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Comparison of histopathological features of *Vibrio cholerae* O1 El Tor and O139 Bengal infections in rabbit intestinal mucosa

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Summary. *Vibrio cholerae* is the causative agent of the infectious disease, cholera. The bacteria adhere to the mucosal membrane and release cholera toxin, leading to watery diarrhea. There are >100 serovars of *V. cholerae*, but the O1 and O139 serovars are the main causative agents of cholera. The present study aimed to compare the severity of intestinal mucosal infection caused by O1 El Tor and O139 *V. cholerae* in a rabbit ileal loop model. The results showed that although the fluid accumulation was similar in the loops inoculated with O1 and O139 *V. cholerae*, the presence of blood was detected only in the loops inoculated with the O139 serovar. Serosal hemorrhage was confirmed by histopathological examination and the loops inoculated with O139 showed massive destruction of villi and loss of intestinal glands. The submucosa and muscularis mucosa of the ileum showed the presence of edema with congested blood vessels, while severe hemorrhage was seen in the muscularis propria layer. The loops inoculated with O1 El Tor showed only minimal damage, with intact intestinal villi and glands. Diffuse colonies of the O139 serovar were seen to have infiltrated deep into the submucosal layer of the intestine. Although the infection caused by the O1 serovar was focal and invasive, it was more superficial than that due to O139, and involved only the villi. These observations were confirmed by immunostaining with O1 and O139 *V. cholerae*-specific monoclonal antibodies. The peroxidase reaction demonstrated involvement of tissues down to the submucosal layer in O139 *V. cholerae* infection, while in O1 El Tor infection, the reaction was confined mainly to the villi, and was greatly reduced in the submucosal region. This is the first reported study to clearly demonstrate the histopathological differences between infections caused by the O139 Bengal and O1 El Tor pathogenic serovars of *V. cholerae*.

Key words: *Vibrio cholerae*, Histopathology, El Tor, Bengal, Intestinal mucosa, Rabbit

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Introduction

Cholera, sometimes referred to as "Asiatic cholera", has been endemic in South Asia, especially in the Ganges delta region. In 1817, the first cholera pandemic was reported, followed by six further pandemics, ending in 1923, and affecting mostly the continents in the southern hemisphere, as well as North America and Europe (Barua, 1992). In 1961, a seventh pandemic began in Indonesia, spread to the Indian subcontinent and the Middle East, then to Africa in the 1970s, and finally reached South America in the early 1990s (Blake, 1994; Swerdlow and Isaäcson, 1994; Tauxe et al., 1994). The etiologic agent responsible for cholera was identified by Robert Koch in 1883, who described it as a 'comma-shaped bacterium', later designated as *Vibrio cholerae*. The fifth and sixth pandemics, however, were shown to have been caused by the *V. cholerae* serogroup O1 of the 'classical' biotype, while the seventh pandemic was caused by serogroup O1 biotype 'El Tor' (Barua, 1992). However, in 1992, a serogroup conversion event led to the emergence of a new *V. cholerae* serogroup, O139, which was responsible for large epidemics in Bangladesh and India. This bacterium

resembled the *V. cholerae* O1 in both culture and biochemical characteristics, and produced the same virulent cholera toxin, but did not agglutinate with the O1 antiserum, or with the other non-O1 serogroup antisera (Ramamurthy et al., 1993). This new strain proved more virulent and its emergence was followed by a large outbreak of clinical cholera in southern Bangladesh and several other areas in India that was described as the eighth pandemic (Garg et al., 1993).

Cholera infection starts with the oral ingestion of food or water contaminated with vibrios. The vibrios must survive the acidic conditions in the stomach before colonizing the upper small intestine. Colonization is aided by fimbriae, composed of filamentous protein structures called toxin co-regulated pili, which extend from the cell wall and help to attach the bacterium to receptors on the mucosa. The motility of the bacterium helps it to penetrate the overlying mucus to reach the mucosa (Taylor et al., 1987). The pathogenesis of cholera involves the expression of several genes encoding for virulence factors that aid colonization, coordinated expression of virulence factors and toxin action (Wachsmuth et al., 1994). Genetic analysis has revealed the presence of two important genetic elements that distinguish pathogenic strains of *V. cholerae* from innocuous ones. These are called the CTX genetic element and the Vibrio pathogenicity island (VPI) (Taylor et al., 1987). Although *V. cholerae* O1 serogroup was the major etiological agent of cholera epidemics in the 1980s, the O139 serogroup replaced the O1 serogroup in South East Asia in the 1990s (Albert, 1993). The phenotypic and genotypic characteristics of the O139 Bengal strain suggested a close resemblance to the O1 El Tor biotype (Calia et al., 1994; Bik et al., 1995).

This study compared the colonization patterns of *V. cholerae* O1 El Tor and O139 Bengal in relation to fluid accumulation, mucosal invasion, gross pathology, histology and immunohistopathological changes in a rabbit ileal loop model.

Materials and methods

Bacterial strains

One representative strain each of O1 El Tor and O139 Bengal *V. cholerae* were used in this study. These strains were available from the strain collection unit in the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia. The strains were phenotypically characterized by routine microbiological tests and serotyping with commercially available specific antisera (Denka Seikan, Tokyo, Japan). The El Tor strain used in this study was determined to be non-hemolytic using conventional hemagglutination and hemolysis assays (Barret and Blake, 1981). The genotypic characterization of these strains was carried out using a multiplex polymerase chain reaction (PCR) assay developed in our laboratory that is able to simultaneously identify eight *V. cholerae* genes. These eight detectable genes include (i) the non-virulent gene *lol*B for *V. cholerae* serogroups of O1, O139, non-O1 and non-O139 (ii-iii) *tcpA* for biotyping (iv-vi) *ctx, zot, ace* as virulence genes, (vii) the *rfb* gene, which is specific for the O139 serogroup, and (viii) the *tet*A gene, as a tetracycline antibiotic resistance determinant.

Preparation of V. cholerae strain for inoculation studies

The bacteria were grown in Luria Bertani broth overnight at 37°C. The overnight culture was harvested by centrifugation at 8000 rpm for 10 min. The pellet was then resuspended in 1 ml sterile normal saline and the optical density was measured at 600 nm using a spectrophotometer. The culture was then diluted to concentrations of 10^2, 10^4, 10^6 and 10^8 CFU/ml.

Rabbit ileal loop procedure

Adult New Zealand white rabbits weighing 1.6–2.5 kg were used in this study. Ethical approval for the study design was obtained from the institutional ethical committee. The rabbit ileal loop assay was performed as previously described (Thungapathra et al., 1999), with minor modifications. The experiment was carried out in duplicate. Before the experiment, the animals were starved for 24-36 h but water was provided *ad libitum*. The abdomen of the anesthetized rabbit was shaved and cleaned. A midline incision was made along the *linea alba* of the abdomen and the small intestine was ligated 10 cm from the ileocecal junction. Five centimeter loops, separated by 1 cm, were made by ligation using 3-O catgut. Care was taken to ensure that the blood vessels remained intact. The loops were injected with 10^2-10^8 CFU/ml of either the El Tor or O139 *V. cholerae* in 1.0 ml normal saline, using a 27G needle attached to a 1 ml disposable syringe. Normal saline was used as a control. The small intestine was returned to the bowel and the incision was closed using catgut and silk sutures. A sterile dressing was applied to the wound. The animal was then returned to its cage and provided with limited water, but no food. The animal was euthanized after 18 h and the ligated loops were recovered. The length of the loop (cm) and the volume of accumulated fluid (ml) in each loop were measured. The fluid accumulation ratio (FAR) was calculated by dividing fluid accumulation (in ml) in each loop by the length of the loop.

Gross examination, cytology, histopathological and immunohistopathological studies

The cytology of the accumulated fluid was studied using Giemsa and Papanicolaou (PAP) staining. The fluid was spun in a cytospin at 1000 rpm for 2 min and the cells were fixed with either 95% alcohol for PAP staining, or air-dried for Giemsa staining.

Each ligated intestinal loop was washed in

phosphate-buffered saline, and appropriately 2 cm in length was sectioned and fixed in 10% formalin for histological examination under a light microscope. Tissues were dehydrated in a series of graded alcohols, further processed, embedded in paraffin and mounted into paraffin blocks. The 4-µm tissue sections were stained with haematoxylin and eosin (H&E). Similar tissue sections were used for immunoperoxidase staining. The protocols for H&E staining were as described by Bancroft and Stevens (1990). Duplicate sections of all tissues were stained for *V. cholerae* using standard immunohistochemical procedures with anti-lipopolysaccahride (LPS) monoclonal antibodies specific for 0139 (9A11D6) and 01 El Tor (2B4) *V. cholerae*. These antibodies were obtained as gifts from Prof. Armando Acosta, Finlay Institute, Havana, Cuba and were used as primary antibodies at a dilution of 1:200. The tissue sections underwent an antigen unmasking step by the pressure cooker method, as recommended by the DAKO instrution manual. The slides were then incubated with the primary antibodies for 30 min. This was followed by incubation of the slides in polyclonal goat anti-mouse lgG-biotin at a dilution of 1:200 for 30 min. The slides were washed and incubated with streptavidin-horseradish peroxidase conjugate and developed with 3,3'-diaminobenzidine for 5 min. Sections were then counterstained with hematoxylin stain. Tissue sections incubated with secondary antibodies alone were used as negative controls.

Results

Fluid accumulation ratio

The characteristic features of the O1 El Tor and O139 strains were confirmed using the standard biochemical and agglutination tests carried out with O1- and O139-specific polyvalent antisera. The multiplex PCR assay confirmed the presence of all three toxin genes, namely *zot, ace* and *ctx*, in the strains used in this study. The presence of the ctx operon was confirmed by the accumulation of fluid in the loops inoculated with both the O1 and O139 strains, compared to the control loop. There was a dose-dependent increase in the FAR from 10^2 to 10^6 CFU. The FAR was highest (2.2 ml/cm) in the loops inoculated with either O139 or El Tor strains at 10^6 CFU/ml (Fig. 1). However, the loops inoculated with higher numbers of CFUs (10^7 or 10^8) showed no significant increase in the FAR. This was probably due to a fluid accumulation threshold in the 5-cm loops. It was also noteworthy that the loops inoculated with 10^6 CFU of O139 *V. cholerae* showed the presence of bloody mucus (serosal hemorrhage) and inflammation, while the loops inoculated with 10^8 CFU O1 *V. cholerae* were devoid of blood (Fig. 2). The presence of blood in the loops inoculated with 10^6 CFU O139 *V. cholerae* suggests that this strain might express some virulence factors or capsular antigens that are not expressed by the O1 strain. The cytology of the accumulated fluid from the loops inoculated with either 10^6 CFU O139 or O1 El Tor *V. cholerae* showed clumps of degenerated cells with poorly defined cellular outlines. The degenerated cells were mainly inflammatory cells, neutrophils and marcrophages, but a few intact columnar epithelial cells found in the loops inoculated with O1 El Tor supported the fact that it was less virulent than the O139 strain (data not shown).

Histopathological and immunohistopathological changes in rabbit ileum infected with O139 V. cholerae and O1 El Tor

Gross examination revealed that the loops infected

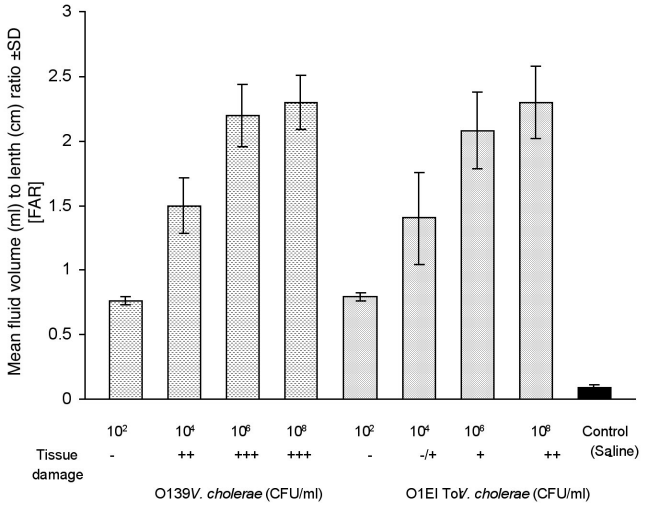

Fig. 1. Mean fluid accumulation ratio in relation to fluid volume and loop length of rabbit ileal loops when different doses of O139 or O1 El Tor *V. cholerae* were inoculated.

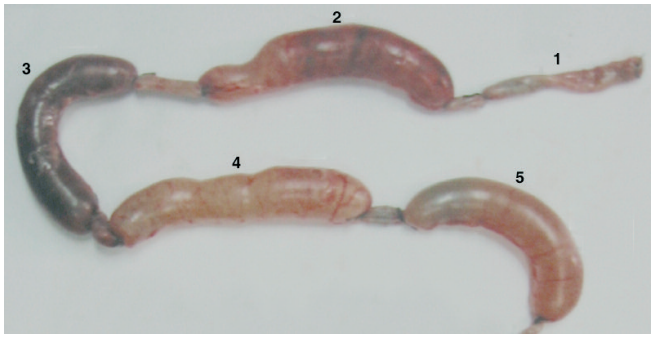

Fig. 2. Gross examination of rabbit ileal loops revealed greater serosal hemorrhage with fluid accumulation in rabbit ileal loops inoculated with $1x10^6$ and $1x10^8$ CFU/ml O139 *V. cholerae* (Loop 2-3), but no serosal hemorrhage with fluid accumulation was seen in rabbit ileal loops inoculated with $1x10^8$ and $1x10^6$ CFU/ml O1 El Tor *V. cholerae* (Loop 4-5). The control loops injected with phosphate-buffered saline yielded no fluid (Loop 1). These results were consistent in different rabbits (n=3).

with O139 had more serosal hemorrhage than the loops infected with O1 El Tor (Fig. 2). The important histopathological features included a severely degenerated, damaged and sloughed ileal mucosa (thin arrow, Fig. 3A) in the loops infected with O139 *V. cholerae*. There was marked congestion of blood vessels and infiltration of polymorphonuclear neutrophils (PMN) in the lamina propria and at the surface of the crypt epithelium (thick arrow, Fig. 3A). Congested blood vessels, edema and significant numbers of PMNs were observed in the submucosa at higher magnification. Congested blood vessels and mild edema were also seen in the muscularis mucosa in the loops infected with O139 (thick arrow head, Fig 3A), while the

Table 1. Comparision of histopathological findings seen in O139 Bengal and O1 El Tor *Vibrio cholerae* pathogenesis.

Histopathological changes	*Vibrio Cholerae* O139 Bengal	*Vibrio Cholerae* O1 El Tor
Mucosal ulceration	Extensive/Diffuse	Focal
Remaining normal mucosa	±	++
Submucosal congestion	Prominent	minimal
Mucosal/submucosal congestion	Prominent	minimal
Mucosal/submucosal edema	+++	+
Acute/chronic inflammation	Transmural (mucosa/submucosa/muscle)	Confined to mucosa
Muscle edema	++	+
Bacterial Invasion	Up to muscular layer (mucosa/submucosa/muscle)	Up to muscular layer

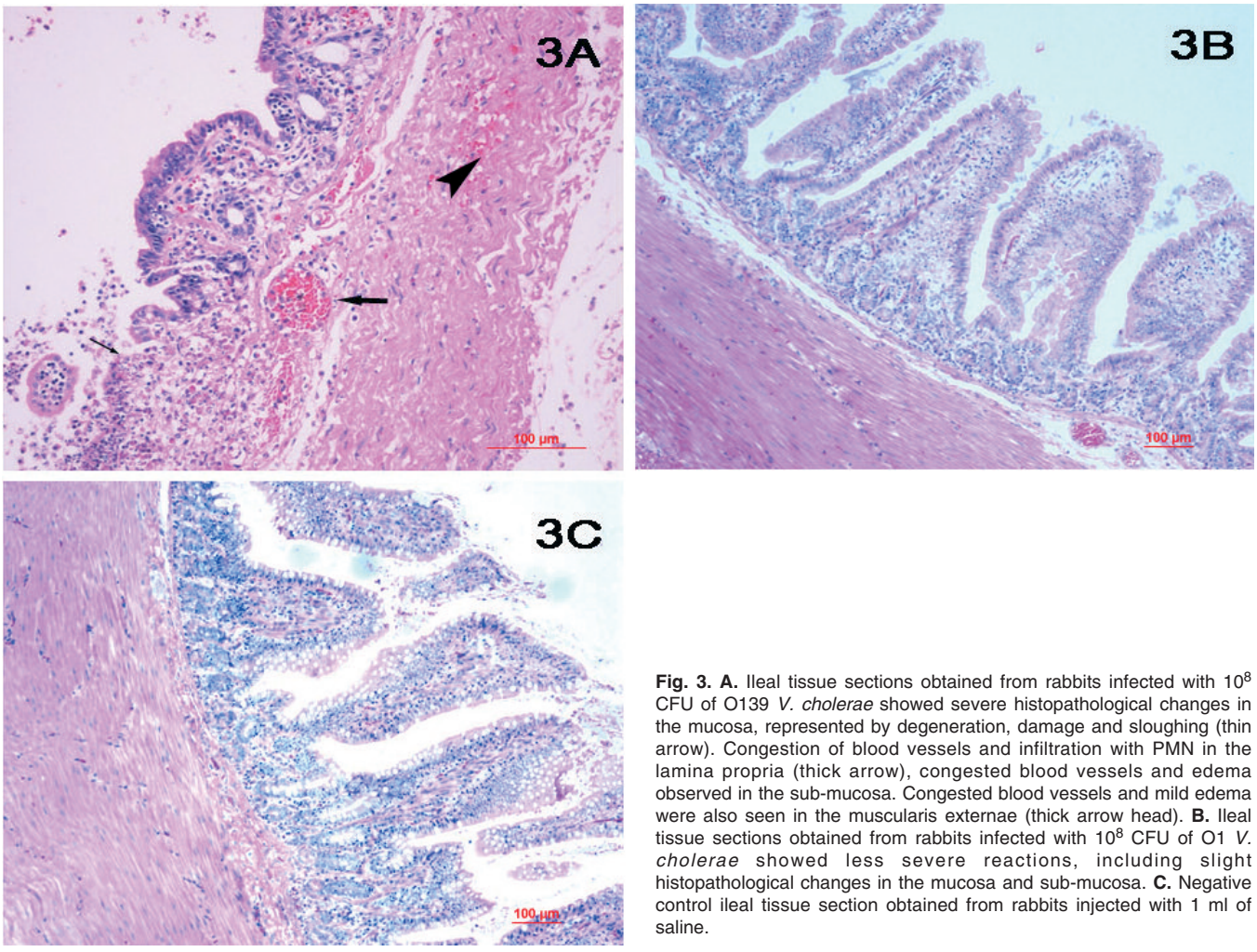

Fig. 3. A. Ileal tissue sections obtained from rabbits infected with 10^8 CFU of O139 *V. cholerae* showed severe histopathological changes in the mucosa, represented by degeneration, damage and sloughing (thin arrow). Congestion of blood vessels and infiltration with PMN in the lamina propria (thick arrow), congested blood vessels and edema observed in the sub-mucosa. Congested blood vessels and mild edema were also seen in the muscularis externae (thick arrow head). **B.** Ileal tissue sections obtained from rabbits infected with 10^8 CFU of O1 *V. cholerae* showed less severe reactions, including slight histopathological changes in the mucosa and sub-mucosa. **C.** Negative control ileal tissue section obtained from rabbits injected with 1 ml of saline.

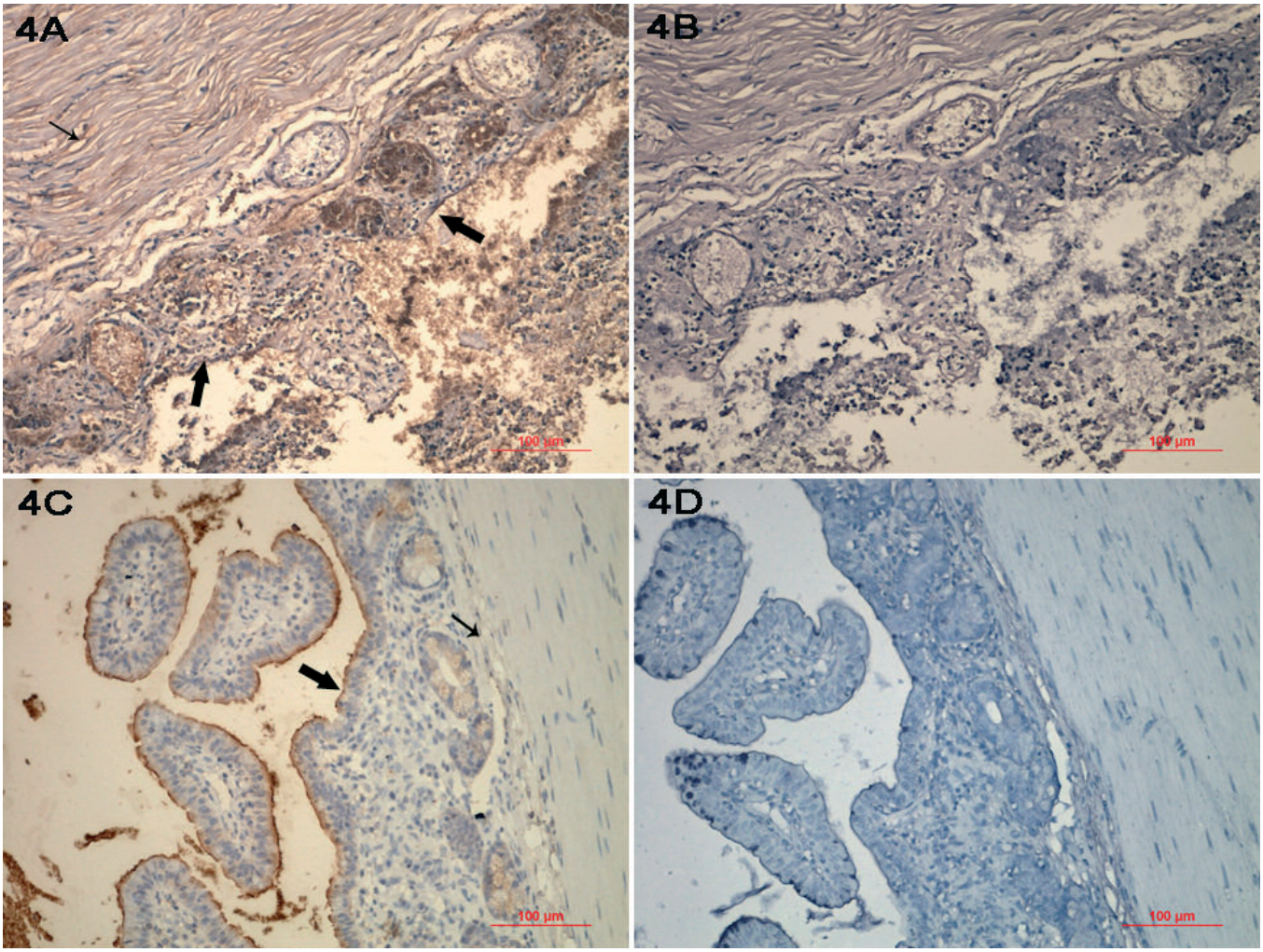

Fig. 4. Immunostaining was carried out using either O1- or O139-specific mouse monoclonal antibodies followed by treatment with biotinylated anti-mouse IgG. Streptavidin–horseradish peroxidase was used as a conjugate and sections were developed with 3,3'-diaminobenzidine with hematoxylin counterstaining. **A.** Immunohistopathological staining of rabbit ileal tissue sections infected with 10^6 CFU of O139 *V. cholerae* after 18 hours. There was an intense immunoperoxidase reaction in the mucosa (thick arrow) and intense labeling was seen within the submucosa and muscularis externae (thin arrow). **B.** Negative control where treatment with O139 *V. cholerae*-specific primary antibodies was omitted showed no immunoperoxidase staining in the inflammatory and degenerate areas of the ileum. **C.** Immunohistopathological studies of rabbit ileal tissue sections infected with 10^6 CFU of O1 El Tor *V. cholerae* after 18 hours. The ileal tissue sections showed a significant immunoperoxidase reaction in the mucosa (thick arrow), and also labeling within the submucosa (thin arrow), but the degree of inflammation and degeneration was less than in the O139 infected tissues. **D.** Negative control where treatment with O1 El Tor *V. cholerae*-specific primary antibodies was eliminated showed no immunoperoxidase staining in the inflammatory and degenerate areas of the ileum.

histopathological changes in loops infected with O1 El Tor were less severe, with only patchy ulceration in the mucosal layer (Fig. 3B) and intact intestinal glands were seen under the light microscope. The severity of the important histopathological features and the damage caused by the O139 and O1 *V. cholerae* strains were compared and the findings are summarized in Table 1. From Table 1 it is clear that mucosal ulceration, submucosal congestion and edema were extensive in O139-infected loops compared to O1 loops. The chronic inflammation from the submucosa to the muscle layer was also more evident in the loops infected with O139, leading to thinning of the muscle layer. These results suggest that the O139 *V. cholerae* strain was more virulent than the O1 El Tor strain (Table 1). The tissue sections from the control loop showed normal histological features (Fig. 3C)

The ileal tissue sections were immunostained with either O139 anti-LPS or O1 El Tor anti-LPS *V. cholerae* antibodies. In tissues infected with O139, immunoperoxidase labeling was intense in the mucosal region (thick arrow, Fig. 4A), and this intense labeling extended into the muscularis and submucosa externae (thin arrow, Fig. 4A). Ileal tissue sections

immunostained to detect O1 *V. cholerae* antigen showed less immunoperoxidase labeling in the mucosa (thick arrow, Fig. 4C) and submucosa (thin arrow, Fig. 4C). The tissue sections used as negative controls for both the strains were not treated with primary *V. cholerae* antibodies, hence no immunoperoxidase labeling was seen in the inflamed or degenerated areas of the ileal sections (Fig. 4B,D). These results indicate the presence of LPS antigens or colonization by the bacteria in the submucosa.

Discussion

V. cholerae has been considered to be a non-invasive organism, which causes cholera through the diarrheagenic effects of its enterotoxins, the most important of which is cholera toxin. Enterotoxin action in the intestine is believed to be mediated by stimulation of the mucosal adenyl cyclase-cyclic AMP system (Field, 1971). The work of Polotsky (1977) showed that El Tor cholera vibrios attached themselves to the enterocytes and multiplied, most probably on the intestinal epithelial surface, in ligated rabbit gut loops, but they did not penetrate deeper and were unable to cause marked inflammation or destruction of the epithelium. This work also revealed that the enterotoxin-induced epithelial hypersecretion causing fluid accumulation in the gut loops resulted in the attached vibrios being cleared off the epithelium lining into the gut lumen (Polotsky et al., 1977). However, few studies have investigated the nature of the invasiveness of O139 *V. cholerae*, since it emerged and became epidemic only in 1993. Recent studies by Farthing (2000) have shown that choleric diarrhea is due to bacterial products that have an effect not only on the enterocytes, but also on other structures within the gut mucosa and submucosa.

Our study compared, for the first time, the immunohistopathological events that occurred in intestines infected by O139 and El Tor *V. cholerae*, in a rabbit model. There was an almost complete loss of mucosal architecture and diffuse necrosis of enterocytes, with evidence of neutrophil infiltration in the lamina propria or epithelium in the loops inoculated with O139 Bengal *V. cholerae*. Loops inoculated and infected with wild-type O139 Bengal *V. cholerae* showed more redness and hemorrhage as compared to those inoculated with wild-type O1 El Tor *V. cholerae*. Both first adhered to and then colonized the intestinal tissue, but the local injury and inflammatory reaction to O139 *V. cholerae* type was greater than to the El Tor type *V. cholerae*. This was confirmed by histological examination showing extensive and severe necrosis of the mucosa in the ileal loops and hemorrhage associated with severe ulceration in the tissue sections from the ileal loops infected with O139 *V. cholerae*, while only patchy ulceration was observed in the sections from ileal loops infected with El Tor O1 *V. cholerae*. Focal areas of necrosis of the luminal epithelium with local areas of mucosal congestion were prominent in El Tor infection, compared to Bengal serotype infection. One of the differences observed was in the spreading pattern of El Tor and O139 *V. cholerae* in the intestinal layers. No intact glands were seen in ileal loop sections inoculated with O139 *V. cholerae* due to the extensive damage, but intact glands were observed in loops inoculated with O1 El Tor (Fig. 3A,B). Ileal loops infected with O139 bacteria were severely damaged, as detected by O139-specific anti-LPS antibodies which showed the presence of bacteria down to the level of the submucosa and muscularis externae, while the O1 El Tor-specific antibodies were attached only to the submucosal layer, and the villi remained intact (Fig. 4A,C).

The increased virulence of the O139 strain may be attributed to the presence of a thin capsular layer in the cell wall. This capsule has been shown to be responsible for its virulence and for its resistance to complement-mediated killing by normal human serum (Johnson et al., 1994). However, the capsule does not play an antiphagocytic role, since both capsular and acapsular mutants of O139 strains were equally taken up by macrophages (Meno et al., 1998). Earlier reports by Owen et al. (1986) showed that, when viable vibrio (classical) were inoculated into the intestinal lumen of nonimmune rabbits, they were phagocytosed by M cells over Peyer's patch lymphoid follicles, carried in vesicles through the epithelium, and discharged among the underlying lymphocytes and macrophages. Uptake and transport by M cells may also assist pathogenic bacteria in crossing the mucosal barrier. M cells can thus convey viable *V. cholerae*, which are not otherwise invasive, into the intestinal lymphoid tissue, where mucosal immune responses are initiated. Transmission electron microscopy studies in rats showed that viable *V. cholerae* O1 were initially taken up by the M cells which overlay Peyer's patches, and intact vibrios were subsequently delivered to phagocytic cells in the Peyer's patches (Sincharoenkul et al., 1993). Studies have also shown that cholera toxin induces the production of proinflammatory cytokines, such as interleukin-6, thereby activating the enteric immune system and potentially generating arachidonic acid metabolites, such as prostaglandins or leukotrienes, which stimulate chloride secretion (Klimpel et al., 1995).

Electron microscopy revealed the disruptive effect of the Bengal O139 strains on the apical membrane of the epithelial cells, even though it proliferates and colonizes the mucosal surface of the rabbit small intestine (Koley et al., 1995). Thus, if the apical surface is damaged, then *V. cholerae* O139 would be able to invade the small intestine. Saha et al. (1997) have shown, in a rabbit model, that adhesion and subsequent colonization were important events in infection by *V. cholerae* O139 Bengal, and electron microscopy revealed the cellular invasive processes, with bacteria detected in the lamina propria and other associated inflammatory changes (Saha et al., 1997).

The focus of the present study was to investigate the colonization and pathophysiological nature of O139

compared to O1 El Tor. We found that O139 damaged the mucosal and submucosal layers more aggressively and was more invasive than the O1 El Tor strain. Further studies using transmission electron microscopy and histopathological examination will be carried out to determine if the O139 *V.cholerae* reach the lamina propria through M cells, where they can be processed by antigen presenting cells, so leading to an immune response.

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