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REVIEW

Major virulence factors of enterotoxigenic *Escherichia coli* in pigs

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Abstract Enterotoxigenic *Escherichia coli* (ETEC) infection is the most common type of colibacillosis of young animals, and it is also a significant cause of food- and waterborne *E. coli*-mediated human diarrhea worldwide. ETEC is a pathotype characterized by the production of adhesins that mediate bacterial adherence to the intestinal epithelium and enterotoxins that interact with the intestine to cause diarrhea. In addition to adhesive and enterotoxic virulence factors, pathogenesis also involves host factors, the most important of which are receptors for adhesins and enterotoxins. The aim of this review is to summarize current knowledge and to highlight new developments and the most actual research topics in the area of ETEC infections in pigs. Attention is also paid to recently described new virulence factors and new vaccines against ETEC bacteria.

Keywords ETEC · Fimbrial adhesins · Enterotoxins · Non-fimbrial adhesins · Virulence factors

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are an important cause of severe, watery diarrhea in the newborn of some animal species and also a main cause of diarrhea among travelers and children in developing countries. The virulence of ETEC is characterized by the production of fimbrial adhesins and enterotoxins. Fimbrial adhesins mediate the attachment of bacteria to the surface of host epithelium cells and allow bacterial colonization. The majority of ETEC isolates for swine express one or more of five antigenically distinct adhesins, denoted K88 (F4), K99 (F5), F41, 987P (F6), and F18, respectively. Enterotoxins, including heat-stable enterotoxins (ST) and heat-labile enterotoxin (LT), have been found to disrupt intestinal fluid homeostasis and to cause watery diarrhea (Vu Khac et al. 2006). In the past decade, a number of new potential virulence factors of ETEC have been isolated from pigs, including adhesin, involved in diffuse adherence (AIDA-I) (Benz and Schmidt 1989), enteroaggregative heat-stable factor (EAST1) (Savarino et al. 1993), new pili factor (type IV) (Pichel et al. 2002), and porcine attaching and effacing-associated factor (paa) (Batisson et al. 2003). The characteristics and mechanisms of several of the adhesive and enterotoxic virulence factors are well known, but others are only partially understood or completely unknown. In addition to adhesive and enterotoxic virulence factors, pathogenesis also involves host factors, the most important of which are the receptors for adhesins and enterotoxins.

Methods for the treatment and control of ETEC diarrhea are still a matter of debate among veterinarians and livestock producers and in the animal industry in general. At the present time, the use of immune-based therapy is considered to be a promising approach for preventing ETEC. While several reagents are in use against ETEC in pigs, most of these reagents are based on the most prevalent fimbriae and, consequently, do not confer broad protection against all ETEC strains due to the antigenic diversity and

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high prevalence of unidentifiable forms of specific fimbriae of ETEC (Thomas and Rowe 1982). The more recent development of modern vaccines, such as recombinant subunit vaccines, DNA vaccines, and plant-derived edible vaccines has raised hope of a more universal protection, but their practical application in production systems requires further study.

Fimbrial adhesins

K88 fimbriae

In pigs, ETEC strains expressing K88 fimbriae are responsible for much of the neonatal and post-weaning diarrhea (PWD) infections. Zhang et al. (2007) reported that roughly 65% of the fimbriaed *E. coli* isolates carry the K88 fimbrial gene in the USA. Three antigenic variants of K88 fimbriae from porcine ETEC strains are K88ab, K88ac, and K88ad. K88ac fimbrial ETEC strains are the most common in young piglets with clinical diarrhea. The genetic determinant for the biosynthesis of K88 fimbriae is located on large and usually nonconjugative plasmids (Van Den Broeck et al. 2000). K88 fimbriae are composed of multiple copies of the *faeG* major subunit, and one copy of the *faeC* minor subunit. The *faeG* subunit is highly conserved among $K88^+$ *E. coli* isolates (Verdonck et al. 2004). Other genes of the K88 operon, including *faeA*, *faeB*, *faeD*, *faeE*, *faeF*, and *faeH*, could also be involved in fimbrial biosynthesis, but these genes contribute largely to local regulation, anchorage, transmembrane transport, and modification of the major protein subunit and they do not affect fimbrial binding activity (Van Den Broeck et al. 2000).

Interaction of K88 fimbriae with K88-specific receptors (K88R) enables $K88^+$ ETEC to attach to the host epithelial cells and colonize the small intestine (Van den Broeck et al. 1999). There are three receptors specific for $K88^+$ ETEC, namely, high-molecular-weight intestinal mucin-type sialoglycoproteins (IMTGPs) (Billey et al. 1997), an enterocyte membrane-associated transferrin (Grange and Mouricout 1996), and an intestinal neutral glycosphingolipid (IGLad) receptor (Grange et al. 1999). Structural differences in the host receptors and the differential ability of the K88 fimbrial variants to recognize these structures may explain the binding differences among the three K88 fimbrial variants. In addition, the polymeric K88 fimbriae are necessary for the attachment of $K88^+$ ETEC to the intestinal epithelium (Devriendt et al. 2010).

Parenteral vaccination with purified K88 fimbriae is able to prevent ETEC infections in suckling piglets because the protective immunoglobulin A (IgA) antibodies can be transmitted via colostrum and milk to suckling piglets. However, this method is not efficient in preventing PWD no longer protected by passive lactogenic immunity since it stimulates a systemic rather than a K88-specific immune response. Oral immunization of $K88R^+$ piglets with purified K88 fimbriae induces a K88-specific intestinal immune response that protects them against a subsequent ETEC challenge (Verdonck et al. 2004). In contrast, oral immunization with K88 fimbriae purified from $K88^+$ ETEC mutants, in which the polymeric stability of the fimbriae is disrupted, results in reduced mucosal immune responses (Joensuu et al. 2006; Verdonck et al. 2008) due to the polymeric stability of K88 fimbriae being very important for its biologic activity (Devriendt et al. 2010).

F18 fimbriae

F18 fimbrial *E. coli* strains adhere to the microvillus of small intestinal cells in piglets and are associated with PWD or porcine edema disease (ED). There are two F18 antigenic variants: F18ab and F18ac. The F18ab variant is often expressed in strains producing Shiga toxins (STEC) and causing ED. Many of these strains belong to serogroup O139, whereas strains expressing F18ac fimbriae are ETEC, often belonging to serogroups O141 and O157, which cause diarrhea by elaborating enterotoxins (Nagy et al. 1997). They bind to the same receptor on the brush border although biologically distinct (Tiels et al. 2005).

The *fed* gene cluster, encoding the F18 fimbriae, is only about 5.6 kb and is composed of five genes. The major subunit *fedA* forms the backbone of F18 fimbriae, with both the *fedE* and *fedF* genes encoding the minor subunits. Unlike the K88 fimbriae, F18 adhesin is the minor subunit *fedF*, which is conserved among all field isolates from different locations around the world (Tiels et al. 2005). *FedB* and *fedC* were recently characterized and found to encode the putative usher and chaperone. The gene encoding the afimbrial adhesin AIDA is also frequently found on the same plasmid as the *fed* gene cluster (Mainil et al. 2002), suggesting that these two adhesins may have some innate relationship with each other.

The structure of the F18 receptor is not yet characterized. The alpha(1,2)-fucosyltransferase genes (*FUT1* and *FUT2*) have been proposed as candidate genes for the *E. coli* F18 receptor (ECF18R) (Meijerink et al. 1997). A guanine (G)/adenine (A) polymorphism at nucleotide 307(M307) in the *FUT1* gene has been shown to be highly correlated with the ECF18R genotypes, with genotype $M307^{AA}$ representing resistance and genotypes both $M307^{GG}$ and $M307^{GA}$ representing susceptibility to colonization (Meijerink et al. 1997). Monoclonal H-2-specific antibody is able to strongly inhibit the adhesion of $F18^+$ *E. coli* to intestinal villus enterocytes (Snoeck et al. 2004). These data suggested that the F18 receptor contains the

blood group antigen H-2 [α-fuc-(1–2)-β-Gal-(1–4)-GlcNAc] as a major carbohydrate.

To date, there is no good preventive strategy for protecting pigs from $F18^+$ *E. coli* infections. One of the most attractive approaches for eliminating $F18^+$ *E. coli* infections is selection for $F18^+$ *E. coli*-resistant genetically pigs. However, this strategy was initially believed to be largely unacceptable because of reports of genetic association with the stress-susceptibility gene in the Swiss Landrace (Meijerink et al. 1997). However, in contrast to these earlier findings, it has recently been reported that there is no association between the $F18^+$ *E. coli* resistance allele and the stress-susceptibility allele in the Belgian pig population. Consequently, selection for breeding pigs with genetic resistance to $F18^+$ *E. coli* infections can be considered (Coddens et al. 2008). Since there is no available commercial vaccine against $F18^+$ *E. coli*, this protective strategy against $F18^+$ *E. coli* infections has the potential to be of great economical importance for the pig industry.

987P fimbriae

The 987P antigen was first described by Isaacson and Richter (1981) as a long, straight structure surrounding the bacterial cell. The growing family of 987P-like fimbriae include the CS12, CS18, and, most probably, CS20 fimbriae (Valvatne et al. 1996). The 987P gene cluster is composed of eight genes (*fasA–fasH*) located on both the large plasmid and chromosome adjacent to a Tn1681-like transposon encoding the STIa. It is a heteropolymeric structure consisting of one major subunit, *fasA*, and two minor subunits, *fasF* and *fasG* (Khan and Schifferli 1994). The *FasG* minor subunit is the adhesin for the attachment of 987P ETEC to the porcine intestine. *FasD* and *fasB* are both closely involved in exporting and assembling the structural components of the fimbriae. The regulator gene of *fasH* is able to activate transcription of the structural subunit (Klaasen and De Graaf 1990).

The 987P fimbriae mediate bacterial adherence to both glycoprotein and glycolipid receptors found on porcine intestinal epithelial cells (Choi and Schifferli 2001). The adhesin *fasG* harbors two distinct functional domains for its different types of receptors. The *fasG* K117 residue is required only for binding to the glycolipid receptor, whereas two fragments from the amino acid residues at position 211 (glutamine) to 220 (serine) and from position 20 (aspartic acid) to 41 (serine), respectively, are required specifically for the recognition of the glycoprotein receptor (Choi and Schifferli 2001). Zhu et al. (2005) indicated that the intestinal protein receptors for 987P are histone H1 proteins. The histone H1 molecules stabilize the sulfatide–fimbriae interaction by simultaneously binding to the membrane and to 987P. The receptors for 987P become overexpressed with age, leading to the shedding of free receptors into the intestinal lumen to cover the fimbriae 987P, thereby blocking adhesion.

K99 and F41 fimbriae

The K99 fimbriae specifically mediate the attachment of ETEC to mucosal surfaces of calves, lambs, and piglets. A 7.1-kb operon of eight genes (*fanA–fanH*) on a large plasmid encode the pilus and other associated proteins responsible for the regulation, transport, and assembly of the K99 pili. The major subunit, *fanC*, comprising 159 amino acids, is responsible for binding to the ganglioside receptor (Garg et al. 2007). The genes encoding the K99 fimbriae can be categorized into three independently regulated regions. Region 1 encodes *fanA–fanD* and is dependent of cyclic adenosine 3', 5'-monophosphate (cAMP), cAMP receptor protein (CRP), and leucine-responsive protein (LRP); region 2 encodes *fanE* and *fanF* and is cAMP and CRP dependent but independent of LRP; region 3 encodes *fanG* and *fanH* and is independent of the cAMP, CRP, and LRP (Inoue et al. 1993). K99 expression was affected by numbers of different environmental conditions such as the degree of aeration provided and the growth rate of the cells. Aerated and logarithmically growing bacteria produced large amounts of fimbriae. The glycoprotein receptor *N*-glycolylneuraminic acid-GM3 (NeuGc-GM3) is necessary for K99 to bind to the host enterocytes. This ganglioside receptor located on the surface of bovine enterocytes is also found on equine red blood cells (Teneberg et al. 1990).

Conventional vaccines against $K99^+$ETEC include parenteral or subcutaneously administered purified K99 fimbrial protein or formalin-inactivated ETEC, but they have been shown to achieve only limited protection due to their inability to stimulate the appropriate mucosal immunity. Edible transgenic plants producing K99 fimbrial subunit proteins is a potential means to vaccinate animals against these diseases (Piller et al. 2005; Garg et al. 2007). Piller et al. (2005) used transgenic soybean to develop a K99-based plant-edible vaccine that could elicit special anti-K99 humoral and cellular immune responses in mice. The mice immunized intraperitoneally with a protein extract derived from transgenic soybean leaves expressing chloroplast-targeted *fanC* also developed significant antibody titers against *fanC* (Garg et al. 2007).

F41 fimbriae combined with K99 are often found on the same strains with serogroups O8 or O9. Like K99 fimbriae, F41antigen expression is also affected by a variety of environmental factors. The genes code for F41 and its stucture is not yet well elaborated. Glycoproteins from human erythrocytes and glycophorin have been found to act

as an erythrocyte receptor for F41fimbriae (Lindahl and Wadstrom 1986).

Non-fimbrial adhesins

In addition to fimbrial adhesins, a number of non-fimbrial adhesins also play an important role in ETEC infection. AIDA-I and *pAA* are two potential non-fimbrial virulence factors that have recently been implicated in porcine diarrhea. AIDA-I was originally detected from a human isolate (strain 2787) with diarrhea by Benz and Schmidt (1989). In 2001, Niewerth and colleagues first detected *AIDA-I* from a strain isolated from pigs with ED and PWD. In a study carried out in the USA, about 26.9% *E. coli* strains isolated from young pigs with diarrhea carried the *AIDA-I* gene (Zhang et al. 2007). In China, the *AIDA-I* gene was detected in about 6.5% *E. coli* isolated from piglets with diarrhea (Zhao et al. 2007). Because of its capacity to autoaggregate and form biofilms, in addition to its adhesive function, the *AIDA-I* gene is classified as belonging to the Self-Associating Auto Transporter (SAAT) family. The AIDA is encoded by *AidA* (*orfA*) and *aah* (*orfB*) genes. *AidA* codes for the precursor of AIDA-I and requires the adjacent *aah* (autotransporter adhesion heptosyl-transferase) genes whose product adds heptoses to the AIDA protein. AIDA-I adhesin from porcine is an acidic protein consisting of five isoforms, and the sequences of AIDA-I from human and porcine origins are very similar (Fang et al. 2005). The observed similarities between human and porcine AIDA-I may represent a potential hazard for cross-infection between humans and pigs. The detection of AIDA-I in association with other virulence factors has been frequently reported. Niewerth et al. (2001) reported a frequent association of AIDA, F18, and Stx2e in *E. coli* isolates from pigs with ED and PWD. A recent study clearly established the importance of AIDA-I adhesin in conjunction to STb production by *E. coli* in the development of swine diarrhea (Ravi et al. 2007). However, it was not determined whether porcine *E. coli* strains carrying AIDA-I only and lacking enterotoxin genes are able cause diarrhea in pig. Therefore, the STb/AIDA linkage may only be a missing piece of information to add to our current knowledge of porcine colibacillosis (Dubreuil 2010). Although pAA was reported to be necessary for the development of the attaching/effacing (AE) lesion by human EPEC strains (Batisson et al. 2003), the significance of paa in ETEC-associated diarrhea in pigs remains unknown. A third potential virulence factor, the *E. coli* attaching and effacing factor (EAE), has also been reported to be associated with porcine PWD (Ngeleka et al. 2003). However, none of these potential virulence factors have been well characterized in terms of their significance role in porcine diarrhea.

Enterotoxins

Heat-labile enterotoxin

Heat-labile enterotoxin consists of a catalytic A subunit (LTA) and a pentamer of receptor-binding B subunits (LTB). The homopentameric B subunit is responsible for binding to the host GM1 ganglioside receptors (Spangler 1992). The A subunit is composed of two functionally different subunits, namely, an enzymatic subunit A_1 and a short connector A_2. Release of A_1 from the holotoxin by nicking and disulfide-band reduction plays an important role in the expression of ADP-ribosyltransferase activity and toxicity (Spangler 1992). Two subtypes, LT-I and LT-II, are known. LT-I is associated with diarrhea diseases of both humans and animals, while LT-II is typically associated with diarrheal diseases in animals.

LT is very similar to cholera toxin (CT) in terms of its antigenic properties and mechanism of action. Following the binding of the B subunits predominantly to the GM1 ganglioside receptor, the A subunit covalently modifies the α subunit of Gs GTP-binding protein, resulting in constitutive activation of adenylate cyclase and the production of cAMP. Intracellular increases in cAMP lead to activation of the cAMP-dependent protein kinase A (PKA), which phosphorylates the R domain of the cystic fibrosis transmembrane conductance regulator (CFTR). Ensuing chloride and water efflux into the intestinal lumen leads to significant volumes of watery diarrhea (Johnson et al. 2009).

LT is recognized as one of the most potent mucosal immunogens and adjuvants. The B subunit has good immunogenicity and is the sole LT-B subunit or recombinant-expressing LT-B subunit that can induce a specific antibody against LT. Dickinson and Clements (1995) designed a nontoxic LT (R192G) of the LT derivative that contained an amino acid exchange (R192G) at the A subunit trypsin-sensitive site. The resulting molecule lacked detectable ADP-ribosylating activity but retained the LT adjuvant effects. The LT (R192G) adjuvant has also been reported to be a powerful adjuvant for mucosal immunizations in mice and other animals (Verdonck et al. 2007). In pigs, LT (R192G) was also shown to be an effective adjuvant for nasal immunization (Yuan et al. 2001).

Heat-stable enterotoxin

Heat-stable enterotoxins are small and monomeric molecules. They are classified into two structurally, functionally

and immunologically unrelated types, namely STa (STI) and STb (STII). Two STa variants are known: STaH found in human ETEC strains and the STaP isolated from both human and porcine strains. The STa genes (*estAs*) of ETEC have a highly conserved 72-amino acid open reading frame. Although the STaP and STaH genes were predicted to encode peptides of the same length, a considerable nucleotide sequence divergence has been observed among these genes. The biological potency of STa depends to a great extent on the correct formation of the three intramolecular disulfide bridges. The biological activity of STa is exerted through activation of guanylate-cyclase-C (GC-C) receptor, which stimulates activity of the CFTR and inhibition of sodium/hydrogen exchangers by a cGMP-dependent protein kinase (PKGII)-dependent pathway, thus resulting in water secretion (Vaandrager 2002). EAST1 is another ST that has been found to be highly prevalent in ETEC strains isolated from humans and animals with diarrhea (Choi et al. 2001). EAST1 shares about 50% protein identity with STa and may act in a similar way to STa toxin; however, the effects of EAST1 on the induction of electrolyte loss from the intestine have not yet been determined.

STb, which is found mainly in association with porcine ETEC, has also been found occasionally in *E. coli* strains isolated from various animals and also in humans (Dubreuil 2008). STb, a 48-amino acids peptide of 5.2 kDa, possesses four cysteine residues forming two disulfide bridges. The gene for STb (*estB*) is frequently observed in ETEC recovered from PWD in pigs and associated with AIDA-I (Ngeleka et al. 2003). A variant for STb toxin from ETEC strains was recently identified in Canada. This variant shows a single base change in a codon, resulting in the His12 to Asn variant in 23 of 100 randomly tested strains carrying *estB*. In this study, no association was observed between AIDA-I and the variant, but a positive association was seen for wild-type STb toxin (Taillon et al. 2008).

The STb toxin has to be internalized to stimulate fluid secretion. STb endocytosis is mediated by two endocytic pathways; one is clathrin-dependent and the other is caveolae-dependent (Goncalves and Dubreuil 2009). Once inside the cell, STb induces nonspecific pore formation in brush border vesicles from pig jejunal tissue. These pores may account for the secretion of electrolytes observed in STb-provoked colibacillosis (Goncalves et al. 2007). In fact, although STb is often associated with pig diarrhea, up to recently, it has been difficult to experimentally reproduce a profuse diarrheal disease with a bacterial strain producing only the STb toxin.

To generate a successful toxoid, a ST must be made that is both immunogenic and non-toxic. ST is non-immunogenic in its natural form because of its small size, but it becomes immunogenic when ligated to an appropriate large-molecular-weight carrier (Taxt et al. 2010). This has been successfully achieved with several carriers using either chemical conjugation or recombinant fusion techniques. The first report of a successful attempt to make ST immunogenic was the chemical conjugation of ST to porcine IgG. This ST–IgG conjugate was found to have the potential to provide protection against ST-only-producing ETEC in rats when tested using the ligated ileal loop assay (Klipstein et al. 1981). The synthetic ST was then conjugated to LT-B and shown to be immunogenic in both rats and rabbits. Aref and Saeed (2011) demonstrated that the dimethyformamid (DMF)-based STa conjugation protocol is more efficient for designing highly immunogenic STa conjugate than other conjugation protocols, based on total mouse units and conjugation ratio. Additionally, the immune response against STa elicited by the DMF-based STa conjugate was higher than those reported in previous studies. Based on these high levels of STa binding and neutralizing antibodies titers, the DMF-based STa conjugate would seem to have a great potential in developing immunotherapeutic reagents and/or STa-based vaccine against ETEC.

Conclusion

The question of how to treat and control ETEC diarrhea has not yet been conclusively answered to date, but immune-based therapy is considered to be a promising approach for combating ETEC. Most vaccines currently on the market are inactivated bacteria with protective antigens (fimbrial adhesins with or without LT enterotoxins) or purified antigens which do not confer broad protection against ETEC strains. Modern vaccines based on recombinant proteins or synthetic peptides are considered to be safer and to cause fewer side effects than inactivated and live attenuated vaccines. However, these well-defined vaccine candidates are usually poorly immunogenic and require either appropriate adjuvantation or the formation of virus-like particles to be efficacious (Pardoll 2002). The lack of the suitable animal models for testing the efficacy of vaccine candidate antigens is another problem encountered during efforts to develop ETEC vaccines because the highly complex cellular and molecular interactions involved can only be analyzed within a physiological environment. It is always difficult to accurately mimic human or veterinary pathology so there is often a discrepancy between results from in vitro and in vivo studies. The in vitro adherence conditions are excellent for bacterial growth and less stressful and lack the full host response component, while the in vivo environment includes the harsh conditions in the intestinal tract, such as the immune response, the indigenous microflora, and competition for nutrients (Shin et al. 2002).

An oral ETEC vaccine consisting of the recombinant cholera B subunit (rCTB) and formalin-inactivated *E. coli* bacteria expressing major colonization factors (CFs) has been shown to be safe and immunogenic in adults and children in different countries (Svennerholm 2011). The vaccine also induces significant protection against non-mild ETEC diarrhoea, i.e., diarrhoea interfering with daily activity in American travelers, but not against ETEC diarrhoea in young children in Egypt. Against this background, a modified ETEC vaccine consisting of recombinant *E. coli* strains overexpressing the major CFs and a more LT-like hybrid toxoid (LCTBA) has been developed. This vaccine will be tested soon alone and together with a mucosal adjuvant, i.e., double-mutated LT (dm LT), in clinical trials (Svennerholm 2011). Although no effective ETEC vaccine is yet available for humans, there is strong evidence that such a vaccine will be developed.

The future vaccines against ETEC should provide broad protection against ETEC-induced diarrhea. The strategies for developing such new vaccines include the use of the novel technologies and adjuvants aimed at improving the efficacy of conventional vaccines. Other strategies are the identification of major protective antigens preventing the binding of bacteria in the intestine, suitable toxoids, and optimal ways of inducing intestinal immune responses. Highly conserved flagellin plays an important role in ETEC adherence, so it could serve as viable antigenic targets in the design of novel vaccines to prevent infections caused by ETEC and other important motile pathogens (Roy et al. 2009). The ETEC-derived flagellin also has a potential mucosal adjuvant capacity, so it may be used as an adjuvant to improve the design of rational vaccines against ETEC infections (Devriendt et al. 2010). Chen et al. (2009) used the mutant *E. coli* strain C43 (DE3) in the large-scale production of both bacterial lipoproteins and lipoimmunogens with intrinsic adjuvant properties. The lipo-recombinant proteins are able to elicit stronger immune responses than those formulated with the alum adjuvant. Give this property, lipoimmunogens can be used for the development of novel vaccines. The multivalent or polyvalent vaccine designed to immunize against two or more strains of the ETEC could be also used in the future vaccine development. The new vaccines against ETEC in pigs that are now available raise some hopes, but their protective effects are remain to be seen.

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