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8 Adhesins and receptors for colonization by different pathotypes of *Escherichia coli* in calves and young pigs[1]

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Enteric diseases of pigs and calves owing to *Escherichia coli* typically appear during the first few days (and weeks) of life. The so far recognized pathotypes of *E. coli* involved are the enterotoxic *E. coli* (ETEC), verotoxic *E. coli* (VTEC), enteropathogenic *E. coli* (EPEC), and necrotoxic *E. coli* (NTEC). The first step in the pathogenesis of all these types is to adhere to the intestinal microvilli with or without inducing morphological lesions and produce specific toxins acting locally on enterocytes and/or absorbed into the bloodstream. This action is assured by specific ligand (adhesins) and receptor interactions which are characteristic of the pathotypes involved and may vary according to the animal species. The host-adapted adhesin/receptor systems are targets of several preventive measures including vaccines, receptor blocking and breeding for genetic resistance. They also provide the basis for cross-species infections including zoonoses. Therefore they deserve the attention of epidemiologists, research scientists and technologists. Besides, they provide tools for increased understanding of molecular pathogenesis, and offer excellent models for comparative studies of different disease entities of enteric colibacillosis in humans.

1. INTRODUCTION

Enteric colibacillosis of pigs and calves has decreased as a devastating problem of intensive animal farming during the past two decades but even today it represents

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one of the major health issues in the pig or cattle industry of several developed and of most less developed countries. The reasons for the decreased losses are primarily the new diagnostic tools and vaccines that have been developed and widely used as a result of intensive research efforts on enteric *E. coli* infections of animals during the 1970s and 1980s. The major breakthroughs in the diagnosis and prevention of enteric colibacillosis of growing pigs and calves were mainly due to our increased understanding of colonization and adhesion mechanisms of these enteric pathogens to the intestinal mucosae. In spite of these positive developments there is still room for research in this area partly because of the obvious human implications (several pathotypes of *E. coli* are also prevalent in humans), and partly because our knowledge is still quite limited. This is especially true for the area of intestinal receptors of bacterial adhesions.

The main pathotypes involved in enteric colibacillosis of pigs and calves are the enterotoxigenic *E. coli* (ETEC), verotoxigenic *E. coli* (VTEC), enteropathogenic *E. coli* (EPEC), and necrotoxigenic *E. coli* (NTEC).

This review aims to give a general overview of the virulence factors and their genetic regulators in *E. coli*. Furthermore it aims to describe the most important adhesins and their receptors playing a role in the pathogenesis of different pathotypes of enteric *E. coli*. It also points out some of the areas where future research is needed.

As there is a lot of analogy in the regulation of virulence factors of the above pathotypes and as the most abundant information is available on ETEC, we will use ETEC as the "veterinarians's horse" to describe the basic organization of virulence genes. Therefore we will start with the description of adhesions and receptors with the ETEC pathotype and will refer to them in the case of analogies at appropriate sections of other pathotypes. We will be able to describe the practical applications of present knowledge essentially also on ETEC. We have to admit that little information is available on that aspect for EPEC or NTEC.

2. ENTEROTOXIGENIC *E. COLI*

In enteric *E. coli* infections, especially in enterotoxic *E. coli* (ETEC) infections of different species, bacteria adhere to the small intestinal epithelial cells (overwhelmingly in newborn or very young animals), thereby colonizing the gut. They also secrete proteins or peptides (enterotoxins) which stimulate the small intestine for increased water and electrolyte secretion and/or decreased fluid absorption. The ability of adhesion of ETEC to intestinal epithelial cells is mainly due to the production of thin (3–7 nm) proteinaceous surface appendages (fimbriae or pili) which can be morphologically, biologically and antigenically different on various strains. Some of them morphologically resemble the common fimbriae ("Type 1" fimbriae or pili) of *E. coli* (Duguid et al., 1955). With the help of these adhesins (fimbriae), the bacteria are able to attach themselves to the microvilli of small intestinal epithelial cells, thereby more intensively transferring the enterotoxins to the target cells. There is no characteristic

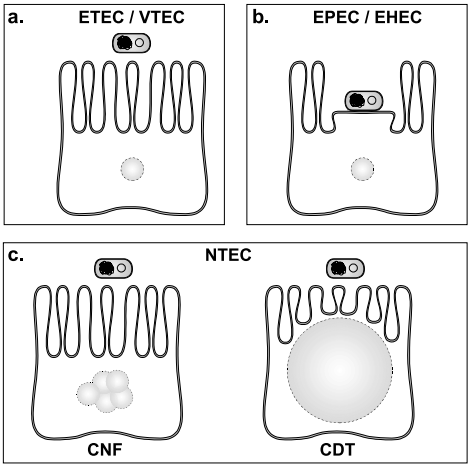

Fig. 1. Schematic presentation of cellular changes due to interaction of *E. coli* bacteria pathotypes with intestinal epithelial cells: (a) ETEC/VTEC: no obvious change in cellular microvillus morphology. (b) EPEC/EHEC: attaching to cell membrane and effacing of microvilli with pedestal formation. (c) NTEC: multinucleation, induced by CNF; distension of the cell and nucleus induced by CDT.

histological or ultrastructural morphology of adhesion or colonization by ETEC. The microvilli and the epithelial cells remain intact (fig. 1a).

2.1. Adhesins and other virulence factors in the pathogenesis of ETEC

According to our present understanding, the pathogenesis of enterotoxic colibacillosis starts with the adhesin–ligand interaction on the small intestinal microvilli, resulting in a strong but morphologically non-destructive attachment of bacteria to the microvilli. Therefore the virulence characteristics of ETEC are strongly dependent on the production of adhesins (fimbriae) and enterotoxins. In addition to adhesive and enterotoxic virulence factors, pathogenesis due to ETEC infection also involves host factors among which the most important ones are the receptors for adhesin (and/or enterotoxin). Species specificity – which is a general characteristic of ETEC infections – is largely due to the presence of specific receptors in only one (or in a limited spectrum of) animal species. Several of these adhesive virulence factors of ETEC and some of their receptors are known and will be discussed in detail below, but some of them are still unknown. Future research in this area is clearly needed and could bring further understanding of pathogenesis, thereby it would contribute to more successful strategies in the prevention and treatment of enteric enterotoxic colibacillosis due to ETEC.

The most common adhesive fimbriae of animal ETEC strains can be differentiated as surface antigens such as K88 or K99, 987P or F41 or F107 and 2134P in pigs and calves, also designated as F4, F5, F6, F41, or F18ab and F18ac, respectively (Ørskov and Ørskov, 1983; Moon, 1990; Rippinger et al., 1995) (table 1).

Table 1. Adhesins and their receptors of different pathotypes of enteric *E. coli* in calves and in young pigs

Pathotype	Adhesin	Gene/Operon	Location	Receptor
ETEC	F4 (K88)	*fae*	plasmid	glycoprotein / mucin
	F5 (K99)	*fan*	plasmid	glycolipid
	F6 (987P)	*fas*	plasmid	glycoprotein
	F18 (F107)	*fed*	plasmid	?
	F41	*fimf41*	chromosome	?
EPEC	Bfp pili	*bfp*	plasmid	? PE (phosphatidylethanolamine)
(EHEC[a])	Intimin	*eae*	chromosome	Tir (bacterial protein)
NTEC	P (Pap)	*pap*	chromosome	α-dGal(1-4)-β-Galactose
	fimbria	*sfa*	chromosome	(glycolipids)
	S fimbria			α-sialyl(2,3) β-Galactose
Afimbrial Adhesions	AFA	*afa*	chromosome/ plasmid	Dr blood group antigen

[a] EHEC does not have Bfp.

These fimbriae are characterized as straight, bent or kinked proteinaceous appendages originating from the outer membrane of the bacterial cells. They have various molecular weights (from 15 to 25 kDa). In general, fimbriae are composed of "major" and "minor" subunit structures governed and assembled under the direction of structural and accessory genes respectively. For adhesive fimbriae, the adhesive function is often represented by molecules at the tip of the filaments. The ability of fimbriae (pili) to agglutinate red blood cells of different species was recognized very early (Elsinghorst and Weitz, 1994) and it has been used for classification along with the effect of 0.5% D-mannose: MS = mannose sensitive (adhesion blocked by mannose) or MR = mannose resistant adhesion. Among fimbriae of animal ETEC bacteria we can recognize the following categories: MS haemagglutinating fimbriae (Type 1), MR haemagglutinating fimbriae (K88, K99, F41), and MR non-haemagglutinating fimbriae (987P, F18ab, F18ac). Adhesive fimbriae provide the necessary first step for the enterotoxins to act efficiently.

Enterotoxins can be described as extracellular proteins or peptides (exotoxins) which are able to exert their actions on the intestinal epithelium. ETEC strains are characterized by the production of one or both of the following enterotoxin categories (Sherman et al., 1983), all of which are plasmid regulated:

large molecular weight (88 kDa) heat-labile enterotoxins (LT);

small molecular weight (11–48 amino acid containing) heat-stable peptide toxins (ST) resistant to 100°C for at least 15 min.

LT enterotoxins are produced predominantly by human and porcine ETEC, while ST enterotoxins are produced by ETEC of human, porcine and bovine origin.

LT toxins have good antigenicity while ST toxins do not. LT toxins can be divided into two antigenically and biologically distinct but structurally similar groups: LTI and LTII. Within LTI are the LTh–I (human) and LTp–I (porcine) strains, while within LTII two antigenic variants (LTIIa and LTIIb) can be distinguished (O'Brien and Holmes, 1996).

ST toxins fall into two classes: STa and STb (also referred to as STI and STII, respectively). STa toxins have variants which are STaH and STaP [indicating human (H) or porcine (P) type of the STa enterotoxins]. STa toxins are further characterized by method solubility and by the ability to induce small intestinal fluid secretion in baby mice and to a lesser extent in weaned pigs. STb is not soluble in methanol and does not react in baby mice, however it can induce small intestinal fluid secretion in newborn and weaned pigs.

2.2 Expression and regulation of adhesive virulence factors of ETEC

Genetic regulation and byosynthesis of fimbrial adhesins is of course different according to different fimbriae. There is, however, a general scheme under which these operons are constructed and function. There are regulator elements that code for transacting polypeptides involved in the biogenesis of the whole fimbria and there are several structural genes encoding polypeptides that partly form major structural units ensuring fimbrial (pilus) formation or minor fimbrial units ensuring adhesive capacity and variant specificity (de Graaf, 1990). Changes in the gene expression can be the result of a random genetic event (stochastic process), but expression of virulence factors is usually linked more to environmental signals, such as temperature, ion concentration, osmolarity, carbon source, Fe^{++}, pH, O_2 etc. These signals can also be sensed by ETEC bacteria in order to more appropriately accommodate the *in vitro* and *in vivo* environment (stereotypic response). Under *in vivo* conditions some of the above factors can induce a whole cascade of virulence functions, turning on different genes while turning off others at different steps of the infectious process (for instance: invasion genes are turned on early in the infection but are repressed once bacteria are within the host cell) (Finlay and Falkow, 1997). For ETEC, and for some other pathotypes mentioned below much less is known about regulation. Virulence factors are influenced by the above signals through the "regulator elements". Some of these control the fimbrial synthesis only, some others control the expression of many unrelated genes and are therefore called "global regulators". Virulence genes of enteropathogenic strains of *E. coli* are mainly genes "foreign" to *E. coli* and they can be controlled by several regulators. These regulators are therefore a possible exciting area of research for ETEC in terms of pathogenesis (*in vivo* functions) and diagnosis.

Expression and regulation of virulence determinants are also dependent on *secretion mechanisms*: there are three general secretion pathways recognized in Gram-negative bacteria that export virulence factors (I–III). Another group of bacterial

proteins (IV) mediate their own transport and are therefore called autotransporter systems (Finlay and Falkow, 1997). It is also known that the secretion of STa and STb involves an energy and secA-dependent (type II) conversion of the performed toxins to the extracellular toxins (Kupersztoch et al., 1990; Yamanaka et al., 1997). However, several steps of enterotoxin and adhesin production as related to secretion systems have yet to be clarified. It should be noted that the maturation of virulence proteins is also part of the different secretion and expression mechanisms, i.e. formation of disulphide bonds within the periplasm (for cholera toxin and for LT).

2.3. Adhesins and receptors for ETEC of calves

Most of the ETEC strains responsible for diarrhoea of newborn calves are characterized by K99 (F5) and F41 and by STaP enterotoxins. They usually belong to the O8, O9, O20 and O101 serogroups and often produce an acidic polysaccharide type of K(A) antigen (K25, K28, K30, K35), making the colonies of such strains more compact and less transparent. It seems that such capsular polysaccharide antigens enhance colonization induced by K99 (Isaacson et al., 1977; Hadad and Gyles, 1982). K99 and other fimbrial adhesins mediate attachment of the ETEC to the small intestinal (mainly ileal) microvilli, thereby resisting removal and facilitating colonization. Thus bacteria are able to efficiently transmit the STa that they typically produce, which in turn induces extensive excretion and loss of water and electrolytes, rapidly leading to dehydration. Other, less frequently occurring adhesins are the so-called F17 (earlier known as FY and Att25) (Lintermans et al., 1988). Adhesions mediated by these surface proteins are generally dependent on the presence of glycoprotein or glycolipid receptors, which are abundantly present in newborn calves and lambs. In the case of K99, for instance, the receptors are acidic glycolipids (gangliosides) like N-glycolyl-GM3, which gradually decrease with age (Runnels et al., 1980; Willemsen and de Graaf, 1993; Teneberg et al., 1994). Although K99 and F41 are frequently produced simultaneously by bacteria of the same ETEC strain, there are different receptors for K99 (sheep and horse haemagglutinin) and for F41 (guinea pig and human-A haemagglutinin). K99 and F41 also differ in their genetic regulation (K99 is regulated by a plasmid while F41 is regulated by a chromosome). Both K99 and F41 as well as F17 can, however, also adhere to the porcine small intestinal brush border and can induce porcine enterotoxic colibacillosis. Receptors for these adhesins are of course different. K99 receptors are certain glycolipids (as mentioned above), F41 receptors are glycoproteins (i.e. glycophorin) (Brooks et al., 1989), while the receptors for F17 (FY/Att25) are on the sialyated mucus (Mouricout and Julien, 1987). It must be mentioned that association of F17 (FY/Att25) with ETEC is not quite clear. Original descriptions of F17$^+$ *E. coli* reported enterotoxic activities (Pohl et al., 1986; Lintermans et al., 1988). Studies in recent years revealed that F17 fimbrial adhesins are somewhat heterogeneous and they form a so-called F17 family of fimbriae

(F17a, F17b, F17c, F17d and G fimbriae) based on their receptor specificities (Le Bougouénec and Bertin, 1999). Another (non-fimbrial) surface protein (CS31A) has also been associated with calf diarrhoea (Girardeau et al., 1988) but it is also detected on septicaemic *E. coli* from calves, in contrast to K99 or F41. Interestingly, CS31A is genetically related to K88 fimbria (known as a typical porcine adhesin) (Girardeau et al., 1988). In fact, the N-terminal sequence of purified CS31A shows a homologous protein of 26.77 kDa between CS31A, F41 and K88, indicating an evolutionary relationship between these fimbrial and afimbrial adhesions (Girardeau et al., 1991). More on the F17 fimbriae will be mentioned in the section on necrotoxigenic *E. coli* (NTEC).

In connection with ETEC of calves there should also be a few words on ETEC of goat kids and lambs. As mentioned above, lambs have a very similar clinical diarrhoeal disease and similar strains of ETEC as calves. However, this seems much less certain in goat kids. In general, it is true for both animal species that we have much more limited information about their ETEC infections as compared to those of calves. For instance, the adhesins F17 (FY/Att25) and CS31A detected on calf diarrhoea strains have not been described so far for *E. coli* bacteria from lamb or goat diarrhoea, but such isolates can be prevalent among septicaemic strains of lambs and goat kids (Le Bougouénec and Bertin, 1999). Information about ETEC infection in goats is even more limited. According to our earlier studies (Nagy et al., 1984), infection by K99 + ETEC may also cause diarrhoea of young goat kids in some herds but cryptosporidiosis and rotavirus infections seem to be the main aetiological agents. This observation is supported by the experimental infection of goat kids with K99 + ETEC strains and by successful prevention of diarrhoea by the K99 vaccine (Contrepois et al., 1993). In contrast to ETEC, verotoxic *E. coli* (VTEC) strains have been isolated more frequently from 1–2-month-old goat kids with diarrhoea and they seem to be the major diarrhoeal agent of this age group (Duhamel et al., 1992). More information is needed, however, about ETEC (and in general about enteric *E. coli*) infection of goat kids and lambs.

2.4. Adhesins and receptors for ETEC of pigs

Enteric enterotoxic colibacillosis produces significant losses in two different age groups of pigs: first among newborn pigs and later at the post-weaning age. Aetiology, pathogenesis and epidemiology should be discussed separately for the two age groups, but diagnosis, treatment and prevention have enough in common to be described under one separate heading for pigs and calves.

E. coli strains of enterotoxic colibacillosis in suckling piglets are characterized by one or the other of the K88 adhesins (in variants K88ab, K88ac, and K88ad) also known as the (F4), by K99 (F5) or 987P (F6) adhesins and occasionally by the F41 (Vazquez et al., 1996), F165 (Fairbrother et al., 1986) or F42 adhesins (Sperandio and da Silveira, 1993). Among these adhesins K88 (F4) and 987P (F6) are specific

for pigs, while K99 (F5) and F41 seem to have receptors in both pigs and calves. ETEC strains possessing K88 (especially K88ac) are the most common cause of diarrhoea and they usually produce LT in addition to STaP or STb. K88 + ETEC are also characterized by haemolysin production *in vitro*. ETEC strains carrying K99 and/or F41 or 987P produce only STaP and are non-haemolytic. While K88 + ETEC may represent about 40–60% of the *E. coli* strains causing diarrhoea in piglets, the above non-K88 strains make up between 20–30% (Woodward and Wray, 1990; Nagy, 1993). The typical O serogroups for neonatal porcine ETEC infections are O8, O9, O20, O101, O141, O147 and O157 representing both K88+ and non-K88 ETEC. In our experience, the two groups (K88+ and non-K88) of ETEC have a somewhat different clinical picture: K88 strains cause more severe diarrhoea at a younger age (1–5 days) while non-K88 strains give rise to milder diarrhoea with a later onset (approximately 4–14 days of age). It should also be noted that the rotavirus infection often complicates neonatal colibacillosis of pigs, especially in non-K88 ETEC infections at the second week of age.

Small intestinal receptors of adhesins are the other essential element of intestinal colonization by ETEC. It has been shown that the receptors for K88 are glycoproteins and the lack of production is a recessive trait. Thus, homozygous piglets are resistant to K88 mediated adhesions, to colonization and to disease (Sellwood et al., 1975). The genes responsible for production of intestinal receptors belong to the TF blood group linkage group (Gibbons et al., 1977). Receptor functions seem to be dependent on the "b", "c" and "d" components, and in genetically resistant piglets the receptors are usually absent for both of these components of the K88 variants (Hohmann and Wilson, 1975; Bijlsma et al., 1982). *In vitro* adhesion tests have revealed a polymorphism of intestinal receptors for K88 and indicated that there are 5–6 different adhesion patterns (A–F) among piglets according to the K88ab, K88ac and K88ad variants (Bijlsma et al., 1982; Rapacz and Hasler-Rapacz, 1986; Billey et al., 1998). Unfortunately, this phenomenon of genetically determined resistance could not gain a wide practical application. It may, however, complicate epidemiological pictures, by partially producing non-diarrhoeal homozygous recessive (ss) litters, and by partially leaving heterozygous (Ss) piglets (which are born to resistant sows and sensitive boars) without colostral immunity (such sows would not have acquired the infection and could not produce specific antibodies in their colostrum). The practical application of this knowledge is further complicated by the fact that the correlation of the adhesion of K88 variants to the small intestinal brush borders with susceptibility to colonization and diarrhoea may be lacking. This can be explained by the findings of Francis et al. (1998), suggesting that the intestinal mucin-type glycoprotein (IMTGP) is a biologically more relevant receptor for K88ab and K88ac as compared to the so far widely accepted enterocyte brush border glycoprotein.

So far, no information is available about the genetic determination of receptors for K99, F41 or 987P in pigs, but there are mice that are genetically resistant to colonization by K99 (Duchet-Suchaux et al., 1990). Future research on these areas of mammalian genetics would clearly be needed.

The production of receptors also influences age-related resistance to the disease. This is, however, manifested in different ways for different adhesins. Receptors for K88 are abundant in newborn pigs and will decrease with age but remain relatively stable throughout the weaning and post-weaning periods. Receptors for K99 gradually decrease with age (Runnels et al., 1980). In contrast, production of receptors for 987P, does in fact increase with age (Dean et al., 1989). This invariably leads to a lower intensity of adhesion and colonization because the receptors are shed into the lumen and block bacterial adhesion before contacting intestinal epithelial cells. The ageing nature of receptors for F41 is unknown but data indicate that they may be produced all through the weaning age in pigs (Nagy, unpublished results).

Post-weaning diarrhoea (PWD) is usually the most constant disease problem of large-scale farms, especially of those that wean around 3–4 weeks of age. PWD starts a few days after lacteal protection completely ceases, and pigs are placed in an environment that is completely new from a technical, social and microbiological point of view. It is widely accepted that specific serotypes and pathotypes of ETEC are responsible for the major part of PWD. It is also without debate that the disease is a highly complex one in which ETEC only plays a part (although an essential one). It is frequently seen in almost all large-scale piggeries but it is one of the most difficult diseases to reproduce experimentally. Diarrhoea and reduction of weight are only part of the losses. Retarded growth, which usually follows diarrhoeal episodes in weaned pigs, makes the losses even worse. The main cause of post-weaning diarrhoea is the weaning itself. Only on this basis can we understand the aetiology and pathogenesis more realistically and can we be more humble about our capacities to bring real (economically feasible) improvement to this enigma. The ETEC strains involved are most frequently of the O serogroup: O8, O141, O138, O147, O149, O157, of which O149:K88 seems to be the predominant serotype in most countries (Hampson, 1994). So far, all the typical PWD strains of ETEC are haemolytic, although haemolysin does not play an essential part in the virulence of porcine ETEC (Smith and Linggood, 1971).

The most frequent adhesive virulence factors of ETEC strains in the case of PWD are K88 (mainly K88ac) fimbria. Furthermore, K99, 987P and F41 have also been described on some PWD strains (Nakazawa et al., 1987; Nagy et al., 1990a, 1996a) but they seem to be rarely involved in diarrhoea at that age. Recently, a new fimbrial adhesin has been recognized under the F18 designation.

The *F18 fimbriae* have been described under different names, and misunderstandings are frequent in the use of the earlier names and new designations. During the past few years, three new colonization factors or adhesive fimbriae have been described for groups of *E. coli* involved in PWD or oedema disease: F107 on oedema strains (Bertschinger et al., 1990), 2134P on ETEC strains (Nagy et al., 1992b), and "8813" also on ETEC strains (Salajka et al., 1992). Additionally, fimbriae of two ETEC strains of serogroup O141 have also been described (Kennan and Monckton, 1990), although no data have been given on their adhesive or

pathogenetic significances. As a first attempt to clarify the relationships between these factors, pili 2134P were compared to fimbriae F107 by means of polyclonal and monoclonal antibodies. It was provisionally concluded that these two adhesins were morphologically similar and shared a common antigenic determinant in addition to a type-specific one (Nagy et al., 1992a). These findings were confirmed (Wittig et al., 1994) and it was suggested that the symbol "a" should be used for the common determinant and the symbols "b" and "c" for the specific determinants of F107 and 2134P respectively. Furthermore, Rippinger et al. (1995) investigated the morphological, immunological, genetic and receptor-binding relatedness of fimbriae F107 and 2134P, together with the colonization factor "8813". Based on earlier suggestions made by Ida and Ørskov (International *Escherichia coli* Centre, Copenhagen, 1992) for a new F18 fimbria, it was shown that two serological variants were determined and should be designated as follows: F18ab (for F107) and F18ac (for 2134P and 8813) (Rippinger et al., 1995). The genetic relatedness of the above family of F18 fimbriae was described by Imberechts et al. (1994), supporting the above grouping, and adding the fimbriae of Kennan's O141 strains (Kennan and Monckton, 1990) to the group of F18ac. In a recent study, it was pointed out that F18ab and F18ac fimbriae are biologically distinct: F18ab fimbriae are poorly expressed both *in vitro* and *in vivo*. They are frequently linked with the production of SLT-IIv (VTEC strains), while F18ac are more efficiently expressed both *in vitro* and *in vivo* and they are more characteristic of ETEC strains (Nagy et al., 1997). It should also be mentioned that some ETEC strains may produce multiple adhesins such as K88, F18ac or K88, F41 or even K88, F18ac, and F41 (Nagy et al., 1996a). It remains to be shown if such strains have a pathogenetic advantage over strains with one kind of adhesin. It may also be questioned under what conditions there are receptors for these rarely occurring adhesins (K99, 987P, F41) available in the right amount on the small intestinal mucosae.

In weaned pigs receptors for K88 are produced, although to a somewhat reduced extent, all through the weaning age, while receptors for the variants of F18 (F18ab and F18ac) are increasingly produced up to the weaning age (Nagy et al., 1992a, 1997) and the fimbriae F18ac seem to have more receptors around the ileal Peyer's patches (Nagy et al., 1992a). The lack of receptors for F18ab and F18ac in newborn pigs offers an explanation why these VTEC and ETEC strains (and why the oedema disease itself) are only prevalent in weaned pigs.

Inherited resistance to PWD owing to production of intestinal receptors of fimbria F18ab has also been investigated by oral inoculation of weaned pigs and by *in vitro* adhesion tests (Bertschinger et al., 1993), and it seems that phenotypes susceptible or resistant to F18 adhesion can be differentiated. Pigs with at least one copy of a dominant allele for receptors are susceptible to colonization and *in vitro* adhesion (which is similar to the K88 receptors). Additional genetic marker studies localized the receptor gene on the porcine chromosome 6, closely linked to the gene

encoding for halothane sensitivity (Vogeli et al., 1994). It seems that the lack of receptors will coincide with halothane (stress) sensitivity, making it difficult to select and raise pigs without small intestinal receptors for F18 fimbria. Small intestinal receptors for K88 and for F18 seem to be different, on the basis of comparative *in vitro* studies (Nagy et al., 1997) and the different localization of their regulation on the porcine chromosome (Gibbons et al., 1977; Vogeli et al., 1994).

Breeding pigs resistant to ETEC adhesion seems to be very difficult. First of all it is difficult to select subdominant alleles of two different, independently inherited traits (lack of receptors for K88 and F18) but we should also consider that the *E. coli* bacteria are genetically much more flexible than their host. This would ultimately lead to the emergence and proliferation of new ETEC pathotypes. Furthermore, we should take into account the possible co-selection of unwanted traits (such as halothane sensitivity).

3. VEROTOXIGENIC *E. COLI* IN PIGS AND CALVES

Verotoxigenic *E. coli* (VTEC) is one of the alternative names for *E. coli* bacteria producing a cytotoxin detectable on Vero (African green monkey kidney) cell culture (Konowalchuk et al., 1977) and sharing a number of properties with Shiga toxin (O'Brien et al., 1977). Therefore they are also called "Shiga-like" toxin producing *E. coli* (SLTEC). The toxins of this group are generally characterized by the same or similar structure and a pathomechanism like that of the toxin of *Shigella dysenteriae* (composed of one A and five B subunits). As a result of enteric infection with VTEC strains, these toxins (VT1 and VT2) produce enteric (haemorrhagic colitis) and systematic disease (haemolytic uraemic syndrome) in humans, practically only enteric disease in calves (calf dysentery) and systemic disease in pigs (oedema disease). The pathomechanisms of these toxins are characterized by a receptor-mediated endocytosis of the A subunit of the VT, followed by a fusion in lysosomes and release of the enzymatically active fragment A1, leading to inhibition of protein synthesis and cell death.

Most of the VTEC (SLTEC) bacteria of ruminants and humans produce a characteristic attachment and effacement (AE) type of microvillous degeneration and bacterial adhesion (fig. 1b) (as will be described later in the section on EPEC and EHEC). However, there are verotoxin-producing *E. coli* strains which do not have the AE phenotype, and therefore do not produce such characteristic lesions but possibly adhere to the brush border, leaving the microvilli intact. In order to differentiate these verotoxic bacteria from those which also produce characteristic lesions (and haemorrhagic colitis) in this chapter we refer to them as verotoxigenic *E. coli* (VTEC). Such VTEC bacteria produce oedema disease in pigs and there are some others that produce milder diarrhoea in humans and in calves (Wieler, 1996; Mainil, 1999).

3.1. Adhesins of VTEC for calves and pigs

In calves most of the VTEC strains also have the attachment effacement capacity (Gyles, 1994; Wieler, 1996) and could therefore be designated as enterohaemorrhagic *E. coli* (EHEC) (see below). The non-AE strains of VTEC of calves have not been thoroughly studied for adhesins yet. Wieler (1996) demonstrated that bovine VTEC strains without AE genes were relatively frequent among VT2 strains of calves. Wieler has also demonstrated that many of these strains also adhered to cultured Hep2 cells, and they were all negative for bundle-forming pili (Bfp) characteristic of human EPEC. At this point it remains an interesting question how these strains could colonize the bovine intestine and continue being shed into the environment. It can be speculated whether such VTEC strains of calves have lost their earlier capacity to produce AE lesions or they have not gained it yet, or they have developed as a clonally different lineage.

In pigs at present the only VTEC known are the ones producing oedema disease. They produce a variant of VT2 (VT2e). This toxin leaves the intestinal epithelial cells without damage but enters the bloodstream, using receptors on red blood cells, and damages the endothelial cells of the small blood vessels by inhibiting protein synthesis. This leads to perivascular oedema and hyalinization in several organs and to death. The only known adhesins of the porcine VTEC are the F18 fimbria (as described above). Adhesion and colonization mediated by F18 do not cause characteristic damage to the morphology of the intestinal cells, resembling the morphology of adhesion by ETEC (Bertschinger et al., 1990; Nagy et al., 1997).

4. ENTEROPATHOGENIC *E. COLI* AND ENTEROHAEMORRHAGIC *E. COLI*

Enteropathogenic *Escherichia coli* (EPEC) were first described in the 1940s and 1950s as the causative agents of infantile diarrhoea, and are still a major cause of infant diarrhoea in the developing world. EPEC do not produce enterotoxins and are not invasive; instead their virulence depends on causing characteristic intestinal histopathology called attaching and effacing (AE), which can be observed in intestinal biopsy and *in vitro* (Moon et al., 1983; Knutton et al., 1987). The AE phenotype is characterized by effacement of microvilli and intimate adherence between the bacterium and the epithelial cell membrane. The AE phenotype develops due to a specific signalling pathway and the AE lesions are characterized by localized effacement of the brush border of enterocytes with intimate bacterial attachment and pedestal formation beneath the adherent bacteria. EPEC have a set of adhesins (reviewed by Nataro and Kaper, 1998). Intimin is essential, but not enough for the pathogenesis of EPEC, and it is encoded by a chromosome (Jerse et al., 1990). Most of the EPEC strains possess a plasmid of about 60 Mda which promotes the adherence to cultured epithelial cells in a localized adherence (LA) pattern. Early studies proved the importance of this

plasmid, named EAF (EPEC adherence factor) (Baldini et al., 1983). The EAF plasmid encodes a fimbrial adhesin called bundle-forming pilus (Bfp, Giron et al., 1991).

4.1. Intimin and translocated intimin receptor (Tir)

The first gene to be associated with the AE phenotype was the *eae* (for *E. coli* attachment effacement) encoding intimin, a large molecular weight outer membrane protein (Jerse et al., 1990; Jerse and Kaper, 1991). Subsequently, the *eae* gene was shown to be part of a large chromosomal region of DNA that encodes all the necessary determinants for the AE phenotype (McDaniel et al., 1995). This chromosomal region is named the locus of the enterocyte effacement (LEE) pathogenicity island. The LEE is responsible for the AE intestinal histopathological changes caused by EPEC and enterohaemorrhagic *E. coli* (EHEC) and related animal pathogens, first of all rabbit EPEC. The LEE is organized into three main parts (gene clusters).

The middle part of the LEE contains the *eae* and *tir* genes as well as the *cesT* gene. The right side encodes for the proteins secreted via the type III secretory system (espA, espB, espD, espF genes), while the left side encodes for the genes of the type III secretory system itself (partly functioning like a molecular syringe). Details of the functions of the three areas of the LEE follow.

On the middle part the *eae* codes for intimin (Eae), a 94–97 kDa outer membrane protein that is an intestinal adherence factor to epithelial cells, and *tir* (translocated intimin receptor) encodes the Tir, the intimin receptor protein (Kenny et al., 1997; Deibel et al., 1998). *E. coli eae* genes have been cloned and sequenced from different EPEC and EHEC strains isolated from humans and animals including calf (Goffaux et al., 1997). Sequence comparisons of different *eae* genes revealed that the N-terminal regions show high conservation but the C-terminal regions encoding the last 280 amino acids are heterogeneous. The cell-binding activity of intimin is localized at the C-terminal 280 amino acids of polypeptide "Int280" (Frankel et al., 1995; Liu et al., 1999).

Immunological and genetic studies revealed the existence of pathotype-specific intimin subtypes. Agin and Wolf (1997) identified three intimin types, α, β, γ, Adu-Bobie et al. (1998a) detected four distinct subtypes of intimin, α, β, γ, δ, and recently Oswald et al. (2000) characterized an additional new intimin variant, intimin ε. Molecular studies revealed that these intimin types are pathotype (and species) specific. Intimin α was specifically expressed by human EPEC strains belonging to classical EPEC (clone 1) serotypes of O55:H6, O125:H, O127:H6, O142:H6 and O142:H34 (Adu-Bobie et al., 1998b). Intimin β appears to be the most ubiquitous type: it is associated with EPEC strains belonging to clone 2 (O26:H$^-$, O111:H$^-$, O111:H2, O142:H2, O119:H2, O1219:H6, and O128:H2) and EHEC O26:H11; intimin β was detected in rabbit O15:H$^-$, O26:H11, and O103:H2 strains; and this subtype was present in O26:H11 bovine strains as well (Oswald et al., 2000). Intimin γ is associated mainly with human and cattle Shiga-like toxin

producing *E. coli* (SLTEC) strains including sorbitol-fermenting and sorbitol non-fermenting EHEC O157:H7, O157:H$^-$ strains, and SLTEC strains of serotypes O111:H8, O111:H$^-$, O86:H40, O145:H$^-$, and EPEC O55:H$^-$ and O55:H7 strains also harbour intimin γ. Intimin δ was associated with human EPEC O86:H34 (Adu-Bobie et al., 1998a). Intimin ε was present in human and bovine EHEC strains of serogroups O8, O11, O45, O103, O121 and O165 (Oswald et al., 2000).

The observation that different intimin subtypes are associated with different pathogenic clones can explain why these strains colonize different segments of the intestine in different host species. Tzipori et al. (1995) infected pigs with human strains having different types of intimin and demonstrated that the intimin α-producing strain caused AE lesions in both the large and the small intestine, while the intimin γ-producing EHEC strain caused AE lesions only in the large intestine. When the pigs were infected with an *eae*γ$^-$ but *eae*α$^+$ EHEC recombinant strain, AE lesions were observed in both the small and the large intestine (Tzipori et al., 1995).

Interestingly, in the case of EPEC, the receptor for bacterial adhesin (intimin) is another protein of the same bacteria called translocated intimin receptor (Tir). Tir is a bacterial protein that is translocated into the host cell via a type III secretion system and upon entry into the eukaryotic cell it serves as the receptor for the intimin. Initially it was believed that the intimin receptor protein is a mammalian membrane protein that was originally called Hp90 ("host protein") and that was tyrosine phosphorylated in response to EPEC infection (Rosenshine et al., 1996). The combined interactions between host kinases and EPEC proteins result in additional host signalling events such as actin aggregation and polymerization leading to the characteristic cellular pathology. In *E. coli* O157:H7 infection the Tir protein has an analogous function, but it is not phosphorylated after translocating to the eukaryotic cell (DeVinney et al., 1999).

As mentioned above, the LEE contains two additional main functional clusters: on the right side of the LEE are the espA, espB, espD, espF genes, of which the first three genes are necessary for the AE phenotype. The EspA is a structural protein and a major component of a large organelle; it is transiently expressed on the bacterial surface and interacts with the host cell during the early stage of AE lesion formation. EspA forms a physical bridge between the bacterium and the infected eukaryotic cell surface and is required for the translocation of EspB into infected epithelial cells, and may contribute to bacterial adhesion as well (Knutton et al., 1998). EspB protein is translocated into the host cell membrane by EspA and cytoplasm and serves as the distal end of EspA filament, and it might have a function in the host signal transduction events. EspB promotes tyrosine phosphorylation of Tir and induction of inositol phosphates and calcium fluxes. The increased calcium levels can induce cytoskeletal rearrangements and activate calcium-dependent kinases resulting in morphological changes including microvillus effacement and pedestal formation.

McNally et al. (2001) observed clear differences in the expression of LEE-encoded factors between O157 strains, with the same *stx*$^+$ *eae*$^+$ genotype, isolated from

human disease cases and those isolated from asymptomatic cattle. All strains produced a detectable amount of EspD when grown in tissue culture medium, but in the case of the human O157 strains that amount was on average 90-fold higher than for the bovine O157 strains. The level of secretion also correlated with the ability to form AE lesions on HeLa cells, and with only high-level protein secretors in tissue culture medium exhibiting a localized adherence phenotype (McNally et al., 2001). These data correlate with earlier findings based on the results of a comprehensive molecular analysis (Kim et al., 1999). That analysis revealed the existence of two distinct lineages of *E. coli* O15:H7 in the United States. Human and bovine isolates are non-randomly distributed among the lineages, suggesting that lineage II strains may not readily cause disease or may not be transmitted efficiently to humans from bovine sources. Alternatively, the distribution may reflect a loss of characteristics in lineage II that are necessary for virulence in humans, perhaps as a consequence of adaptation to the bovine environment.

On the left side of the LEE is a set of genes coding for the type III secretion system itself. These genes share sequence homology with the type III secretion systems of *Yersinia enterocolitica*, *Shigella flexneri* and *Salmonella typhimurium* (reviewed by Mecsas and Strauss, 1996). The type III secretion systems are responsible for secretion and translocation of different virulence determinant proteins such as the espA, -B, -D, -F encoded proteins and the Tir protein.

4.2. EPEC adherence factor plasmid

The majority of EPEC strains possess a plasmid 50–70 MDa in size, named the EAF (EPEC adherence factor) plasmid. These plasmids share extensive homology among various EPEC strains. The typical EPEC strains associated with diarrhoea possess EAF, while EPEC strains that do not have the EAF plasmid are referred to as atypical EPEC (Nataro and Kaper, 1998). The importance of EAF was demonstrated *in vivo* by Baldini et al. (1983) and a volunteer study revealed that EAF is essential for the full virulence (Levine et al., 1985). Giron et al. (1991) identified an EAF encoded adhesin, called bundle-forming pilus (BFP), which is a member of the type IV pilus family. The expression of BFP was associated with localized adherence to HEp-2 cells and the presence of the EPEC adherence factor plasmid (Giron et al., 1991).

Barnett-Foster et al. (1999) demonstrated that phosphatidylethanolamine (PE) serves as a receptor for EPEC and EHEC. These bacteria bind to PE specifically and in a dose-dependent manner, and this binding was consistently observed whether the lipid was immobilized on a thin-layer chromatography plate, in a microtitre well or incorporated into a unilamellar vesicle suspended in aqueous solution. Bacterial binding to two epithelial cell lines also correlated with the level of outer leaflet PE and it was reduced following preincubation with anti-PE. The PE-binding phenotype of EPEC correlated with the *bfp* genotype of a number of clinical isolates.

4.3. EPEC (and EHEC) in pigs and calves

In calves the first description of attachment effacement (AE) lesions due to "atypical *E. coli*" was given in the UK by Chanter et al. (1984), in relation to natural cases of "calf dysentery". The *E. coli* O5 strain produced bloody diarrhoea and typical AE lesions in gnotobiotic piglets (Chanter et al., 1986) and it turned out to be verotoxigenic as well. Further observations in the United States indicated that verotoxigenic and AE lesion-producing strains of *E. coli* O26 are a relatively frequent cause of calf diarrhoea (Janke et al., 1990) and such strains have also been detected to cause natural infections in the UK (Gunning et al., 2001). On the basis of the fact that most of the bovine AE lesion-producing strains also produce verotoxins, they could also be named enterohaemorrhagic-like *E. coli* (or EHEC-like strains). As is well known, the classical human EHEC strains O157:H:H7 and O157:NM are frequently carried asymptomatically by calves and older cattle as well as by small ruminants and it is usually among the least frequent serotype occurring in cattle, in contrast to humans where O157 is the leading serogroup of EHEC (reviewed by Dean-Nystrom et al., 1998). The EHEC strains causing bovine diseases (O26, O103, O111, O118 and O157) can also be transmitted to humans and, thus, these strains have a serious zoonotic potential. So far it seems that several bovine and human strains have beta intimin (supporting the zoonotic significance of bovine EHEC).

Non-verotoxigenic AE *E.coli* seem to be relatively rare in calves, although they can also produce watery diarrhoea (Pearson et al., 1989), and can be regarded as the bovine EPEC. The intimin type of these bovine EPEC strains is usually also the beta intimin (Oswald et al., 2000). At present there is no solid information available on any additional adhesive factor of bovine EPEC or EHEC strains, although it seems quite likely that there are some peculiarities in the adhesins of these strains as well.

EPEC strains of porcine origin were first detected by Janke et al. (1989) and porcine EPEC infection was studied on newborn pigs by Helie et al. (1991) who have shown colonization and typical AE lesions in the ileum and jejunum as early as 12–24 h after infection with a porcine O45:K"E65" *E. coli*, while the caecum and colon were colonized at 24–48 h post infection. This group demonstrated that porcine EPEC have virulence characteristics similar to those of human strains (Zhu et al., 1994, 1995) and, by using transposon mutagenesis, identified a porcine attaching-effacing-associated (paa) factor associated with the presence of the *eae* gene. Interestingly this paa was found in EHEC O157:H7 and in O26 strains and a strong association was with the heat-labile enterotoxin (LT) gene (An et al., 1999). Further studies have proven that the *eae* gene of porcine EPEC prototype *E. coli* 1930 (O45) strain was a member of the beta intimin group and showed the highest similarity with the rabbit EPEC strains (An et al., 2000). Such strains may be present in small numbers in the pig population not only in North America but also in Europe as well (Osek, 2001). However, the overall significance of porcine EPEC strains cannot be judged on the basis of the available data. It seems that further epidemiologic studies are needed to establish their significance in porcine enteric disease.

It is interesting to see that in pigs two genetic lineages have diverged: one is VTEC (verotoxin production without AE lesion) and the other is EPEC (AE lesion without verotoxin production). The first is responsible for oedema disease of weaned pigs (with well-identified fimbrial adhesin) while the other may induce diarrhoea in pigs (with beta intimin and paa antigen).

5. NECROTOXIGENIC *E. COLI*

Necrotoxigenic *Escherichia coli* (NTEC) are defined as *E. coli* strains producing a large molecular weight toxin named cytotoxic necrotizing factor (CNF). NTEC are associated with intestinal and extraintestinal diseases in animals and human beings (DeRycke et al., 1999). CNF was first identified from children with enteritis by Caprioli et al. (1983). The large monomeric protein toxin causes necrosis in rabbit skin and induces formation of multinucleation and thick bundles of actin stress fibres in HeLa, CHO and Vero cells (Caprioli et al., 1984) (fig. 1c). Two types of CNF (CNF1 and CNF2) have been identified, each of them being genetically linked to several other specific virulence markers (DeRycke and Plassiart, 1990). The CNFs covalently modify Rho proteins (small GTPases) that regulate the physiology of the cell cytoskeleton of mammalian cells, and lead to polymerization of actin fibres (Oswald et al., 1994; Fiorentini et al., 1995). CNFs are encoded by a single structural gene. The CNF1 operon is located on the chromosome (Falbo et al., 1992) and CNF2 is determined by a conjugative plasmid (Oswald et al., 1994).

NTEC1 strains can be found in humans and in all species of domestic mammals (DeRycke et al., 1999). The CNF1 operon is frequently associated with other virulence factor genes and these genes constitute large chromosomal regions called pathogenicity islands (PAIs, Hacker et al., 1997). One of these PAIs (PAI II) encodes CNF1, alpha-haemolysin and P-fimbriae and it was first identified in a human uropathogenic *E. coli* (UPEC) strain. This virulence gene pattern was reported in intestinal strains isolated from suckling (Garabal et al., 1996; Dozois et al., 1997) and from weaned pigs (Tóth et al., 2000), which may be explained by the unusual mobility of the PAIs.

NTEC2 strains have only been reported in ruminants (DeRycke et al., 1999). In NTEC-2 strains, CNF2 is encoded by a virulence plasmid (pVir, Oswald et al., 1994). pVir also codes for a new member of the cytolethal distending toxin family (CDTIII, Peres et al., 1997) and for the F17b or F17c fimbrial adhesin that confers the ability to adhere to calf intestinal villi (Oswald et al., 1994) and enter the bloodstream (Van Bost et al., 2001). It is tempting to speculate that the large conjugative plasmid (pVir) is also carrying a PAI containing the operons for CNF2, F17b, and CDTIII.

5.1. Adhesins and receptors of NTEC isolated from animals

Molecular epidemiological studies revealed that most of the human and animal NTEC strains have different fimbrial (*pap*, *sfa*, *f17*) and afimbrial adhesin (*afa*) genes. Mainil et al. (1999) reported that most NTEC1 extraintestinal calf isolates

hybridized with the PAP probe and additionally either with the SFA probe (37%) or with the AFA probe (49%). In contrast, the NTEC2 isolates hybridized with the F17 probe (45%), with the AFA probe (19%), or with the F17 and AFA probes simultaneously (22%). In correlation with the CNF2 prototype strains *E. coli* S5 (Smith, 1974) and *E. coli* 1404 (Peres et al., 1997) all the 19 NTEC2 cattle isolates had a virulence plasmid coding for CNF2 and most of them coded for fimbrial (F17) or afimbrial (AFA) adhesins as well, for which the PCR results suggested the existence of a new variant of AFA (Mainil et al., 1999).

Examining 32 herds for NTEC, it was found that CNF2 was more frequently detected than CNF1 in the faecal samples of healthy cattle. CNF2-producing NTEC strains were significantly more frequently isolated from calves (24%; 17 of 71) than from cows (4%; 11 of 257). Reports confirmed that healthy calves are a reservoir of NTEC producing CNF2 (Blanco et al., 1998), and NTEC that produced CNF2 may be part of the normal intestinal flora of cattle (Blanco et al., 1993). A sero-epidemiological study revealed that the O groups of CNF2+ strains isolated from cows (O2, O8, and O14) were different from those found in calves (O8-O75, O15, O55, O86, O88, O115 and O147) (Blanco et al., 1998). Depending on the serotypes the CNF1-producing strains isolated from human extraintestinal infection had different adhesins (Blanco et al., 1994). These latter authors also suggested that extraintestinal infections are caused by a limited number of virulent clones. CNF1 strains of serotypes O2:K7:H$^-$ and O4:K12:H1 express P fimbriae, whereas CNF1 strains of serotypes O2:K?:H1, O2:K1:H6 and O75:K95:H5 possess the adhesin responsible for the so-called MRHA type III. In the following section the above mentioned P and S fimbriae and the afimbrial adhesions (AFA) will be discussed in some detail. Information on the F17 fimbrial family has partly been provided in the bovine ETEC section.

5.2. P (pap) fimbriae

Type P fimbriae are also named pyelonephritis-associated pili (pap) and have been recognized as P blood-group-specific adhesins (Kallenius et al., 1981). They are composed of a thin fibrillum (carrying the adhesin) at the proximal end of a more rigid pilus rod 7 nm in diameter (Kuehn et al., 1992). P fimbriae are part of a family of adhesive organelles that are characterized by an assembly machinery consisting of a periplasmatic chaperone (PapD) and a pore-forming outer membrane (PapC) usher protein (Hultgren et al., 1996). The 11 genes coding for functional P fimbrial adhesin are clustered in an operon encoding the main component of the pilus rod (PapA) and several minor fimbrial subunits (PapH; K; E; F), the PapG which is the adhesin and the assembly machinery (PapC; D; J), and the two regulatory proteins (Pap J; B) (reviewed by Hultgren et al., 1996). PapG adhesin located at the tip of the fimbriae binds to the alpha-D-galactopyranosyl-(1–4)-beta-D-galactopyranose or Gal alpha (1–4)Gal disaccharides (Kuehn et al., 1992), while the receptor for the P-related sequences (prs) is the GalNAc-α-(1–3)-GalNAc which is related to fimbriae of serotype F13.

There are known alleles of PapG, referred to as classes I, II, and III. These classes have different haemagglutination patterns. PapG I agglutinates only human erythrocytes, PapG II agglutinates human erythrocytes very well and sheep erythrocytes only poorly, and PapG III agglutinates only sheep erythrocytes (reviewed by Hultgren et al., 1996).

The *pap* operon is located on the bacterial chromosome mostly associated with other virulence factor genes forming PAIs in uropathogenic *E. coli* strains. At least four PAIs are present in the genome of UPEC 536 of O6:K15:H31 prototype, and three of them encode different adhesions: PAI I and II carry genes for P fimbriae and haemolysin, while PAI III encodes the S fimbrial adhesin. UPEC J96 of serotype O4:K6 has two PAIs. One PAI carries virulence determinants pap and hlyI. The second PAI encodes CNF1, hlyII and harbours *prs* (pap-related sequence) genes. Because these islands represent a mechanism for spreading the pathogenicity factors between strains belonging to the same and different species, the presence of classical UPEC specific adhesins in intestinal isolates such as NTEC1 strains is understandable (reviewed by Hacker et al., 1997).

5.3. S fimbriae

S fimbrial adhesins I and II (SfaI and SfaII), produced by extraintestinal *Escherichia coli* pathogens that cause urinary tract infections (UTI, Hacker et al., 1985) and newborn meningitis (NBM, Hacker et al., 1993), respectively, mediate bacterial adherence to sialic acid-containing glycoprotein receptors (Moch et al., 1987) present on host epithelial cells and on extracellular matrix. The S fimbrial adhesin (sfa) determinant of *E. coli* comprises nine genes (Schmoll et al., 1990). Both SfaI and SfaII adhesin complexes consist of four proteins: SfaA (16 kDa) is the major subunit protein and the minor subunit proteins are SfaG (17 kDa), SfaS (15 kDa), and SfaH (29 kDa).

Genetic and functional analysis of the sfa I complex conducted by Khan et al. (2000) revealed that sialic acid-specific binding is mediated by the minor subunit protein SfaI-S, which is located at the tip of the fimbriae. The SfaI-S was the only minor protein gene which increased the degree of fimbriation and provided adhesion properties for a non-adhesive derivative K-12 strain which had the sfaI-A major subunit gene but had neither the *sfa*I-G nor the *sfa*I-H gene. *sfa*EF genes are part of the assembly and transport apparatus, while *sfa*C and *sfa*B genes are regulators. The receptor of the S fimbrial adhesions is α-sialyl (2,3)-β-galactose.

Although both the P and S fimbrial families are recognized as typical extraintestinal (mainly uropathogenic) adhesive virulence factors, the fact that they can be detected relatively frequently on intestinal isolates, indicates that they may have a role in the intestinal colonization of animals (including pigs and calves) and humans.

5.4. Afimbrial adhesins

Afimbrial adhesins (AFA) are the first adhesin structures that are not associated with fimbriae. They were observed for the first time on a uropathogenic *E. coli* strain (Labigne-Roussel et al., 1984). At present at least eight different *afa* gene clusters are known. The *afa*I gene cluster identified first from *E. coli* strains associated with urinary and intestinal infections encodes AfaABCDE proteins and it is involved in adhesion to epithelial cells and haemagglutination (Labigne-Roussel and Falkow, 1988). Three Afa proteins, AfaB, AfaC and AfaE, are required for the mannose-resistant haemagglutination (MRHA) and for adherence to uroepithel cells (Labigne-Roussel et al., 1984; Labigne-Roussel and Falkow, 1988). Among these three proteins AfaA and AfaF are transcriptional regulators, AfaB functions as a chaperone, AfaC is an outer membrane usher, AfaD is an invasin, and AfaE is the adhesin protein (Walz et al., 1985).

Immunological and DNA hybridization studies revealed the existence of at least four *afa* operons encoding different adhesins in which the *afa*B, *afa*C, and *afa*D genes are highly conserved but the *afa*E genes (encoding the adhesin proteins) are variable (Labigne-Roussel and Falkow, 1988). All these Afa I–IV variants were identified in human UPEC strains but later a hybridization and PCR analysis based study revealed the existence of related sequences in pathogenic *E. coli* isolates of bovine and porcine origin (Harel et al., 1991). Further studies suggested that these operons are different from the *afa* operons of human isolates (Maiti et al., 1993; Mainil et al., 1997).

Lalioui et al. (1999) cloned and characterized *afa*-7 and *afa*-8 gene clusters encoding afimbrial adhesins from diarrhoeagenic and septicaemic *E. coli* strains of bovine origin. The AfaE-VII and AfaE-VIII adhesin proteins are genetically different from the AfaE adhesins produced by human pathogenic strains, and they also have different binding specificity. The AfaE adhesins of human pathogenic strains mediate the MRHA of human erythrocytes and specific attachment to HeLa, uroepithel cells and Caco-2 cells via recognition of the so-called decay-accelerating factor (DAF) molecule as a receptor. AfaE-VII mediates MRHA of human, bovine and porcine erythrocytes and the adhesion of bacteria to HeLa, Caco-2 and uroepithel cell lines, and to MBDK bovine kidney cell line and does not bind to canine kidney. AfaE-VII does not recognize the SCR-3 domain of DAF, which is the receptor of the human AfaE adhesins (Nowicki et al., 1993). AfaE-VIII binds to different still unidentified receptors. *In vitro* assays showed that it binds to uroepithel cells and to canine kidney cell line, but does not bind to HeLa and Caco-2 cell lines. AfaE-VII is slightly similar to fimbrial adhesin AAF/I produced by enteroaggregative *E. coli* isolates and AfaE-VIII is very similar to the M agglutinin (Lalioui et al., 1999). Further, the afaE-VIII gene is frequent and highly conserved among *E. coli* strains isolated from calves, particularly in NTEC strains in association either with the *cnf1* or the *cnf2* gene. The fact that the *afa*-VIII gene cluster is located on the chromosome or on the plasmid suggests that it could be carried by a mobile element, facilitating its dissemination among bovine pathogenic *E. coli* strains.

6. PRACTICAL APPLICATIONS

The above information on basic mechanisms of pathogenesis of enteric *E. coli* infections of calves and pigs has led to practical applications mainly in the area of diagnosis and prevention of these diseases. Unfortunately, specific preventive measures have only been worked out against ETEC infection of newborn calves and pigs as discussed below.

6.1. Diagnosis of enteric *E. coli* infections

Diagnosis of ETEC infections requires the phenotypic detection of virulence factors (adhesins, enterotoxins) using *in vitro* tests (slide or latex agglutination or ELISA) in most cases (Thorns et al., 1989). Adhesive fimbriae can, however, be most efficiently detected *in vivo*, by an immunofluorescent method using absorbed polyclonal or monoclonal antifimbrial antibodies (Isaacson et al., 1978). In contrast to fimbriae, enterotoxins produced *in vivo* are much more difficult to detect. Therefore, in early ETEC studies *in vitro* produced toxins could only be tested by biological assays: ligated small intestinal segments (for all enterotoxins) or baby mouse assay (for STa), followed by cell cultures (for LT), and later on by ELISA assays (for LT and ST) (Czirok et al., 1992). Now, with the advent of molecular methods in the diagnostic laboratories, the cumbersome biological assays can be replaced by so-called gene probes: DNA hybridization and PCR (recently in a complex form) for detecting the genes of different virulence characters (Mainil et al., 1990; Franck et al., 1998; Tsen and Jian, 1998). The question can be raised, however, of whether our chances to discover new adhesive and other virulence attributes will not be limited if we disregard classical biological assays in the long run.

6.1.1. Diagnosis in calves

According to our present knowledge, the diagnosis of ETEC infection in calves is greatly facilitated by the high frequency of K99 antigens on bovine ETEC. The presence of K99 can, however, be covered by the K(A) antigens. Besides, the production of K99 may also be repressed by the presence of glucose, while for other strains glucose may even enhance K99 production (Girardeau et al., 1982). Therefore, special media such as Minimal Casein Agar with Isovitalex® added (MINCA-Is) are required (Guinee et al., 1977) for the detection of K99 *in vitro*. Alternatively, the immunostaining of small intestinal segments from calves that died as a result of diarrhoea proved to be more efficient (Isaacson et al., 1978; Nagy and Nagy, 1982). Monoclonal based latex reagents (Thorns et al., 1989, 1992) and DNA probes (hybridization and PCR) that detect the above fimbrial genes are available for more efficient diagnosis (Mainil et al., 1990) not only for ETEC but for other pathotypes as well.

6.1.2. Diagnosis in pigs

Piglet diarrhoea is almost always accompanied by some type of non-commensal *E. coli* infection at the suckling age and within the first 2 weeks after weaning. Today, we already know of several types of porcine ETEC (although it seems that other pathotypes can also complicate and partly induce diarrhoea in newborn and especially in weaned pigs). Furthermore, it should be remembered that on the herd level, diarrhoeal episodes are infrequently monocausal. The presence of one or more types of ETEC (for example) can often be accompanied by rotaviruses, caliciviruses, coccidia, or by the coronavirus of porcine epidemic diarrhoea (PED) in both age groups but especially in weaned pigs (Hampson, 1994; Nagy et al., 1996b). In this chapter only the diagnosis of infections due to known and established types of ETEC will be discussed, which are in most cases the dominant elements of sporadic diarrhoeal diseases on the herd level. Diagnosis of ETEC infection is based on the detection of known virulence factors (and of the serogroup) of the suspected ETEC. This would not necessarily require culturing of bacteria (see below), but the need to determine antibiotic resistance patterns simultaneously makes culture and test of bacterial attributes *in vitro* an accepted routine for diagnostic laboratories. For cultures, usually small intestinal or faecal samples are available, from which it is advisable to inoculate specific media (besides classical media) required for preferential growth of some adhesins (such as MINCA-Is for K99, or Difco Blood agar Base with sheep blood for 987P) (Guinee et al., 1977; Nagy et al., 1977). To test if the isolates are ETEC, the fimbrial antigens K88, K99, F41 and 987P can be detected by slide agglutination using specific absorbed sera or by latex agglutination for which there are monoclonal antibody based kits available (Thorns et al., 1989, 1992). Adhesive fimbriae produced *in vivo* can be more efficiently detected by testing small intestinal smears of diarrhoeal pigs using fluorescence antibody assays. As there may be ETEC strains without known (or detectable) adhesive virulence factors, it is advisable to perform tests for enterotoxins as well. LT and STa toxins can be identified by ELISA or by latex agglutination; unfortunately no such tests are available for STb. DNA probes (hybridization and PCR) are also in use for *in vitro* detection of almost all known virulence genes of porcine ETEC (Mainil et al., 1990; Nagy et al., 1990a; Franck et al., 1998).

Besides bacteriological results, there is almost always a need for differential diagnostic investigations (such as virus detection) as well. Therefore, in the case of weaning pigs it is strongly advised not to be content with a possible bacteriological result detecting some types of ETEC (carrying K88 or F18 surface antigens), but it is also necessary to consider other physiological, environmental, dietary and viral factors that may sometimes be as important as the given ETEC bacteria themselves. Therefore, differential diagnosis should frequently include the detection of rota- and coronaviruses as well as spirochaetes and *Salmonella* (Hampson, 1994; Nagy et al., 1996b). Culturing and/or immunofluorescent *in vivo* identification of ETEC strains from the ilea of diarrhoeal pigs is the most effective and simplest way of making a

bacteriological diagnosis (as described for diarrhoea of newborns). The bacteriological analysis of faecal samples for ETEC is more difficult because the bacteria present in the faeces may not reflect the microbial status of the small intestine. There are a variety of *in vitro* techniques that detect virulence factors (adhesins and toxins) of ETEC including immunological and biological assays, molecular probes (DNA hybridization and PCR) as mentioned above for newborn diarrhoea.

6.2. Prevention by vaccination (using adhesins as protective antigens)

ETEC infections can, and should, be prevented by several hygienic and management techniques which are outside the scope of this chapter. Among these, the most important factor, in the case of newborn animals, remains the early and sufficient colostral supply. The protective value of colostrum against diarrhoeal diseases of the offspring caused by ETEC can be increased essentially by maternal immunization. For that purpose several vaccines are used mainly by parenteral application (which can be adjuvanted by oral immunization). These vaccines contain the so-called protective antigens (virulence factors – fimbrial adhesins with or without LT enterotoxins). Vaccinations should usually take place in late pregnancy and can be repeated as "reminder" vaccinations before each subsequent farrowing. As a result, colostral antibodies would block virulence factors and propagation of bacteria in the intestine. Similar effects can be expected in the case of passive immunization, i.e. the oral application of polyclonal or monoclonal antibodies (Sherman et al., 1983). Immune colostrum or specific antibodies can also be applied metaphylactically, however, with much less success. Amongst the mechanisms of action described above, the success of colostral vaccines depends largely upon matching the right protective antigens with the pathogens present in a given animal population. Our knowledge about the possible existing virulence factors is, however, still limited and further improvements in this area are to be expected.

Vaccines against enterotoxic colibacillosis of calves or small ruminants contain both K99 and F41 (Contrepois et al., 1978; Acres et al., 1979; Nagy, 1980). In countries where F17(FY/Att25) fimbriae are prevalent, vaccines should also contain the F17(FY/Att25) antigens (Contrepois and Girardeau, 1985; Lintermans et al., 1988). As ETEC infections of calves and small ruminants frequently occur simultaneously with rotavirus infection, most of the vaccines used today contain bovine rotavirus antigens as well (Bachmann et al., 1984; Köves et al., 1987). So far, no information is available about a possible shift in fimbrial characteristics of ETEC in herds or areas where K99 and/or F41 containing vaccines are used. There is evidence, however, suggesting that the strongly reduced incidence of K99 and F17 may be explained by the use of vaccines containing these antigens (Contrepois and Guillimin, 1984). During the past decade, no new adhesins or toxins of calf or ruminant ETEC strains were discovered, although it seems almost impossible that the adhesin (and toxin) spectrum in these animal species is that limited all over the world.

Vaccinations against neonatal diarrhoea of pigs caused by ETEC have been very successful especially since the most prevalent adhesins (K88, K99, 987P) and toxin (LT) became standard components of the vaccines (Moon and Bunn, 1993). It seems that LT could act not only as a protective antigen, but also as an oral adjuvant (Ahren et al., 1998). Such vaccines are almost always used to provide maternal immunity through immune colostrum to the offspring. This requires parenteral (or oral) application of the above antigens well before farrowing. As a result, passively acquired antibodies through colostrum will protect piglets for about a week against most types of ETEC under normal farming conditions, provided that the piglets ingest immune colostrum early enough and in an adequate quantity during the first 12 h of life (before the sharp decline of their absorptive capacity for colostral immunoglobulins). There have been several ways to improve the efficiency of maternal parenteral vaccines against ETEC (Morein et al., 1984; Nagy et al., 1990b).

Some companies advise the use of "in-feed" vaccines (containing killed or live bacteria) for sows or to combine them with parenteral vaccines. The results of Moon et al. (1988) suggest that effective presentation of the protective antigens would require the use of live oral vaccines for such purposes. Such oral vaccines, if licensed, could efficiently stimulate the mucosa-associated lymphoid system (GALT) so that secretory antibodies (especially SIgA) – which are protected from digestion – could be produced and provide the firmest protection. Strong lactogenic immunity mediated in this way lasts for about the first 10–14 days of life. It should be noted that first farrowing gilts are less able to produce high levels of antibodies whatever the route of immunization. The combination of "in-feed" and parenteral vaccines can be recommended for first and second pregnant gilts as well (Moon et al., 1988). It should be remembered, however, that licensing of live oral bacterial vaccines for use in veterinary medicine, especially those produced by genetic engineering, is difficult in most countries. Killed oral vaccines are, however, of limited value. Live oral vaccines still represent a more controlled and more effective way of specific immune prevention of neonatal diarrhoea as compared to the so-called "feed back" (feeding of diarrhoeal faecal material to pregnant sows, as practised on some farms). The use of recombinant *Salmonella*-vector vaccines expressing the necessary adhesive epitopes could also come into question (Attridge et al., 1988; Morona et al., 1994). Finally, it is hoped that more progress in the area of genetically engineered plants (containing the required antigens produced for feeding) will be made in the future.

Vaccinations against post-weaning diarrhoea of pigs have not shown much progress lately, although the theoretical basis is clear and the need is unquestionable. In-feed vaccines containing heat-treated ETEC bacteria have not been consistently effective and most have been removed from the market. Parenteral vaccination of piglets before weaning is advised by some companies but its efficacy against PWD has not been convincingly demonstrated. At present the most promising experiments are in the area of live oral vaccines applied before weaning. Bertschinger et al.

(1979) demonstrated the efficacy of such a vaccine when a low-energy diet was also given. Further experiments of this group provided evidence about the protection of pigs against PWD and oedema disease by a live oral vaccine containing F18 fimbria. A combined (live oral plus killed parenteral) vaccine against PWD also seems to be successful in preventing losses (Alexa et al., 1995).

7. CONCLUDING REMARKS

Adhesion and colonization are the first (but not the only) functional prerequisites for a mucosal bacterium to be pathogenic. The previous sections have shown the vast genetic and phenotypic arsenal of adhesins of *E. coli* bacteria for successful colonization of small intestinal mucosal surfaces in calves and young pigs. These adhesins represent surface proteins, governed by specific operons and constructed in ways according to the particular adhesin (with fimbrial or afimbrial structures). Beside their structure, these adhesins can also be grouped according to their receptors usually present on the intestinal mucosal epithelium (but also on red blood cells of different animal species and humans) and on the urinary epithelium. Our knowledge of the genetics and function of these adhesins has helped so far to reduce losses due to enterotoxic colibacillosis of calves and young pigs and may bring further success in the prevention of diseases due to other pathotypes (EHEC, EPEC and NTEC), which at present seem to be a greater threat to human health. Our tools in combating these losses are better and more specific diagnostic reagents (including DNA-based diagnostic tests) and vaccinations (mainly using the proteinaceous adhesins as protective antigens). The knowledge on genetics of receptors for adhesins of different *E. coli* pathotypes and subtypes has raised great hopes for breeding genetically resistant animals – in the case of newborn piglet diarrhoea (receptors for K88) and in the case of weaned pig diarrhoea or oedema (receptors for F18). As the classical selection in breeding would not be practical (disadvantageous linkage groups with other important genes), it seems that the utilization of these genes will have to await further technological developments.

8. FUTURE PERSPECTIVES

Because *E. coli* is a highly flexible organism (acquiring new virulence characters or masking the ones that may be disadvantageous for survival) (Mainil et al., 1987), and because there are several kinds of infections (due to viruses and protozoa as described above) and conditions that may predispose the host to colonization by ETEC, thereby enhancing the chances for *E. coli* to utilize its pathogenic potential, the protection of pigs and calves from pathogenic *E. coli* is a constant challenge for farmers and veterinarians alike. As described in the previous sections, the knowledge on adhesins and receptors for colonization by different pathotypes of *E. coli* has been utilized quite extensively for diagnostic purposes (antifimbrial diagnostic

sera and reagents) and for the prevention of diarrhoeal diseases (mainly in the form of killed maternal vaccines containing fimbrial antigens). In the future, further applications can be expected in the development of live oral vaccines (to establish more efficient local immunity in the intestine). This would imply using non-virulent but adherent *E. coli* strains with apropriate adhesins for the species and age of the target animal population. Furthermore – in spite of the difficulties described above – progress may also be expected in the area of application of genetic resistance against enterotoxic colibacillosis of pigs. Apart from direct practical applications, there are further significant scientific developments and applications expected in the area of neonatal biology and comparative human pathobacteriology. The most likely areas for further advancements will be (and in some cases are) the applications of real-time PCR and DNA chip technology in studying quantitative aspects of gene expression and functional analysis of the genes discussed above. The results of these studies will reveal more complex interactions between the pathogenic bacteria and the host on the gene expression level.

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