Enteropathogenic *Escherichia coli* enteritis: evaluation of the gnotobiotic piglet as a model of human infection

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SUMMARY The pathogenicity of classical enteropathogenic *Escherichia coli* strains of human origin was investigated in gnotobiotic piglets. One to two day old piglets in groups of four were infected perorally with approximately 10^8 colony forming units of one of eight enteropathogenic *E coli* strains or a non-pathogenic control strain. Animals were necropsied 24 or 48 hours after infection and their intestines were subjected to histological examination, quantitative bacterial culture and estimation of lactase activity. Four enteropathogenic *E coli* strains caused mild to moderate diarrhoea in nine of the 16 piglets inoculated with them. Piglets given two of these strains later became moribund. One enteropathogenic *E coli* strain caused a severe illness unaccompanied by diarrhoea. Inflammation of the intestinal mucosa occurred with all eight enteropathogenic *E coli* strains, but not with the control strain. Pathological changes were most pronounced in the distal ileum and colon and adherent bacteria were seen on the surface of the inflamed mucosa. The extent of the inflammatory response in infected piglets for the most part paralleled the severity of the clinical signs, the degree of bacterial colonisation and the reduction in lactase activity. Electron microscopic examination of tissue from piglets infected with three different strains showed that bacterial adherence to the apical plasma membrane of epithelial cells was accompanied by distinctive ultrastructural changes. These included degeneration of the microvillous brush border, together with cupping and pedestal formation of the plasma membrane at sites of bacterial attachment. The same changes have been seen in naturally occurring enteropathogenic *E coli* diarrhoea in humans and rabbits. The combined clinical and pathological findings indicate that the neonatal gnotobiotic piglet is a suitable model of infection with enteropathogenic *E coli*.

Escherichia coli is both the predominant member of the normal aerobic colonic flora and a prominent cause of gastroenteritis.¹ Strains of *E coli* that cause diarrhoea are divided into three categories according to their pathogenic mechanisms.^{1,2} The pathogenicity of enterotoxigenic *E coli* resides in their ability to colonise the surface of the small intestine and to produce heat-labile and/or heat-stable enterotoxins.² Enteroinvasive *E coli*, by contrast, penetrate and proliferate within the large bowel epithelium.^{1,2}

A third category of diarrhoeagenic *E coli*

comprises bacteria that attach intimately to the intestinal epithelium and disrupt the microvillous brush border of intestinal epithelial cells. These bacteria, which Moon *et al*³ have termed attaching effacing *E coli*, appear to include the traditional or 'classical' enteropathogenic *E coli*⁴ that have been responsible for numerous nosocomial outbreaks of severe infantile diarrhoea^{1,4} and that, in some parts of the world, play an important role in sporadic diarrhoea of infants and young children.⁵

The underlying mechanism of enteropathogenic *E coli*-induced diarrhoea is not clear. Enteropathogenic *E coli* possess no well defined virulence factors. They do not produce conventional enterotoxins⁶ or invade the intestinal mucosa, and generally they are identified by serotyping alone.⁷ An animal model would not

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only facilitate investigation of the pathogenic mechanisms of enteropathogenic *E coli* diarrhoea, but could also be used to examine methods of identifying enteropathogenic *E coli* more convenient and reliable than serotyping.

Pigs have previously served as models of diarrhoea caused by various bacteria, viruses, and protozoa,⁸⁻¹⁰ and in view of recent observations that piglets may also be susceptible to infection with enteropathogenic *E coli*,^{3,11} we inoculated neonatal gnotobiotic piglets with several enteropathogenic *E coli* strains of human origin and determined their clinicopathological response. Our findings are reported in this paper.

Methods

BACTERIA

Seven enteropathogenic *E coli* strains isolated during outbreaks of infantile diarrhoea and one from a sporadic case were used^{12,13} (Table 1). A non-pathogenic *E coli* strain, recovered from a healthy pig, served as a control. All strains were unreactive in conventional assays of *E coli* pathogenicity, including tests for invasive capacity and for the production of enterotoxins and colonisation factor antigens. Two of the enteropathogenic *E coli* strains were previously shown to produce diarrhoea in adult volunteers.¹⁴

Bacteria were maintained freeze dried or in 30% glycerol at -20°C. Before inoculation of experimental animals, test strains were cultivated on 5% sheep blood agar overnight at 37°C and harvested in sterile physiological saline.

EXPERIMENTAL ANIMALS

Thirty-six gnotobiotic piglets were derived as described previously¹⁵ and maintained in groups of four in sterile plastic containers. They were bottle fed five or six times daily a total of 200 to 250 ml

reconstituted evaporated cow's milk. For the experimental challenge, one to two day old piglets in each group were inoculated perorally with between 10⁸ and 2×10⁸ colony forming units of a test strain. Twenty four or 48 hours later, two piglets in each group were given a lethal intracardiac dose of sodium pentobarbitone and necropsied.

NECROPSY PROCEDURE AND TISSUE EXAMINATION

Segments from the proximal, mid and distal small intestine as well as one each from the caecum and colon were obtained and fixed in neutral buffered formalin. Sections were stained with haematoxylin and eosin, and by the peroxidase- antiperoxidase (PAP) technique¹⁶ using specific *E coli* O-antisera. Pathological changes in haematoxylin- and eosin-stained sections were graded on a scale of - to +++ (Table 2). PAP-stained sections were used to estimate the extent of bacterial attachment (Table 2).

Tissue for transmission electron microscopy was fixed in 2.5% gluteraldehyde, postfixed in 2% aqueous osmium tetroxide and stained *en bloc* in 2% uranyl acetate in 70% acetone.

The proximal, mid and distal small intestine were also sampled for bacterial counts and for estimation of lactase activity. For bacterial counts, serial 10-fold dilutions of homogenised gut contents and mucosal scrapings were prepared in sterile physiological saline, plated on paired 5% sheep blood and MacConkey agar plates, and incubated overnight at 37°C. Lactase activity in homogenised mucosa was determined by the method of Dahlqvist.¹⁷

Results

CLINICAL FINDINGS

Piglets infected with enteropathogenic *E coli*

Table 1 *Escherichia coli* strains used in this study

Strain	Designation	Serotype	Origin	Pathogenicity for adult volunteers	References
E508/69	EPEC	0114:H2	Manchester, England	NT	12
E2347/69	EPEC	0114:H2	Taunton, England	NT	12
UR	EPEC	0125ac:H?	Chapel Hill, NC, USA	NT	13
E63/68	EPEC	0128:H2	Teesside, England	NT	12
E74/68	EPEC	0128:H2	Teesside, England	—	12, 14
E2348/69	EPEC	0127:H6	Taunton, England	+	12, 14
E2381/70	EPEC	0142:H6	Glasgow, Scotland	NT	12
E851/71	EPEC	0142:H6	Glasgow, Scotland	+	12, 14
P3175	Normal flora	07:H32	Bendigo, Australia	NT	

NT = Not tested. EPEC = enteropathogenic *E Coli*.

Table 2 Major clinical and histopathological findings in pairs of gnotobiotic piglets inoculated with an enteropathogenic *Escherichia coli* strain of human origin or with non-pathogenic pig *E coli*

24 hours after inoculation					48 hours after inoculation			
E coli strain	Clinical signs	Histopathology*			Clinical signs	Histopathology		
		Proximal small intestine	Distal small intestine	Large intestine		Proximal small intestine	Distal small intestine	Large intestine
E508/69	Normal	+/+	++/+ +	++/+ +	Moribund	++/+	+++/+	++/++
E2347/69	Normal	+/-	++/+	++/+ +	Normal	-/-	+/+	++/++
UR	Normal/Moribund	++/+	++/+	++/+ +	Diarrhoea	+/-	++/+	++/+ +
E63/68	Normal	-/-	+/-	-/+	Normal	+/-	+/+	-/+
E74/68	Normal	-/-	-/-	+/+	Normal	+/-	+/+	+/+
E2348/69	Normal	+/+	+/+	+++ /+++ +	Diarrhoea	+/-	++/+	+++ /+++ +
E2381/70	Diarrhoea	+/-	+/+	+++ /+++ +	Moribund	+++/-	+++ /+++ +	+++ /+++ +
E851/71	Diarrhoea	+/+	+++ /+++ +	+++ /+++ +	Diarrhoea	+/-	+++ /+++ +	+++ /+++ +
P3175	Normal	-/-	-/-	-/-	Normal	-/-	-/-	-/-

* Each result is the combined findings for 2 piglets expressed semiquantitatively as:

mucosal changes/bacterial attachment, where:

- represents no change; + scattered submucosal inflammatory cell infiltrate; ++ scattered submucosal inflammation, mild congestion and submucosal oedema; +++ extensive inflammatory cell infiltrate throughout mucosa, intense congestion and submucosal oedema; and

- represents no attachment; + few scattered foci of attachment; ++ extensive focal attachment; +++ heavy continuous attachment.

displayed widely differing clinical responses, ranging from no apparent effect, through diarrhoea, to severe generalised illness (Table 2). With the exception of *E coli* UR, however, each bacterial strain elicited a reproducible clinical response in piglets.

Twenty four hours after inoculation, increased frequency of discharge and fluid contents in the faeces, interpreted as mild diarrhoea, was observed in three of four piglets inoculated with enteropathogenic *E coli* E851/71 and in two of four piglets given strain E2381/70. In contrast, piglets inoculated with other strains, including the non-pathogenic control strain, produced semi-solid or pasty yellow faeces. One piglet inoculated with enteropathogenic *E coli* UR became moribund within 20 hours, and another, given enteropathogenic *E coli* E63/68, died of causes unrelated to infection.

Forty eight hours after inoculation, the two remaining piglets inoculated with strains E508/69 and E2381/70 were moribund, while mild diarrhoea was observed in both piglets inoculated with enteropathogenic *E coli* E851/71 and UR, and in one piglet given strain E2348/69. The illness caused by enteropathogenic *E coli* E508/69, although severe, was not accompanied by diarrhoea. The clinical findings are summarised in Table 2.

NECROPSY FINDINGS

Necropsy findings were unremarkable. The

intestine of a few piglets, notably those infected with enteropathogenic *E coli* 508/69, E2348/69, E2381/70, and E851/71 contained liquid faeces, but otherwise all appeared grossly normal.

HISTOPATHOLOGY

Despite the range of clinical responses of piglets to the different enteropathogenic *E coli* strains, the pathological changes resulting from infection were broadly similar, differing only in severity and extent. All animals that received enteropathogenic *E coli*, but not those infected with the normal flora strain, showed focal inflammation of the lamina propria of the large and small intestine. Pathological changes occurred throughout the intestine, but were most pronounced in the distal ileum and colon.

In general, the severity and extent of the pathological changes corresponded to the duration of infection and the clinical presentation. Thus, in piglets infected with enteropathogenic *E coli* that produced no ill effects - for example, strains E2347/69 and E74/68, histological changes were restricted to widely scattered foci of submucosal inflammation (Figs. 1a, and 2a, Table 2). In contrast, enteropathogenic *E coli* that caused clinical disease - for example, strains E851/71, E2381/70, and E508/69, elicited extensive mucosal and submucosal polymorphonuclear leukocytic infiltration, hyperaemia and oedema (Figs. 1b, c and 2b, c). The consistency of the changes induced

Fig. 1 Light micrographs of the large bowel of gnotobiotic piglets 24 hours after inoculation with different strains of human enteropathogenic *Escherichia coli* showing variable degrees of mucosal changes. (a) Strain E2347/69 had no effect on the integrity of the epithelial lining, and the lamina propria is only mildly infiltrated with inflammatory cells. Note bacterial clumps intimately associated with the epithelium (arrows) ($\times 200$ original magnification). (b) Strain E851/71 induced moderate changes. The epithelium is irregular, slightly flattened and sloughed at the villous tips. The lamina propria is infiltrated with inflammatory cells ($\times 200$ original magnification). (c) *E. coli* E2381/70 caused much more severe changes. The epithelium is flat or absent with focal vacuolation and nuclear stratification. Cellular infiltration in the mucosa can be seen exuding into the lumen through ulcerated villi ($\times 192$ original magnification). Crypt cells in all three sections appear normal. Haematoxylin-eosin.

by each strain made it possible to rank the bacteria in decreasing order of pathogenicity as follows: E508/69 > E2381/70 > E851/71 > E2348/69 > UR > E2347/69 > E63/68 and E74/68.

The extent of bacterial colonisation observed in PAP-stained sections paralleled the histopathological findings. Enteropathogenic *E. coli* numbers appeared greatest in those piglets that showed the most pronounced morphological changes and in those regions of the intestine that were most severely affected (Table 2). This observation was confirmed by the bacterial counts in the small intestine, which revealed an increase in bacterial numbers from the proximal to the distal small intestine.

ELECTRON MICROSCOPY

Electron microscopic examination of sections of

large and small intestine from piglets given strains E2381/70, E851/71, and E2347/69 showed that these bacteria induced similar, distinctive lesions of the mucosa, which varied only in extent and severity in keeping with the light microscopic findings. In general, lesions with adherent bacteria were multifocal in the ileum and more diffuse in the colon, but otherwise were similar throughout the intestine.

The brush border and apical cytoplasm of infected epithelial cells showed varying degrees of degeneration. At sites of bacterial attachment, microvilli were absent; between attachment sites they were elongated, shortened or absent (Fig. 3). Increased density of the terminal web directly beneath attached bacteria was frequently seen (Fig. 3). The plasma membrane was cupped or raised in the form of pedestals beneath attached

Fig. 2 Light micrographs of the small intestine of gnotobiotic piglets 48 hours after inoculation with different strains of human enteropathogenic *Escherichia coli* showing mucosal changes of differing severity. (a) and (b) show the contrasting effect of strains E74/68 and E851/71 respectively on the mucosa in the midileum. (a) *E. coli* E74/68 induced no apparent changes. Extensive vacuolation of enterocytes is normal ($\times 85$ original magnification). (b) By contrast *E. coli* E851/71 caused marked shortening of villi and lengthening of crypts. Villi are heavily infiltrated with inflammatory cells, and absorptive cells are flat or absent at some villous tips ($\times 200$ original magnification). (c) Strain E508/69 induced mucosal changes throughout the small intestine. This section through the proximal jejunum shows swollen villi heavily infiltrated with inflammatory cells. The tissue appears extremely friable ($\times 200$ original magnification). Haematoxylin-eosin.

bacteria (Fig. 4). Apart from brush border degeneration, damage to the epithelium was evidenced by loss of apical cytoplasm (Fig. 4) and extrusion of severely affected cells.

Occasional absorptive cells containing bacteria within membrane-bound vacuoles were observed in the ileum and colon (Fig. 5). Bacteria were not seen to penetrate between epithelial cells. Some bacteria were seen in the cytoplasm of phagocytes in the intestinal lumen, but not in those in the lamina propria.

LACTASE ACTIVITY

Alterations to small intestinal integrity and function, as evidenced by reduced lactase activity, corresponded with the extent and severity of the morphological changes. In piglets inoculated with

the more virulent enteropathogenic *E. coli* strains, lactase concentrations in the proximal and mid small intestine were generally less than half those in piglets given enteropathogenic *E. coli* strains of reduced pathogenicity or the normal flora strain (Spearman's rank order correlation coefficient [ρ] = -0.70 and -0.59 for the jejunum and mid ileum respectively; $p < 0.05$). In the terminal ileum, where lactase concentrations are physiologically low, no significant correlation between histological findings and lactase activity was found ($\rho = -0.32$; $p > 0.1$).

Discussion

Early descriptions of enteropathogenic *E. coli*-induced changes in experimental animals are

Fig. 3 Electron micrograph showing bacteria adhering to the apical surface of enterocytes in the midileum. Note the elongated microvilli between bacteria, and the absence of microvilli and increased terminal web density at sites of bacterial attachment. ($\times 10\,000$ original magnification).

Fig. 4 Electron micrograph of the colon showing bacteria adhering to surface epithelial cells and to cellular material shed into the lumen. There is some loss of apical cytoplasm and disruption of cellular organelles ($\times 7500$ original magnification).

Fig. 5 Electron micrograph of the ileum showing bacteria within a membrane bound vacuole in the cytoplasm of an absorptive cell. The separation of bacterial cell contents from the cell wall may have occurred during processing ($\times 6000$ original magnification)

conflicting.^{18–20} Although these differences could be explained by variations in enteropathogenic *E coli* pathogenicity, it seems more likely that some researchers were working with both enteropathogenic and enterotoxigenic *E coli*, which at that time were not recognised as separate entities that cause diarrhoea by distinctive mechanisms and therefore produce different pathological effects. Failure to distinguish between *E coli* of different pathogenic groups may also account for some of the contradictory reports of the pathology of *E coli* diarrhoea in children, in whom findings ranging from normal^{21 22} to extremely severe, with intestinal perforation and gangrene,^{23 24} have been described. The range of intestinal pathology found at necropsy of neonates suggested to Rho *et al* that host rather than bacterial factors influenced the course of infection more strongly.²⁵ Another explanation is that enteropathogenic *E coli* comprise a heterogeneous collection of bacteria lacking easily identifiable virulence characteristics. The finding that enteropathogenic *E coli* strains of different serotypes and of diverse origin produced similar pathological changes in experimental animals indicates, however, that different enteropathogenic *E coli* strains probably produce

enteritis by the same mechanism.

Notwithstanding the reported variations in enteropathogenic *E coli*-associated pathology, light microscopic examination of the intestine of most children with enteropathogenic *E coli*-induced diarrhoea shows infiltration of the mucosa and submucosa by polymorphonuclear leucocytes, plasma cells, and macrophages, accompanied by varying degrees of oedema and capillary congestion.^{13 24–26} This picture resembles that seen in experimentally infected piglets.

Recent electron microscopic studies of enteropathogenic *E coli*-induced changes in experimental and naturally occurring infections have provided a more coherent picture of enteropathogenic *E coli* enteritis. In independent studies of human enteropathogenic *E coli* infection of rabbits, Polotsky *et al*²⁷ and Prescott²⁸ reported ultrastructural changes in enterocytes, including microvillous degeneration, and cupping and pedestal formation, similar to those found in piglets in the present study. These changes also characterise the disease in naturally infected children^{13 26} and in rabbits infected with *E coli* RDEC-1, a non-enterotoxigenic, naturally-occurring rabbit pathogen.^{29 30} Recently, Moon *et al*,³ have shown that strain RDEC-1 and several human enteropathogenic *E coli* strains give rise to similar light and electron microscopic changes in gnotobiotic piglets and in rabbit and piglet ligated ileal loops.

Our findings agree largely with those of Moon *et al*,³ except that we found considerably less animal-to-animal variation in response to each enteropathogenic *E coli* strain than that reported by Moon's group.³ This apparent conflict can be explained by the fact that, in our study, piglets inoculated with each enteropathogenic *E coli* strain were litter mates, given a measured bacterial inoculum, reared together under identical conditions and necropsied at specified time intervals. It is noteworthy, however, that different enteropathogenic *E coli* strains, including some of the same serotype – for example, E508/69 and E2347/69, both of which are 0114:H2, produced clinical and pathological changes of varying severity. Whether this disparity reflects differences in the inherent pathogenicity of different enteropathogenic *E coli* or variations in host susceptibility remains uncertain.

The close resemblance of enteropathogenic *E coli*-induced changes in piglets to those reported in naturally infected humans and rabbits indicates that gnotobiotic piglets are a useful model of human disease. Equally striking was the finding that the relative pathogenicity of human

enteropathogenic *E coli* for piglets corresponded to the pathogenicity of the same strains in adult volunteers.¹⁴

A possible drawback of the gnotobiotic piglet model, apart from its lack of general availability, is that diarrhoea did not always occur in animals infected with enteropathogenic *E coli*. This may be explained by the fact that, in neonatal piglets, most absorption of fluid and electrolytes takes place in the proximal small intestine, whereas most enteropathogenic *E coli*-induced pathology occurred in the terminal ileum and colon. This explanation is supported by the finding that those enteropathogenic *E coli* strains which caused diarrhoea generally colonised the small intestine more extensively and gave rise to more severe and widespread pathological changes in the intestine than strains that did not cause diarrhoea. It is also possible, however, that diarrhoeagenic enteropathogenic *E coli* may possess virulence factors, such as enterotoxins, in addition to those necessary for adherence and the production of tissue damage.

In summary, this report documents the usefulness of gnotobiotic piglets as a model for enteropathogenic *E coli* enteritis. Support for this conclusion stems from (i) the similar clinical signs in piglets and humans infected with enteropathogenic *E coli*, apart from the absence of diarrhoea in some experimental animals; (ii) the similar light and electron microscopic observations in experimental and naturally occurring enteropathogenic *E coli* infections; (iii) the similar bacterial colonisation of the small intestine as revealed by culture and PAP staining; (iv) the occurrence of brush border damage and reduced lactase activity in experimentally infected piglets and naturally infected children,^{13 26} and (v) the corresponding severity of enteropathogenic *E coli*-induced disease in piglets and adult volunteers. The specificity of the piglet model was further shown by the failure of an avirulent normal flora *E coli* to induce any morphological or functional abnormalities in infected animals.

The availability of the piglet model will enable investigation of the pathogenic mechanisms of enteropathogenic *E coli*, including the relative contribution of adhesion, invasive ability and enterotoxigenicity to virulence. Recently, piglets were used to investigate the role of plasmids in enteropathogenic *E coli* pathogenicity.¹¹ It will now also be possible to examine factors influencing the immunity to and recovery from enteropathogenic *E coli* disease so that effective means of treating and preventing this condition may be developed.

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