing overlap extension (SOE) PCR with primers listed in Table 2. Digested with *Nhe*I and *Eag*I, each epitope fusion PCR product was cloned into expression vector pET28a and expressed in *E. coli* BL21.

Epitope fusion recombinant strains were cultured in LB. Culture was grown overnight and transferred in 200 mL LB at a 1:50 ratio and continued to be cultured at 37°C. After OD_{600} reached 0.5-0.6, bacteria were induced with 0.1 mM isopropyl-1-thio-β-d-galactoside (IPTG) for 4 more hours. Induced bacteria were collected and used for total protein extraction using B-PER (bacterial protein extraction reagent in phosphate buffer).

Epitope fusion proteins were examined in a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with anti-K88 antiserum. Epitope fusion proteins were also examined in ELISA with anti-K88 antiserum. The K88 fimbriae major subunit FaeG gene was amplified by PCR, cloned in pET28a, and expressed in *E. coli* BL21. Competitive ELISAs were carried out by coating K88 fimbriae (10 ng/well) to Immulon 2HB plates. Wells were incubated with anti-K88 mouse antiserum and each epitope fusion protein (800 ng/well) at 37°C for 1 h. The HRP-conjugated goat-anti-mouse IgG (1:3000 dilution) was used as the secondary antibody. Optical densities (OD_{650}) were measured using an ELISA plate reader.

[8] Zhang, W., D.A. Sack. 2015. Current progress in developing subunit vaccines against enterotoxigenic *Escherichia coli*-associated diarrhea. Clinical and Vaccine Immunology, 22(9), 983-991.

[9] Ruan, X., D.A. Sack, W. Zhang. 2015. Genetic Fusions of a CFA/I/II/IV MEFA (Multiepitope Fusion Antigen) and a Toxoid Fusion of Heat-Stable Toxin (STa) and Heat-Labile Toxin (LT) of Enterotoxigenic *Escherichia coli* (ETEC) Retain Broad Anti-CFA and Antitoxin Antigenicity. PLOS ONE 10(3): e0121623. https://doi.org/10.1371/journal.pone.0121623.

Mouse Immunization

To immunize each 8-week old BALB/c mouse (5 mice per group) subcutaneously, 40 μg of each epitope-CfaB fusion protein were used with 0.2 μg double mutant LT (dmLT) adjuvant. Immunized mice received two booster injections (the same dose of the primary) biweekly. Two weeks after the second booster injection, mice were euthanized. A group of five mice without immunization was used as the control.

Antibody Titration and Neutralization

Mouse serum samples collected two weeks after the final booster were utilized for antibody titration. Immulon 2HB plates coated with K88 fimbriae (100 ng/well) or FaeG recombinant protein (100 ng/well) were used to titrate epitope-specific antibodies. Mouse serum samples were further examined for antibody neutralization activity against adherence of K88 fimbriae using porcine cell line IPEC-J2. Western blot with FaeG recombinant protein or denatured K88 fimbriae were used to examine reaction with the serum of mice immunized with each epitope fusion.

Data Analysis

Two-way ANOVA was performed to analyze all ELISA data, and one-way ANOVA was used for antibody titration data and antibody adherence inhibition assay data. A P value of < 0.05 was considered as statistically significant. All experiments were repeated two times using duplicate samples.

Results and Discussion

Nine epitopes–MTGDFNGSVD, LNDLTNGGTK, GRTKEAFATP, ELRKPDGGTN, PMKNAGGTKVGAVKVN, LGRGGVTSADGEL, PRGSELSAGSA, RENMEYTDGT, and FNQAVTTSTQ–were identified from the FaeG protein (GenBank AJ616256.1) by BepiPred-2.0 (http://www.cbs.dtu.dk/services/BepiPred/) and IEDB Analysis Resource (http://tools.immuneepitope.org/bcell/) (Figures 1A and 1B). Each epitope was used to substitute peptide $_{80}$-KVIVKLAD-$_{87}$ of CfaB for CfaB-epitope fusions. Each epitope fusion gene was DNA sequencing verified for its cloning in vector pET28α and expression in *E. coli* BL21 (Figure 1C).

The ELISA and Western Blot analyses showed that epitopes MTGDFNGSVD, LNDLTNGGTK, GRTKEAFATP, PMKNAGGTKVGAVKVN, and FNQAVTTSTQ were well recognized by mouse anti-K88 antiserum (Figures 2A and 2B). Competitive ELISA showed that epitope fusions CfaB-K88-$_{\text{MTGDFNGSVD}}$, CfaB-K88-$_{\text{LNDLTNGGTK}}$, CfaB-K88-$_{\text{ELRKPDGGTN}}$, CfaB-K88-$_{\text{PMKNAGGTKVGAVKVN}}$, and CfaB-K88-$_{\text{PRGSELSAGSA}}$ blocked the reaction between K88 fimbriae and anti-K88 antiserum (Figure 2C).

Mice immunized with CfaB-K88-$_{\text{epitope}}$ fusion developed IgG antibodies. Anti-K88 IgG titers from the serum samples of the mice immunized with CfaB-K88-$_{\text{MTGDFNGSVD}}$, CfaB-K88-$_{\text{LNDLTNGGTK}}$, CfaB-K88-$_{\text{GRTKEAFATP}}$, CfaB-K88-$_{\text{PMKNAGGTKVGAVKVN}}$, and CfaB-K88-$_{\text{FNQAVTTSTQ}}$ fusion were significantly greater than those from the mice immunized with the other epitope fusions (Figure 3A). Moreover, antibodies in the serum of the mice immunized with CfaB-K88-$_{\text{MTGDFNGSVD}}$, CfaB-K88-$_{\text{LNDLTNGGTK}}$, CfaB-K88-$_{\text{GRTKEAFATP}}$, CfaB-K88-$_{\text{ELRKPDGGTN}}$, CfaB-K88-$_{\text{PMKNAGGTKVGAVKVN}}$ and CfaB-K88-$_{\text{RENMEYT-}}$

$_{DGT}$ fusion significantly inhibited adherence of ETEC strain 3030-2 to IEPC-J2 cells (Figure 3B).

To determine whether epitope fusion conformation alterations resulted in the loss of reaction with anti-K88 antiserum or if they fail in inducing antibody responses, ELISA and Western blot analyses were conducted using FaeG protein and denatured (boiled) K88 fimbriae (with FaeG subunits that were separated after boiling). Data showed that FaeG protein and the boiled K88 fimbriae, unlike the whole K88 fimbriae, were recognized by antibodies in the serum samples of all immunization groups (Figure 4). These results indicate that epitopes LGRGGVTSADGEL, PRGSELSAGSA, and RENMEYTDGT may locate at the region connecting adjacent FaeG subunits for K88 fimbriae.

In conclusion, epitopes identified from this study reacted differently to anti-K88 antiserum. Epitopes MTGDFNGSVD, LNDLTNGGTK, GRTKEAFATP, and PMKNAGGTKVGAVKVN are identified as the immunodominant epitopes and the neutralizing epitopes. These epitopes can represent K88 fimbria as the antigens for developing vaccines against PWD. Data also suggested incongruence between epitope immunodominance and antibody neutralization activity. The results are illustrated by fusion CfaB-K88-$_{ELRKPDGGTN}$, which induced strongly neutralizing antibodies but with lower immunogenicity, while CfaB-K88-$_{FNQAVTTSTQ}$ had higher immunogenicity but induced weakly neutralizing antibodies. Data also showed that epitopes LGRGGVT-SADGEL, PRGSELSAGSA, and RENMEYTDGT were less immunogenic and less effective in inducing neutralizing antibodies. This study is important as these data can be used to develop precision vaccines against ETEC associated with PWD.

Table 1. *E. coli* strains and plasmids used in this 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
3030–2	Porcine field isolate, K88ac/LT/STb/STa	(6)
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$_{\text{-MTGDFNGSVD}}$ in BL21	This study
9677	pET28α-CfaB-K88$_{\text{-LNDLTNGGTK}}$ in BL21	This study
9678	pET28α-CfaB-K88$_{\text{-GRTKEAFATP}}$ in BL21	This study
9679	pET28α-CfaB-K88$_{\text{-ELRKPDGGTN}}$ in BL21	This study
9680	pET28α-CfaB-K88$_{\text{-PMKNAGGTKVGAVKVN}}$ in BL21	This study
9681	pET28α-CfaB-K88$_{\text{-LGRGGVTSADGEL}}$ in BL21	This study
9682	pET28α-CfaB-K88$_{\text{-PRGSELSAGSA}}$ in BL21	This study
9683	pET28α-CfaB-K88$_{\text{-RENMEYTDGT}}$ in BL21	This study
9676	pET28α-CfaB-K88$_{\text{-FNQAVTTSTQ}}$ in BL21	This study
Plasmids		
pET28α		Novagen

Table 2. PCR primers used in this study

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
K88e1-L	CGAACCATTGAAATCACCAGTCATTTTTT-TAGTTGCATCGTTTGT	Overlapping with K88$_{\text{-MTGDFNGSVD}}$
K88e1-R	GGTGATTTCAATGGTTCGGTCGATGATA-CACCACAGCTTACAGAT	
K88e2-L	TCCACCATTGGTCAGGTCATTCAATTTTT-TAGTTGCATCGTTTGT	Overlapping with K88$_{\text{-LNDLTNGGTK}}$
K88e2-R	GACCTGACCAATGGTGGAACCAAAGATA-CACCACAGCTTACAGAT	
K88e3-L	AGCAAATGCTTCTTTGGTTCGGCCTTTTT-TAGTTGCATCGTTTGT	Overlapping with K88$_{\text{-GRTKEAFATP}}$
K88e3-R	ACCAAAGAAGCATTTGCTACGCCAGATA-CACCACAGCTTACAGAT	
K88e4-L	TCCACCATCAGGTTTTCTGAGTTCTTTTT-TAGTTGCATCGTTTGT	Overlapping with K88$_{\text{-ELRKPDGGTN}}$
K88e4-R	AGAAAACCTGATGGTGGAACTAATGATA-CACCACAGCTTACAGAT	
K88e5-L	AACTTTAGTGCCCCCTGCATTTTTCATCG-GTTTTTTTAGTTGCATC	Overlapping with K88$_{\text{-PMKNAGGTKVGAVKVN}}$
K88e5-R	GCAGGGGGCACTAAAGTTGGTTCAGT-GAAAGTGAATGATACACCA	
K88e6-L	CGCAGAAGTAACCCCACCTCTCCCTA-ATTTTTTAGTTGCATCGTT	Overlapping with K88$_{\text{-LGRGGVTSADGEL}}$
K88e6-R	GGTGGGGTTACTTCTGCGGAC-GGGGAGCTGGATACACCACAGCTT	
K88e7-L	CCCAGCCGAGAGTTCAGAACCCCTCG-GTTTTTTAGTTGCATCGTT	Overlapping with K88$_{\text{-PRGSELSAGSA}}$
K88e7-R	TCTGAACTCTCGGCTGGGAGTGCCGATA-CACCACAGCTTACAGAT	
K88e8-L	ATCAGTGTACTCCATGTTTTCCCTTTTTT-TAGTTGCATCGTTTGT	Overlapping with K88$_{\text{-RENMEYTDGT}}$
K88e8-R	AACATGGAGTACACTGATGGAACTGATA-CACCACAGCTTACAGAT	
K88e9-L	GCTGGTAGTTACAGCCTGATTAAATTTTT-TAGTTGCATCGTTTGT	Overlapping with K88$_{\text{-FNQAVTTSTQ}}$
K88e9-R	CAGGCTGTAACTACCAGCACTCAGGATA-CACCACAGCTTACAGAT	
FaeG-F	CGGGCTAGCTGGATGACTGGTGATTTC	Upstream of FaeG gene, with *Nhe*I site
FaeG-R	TTACGGCCGTTAGTAATAAGTAATTGC	Downstream of FaeG gene, with *Eag*I site

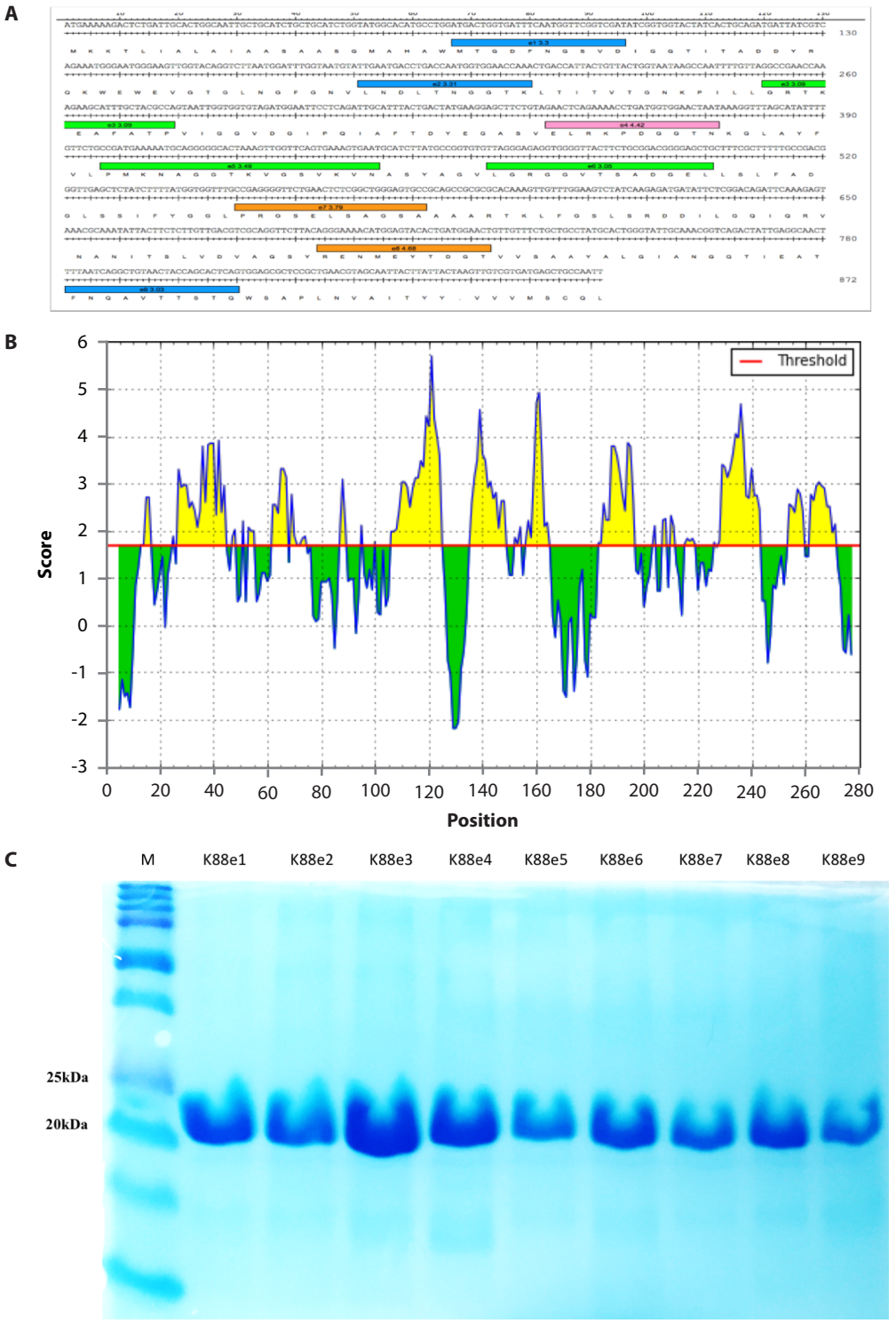

Figure 1. Construction and expression of CfaB-K88$_{\text{-epitope}}$ fusions.
Panel A: B-cell epitope *in silico* identification from K88 fimbrial major subunit FaeG. Seven epitopes were shown by different colors and labeled with their parker hydrophilicity prediction scores accordingly. The epitopes were chosen based on their parker hydrophilicity prediction scores which needed be higher than 3.0.
Panel B: Parker hydrophilicity prediction results of FaeG subunit protein. Average score: 1.709 Minimum: -2.180 Maximum: 5.700.
Panel C: SDS-PAGE detection of CfaB-K88$_{\text{-epitope}}$ fusions by Coomassie blue staining. Each CfaB-K88-$_{\text{epitope}}$ fusion gene was constructed by SOE PCR and cloned into vector pET28α and expressed by *E. coli* BL21.

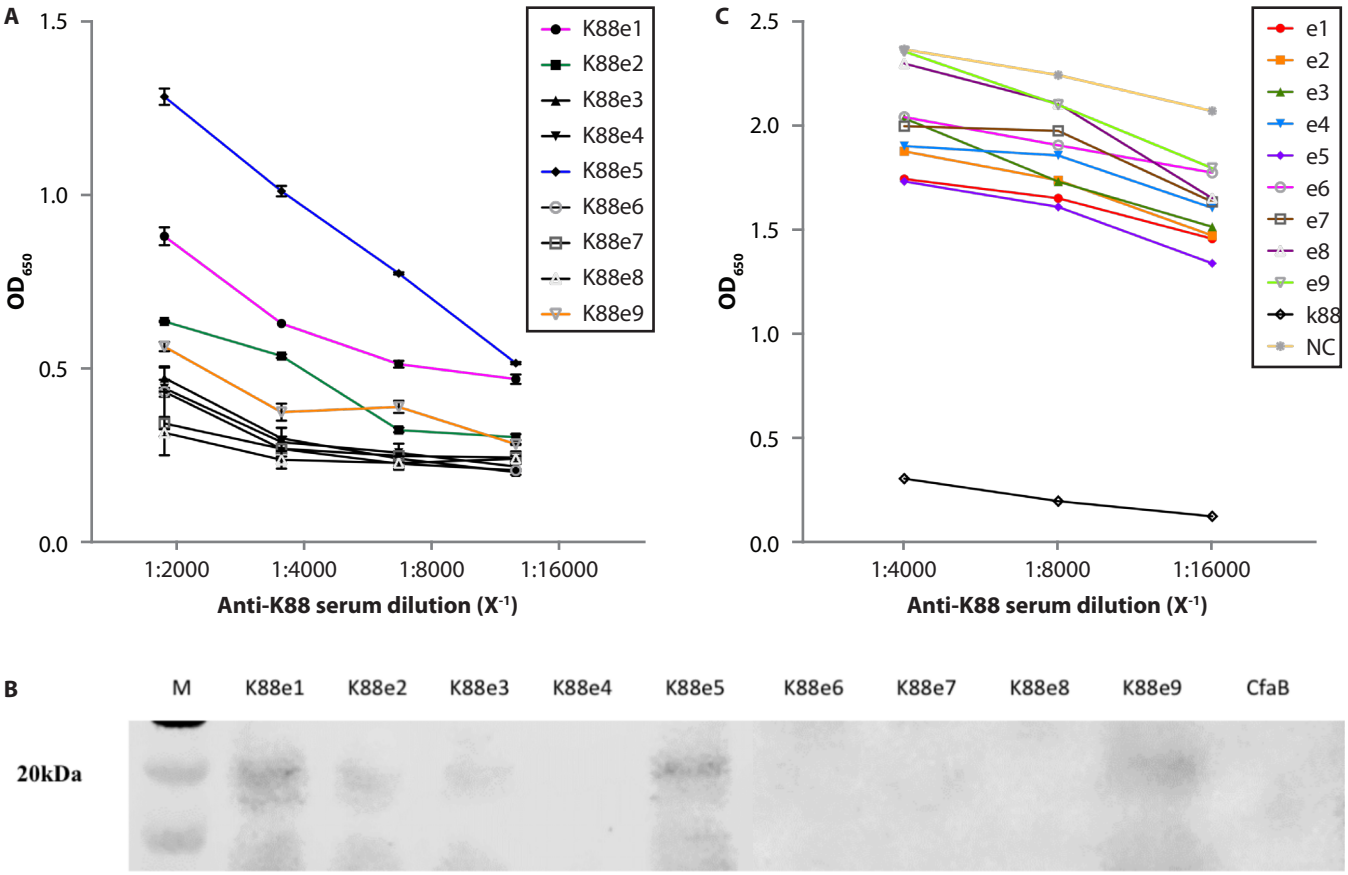

Figure 2. Detection of CfaB-K88$_{\text{-epitope}}$ fusions proteins with mouse anti-K88 anti-serum.
Panel A: ELISA with serially diluted mouse anti-K88 antiserum. The OD_{650} of CfaB-K88-$_{\text{PRGSELSAGSA}}$ (mean of K88e7=0.235) and CfaB-K88-$_{\text{RENMEYTDGT}}$ (mean of K88e8=0.205) fusion proteins were lower than the cutoff OD (0.3). The OD_{650} of CfaB-K88-$_{\text{GRTKEAFATP}}$ (mean of K88e3=0.376), CfaB-K88-$_{\text{ELRKPDGGTN}}$ (mean of K88e4=0.345), CfaB-K88-$_{\text{LGRGGVTSADGEL}}$ (mean of K88e6=0.334) and CfaB-K88-$_{\text{FNQAVTTSTQ}}$ (mean of K88e9=0.476) fusion proteins were higher than 0.3 but lower than 0.5. The OD_{650} of CfaB-K88-$_{\text{MTGDFNGSVD}}$ (mean of K88e1=0.822), CfaB-K88-$_{\text{LNDLTNGGTK}}$ (mean of K88e2=0.555) and CfaB-K88-$_{\text{PMKNAGGTKVGAVKVN}}$ (mean of K88e5=1.26) fusion proteins significantly higher than other proteins. ($P < 0.05$, n = 4).
Panel B: Western blot to characterize epitope fusion proteins with mouse anti-K88 antiserum. CfaB-K88-$_{\text{MTGDFNGSVD}}$, CfaB-K88-$_{\text{LNDLTNGGTK}}$, CfaB-K88-$_{\text{GRTKEAFATP}}$, CfaB-K88-$_{\text{PMKNAGGTKVGAVKVN}}$, CfaB-K88-$_{\text{FNQAVTTSTQ}}$ were detected, while other epitope fusion proteins showed no detection by anti-K88 antiserum.
Panel C: Competitive ELISA with serially diluted mouse anti-K88 antiserum and K88 fimbriae protein (K88: fusion protein=1:80). The OD_{650} of all CfaB-K88-$_{\text{epitope}}$ fusion proteins were lower than that without competing protein (mean of NC=2.363) and higher than that with K88 as competing protein (mean of K88=0.302). The OD_{650} of CfaB-K88-$_{\text{MTGDFNGSVD}}$ (mean of K88e1=1.74), CfaB-K88-$_{\text{LNDLTNGGTK}}$ (mean of K88e2=1.873), CfaB-K88-$_{\text{ELRKPDGGTN}}$ (mean of K88e4=1.898), CfaB-K88-$_{\text{PMKNAGGTKVGAVKVN}}$ (mean of K88e5=1.729) and CfaB-K88-$_{\text{PRGSELSAGSA}}$ (mean of K88e7=1.994) fusion proteins significantly lower than the ODs in wells without competing protein ($P < 0.05$, n = 4).

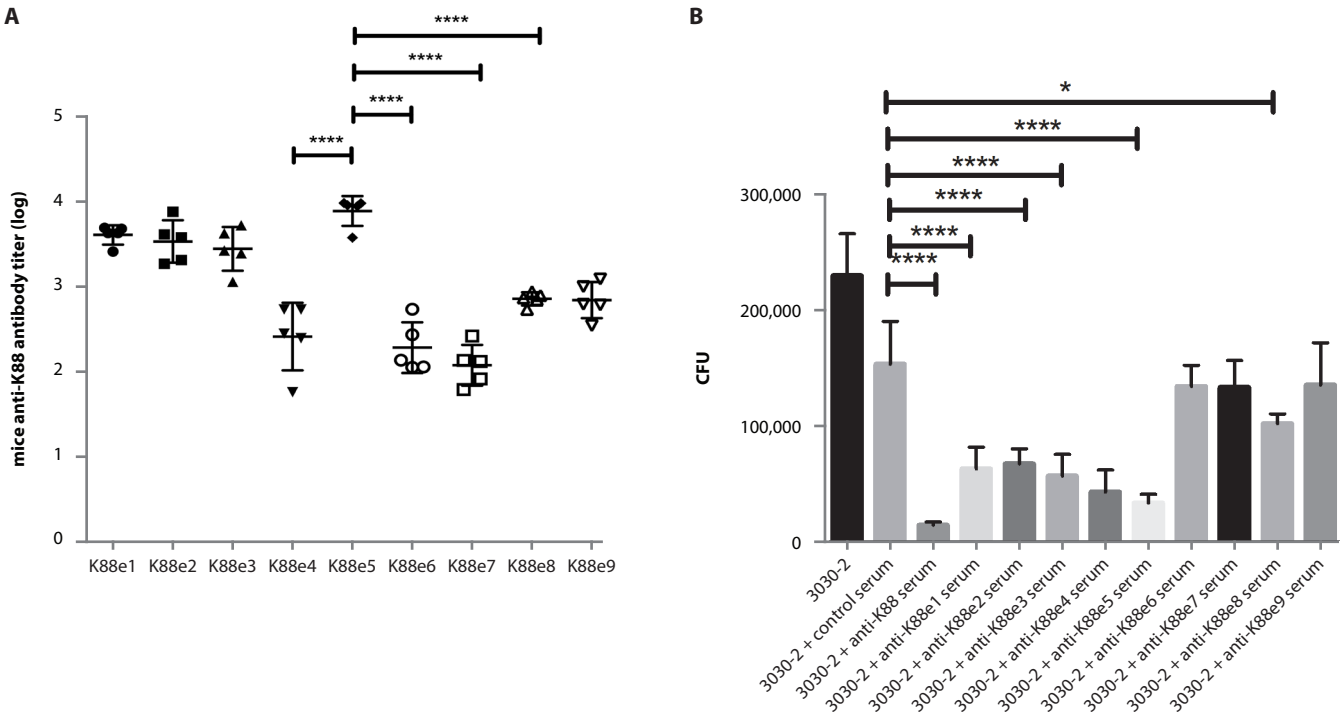

Figure 3. Mouse serum anti-CfaB-K88-$_{epitope}$ IgG antibody titers and antibody adherence inhibition assays.

Panel A: Mouse serum anti-CfaB-K88-$_{epitope}$ IgG titers (log_{10}), with K88 fimbriae used as ELISA coating antigens. Each dot represents the IgG titer from an individual mouse; bars indicated the mean titer of the group. CfaB-K88-$_{MTGDFNGSVD}$ (titer of K88e1= 3.19±0.21), CfaB-K88-$_{LNDLTNGGTK}$ (titer of K88e2=3.23±0.41), CfaB-K88-$_{GRTKEAFATP}$ (titer of K88e3=2.76±0.22), CfaB-K88-$_{PMKNAGGTKVGAVKVN}$ (titer of K88e5=3.55±0.19) and CfaB-K88-$_{FNQAVTTSTQ}$ (titer of K88e9=3.39±0.13). These antibody titers were not significantly different from each other. IgG titers were presented as means ± SEM of three independent experiments. Note: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Panel B: Mouse anti-CfaB-K88-$_{epitope}$ serum antibody adherence inhibition assays using ETEC bacteria 3030-2 and porcine cell line IPEC-J2. The number of ETEC 3030-2 bacteria adhered to IPEC-J2 cells was used to indicate activity of anti-CfaB-K88-epitope antibodies against bacteria adherence. The serum antibodies from the mice immunized with CfaB-K88-$_{MTGDFNGSVD}$, CfaB-K88-$_{LNDLTNGGTK}$, CfaB-K88-$_{GRTKEAFATP}$, CfaB-K88-$_{ELRKPDGGTN}$, CfaB-K88-$_{PMKNAGGTKVGAVKVN}$, and CfaB-K88-$_{RENMEYTDGT}$ s significantly inhibited bacteria adherence compared to the serum from the control mice. Data are presented as means ± SEM of three independent experiments. Note: *, $P < 0.05$; **, P < 0.01; ***, $P < 0.001$; ****, $P < 0.0001$.

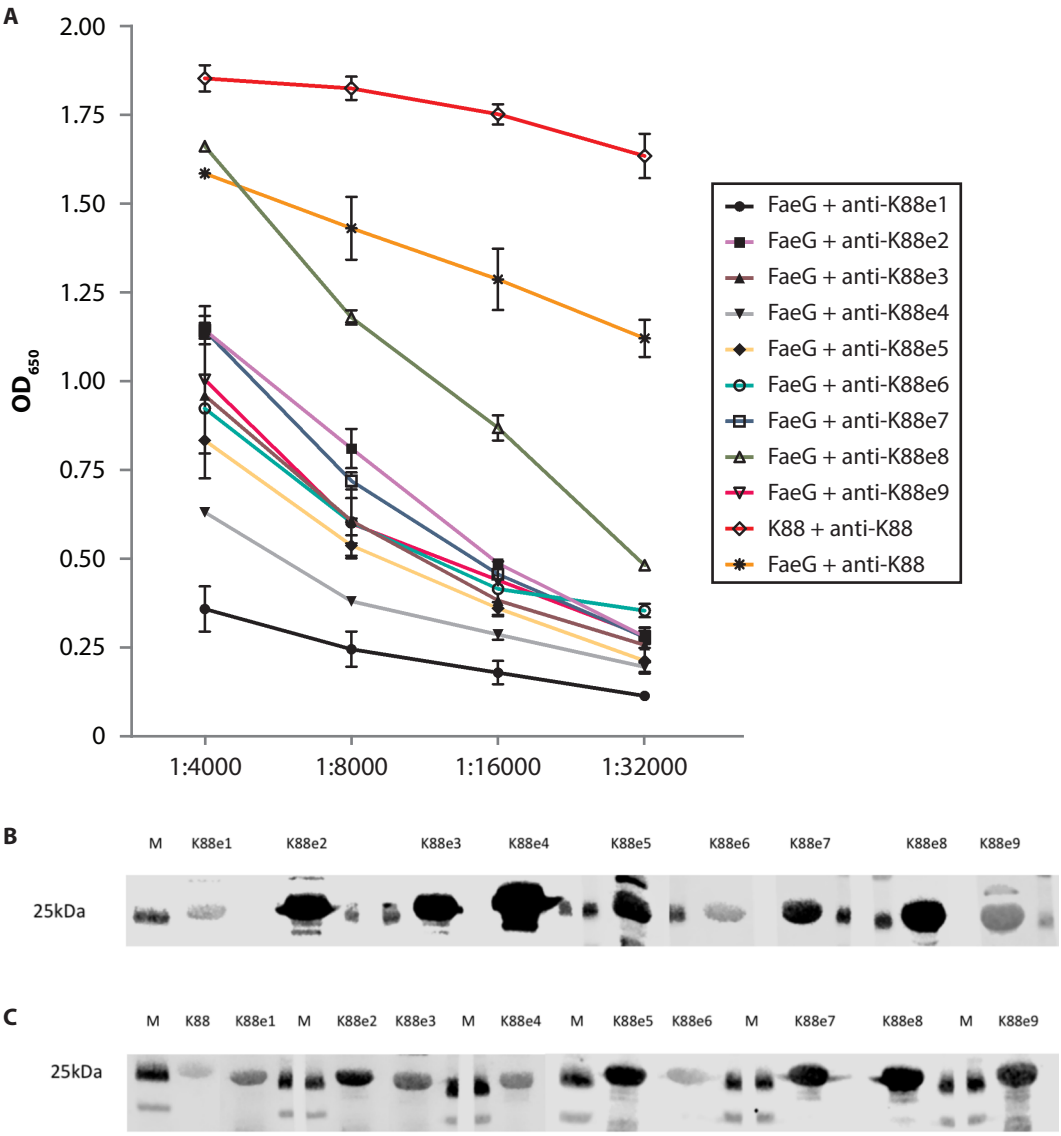

Figure 4. Mouse anti-CfaB-K88-$_{epitope}$ antiserum detection of FaeG proteins.
Panel A: ELISA with serially diluted mouse anti-CfaB-K88-$_{epitope}$ antiserum using FaeG recombinant proteins as the ELISA coating antigen. OD_{650} values were significantly lower than the ODs in wells using mouse anti-K88 antiserum or in wells using K88 fimbriae as the coating antigen and mouse anti-K88 antiserum ($P < 0.05$; n = 4).
Panel B: Western blot using mouse anti-CfaB-K88-$_{epitope}$ antiserum to detect denatured K88 fimbriae.
Panel C: Western blot using mouse anti-CfaB-K88-$_{epitope}$ antiserum to detect K88 fimbrial subunit FaeG proteins.