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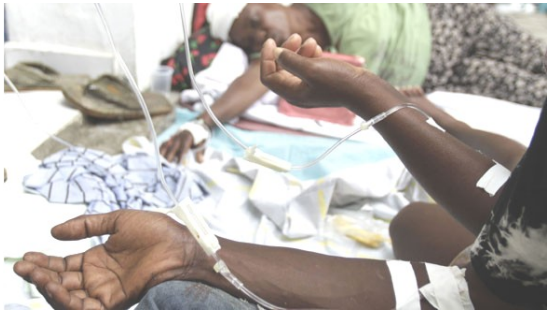


Histology and Histopathology
Cellular and Molecular Biology

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

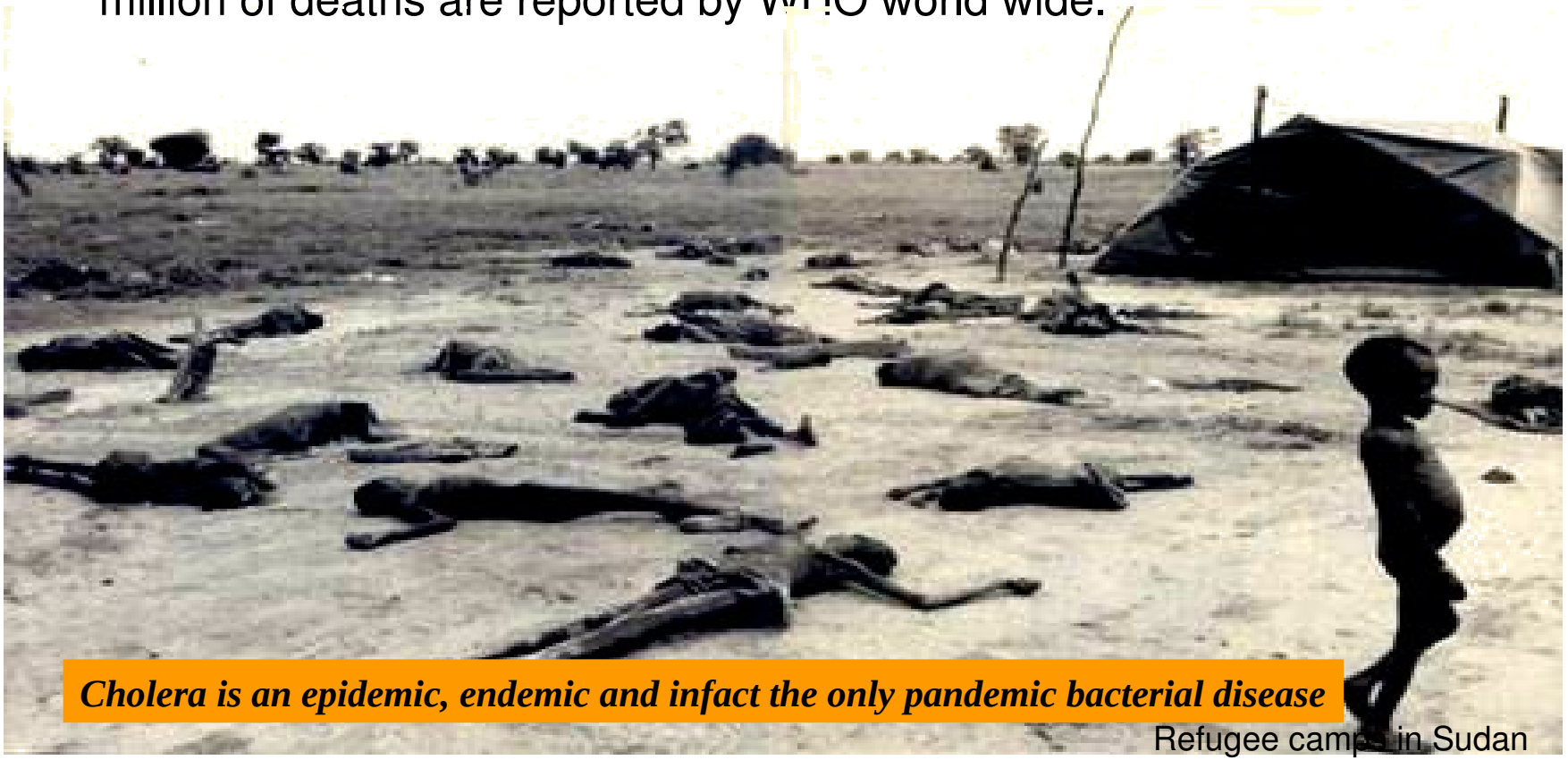
Atif Amin[1], Atif Ali[1], Sinnah Kurunathan[1], Tan Gim Cheong[1], Karim Alwan Al-Jashamy[1], Hasnan Jaafar[2], Zainul F. Zainuddin[3], Manickam Ravichandran[4] and Pattabhiraman Lalitha[4]

[1]Department of Medical Microbiology and Parasitology, School of Medical Sciences, University Sains Malaysia Health Campus, Kelantan, Malaysia, [2]Department of Pathology, School of Medical Sciences, University Sains Malaysia Health Campus, Kelantan, Malaysia, [3]School of Health Sciences, University Sains Malaysia Health Campus, Kelantan, Malaysia, [4]Department of Biotechnology, Faculty of Applied Sciences, AIMST University, Semeling, 08100 Bedong, Kedah, Malaysia

Presenter: Atif Amin Baig

Cholera

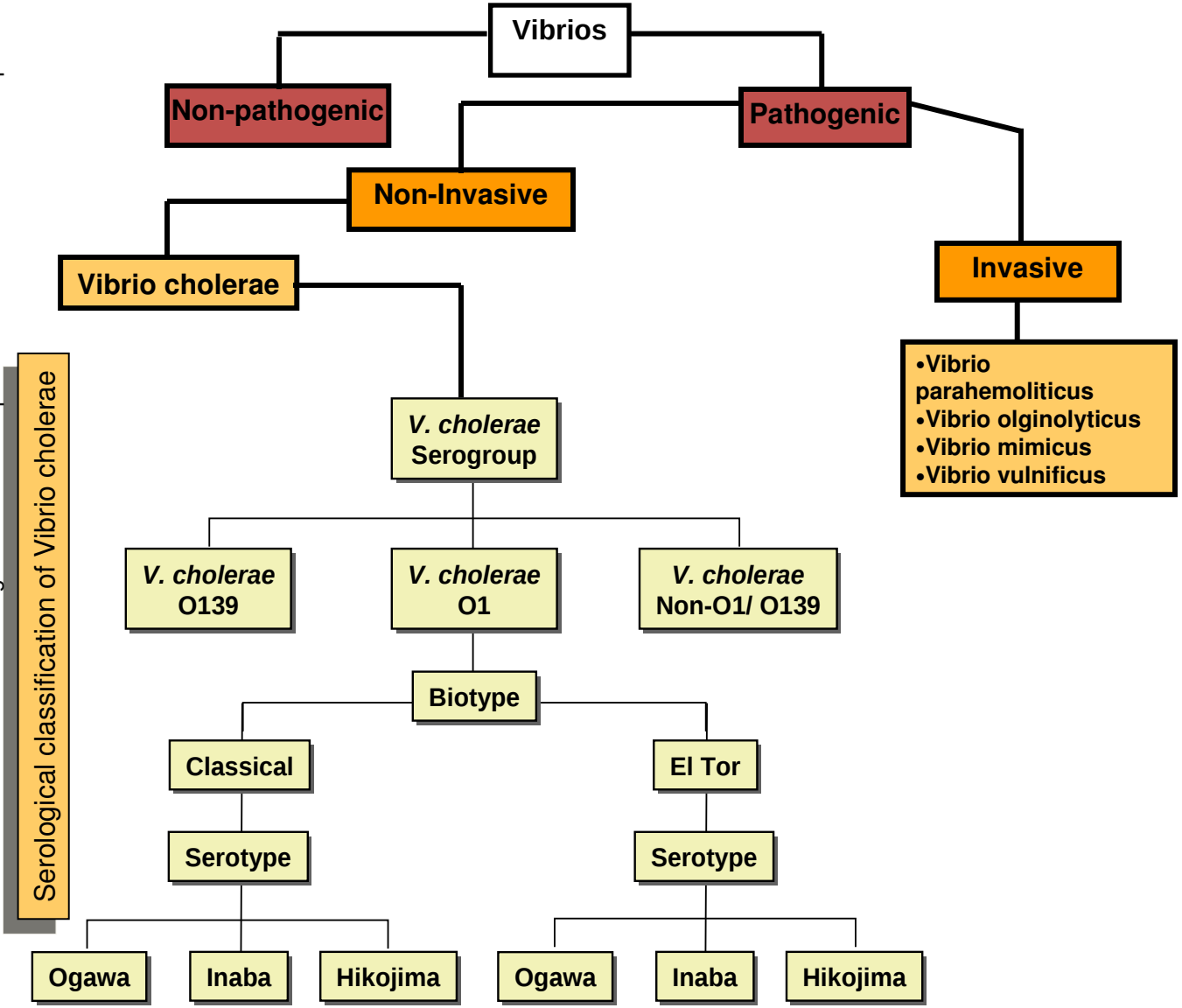
- Cholera is an acute toxigenic diarrheal disease caused by the bacterium *Vibrio cholerae.*.
- It affects mostly the under-developed countries associated with war, natural disaster and poor sanitation.
- Approximately 3 million cases of cholera and approximately 1.5 million of deaths are reported by WHO world wide.

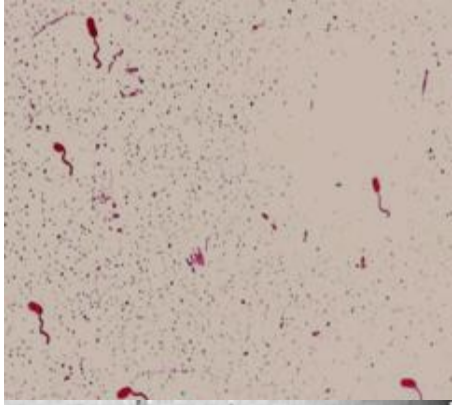


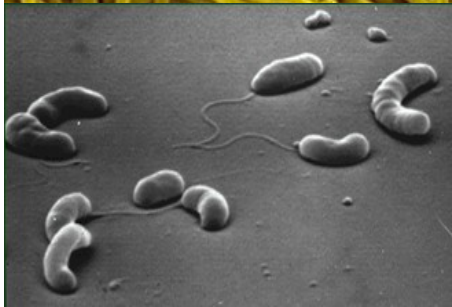
Cholera is an epidemic, endemic and infact the only pandemic bacterial disease

Refugee camps in Sudan

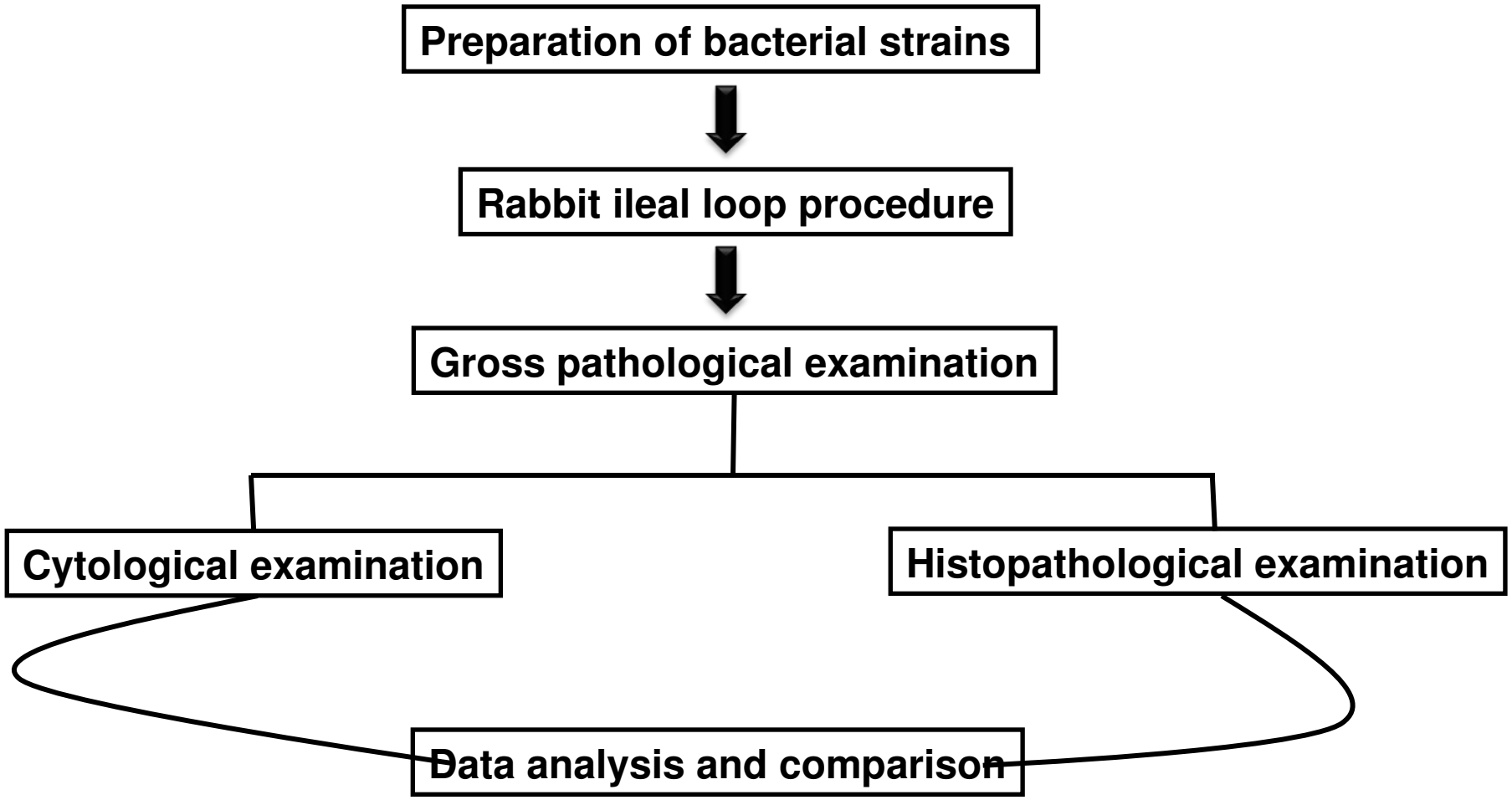
Classification of *Vibrio cholerae*







Experimental Flow



Material 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.

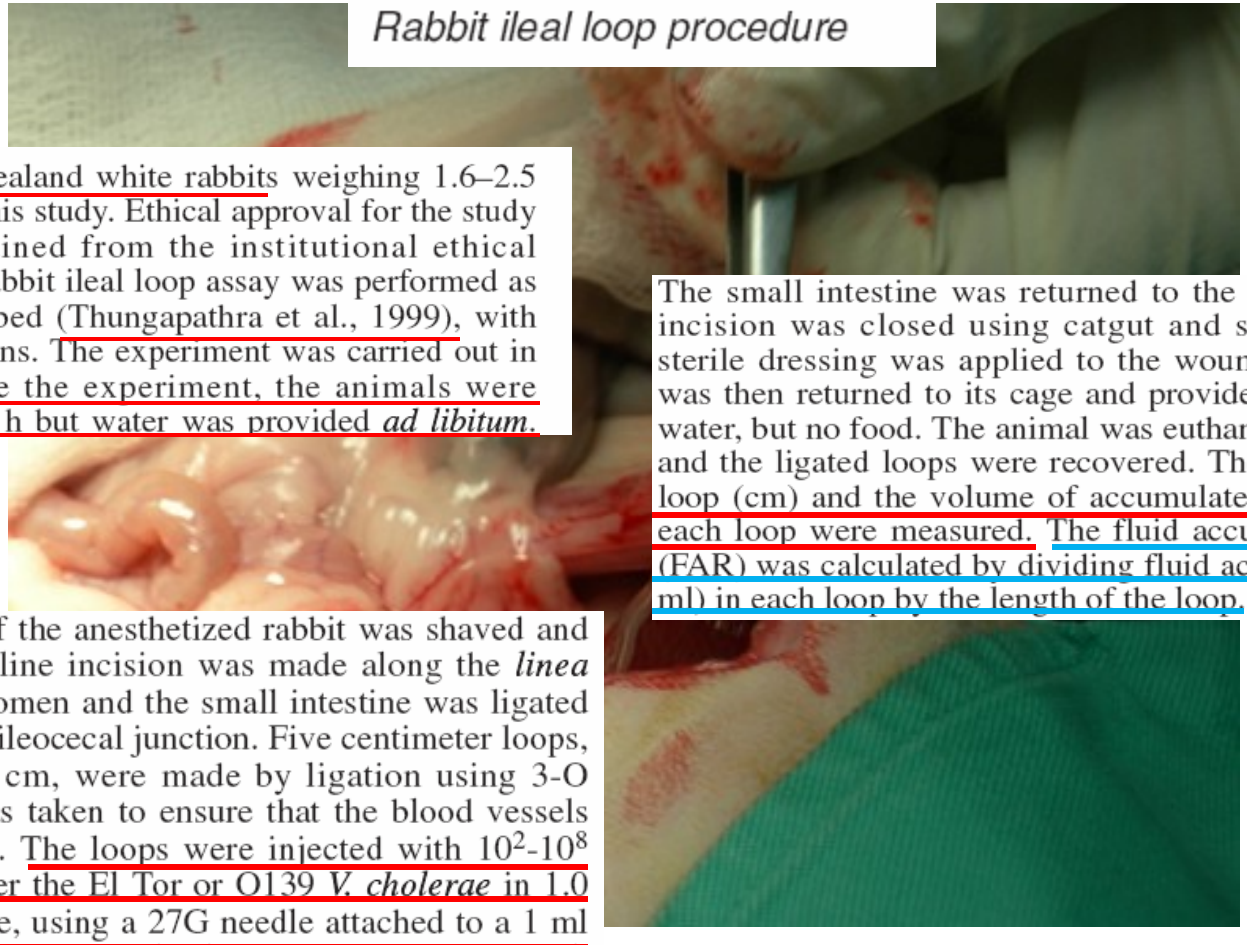
Material and Methods

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.

The count was based on the growth curve which was determined for the *V. cholerae*

Material and Methods



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.

Material and Methods

Gross examination, cytology, histopathology and immunohistopathological studies

Gross examination

Cytology

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.

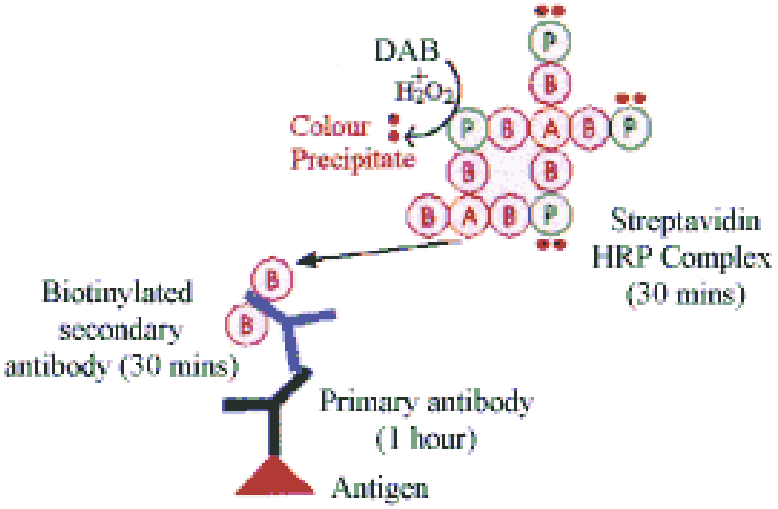
H and E staining

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

Histopathology

Immunohistochemical staining

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.



Microtechniques in Histopathology

Tissue preparation

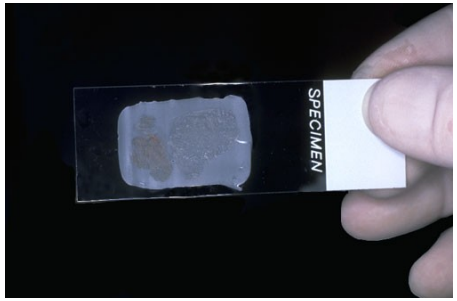
1. Labeling
2. Record of specimen
3. Fixation

Tissue processing

1. Dehydration
2. Clearing
3. Impregnation
4. Embedding

Tissue sectioning

1. Microtomy
2. Slide preparation



Staining and Microscopy

1. Staining of nucleus (Hematoxylin)
2. Staining of cytosol (Eosin)
3. Staining of cell membrane of microorganism (Gram)

Results

Fluid accumulation ratio

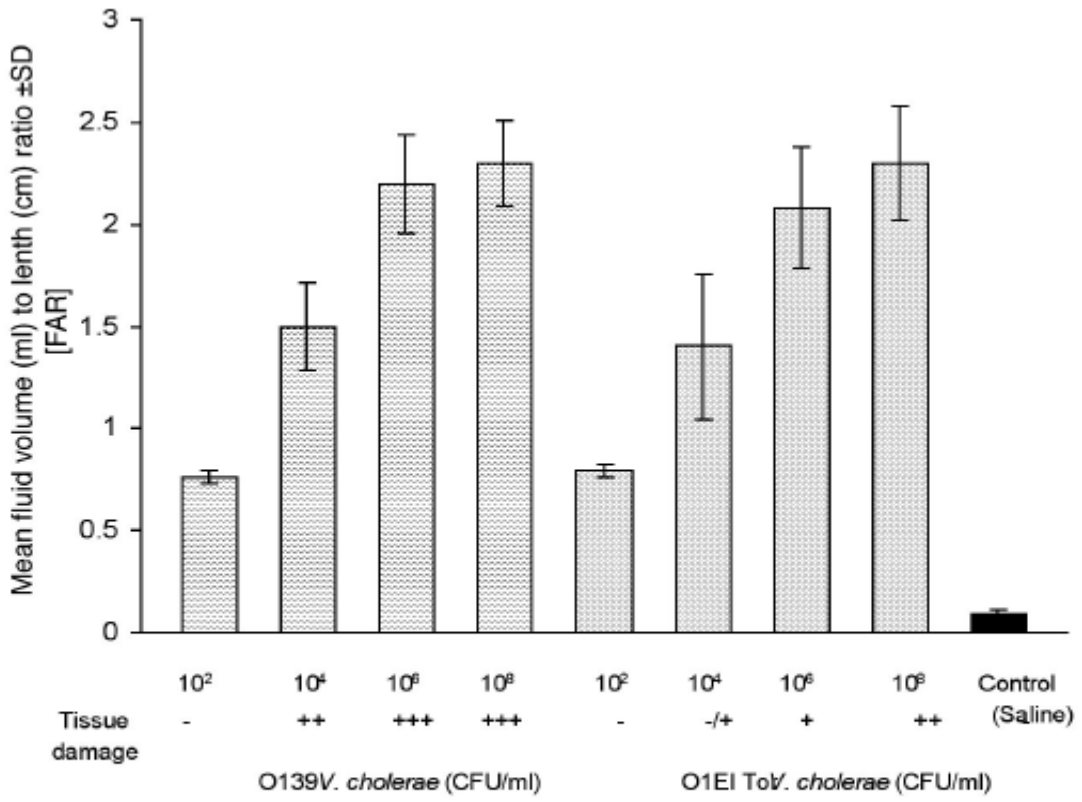


Fig. 1. Mean fluid accumulation ratio in relation to fluid volume and loop length of rabbit ileal loops when different doses of O139 or O1 El Tor *V. cholerae* were inoculated.

Results

Histopathological and Immunohistochemical changes

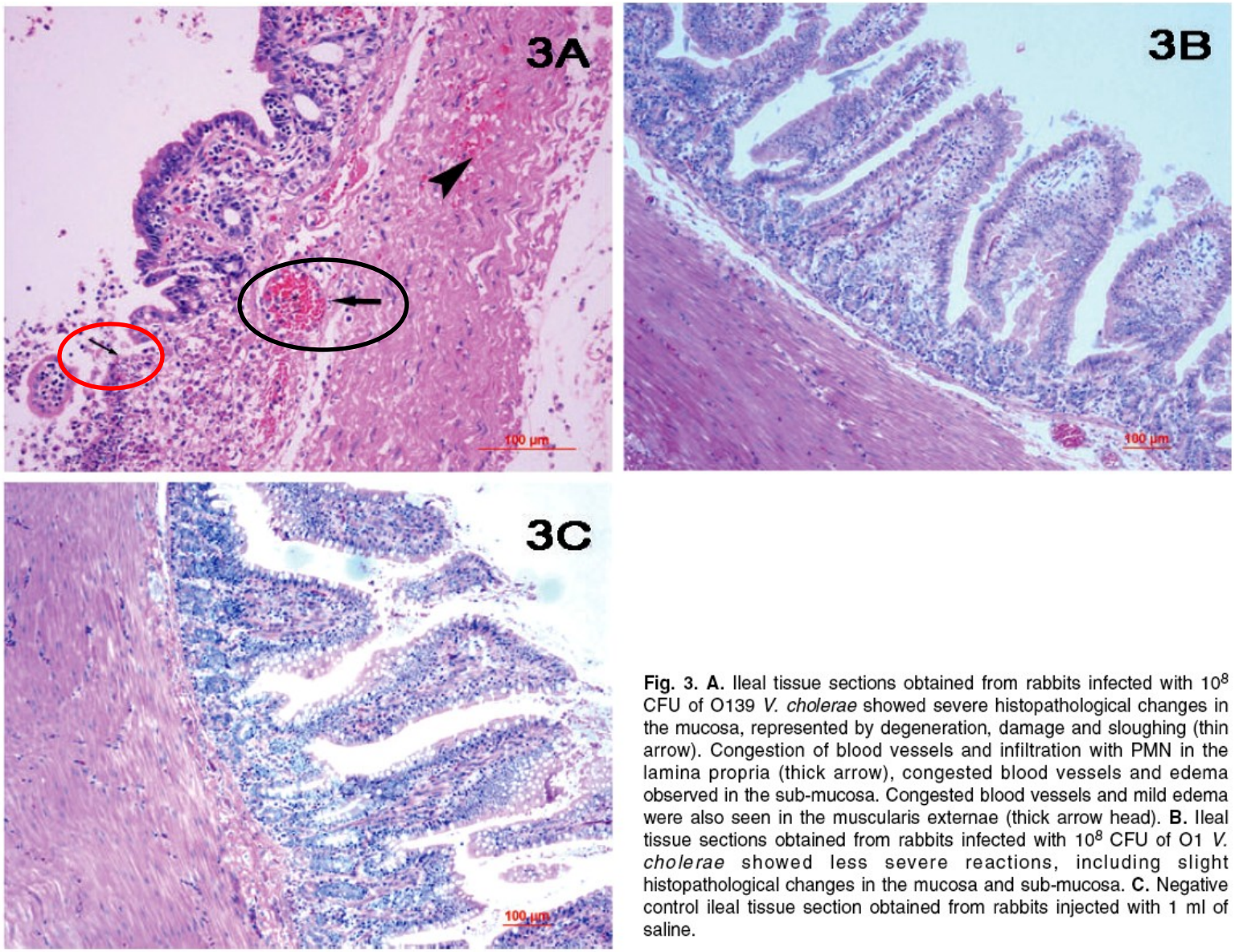


Fig. 3. A. Ileal tissue sections obtained from rabbits infected with 10^8 CFU of O139 *V. cholerae* showed severe histopathological changes in the mucosa, represented by degeneration, damage and sloughing (thin arrow). Congestion of blood vessels and infiltration with PMN in the lamina propria (thick 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.

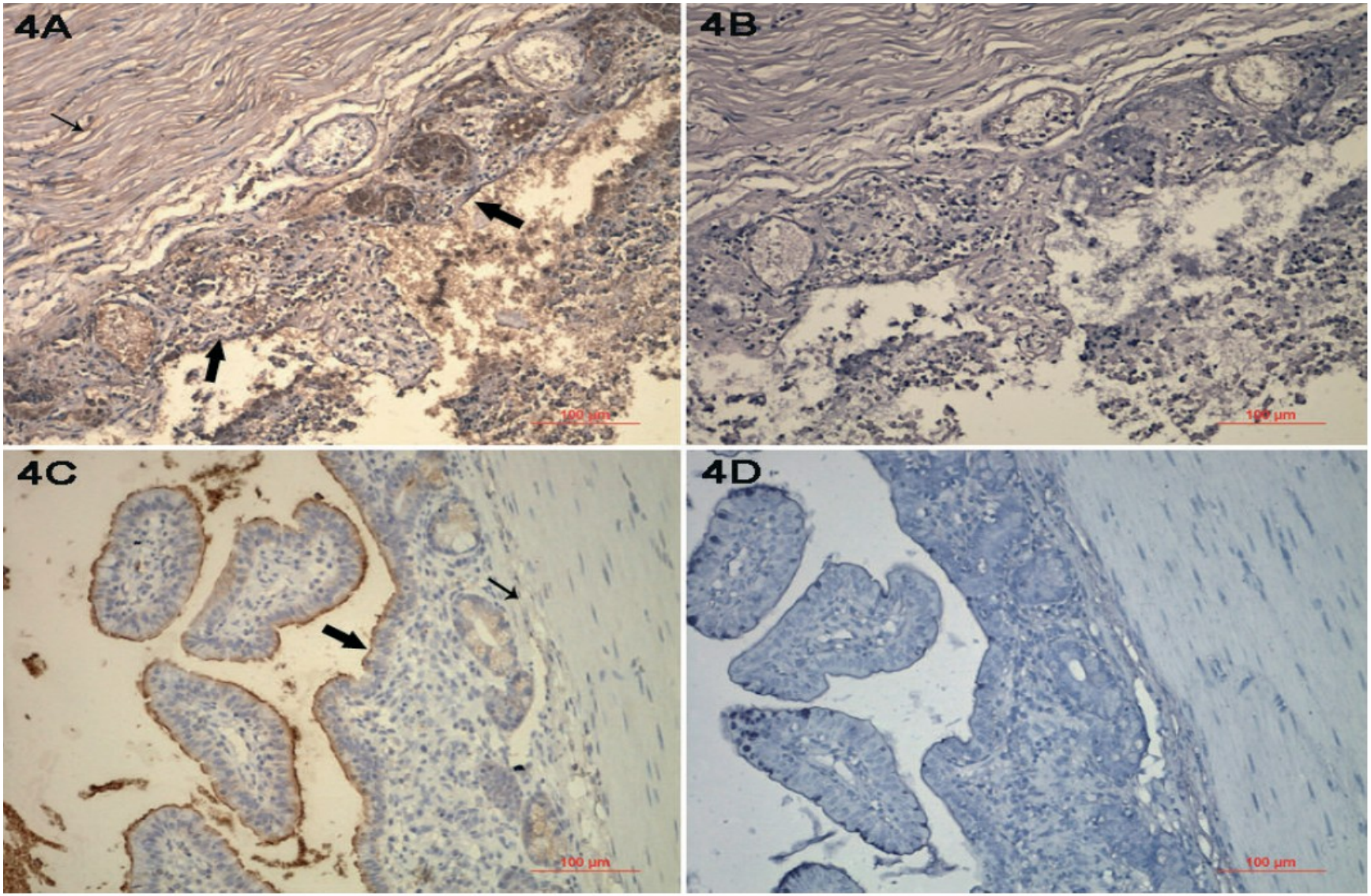


Fig. 4. Immunostaining was carried out using either O1- or O139-specific mouse monoclonal antibodies followed by treatment with biotinylated anti-mouse IgG. Streptavidin–horseradish peroxidase was used as a conjugate and sections were developed with 3,3'-diaminobenzidine with hematoxylin counterstaining. **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.

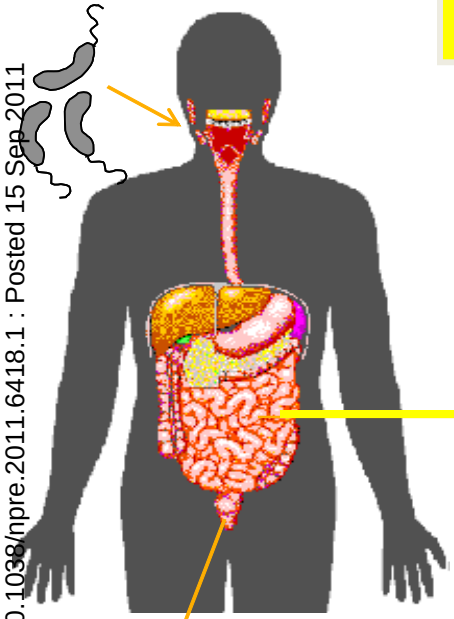
Results

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

Discussion

Vibrios are ingested



purging & excretion of *vibrios*

1. Passing of Vibrio cholerae through the gastric acid barrier and mucosal barrier (mucin layer)

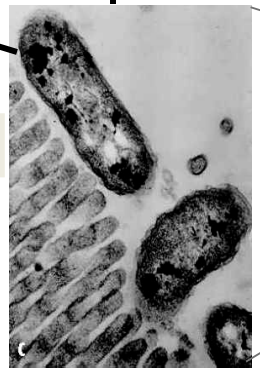
2. Intestinal adherence and colonization

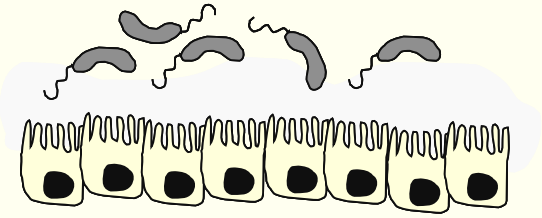
3. Toxin Secretion followed by toxins' activations

4. Vibrio cholerae detachment from the mucosal epithelium followed by the intestinal adherence and colonization.

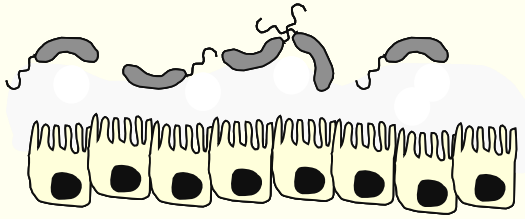
Toxigenic effects

Histo pathological effects

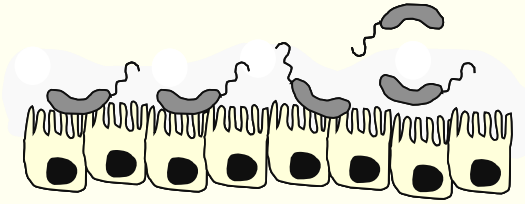




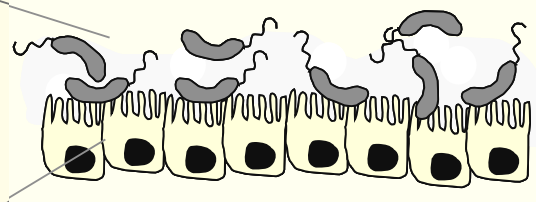
(a) *Vibrios* reach to the surface of the lumen which is covered with mucous



(b) *Vibrios* secrete mucinase(s) to overcome mucous barrier



(c) *Vibrios* align themselves to the surface of enterocytes with the help of colonization factors



(d) *Vibrios* multiply, establish new infection foci and secrete cholera and accessory toxins

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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.

Gross pathological analysis

There was an almost com
mucosal architecture and diffuse necrosis o
with evidence of neutrophil infiltration i
propria or epithelium in the loops inoculat
Bengal *V. cholerae*. Loops inoculated and
wild-type O139 Bengal *V. cholerae* s
redness and hemorrhage as compared to th
with wild-type O1 El Tor *V. cholerae*. Both
to and then colonized the intestinal tissue,
injury and inflammatory reaction to O13
type was greater than to the El Tor type *V. c*
was confirmed by histological examinat

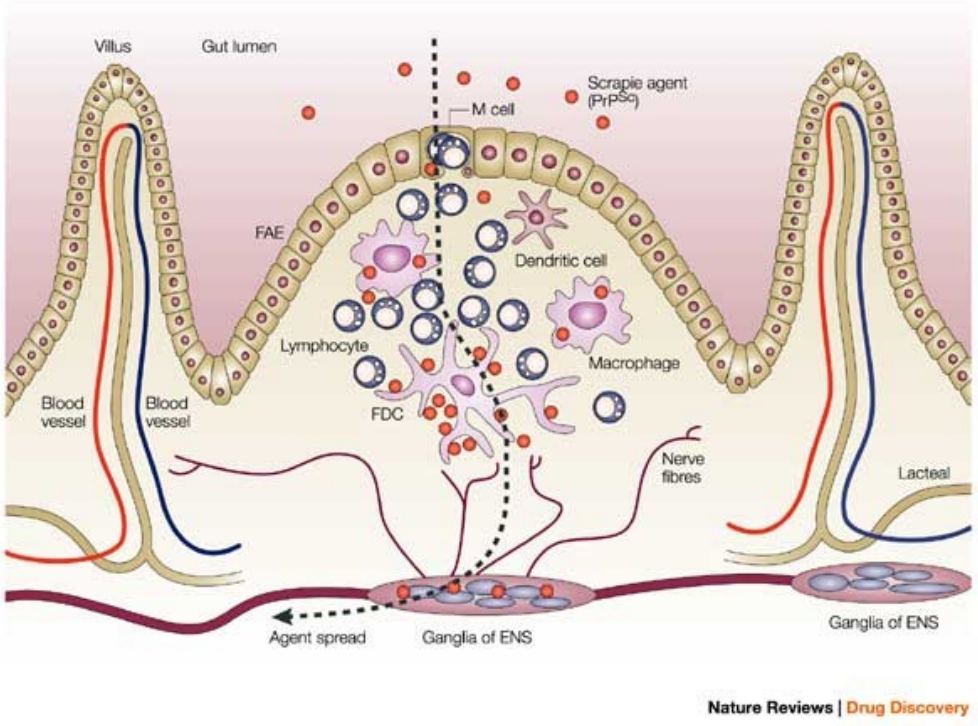
compared to Bengal serotype infection. One of the differences observed was in the spreading pattern of El Tor and O139 *V. cholerae* in the intestinal layers. No intact glands were seen in ileal loop sections inoculated with O139 *V. cholerae* due to the extensive damage, but intact glands were observed 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).

Reason of High pathogencity of O139

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

O1 Eltor



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).

Cellular interactions

Molecular interactions

O139

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).

Conclusion

- O139 damaged the mucosal and submucosal layers more aggressively and was more invasive than the O1 El Tor strain.

About Journal

Description

Histology and Histopathology (cellular and Molecular Biology) is an international journal, the purpose of which is to publish original works in English in histology, histopathology and cell biology; high quality is the overall consideration.

Indexing of journal

Indexed in PubMed, ISI WoK, Biosis, CABLE, Center for Clinical Computing, Chemical Abstracts Service, Current Awareness in Biological Sciences, Current Contents, Euroscience, Excerpta Medica, Medline, Research Information Systems, Science Citation Index, etc.

Impact factor: 2.502

Country: Spain

Strength of research

- Very Strong
- The novelty lies in the fact that the authors are actually indirectly trying to state that the *Vibrio choelrae* could be invasive considering its invasion pattern in rabbit ileum. This is against the current literature regarding the pathogenesis of *Vibrio cholerae* which states *V. cholerae* to be non invasive.
- The exploration about the difference in invasion pattern of O1 ElTor and O139 opened new horizon towards the development of vaccine for Vibrio cholerae. The vaccine must be a bivalent with the capability of targeting both the strains of *Vibrio* cholerae.

Acknowledgements: This study was supported by a grant from the Scientific Advancement Grant Allocation (SAGA) Academic Sains Malaysia, no: 304/PPSK/615304

Strength and errors in article

- Strong
- No grammatical mistakes
- No spelling mistakes
- Up to date references for the topic of interest.
- Figure 2 must be presented before figure 1.
- The gross pathological examination has been discussed and is mentioned in abstract but not mentioned properly in result section.
- The results of TEM and SEM are not included in this article even though the article did explain in the discussion portion regarding TEM studies.

Recommendations to read this article

- Highly recommended
- Especially to medical microbiologists and researchers who have been working with Enterobacteriaceae in any field.
- Vaccine developers.
- To do further research to see the invasion of *Vibrio cholerae* in Liver, gall bladder or Bile and other viscera.

Thank you

Session for Critics and Discussion

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