

Review Article

Progress and Challenges in Vaccine development Against Enterotoxigenic Escherichia Coli (ETEC)-Associated porcine Post-Weaning Diarrhoea (PWD)

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Abstract

Porcine Post-Weaning Diarrhea (PWD) continues to be one of the most important swine diseases worldwide. Enterotoxigenic *Escherichia coli* (ETEC) strains are the primary cause of PWD. The key virulence factors of ETEC in PWD are bacterial fimbrial adhesins and enterotoxins. Adhesions mediate ETEC bacteria initial attachment to pig epithelial cells and subsequent colonization at pig small intestines. Enterotoxins including heat-labile toxin (LT) and heat-stable toxin (ST) disrupt fluid homeostasis in small intestinal epithelial cells to cause electrolyte-rich fluid hyper-secretion and diarrhea. Currently there are no effective prevention measures to protect weaned pigs against PWD. Vaccination would be the most practical and effective prevention approach, and vaccines inducing anti-adhesion immunity to block ETEC attachment and colonization and also antitoxin immunity to neutralize enterotoxicity are considered optimal against ETEC-associated PWD. Although progress has been made in past decades in developing effective vaccines against PWD, challenges continue to exist due to the disease complexity and immunological heterogeneity among ETEC strains. Recent progress in using safe toxoid antigens, toxoid fusion antigens and MEFA (multi-epitope fusion antigen) approach to develop multivalent vaccines for broad protection, however, shows promising in developing new vaccines for effective protection against ETEC-associated PWD.

ABBREVIATIONS

PWD: Post-Weaning Diarrhea; ETEC: Enterotoxigenic *Escherichia coli*; DAEC: Diffusely Adherent *Escherichia coli*; EPEC: Enteropathogenic *Escherichia coli*; EAEC: Enteroaggregative *Escherichia coli*; EHEC: Enterohemorrhagic *Escherichia coli*; TGE: Transmissible Gastroenteritis; PEDV: Porcine Epidemic Diarrhea Virus; LT: Heat-Labile Toxin; STa: Heat-Stable Toxin Type Ia; STb: Heat-Stable Toxin Type II; EAST1: Enteroaggregative Heat-Stable Toxin 1; Stx2e: Shiga Toxin Type 2e; AIDA-I: Adhesion Involved In Diffuse Adherence; Paa: Porcine Attaching-And-Effacing Associated; EAE: Attaching And Effacing; MEFA: Multi-epitope Fusion Antigen

INTRODUCTION

Piglets commonly develop diarrhea 3-10 days after they weaned, a clinical condition commonly known as porcine Post-Weaning Diarrhea (PWD). PWD continues to be one of the most important swine diseases worldwide [1-3]. PWD is caused

by pathogenic bacteria and viruses including enterotoxigenic *Escherichia coli* (ETEC), corona viruses [both Transmissible Gastroenteritis (TGE) and Porcine Epidemic Diarrhea Virus (PEDV)] and rotaviruses, but ETEC strains are the predominant cause of PWD [4]. PWD results in weight loss, slow growth and acute death in recently weaned pigs, that cause substantial economic losses to swine producers in the US and worldwide [5-8]. Currently, there are no effective prevention strategies available to protect against PWD. Oral administration of specific-antibody-containing egg yolk, sow milk or plasma proteins, treatment with dietary, diet supplementary or probiotics have been attempted. But these treatments are less or not effective, or not economically practical. Prophylactic treatment with antibiotics may relieve disease burden [9], but excessive use of antibiotics is linked to the surge of antimicrobial resistance and potentially poses a threat to public health and the environment [10-13]. On the other hand, implementation of a ban on the use

of food animal growth promoting antibiotics in Scandinavia and Europe has spiked an increase of PWD outbreaks [14]. Therefore, alternative prevention strategies are urgently needed. Vaccination would be the most practical (economically) and likely the most effective approach to control PWD. Unfortunately, although a couple of vaccine products gained licensed in a few countries, there are no vaccines currently available to effectively protect against PWD [2,15,16].

Since ETEC strains are by far the most common and important cause of PWD, this group of pathogenic *E. coli* has been primarily targeted in vaccine development. ETEC are *E. coli* strains that produce enterotoxins. These ETEC strains proliferate in pig small intestines and disrupt host cell fluid homeostasis to cause fluid hyper-secretion and PWD. Stress of weaning, decreased maternal antibodies and dietary adjustment are considered important but indirect factors influencing the course of PWD, since these conditions create a favorable environment for ETEC manifestation [2]. ETEC strains causing PWD express adhesions and enterotoxins (Table 1). These adhesins mediate ETEC bacteria initial attachment to host receptors at epithelial cells and subsequent colonization in pig small intestine [17]. Colonized ETEC bacteria produce enterotoxins and deliver them to small intestinal epithelial cells to cause fluid and electrolyte hyper-secretion that leads to diarrhea [18]. Therefore, bacterial adhesins and enterotoxins are the virulence determinants of ETEC in PWD, and have been the main targets in PWD vaccine development.

Porcine ETEC adhesins

Adhesins expressed by ETEC causing PWD include fimbrial

adhesins and non-fimbrial adhesins (Table 1). Bacteria fimbriae are fimbrial or fibrillar bio polymeric filaments displayed at the external surface. A typical ETEC fimbria is composed of hundreds of copies of fimbrial major structural subunits and a single or a few copies of one or two minor structural subunits. Non-fimbrial adhesins are often bacteria outer membrane proteins, and do not appear in a filamentous structure. ETEC fimbriae attach host cell receptors and facilitate an initial adherence between bacteria and pig epithelial cells at small intestines. Indeed without the fimbrial attachment, PWD cannot occur. This initial attachment leads to bacteria colonization and proliferation in pig small intestines and also brings ETEC bacteria to close proximity to pig epithelial cells so that bacteria can effectively deliver produced enterotoxins to pig small intestinal epithelial cells. Therefore, adherence between ETEC bacteria and pig epithelial cells initiates PWD disease, and fimbriae play a critical role in ETEC-associated PWD.

Fimbriae of the ETEC strains causing PWD are K88 (F4), K99 (F5), 987P (F6), F41 (F7) and F18 [19-25]. But K88 and F18 are by far the most predominant and important fimbriae, since ETEC strains expressing K88 or F18 cause nearly all PWD cases. Fimbriae K99 [26,27], 987P [28,29], and F41 [30] are only occasionally detected in ETEC strains associated with PWD [24,25,31].

K88

K88 fimbriae consist of three antigenic variants: K88ab, K88ac and K88ad [32-34]. K88ab ETEC was once the main variant causing PWD in the Europe, but variant K88ac becomes the predominant one worldwide [2,16], accounting for over 96% to all K88-positive ETEC strains associated with PWD [25,35,36], and is the only one in the US associated with PWD [37]. The major

Table 1: Virulence factors of ETEC associated with PWD.

I. Adhesins		References
Fimbrial adhesins	Morphology (diameter) & structural subunit (kDa)	
K88 (F4)	Fibrillar (2.1 nm), FaeG ^a (27.6)/FaeC ^b (16.9)	Bakker <i>et al.</i> , 1991.
K99 (F5)	Fibrillar (4.8 nm), FanC ^a (16.5)/FanF ^b (31.5)	Isaacson, 1977; Bakker <i>et al.</i> , 1991.
987P (F6)	Fimbrial (7.0 nm), FasA ^a (23.0)/FasF ^b (17.5)/FasG ^b (40)	Isaacson & Richter, 1981; Edwards <i>et al.</i> , 1996.
F41 (F7)	Fibrillar (3.2 nm), Fim41a ^a (29.5)	deGraaf, 1982.
F18 (F107;2134P; CFA8813)	Fibrillar (3 - 4 nm), FedA ^a (15.1)/FedF ^b (30.3)	Imberechts <i>et al.</i> , 1996; Nagy <i>et al.</i> , 1997 #17795; Smeds <i>et al.</i> , 2001.
Non-fimbrial adhesins		
AIDA-I	Autotransporter protein, AidA (79.5 ^c)	Benz & Schmidt, 1992; Benz & Schmidt, 2001.
paa	Outermembrane protein, Paa (27.6)	Batissou <i>et al.</i> , 2003.
EAE	Outermembrane protein, EaeA (94- 97)	Jerse & Kapper, 1991.
II. Toxins	Gene, mature toxin protein	
LT	<i>eltAB</i> , 1:5 AB holotoxin (86 kDa)	Sixma <i>et al.</i> , 1991; Streatfield, 1992.
STa	<i>estA</i> , 18 amino acid peptide (2 kDa)	Dreyfuset <i>et al.</i> , 1983.
STb	<i>estB</i> , 48 amino acid peptide (5.1 kDa)	Leeet <i>et al.</i> , 1983; Dreyfuset <i>et al.</i> , 1992.
EAST1	<i>astA</i> , 38 amino acid peptide (4.1 kDa)	Nataroet <i>et al.</i> , 1987.
Stx2e	<i>stx2e</i> , 1:5 AB toxin (70 kDa)	Pierardet <i>et al.</i> , 1991.

Note: a: major structural subunit;

b: minor structural and/or fimbrial tip subunit.

c: mature AIDA-I adhesin is detected as 100 kDa by SDS-PAGE.

structural subunit of K88 fimbriae is FaeG, which is the main structural component of the fimbria and also the adhesin, the adhesive unit [27,38].

F18

F18 fimbriae include two antigenic variants: F18ab and F18ac [39]. F18ab, previously called F107, is often expressed by *E. coli* strains producing shiga-like toxin Stx2e and mainly causing edema disease in young pigs [40,41]. F18ac, previously known as 2134P or CFA8813, is expressed by ETEC strains that produce STa and STb enterotoxins, (and sometimes also Stx2e,) and cause PWD [42]. F18 fimbriae bind specific receptors on pig small intestinal epithelial cells [43,44]. The major structural subunit of F18 is FedA, and the minor subunit is FedF which serves as the adhesive subunit and plays a central role in binding to pig receptors [45-47].

Non-fimbrial adhesins

Non-fimbrial adhesins expressed by ETEC strains associated with PWD include AIDA-I (adhesin involved in diffuse adherence), Paa (porcine attaching-and-effacing associated) and EAE (attaching and effacing). AIDA-I adhesin originally identified from a different group of diarrheal *E. coli*, diffusely adherent *E. coli* (DAEC), is also carried by ETEC strains causing PWD. AIDA-I is a bacterial auto transporter protein which associates non-covalently at bacterial surface, and mediates diffusing adherence to host receptors at small intestinal epithelial cells [48,49].

Paa and EAE adhesins are bacterial outer membrane proteins. The virulence significance of Pa in PWD has not been well characterized. Paa was initially thought to be expressed together with the EAE adhesin gene and was suggested to play an important role at the early stage of development of attaching and effacing (A/E) lesion [50]. However, *paa* gene was detected in 60% of ETEC strains isolated from pigs with PWD, but the *eae* gene was carried by only 1% of the ETEC strains [25]. The lack of concordance in prevalence of these two genes suggests Paa in ETEC strains likely play a role other than assisting EAE for attaching and effacing. The EAE adhesin, on the other hand, has been confirmed for association with A/E lesion [51], but mostly by the enter hemorrhagic *E. coli* (EHEC) or porcine enteropathogenic *E. coli* (EPEC), and not the ETEC strains [52]. Additionally, the low prevalence of EAE in ETEC strains suggests EAE unlikely play a major role in ETEC adherence to pig small intestines.

ETEC toxins

Initial attachment and colonization at host small intestines is the first step of ETEC-associated PWD, but it is the toxins produced by ETEC strains that enter host cells and enzymatically disrupt fluid homeostasis in pig small intestinal epithelial cells to essentially cause electrolyte-rich fluid hyper-secretion and diarrheal [18]. Enterotoxins produced by ETEC strains associated with PWD are heat-labile toxin (LT), heat-stable toxin type Ia (porcine-type STa, pSTa) which differs from the type Ib STa (hSTa, that associates with human diarrhea), heat-stable toxin type II (STb), and enter aggregative heat stable type 1 (EAST1). In addition, shiga-like toxin Stx2e is also detected in ETEC strains associated with PWD [25].

LT

LT or LTI, a typical 1:5 AB-type holotoxin, which is closely related to cholera toxin (CT) produced by *Vibrio cholerae* [53,54], is composed of one 28-kDa subunit A (LT_A) and five identical 11.6-kDa B (LT_B) subunits [55]. The LT_A subunit is the enzymatic activity unit of the toxin, whereas the B subunits bind host receptor GM₁ located at small intestinal epithelial cells. After binding GM₁ receptor, LT enters host small intestinal epithelial cells and stimulates adenylate cyclase activity to elevate intracellular cyclic AMP levels. That directly disrupts osmotic balance in pig small intestinal epithelial cells to cause net water secretion to the gut lumen, resulting in PWD.

A different type of LT, LTII, found in *E. coli* strains [56-58] is also holotoxin-structured and homologous to LTI structurally. Although the role of LTII playing in ETEC is not determined, it is thought to be not associated with diarrhea. Purified LTII was found to stimulate fluid accumulation in ligated intestinal loops of calves but not of pigs or rabbits [59], suggesting LTII is unlikely associated with PWD.

STa

The mature porcine-type STa (heat-stable toxin type Ia) is a 2 kDa peptide with only 18 amino acids [60]. STa, coded by gene *estA*, possesses 6 cysteine residues which form 3 disulfide bonds to maintain the toxin structure [61]. STa binds a trans membrane guanylate cyclase C (GC-C) receptor at apical membrane of pig intestinal epithelial cells. STa toxin activates guanylate cyclase pathway, leading to an elevation of intracellular cyclic GMP levels in pig small intestinal epithelial cells, fluid hyper-secretion and diarrhea.

STb

STb (heat-stable toxin type II) differs from STa genetically and antigenically. Coded by gene *estB*, the mature STb is a 48 amino acid peptide (5.1 kDa) [62,63]. STb binds receptor sulfatide at pig small intestinal epithelial cells [64,65]. STb does not stimulate an increase of intracellular cAMP or cGMP levels in host epithelial cells [66], rather stimulates a pertussis toxin-sensitive GTP-binding regulatory protein and promotes water and electrolytes transported out of intestinal cells, that leads to diarrhea [66-68].

EAST1

A mature EAST1 peptide is of 38 amino acids (4.1 kDa) coded by *astA* gene. Interactions between EAST1 and host cells are not well studied. EAST1 is originally found from a different group of diarrheal *E. coli*, enter aggregative *E. coli* (EAEC), and was suggested to be associated with human diarrhea [69,70]. But EAST1 gene is also highly prevalent in ETEC strains isolated from pigs with PWD [25]. Recent studies, however, indicate that EAST1 does not stimulate an increase of intracellular cAMP or cGMP levels in T-84 cells or porcine cell line IPEC-J2 [71] and does not cause diarrhea in young pigs [71,72].

Stx2e

Stx2e is a member of shiga toxin family, a group of ribosome-inactivating toxins that cause vascular damage, and is associated with diarrhea (together with LT and ST enterotoxins). Stx2e toxin is composed of one A subunit (32 kDa) and five B subunits

(7.6 kDa each) [73] and binds globotriaosylceramide (Gb4) at pig small intestine and causes edema disease in young pigs [18,74]. Although it is often found (together with LT and/or ST enterotoxins) in ETEC strains isolated from pigs with PWD, Stx2e is thought to primarily cause edema disease in young pigs [41,75].

CHALLENGES IN PWD PREVENTION

Despite the fact that the virulence determinants of ETEC-associated PWD and the disease mechanism have been well studied, effective prevention measure against PWD is still lacking [2]. Various prevention approaches, including treatment with antibiotics, passive administration with specific antibodies, dietary supplementation including prebiotics, probiotics, as well as genetic breeding for ETEC-resistant herds have been attempted (along with vaccine development) to control PWD. These prevention approaches (alternative to vaccination) have shown some promise, but cost effectiveness or concerns of environmental risk make them less favorable or not practical.

ALTERNATIVE PREVENTION APPROACHES

Antibiotics

Giving antibiotics in feeding materials was once commonly practiced to treat *E. coli* infected herds, and it remains in practice in some countries. Unfortunately, K88 and F18 fimbrial ETEC strains which cause nearly all PWD cases are usually not sensitive to routinely used antibiotics, therefore, the prophylactic treatment with antibiotics has not been effective in controlling PWD. But the excessive use of antibiotics raises growing concerns at antimicrobial resistance in pathogens, impaired buildup of host immunity and selection of antibiotic resistant bacteria. Indeed, antimicrobial resistance (to apramycin, neomycin, trimethoprim or trimethoprim-sulfamethoxazole, and to Colistin) has been increasingly observed in ETEC strains causing PWD [76-79] and *E. coli* strains isolated from pigs are largely resistant to ampicillin and tetracycline [79-81].

Specific antibodies

Oral administration of specific-antibody-containing egg yolk, sow milk, or spray-dried plasma proteins to weaned piglets showed some protection against PWD by reducing disease severity [82-92]. Limitation is that antibody-containing spray-dried porcine blood plasma and sow milk protect piglets only during feeding [93,94], and the large quantity used in feeding is not economically effective in pig production, or in worse situation showed no protection against ETEC challenge or PWD outcomes [95-97], likely because the contained antibodies are not specific against the infected ETEC strains.

Dietary supplements and prebiotics

Dietary and diet supplementary treatment have also been practiced to control PWD. Soybean-based feed was reported to favor PWD occurrence [98], but fermented soya beans [99] and a barley- or rice-based diet helped to reduce PWD incidence [100,101]. In addition, feeds with decreased protein content or with special diet supplements were shown helpful against PWD outcomes [101-109], and the addition of organic acid to reduce gastric pH was found to decrease *E. coli* colonization and to minimize PWD [102,110-113]. In addition, zinc oxide was reported

to be an alternative of antimicrobials. Feeds containing zinc oxide reduced PWD cases and promoted pig health and growth [114]. The setback is that the use of high levels of zinc oxide led to heavy metal contamination in soil that raised environmental concerns [78,115]. Other supplements including methionine [116], bovine lacto ferrin peptides [117], glutamine [118,119], protease and lysozyme were also examined for promotion of piglet health or prevention against PWD [120,121]. However, different studies showed contradictory results, concluding that a reduced protein content level in feeds compromised pig growth performance but have no effect at reduction of ETEC in gastrointestinal bacteria population and that diet supplementary intakes did not alter PWD outcomes [122-126].

Prebiotics selectively stimulating proliferation and activities of gut-healthy bacteria was suggested to promote growth and health of weaned pigs [127]. Feeding weaned pigs with selectively fermented ingredients, such as non-starch polysaccharide hydrolysis was shown to reduce severity of K88 ETEC infection [128,129]. But other studies indicated that non-starch polysaccharide hydrolysis had no effect on some prebiotic bacteria [130].

Probiotics

Probiotic treatments with yeast and some bacterial strains which competitively inhibit adherence of ETEC and other pathogens and produce host-beneficial microbicidal substances have been reported to have some beneficial effects against PWD [131-139]. For an example, treating herds of high PWD incidence with commercially available *Bacillus* viable spores reduced PWD incidence, severity of disease, mortality and shedding of ETEC strains [140]. But contradictory results were shown by other studies as they indicated that feeding with probiotic bacterial strains of *Lactobacillus*, *Enterococcus*, and *Bacillus* or yeast had no or no-consistent protection against PWD [96,141-145].

GENETIC BREEDING FOR ETEC RESISTANT HERD

Pigs with different genetic backgrounds were found naturally susceptible or resistant to K88 ETEC infection [146-148]. Recently, DNA markers were developed to identify K88ac-resistant [149-151] or F18-resistant pigs [152,153]. These markers, however, might be population specific, since they can be used to identify pigs with the resistant phenotype in some herd populations but show low correlation in other populations (communication with Dr. David Francis). In addition, an early study suggested that K88ac susceptible piglets tend to have better growth performance over K88ac resistant pigs [154].

PWD VACCINES

Along with seeking antimicrobial, ETEC antibody, dietary, probiotics and breeding approaches to control PWD, development of live and subunit vaccines has been explored in the past decades. Despite ETEC-causing neonatal diarrhea has been largely controlled by passive maternal antibody protection through vaccination of pregnant sows, vaccination to effectively prevent PWD has not been achieved. Key challenges in developing effective vaccines against PWD include: 1) ETEC strains causing PWD express immunologically heterogeneous fimbriae and enterotoxins, only vaccines inducing broadly

protective anti-adhesin immunity and antitoxin immunity could effectively protect against PWD; 2) piglets have divergent genetic background, pigs expressing receptors which are not targeted by the vaccine fimbrial antigen will not or less effectively develop specific immune responses; 3) enterotoxins are virulence determinants in PWD and thus must be included as vaccine components, but LT, STa and STb are potently toxic and cannot be used as safe antigens; 4) STa and STb are poorly immunogenic, themselves alone are unable to induce anti-STa and anti-STb immunity, also F18 fimbriae are not effective in inducing protective anti-F18 immunity; 5) challenges in developing cost-effective vaccines to induce active-acquired immunity by the time of weaning.

Immunological heterogeneity of ETEC virulence factors

ETEC strains causing PWD expressing K88 and F18 fimbriae and toxins including LT, STa, STb and also Stx2e [24,25]. These fimbriae and toxins are genetically and immunologically heterogeneous. Therefore, immunity induced by one fimbria or toxin provides protection only against ETEC strains expressing the same fimbria or toxin, but cannot cross protect against ETEC strains expressing a different fimbria or a different toxin [155-157]. Experimental challenge studies indicated that an ETEC strain expressing K88 and either LT, STa, or STb caused diarrhea in pigs [158-161]. Thus an effective PWD vaccine would have to induce anti-adhesin immunity against both K88 and F18 fimbriae and also antitoxin immunity against LT, STa, STb and perhaps also Stx2e toxins.

Different genetic background of pigs

Pigs of various genetic backgrounds express different receptors, and these receptors are recognized and adhered by specific fimbriae expressed by different ETEC strains. Orally immunized with a fimbrial vaccine product, only pigs expressing receptors specific to this fimbria will effectively develop immune responses. For an example, oral administration of purified K88 adhesins induced K88-specific antibodies in K88-receptor positive piglets and protected against a K88-positive ETEC strain [162]. But the same administration induced no immune responses in K88-receptor negative pigs [163]. In addition, pigs expressing different loci of K88 receptors vary in developing anti-K88 immune responses [163].

Potent toxicity of enterotoxin antigens

LT, STa, STb are potently toxic. ETEC strains expressing LT, STa or STb alone cause diarrhea in piglets. Therefore, native toxins cannot be directly used as antigens for safe PWD vaccine development. But since they are the virulence determinants of PWD, these toxin antigens must be included as vaccine components to induce antitoxin antibodies. While the nontoxic B subunits can be used as safe antigens for LT and Stx2e, STa or STb has never been included in ETEC vaccine development until very recently. Moreover, immune responses induced by the B subunits protect against only the binding of LT or Stx2e to pig receptors GM1 or Gb4, but do not protect against the toxicity of LT or Stx2e.

Difficulties in inducing anti-ST immunity and protective anti-F18 immunity

Toxins STa and STb are small molecules and are poorly

immunogenic. Therefore, STa and STb antigens alone cannot induce pig anti-STa and anti-STb immune responses which are however required for effective protection against PWD. It was also found that purified F18 fimbriae or whole F18 fimbriae expressing by an *E. coli* strain are not effective at inducing protective anti-F18 antibody response [164,165]. Thus, vaccine candidate products composed of purified F18 fimbriae or an *E. coli* strain expressing F18 fimbriae cannot protect against PWD caused by F18-fimbrial ETEC strains or edema disease.

Optimal immunization to eliminate maternal antibody interference and to induce active immunity against PWD upon weaning

Passive maternal antibodies protect suckling piglets against ETEC-associated neonatal diarrhea, but the existing antibodies in suckling piglets block immunized vaccine antigens from inducing host ETEC-specific antibody responses, thus compromising efficacy of PWD vaccination. The dilemma is that on one hand suckling piglets need to acquire passive maternal antibodies at a certain level to protect against ETEC neonatal diarrhea, but on the other hand they need to have active-acquired antibodies developed in order to protect against PWD the moment entering weaning. In order to have active-acquired antibodies ready at weaning, piglets need to be immunized during suckling period, ideally ten days to two weeks before weaning. It becomes a practical challenge to maintain passive maternal antibodies in suckling piglets at a level sufficiently high to protect against neonatal diarrhea and also low enough to not interfere immunized antigens in effectively inducing specific antibodies against PWD.

Development of cost-effective and protective vaccines against PWD

Live oral PWD vaccines would be preferred over parenteral vaccine products. Oral products are more cost-effective in production, require no professional training for administration, and more importantly induce host local mucosal immunity and provide herd protection. Since pigs become vulnerable to ETEC infection the moment entering weaning, they will need to be immunized during the suckling period. But immunizing individual suckling piglets is labor tedious. Oral immunization at weaning (by adding vaccine products in feeds or drinking water), although is labor efficient, will leave newly weaned piglets vulnerable to PWD during the first several days. Other technical and practical challenges include effective expression and delivery of multiple adhesin and toxin antigens by a live vaccine product to induce immunity broadly against K88, F18, LT, STa, STb and potentially Stx2e, elimination of cold chain transportation and storage, and prolongation of product shelf life. Live vaccine may carry antibiotic resistance gene(s), alternative selection markers would be needed to ease environment concerns.

PROGRESS IN PWD VACCINE DEVELOPMENT

Although still exploratory, vaccination with a live and a subunit product to protect against PWD has been attempted in the past decades, and remains the most promising for PWD prevention [2,16]. Since ETEC strains expressing K88 or F18 fimbrial adhesins cause nearly all PWD cases, F18 and K88 fimbriae have been the main targets in anti-adhesin vaccine

development. For protection against enterotoxins, only the nontoxic LT_B subunit was previously included in PWD vaccine experimental studies. However, progress has been made recently. By applying toxoid antigens and genetic fusion antigens, we are able to include LT and ST antigens for antitoxin vaccines against PWD. Additionally, the recently developed multi epitope fusion antigen (MEFA) strategy enables us to include multiple adhesin and toxin antigens into a single product for development of broadly protective PWD vaccines.

Anti-fimbria experimental vaccine products

Bacterial fimbriae physically extracted or expressed by live *E. coli* strains were found to induce anti-adhesin immunity in pigs especially sows to protect born piglets [166-170]. But passive-acquired anti-fimbria antibodies protect only the suckling but not weaned piglets against ETEC diarrhea. Feeding pigs with non-pathogenic *E. coli* strain (s) expressing one or two fimbriae remains in practice in some farms to protect against PWD. Immunization of piglets with purified fimbriae, however, is not favored because of the high cost in fimbria extraction. In addition, purified fimbriae do not stand well against the acid environment in pig stomach. Microencapsulation is found helpful to prevent K88 fimbriae from acid degradation [164], but the encapsulation process itself adds up production cost and is often not great in general for the release of fimbria antigens and stimulation of host anti-fimbria immune responses [171].

Anti-K88 (F4) fimbrial candidates

K88 fimbriae, expressed by a live strain or purified from bacteria culture, can induce protective anti-K88 antibodies in pigs. Oral immunization of piglets with a live *E. coli* or *Salmonella* strain that expresses K88 fimbriae was found to protect against K88-fimbrial ETEC infections [172-175], and piglets immunized with purified K88 fimbriae developed anti-K88 antibody responses at levels similar to those in pigs immunized with a live strain expressing K88 fimbriae [162,176]. Furthermore, K88 fimbria major structural subunit, FaeG, expressed as recombinant protein [177,178], plasmid DNA [179-181] or plant-based protein [182,183], also induced anti-FaeG specific antibodies and showed protection against K88 ETEC infection. But anti-K88 antibody responses alone protect against only K88 fimbrial ETEC strains, they do not protect piglets against PWD caused by ETEC expressing F18 or other fimbriae, or piglets that are K88-receptor negative [184,185].

Anti-F18 vaccine candidates

Unlike K88 fimbriae that induce protective anti-K88 immunity, oral administration of encapsulated *E. coli* bacteria expressing F18 fimbriae or purified F18 fimbriae did not induce anti-F18 antibody response or protected against F18 ETEC infection [6,164,165,186]. In addition, piglets less than 20 day old do not have the F18 receptor fully expressed [187]. Therefore, oral immunization of suckling piglets with F18 fimbriae or a strain expressing F18 fimbriae cannot effectively induce anti-F18 antibody response, and thus cannot fully protect against F18 ETEC infection. Recent studies, however, demonstrated that the F18 minor subunit FedF, the adhesin of F18 fimbriae [47], alone [188] or genetically fused to K88 FaeG subunit or conjugated to K88 fimbriae, induced protective anti-F18 antibodies [15,189,190].

Broad anti-adhesin vaccine candidates

Vaccines inducing anti-adhesin immunity against both K88 and F18 would be needed for broad protection against adherence and colonization of ETEC strains and likely PWD. But even a regionally licensed cocktail product carrying live *E. coli* strains expressing K88 fimbriae and F18 fimbriae is not expected to be effective against PWD, since F18 fimbriae expressed by a live strain (or purified) are not effective in inducing protective anti-F18 antibodies. However, the antigen fusion strategy, demonstrated by recent studies, showed that 1) a fusion antigen including K88 FaeG major subunit and F18 FedF minor subunit (with LT toxoid) induced neutralizing anti-K88 and anti-F18 antibodies [15], and 2) piglets orally immunized with a live *E. coli* strain expressing this fusion antigen developed protective (*in vitro*) anti-K88 and anti-18 (& anti-LT) immunity and were protected when challenged with a K88-positive ETEC strain [190]. If future challenge studies show the induced anti-adhesin immunity also protects against F18-positive ETEC infection, this live strain can be the candidate for a broadly protective anti-adhesin vaccine.

Anti-toxin vaccine candidates

Developing antitoxin vaccines against PWD has been much hampered in the past largely because of the potent toxicity of ETEC toxins, the lack of cross protection of antitoxin immunity, and the difficulty in inducing anti-ST antibodies. But recent success in using toxoids as safe antigens and toxoid fusion antigens to enhance anti-ST immunogenicity shows feasibility at development of broadly protective antitoxin vaccines against PWD [191].

Vaccine candidates against individual toxin

Vaccine candidates carrying an individual toxin or an toxin derived antigen are shown to provide homologous protection against LT [15,156,190,192-194], ST_a [195] or Stx_{2e} [188,196]. However, since ETEC toxins are immunologically heterogeneous and ETEC strains expressing a different toxin cause PWD, vaccine candidates carrying an individual toxin antigen is never effective against PWD.

Broad antitoxin vaccine candidates

As an ETEC strain expressing LT or ST can cause PWD, an effective antitoxin vaccine should induce broad immunity against LT and ST toxins. It has been demonstrated recently that LT with a mutation of one or two amino acids becomes less toxic and retains anti-LT immunogenicity, and these LT derivatives can be used as a protein carrier to enhance immunogenicity of ST toxin antigens (and also to induce protective anti-LT antibodies). Genetic fusions of LT_{R192G} with a ST_a toxoid or a ST_b peptide induced protective antibodies against LT and also ST_a or ST_b toxin [197,198]. Moreover, a toxoid multi epitope fusion antigen (MEFA) that uses LT_{R192G} as a backbone to carry ST_a toxoid ST_a_{N115'}, a ST_b epitope and a Stx_{2e} A subunit epitope is found to induce neutralizing antibodies against toxins LT, ST_a and ST_b and Stx_{2e} (data not shown). This LT-ST_a-ST_b-Stx_{2e} toxoid MEFA should serve as a candidate for future development of a broadly protective antitoxin vaccine against PWD.

Broad PWD vaccine candidates

It has been stipulated that neutralizing antitoxin antibodies, without assistance from antibodies against fimbrial adhesins, may be less effective against ETEC strains which attach host cells and release enterotoxins directly to small intestinal epithelial cells [199]. Thus vaccine candidates inducing antitoxin immunity but not anti-adhesin immunity may still not be sufficiently effective against ETEC infection [171]. Therefore, an effective PWD vaccine should induce protective anti-adhesin immunity against K88 and F18 fimbriae but also antitoxin immunity against LT and ST toxins. We have demonstrated that a live *E. coli* strain expressing K88-StA_{-epitope}, K88-LT_{-epitope}, or K88-StA_{-epitope}-LT_{-epitope} chimeric fimbriae induced neutralizing antibodies against K88 fimbria, STa toxin and/or LT [195], and a live strain expressing FaeG-FedF-LT_{A2:B} fusion antigen induced protective immunity against adherence from K88 and F18 fimbriae and LT enterotoxigenicity [190]. If the live strain expressing FaeG-FedF-LT_{A2:B} can also carry STb, STa and Stx2e antigens to induce protective antibodies against these three toxins, this strain would become a primary oral vaccine candidate for truly broad-spectrum protection against PWD.

To express multiple fimbrial and toxin antigens in a single PWD vaccine product seems challenging. Helpfully, we recently developed the multi epitope fusion antigen (MEFA) strategy and successfully constructed a single MEFA antigen to induce protective immunity against seven human ETEC CFA adhesins [200]. Moreover, this CFA MEFA was able to be further fused to an LT-STa toxoid fusion, and the resultant CFA-toxoid MEFA induced protective antibody responses to seven CFA adhesins and both LT and STa toxins. This MEFA approach likely enable us to include antigenic elements of K88 FaeG, F18 FedF, LT, STa, STb and also Stx2e in a single antigen, and an *E. coli* strain expressing this fimbria-toxoid MEFA then can surely serve as a vaccine candidate for broad protection against PWD.

CONCLUSION

PWD is one of the most important swine diseases and causes substantial economic losses to the swine producers in the US and worldwide. Currently, there are no prevention approaches available to effectively protect against PWD. Development of broadly protective vaccines against PWD should remain as a top priority for the swine industry. Due to the nature of the disease, challenges in PWD vaccine development will continue to exist. But with the progress made recently, particularly the application of toxoid antigens, toxoid fusion antigens and MEFA strategies, a broadly protective PWD vaccine should be developed in a very near future.

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