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Escherichia coli in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies

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Abstract

Escherichia coli is one of the most important causes of postweaning diarrhea in pigs. This diarrhea is responsible for economic losses due to mortality, morbidity, decreased growth rate, and cost of medication. The *E. coli* causing postweaning diarrhea mostly carry the F4 (K88) or the F18 adhesin. Recently, an increase in incidence of outbreaks of severe *E. coli*-associated diarrhea has been observed worldwide. The factors contributing to the increased number of outbreaks of this more severe form of *E. coli*-associated diarrhea are not yet fully understood. These could include the emergence of more virulent *E. coli* clones, such as the O149:LT:STa:STb:EAST1:F4ac, or recent changes in the management of pigs. Development of multiple bacterial resistance to a wide range of commonly used antibiotics and a recent increase in the prevalence and severity of the postweaning syndromes will necessitate the use of alternative measures for their control. New vaccination strategies include the oral immunization of piglets with live avirulent *E. coli* strains carrying the fimbrial adhesins or oral administration of purified F4 (K88) fimbriae. Other approaches to control this disease include supplementation of the feed with egg yolk antibodies from chickens immunized with F4 or F18 adhesins, breeding of F18- and F4-resistant animals, supplementation with zinc and/or spray-dried plasma, dietary acidification, phage therapy, or the use of probiotics. To date, not a single strategy has proved to be totally effective and it is probable that the most successful approach on a particular farm will involve a combination of diet modification and other preventive measures.

Keywords: *Escherichia coli*, diarrhea, postweaning, pig, vaccination, receptor, fimbriae, F4, K88, F18

Introduction

Escherichia coli postweaning diarrhea (PWD), also called postweaning enteric colibacillosis, is an important cause of death in weaned pigs and occurs worldwide. Enteric *E. coli* infection in weaned piglets may also manifest as a diarrhea which usually occurs during the first week of postweaning and often results in decreased weight gain. Several factors, such as the stress of weaning, lack of antibodies originating from the sow's milk, and dietary changes, contribute to the severity of this disease.

Recently, an increase in incidence of outbreaks of severe *E. coli*-associated diarrhea has been observed worldwide. These are manifested as sudden death or severe diarrhea, often associated with F4 $(K88)^+$ *E. coli*. Most outbreaks have occurred in early-weaned piglets although traditional herds are being increasingly affected. The *E. coli* isolates often demonstrate multiresistance to three or more of a wide range of antimicrobials including apramycin, trimethoprim-sulfonamide, spectinomycin,

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and neomycin (Fairbrother *et al.*, 2000; Amezcua *et al.*, 2002; Lanz *et al.*, 2003; Maynard *et al.*, 2003). The prophylactic use of antibiotics probably plays a role in the development of this antimicrobial resistance (Docic *et al.*, 2003). Another interesting concept is that the ban on the use of food animal growth promoting antibiotics such as avoparcin, bacitracin, spiromycin, tylosin, and virginiamycin in Scandinavia and Europe has demonstrated that these agents possess prophylactic activity and their withdrawal has been associated with an increase in diarrhea, weight loss, and mortality due to *E. coli* in postweaning pigs (Casewell *et al.*, 2003). This has led to an increase in the therapeutic use of various antimicrobial agents, such as aminoglycosides, trimethoprim-sulfonamide, macrolides, and lincosamides, which may exacerbate the problem of antimicrobial resistance.

Other factors contributing to the increased number of outbreaks of this more severe form of *E. coli*-associated diarrhea are not yet fully understood. These could include a more effective *E. coli* colonization of the intestine due to changes in the feed regimens following weaning. For instance, in farms where husbandry measures such as addition of higher levels of protein of animal source, plasma, acidifying agents, and zinc oxide are being used at weaning, peaks of diarrhea and enteric colibacillosis complicated by shock may be delayed to 3 weeks after weaning, or even at 6–8 weeks after weaning, at the time when the pigs enter the growing barns (Fairbrother, unpublished results).

Diet is one of the most important factors influencing the course of the disease in these animals. A diet rich in milk products and energy reduces the duration of the period of lowered feed intake and associated mortality and delays the onset of clinical signs (Tzipori *et al.*, 1980). Other products of animal source, such as dried plasma added to the feed, also have a protective effect in reducing the incidence and severity of the diarrhea (Van Beers-Schreurs *et al.*, 1992). In contrast, the presence of other ingredients in the feed, such as soybeans, seems to favor the occurrence of PWD. This could be due to the presence of trypsin inhibitors or antigens inducing a localized immune response (Dréau *et al.*, 1994). The latter could result in changes such as a decrease in villus height, deepening of the crypts, and an increase in anti-soya immunoglobulins in the serum, possibly predisposing to a proliferation of *E. coli* (Li *et al.*, 1991a, b). The presence of organic acidifiers in the feed can promote a higher mean daily weight gain, feed conversion, and decreased incidence of PWD (Giesting and Easter, 1985). The addition of zinc oxide at levels above 2400 ppm in the feed decreases the severity of PWD although zinc sulfate and organic zinc are potentially toxic (Holm and Poulsen, 1996). The presence of enterotoxigenic *E. coli* (ETEC) in the environment of pigs is an important factor in the development of diarrhea, these bacteria being able to survive for at least 6 months in the environment if they are protected by manure (Van Beers-Schreurs *et al.*, 1992). The ETEC may be disseminated by the feed, other pigs, or other animal species (Bertschinger, 1999).

Bacterial factors contributing to the recent upsurge in outbreaks of severe disease could include changes in characteristics of the *E. coli* isolates associated with PWD and the possible emergence of more virulent clones. Irrespective of the cause of this upsurge, it will be primordial to develop alternative prophylactic strategies for the efficacious control of this disease in pigs, to alleviate the problem of the high level of antimicrobial multiresistance in the causative *E. coli* isolates. These aspects will be the focus of this review.

Virulence factors of *E. coli* associated with PWD

PWD due to *E. coli* is caused primarily by ETEC, a pathotype that is characterized by production of adhesins that mediate bacterial adherence to the intestine and enterotoxins that cause diarrhea. The ETEC that cause PWD typically produce alpha-hemolysin and give rise to colonies with clear zones of hemolysis on blood agar. In a recent study, 87.8% of 563 *E. coli* isolated from weaned pigs with diarrhea were hemolytic (Frydendahl, 2002). A recent study from China had results that were quite different, as only 25 (11.6%) of 215 strains from pigs with PWD were hemolytic, although not all of these strains were ETEC (Chen *et al.*, 2004).

Alpha-hemolysin, an approximately 110 kDa pore-forming cytolysin, belongs to the RTX family of toxins. The *hlyA* gene that encodes the hemolysin is part of an operon that is found on plasmids in ETEC but on the chromosome in human uropathogenic *E. coli*. Alpha-hemolysin is a potent cytotoxin that can damage a variety of cells, but its role in PWD has not been demonstrated. Smith and Linggood (1971) found that a strain of O141 ETEC that had been cured of its hemolysin plasmid did not seem to be impaired in its ability to induce disease in pigs. However, a subtle effect of the hemolysin may have gone undetected as the pigs that were challenged were unusually susceptible to infection. Similarly, inactivation of the hemolysin structural gene (*hlyA*) in an ETEC strain did not increase the incidence of septicemia in orally challenged neonatal gnotobiotic piglets (Moxley *et al.*, 1998).

Before the virulence factors of ETEC had been identified, serological typing of outbreak strains had shown that ETEC strongly associated with PWD in pigs belonged to a few serotypes and that serotype was a good marker for ETEC (Sojka, 1965). Serological typing has been expanded to include fimbrial antigens, which are virulence factors, as well as O and H antigens which are virulence markers. Some strains of ETEC that cause PWD possess additional genes that encode Shiga toxin 2e (Stx2e or verotoxin 2e, VT2e), allowing them to cause edema disease (ED) as well. *E. coli* that produce shiga

toxin (verotoxin) are called Shiga toxin-producing *E. coli* (STEC) or verotoxin-producing *E. coli* (VTEC) and the ETEC strains that produce Stx (VT) are appropriately called ETEC/STEC or ETEC/VTEC (Nagy and Fekete, 1999).

A second type of *E. coli* that is implicated in PWD is enteropathogenic *E. coli* (EPEC) that are able to cause attaching and effacing (AE) lesions and are therefore called attaching and effacing *E. coli* (AEEC) (Janke *et al.*, 1989; Zhu *et al.*, 1994; An *et al.*, 2000). Identification of porcine EPEC (PEPEC) is very difficult and veterinary diagnostic laboratories do not routinely seek to identify this pathotype of *E. coli*. Where investigations have been carried out, EPEC appears to be involved in about 6% of cases of PWD (Fairbrother, 1999). In a recent study, a few *eae*-positive *E. coli* belonging to O groups 45, 26, and 116 were identified among isolates from weaned pigs with diarrhea in Denmark (Frydendahl, 2002). The *eae* (*E. coli* AE) gene is a marker for PEPEC, but some *eae*-positive porcine *E. coli* isolates may be non-pathogenic. PEPEC of O group 45 have been clearly shown to possess genes of the locus of enterocyte effacement (LEE), a locus that is well established as conferring the ability to cause AE lesions (Helie *et al.*, 1991; Zhu *et al.*, 1994; An *et al.*, 2000). PEPEC isolates lack the *bfpA* gene that is required for production of bundle-forming pili (An *et al.*, 2000), making them similar to human atypical EPEC (Nataro and Kaper, 1998).

Fimbriae associated with ETEC in PWD

Fimbriae-designated F18 and F4 (K88) are the types that are commonly found on ETEC from PWD in pigs. The designation F4 will be subsequently used in this review. Genes for F4 and F18 were identified in 92.7% of all ETEC from PWD (Frydendahl, 2002). F18 fimbriae are typically associated with diarrhea of weaned pigs, whereas F4 fimbriae are associated with diarrhea in nursing pigs as well as in weaned pigs. Recently, the gene for an afimbrial adhesin called AIDA (adhesin involved in diffuse adherence) has been detected in *E. coli* from weaned pigs with PWD and ED (Niewerth *et al.*, 2001; Fekete *et al.*, 2002; Mainil *et al.*, 2002; Ha *et al.*, 2003; Ngeleka *et al.*, 2003). AIDA may be present as the only known adhesin or it may be present along with F18 fimbriae, and has been reported to play a role in colonization of certain ETECs in PWD (Ngeleka *et al.*, 2003). Another surface protein called Paa (porcine AE associated) has also been proposed as a potential adhesin of both PEPEC and ETEC in PWD (Batisson *et al.*, 2003).

F18 fimbriae

F18 fimbriae are long flexible appendages that show a characteristic zigzag pattern (Nagy *et al.*, 1997) and occur as two antigenic variants, F18ab and F18ac. The designation F18 is relatively recent (Salajka *et al.*, 1992), and prior to 1995 fimbriae of this type were designated F107 (now F18ab), 2134P (now F18ac) or 8813 (now F18ac) (Imberechts *et al.*, 1992, 1994; Rippinger *et al.*, 1995). F18ab fimbriae are usually found on STEC, ETEC, or ETEC/STEC and are poorly expressed *in vitro*, whereas F18ac fimbriae are usually found on ETEC and are more readily expressed *in vitro* (Wittig *et al.*, 1995; Nagy *et al.*, 1997). F18-positive ETEC strains most frequently produce heat-stable enterotoxins STa and STb, with or without Stx2e, and infrequently produce heat-labile enterotoxin (LT) as well (Rippinger *et al.*, 1995; Francis, 2002). Genes that encode the F18ab fimbriae have been reported to be carried on plasmids that also carry genes for the AIDA in STEC strains and vary from 42 to 98 MDa (Fekete *et al.*, 2002; Mainil *et al.*, 2002), whereas the genes that encode the F18ac fimbriae are found on 98 MDa plasmids (Fekete *et al.*, 2002).

Prior to detection of F18 fimbriae, strains of ETEC that possessed them were referred to as 4P-, because they lacked F4, F5 (K99), F6 (987P), and F41 pili (Nagy *et al.*, 1992). The F18 fimbriae are expressed at 37 °C but not at 18 °C and mediate adherence to the intestinal epithelium of pigs older than 3 weeks of age but not to the intestinal epithelium of younger pigs.

Five genes have been identified as members of the operon that encodes F18 fimbriae: *fedA* (major pilus subunit), *fedB* (usher), *fedC* (chaperone), *fedE* (minor protein of unknown function), and *fedF* (adhesin) (Imberechts *et al.*, 1996; Smeds *et al.*, 2001, 2003). The *fedF* gene is highly conserved among F18-positive *E. coli*, and FedF or its binding region has been proposed as a potential vaccine candidate (Smeds *et al.*, 2003). F18ab and F18ac may be differentiated by molecular genetic methods (Bosworth *et al.*, 1998) or by serological methods (Nagy *et al.*, 1997).

The frequency of association of F18 fimbriae with PWD varies over time and location. A thorough recent study by Frydendahl (2002) showed that 27% of 173 ETEC from PWD in Denmark were positive by PCR for the *fedA* gene, whereas 89% of the isolates from ED were positive. In North Carolina, F18-positive ETEC constituted 53% of 175 ETEC in PWD in 2000 (Post *et al.*, 2000), whereas in Ontario, Canada, F18-positive ETEC accounted for PWD on only 7% of 28 farms that were investigated in 2000 (Amezcua *et al.*, 2002). In Poland, genes for F18 fimbriae were detected on 62% of strains from diarrhea and 84% of strains from ED (Osek *et al.*, 1999).

Some pigs lack the receptor for the F18 fimbriae and are therefore resistant to colonization by F18-positive ETEC (Frydendahl *et al.*, 2003). A simple PCR test is capable of identifying whether pigs lack or possess the F18 receptor (see the section Breeding of resistant pigs).

F4 (K88) fimbriae

F4 are flexible fimbriae that occur as F4ab, F4ac, or F4ad variants, but the F4ac variant is by far the most common type that is seen. The 'a' antigenic region is conserved and a second antigenic region is variable and designated 'b', 'c', or 'd'. F4ab used to be detected at relatively high frequency and F4ad was reported to appear in Europe in 1973 (Guinee and Jansen, 1979), but F4ac is now the dominant type, worldwide. For example, Choi and Chae (1999) examined 44 F4-positive *E. coli* isolates from pigs with diarrhea for the presence of genes for F4ab, F4ac, and F4ad fimbriae. They found that 96% carried the F4ac fimbrial genes and 4% carried the F4ab fimbrial genes. Similarly, Alexa *et al.* (2001) used PCR to show that the F4ac variant was present in 98% of 237 F4-positive porcine ETEC from PWD; F4ab was present in 0.8%, and F4ad in 1.3%. All F4 antigenic types bind carbohydrates on glycoconjugates present on intestinal epithelial cells, intestinal mucus, or red blood cells (Erickson *et al.*, 1994; Grange *et al.*, 2002). The three varieties represent not only antigenic variants but also exhibit differences in binding specificity as some pigs are susceptible to all three types, some are susceptible to two types (ab and ac or ab and ad), and some are susceptible to a single type (ad or ab) and some are resistant to binding by all three types (Francis *et al.*, 1999).

The F4 fimbriae are encoded by genes on large non-conjugative plasmids that frequently also carry genes for raffinose fermentation (reviewed by van den Broeck *et al.*, 2000). The genes that encode F4ab and F4ac have been analyzed and shown to be organized into an operon of 10 genes that differ between the variants in the *faeG* gene that encodes the adhesin. In contrast to the F18 fimbriae, in which the adhesin is a minor protein distinct from the major fimbrial subunit, FaeG is both the major fimbrial subunit and the adhesin. The functions of all the genes have not been determined but genes that play roles in initiation of fimbrial biosynthesis, as usher, as chaperone, and as minor structural components of the fimbrial shaft have been identified. Interestingly, incubation of F4-positive *E. coli* with anti-F4 antibody results in loss of the F4 plasmid under conditions in which the F4 antigen is expressed (Nagy *et al.*, 1986). It is likely that this occurs because binding of antibody to the F4 fimbriae impairs growth or accelerates removal of clumps that are formed, resulting in a selective advantage for those bacteria in which there is spontaneous loss of the plasmid.

A number of investigators have examined regulation of expression of the F4 operon (Huisman and de Graaf, 1995; Huisman *et al.*, 1996; Mol and Oudega, 1996). The genes are temperature-regulated and are expressed at 37 °C but not at 18 °C, a feature consistent with a need for these fimbriae when the bacteria are in the intestine of their host but not in the environment. Expression of F4 fimbriae is negatively regulated globally by the leucine responsive regulatory protein (Lrp) and locally by the FaeA protein. Co-operative binding of Lrp and FaeA to the regulatory region of the *fae* operon occurs or fails to occur depending on the methylation status of GATC sites that are present (Huisman and de Graaf, 1995; Huisman *et al.*, 1996).

AIDA

The AIDA, first described in association with certain human diarrheagenic *E. coli*, is an autotransporter protein that is active in its glycosylated form on the bacterial surface (Benz and Schmidt, 2001; Sherlock *et al.*, 2004). The *aidA* gene that encodes AIDA therefore requires the adjacent gene *aah* (autotransporter adhesion heptosyltransferase), whose product adds heptose residues to the AIDA protein. AIDA mediates adherence to a variety of surfaces and promotes bacteria-to-bacteria adherence and biofilm formation (Sherlock *et al.*, 2004). In 2001, Niewerth and co-workers determined that the *aidA* and *aah* genes were present in 26% of a collection of 104 *E. coli* from ED and PWD but absent from *E. coli* of bovine, equine, sheep, and rabbit origins. The genes were shown to be located on a large plasmid and were most prevalent in strains that were positive for the *fedA* and *stx2e* genes. Recently, AIDA has been associated with ETEC recovered from weaned pigs with diarrhea and there is evidence that it plays a role in colonization of the intestine of pigs (Ha *et al.*, 2003; Ngeleka *et al.*, 2003). An in-frame deletion mutation in the *aidA* gene of an AIDA-positive ETEC resulted in a loss of ability to colonize the intestine and induce disease (M. Ngeleka, personal communication).

Paa

The gene encoding the porcine AE-associated (Paa) protein was first identified in porcine enteropathogenic *E. coli* (PEPEC) as a gene whose inactivation led to a loss of AE activity (An *et al.*, 1999). The Paa protein in the PEPEC strain was 100% identical in amino acid sequence with Paa of enterohemorrhagic *E. coli* O157:H7. The *paa* gene has recently been found in ETEC of O149:H10 serotype (Fontaine *et al.*, 2002; Noamani *et al.*, 2003) and has been localized to a virulence region on a plasmid that encodes both drug resistance and enterotoxin STa (Boerlin and Gyles, 2004). Paa has been demonstrated to be necessary for development of the AE lesion in PEPEC strains of O45 group and it has been suggested to be a surface-exposed protein that may play a role as an adhesin (Batisson *et al.*, 2003). The role, if any, of Paa in pathogenesis of porcine ETEC is not known.

Other adhesins

Other adhesins that are implicated in ETEC diarrhea in pre-weaned pigs are sometimes found, independently of or along with F18 or F4, on ETEC from PWD. For example, Kwon *et al.* (2002) found that genes for F5 (K99), F6 (987P), and F41 were present in 4, 10, and 2%, respectively, of ETEC from weaned pigs with diarrhea in Korea. These fimbriae are usually associated with ETEC from neonatal pigs and it is not surprising that they are occasionally detected on ETEC from PWD, as the age of weaning has been reduced substantially over the years.

Enterotoxins of ETEC from PWD

Heat-labile enterotoxin (LT)

The *E. coli* LT is an 84 kDa A:B_5 protein structure, in which a single enzymatic A subunit is non-covalently associated with a pentamer of B subunits that bind the toxin to its receptor (de Haan and Hirst, 2004). The A subunit consists of an ADP–ribosyl transferase (A1) fragment linked by a disulfide bond to an A2 fragment, which passes through a central pore created by a doughnut-like arrangement of the B subunits. The A and B subunits of LT are synthesized in the cytoplasm, transported through the inner membrane, and assembled into holotoxin in the periplasm. Toxin may be released into the membrane vesicles, following its association with lipopolysaccharide (LPS). LT is highly related to cholera toxin (CT), with approximately 77% identity at the nucleotide level (there are slight differences in sequence between LT from human origin *E. coli* and LT from porcine origin *E. coli*). Both toxins bind GM1 ganglioside, but LT can also bind to other receptors with a terminal galactose.

LT binds to receptors on the surface of intestinal epithelial cells. It binds with very high affinity to GM1 ganglioside (Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1ceramide) with each B subunit binding one GM1 ganglioside molecule. Although LT can bind to other gangliosides and to N-acetyl lactosamine residues on glycoprotein receptors, it is GM1 that is critical for its activities (de Haan and Hirst, 2004). LT is internalized by receptor-mediated endocytosis, undergoes retrograde transport through the endoplasmic reticulum, and the A1 fragment is released into the cytosol. The A1 fragment moves to the cytoplasmic face of the basolateral membrane and transfers the ADP–ribose moiety from nicotinamide adenine dinucleotide (NAD) to the G_s protein that regulates the catalytic activity of adenylate cyclase. The G_s protein is part of a complex consisting of a hormone stimulatory receptor (Rs), regulatory G_s protein, and adenylate cyclase (C) in the basolateral membrane. Stimulation and inhibition of adenylate cyclase activity are effected by hormones that interact with stimulatory and inhibitory receptors, respectively. Activation occurs when GTP is bound to G_s and inactivation occurs through the conversion of GTP to GDP by the intrinsic GTPase activity of G_s. ADP ribosylation inhibits the GTPase activity, so that GTP remains bound and adenylate cyclase is continuously activated (Moss and Richardson, 1978). The end result is an excessive level of cyclic adenosine monophosphate (cAMP), as ATP is converted by adenylate cyclase to cAMP.

The name LT is used synonymously with LTI, a term developed to recognize a second type of LT, called LTII (Holmes *et al.*, 1986). LT-IIa and LT-IIb are antigenic variants of LT-II (Guth *et al.*, 1986). LTII is antigenically distinct from LT-I, binds ganglioside GD1a or GD1b, lacks enterotoxic activity, and is not associated with diseases in animals (Holmes *et al.*, 1986). The differences between LTI and LTII are largely due to differences in their B subunits.

Functions other than enterotoxicity have been ascribed to LT. Horstman *et al.* (2004) suggested that because LT binds to LPS and remains associated with the bacterial surface, it may act as an adhesin that binds the bacteria to GM1 ganglioside on the intestinal epithelial cell surface. Interestingly, Berberov *et al.* (2004) have recently shown that elimination of the genes for LT was associated with not only a reduction in severity of diarrhea but also a reduction in colonization of the intestine of gnotobiotic pigs. These findings strongly suggest that LT may play a role as an adhesin. There is also a clear evidence that LT can be secreted by some strains of ETEC, and a type II protein secretion pathway that is highly related in homology to a pathway that secretes CT in *Vibrio cholerae* has been reported in human ETEC strain H10407 (Tauschek *et al.*, 2002). There have been no reports of such a pathway in porcine ETEC. Previously, the PAI-encoded gene *leoA* (labile enterotoxin output) was shown to be required for maximal transport of LT from the periplasm of human ETEC strain H10407 (Fleckenstein *et al.*, 2000).

LT is a potent modulator of the immune system and is being actively investigated for use as a dermal patch vaccine and as a mucosal adjuvant in humans. Interestingly, the B subunit of LT (LTB) by itself has been shown to have immunomodulatory properties as it can bind GM1 ganglioside and modulate immune function, through signaling events (Salmond *et al.*, 2002). Activities that result from these events include apoptosis of CD8-positive T cells, activation of B cells, and alteration of cytokine secretion by monocytes. Mixing of antigens with LTB prevents the development of oral tolerance following oral administration (Williams *et al.*, 1999). The adjuvant properties of LTB were effectively demonstrated by Francis and Willgohs (1991), who demonstrated that there was superior protection against ETEC diarrhea in weaned pigs by a live *E. coli* vaccine that had the genes for LTB and K88ac compared with an isogenic strain that

lacked the gene for LTB. The strain that had LTB alone was ineffective in eliciting protective immunity.

Typically, LT+ strains also produce STb as both genes are frequently on the same plasmid (Broes *et al.*, 1988; Berberov *et al.*, 2004). For example, of 111 LT+ ETEC examined by Frydendahl (2002), 110 also possessed the gene for STb.

Heat-stable enterotoxin STa

Heat-stable enterotoxin, Sta, is a low molecular weight (approximately 2 kDa) peptide that consists of 18 or 19 amino acids with three disulfide bonds. STa is water and methanol soluble; resists boiling for 15 min, digestion by proteolytic enzymes, and exposure to acid; and is inactivated by agents that destroy disulfide bonds. The type of STa produced by porcine ETEC is made up of 18 amino acids and is called STaP.

STa, a structural analog of the hormone guanylin, is produced in the intestine by some ETEC and binds guanylyl cyclase C (GC-C), a protein that spans the membrane, having an extracellular binding domain and intracellular protein kinase and catalytic domains. Binding of STa to GC-C results in activation of guanylate cyclase and increased levels of cyclic guanosine monophosphate (cGMP) inside enterocytes.

The *estA* gene which encodes STa is a part of transposon Tn*1681* and is found on plasmids of a wide range of molecular weights. Fekete *et al.* (2003) reported their detection of the *estA* gene linked to the *estB* gene on a newly discovered 40-kb pathogenicity island (PAI) in a plasmid in F18-positive ETEC and ETEC/STEC strains. The *estA* and *estB* genes were found in close proximity to each other and were part of an approximately 10-kb 'toxin-specific locus'. The plasmid also encoded tetracycline resistance. The researchers demonstrated that the PAI was widespread, being present in porcine ETEC isolated in Hungary, Austria, and the USA. Recently, the estA gene has been associated with the *tetA* gene for tetracycline resistance on certain F-like plasmids of O149 ETEC (Boerlin and Gyles, 2004).

STa appears to be particularly associated with ETEC that cause disease in neonatal animals. Thus bovine ETEC and a subset of ETEC that affect neonatal pigs typically possess the F5 fimbrial antigen and produce STa as the only enterotoxin. Nonetheless, STa is also produced by some ETEC implicated in PWD in pigs, but rarely as the sole enterotoxin.

EAST1

Enteroaggregative *E. coli* heat-stable enterotoxin I (EAST1) is a low molecular weight enterotoxin, which belongs to the STa family of enterotoxins. It is of interest that whereas STa requires three disulfide linkages for full biological activity, EAST1 and guanylin both possess only four cysteine residues.

The EASTI toxin was discovered as a product of certain human diarrheagenic *E. coli*, but the *astA* gene that encodes EAST1 has subsequently been shown to be widespread among porcine ETEC (Yamamoto and Nakazawa, 1997; Choi *et al.*, 2001; Frydendahl, 2002; Noamani *et al.*, 2003; Osek, 2003). Several of these studies have shown that this gene is most commonly detected among F4-positive ETEC, especially of O group 149. F4+ LT+ EAST1 is a common combination in porcine ETEC (Yamamoto and Nakazawa, 1997). However, the gene for EAST1 was detected in several virotypes of F18-positive ETEC: 21/45 (47%) F18+ ETEC, 14/20 (70%) of ETEC/STEC, but not in 0/15 Stx2e-positive, enterotoxin-negative isolates (Frydendahl, 2002).

The *astA* gene is on a large plasmid of variable size (58 kb in an O141 and 85 kb in an O149 ETEC) and has more than 98% homology with the nucleotide sequence of the corresponding gene from human isolates (Yamamoto and Nakazawa, 1997; Berberov *et al.*, 2004). The *astA* gene has also been reported in EHEC O157:H7 (in which it may be inactive), in a high percentage of human ETEC and EPEC, and in *E. coli* from children without diarrhea (Savarino *et al.*, 1996).

Interestingly, purified EAST1 did not show biological activity in the suckling mouse assay that detects STa activity (Menard *et al.*, 2004), but was enterotoxic as measured by electrogenic changes in Ussing chambers containing rabbit ileum and was associated with increased cGMP levels in rabbit ileum (Savarino *et al.*, 1993). A strong association between diarrhea and possession of the gene for EAST1 in human atypical EPEC prompted Dulguer *et al.* (2003) to suggest that this toxin may be a virulence marker that could be used in detection of atypical EPEC. It would be interesting to determine whether PEPEC carry the gene for EAST1 enterotoxin.

Heat-stable enterotoxin STb

STb is a 48-amino acid peptide of about 5 kDa, which has four cysteine residues involved in two disulfide bridges and is heat-stable but susceptible to degradation by proteolytic enzymes. This enterotoxin is associated almost exclusively with porcine ETEC but has occasionally been detected in ETEC of human origin. The human isolates may represent infection of people with porcine strains. The mechanism by which STb causes accumulation of fluid in the intestine is not known but it appears to involve elevation of the levels of prostaglandin E2 in the intestine (see reviews by Dubreuil, 1997; Menard and Dubreuil, 2002).

The gene for STb is most frequently detected among enterotoxin genes in ETEC recovered from PWD in pigs (Moon *et al.*, 1986). This is not surprising as the gene for STb is usually present on a plasmid that encodes LT in

LT+ F4+ ETEC and is also common in F18+ ETEC, most of which also produce STa (Francis, 2002). Interestingly, in the study by Moon *et al.* (1986), 33% of the ETEC that had been isolated from older pigs had the gene for STb (*estB*) as the only enterotoxin gene, whereas a later study by Post *et al.* (2000) detected six of 175 ETEC from PWD in pigs as being of the STb type only, and a more recent report by Francis (2002) had no isolates that were STb only (Francis, 2002). These findings may indicate changes over time. As noted in the section on STa, the gene for STb was part of a 40-kb PAI in F18-positive porcine ETEC (Fekete *et al.*, 2003). Interestingly, in that study, the flanking region for STb in F18-positive ETEC was shown to be different from the flanking region in F4-positive ETEC, in which *estB* is a part of transposon Tn*4521*.

Serogroups and serotypes of ETEC in PWD

Specific serogroups of ETEC are often associated with a particular set of virulence genes, with greater variation among the toxin-encoding genes than among the fimbrial genes. Different clones within a serogroup may have evolved by acquiring different virulence genes, resulting in clonal variation associated with a particular region or country. For example, ETEC of serogroup O139 is associated worldwide with the F18ab fimbriae. However, strains of this serogroup in Australia typically cause PWD, whereas those from Europe typically cause ED.

The predominant serogroup of *E. coli* associated with PWD in pigs worldwide is O149. This serogroup was first officially designated by Orskov *et al.* in 1969 and the serotype was called O149:K91:F4ac:H10, but the K91 was later dropped when it was recognized that these organisms do not produce a capsule. O149 ETEC with H:NM (non-motile) and H19 have also been reported (Yamamoto and Nakazawa, 1997). Recently an O149 ETEC with the H43 antigen was reported (Noamani *et al.*, 2003).

Prior to 1964, the dominant serogroups in pigs with PWD were O138, 139, and 141 (reviewed by Sojka, 1965; Dam and Knox, 1974). Since 1964, serogroup O149 has been reported as the major O group in pigs with diarrhea in Ireland (Sweeney, 1970), Switzerland (Sarrazin *et al.*, 2000), USA (Glantz and Kradel, 1971), Denmark (Dam and Knox, 1974), Hungary (Nagy *et al.*, 1990), Spain (Blanco *et al.*, 1997), South Africa (Henton and Englebrecht, 1997), Germany (Wittig and Fabricius, 1992; Sarrazin *et al.*, 2000), and many other countries. O149 ETECs were first detected in Denmark in 1966 and are still dominant today (Frydendahl, 2002). Dam and Knox (1974) were able to trace the spread of O149 ETEC in Denmark, from its first appearance on four farms in 1966 to its rapid spread over the entire country by 1969. In 1971, O149 ETEC constituted 82.6% of the hemolytic *E. coli* recovered from pigs with diarrhea. Initially this O group was associated primarily with neonatal diarrhea but it quickly became associated with weaned pigs as well.

In the mid-1970s, Danish researchers noted that O149 strains implicated in PWD lacked the F4 antigen, whereas those recovered from neonatal pigs with diarrhea were F4-positive (Riising *et al.*, 1975). Frydendahl (2002) suggests that the F4-negative O149 isolates may have been F18-positive and noted that almost 10% of the O149 isolates he detected were F18-positive. Since the F18 fimbriae were not known in the 1970s, it is possible that the Danish strains possessed this type of fimbriae or some other fimbrial or afimbrial adhesins.

Certain O149 ETEC show a delayed positive phenotype for urease (Larsen, 1976; Noamani *et al.*, 2003) and the operon that encodes this activity is almost identical to one found in EHEC O157:H7 (Gyles and Parreira, unpublished work). Furthermore, this operon (as in EHEC O157) is a part of an O island which includes genes for tellurite resistance and two adhesin homologs (AIDA and IrgA). ETECs of O group 149 are only rarely *stx*-positive (Gannon *et al.*, 1988; Nagy *et al.*, 1990). Typically, members of this O group possess genes for EAST1, LT and STb enterotoxins, but they may also produce STa (Blanco *et al.*, 1997; Frydendahl, 2002; Noamani *et al.*, 2003). Rarely, they have been reported to lack the genes for STb or for both STb and STa (Yamamoto and Nakazawa, 1997).

Other serogroups that are frequently implicated in PWD of pigs are O8, O138, and O141 (Sojka, 1965; Salajka *et al.*, 1992; Nagy *et al.*, 1997; Nagy and Fekete, 1999; Francis, 2002; Frydendahl, 2002) (Table 1). There are many other serogroups that are less frequently reported in

Table 1. O serogroups most frequently implicated as enterotoxigenic *E. coli* that cause postweaning diarrhea in pigs

O serogroup	Associated fimbrial antigens	Associated H antigens	Comments
8	F4ab(K88ab), F4ac(K88ac)	H19	Less common than in earlier years
138	F18, F4ac*	H−, H4	Sometimes Stx2e+
139	F18	H1	Most commonly associated with ED
141	F18, F4ab, F4ac	H4	Sometimes Stx2e+
147	F4ac, F18	H6, H19	Sometimes Stx2e+
149	F4ac, F18*	H10, H19, H43, H−	Occasionally Stx2e+
157	F4ac	H19, H43	Occasionally Stx2e+

Sources: Nagy *et al.*, 1990; Harel *et al.*, 1991; Salajka *et al.*, 1992; Francis, 2002; Frydendahl, 2002; Prager *et al.*, 2004.
*Infrequently.

association with PWD. The genes for fimbriae and for enterotoxins in ETEC from PWD in pigs are plasmid-encoded, allowing for rapid evolution of virulence types (virotypes). However, with a few exceptions, there is little evidence that this rapid evolution has occurred to create a large number of serogroups of porcine ETEC that cause PWD.

Strains of a large number of serogroups have been isolated from pigs with PWD but evidence that the strains were likely ETEC (i.e. possessed functional genes for fimbriae and enterotoxins) is lacking for all but those serogroups that are well established as porcine ETEC. Among 219 isolates from PWD or ED, Frydendahl (2002) detected genes for both fimbriae and enterotoxins in 148 isolates that belonged to O groups 138, 141, 147, 149 and 157 and in 18 isolates that belonged to O groups 32, 59, 121 and 175 or were O-rough or non-typeable. 14.6% of the isolates had no genes for F4, F5, F6, F18 or F41 fimbriae; these included 10% that had no genes for either enterotoxins or fimbriae. Chen *et al.* (2004) reported that there were 45 O groups among 215 *E. coli* recovered from pigs with PWD in China. The dominant O group was 107. A total of 107 of the isolates did not possess any of the five fimbrial types commonly associated with porcine ETEC (F4, F5, F6, F41, and F18).

Virulence gene profiles (virotypes) of ETEC in PWD

The virotypes of *E. coli* associated with PWD usually have either F4 or F18 as fimbrial adhesin, but some *E. coli* recovered from pigs with PWD and analyzed by The *Escherichia coli* Laboratory in St. Hyacinthe, Quebec, and by others (e.g. Frydendahl, 2002) lack F4 and F18 fimbriae. It is not known whether these are strains that have lost genes for these fimbriae or have never possessed these genes. Nor is it known whether these strains produce other adhesins that enable them to colonize and cause disease or whether they are incapable of causing disease and represent isolates from pigs whose diarrhea was caused by another agent. These strains are worthy of further investigation to determine whether they are capable of inducing diarrhea in pigs and to identify adhesins that may mediate attachment to the intestine. It is noteworthy that some of these strains of the STb or STb:EAST1 virotypes from neonatal or weaned pigs may also be AIDA-positive and do induce diarrhea, at least in experimental infection of neonatal pigs (Ngeleka *et al.*, 2003).

An interesting evolution in the virotype of O149 isolates has occurred in central Canada. In retrospective studies, it was observed that O149:F4 strains isolated before 1990 were predominantly of virotype LT:STb:EAST1 (Fairbrother *et al.*, 2000; Fontaine *et al.*, 2002; Noamani *et al.*, 2003). Since 1990, a new virotype, LT:STa:STb:EAST1, has appeared and is now either as prevalent as (Fairbrother *et al.*, 2000; Fontaine *et al.*, 2002) or has almost replaced (Noamani *et al.*, 2003) the old virotype. This trend may not be universal, as most O149 strains isolated from 4- to 6-week-old weaned pigs with diarrhea in Poland (Osek, 2003) were of the LT:STb:EAST1 virotype, and the new virotype did not appear to be prevalent in a 2001–2002 South Dakota study (Francis, 2002).

The predominant virotypes vary from country to country and over time. In a study of Danish isolates from 1999–2000, genes for the fimbrial adhesins F4 and F18 were detected in 45 and 39%, respectively, of isolates from pigs with PWD or ED in Denmark (Frydendahl, 2002). All the F4-positive isolates that were examined were O149, two-thirds of which were virotype LT:STb:EAST1:F4 and one-third possessed the gene for STa as well. The F18 isolates belonged to several O groups and were more heterogeneous, the most common serotype/virotype profiles being O149:LT:STb:EAST1:F18, O138:STa:STb:F18, O138:LT:STb:EAST1:Stx2e:F18, and O139:Stx2e:F18. In South Dakota, USA, in 2001–2002, more than half of the ETEC isolated from pigs with diarrhea were of virotype LT:STb:F4, STa:STb:F18, or STa:STb:Stx2e:F18, F4-positive isolates being slightly more prevalent than F18-positive ones (Francis, 2002). In Quebec, in a 1992–1993 study of pigs with diarrhea in the period up to 14 days following weaning on farms with problems of PWD, F18-positive isolates were more prevalent, only 9% of ETEC isolates being F4-positive, whereas 11% of ETEC isolates were F18-positive (Fairbrother *et al.*, 1994). On the other hand, F4 was the predominant fimbrial adhesin in ETEC from pigs with PWD in Quebec, both in diagnostic cases from 1994 to 1998 (Fairbrother *et al.*, 2000) and in a recent study of 17 farms with diarrhea in at least 15% of pigs in the first 3 weeks of postweaning (Fairbrother, 2002, personal observations). In both of these latter studies, about half of the ETEC isolates produced F4 fimbriae. All F4-positive isolates were O149, most being of virotype LT:STb:EAST1:F4 in diagnostic cases of 1994, whereas half were of virotype LT:STb:EAST1:F4 and half were of virotype LT:STa:STb:EAST1:F4 in the later studies. Overall, about 2% of isolates were F18-positive and were mostly of virotype STa:STb. All other ETEC produced neither F4 nor F18, the most common virotypes being STa:STb and STb:EAST1:AIDA.

In Denmark, ETEC/STEC of serogroups O121, O138, O141, O147, and O-rough were associated solely with diarrhea, whereas STEC of serogroup O139 and O-rough that lacked genes for enterotoxins were the only types implicated in ED (Frydendahl, 2002). Blanco *et al.* (1997) found that O149:H10:F4 (LT+, STb+) was the dominant sero/virotype among porcine ETEC in Spain. Sarrazin *et al.* (2000) also found that O149 of virotype LT+, STb+ was dominant in Switzerland. Among F18+ isolates, ETEC/STEC were Stx2e:STa:STb or Stx2e:EAST1:LT:STb. Other F18+ virotypes include STb, EAST1, STa:STb, EAST1:STb, EAST1:LT:STb, and EAST1:STa:STb:LT. These

observations indicate a strong association among LT, STb, EAST1, and F4 and among STa, STb, and F18.

Clonality

Identification of clones of ETEC and ED isolates has proven to be useful in tracing the spread of these organisms from farm to farm (Aarestrup *et al*., 1997) and in providing insights into the evolution of these pathogens. Pulsed-field gel electrophoresis (PFGE) and multilocus enzyme electrophoresis (MLEE) are two of the more common methods for assessing relatedness of isolates.

Osek (2000) used PFGE of DNA from O138, O139, O141, and O149 ETEC that had been digested with *Not*I and *Xba*I restriction enzymes. The researcher found that PFGE patterns were highly serogroup related, but that relatedness within a serogroup varied, depending on the O group. Hampson *et al*. (1993) found that isolates of O groups 8 and 138 varied considerably by electrophoretic type (ET), whereas isolates of O141 and of O149 from unweaned pigs did not show much intra-serogroup variation. Nagy *et al*. (1999) also used MLEE to examine clonality in F18-positive strains of O serogroups X, 5, 21, 109, 138, 139, 141, 147, and 157 from diarrhea and ED in weaned pigs. They determined that several clones could exist within an O serogroup but that clones of the same O group tended to cluster closely. Interestingly, isolates of O serogroup 138 were found to cluster very closely. Osek (1999) used randomly amplified polymorphic DNA (RAPD) analysis to examine clonality within O149 ETEC and reported that there were three RAPD profiles among 72 isolates that were examined. Noamani and Gyles (unpublished work) found that O149:H10 ETEC with LT, STa, and STb genes had a distinctly different PFGE profile from O149:H43 ETEC that lacked the gene for STa. It is evident that there is some variation in findings associated with the use of different methodologies, but a consistent finding is that ETEC of the same O serogroup may be usefully separated into various clones that tend to cluster together.

Experimental reproduction of *E. coli* PWD

Only a small number of serotypes of ETEC have been used in experimental reproduction of disease. This is largely due to the difficulty in experimental reproduction of a disease similar to that seen in weaned pigs in the field. Many of the unsuccessful attempts go unreported. Cox *et al*. (1991) and Madec *et al*. (2000) have provided excellent reports of attempts to reproduce the disease experimentally. Cox and colleagues reported that they needed to infect pigs with the transmissible gastroenteritis virus prior to administration of an ETEC in order to produce a severe disease with dehydration and high mortality. There is some corroborating evidence from investigations of natural disease that certain viral infections may exacerbate disease due to ETEC in weaned pigs. Nakamine *et al*. (1998) noted that concurrent infection of weaned pigs with the porcine reproductive and respiratory syndrome PRRS virus and ETEC resulted in a severe disease characterized by septicemia and death following diarrhea of short duration. Recent studies have reported on the use of pre-treatment of weaned pigs with antibiotics prior to infection with ETEC (Verdonck *et al*., 2005). However, despite the antibiotic-pretreatment and a challenge of 10^{10} ETEC, no pigs developed diarrhea.

Madec and co-workers induced short-lived diarrhea in about 50% of 124 piglets that were challenged with ETEC. The average duration of diarrhea was 1.7 days. However, when these researchers used a high challenge inoculum (10^{12} colony forming units (cfu)), 10 of 16 pigs died. Interestingly, although the percentage of pigs that developed diarrhea was higher in F4-adhesive pigs compared with those that were adhesin-negative (56% compared with 34%), it is surprising that one-third of the adhesin-negative pigs developed diarrhea. None of the control pigs developed diarrhea.

A reproducible model of *E. coli* PWD is important, as the ease with which PCR may be applied has led to identification of many strains of serogroups that have not been implicated in disease but possess sequences of virulence genes. Furthermore, studies on pathogenesis, prevention, and treatment of the disease will be facilitated by such a model.

Pathogenesis of *E. coli* PWD

Colonization of the small intestine

E. coli causing PWD and/or ED enter the animal by ingestion and, when present in sufficient numbers, colonize the small intestine following bacterial attachment to receptors on the small intestinal epithelium or in the mucus coating the epithelium, by means of specific fimbrial adhesins. These bacteria then proliferate rapidly to attain massive numbers in the order of 10^9 cfu per gram of tissue in the mid-jejunum to the ileum. The degree of colonization determines whether or not disease results from infection. Fimbriae adhere to specific receptors on the cell membrane of intestinal epithelial cells and to specific receptors or non-specifically in the mucus coating the epithelium. ETEC producing fimbriae F5, F6, and F41 mostly colonize the posterior jejunum and ileum, whereas F4-positive ETEC tends to colonize the length of the jejunum and the ileum. F4 fimbriae mediate bacterial adherence to the intestinal epithelium throughout most of the small intestine of pigs of all ages. Hence, colonization of the intestinal mucosa by F4-positive ETEC occurs in both neonatal and postweaning pigs and may be observed in finisher pigs. Adherence due to F4 is species-specific, occurring mostly in pigs.

Certain pigs do not have receptors for the F4 adhesin on intestinal epithelial cells and are thus resistant to infection by F4-positive ETEC (Sellwood *et al.*, 1975). This genetic resistance to infection is inherited in a simple Mendelian way, and the allele for the receptor is dominant. Hence, three genotypes exist: ss (resistant), SS, and Ss (sensitive). Subsequent studies have demonstrated at least five pig phenotypes, based on susceptibility of brush borders of different pigs to adherence of isolates producing the different variants F4ab, F4ac, and F4ad (Bijlsma *et al.*, 1982; Hu *et al.*, 1993). An additional phenotype demonstrating binding only to F4ab isolates has also been identified (Baker *et al.*, 1997).

The genes for the F4 receptor were determined to be on chromosome 13 (Guérin *et al.*, 1993) and the loci encoding porcine intestinal receptors for F4ab and F4ac are closely linked on this chromosome (Edfors-Lilja *et al.*, 1995). The map position of this locus has now been refined to a region between the microsatellite markers Sw207 and Sw225 (Jorgensen *et al.*, 2003). Van den Broeck *et al.* (2000) described a receptor model (receptors *bcd*, *bc*, *b*, *d*) according to the six porcine phenotypes that can be distinguished with regard to the brush border adhesiveness to the three F4 serotypes (F4ab, F4ac, and F4ad), F4ac being the most prevalent variant isolated from affected pigs. The receptor *bcd* is proposed as a collection of glycoproteins (45–70 kDa). The *bc* receptor was initially identified by Erickson *et al.* (1992) as two glycoproteins (210 and 240 kDa) which were subsequently characterized as intestinal brush border mucin-type sialoglycoproteins (Erickson *et al.*, 1994). The *b* receptor was identified by Grange and Mouricout (1996) as a glycoprotein of 74 kDa, belonging to the transferrin family and associated with the brush-border membrane. The *d* receptor was later identified by Grange *et al.* (1999) as a brush-border glycosphingolipid with unknown molecular mass. *In situ* hybridization revealed that the loci for the F4ac and F4ab receptors (F4acR) are linked to the transferrin locus, mapped to the porcine chromosome 13 (Chowdhary *et al.*, 1993; Python *et al.*, 2002; Python, 2003). Animal challenge studies conducted by Francis *et al.* (1998) indicated that the sialoglycoproteins (i.e. the *bc* receptor) were likely the biologically relevant receptor for F4ab and F4ac.

The receptor for F18 is distinct from the receptor for F4. The receptor for F18 fimbriae is also controlled in a single locus, and the presence of a receptor is dominant over absence (Bertschinger *et al.*, 1993). The genes for the F18 receptor were located on chromosome 6 close to the locus for stress susceptibility. In a high proportion of the Swiss pig population, resistance to stress is linked to susceptibility to adhesion of *E. coli* with F18 fimbriae (Vögeli *et al.*, 1996). In view of the low prevalence of stress-susceptible pigs, the frequency of pigs with the F18 receptor would be predicted to be high. This has been confirmed in a number of studies. Genotypes susceptible and resistant to F18 adherence have been differentiated, and pigs with at least one copy of the dominant allele for receptor are susceptible to epithelial adherence *in vitro* and hence to intestinal colonization. Polymorphisms in the α(1,2)fucosyltransferase gene FUT1 have been closely linked with the locus controlling resistance and susceptibility to *E. coli* F18 adhesion and colonization in the small intestine (Meijerink *et al.*, 1997). It is highly probable that the enzyme FUT1, and particularly the amino acid at position 103, is involved in the adherence of F18-positive ETEC to small intestine epithelial cells (Meijerink *et al.*, 2000). Nevertheless, the receptor for F18 has not yet been fully characterized. Recently, it has been demonstrated in adherence inhibition studies using specific monoclonal antibodies that the F18 receptor contains the blood group antigen H-2 (α-fuc-(1-2)-β-Gal-(1-4)-GlcNAc) as major carbohydrate (Snoeck *et al.*, 2004a).

Fimbrial receptors are subject to modulation by feed lectins such as constituents of leguminous plants (Kelly *et al.*, 1994). It may be speculated that feed-induced changes of the receptor are involved in the reduced susceptibility to colonization by F18-positive *E. coli* in the first days after weaning (Bertschinger *et al.*, 1993). Endogenous as well as orally administered proteases may reduce the receptor activity for F4 fimbriae (Mynott *et al.*, 1996). Receptors for F4 are fully expressed from birth to adult age, whereas the F18 receptor is not yet fully expressed by piglets under about 20 days of age (Nagy *et al.*, 1992). Hence, *E. coli* with F18 fimbriae do not cause disease in neonatal pigs. Constant expression of receptors may underlie the earlier appearance after weaning of *E. coli* strains with F4 in herds where F4- and F18-positive strains are endemic.

Loss of milk antibodies appears to contribute significantly to the susceptibility of pigs to *E. coli* enteric infections in the postweaning period (Deprez *et al.*, 1986; Sarmiento *et al.*, 1988). A variety of viruses infect the porcine intestine and may thereby change the bacterial environment. Dual infection of pigs with rotavirus and with an ETEC strain without F4 results in a more severe diarrhea than inoculation with either agent alone (Lecce *et al.*, 1982). The investigators concluded that viral damage of the epithelium favors colonization by *E. coli*.

Porcine AEEC attach to the intestinal mucosa and cause lesions similar to those observed for EPEC isolated from human infantile diarrhea (Hélie *et al.*, 1991). They attach intimately to the intestinal epithelial cell membrane by means of a bacterial outer-membrane protein termed 'intimin' or 'EPEC attaching and effacing factor' (Eae), efface the microvilli, and invade the epithelial cells (Zhu *et al.*, 1994). Experimental infection of gnotobiotic pigs allows reproduction of the lesions. Several predisposing factors, such as a weaner diet containing soybean and field peas or PRRS virus infection, may enhance bacterial colonization and development of AE lesions (Neef *et al.*, 1994; Fairbrother, personal observation).

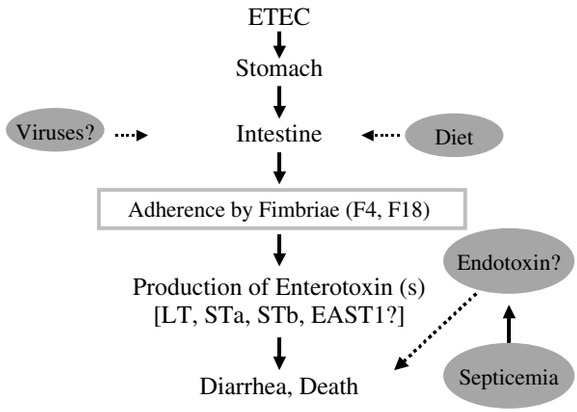

Fig. 1. Overview of steps in development of *E. coli* postweaning diarrhea in pigs. Death is generally due to dehydration. However, endotoxin may be absorbed from the intestine or may be released from bacteria that are in the bloodstream, and septicemia and/or endotoxemia may lead to death in the absence of severe dehydration.

Mechanisms by which enterotoxins induce diarrhea

Pigs with *E. coli* PWD typically have watery diarrhea that lasts from 1 to 5 days, but some pigs die suddenly without diarrhea having been observed. This latter group of pigs usually have an accumulation of fluid in the intestine and invasion of the blood and tissues. The steps in development of *E. coli* postweaning diarrhea in pigs are outlined in Fig. 1.

Diarrhea involves a significant increase in excreted fluid volume, either due to a failure of the bowel to reabsorb or absorb fluid or due to a great increase in fluid secreted into the bowel. Diarrhea due to ETEC is attributable to the action of one or more enterotoxins produced by the bacteria that have colonized the small intestine. LT, as noted above, leads to an accumulation of cAMP in the enterocyte. The steps leading from elevated levels of intracellular cAMP to hypersecretion are not very clear. However, cAMP-stimulated PKA (protein kinase A) phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR), thereby causing chloride secretion from the apical region of enterocytes (Thiagarajah and Verkman, 2003; de Haan and Hirst, 2004). Other c-AMP-mediated changes include activation of an apical chloride channel and a basolateral Na/K/2Cl co-transporter, stimulation of platelet-activating factor leading to synthesis and release of prostaglandin E2 (PGE_2), release of vasoactive intestinal peptide (VIP), and loosening of tight junctions (de Haan and Hirst, 2004). These activities all contribute to increased chloride secretion, reduced sodium absorption, and a concomitant massive loss of water into the intestinal lumen.

The role of the enteric nervous system in response of the intestine to LT has been investigated by a number of researchers. Mourad and Nassar (2000) found that a VIP antagonist reversed the secretory effects of both LT and STa in the rat jejunum. Possible mechanisms suggested by the researchers include a direct binding of LT to VIP-containing neurons, as occurs with CT, or release of an unidentified transmitter that activates the neuronal reflex. Following stimulation of secretomotor reflexes, VIP is released from intestinal enterochromaffin cells and acts on the mucosa to activate adenylate cyclase. Although 5-hydroxytryptamine (5-HT) is implicated in the action of CT, it appears that it is not involved in the action of LT (Mourad *et al.*, 1995).

STa causes excessive levels of cGMP in enterocytes. Signals resulting from cGMP accumulation lead to activation of CFTR and to increased secretion of Cl^- ions from crypt cells and inhibition of Na^+ and Cl^- absorption from cells at the tips of villi (Forte *et al.*, 1992). Fluid accumulation in the lumen of the intestine in response to STa is also known to be under neuroendocrine influence as described above. Because STa is known to act through a nitric oxide-dependent neuroendocrine pathway, Mourad and Nassar (2000) suggested that nitric oxide and VIP (which are known to have interactions) interact in promoting secretion by STa.

STb induces diarrhea by mechanisms that are not well understood but are independent of the elevation of cyclic nucleotides that characterizes the actions of LT and STa. Harville and Dreyfus (1995) have presented good evidence that STb acts through the secretagogues 5-HT and PGE2. Administration of STb to rat intestine resulted in elevation of the levels of 5-HT and PGE2 in a dose-dependent manner, and inhibitors of 5-HT and PGE2 resulted in a reduction of the fluid response to STb.

Prevention strategies

Much less is known about strategies for the treatment and control of PWD than about pathogenesis and treatment of neonatal colibacillosis. F4- and F18-positive ETEC strains responsible for these infections are not well controlled by prophylactic antibiotic treatments, mainly due to the emergence of antimicrobial resistance. There is growing concern about the increasing pool of drug-resistance genes in bacteria in the intestine of animals, and pressure is mounting to reduce the use of antibiotics in animal production (Shea, 2003). This has led to increasing interest in alternatives to antibiotics in agriculture and to a number of initiatives to evaluate vaccines, probiotics, prebiotics, bacteriophages, non-antibiotic chemicals, and management procedures that may minimize the use of antibiotics (Joerger, 2003).

Breeding of resistant pigs

Augmentation of the presence of both the F18 and F4 resistance loci in the pig population through breeding is

an attractive approach to prevent PWD. However, it will be important to avoid co-selection of unwanted traits closely linked with loci coding for the F4 and the F18 receptors. It cannot be predicted if additional types of adhesive fimbriae or new variants of known types will emerge which could bind to yet unidentified receptors. Availability of techniques for large-scale selection of resistant animals is lacking and will be the main challenge in the near future. The feasibility of breeding for disease resistance can be illustrated by the *E. coli* F18-associated PWD. A PCR-RFLP test detecting FUT1 M307 polymorphism, correlated with the gene controlling expression of the *E. coli* F18 receptor, could be a simple and inexpensive method for large-scale selection of animals (Meijerink *et al.*, 2000; Frydendahl *et al.*, 2003). This test could be employed as a predictor of susceptibility to *E. coli* F18-associated diarrhea. Frydendahl *et al.* (2003) confirmed the relationship between the *E. coli* F18 receptor genotypes and susceptibility to *E. coli*-associated PWD. The introduction of this molecular test in Switzerland in 1996 tripled the percentage of Large White resistant pigs in 5 years and reduced the proportion of susceptible pigs from 44 to 18% (Vögeli *et al.*, 2002).

Less is known about the genes associated with the expression of the F4 receptors and pig resistance. At present, only such onerous approaches for determination of the phenotype as challenge of pigs with an F4+ strain followed by treatment are available. Encouragingly, genotyping methods for the identification of pigs resistant to F4-positive ETEC have recently been proposed based on genetic polymorphisms, possibly in the porcine gene for MUC4 (Jorgensen *et al.*, 2004), in the region of chromosome 13 recently identified to carry the loci encoding the porcine intestinal receptors for F4ab and F4ac (Jorgensen *et al.*, 2003). On the other hand, the immunity aspect should be considered if breeding for resistance becomes available for F4-positive ETEC, since resistant sows (F4 receptor-negative sows) do not develop and transfer F4-specific antibodies in their colostrum and thus, heterozygous piglets are not passively protected from development of neonatal diarrhea due to these strains. However, on such farms F4+ *E. coli* may be less prevalent and be of minor problem due to the absence of F4R+ sows which maintain the F4+ *E. coli*.

Immunoprophylaxis: *mucosal immunity, live and subunit vaccines*

The fetus and newborn pig have the capacity to develop immune reactions against mucosal antigens and the response may be either tolerance or defense against the antigen (Rothkotter *et al.*, 2002). Weaned pigs can be protected passively (passive immunotherapy) or actively (active immunotherapy). Active intestinal mucosal immunization is needed for the protection of newly weaned pigs since they have been deprived of passive lactogenic immunity. Injectable vaccines, such as those administered to sows for the prevention of neonatal diarrhea, stimulate mostly systemic rather than mucosal immunity, giving rise to circulating antibodies which do not reach intestinal bacteria in high enough levels to be very effective (Van de Broeck *et al.*, 1999a). Such vaccines may even suppress the mucosal immune response following subsequent oral infection with a pathogenic *E. coli* (Bianchi *et al.*, 1996). Nevertheless, intramuscular immunization with F4 fimbriae induced a systemic IgA response similar to that resulting from an oral challenge with live F4-positive ETEC (Van den Broeck *et al.*, 1999a). Van der Stede *et al.* (2002) reported that intramuscular injections of low doses (0.1 mg) of F4 fimbriae are optimal for the induction of F4-specific IgA, as demonstrated by high levels of systemic F4-specific IgA and high numbers of F4-specific IgA antibody secreting cells in the local draining lymphoid tissues of immunized animals. Depending on the dose injected intramuscularly, F4 fimbriae would probably induce distinct patterns of cytokines, promoting different T-lymphocyte functions (Van der Stede *et al.*, 2002). Supplementation of intramuscular injection of F4 fimbriae with 1α,25-dihydroxyvitamin D3 enhanced the reduction of excretion of F4-positive *E. coli* following subsequent oral infection with a pathogenic *E. coli*, which was correlated with the presence of a secondary mucosal IgA response (Van der Stede *et al.*, 2003).

Vaccines for preventing PWD should activate the mucosal immune system and evoke antigen-specific IgA or IgM responses in order to induce a protective mucosal immunity (Bianchi *et al.*, 1996; Van den Broeck *et al.*, 1999a). In pigs, the small intestine is the major site of IgA and IgM production. The jejunal Peyer's patches were identified as the major site of induction of mucosal immune response to F4 fimbriae (Huyghebaert *et al.*, 2005). In mice, the only isotype secreted in the mucosa is IgA whereas in the pig, initially IgM and later IgA is the predominant isotype secreted in the intestine (Bianchi *et al.*, 1996). In pigs, IgM is found more mucosally rather than systemically, in contrast to the situation in other animal species, and probably has an important role in mucosal immunity (Bianchi *et al.*, 1999). In the lamina propria of 4-week-old pigs, the proportion of IgM-secreting cells is similar to that of IgA-secreting cells, and increases with the age of pigs. The shift from IgM to IgA as the predominant mucosal isotype occurs at about 12 weeks of age.

Several approaches for the control of *E. coli*-associated PWD are currently being investigated. Most of these control strategies are specific for the adhesin or O serogroup of the causative *E. coli*. Hence, an accurate diagnosis and identification of the adhesin type is essential to assure a more effective control of the diarrhea. Vaccination strategies include the oral immunization of pigs with live attenuated or live wild-type avirulent *E. coli*

strains carrying the fimbrial adhesins. Such vaccine strains may be administered to weaned piglets in the drinking water or to unweaned piglets by oral dosing at least 1 week prior to the expected onset of diarrhea, to allow intestinal colonization by these bacteria and induction of local intestinal antibodies which will block the adherence of the pathogenic *E. coli* and hence prevent the development of diarrhea. This approach appears to be effective for the control of both F4 and F18 *E. coli*-associated PWD. However, colonization of the intestinal tract by the vaccine strain and consequently by immunization may be inhibited by the presence of lactogenic immunity in the piglets.

A large-scale on-farm study in the USA has demonstrated a decreased mortality and reduced use of antimicrobials following oral administration of a live non-enterotoxigenic F4 *E. coli* strain to pigs immediately after weaning (Fuentes *et al.*, 2004). Bianchi *et al.* (1996) reported that only oral immunization with live F4-positive *E. coli* was able to prime for a secondary mucosal immune response against F4 upon secondary oral infection with an F4-positive ETEC challenge strain. Parenteral immunization with F4 fimbriae induced a state of suppression evidenced by the lack of a mucosal immune response on subsequent oral challenge with a live F4-positive ETEC. Use of levamisole as an immunomodulator to prime for oral vaccination of pigs with a live F4-positive avirulent *E. coli* resulted in stimulation of the intestinal immune system upon virulent challenge and reduction of PWD clinical signs, as compared to non-primed pigs (Bozic *et al.*, 2003). However, the F4-receptor status of pigs was not evaluated in these studies.

Pigs that have been colonized by an F18-positive ETEC are protected against subsequent colonization by a heterologous ETEC, which have only the F18 fimbrial antigen in common with the immunizing strain. However, the cross-protection between strains with fimbrial variants F18ab and F18ac may not be very high (Bertschinger *et al.*, 2000). The role of F18 fimbriae as protective antigens was illustrated by the need to immunize with an F18-fimbriated culture (Bertschinger *et al.*, 2000).

Current research is aimed at the strategy of oral administration of purified F4 fimbriae, instead of the whole bacteria, as a vaccine for the control of outbreaks of *E. coli*-associated PWD (Van den Broeck *et al.*, 1999a). Use of such oral subunit vaccines results in a specific mucosal immune response in the intestines and, in some cases, in a significant reduction in fecal excretion of the pathogenic F4-positive ETEC. Van den Broeck *et al.* (1999a) demonstrated that purified F4 is a powerful oral immunogen, inducing antibody-secreting cells, mainly of IgM isotype, in Peyer's patches, mediastinal lymph nodes, blood, and lamina propria. This induction was observed shortly after immunization. Furthermore, the immunization induced systemic F4-specific IgA and IgG responses comparable to those observed following oral infection with viable ETEC and intramuscular injection of F4 fimbriae, respectively. F4 receptors seem to be a prerequisite for an immune response following oral immunization (Van den Broeck *et al.*, 1999b). On the other hand, Bosi *et al.* (2004) observed that susceptible phenotype was not a determinant for the production of salivary IgA, which increased in both receptor-positive and -negative animals after challenge with a live F4-positive ETEC strain, whereas F4-specific IgA levels in the plasma increased only for receptor-positive pigs.

Despite the demonstration of induction of an immune response following oral immunization with purified F4 fimbriae, there have been few reports on the protective effect against colonization and disease associated with F4-positive ETEC. Van den Broeck *et al.* (1999b) reported that only unvaccinated F4-receptor-positive animals developed an immune response and excreted F4-positive ETEC after challenge, in contrast to a lack of observed postchallenge response in both receptor-positive and receptor-negative pigs orally vaccinated with F4 fimbriae and in unvaccinated receptor negative animals, indicating an inhibition of colonization and lack of restimulation and consequently suggesting a protective effect. On the other hand, unvaccinated receptor-positive animals did not show clinical signs and excreted only low numbers of ETEC (approximately 1×10^4 per g^{-1} of feces) after challenge, suggesting that other factors may have affected the colonization of the challenge strain in the trial. Generally, a level of 10^8 cfu of F4-ETEC is observed after challenge (Snoeck *et al.*, 2003; van der Stede *et al.*, 2003). According to Van den Broeck *et al.* (2002), low oral doses (2 mg) of F4 fimbriae would be sufficient to induce a protective mucosal immune response in receptor-positive pigs without a risk of provoking oral tolerance in receptor-negative pigs. Contrary to F4-associated infection, oral immunization of pigs with micro-encapsulated F18 fimbriae does not induce a protection against F18 infection (Felder *et al.*, 2000; Verdonck *et al.*, 2002).

According to Snoeck *et al.* (2003), piglets must be immunized during the suckling period to prevent PWD, although the presence of F4-fimbriae-specific antibodies in milk can inhibit adhesion of F4 to their receptors and subsequent immune induction. Therefore, they compared the use of purified F4 fimbriae in solution with enteric-coated F4-fimbriae for oral immunization of suckling piglets. Despite the induction of an immune response following the use of both coated and unprotected formulations, the intestinal colonization by F4-positive ETEC after challenge could not be prevented, although it was reduced. Furthermore, contrary to the findings of Van den Broeck *et al.* (1999a), the oral administration of F4 fimbriae in solution did not reduce the fecal excretion of F4-positive ETEC after challenge whereas a marginal, but significant, reduction was observed in the pigs vaccinated with coated fimbriae. *In vitro* studies showed that F4 fimbriae are completely digested after 3 h in pH 2 simulated gastric fluids (Snoeck *et al.*, 2004b). Since

stomacal pH values of 1 to 2-week-old postweaning pigs could reach 1.6 and stress of weaning leads to delayed gastric emptying (Snoeck *et al.*, 2004b), the protection of purified F4 fimbriae against gastric conditions could lead to more efficacious oral immunization during suckling and periods shortly after weaning. An enteric-coated multiparticulate formulation of F4 fimbriae for oral vaccination of suckling piglets has been recently developed (Huyghebaert *et al.*, 2005). This formulation could be mixed with creep feed. On the other hand, optimization of this formulation is needed since incompatibility between the fimbriae and the enteric coating was observed. An oral tablet formulation consisting of carboxymethyl high amylose starch and a live oral F4-positive avirulent *E. coli* strain was recently developed (Calinescu *et al.*, 2005). This excipient carrier is now being tested with purified F4-fimbriae. The bacteria thus formulated displayed higher survival rates, and for longer periods, in simulated acidic gastric conditions, than free bacteria. Furthermore, the bacteria thus protected were totally, but gradually released during the first 6-h period in the simulated intestinal fluid. Joensuu *et al.* (2004) showed that FaeG, the major F4ac fimbrial subunit protein, produced in the tobacco plant was stable in fluids simulating piglet gastric and intestinal conditions and could bind isolated piglet villi and inhibit subsequent F4 ETEC binding. Hence, the use of transgenic plants may be an interesting alternative for the large-scale production of F4 protein as an oral vaccine for the control of ETEC diarrhea in pigs. Nevertheless, despite important advances with regards to immune response against F4 fimbriae, there is still a need for competent oral vaccines for induction of mucosal protection.

Administration of specific egg yolk antibodies and spray-dried plasma proteins

A sufficient induction of the immune system following active immunotherapy will depend on the maturity and/or optimal function of the immune system. Furthermore, an active immune response takes several days to develop. Hence, for very young, sick, and/or already infected animals, active vaccination is an unsatisfactory approach for the prevention or treatment of infection. On the other hand, passive immunotherapy bypasses the requirement of the induction of the immune system for the control of infections by pathogens. In the same manner as lactogenic F4- or F18-specific antibodies can control neonatal diarrhea associated with F4-positive ETEC, oral administration of F4- or F18-specific antibodies has been investigated as a control strategy.

Previous studies demonstrated that feeding of weaned pigs with an appropriate quantity of milk from sows in late lactation completely inhibited intestinal colonization of an F18 EDEC strain, whereas high numbers of EDEC bacteria were shed by pigs fed with the same amount of cow's milk (Deprez *et al.*, 1986). Feeding of spray-dried porcine blood plasma (SDPP) to pigs had a similar inhibitory effect that lasted only as long as the plasma was fed, this inhibitory effect being improved by vaccination of the donor pigs (Deprez *et al.*, 1990, 1996). A recent report has demonstrated improved weight gains and lower frequency of ETEC-associated scours in early weaned pigs (10 days of age) fed on an SDPP-based diet (Owusu-Asiedu *et al.*, 2002). These effects were partly attributed to the presence of specific anti-ETEC antibodies in the SDPP. Nollet *et al.* (1999) also demonstrated that the feeding of SDPP derived from non-vaccinated pigs reduced the excretion of pathogenic ETEC. However, the quantity of SDPP used in this study would not be cost-effective in pig production. Van Dijk *et al.* (2002) evaluated the effects of a relatively low level of SDPP (8%) in the diet of pigs in an *in vivo* ETEC challenge model. The SDPP-based diet did not prevent losses due to the experimental ETEC challenge in the weaned pigs, although a more favorable fecal score, a healthier appearance of treated than control pigs, and improved weight gain were observed. However, the lack of effect on mortality rate in this study could have been due to the severity of the experimental model. Recently, Bosi *et al.* (2004) reported that a non-medicated SDPP diet improved growth performance, protected F4-receptor-positive pigs against ETEC infection, inhibited ETEC excretion, and reduced the *E. coli*-induced inflammatory response of pigs. The addition of SPDD to the diet was as effective as the addition of antibiotics for combating ETEC infection and even more efficacious than addition of antibiotics in reducing the expression of pro-inflammatory cytokines. However, use of SDPP is an expensive strategy for the control of PWD in pig production and is banned in Europe, being an animal origin sub-product.

Oral passive immunization of pigs with antibodies specific to ETEC fimbriae has offered a potential prophylactic and therapeutic approach (Marquardt *et al.*, 1999). The chicken egg yolk is a source of large quantities of relatively inexpensive IgY antibodies (Marquardt *et al.*, 1999). Protection against intestinal colonization by F4- and F18-positive *E. coli* may be attained by addition to the feed of eggs from hens immunized with specific antigens (Imberechts *et al.*, 1997; Marquardt *et al.*, 1999). Specific chicken antibodies have been shown to provide protection against ETEC infections (Marquardt *et al.*, 1999). Weaned pigs fed with egg-yolk antibodies from chickens immunized with purified F4 demonstrated only transient diarrhea and no mortality, whereas control piglets demonstrated severe diarrhea, dehydration, and mortality following challenge with an F4-positive ETEC (Marquardt *et al.*, 1999). Furthermore, occurrence of diarrhea and mortality was significantly lower in pigs fed with the chicken egg yolk supplemented diet on a commercial farm. On the other hand, testing for the F4-receptor status of pigs would

have confirmed that differences observed between treated and untreated groups in the challenge trial were not attributed to disparity in the number of susceptible pigs. Similarly, Owusu-Asiedu *et al.* (2003a) reported that supplementation of a pea protein isolate diet with F4-specific antibody reduced the incidence and severity of diarrhea, mortality and excretion of the challenge strain in pigs. A similar improvement in performance of pigs was observed for SDPP-supplemented diets. Owusu-Asiedu *et al.* (2003b) concluded in another study that addition of SDPP to the diet or supplementation of a pea protein isolate diet with egg yolk containing specific anti-F4 and anti-F18 antibodies, zinc oxide, or fumaric acid would each be effective alternatives to control PWD associated with ETEC.

The latter supplements potentially enabled weaned pigs to efficiently utilize less expensive plant-based proteins, thus substituting animal protein-based diets. Weaned pigs fed on a diet supplemented with egg yolk containing F18-specific antibody from the day of challenge showed a dose-dependant response to antibody treatment (Yokoyama *et al.*, 1997). These pigs demonstrated a lower frequency of diarrhea, higher rate of weight gain, and lower intestinal isolation of the F18-positive ETEC challenge strain. These results suggested that fimbriae-specific egg yolk antibodies could not only be a preventive approach for PWD but also a suitable treatment for pigs already infected with ETEC.

On the other hand, Chernysheva *et al.* (2003) observed no difference in the incidence of diarrhea or mortality among pigs treated with a commercial product containing chicken egg yolk F4-specific antibodies and control animals under routine management conditions, in two farms with PWD problems. Friendship (2002) also reported that egg yolk antibodies were not efficacious for the control of PWD diarrhea due to *E. coli* following experimental challenge. Further research is needed to demonstrate the economic feasibility of the use of egg yolk containing specific antibodies.

Use of preventive feed medication – antibiotics and zinc oxide

Preventive feed medication is practised in the majority of the affected herds in most countries despite serious drawbacks such as consumer resistance, impaired build-up of immunity, and selection of resistant bacteria. The development of bacterial resistance to a wide range of antimicrobial drugs renders this approach unattractive. It is not possible to provide universal data on resistance, because the situation varies in different pig populations depending on the antimicrobials preferentially used. Sick pigs must be treated parenterally. They eat and drink very little, even if they stand close to the creep and to the drinking nipple. Substances must be selected which reach the intestinal lumen, such as amoxicillin/clavulanic acid, fluoroquinolones, cephalosporins, apramycin, ceftiofur, neomycin, or trimethoprim. Testing bacterial resistance is indispensable if there is a herd problem.

A recrudescence of antimicrobial resistance has been observed in the last 10 years for O149:F4 *E. coli* associated with PWD (Maynard *et al.*, 2003). Amezcua *et al.* (2002) observed that soluble antibiotics, especially apramycin and neomycin, and injectable antibiotics such as trimethoprim with sulfadoxine, are frequently used in farms with PWD problems. In recent years, emergence of resistance to apramycin, neomycin, and trimethoprim or trimethoprim-sulfamethoxazole has been observed for *E. coli* strains associated with PWD (Fairbrother *et al.*, 2000; Maynard *et al.*, 2003). Among *E. coli* isolates from pigs, those from PWD show the highest rate of antimicrobial resistance. Colistin, an antimicrobial that is bactericidal for gram-negative bacteria and has a detergent-like mechanism (Hancock and Chapple, 1999), is now widely used in pig production. It has high stability, low toxicity, no cross-resistance with other antibiotics, and slow development of resistance. Colicins are effective against many pathogenic *E. coli* strains, but their efficacy against PWD-associated *E. coli* strains has not been clearly demonstrated. On the other hand, efficacy of two colicins, ColE1 and ColN, against both F4- and F18-positive *E. coli*, was demonstrated *in vitro* (Stahl *et al.*, 2004). ColE1 was more effective against the F18 strain than the F4 strain, whereas ColN was more effective against the F4 strain. A combination of these colicins may be more effective than either colicin alone for use in animal production.

Zinc oxide offers an alternative to antimicrobials. Feeds containing between 2400 and 3000 ppm of zinc reduce diarrhea and mortality and improve growth in pigs. This activity is explained by an antibacterial effect (Holm and Poulsen, 1996). Amezcua *et al.* (2002) reported an important proportion of farms with PWD problems using high levels of zinc oxide. However, environmental considerations should be taken into account when using zinc oxide at such high levels, since this leads to heavy metal contamination of the soil. The mechanisms of the protective effect of zinc oxide have not yet been elucidated. It has been suggested that the prophylactic use of zinc oxide in preventing diarrhea may be due to a bactericidal effect of zinc. Roselli *et al.* (2003) investigated the potential benefits of zinc oxide in protecting *in vitro* intestinal cells from damage induced by F4-positive ETEC. They concluded that the protective effects of zinc oxide were not due to any antibacterial activity but due to a protection of intestinal cells from ETEC infection by inhibition of bacterial adhesion and internalization, thus preventing the increase in tight junction permeability and modulation of cytokine gene expression induced by ETEC. The authors proposed that the zinc oxide could prevent the cytokines from inducing an inflammatory response in infected cells and the disruption of membrane integrity.

Organic acids, proteases, and antisecretory factor

A lower mortality, due to *E. coli*, enterotoxemia and improved weight gains were reported after introduction of rations with a reduced acid-binding capacity. A similar effect is ascribed to organic acids. Improvements in growth rate and efficiency of feed conversion have been achieved after weaning by supplementing starter diets with a pure organic acid, or mixtures of organic acids and/or their salts (Ravindran and Kornegay, 1993; Kyriakis *et al.*, 1996; Tsiloyiannis *et al.*, 2001). Tsiloyiannis *et al.* (2001) reported that weaned pigs from a farm with an uncomplicated problem of PWD, fed with a diet supplemented with organic acids, had reduced incidence and severity of diarrhea and performed significantly better than the negative control group. Furthermore, at the end of the trial, ETEC strains were detected only in the control group.

In vitro and *in vivo* studies showed that proteolytic treatment of F4 intestinal receptors prevents the attachment of F4-positive ETEC to pig intestines. Bromelain, a protease from pineapple stems, administered orally to pigs, reduced the binding of F4-positive ETEC to brush borders in a dose-dependent manner (Mynott *et al.*, 1996). Treatment with enteric-coated Bromelain reduced the incidence of diarrhea following challenge with F4-positive ETEC; all untreated pigs had moderate to strong diarrhea whereas only about 50% of pigs of treated groups demonstrated mild diarrhea (Chandler and Mynott, 1998). On the other hand, Bromelain only temporarily inhibited the F4 receptor activity for approximately 30 h, suggesting the necessity of a continuous treatment.

Scandinavian workers have shown that antisecretory factor reverses secretory diarrhea induced by LT. The levels of antisecretory factor in the blood plasma can be increased by addition of glucose and some amino acids to the feed. Treated weaner pigs were reported to have a lower incidence of diarrhea and a greater weight gain (Göransson *et al.*, 1993). Antisecretory factor probably exerts its effects on the enteric nervous system (Hansen and Skadhauge, 1995).

Bacterial probiotics

In recent investigations, feeding of a diet supplemented with fermented soybeans (especially *Rhizopus*-fermented soybean, but also *Bacillus*-fermented soybean) reduced the excretion of ETEC and the incidence, severity, and duration of diarrhea for weaned pigs (Kiers *et al.*, 2003). Piglets fed with *Rhizopus*- or *Bacillus*-fermented soybeans showed increased feed intake, average daily weight gain, and feed efficiency. Treatment of pigs from a low health-status farm with high incidence of PWD problems, with viable spores of *Bacillus licheniformis* (LSP 122, Alpharma) or viable spores of *Bacillus toyoi* (Toyocerin®) also reduced incidence and severity of diarrhea, mortality, and excretion of an ETEC strain (Kyriakis *et al.*, 1999). Probiotics, especially LSP 122, improved the weight gain and the feed conversion ratio. On the other hand, others reported no efficiency of feeding of *Lactobacillus spp.*, *Enterococcus faecium*, and *Bacillus cereus* strain 'toyoi' to experimentally and/or naturally infected pigs (De Cupere *et al.*, 1992; Johansen *et al.*, 1996; Friendship, 2002).

Phages

Bacteriophages (phages) are viruses that are excellent agents for killing pathogenic bacteria because they are not toxic to the animal host and they multiply in the bacterial host, leading to an increase in titer of the phage as they destroy the bacterial population. Several reports have shown the ability of phages to destroy bacteria that cause infection in humans and animals (reviewed in Summers, 2001). Smith and colleagues in the UK began a new era of investigation of phages in the 1980s. These researchers first demonstrated the effectiveness of phages for control of septicemic *E. coli* infections (Smith and Huggins, 1982), then showed that single phage or mixture of two phages were effective in both prophyaxis and therapy of experimental ETEC infections in neonatal pigs, calves, and lambs (Smith and Huggins, 1983). Bacterial resistance to phages that arose in calves during the course of therapy was due to capsule-negative mutants that were highly reduced in virulence. Additional studies by Smith's group (Smith *et al.*, 1987a, b) confirmed the effectiveness of phages against ETEC infection in calves. These studies firmly established the potential for phage therapy against ETEC infections in newborn pigs, but there have been no studies of phage therapy against O149:F4 ETEC in weaned pigs. There is a possibility that resistant mutant ETEC may be selected when the bacteria are exposed to phages, but *in vivo* findings suggest that this is not likely to be a problem (Smith and Huggins, 1983). Likewise, R.P. Johnson (personal communication, 2004) found that no phage-resistant mutant O157:H7 *E. coli* was detected after treating calves with a mixture of phages followed by O157:H7 *E. coli*. These observations are likely due to the fact that bacterial resistance to phages is often associated with mutations that alter bacterial surface structures used as receptors by the phages and the mutants are therefore often at a competitive disadvantage in the intestine, and/or the use of a mixture of phages results in killing of bacterial mutants that are resistant to one phage by other phages to which they are still susceptible.

The most significant barrier to the deployment of phages against PWD in pigs is likely to be the requirements for licensing of phage products. The concern is that phages might become involved in transferring virulence and/or antimicrobial drug-resistance genes in

the intestine. It will therefore be necessary to demonstrate that phages used for prophylaxis and/or therapy lack this capability.

Conclusion

Postweaning *E. coli* diarrhea in pigs remains a major cause of economic losses for the pig industry. Research has aimed at a better understanding of pathogenesis of the disease, which may lead to improved methods of prevention and treatment. The fact that the major virulence genes for ETEC are on plasmids means that various combinations of these genes are possible but there is a tendency for certain virulence genes to cluster together, suggesting that there are functional relationships that are not yet understood. ETEC of O group 149 has been recognized as the dominant type of ETEC worldwide, but the basis for this dominance is not known. F4 and F18, the major fimbrial antigens present on the *E. coli* that cause PWD in pigs, have been identified as good targets for active and passive immunization and have been the basis for schemes for selection of intestinal receptor-negative pigs. Several approaches to vaccination designed to induce mucosal immunity against the fimbrial antigens or to provide antibodies in the feed are being actively investigated. In addition, various additives intended to impede colonization of the intestine of the weaned pig by ETEC are being employed. To date, not a single strategy has proved to be totally effective and it is probable that the most successful approach on a particular farm will involve a combination of diet modification and other preventive measures.

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