

# Immunocytochemical studies of *Salmonella* Typhimurium invasion of porcine jejunal epithelial cells

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Received 7 January 2004  
Accepted 25 February 2004

Although infection of pigs with *Salmonella* Typhimurium represents a serious problem, most studies on *Salmonella* infection have been carried out in other species. The purpose of the current study was to examine the route(s) of entry of *Salmonella* Typhimurium in pigs, using a jejunal loop model. The infection process was followed over 240 min using single to triple immunocytochemical detection of *Salmonella* and intestinal cell markers. *Salmonella* invasion was observed in both cytokeratin-18-positive and -negative cylindrical absorptive cells within 5–10 min. Subepithelial invasion of ordinary villi was consistently less marked than invasion of the subepithelial layer of Peyer's patches. Our results show that several epithelial cell types were invaded by *Salmonella*, and that Peyer's patches represent the main portal of entry in early *Salmonella* infection. Additionally, infection was associated with alterations in the keratin and F-actin cytoskeleton of intestinal epithelial cells, probably reflecting toxin-mediated actions. Such changes were confined to the proximal region of the jejunum, demonstrating a regional heterogeneity of intestinal epithelial cell responses to *Salmonella* infection.

## INTRODUCTION

Salmonellosis is a major disease of pigs and is mainly manifested as enterocolitis and/or septicaemia. Most cases of porcine salmonellosis in Europe are caused by *Salmonella enteritica* serovar Typhimurium (hereafter referred to as *S.* Typhimurium) (Kaesbohrer, 1999). Even though salmonellosis is a common disease of pigs, there have been few attempts to determine which intestinal epithelial cell types are invaded *in vivo*. Studies in mice have indicated that membranous (M) cells represent the main portal of entry (Jones *et al.*, 1994), while studies in calves and pigs have shown that enterocytes and goblet cells are also invaded within minutes (Frost *et al.*, 1997; Meyerholz *et al.*, 2002). The latter studies were carried out by electron microscopy of calf and pig ileal loops. A problem with electron microscopy is the use of small samples that conceivably could lead to bias. We therefore decided to undertake a light microscopical study of *S.* Typhimurium infection using a previously well-characterized jejunal loop model (Grondahl *et al.*, 1998). Infection was evaluated using single to triple immunocytochemical identification of *Salmonella* and intestinal cell markers. The results show that several epithelial cell types are invaded by *Salmonella*, and that Peyer's patches (PP) represent the main portal of entry in early infection.

Additionally, infection was found to be associated with characteristic alterations in the keratin and F-actin cytoskeleton of epithelial cells in the proximal part of the jejunum.

## METHODS

**Animals.** Danish Landrace/Yorkshire crossbreed, 8–10 week old (16–20 kg) fully weaned female pigs were used for the loop model ( $n = 4$ ) and as a control pig ( $n = 1$ ). Faecal samples from all animals were cultured, and found negative for *Salmonella* (tested by the Danish Veterinary Institute, Copenhagen, Denmark). All experiments followed the ethical guidelines laid down by the Danish Animal Experiment Inspection.

**Surgical procedure.** For surgery, the procedure described by Grondahl *et al.* (1998) was used with slight modifications to fulfil the new ethical guidelines. In brief, animals were fasted for 12 h and then sedated with an intramuscular injection of 4 mg azaperone kg<sup>-1</sup> (Stresnil; Boehringer-Ingelheim) and 0.3 mg butorphanol tritartrate kg<sup>-1</sup> (Torbugesic; Scanvet). After 20 min, general anaesthesia was given by intravenous injection of 2–4 mg propofol kg<sup>-1</sup> (Rapinovet; Schering-Plough) and maintained by inhalation of isoflurane (Isoflo; Schering-Plough). Blood pressure and rectal temperature were monitored and the latter kept between 35.5 and 37.2 °C. Samples for blood-gas analysis were collected and the pCO<sub>2</sub> was adjusted to about 40 mmHg (5332 Pa) by controlled ventilation.

A midline abdominal incision was made and the jejunum was identified. According to the definition in veterinary anatomical textbooks (Nickel *et al.*, 1982), the proximal border towards the duodenum was defined by the ending of the duodenocolic ligament, whilst the distal border was

Abbreviations: EC cell, enterochromaffin cell; E, eosin; FAE, follicle-associated epithelium; H, haematoxylin; M cell, membranous cell; PP, Peyer's patches.

defined by the start of the ileocaecal ligament. For each animal, nine 5 cm jejunal loops were prepared in the proximal and distal region, separated by at least 2 cm spacer loops. Proximal loops started at 0.3 m distal to the duodenocolic ligament and extended 1.5–2.1 m in a distal direction. Distal loops were placed at 0.1 to 0.7 m proximal of the ileocaecal ligament. Care was taken to ensure that all loops contained Peyer's patches. At least 10 m of intestine separated the proximal from the distal part. After the experiment, loops were removed and animals were killed by an intravenous overdose of sodium pentobarbital.

**Experimental procedure.** Strain *S. Typhimurium* 3389-1 (DT12) was isolated from a clinical case of salmonellosis in pigs and prepared for use as inoculum as described by Grondahl *et al.* (1998). Loops were injected with  $10^{10}$  colony-forming units *S. Typhimurium* suspended in 5 ml buffer A (45 mM NaHCO<sub>3</sub>, 10 mM KCl, 70 mM NaCl, 80 mM D-glucose; pH 7.4) or with buffer A alone. Loops were randomized so that they were injected either with bacteria suspended in buffer A or with buffer A alone. Loops were collected at 0, 5, 10, 15, 30, 60, 90 and 240 min post-injection; however, buffer A alone-injected loops were only collected after 240 min. To preserve early stages of *in situ* infection, loops collected between 0 and 10 min had their contents exchanged with 5 ml 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) before removal. The loops were opened, rinsed under running tap water, placed in 4% paraformaldehyde for 19 h at 4 °C and two to three blocks from each loop were routinely embedded in paraffin or were frozen for cryostat sectioning.

**Immunocytochemistry.** Paraffin sections were dewaxed in xylene, hydrated and blocked for endogenous peroxidase activity in 0.03% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in methanol for 30 min. Sections to be stained for cytokeratin-18 were pretreated with 0.015% pepsin (Sigma) in 0.2 M HCl for 20 min. Sections were subsequently preblocked in normal goat serum, diluted 1:10 and exposed to a rabbit antibody against *Salmonella* (Biogenesis, diluted 1:20 000), to rabbit antibody against serotonin (Incstar, diluted 1:8 000) for 20 h at 4 °C or to a monoclonal cytokeratin-18 antibody (clone CY-90, Sigma; diluted 1:8 000) for 1 h at room temperature. The site of antigen-antibody reaction was revealed either with a triple layer method using biotinylated goat anti-rabbit Ig (Dako, preabsorbed in 0.01% normal swine serum for 20 h at 4 °C) and peroxidase-labelled streptavidin (Dako), followed by development in diaminobenzidine-H<sub>2</sub>O<sub>2</sub> or with alkaline phosphatase-labelled goat anti-mouse Ig (Dako, 1:30; preabsorbed in 0.01% normal swine serum for 20 h at 4 °C), followed by development in Fast Red medium (Larsson, 1988). Double-staining for *Salmonella* and cytokeratin-18 employed a mixture of both primary antibodies, followed by the detection methods described above. Parallel sections were stained with haematoxylin and eosin (H & E).

For F-actin staining, cryostat sections were incubated with Alexa 488-labelled phalloidin (Molecular Probes, diluted 1:40) for 1 h at room temperature and counterstained with bisbenzimidazole (Sigma). Triple-staining for F-actin, cytokeratin-18 and *Salmonella*, where F-actin staining was followed by incubation with a mixture of both primary antibodies. The site of antigen-antibody reaction was revealed with goat Alexa 594-labelled anti-mouse IgG and goat Alexa 350-labelled anti-rabbit IgG (Molecular Probes, both diluted 1:400).

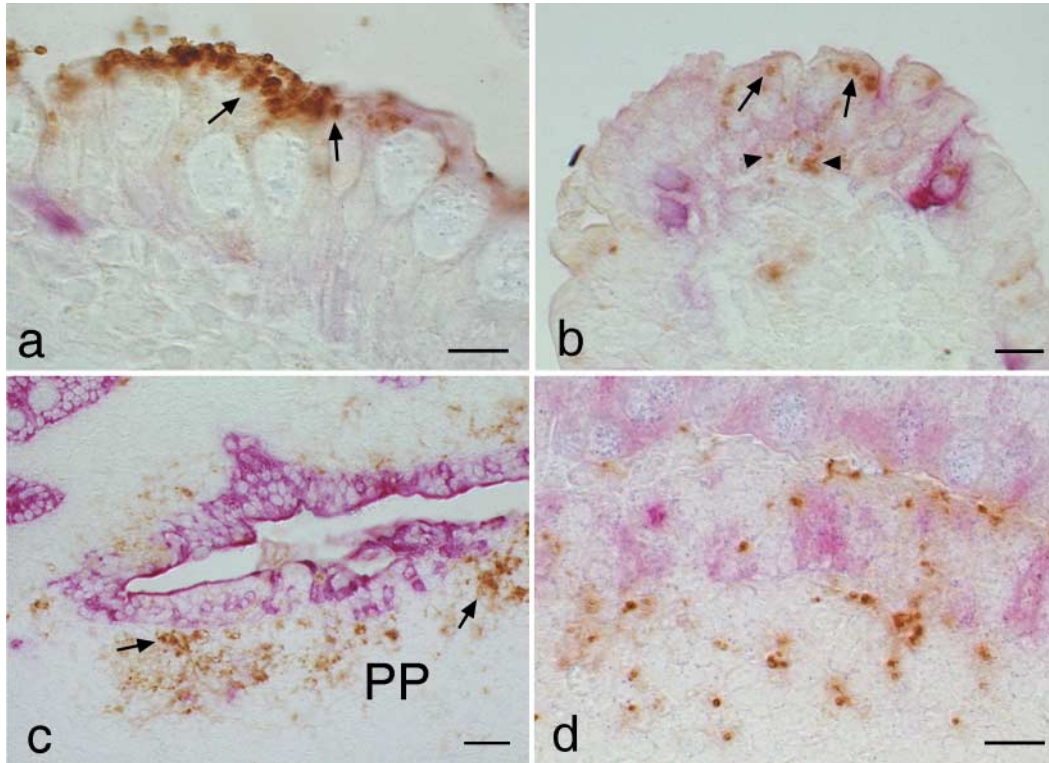
## RESULTS AND DISCUSSION

In the present study, pig jejunal loops were prepared so that they all included PP, which are characterized by follicles of lymphoid tissue forming characteristic domes, and are covered with follicle-associated epithelium (FAE). In both proximal and distal loops, the earliest signs of invasion of epithelial cells were observed within 5–10 min after exposure to *Salmonella*. Early invasion of epithelial cells was char-

acterized by the presence of *Salmonella* in the apical cytoplasm (Fig. 1a). At later stages, when subepithelial invasion was observed, organisms were also detected in the basolateral cytoplasm (Fig. 1b). Extracellular bacteria frequently adhered to the surfaces of the cells and were sometimes found in crevices between epithelial cells. By 5–10 min, scattered epithelial cells of both the FAE and ordinary villi were invaded and invasion of the subepithelial compartment of the PP, but not of ordinary villi, could be detected. By 15–30 min, subepithelial invasion of the PP increased and became marked by 240 min (Fig. 1c, d). By 90 min, subepithelial invasion became marked in a few ordinary villi and in nearly all villi by 240 min. Subepithelial invasion was consistently more marked in the PP than in ordinary villi.

In order to characterize the cell types that were invaded, we used double- and triple-staining for *Salmonella*, cytokeratin-18 [proposed marker for M and brush cells (Gebert *et al.*, 1994; Hofer & Drenckhahn, 1996)] and serotonin [marker for enterochromaffin (EC) cells]. Additionally, HE staining of adjacent thin sections was used for morphological characterization. In the control pig jejunum, staining for cytokeratin-18 revealed scattered-positive cells present both in the FAE and in ordinary villi. The staining pattern was compatible with the reactivity of brush and M cells, but, in addition, goblet and other unidentified cells reacted for cytokeratin-18. The serotonin antiserum stained scattered endocrine-like cells that were present on ordinary villi and crypts, but which rarely occurred in the FAE. Staining of adjacent thin sections for serotonin and cytokeratin-18 demonstrated that a subpopulation of both cell types contained both antigens (Fig. 2a, b). Thus, in addition to brush, M and goblet cells, a subpopulation of EC cells was shown to be cytokeratin-18-positive in the present study.

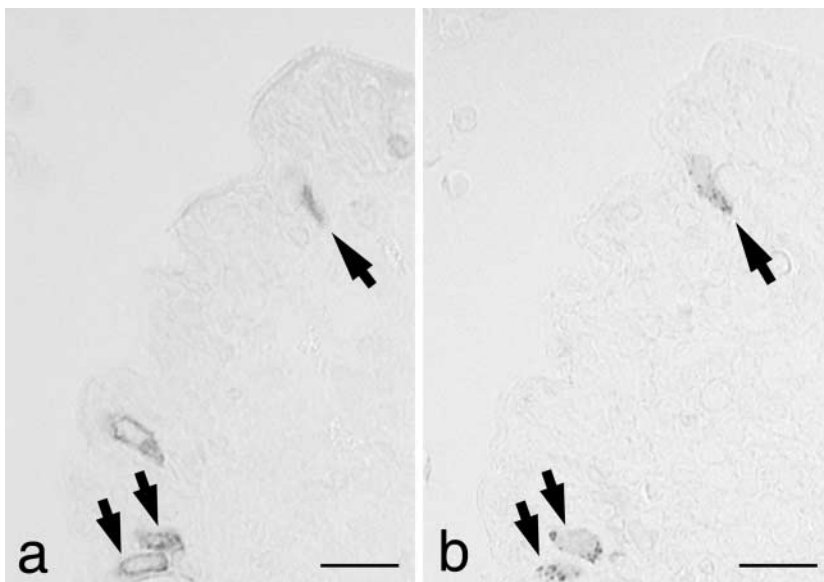
Double-staining for serotonin and *Salmonella* revealed that only very few EC cells were invaded by the organism at all times studied. Double-staining for cytokeratin-18 and *Salmonella* revealed that invasion occurred both in cytokeratin-18-positive and -negative epithelial cells on ordinary villi (Fig. 1a) and PP (Fig. 1d). Thus, in the porcine FAE, invasion was not confined to presumptive M cells but other epithelial cells also became invaded. This contrasts with results obtained in the mouse, which showed that, in this species, M cells were the main cell type invaded (Jones *et al.*, 1994). Our results agree with studies in porcine ileal loop models (Meyerholz *et al.*, 2002; Reed *et al.*, 1986). However, the electron microscopical study by Meyerholz *et al.* (2002) reported that both the villi and PP were markedly invaded in the ileal model. In contrast, the present results show that, in the porcine jejunum, the FAE represents the main portal of entry of *Salmonella* in early infection. According to definitions in veterinary anatomical textbooks, the ileum represents the terminal part of the small intestine to which the plica ileocaecalis is attached (Nickel *et al.*, 1982). Meyerholz *et al.* (2002) employed a definition of the ileum that encompassed a considerably longer stretch of the distal small intestines. Therefore, it is likely that our distal jejunal loops were in the close vicinity of or even overlapped with the loops used by Meyerholz *et al.* (2002). Thus, it is not



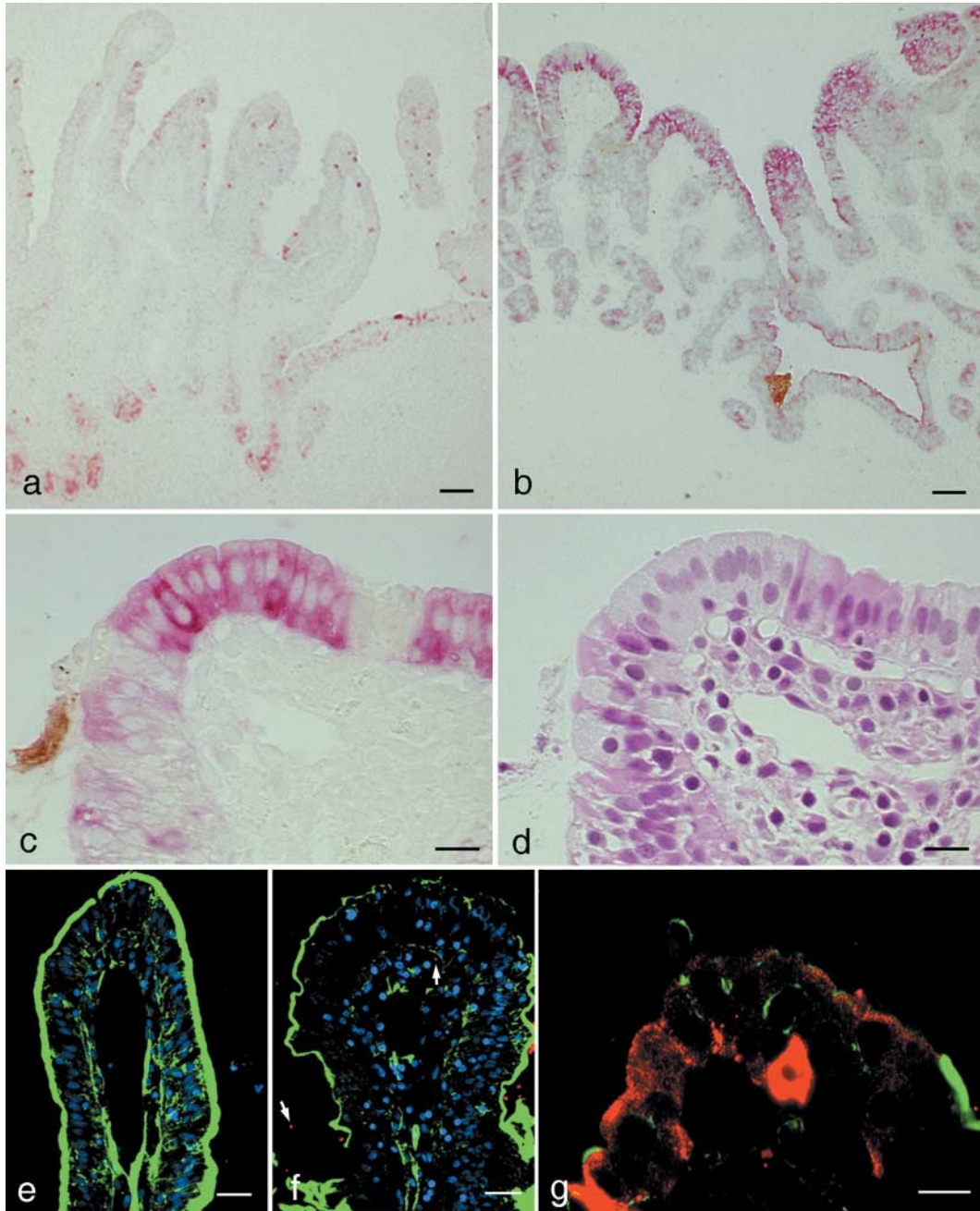
**Fig. 1.** Double-staining for *Salmonella* (brown) and cytokeratin-18 (red). (a) Distal jejunum; loop inoculated for 90 min with *S. Typhimurium*. Note invasion of the apical cytoplasm of epithelial cells (exemplified by arrows). Bar, 5  $\mu\text{m}$ . (b) Distal jejunum; loop inoculated for 240 min with *S. Typhimurium*. Note the presence of *Salmonella* in both apical (arrows) and basolateral positions (arrowheads) in the epithelium. Bar, 10  $\mu\text{m}$ . (c) Proximal jejunum; loop inoculated for 240 min with *S. Typhimurium*. Note heavy invasion of the subepithelial layer of a PP (arrows). Bar, 20  $\mu\text{m}$ . (d) Proximal jejunum; loop inoculated for 15 min with *S. Typhimurium*. Both cytokeratin-18-positive (red) and -negative FAE cells of a PP are invaded by *Salmonella*. Bar, 10  $\mu\text{m}$ .

likely that regional differences account for the discrepancies observed. However, another explanation for the discrepancies may be that Meyerholz *et al.* (2002) inoculated considerably younger pigs with another phage type of *Salmonella*

Typhimurium, whereas we used pigs in the age group where spontaneous infections are most common. However, it has to be emphasized that routes of *Salmonella* invasion might be different elsewhere in the porcine intestine.



**Fig. 2.** Distal jejunum; control loop. Adjacent thin sections stained for cytokeratin-18 (a) and serotonin (b). Note the presence of three cells (arrows) staining for both cytokeratin-18 and serotonin. Bar, 10  $\mu\text{m}$ .



**Fig. 3.** Proximal jejunum; control loop (a, e) and loop inoculated with *S. Typhimurium* (b, d, f and g). (a, b) Staining for cytokeratin-18 (red). Note the increased staining for cytokeratin-18 of the apical epithelial cells on the villi in (b). Bar, 60  $\mu$ m. (c, d) Adjacent sections, double-stained for *Salmonella* (brown) and cytokeratin-18 (red) (c) and stained with HE (d). Apical epithelial cells stain intensely for cytokeratin-18 and exhibit a characteristic pale-staining cytoplasm when stained with HE. Note that the cells are not invaded by *Salmonella*. Bar, 10  $\mu$ m. (e, f) Double-immunofluorescence for F-actin (green) and *Salmonella* (red, exemplified by arrows) counterstained with bisbenzamide (blue nuclei). Note the attenuated or even missing brush border-associated staining for F-actin in (f). Bar, 20  $\mu$ m. (g) Double-immunofluorescence for F-actin (green) and cytokeratin-18 (red). Note that cells with no brush border-associated staining for F-actin correspond to areas with increased staining for cytokeratin-18. Bar, 10  $\mu$ m.

In the proximal, but not the distal, loops, *Salmonella* infection was associated with a marked increase in the degree of staining for cytokeratin-18 in cells close to or at the tips of the villi (Fig. 3b, c). The increase peaked by 15–60 min post-

infection (p.i.) and then decreased. The cells showing increased staining were, however, not consistently associated with bacteria (Fig. 3c). Such cells also showed a characteristic pale-staining cytoplasm in adjacent thin (2  $\mu$ m) sections

stained with HE (Fig. 3d). Similar to the cells staining intensely for cytokeratin-18, the pale-staining cells became numerous by 15–60 min p.i. In contrast, in the FAE, cytokeratin-18 staining of presumptive M cells became markedly reduced, which may reflect destruction of M cells by *Salmonella*, as previously demonstrated in the mouse (Jones *et al.*, 1994).

In the control pig jejunum, staining for F-actin revealed a continuous brush border staining along the villi (Fig. 3e). In addition, weaker staining occurred in the remaining cytoplasm of the epithelial cells and strong staining was detected in myofibroblasts of the lamina propria. In infected animals, the brush border-associated staining for F-actin was frequently thinner or missing entirely in segments close to or at the tip of the villi (Fig. 3f). The attenuated brush border-associated staining for F-actin did not correlate with cells invaded by *Salmonella* but frequently corresponded to areas of increased staining for cytokeratin-18 (Fig. 3g).

Enhanced staining for cytokeratin-18 in epithelial cells at the apical region of ordinary villi was observed only in the proximal part of the jejunum. These data point to regional differences in the response of the gut epithelium to *Salmonella*. Moreover, the cells appear to respond to the presence of *Salmonella* in the lumen rather than being directly invaded by the organisms. Interestingly, it has been suggested that *Salmonella* produces a histotoxin (Lodge *et al.*, 1999), which could potentially contribute to the intestinal alterations observed.

In conclusion, our data demonstrate that *S. Typhimurium* invasion is rapid and not limited to any specific epithelial cell type in the porcine jejunum. Invasion of the lamina propria of ordinary villi was less marked than invasion of the subepithelial layer of the FAE, suggesting that the PP represent the main portal of early entry during *Salmonella* infection. Importantly, this could be related to invasion not only of cytokeratin-18-positive M cells but also to invasion of cytokeratin-18-negative epithelial cells of the FAE. In the epithelium of the proximal, but not distal jejunum, marked changes in the expression of cytokeratin-18 occurred. These changes may reflect possible toxin-mediated effects on apical epithelial cells of ordinary villi. Importantly, these observa-

tions also demonstrate a hitherto unknown regional variation in the responses of the intestinal epithelium to *Salmonella* infection.

## ACKNOWLEDGEMENTS

The technical assistance of Helle Anita Vestergaard Ruby and Tony Bønnelycke is gratefully acknowledged. This study was supported by the Danish Agricultural and Veterinary Research Council.

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