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Isolation of *Vibrio cholerae* in Homogenized Tissues of Liver, Gall Bladder and Bile in Rabbit Model

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Abstract

Vibrio cholerae O139 is well known as the causative agent of cholera. It is noninvasive but many reports suggested it to cause bacteremia which brings in a little controversy with the previously reported old literature especially after reported histopathological invasion pattern of *Vibrio cholerae* O139 and *Vibrio cholerae* O1 ElTor. The rabbits were processes for ileac loops inoculation of *Vibrio cholerae* O139 followed by biochemical and molecular analysis of the presence of *Vibrio cholerae* O139 in liver, gall bladder and bile. We concluded the presence of *Vibrio cholerae* in liver, bile and gall bladder homogenized tissue. Retrograde spreading of bacteria to the liver via the common bile duct was excluded by complete closure of the intestinal lumen distant to the duct in the intestinal lumen. However, the bacteria might traffic, probably via macrophages, to distant area in the body including the liver. Another study in future is a need to identify the lesions of *Vibrio cholerae* invasions within liver and gall bladder by immunohistochemistry and using GFP visibility for *Vibrio cholerae* passage.

Introduction

Vibrio cholerae currently includes more than 180 serogroups [1]. *Vibrio cholerae* of serogroup O1 is well known as the causative agent of cholera. It has become increasingly apparent that in recent years *Vibrio cholerae* of other O groups (non O1 *Vibrio cholerae*) can also cause human disease. Non O1 *Vibrio cholerae* has been isolated from patients with diarrhea throughout the world [2]. Recently, *Vibrio choleraee* O139 Bengal has emerged as the second etiological agent of cholera. It has caused epidemic in the Indian subcontinent and spread to several neighboring countries of the region which included many developed countries. It is now believed that this organism is the causative agent of the eighth cholera pandemic [3].

There are many physiological, biochemical, phenotypic and genotypic similarities between *Vibrio choleraee* O139 and O1 biotype ElTor. The most notable difference is the possession of a capsule by *Vibrio choleraee* O139 like other non O1 Vibrios, which is absent in O1 *Vibrio cholerae* [4]. This possession of capsule by non-O1 Vibrios confers extra virulence characteristics such as resistance to serum killing and ability to invade the bloodstream especially in patients with debilitating conditions or immunosuppression [5]. Laboratory diagnosis reflected that *Vibrio choleraee* O139 cause bacteremia in mice upon intradermal inoculation, whereas this ability was absent in O1 Vibrios [4]. Recent finding by Amin and colleagues [6] showed clearly the invasion pattern of *Vibrio cholerae* in rabbit ilium. The first case of the septicemia due to *Vibrio choleraee* O139 was reported in an adult patient in South India with chronic liver disease [7]. All these observations suggested that *Vibrio cholerae* O139 has the potential to cause bacteremia might be by invading through the intestinal distortion. We have reported the isolation of *Vibrio choleraee* O139 from liver, gall bladder and bile in infected adult rabbit models with bacteremia, based on hypothesis; that, the *Vibrio cholerae*, can invade via hepatic portal system but because of very low CFU, it has not been reported to cause bacteremia. Since, *Vibrio cholerae* has been reported to be a non-invasive organism, once the exact pathogenesis of *Vibrio cholerae* is understood with special focus on its invading capability, it will bring a new horizons to the molecular pathogenesis of *Vibrio cholerae* beside the association of *Vibrio cholerae* to elucidate the MALT and GALT responses towards development of cholera vaccines which is all focused till date towards non-invasive nature of *Vibrio cholerae* and *Vibrio cholerae* toxins.

Materials and Methods

Bacterial strain and culture conditions

Wild type strain of *Vibrio cholerae* O139 was used in this study. The strain was maintained in the lyophilized state and fresh ampoules were used for this study. Bacteria were grown statically and aerobically in LB broth overnight at 37°C. For intestinal invasion bacteria were harvested by centrifugation and resuspended at a concentration of 10^8 bacteria/ml of normal saline for injection into the ileal loops by further dilution into $10^7$and 10^6CFU/ml of *Vibrio cholerae*.

Rabbit ileal loop procedure

The Rabbit ileal loop assays were performed as previously described [8] with minor modifications. A total of four rabbits were used for each of the three groups, (group 1 with $1x10^8$ CFU inoculated, group 1 with $1x10^7$ CFU inoculated, group 1 with 1 x 10^6 CFU inoculated) with

a total of four negative non inoculated controls. The abdomens were cleaned and a midline incision was made along line alba of the abdomen and the small intestine was brought out for each rabbit. The small intestine was ligated 10 cm from the ileocecal junction and was then divided into 5 loops, by ligature using 3-O cat gut, of 5 cm each separated by 1 cm segments. Care was taken that no major blood vessel was ligated. The loops were injected with $1x10^2$-$1x10^7$ *V. cholerae* in 0.5 ml LB medium with a 27G 1¼" needle. The small intestine was returned to the bowel and it was closed by catgut and silk sutures. Sterile dressing was applied on the wound and animals were returned to their cages. Limited water (but no food) was given to the animal. After 18 hours, animals were euthanized as described above. An autopsy was performed and ligated loops were recovered. Total fluid that was accumulated in each loop and the length of the loop was recorded.

Fluid accumulation ratio

The fluid accumulation ratio (FAR=Volume of fluid/length of loop) was determined by measuring the fluid (ml) in the loops and dividing by the length (cm) of the loop. For this purpose; for each loop, the average lengths of loops were measured and its contents were emptied by gravity into a graduated cylinder to determine volume. The results were expressed as volume/length ratio to correct for variability in average loop length compared to the FAR of inoculated ileum loop with normal saline.

Isolation of *Vibrio cholerae* from liver

After recovering the ileums of infected rabbits, the livers of the rabbits were located and were removed from the bodies and were placed in normal saline for 5 minutes. Sections of liver were cut from all rabbits and were homogenized in LB broth; 200 µl of which was platted onto TCBS (Thiosulphate Citrate Bile Salt) agar and the plates were incubated for 16 hours at 37°C. Next day the yellowish-green *Vibrio cholerae* colonies were counted. Another section of liver was inoculated in APW (alkaline peptone water), an enrichment medium for *Vibrio cholerae* and was incubated at 37°C for 8-10 hours. A total of 200 µl of this culture was platted on TCBS agar. Next day the number of yellowish-green colonies was counted. A few of the colonies were picked and were checked for Gram staining, oxidase production and immobilization test. The colonies which gave a negative Gram reaction, positive oxidase reaction and a positive immobilization test were selected to be confirmed as *Vibrio cholerae* by PCR (polymerase chain reaction).

Isolation *of Vibrio cholerae* from gall bladder and bile

The gall bladders of the rabbits were located and were removed from the liver surface carefully. The bile was aspirated from the gall bladder and inoculated into APW. A whole gall bladder was inoculated in APW and was incubated at 37°C for 8-10 hours. Exactly 200 µl of this culture was platted on TCBS agar. Next day the number of yellowish-green colonies was enumerated. A few of the colonies were picked and were checked for gram staining, oxidase production and immobilization test. The colonies which gave a negative Gram reaction, positive oxidase reaction and a positive immobilization test were selected for confirmation to be identified as *Vibrio cholerae* by PCR.

Molecular confirmation of *Vibrio cholerae*

The PCR was carried out by the method described previously [85] For the PCR confirmation of *Vibrio cholerae*, two reported primers (VHMF: 5' TGG GAG CAG CGT CCA TTG TG 3 and VHA-AS5: 5' CAA TCA CAC CAA GTC ACT C 3') specific for *Vibrio cholerae* were used. The PCR conformation was done using 1.2% agarose gel electrophoresis in TBE buffer.

Tissue processing and staining

Tissue section from infected intestine and liver were fixed in formaldehyde and processed [9] followed by micro to my and staining with hematoxylin-eosin (H and E). Few slides were counter stained with Gram staining by the methods described previously [9].

Results

The concentrations of *Vibrio cholerae* inoculated into different loops of ileum were $1x10^6$, $1x10^7$ and $1x10^8$.The gross pathological examination revealed serosal congestion and hemorrhage which increased with increasing concentration of inoculated *Vibrio cholerae* (Figure 1 and 2). The fluid accumulated in each loop was also proportional to the concentration of inoculated Vibrios and was reported in the form of FAR. The result reflects that the fluid accumulated was directly proportional to the concentration of bacteria inoculated. The histopathological sections (Figure 3) of infected ileum stained with H & E showed that the blood vessels were congested, and inflammatory cells were present in the mucosa and sub mucosa. High-powered photomicrograph showed extensive haemorrhage extending till Muscular is propria. Transmural edema and prominent congestion were seen. Muscular is propria was distorted. Later, sections from the same biopsies were stained with H and E followed by Gram staining (Figure 4). Interestingly, it was noted that in those sections, the *Vibrio cholerae* were found cleaving propria and creating a passage for their invasion. The area in the muscular is propria where they faced some resistance, bacteria started colonizing and then because of accumulation of lot of toxins and distortion of the adjacent fibres they started their cleavage-invasion passage pattern once again, which causes the whole ileum dysfunction.

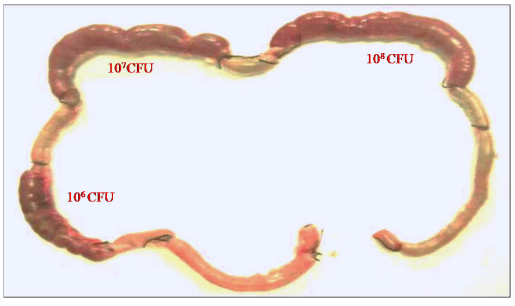

Figure1. The ileum injected with *Vibrio choleraee*.

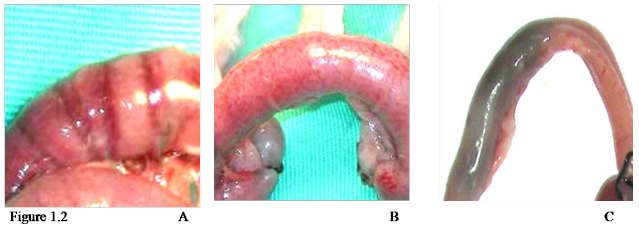

Figure 2: A. Ileum section injected with 108 CFU of *Vibrio choleraee*. **B.** Ileum section injected with 10^6CFU of *Vibrio choleraee*. **C.** Normal ileum section

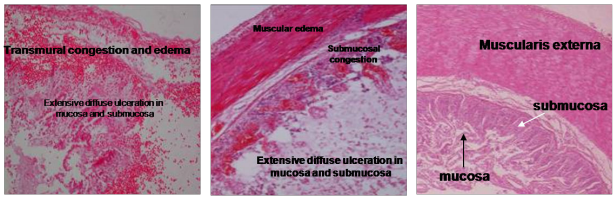

Figure 3: Histopathological features of the infected ileum sections. **A.** Ileum section injected with 10^8CFU of *Vibrio choleraee*. **B.** Ileum section injected with 10^6 CFU of *Vibrio choleraee*. **C.** Normal ileum section.

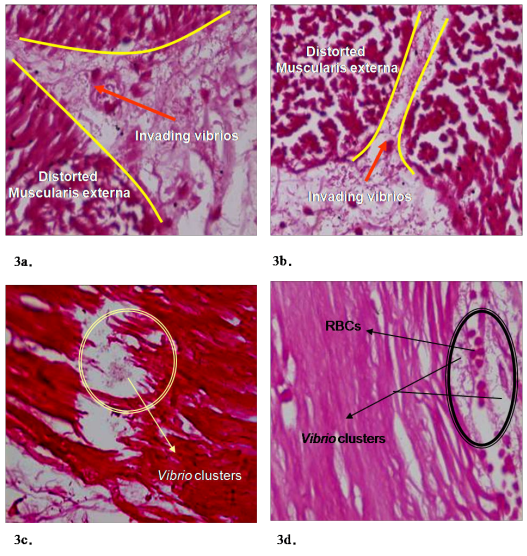

Figure 4: Invasion pattern of *Vibrio choleraee*. **a.** and **b.** Diffuse and focal invasion, stained by H & E followed by Gram staining. **c.** and **d.** Focal invasion followed by the diffuse pattern, stained by H &E followed by Safranin.

Liver biopsy which was homogenized and plated on TCBS agar were incubated at 37°C for 16 hours to count the number of *Vibrio cholerae*. It showed that liver had an average of 130 CFU. At the same time, the tissues from biopsies of liver and gall bladder were inoculated in different bottles of APW media for 8-10 hours followed by platting on TCBS agar. The yellowish-green colonies were picked and platted on LB broth with polymyxin. These colonies were checked for Gram staining, oxidase production and immobility testing. The *Vibrio cholerae* is Gram negative, oxidase positive and positive for immobility testing. The colonies which were Gram negative, oxidase positive and positive for immobility testing were confirmed at molecular level by PCR to be *Vibrio cholerae* (Figure 5). The bile which was aspirated from gall bladder was also inoculated in APW medium followed by platting on TCBS agar, which was followed by Gram staining, oxidase production and immobility testing. The colonies which were Gram negative, oxidase positive and positive for immobility testing were confirmed as *Vibrio cholerae* by PCR.

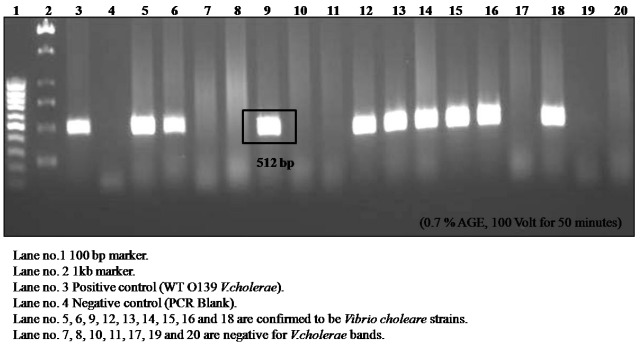

Figure 5: PCR confirmation of the *Vibrio cholerae* isolated from liver, bile and gall bladder.

Discussion

Ingested vibrios from contaminated water or food must pass through the acid stomach before they are able to colonize the upper small intestine. Colonization is aided by way of fimbria, filamentous protein structures called toxin co regulated pilus (TCP) extended from the cell wall, that attach to the receptors on the mucosa and by the bacterium's motility, which is helpful to penetrate the mucosa [10]. Formerly cholera was thought to cause sloughing of the intestinal mucosa by an inflammatory process. However, the intestinal mucosa was known to remain intact and without inflammatory changes [11]. The previous findings of mucosal sloughing were shown to be artifacts, based on autolytic postmortem changes. Koch first postulated in 1884 that the bacteria produced a toxin and this stimulated the massive outpouring of fluid from the intestine. De and Dutta were the first to demonstrate this toxin (now called cholera toxin) by use of culture filtrates in rabbits [12,23]. The toxin was later purified and sequenced [14] to have a molecular mass of 84000 kDa and consists of five binding (B) subunits and one active (A) subunit [15].

As we now understand the mechanism of action, the B subunits are physiologically inactive but bind the holotoxin to the GM1 ganglioside receptors in the small intestinal mucosa, and the A subunit is transported into the cell where it activates adenylate cyclase [16]. This activation leads to an increase in cyclic AMP, followed by an increase in chloride secretion in the crypt cells, and inhibition of neutral sodium chloride absorption in the villus cells, which in turn cause massive outpouring of fluid into the small intestine [17]. The volume secreted exceeds the normal absorptive capacity of the bowel and results in watery diarrhea. Most of the secretions come from the small intestine, although the toxin also inhibits water absorption by the

colon [18]. The diarrheal fluid contains large amounts of sodium, chloride, bicarbonate, and potassium, but little protein or blood cells [19]. The loss of electrolyte-rich isotonic fluid leads to blood volume depletion with low blood pressure and shock and then loss of bicarbonate and potassium leads to metabolic acidosis and potassium deficiency (hypokalemia). The stools of cholera patientscontain high concentrations of cholera vibrios (up to 10^8bacteria/g), and they are highly infectious. When passed into the environment, they can contaminate water sources, food and may seed an environmental reservoir [19].

Vibrio are said to be noninvasive until Calia et al. reported bacteremia by Vibrio parahaemolyticus suckling mice models [20]. It was followed by a report of bacteremia in Gulf Coast community with and without underlying infections. The patients with underlying infections were reported to have soft tissue infections which often progressed to fatal septicemias [21]. The same year a case of bacteremia in an infant was reported in which a 6 day old black male was reported who presented with diarrhea and biochemical evidence of severe electrolyte imbalance. Despite treatment with intravenous fluid and antibiotics, he died within 24 hours of admission. Enterotoxigenic *Vibrio cholerae* El Tor, serotype Inaba was isolated from blood [22]. In 1986, McClesky et al. reported a case of bacteremia by isolating non O1 *Vibrio cholerae* from a patient with cirrhosis after placement of LeVeen shunt [23]. Safrin and colleagues reported a case of non O1 *Vibrio cholerae* and prostatic abscess in a patient with idiopathic aplastic anemia and the data were compared with 23 previously reported cases of non O1 *Vibrio cholerae* bacteremia. The case fatality rate was reported to be 61.5% for 13 cases with majority of cases reported in immune compromised patients particularly those with hematologic malignancy or cirrhosis. Another study also reported the importance of host susceptibility to be potentially important. The *Vibrio cholerae* were identified in sheep blood agar as in previous findings [5]. An interesting case report came from Dhar et al in 1989 which reported the presence of non O1 *Vibrio cholerae* in 50 year old woman and 31 year old man with underlying liver disease presented with fever and signs of liver failure but the source of infection could not be identified in both cases with the only difference that one of the strains after being identified biochemically was non motile [24,25]. It was followed by reports from various authors describing the cases of non O1 *Vibrio cholerae* bacteremia in various parts of world [26-28] many of them using biochemical and microbial cultures for identifying *Vibrio cholerae.* Starting from January 1983 to march 1984, 26 isolates of Vibrio species were recovered from blood of patients admitted to a hospital in India in Siriraj hospital. Only 13 strains were identified as non invasiveO1 *Vibrio cholerae*, 3 were Vibrio vulnificus and 10 were Vibrio species. Most of the patients were adult men with cirrhosis and history of seawater exposure [29]. A similar case associated with prior gastrectomy was reported with the hypothesis that all the cases yet reported were immune deficient and this was the first case reported in healthy patient and susceptibility to such infection may have been enhanced by a prior gastrectomy for duodenal ulcer [30]. Jesudason and colleagues reported *Vibrio cholerae* to be the cause of gastroenteritis and extra intestinal manifestations including septicemia [31] followed by a similar report [32]. A report was published presenting a case of non O1 *Vibrio cholerae* septicemia with myelodysplatic syndrome in Taiwan [33].

In 1992, Jamil and colleagues reported a case of O1 *Vibrio cholerae* septicemia [34]. In the same year, Russel and colleagues made use of new techniques to confirm the invasion of *Vibrio cholerae* in rabbit ileum using different models and they found the intestinal bacterial colonization, intestinal fluid volume and onset of diarrhea caused by non O1 *Vibrio cholerae*. They also reported that the intestinal damage of the bacterial strains were dose dependent with necrosis of the intestinal lamina propria in RITARD rabbits [35]. A case of *Vibrio cholerae* septicemia in 45 year old male with a three year history of liver cirrhosis was reported and the septicemia was associated with severe underlying diseases such as leukemia and liver cirrhosis [36]. Bloodstream invasion of O139 *Vibrio cholerae* was reported in 1993 [7] with a similar report of non O1 meningitis in infant, in Israel and a patients with multiple myeloma respectively [37-39]. Another case of *Vibrio cholerae* invasion was reported in a 44 year old alcoholic man with a fever and bullous cellulites of the lower extremities with no liver cirrhosis [40]. Levine and colleagues also reported cases of septicemia followed by Vibrio infection and invasion in Gulf Coast [41]. Another case of *Vibrio cholerae* bacteremia was reported in patient with multiple myeloma [42]. In 1994, cases of bacteremia with non O1 *Vibrio cholerae* were reported associated with gastroenteritis, meningitis, liver cirrhosis and immnocompromised patients [43-46]. Boyce and colleagues reported a Biomedical Journals-14-763(R-103) Scase of bacteremia by *Vibrio cholerae* O139 after O139 out-break [47] with the same reports from Khan and colleagues [48] and another case of *Vibrio cholerae* non O1 and non O139 associated with hemorrhagic bullous skin lesions of the lower extremities [49]. A case of primary septicemia with *Vibrio cholerae* non O1 was reported in a burn patient [23] with a short gap with a case of nephritic syndrome with non O1 *Vibrio cholerae* bacteremia and peritonitis [50]. Many other cases of septicemia due to non O1 and non O139 were also reported by various researchers in different underlying disorder [51-54]. A case of *Vibrio cholerae* non O1 sepsis in healthy patient was reported in Spain [20] followed by a report on the infection of non O1 *Vibrio cholerae* in Southern Taiwan with bacteremia with concurrent spontaneous bacterial peritonitis or invasive soft tissue infection occurring solely in cirrhotic patients, self-limited acute febrile gastroenteritis that often resulted from a wound on extremities [55]. The same year another case was reported in Mexico in which a 55 year old man developed acute cholera cystitis. West et al., described the first case of septicemic acute acalculous cholecystitis caused by non O1 *Vibrio cholerae* is described in a healthy traveler, and bilary tract infections from *V. cholerae* are reviewed. Immediately after a vacation in Cancun, Mexico, a 55 year old man developed acute cholecystitis. Blood and bile cultures grew non O1 *V. cholerae*. At surgery, the gallbladder was acalculous, inflamed, distended and nearly ruptured. Pathogenetic factors may have included diarrhea prophylaxis with bismuth subsalicylate, distension of the gallbladder from illness induced fasting, and bacterial toxins in the gall bladder. The patients received i.v cephapirin, followed by oral cephradine for a total of 10 days, and he made a quick and complete recovery. *V. cholerae* should be considered in the differential diagnosis of persons from endemic areas who present with cholecystitis or acute jaundice [56]. In 1999, Albert and colleagues reported that the capsulated bacteria exhibited serum resistance and resistance to phagocytosis which resulted in disseminated infections. *Vibrio cholerae* O139 strains possess a thin capsule and have been found to be partially serum resistant and are partially resistant to phagocytosis [3].

The no toxigenic *Vibrio cholerae* O1 was also reported to cause bacteremia [57,58]. There were many similar reports with bacteremia also with non O1 *Vibrio cholerae* in the same year [59-62]. Santamaria and colleagues reported a case of bacteremia with O1 *Vibrio cholerae* in neonate with hypovolemic shock [63]. Many other reports were

published in year 2003 to 2006 stating the invasion of non O1 and O139 *Vibrio cholerae* and bacteremia due to invasion [28,64-68].

In 2007, a case of bacteremia due to O1 *Vibrio cholerae* was again reported in Brazil in 70 years old patient with sepsis [69]. In 2009, Amin and colleagues reported the difference in invasion pattern of O1 and O139 *Vibrio cholerae* in rabbit ileum which stated the level of invasion of *Vibrio choleraee* O139 was more thanO1 El Tor and the pattern of invasion was different [6,70-78]. In this paper we also found that the O139 invaded in a diffuse pattern along with the focal pattern (Figure 3). The FAR is related to the bacterial concentration (Figure 6). Gross serosal hemorrhage (Figure 1) was also found to be similar with previous reports [6,79-83]. The histopathological sections reflected trans mural congestion and excessive ulceration in mucosa and submucosa which also support the previous report [6,84-88]. The detection of *Vibrio cholerae* was confirmed with all possible microbiological and molecular techniques. For the detection of *Vibrio cholerae* in liver, gall bladder and bile the biochemical analysis was done. The molecular analysis was done to confirm the presence of *Vibrio cholerae* in liver, gall bladder and bile by the method of Ravichandran et al., 2007, using specific primers for PCR.

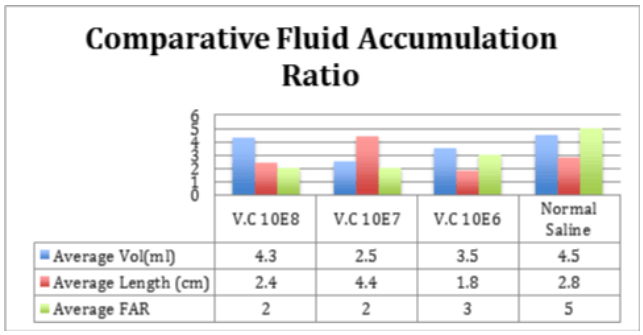

Figure 6: Comparative Fluid Accumulation Ratio in different ileal loops (for every group, n=4).

We concluded the presence of *Vibrio cholerae* in liver, bile and gall bladder homogenized tissue. Retrograde spreading of bacteria to the liver via the common bile duct was excluded by complete closure of the intestinal lumen distant to the duct in the intestinal lumen. However, the bacteria might traffic, probably via macrophages, to distant areas in the body including the liver. Another study in future is a need to identify the lesions of *Vibrio cholerae* invasions within liver and gall bladder by immunohistochemistry and using GFP visibility for *Vibrio cholerae* passage.

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