Histopathological changes in experimental cholera with a non toxigenic non- O1 non-O139 *Vibrio cholerae* strain isolated from Kolkata, India

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This study was conducted to understand the pathophysiological changes in experimental rabbit ileal loop model using the *Vibrio cholerae* strain non-O1 non-O139, isolated as sole pathogen from clinically diagnosed cholera patients in Kolkata. Significant amount of haemorrhagic fluid accumulation was observed in all the test loops of rabbit model where the strain of *V. cholerae* was inoculated as compared to control loops. Microscopic examination of the accumulated fluid showed the presence of erythrocytes and pus cells. Histology revealed structural alteration of the villous epithelium with inflammatory cells infiltration in all the layers of the gut mucosa including the nerve plexus region. Preliminary observation with a haemagglutinin protease extracted from the non-O1 non-O139 strain, was also studied in different concentrations in the same animal model which showed similar type of macroscopic and microscopic response in the ileal loops as seen with the original strain. The results highlight that along with other pathways, inflammatory cells and the enteric neurons have an important role in the pathophysiology of diarrhoea and the isolated protease may be the probable virulence factor in initiating the disease process in this non-O1 non-O139 strain induced cholera.

**Keywords**: Enterotoxigenic, Haemagglutinin protease, Inflammation, Non-O1 non-O139 *Vibrio cholerae*

Cholera, caused by a gram-negative pathogen *Vibrio cholerae* is still a major public health concern because of the severity of the disease and its high epidemic and pandemic potency. Bengal ‘the homeland of cholera’, being a hyper endemic zone, cases prevail throughout the year though insurgency occurs from time to time. *V. Cholerae* O1 and O139 strains are responsible for causing diarrhoea in an epidemic form but *V. Cholerae* non-O1 non-O139 strains are frequently identified as the causative agents of sporadic cases of cholera like diarrhoea, inflammatory enterocolitis and septicaemia in different parts of the developing world including India. National Institute of Cholera and Enteric Diseases, Kolkata has a systematic surveillance system to study established and newer diarrhoeagenic pathogens including *V. cholerae*. The year wise surveillance data recorded from 2001-2004 revealed a shifting trait of *V. cholerae* isolated from hospital patients. *V. cholerae* O1 serogroup at present dominating over the other two whereas isolation rate of non-O1 non-O139 serogroup is more than serogroup O139. *V. cholerae* O1 and O139 are known to produce a variety of extracellular proteins including cholera toxin (CT), which plays an important role in causation of the disease. Choleric diarrhoea is mainly secretory though some recent reports provide an evidence of inflammatory response in association with the disease pathology. Increased production of the mediators like lactoferrin, myeloperoxidase, superoxide dismutase, nitric oxide metabolites, eicosanoids, prostaglandins and leucotrienes have been observed in isolates like stool and urine of cholera patients infected with *V. cholerae* O1 and O139.

Increased level of neutrophils, eosinophils and mast cells have also been reported in cholera patients. Proteases have some important influence in the pathogenic process of choleric diarrhoea and protease deficient mutants are claimed to be less virulent. *V. cholerae* Non-O1 non-O139 strain induced diarrhoea groups belong to a large family of *V. cholerae* strains and differ in their protease activity. Most of the non-O1 non-O139 strains produce a heat labile haemolysin, few produce heat stable toxin and a thermostable direct haemolysin also have been reported. Recently a non-O1 non-O139 strain (serogroup O6) was isolated from diarrhoeal patients
from Infectious Disease Hospital, Kolkata, India. The strain being devoid of ctx genetic element still producing diarrhoea prompted us to undertake this study. From the culture supernatant of the non-O1 non-O139 strain, a protein was identified and purified by gel filtration, hydrophobic interaction and ion exchange chromatography. The protein having mol wt of 35kDa has haemagglutinating activity but no haemolytic activity. Further purification and characterization of the protein is in progress. The influence of haemagglutinin protease (HAP) and the role of innate cells, inflammatory mediators and their effects in the host bacterial interaction in the clinical manifestation of the disease process in V. cholerae non-O1 non-O139 strain induced diarrhoea has not been much studied. A preliminary observation with the HAP protein at different concentrations along with the parent strain was done in rabbit ileal loop model to see the involvement of the innate cells, the mediators influencing the nerve cells and the histopathological changes in details by light and electron microscopy.

**Materials and Methods**

Preparation of V. cholerae strain for inoculation study—Vibrio cholerae non-O1 non-O139 (PL-21) strain isolated from clinically diagnosed cholera patients admitted to the Infectious Disease Hospital, Kolkata, India, was used in the study. The strain was biochemically characterized by the API 20E system (Bio Merieux Science, Montalieu-Vercieu, France) and serotyped at the National Institute of Health, Tokyo, Japan. With specific DNA probe the strain was negative for ctx (cholera toxin), ace (accessory cholera toxin), zot (zona occludens toxin) and sto (heat stable enterotoxin) genes but positive for the structural gene (hlyA) for ELTor haemolysin. The parent strain was acclimatized. Prior to surgery the rabbits were fasted for 24 hr except water ad libitum, and anesthesia was maintained with standard doses of ketamine (35 mg /kg body wt). Under proper aseptic precaution, ileocaecal junction was identified and 3 blind loops of 5 cm segments with 2 cm interspaces were prepared. V.cholerae non-O1 non-O139 strain was inoculated at a concentration of 1 ml of PBS containing 10⁸ log-phase broth cultures in two loops and the PBS was inoculated in the third loop as a negative control. In the 2nd set of experiment, HAP, the purified protein from the culture supernatant of non-O1 non-O139 V.cholerae strain was injected in rabbit ileal loop model at doses of 20, 40, 60 and 100 μg in different loops and 25mM Tris buffer was used in separate loop as a negative control. The two sets of experiments, the 1st one with the V.cholerae O6 strain and the 2nd one with the protease at different concentrations were performed in individual animal and each of the experiment was repeated thrice. The rabbits were sacrificed with Euthanasia 6 solution (pentobarbital sodium) after a designated incubation period of 18 hr of inoculation. The volume of accumulated fluid (ml) and the length of the loop (cm) were measured and a ratio of volume to length was determined for each loop. The ratio greater than 1 was considered as the standard indicator of a positive enterotoxic response. Each ligated loop was sectioned in 2cm in length (approx) and kept in 10 % buffered formalin for histological examination under light microscopy and small pieces of about 1mm in length were fixed in 3% cacodylate buffered glutaraldehyde for ultrastructural study.

Ligated intestinal loop test—Prior to surgery the rabbits were fasted for 24 hr except water ad libitum, and anesthesia was maintained with standard doses of ketamine (35 mg /kg body wt). Under proper aseptic precaution, ileocaecal junction was identified and 3 blind loops of 5 cm segments with 2 cm interspaces were prepared. V.cholerae non-O1 non-O139 strain was inoculated at a concentration of 1 ml of PBS containing 10⁸ log-phase broth cultures in two loops and the PBS was inoculated in the third loop as a negative control. In the 2nd set of experiment, HAP, the purified protein from the culture supernatant of non-O1 non-O139 V.cholerae strain was injected in rabbit ileal loop model at doses of 20, 40, 60 and 100 μg in different loops and 25mM Tris buffer was used in separate loop as a negative control. The two sets of experiments, the 1st one with the V.cholerae O6 strain and the 2nd one with the protease at different concentrations were performed in individual animal and each of the experiment was repeated thrice. The rabbits were sacrificed with Euthanasia 6 solution (pentobarbital sodium) after a designated incubation period of 18 hr of inoculation. The volume of accumulated fluid (ml) and the length of the loop (cm) were measured and a ratio of volume to length was determined for each loop. The ratio greater than 1 was considered as the standard indicator of a positive enterotoxic response. Each ligated loop was sectioned in 2cm in length (approx) and kept in 10 % buffered formalin for histological examination under light microscopy and small pieces of about 1mm in length were fixed in 3% cacodylate buffered glutaraldehyde for ultrastructural study.

Processing of specimens for light and electron microscopy—For light microscopy tissues were dehydrated in graded series of alcohol and further processed and embedded in paraffin. After cutting 3-4
μm thick sections in a rotary microtome (Leica 2145, Germany), staining was done with haematoxyline and eosine stain and examined in a trinocular research microscope (DMLB, Leica). A special immunocytochemical stain (Naphthol chloroacetate esterase) was done for better demonstration of polymorphonuclear leucocytes (PMNs) and mast cells. Collected fluid sample of each loop was also examined microscopically for the presence of erythrocytes and pus cells and photographs were taken by DC 200 digital camera.

For electron microscopy tissues were dehydrated in ethanol after 1% osmium tetroxide treatment, and embedded in Epon 812. Semi thin sections (1μm) were cut by ultramicrotome (Leica Ultracut, UCT) and stained with toluidine blue (1% aqueous solution), examined under light microscope to see the actual area of interest. Ultra thin sections were cut using diamond knife and sections were counterstained with uranyl acetate and lead citrate to study the ultra structural changes in Tecnai12 Bio-Tween Transmission electron microscope (The Netherlands).

Myeloperoxidase (MPO) activity—The biopsy specimens from each loop were homogenized with PBS and centrifuged at 10,000 rpm. For enhancement of the reaction, α- phenylenediamine (600 μg/ml) in phosphate buffer and 300 mM of H2O2 was added and absorbance was observed at 492nm at an interval of 30sec for 5 min. Changes in the optical density were calculated and the results were expressed as percentage increase of MPO activity over the control18.

Statistical analysis—Data were analyzed by employing Paired t-test for MPO activity and P<0.05 was considered as the level of significant difference compared to control.

Results

Fluid response and the histopathological changes in rabbit ileal loop—Significant amount of haemorrhagic fluid accumulation (the fluid volume and loop length ratio > 1) was observed in the ligated rabbit ileal loops inoculated with PL-21 strain, as compared to control loops. Inoculation of the protease from the culture supernatant of the strain also showed haemorrhagic fluid response in the ligated loops with all the test doses, however, it was significantly more from challenge dose of 40μg and onward. No fluid accumulation was observed when EDTA inactivated HA protease was inoculated (Fig.1). The accumulated fluid was thick and viscous and microscopic examination of the fluid revealed the presence of erythrocytes (1-5/HPF) and pus cells (5-10 or more/HPF) in all the challenged loops except in the control loops.

Light microscopic appearance—Three to four well-oriented villous sites of each inoculated loop were examined and following microscopic changes were observed.

Villus epithelium with the PL-21 strain as the inoculum—Gross structural alteration with exfoliation of surface epithelial cells at places, mucous depletion, necrosis at villous tip region with congested blood vessels, red blood cells and inflammatory cells like neutrophil polymorphs, eosinophils and few mononuclear cells infiltration in villous lamina propria were noted (Fig.2). Elongation and dilatation of the crypt region with polymorphs, eosinophils and pericryptal mononuclear cells infiltration were there in all the test loops as compared to control loops. Widening of the submucosa, dilated blood vessels with margination of PMNs and eosinophils were the common finding in the test loops. Inflammatory cells specially eosinophils, few PMNs and mast cells were also observed around meissner’s and myenteric plexus region. Degenerative changes with swelling were present in few of the nerve plexus area with all the inoculum except the control.

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The protease at different concentrations—At 20 μg challenge dose, broadening or fusion of the villi with one another (mild change) above the crypt region, at 40 and 60 μg, blunting and broadening of villi with haemorrhage (moderate change) and at 100 μg, distortion of villous epithelium, necrosis and haemorrhage (severe change) were observed. Villous architecture and mucosal morphology were within normal limit with all the control loops treated with Tris buffer (Fig. 3). Pararosaniline stained sections demonstrated higher number of PMNs cells in villous and crypt area. Scattered mast cells were demonstrated around pericryptal area, around arteriole and nerve plexus region at a minimum challenge dose of 20 μg and onwards (Figs 4, 5).

Electron microscopic changes—Ultrastructural changes with PL-21 strain and the protease at different concentrations confirmed the light microscopic findings. Following 18 hr challenge dose

Fig. 2—Alteration of the villous epithelium with sloughing of surface epithelial cells, haemorrhage and inflammatory changes in the mucosa with the inoculated non-O1 non-O139 strain (10×)

Fig. 3—Normal villous morphology with mucosal glands and submucosa with challenge dose of Tris buffer (control) (10×)

Fig. 4—Increased number of PMNs and eosinophils, dilated crypt lumen with pericryptal mononuclear cells in the lower third of the villi with PMNs (N) and eosinophils (E) in the submucosa, muscle coat and in the myenteric plexus region in challenge dose of 20 μg protease (40×)

Fig. 5—Inflammatory changes along with erythrocytes inside the villous lamina propria, dilated and elongated crypts, oedematous submucosa and presence of PMNs, (N) eosinophils (E) and mast cells around meissner’s and myenteric plexus region in 60 μg inoculum of the protease (40×)
with PL-21 strain, damaged brush border of the villous epithelium with exudates and bacteria in the lumen were observed (Fig. 6). Inflammatory cells like PMNs and eosinophils were present in the villous lamina propria and around the submucosal plexus zone (Fig. 7). Oedematous nerve fibre with neurosecretory granules and eosinophils in close proximity of the nerve fibre at 60μg test dose of the protease was also observed (Fig. 8).

Myeloperoxidase (MPO) activity—Paired t-test showed statistically significant absorbance response indicating the high PMNs activity in all the tested biopsy samples as compared to control loop specimens i.e. Tris-40μg, P<0.001, 95%CI 0.06-0.07 , Tris-60μg, P<0.001, 95%CI 0.179-0.182 and 40μg - 60μg, P<0.001,95%CI–0.0116--0.109. Moreover the Standard Errors were constant between 40 and 60μg doses when compared to control (Tris) which showed the neutrophilic activity was at constant increase with the respective challenge doses (Fig 9).

Discussion

The long continued belief that biochemical and physiological changes in small intestinal function without alteration of intestinal integrity in the pathogenesis of cholera in animal models 19,20 has changed. Recent evidence suggests that the secretory response in choleric diarrhoea is due to the bacterial products, which not only have an effect on the enterocytes promoting chloride secretion but may also affect on other structures within the gut mucosa and

![Fig. 6](image)

Fig. 6—Gross damage of the microvillous brush border with exudates on the surface (arrow 1) and bacteria in the lumen (arrow 2) (bar = 2μm)

![Fig. 7](image)

Fig. 7—PMN in the pericryptal area in lower third of villous lamina propria (arrow 1) and an eosinophil around submucous plexus zone (arrow 2 ) ( bar = 5μm)

![Fig. 8](image)

Fig. 8—Oedematous non myelinated nerve fibre (arrow 1) and an eosinophil in its close proximity (arrow 2) (bar = 2μm)

![Fig. 9](image)

Fig. 9—MPO activities between control-Tris and experimental challenged doses-40 and 60μg. Values are mean ± SE. P <0.001
submucosa including inflammatory cells and the enteric nervous system. In the present study, these findings have been reevaluated with a nontoxicigenic non-O1 non-O139 *V. cholerae* strain in an experimental rabbit ileal loop model. An upregulation of innate cells has been demonstrated in the present study which play a vital role in the initiation of an adaptive inflammatory immune response after inoculation with both the Pl-21 (serogroup O6) strain and with the HAP obtained from the culture supernatant of the strain. The 1st line of defense against host bacterial interaction are the PMNs. The enzyme MPO found mainly in PMNs and to some extent in monocytes, has been considerably increased in the test doses in the present study. Increased level of MPO activity is directly proportional to the number of active neutrophils supporting the direct involvement of PMNs as inflammatory mediator in the disease process. Eosinophils could function as an immunoregulatory cell involved in the release of a number of pro- and anti-inflammatory cytokines and chemokines. Presence of inflammatory cells throughout the gut mucosa and specially eosinophils around nerve plexus area and mast cells around arteriole support the idea that an immune inflammatory mechanism along with neurotransmitters may be the initiating factor in the secretory response in this *V. cholerae* non-O1 non-O139 strain induced diarrhoea.

Structural changes in the nerve plexus region were not much but oedema and mild degenerative changes were noted both by light microscopy and at the ultrastructural level by electron microscopy in the present study. Myenteric plexus along with their interneurons and submucous plexus contain secretomotor fibres that release agonist and either directly or indirectly influence epithelial ion transport by releasing vasoactive intestinal peptide and stimulate intestinal secretions in crypts and inhibit nutrient-independent salt absorption in villi. Both submucosal mast cell and inflammatory effects influence neuronal activity which is supported by the observation that these effects of the toxin are suppressed by neural blockade.

In the present study, accumulation of significant amount of fluid in all the test loops with inoculation of HAP at 40,60 and 100 μg test dose indicated an enterotoxic response of the protease. The damaged villous epithelial surface with structural alteration and accumulation of inflammatory cells and oedematous submucosa with grossly dilated vessels were observed in all the test loops both by light and electron microscopy are suggestive of the cytotoxic and inflammatory response with the strain and also with the protease. The present observations corroborate the findings of infant rabbit ileal loop experiment where damage of villous epithelium with infiltration of polymorph nuclear cells was reported by Joseph et al. with three clinical isolates of non-O1 V.cholerae. In another study, Russell et al. documented presence of inflammatory cells in lamina propria in rabbits infected with *V. cholerae* non-O1 NRT 36S (serogroup O31) in removable intestinal tie adult rabbit diarrhoea model (RITARD). High faecal leucocytes count in stool specimens of choler patients indicating an indirect evidence of inflammation was observed by Saha et al. and these findings are further supported by documentation of inflammatory component in faecal specimens of diarrhoea caused by the wild type strains of *V. cholerae* Eltor, attenuated strains of *V. Cholerae, V. cholerae* O139, CVD 110 vaccine strain and a less reactive vaccine strain CVD 103 HGR2 in human volunteer studies.

HAP secreted by *V. cholerae* strain may be one of the reactogenic factors in these vaccine strains. The role of HAP as a potential reactogenic factor of genetically attenuated vaccine strains has been documented. Culture supernatants from reactogenic vaccine strains showed a decrease in the trans epithelial resistance of T-84 intestinal cells and this decrease correlated with the presence of haemagglutinating protease but not with other potential accessory toxins or proteases.

To summarise, the present observations provide that *V. cholerae* non-O1nonO-139 (serogroup O6) strain produced a protease that was heat labile and had haemagglutinating activity. The protease was cytotoxic and enterotoxic and initiated an upregulation of innate cells like neutrophil, eosinophil and mast cells. Swollen nerve fibre and inflammatory cells like mast cell and eosinophil around arteriole and nerve plexus region suggested the neuronal influence in immediate response to enteric infection and coordination of the host response to such type of infection. Further purification and characterization of the protein, study of chemokines and neurotransmitters will refine our knowledge regarding the pathogenesis of non-O1 non-O139 strain induced diarrhoea and development of strategies for better management of the disease in future.
References


