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Effect of Experimentally-induced Villus Atrophy on Adhesion of K88ac-positive *Escherichia coli* in Just-weaned Piglets

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ABSTRACT

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Three- to four-week-old, just-weaned piglets were infected with transmissible gastroenteritis (TGE) virus and the next day with K88ac⁺ enterotoxigenic *Escherichia coli* (ETEC). Histological examination of caudal jejunum and ileum of piglets killed 2-3 days after virus challenge (1-2 days after ETEC infection) revealed severe villus atrophy especially in the jejunum compared with controls (P< 0.05). Four-5 days after TGE virus infection villus length increased and after 7 days it was near normal. Villi scraped from jejunal and ileal mucosa of the piglets were incubated in vitro with K88ac⁺ *E. coli* and the number of bacteria adhering to 250 µm villus brush border was counted. Attachment of bacteria to villi of piglets killed 2-3 days after TGE virus infection was significantly decreased in comparison with adhesion to villi of non-infected piglets or of piglets killed 7 days after the virus infection. Correlation between in vitro adhesion and villus height was 0.6649 (P<0.001). The results suggest that the experimentally-induced villus atrophy was attended with a temporarily diminished susceptibility of villus enterocytes to adhesion of K88ac⁺ *E. coli*.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are one of the most important causal agents of acute diarrhoeal disease in man and young animals (Morris and Sojka, 1985; Black, 1986; Taylor and Echeverria, 1986; Wilson and Francis, 1986). ETEC strains possess long threadlike protein polymers (fimbriae) on their surface which mediate adhesion to specific membrane receptors or receptor sites on villus enterocytes of the small intestine. Specific adherence
allows the ETEC strains to resist the flushing mechanism in the small intestine and to colonize the tissues (Klemm, 1985). Children in developing countries and domestic livestock throughout the world often show a combination of ETEC and enteric viral infection (mostly rotaviruses, sometimes coronaviruses) (House, 1978; Newsome and Coney, 1985; Wilson and Francis, 1986). This dual infection induces a more severe diarrhoea than monoinfections. In calves even synergistic effects have been reported (Hess et al., 1984). The enteropathogenic viruses damage the small intestinal villi, resulting in villus atrophy (Moon, 1978). Such damage can change membrane nature (Egberts et al., 1984) and might subsequently influence attachment of ETEC to enterocytes. Lecce et al. (1982) postulated that the injury caused by a rotavirus infection could alter the binding sites on villus enterocytes so that the adhesion of ETEC strains to villi is facilitated. In this communication we describe the effect on intestinal villus height during experimentally-combined infections of piglets with a coronavirus (transmissible gastroenteritis virus) and K88ac fimbriae-possessing ETEC (Cox et al., 1986) and the effect of villus shortening on adhesion of K88ac E. coli to villi of piglets.

MATERIALS AND METHODS

Bacterial strains

The characteristics of ETEC strains used are listed in Table 1. The enterotoxin production was determined by L. Okkerman (Faculty of Veterinary Medicine, Ghent, Belgium). The thermo-labile (LT) enterotoxin was identified with a reversed passive latex agglutination kit (VET-RPLA kit, Oxoid), whereas for the production of the thermo-stable enterotoxin type a (STa) the suckling mouse bioassay was used (Scotland et al., 1985). The thermo-stable type b (STb) enterotoxin was determined by DNA colony hybridization with a gene probe by J.G. Mainil as reported (Mainil et al., 1986). The strains were

| Strain | Serotype | Fimbriae | Enterotoxin | Source |
|--------|----------|----------|-------------|--------|
| GIS 25 | 0149:K91:K88ac | K88ac | LT, STa, STb | RIT |
| 156KP83 | 0149:K91:K88ac | K88ac | LT, STa, STb | NIDO |
| E57 | 0138:K81 | None | STa, STb | NIDO |

1LT, heat-labile enterotoxin; STa, heat-stable enterotoxin type a; STb, heat-stable enterotoxin type b.

2RIT, Smith-Kline RIT, Genval, Belgium; NIDO, P. Pohl and P. Lintermans, National Institute for Veterinary Research, Brussels, Belgium.
stored at \(-20^\circ\)C in tryptone soy broth (Oxoid) supplemented with 20% (v/v) glycerol.

**Animals**

Twenty-six, 3- to 4-week-old female piglets from primiparous sows were purchased from the same commercial farm. Sows were vaccinated 6 and 2 weeks prior to delivery with a polyvalent *E. coli* vaccine containing among others, 0149:K91:K88ac antigens (Gletvax®; Wellcome). The vaccination of gilts results in passive immunity of suckling piglets against K88 ETEC-mediated neonatal enteric colibacillosis via colostrum and milk (Kohler, 1978). Two days post-weaning, however, 3-week-old piglets are susceptible again to *E. coli* enteritis (Nagy et al., 1979). Immediately after weaning, piglets were individually housed and during the experiment they were allowed to drink ultra high temperature sterilized whole cow's milk ad libitum.

**Experimental procedure**

During 3 days prior to infection 18 piglets were treated with chloramphenicol (Chloromycetin®, Parke-Davis) suspended in their milk (1875 mg l\(^{-1}\) milk). This antibiotic suppresses the anaerobic microflora and may result in colonization of the intestine with potentially pathogenic microorganisms (Kaufman, 1984). After a starvation period of 3 h, piglets were orally infected with TGE virus (1.66 × 10\(^6\) pig infective dose) on Day 4. The TGE virus was supplied by Prof. Dr. M. Pensaert (Faculty of Veterinary Medicine, Ghent, Belgium). On the fifth day, again after a starvation period of 3 h, these 18 piglets were given 62 ml of a 1.4% NaHCO\(_3\) solution to neutralize gastric acidity (prevention of ETEC destruction); 15 min later each piglet was intragastrically inoculated with 10 ml bacterial suspension of strain GIS 25 and with 10 ml suspension of strain 156KP83. The bacterial suspensions were prepared by inoculating the strains overnight on brain heart infusion agar (Oxoid) at 37°C. Afterwards they were examined for production of K88ac fimbriae with a slide agglutination test. They were then suspended and diluted in sterile physiological saline until an \(A_{620}\) of 0.4 (approximately 1.2 × 10\(^9\) bacteria ml\(^{-1}\)).

Six piglets were killed 2–3 days (Group 1), 3 piglets 4–5 days (Group 2) and the 9 remaining piglets (Group 3) 7 days after the TGE virus challenge. Eight piglets were kept as uninfected controls (Group C) and were killed between 5 and 10 days after weaning. Piglets were killed with an intraperitoneal injection of methomidate (Hypnodil®, Janssen).

**Samples**

Immediately after euthanasia, the abdomens of the piglets were opened and 15-cm long intestinal segments were excised at two sites: at two-thirds of the
distance between the pylorus and the ileocaecal valve (caudal jejunum) and also 2 cm cranial to the ileocaecal valve (ileum). The segments were opened and washed in a Krebs buffer containing 1% formaldehyde after which villi were gently scraped from the mucosa with a glass slide, washed several times with the same buffer and stored in this buffer. The formaldehyde was added to the buffer to stop all enzymatic reaction which would lyse epithelial cells. Within 3 weeks the in vitro villus adhesion assay was performed.

Except for 1 piglet in Group 1 and 1 in Group 3, specimens of the caudal end of the excised small intestinal segments were fixed in 10% phosphate-buffered formalin for histological examination.

**Villus adhesion assay**

This assay was based on the in vitro technique described by Girardeau (1980). The stored villi were placed in a Krebs buffer without formaldehyde and after 1 h the buffer was renewed. This step was repeated 4 times. About 50 villi were transferred with a pipette into 1 ml 0.1 M phosphate buffer (pH 6.7) containing 1% (w/v) D-mannose (Difco), after which 0.1 ml of a bacterial suspension in sterile physiological saline, containing strains GIS 25, 156KP83 or E57 (Table 1), was added. The presence of D-mannose in the buffer results in an inhibition of the cellular adhesion of bacteria by Type 1 pili. Bacterial suspensions were prepared by inoculating the 3 strains overnight on 5% sheep blood agar (Oxoid) at 37°C, examining them for production of K88ac with a slide agglutination test, using a specific antiserum (RIVM, Bilthoven, The Netherlands), reinoculating them on tryptone soy agar (TSA) (Oxoid) during 24 h at 37°C and retesting them for production of fimbriae. The strains on TSA were suspended and diluted in sterile physiological saline until an A620 of 1.2 (approximately 4.5×10⁹ bacteria ml⁻¹ suspension). After gently mixing villi and bacterial suspension for 1 h at room temperature, some villi were removed with a pipette, placed under a cover slip and examined by light microscopy at magnification ×600. When no adhesion was observed piglets were considered as genetically resistant (Bijlsma et al., 1982; Sellwood, 1983). When adhesion occurred, however, it was quantified by counting the number of visible bacteria adhering along 50 μm villus brush border at 5 different places villus⁻¹ (250 μm villus⁻¹) and on 5–6 villi small intestinal segment⁻¹. Villi of each segment were tested twice. The mean bacterial adhesion 250 μm⁻¹ villus length and experimental group⁻¹ was calculated for piglets with a K88ac adhesive phenotype.

**Histological examination**

The small intestinal specimens fixed in formalin were dehydrated, embedded in paraplast, cut at 5 μm and stained with hematoxylin and eosin. Only for
K88ac genetically-susceptible piglets were villus height (from the tip of the villus to the villus/crypt junction) and crypt depth (junction of villus/crypt to base of the crypt) measured, using an ocular micrometer, for 10 well-oriented villi segment\(^{-1}\). Subsequently the mean villus height and crypt depth intestinal segment\(^{-1}\) and experimental group\(^{-1}\) were calculated.

**Statistical procedures**

Data were presented as means ± standard error of the mean (SEM) and were analysed by a two-level nested analysis of variance (ANOVA) with unequal sample sizes. Significance was accepted at the \(P<0.05\) level. Pearson product–moment correlation \((R)\) was calculated between mean villus height in an intestinal segment and the mean number of bacteria adhering in vitro to villi of the same segment.

**RESULTS**

The in vitro adhesion assay revealed that the villi of all piglets in Group 1 and Group 2, of 6 of the 9 piglets in Group 3 and of 5 of the 8 piglets in the control group (Group C) were susceptible to adhesion of K88ac ETEC (Table 2). Only data of susceptible piglets were included in the results. The infected piglets developed severe diarrhoea, whereas some started vomiting. All piglets slaughtered 2–5 days after the TGE virus infection (Groups 1 and 2) were severely dehydrated and markedly depressed. In the other infected piglets, faecal fluid loss and dehydration seemed to be less pronounced and recovery occurred 5–6 days after the virus challenge. These piglets were killed 7 days after TGE virus inoculation (6 days after the ETEC inoculation; Group 3). At that time only one piglet showed diarrhoea. One of the control piglets spontaneously developed diarrhoea during the observation period, which was still present at necropsy. The other control piglets remained healthy.

Mean villus height and crypt depth in jejunum and ileum of K88ac-suscep-

| Pig phenotypes relating to adhesion of K88ac-positive *E. coli* to their villi |
|---|---|---|---|
| Group | \(N\) \(^1\) | Phenotype |
| | | Adhesive | Non-adhesive |
| C | 8 | 5 | 3 |
| 1 | 6 | 6 | 0 |
| 2 | 3 | 3 | 0 |
| 3 | 9 | 6 | 3 |

\(^1\)Number of pigs tested in each group.
tible piglets in the different groups are illustrated in Fig. 1. In Group 1, a significant villus atrophy occurred in the caudal jejunum, whereas in the ileum a similar but not significant villus atrophy was observed. Villi or villus tips were covered with cuboidal enterocytes. Patchy to continuous attachment of coliform bacteria to villi was observed in the jejunum of 4 piglets and the ileum of 3 piglets of Group 1. In one piglet no adhesion of bacteria to jejunal and ileal villi was observed. In intestinal segments where adhesion was absent, villi were extremely short, less than 120 μm. In the other small intestinal segments, villus length was above 180 μm. Four to five days after the TGE virus infection (3–4 days after the ETEC infection; Group 2) villi were still slightly shortened (Fig. 1). Villus enterocytes were low to high columnar. A patchy bacterial adherence with irregularly arranged groups of bacteria of various sizes occurred in the jejunum of one piglet and the ileum of all 3 piglets. In piglets killed 7 days after the TGE virus infection (6 days after the ETEC infection; Group 3), villi were almost as high as in control piglets (Fig. 1). Villus enterocytes were high columnar. No bacterial adhesion was observed. In the control group, on the contrary, multifocal adhesion of coliform bacteria was observed on villi in 2 piglets. One had diarrhoea when euthanatized, the other was healthy. A slight villus atrophy was observed in both piglets. The other control piglets had long slender villi covered with high columnar enterocytes. Crypt depth did not differ significantly between the 4 groups (Fig. 1).

The bacteria adhering after incubating villi for 1 h with strains 156KP83 or

![Graph](image-url)

**Fig. 1.** Mean (± SEM) villus height and crypt depth in caudal jejunum and ileum of non-infected piglets (empty bars: Group C: n=5) and of piglets infected with TGE virus and enterotoxigenic *E. coli* (striped bars). Piglets of Group 1 (n=6), Group 2 (n=3) and Group 3 (n=6) were euthanatized 2–3, 4–5 and 7 days after TGE virus challenge, respectively. Ten villi and crypts were measured small intestinal segment⁻¹. a, in comparison with Group C; b, in comparison with Group 3; P < 0.05.
GIS 25 was a combination of in vivo adhesion (oral infection with both strains) and of in vitro adhesion (in vitro incubation of villi with one of both strains). The in vitro adhesion of the E57 strain was evaluated by comparing the counts of non-incubated villi with values obtained after incubating the villi. Whereas both K88ac-strains adhered equally to villi, no adhesion of the E57 strain was observed. As can be seen in Fig. 2, adhesion of K88ac-positive strains to jejunal villi was significantly decreased for piglets of Group 1 in comparison with non-

![Fig. 2. Number of K88ac-positive E. coli adhering along 250 µm villus brush border of villi isolated from jejunal and ileal segments of piglets. Villi were obtained from non-infected piglets (empty bars: Group C: n = 5) and from piglets infected with TGE virus and enterotoxigenic E. coli (striped bars) and euthanatized 2–3 (Group 1: n = 5), 4–5 (Group 2: n = 3) and 7 days (Group 3: n = 5) after TGE virus inoculation. Bars represent mean number of bacteria 250 µm⁻¹ villi and experimental group⁻¹ ± SEM. a, in comparison with Group C; b, in comparison with Group 3; P < 0.05.](image1)

![Fig. 3. Relationship between adhesion of K88ac-positive E. coli to villi and villus height. Each mark (n = 36) represents the mean values from jejunum or ileum of 1 piglet (n = 18). The fitted line represents the least square linear regression (equation: E. coli 250 µm⁻¹ brush border = -10.84 + 0.302 × villus height), whereas R is the correlation coefficient.](image2)
infected piglets (Group C) and with piglets recovering from the experimental infection (Group 3). Adhesion to ileal villi of Group 1 was significantly decreased in comparison with adhesion in Group 3. In Group 2 the adhesion to jejunal and ileal villi was similar to the adhesion in Groups 3 and C.

The correlation between mean villus height intestinal segment$^{-1}$ and the mean number of bacteria adhering along 250 $\mu$m villus brush border of the same segment was $R=0.6649$ ($P<0.001$), indicating that a significant correlation existed between both parameters (Fig. 3).

**DISCUSSION**

The villus height measured in piglets of Groups 1 (48–72 h after TGE), 2 (96–120 h after TGE) and 3 (168 h after TGE) in our study is comparable with the atrophy measured by Shepherd et al. (1979) at 40, 72 and 144 h, respectively, after experimental infection of 3-week-old piglets with TGE virus. This suggests that villus atrophy measured in this study was primarily a result of the TGE virus infection. However, Hess et al. (1984) observed a more pronounced villous atrophy in calves simultaneously infected with ETEC and rotavirus than in calves infected with ETEC alone or with rotavirus alone, and Rose et al. (1987) proved that the STb enterotoxin is capable of causing partial villous atrophy in young pigs. So, the ETEC infection could have aggravated the virus-induced villus shortening in our experiment.

In Group 1, in vivo adhesion of coliform bacteria to villi was noted in histological specimens of most piglets. No adhesion, however, was observed in specimens with severe atrophic villi (smaller than 120 $\mu$m). Since K88-positive ETEC specifically adhere to brush border membranes from genetically-susceptible pigs, this absence of in vivo adhesion on these severe shortened villi could have indicated that piglets had a non-adhesive phenotype (Bijlsma et al., 1982; Sellwood, 1983). The vitro adhesion assay, however, revealed attachment of K88ac ETEC to the atrophic villi. Thus, the piglets belonged to the adhesive phenotype. Nevertheless, after the in vitro incubation of villi with K88ac E. coli, the number of bacteria adhering along 250 $\mu$m villus brush border was still significantly less in this group than in Groups 3 and C. The atrophic villi in Group 1 were covered with cuboidal to low-columnar cryptlike cells. An electron microscopic study of the ultrastructure of these cells showed a short and sometimes incomplete microvillar border (Cox et al., 1988). As reported by Pensaert et al. (1970b) and Shepherd et al. (1979), the cuboidal shape and the short and irregular brush border are indications of the immature nature of these enterocytes. In Group 2 (4–5 days after TGE virus infection) bacterial adhesion was again more pronounced and reached values comparable with results in Groups 3 and C. At this time villus height increased and villus enterocytes regained their normal structure. Transmission electron microscopic
examination of the enterocytes of piglets in this group revealed microvilli which were longer than in Group 1 (Cox et al., 1988). In Group 3 (piglets killed 7 days after the viral infection) villus length, villus structure and adhesion to the villi were similar to the control group. Total recovery had probably occurred. This is in accordance with the results of Shepherd et al. (1979) who observed recovery of villus structure 3–6 days after TGE virus infection. Recently we evaluated the in vitro adhesion of K88ac E. coli to villi of 5 piglets killed 2 days after an oral inoculation with the 2 ETEC strains used in this study. The number of bacteria adhering to jejunal villi (81 ± 6 E. coli 250 μm⁻¹ villus brush border) and to ileal villi (51 ± 5 E. coli 250 μm⁻¹ villus brush border) was the same as in Group C in this experiment (E. Cox et al., unpublished observation, 1987), which indicates that the decreased in vitro adhesion in Group 1 was not due to a direct effect of the ETEC strains. The decreased in vitro adhesion on villi 2–3 days after TGE virus challenge was probably the reflection of a temporary change in brush border membrane receptors occurring as a result of the viral infection. The enterocytes covering the villi during the phase of cell replacement following the virus infection (Pensaert et al., 1970a, 1970b; Shepherd et al., 1979) are not fully differentiated and probably possess a decreased number of receptors or receptor sites for K88ac-positive ETEC. This is in agreement with earlier studies showing that K88-positive strains adhere to the brush border of villus enterocytes but not to the undifferentiated crypt cells (Arbuckle, 1970; Bertschinger et al., 1972; Hohmann and Wilson, 1975).

Apart from virus-induced villus shortening, the variation in adhesion could have been due to the presence of piglets with a weak-adhesive phenotype (Sellwood, 1983; Bijlsma and Bouw, 1985). This phenotype is also an inherited characteristic.

Our findings indicate that the experimentally-induced villus atrophy probably results in a temporary diminished susceptibility of villus enterocytes to K88ac ETEC adhesion. When villi regenerate, enterocytes seem to regain their normal adhesion capacity. Further experiments are requested to determine the exact mechanism for the observed phenomenon.

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