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Comparative analysis of different oral approaches to treat *Vibrio cholerae* infection in adult mice

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ABSTRACT

In this study, we have established an oral phage cocktail therapy in adult mice model and also performed a comparative analysis between phage cocktail, antibiotic and oral rehydration treatment for orally developed *Vibrio cholerae* infection. Four groups of mice were orally infected with *Vibrio cholerae* MAK 757 strain. Phage cocktail and antibiotic treated groups received 1×10^8 plaque forming unit/ml (once a daily) and 40 mg/kg (once a daily) as an oral dose respectively for consecutive three days after bacterial infection. In case of oral rehydration group, the solution was supplied after bacterial infection mixed with the drinking water. To evaluate the better and safer approach of treatment, tissue and serum samples were collected. Here, phage cocktail treated mice reduced the $\log_{10}$ numbers of colony per gram by 3 $\log_{10}$ ($p < 0.05$); however, ciprofloxacin treated mice reduced the viable numbers up to 5 $\log_{10}$ ($p < 0.05$). Whereas, the oral rehydration solution application was not able to reduce the viable bacterial count but the disease progress was much more diminished ($p > 0.05$). Besides, it was evident that antibiotic and phage cocktail treated group had a gradual decrease in both IL-6 and TNF-α level for 3 days ($p < 0.05$) but the scenario was totally opposite in bacterial control and oral hydration treated group. Histological examinations also endorsed the phage cocktail and ciprofloxacin treatment in mice. Although, in this murine model of cholera ciprofloxacin was found to be a better antimicrobial agent, but from the safety and specificity point of view, a better method of application could fill the bridge and advances the phages as a valuable agent in treating *Vibrio cholerae* infection.

Introduction

Diarrheal disease is a foremost cause of morbidity and mortality in the world today. *Vibrio cholerae* is a gram-negative pathogen that induces diarrhea dependent upon the action of the cyclic AMP-stimulating cholera toxin (CT) and among the key pathogens responsible for inducing diarrhea throughout the world, mostly developing countries. The way of acquiring infection is through contaminated water or food products and once it establishes infection, it induces a severe watery diarrhea that persists for days to weeks. This sickness can lead to dehydration and death if not treated. The majority of bacteria are killed by the acidic pH in the stomach. Those that survive pass in to the lumen of the small intestine and initiate colonization. According to Southeastern and Central Asia reports the annual acute diarrheal cases for *V. cholerae* infection were estimated more than 0.5–0.7 million (WHO, 2013). Recent, outbreak in Sierra Leone has recorded 23,124 cases of cholera, including 299 deaths (WHO, 2013). These situations again reveal the devastating nature of cholera. Moreover, the number of bacterial infection may be increasing gradually due to the resistance to antibiotics.

Phage therapy is among one of the reemerging approaches of treatment and has been considered since the late 1980s (Barrow and Soothill, 1997; Brussow, 2005; Merril et al., 2003). Most of the phages are specific to their target bacterial host cell but are unresponsive to human or eukaryotic cell (ICMR Bulletin 2002; Filippov et al., 2012) whereas antibiotics target both pathogenic microorganisms and normal microflora. This clear distinction, explores the beneficial nature of bacteriophages. Bacteriophage therapy is an approach of harnessing phages as bioagents for the treatment of bacterial infections. Antibiotic resistance is an everlasting problem to the researchers as new antibiotics can be developed through extensive funding but ultimately pathogens will be resistant to it. So, phage therapy may be an alternative and potential weapon against infectious diseases as it has been experimentally

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proved that bacteriophages are therapeutically superior to antibiotic therapy in many ways (Lancet, 1983; Barrow and Soothill, 1997; Lederberg, 1996; Pirisi, 2000). Moreover, the factors that validate the use of phage therapy are, (i) it is still being used for treatment of humans (Górski et al., 2009) and (ii) phage biology is much better understood today than it was in the mid-20th century, when it was ignored in support of antibiotics (Hatfull, 2008; Campbell, 2003; Summers, 2001). Bacteriophages are natural predators of bacteria, abundant in environment, self-limiting and self-replicating in their target bacterial cell (Carvalho et al., 2010). Furthermore, bacteriophages can adapt to resistant bacteria.

Very promising results have been obtained by many researchers regarding the application of phage therapies in animal models (O'Flynn et al., 2004). Different animal models have been used in cholera research but the true fact is no individual animal model reproduces all the characteristics of human diseases (Richardson, 1994). A variation of nutritional, chemical, and antibiotic treatments allows the colonization of adult rabbits by *V. cholerae*, but such manipulations may not be physiologic. In case of suckling mice model *V. cholerae* can infect suckling animals but their immature host defenses and reduced intestinal floras is often fatal and is a poor model to study the development of immune responses (Butterton et al., 1996). In contrast, adult mice model would convey a mature immune system for assessing the immune responses. Mouse model can perform as a more versatile model than the rabbit because of the availability of different genetic backgrounds of mice as well as ease of handling compare to other animals. Different therapeutic experiments establish the importance of mouse model in the field of research (Chhibber et al., 2008; Sheng et al., 2006; Maura and Debarbieux, 2012; Capparelli et al., 2010; Sunagar et al., 2010). Presently, there are two different ways to treat *V. cholerae* infection, which include oral rehydration solution (ORS) treatment and antibiotic treatment. From the late 1960s establishment of the efficacy of oral rehydration solutions has gained importance in treating cholera (Hirschhorn et al., 1968; Pierce et al., 1968). The mechanism of action of ORS is to enhance the absorption of sodium and fluid in the small intestine, even in cases of enterotoxic diarrhea, where fluid loss is often substantial. It is an important intervention for reducing the morbidity and mortality associated with diarrheal disease, regardless of etiology (WHO, 2000) but lack of clean water supply often diminishes the effectiveness of ORS treatment. Antibiotics are not obligatory to successfully treat patients, but they can reduce the duration of disease, diminish the volume of rehydration fluids needed, as well as reduces the duration of shedding of the pathogen. Different classes of antibiotics such as doxycycline, ciprofloxacin and azithromycin are effective in the treatment of cholera in treating *V. cholerae* infection (Nelson et al., 2011). Fluoroquinolone drugs are commonly used in the treatment of enteric infections, because of their good in vitro activity, the high serum and stool concentrations achieved after ingestion, and their safety (DuPont et al., 1993). Several studies were performed earlier to validate the in vivo efficacy of fluroquinolone drugs against *V. cholerae* infection in human clinical trials (Bhattacharya et al., 1990; Gottuzo et al., 1995; Khan et al., 1995).

The aim of this present study was to investigate the potential role and feasibility of phage cocktail therapy compare to the traditional antibiotic and oral rehydration therapy (ORT), as an alternative regimen against the multidrug resistant *V. cholerae* infection. For this purpose inflammatory cytokines, tissue histology, bacteria and phage counts were taken as the parameters.

Materials and methods

All personnel followed strict biosafety procedures when handling *Vibrio cholerae* O1, Ogawa, MAK 757 and/or animals, and all procedures were approved by the Institutional Animal Ethical and Biosafety committees. Independent trials were executed three times in each experiment. The error bars in the graphs are represented of the standard deviation in each experiment. The mice were weighed before inoculation and thereafter daily as an indicator of health.

Bacterial strain and culture

Vibrio cholerae O1 biotype El Tor strain Ogawa MAK 757 (ATCC 51352) was stored at −80 °C in Luria–Bertani (LB) (Merck, Germany) medium containing 15% glycerol (Merck, Germany) and grown in LB medium at 37 °C with agitation for 18 h, used here as the challenge strain. Before using the strain for experiment the virulence of the strain was confirmed in vitro by determining growth characteristics and detecting virulence markers by the polymerase chain reaction (PCR) and by in vivo passage in mice. The strain was plated directly onto thiosulfate citrate bile salt sucrose agar (TCBS) medium (Eiken Chemical Co. Ltd., Tokyo, Japan) and incubated at 37 °C for 18 h. *V. cholerae* was identified and confirmed using standard techniques (WHO, 1993). The strain was successively serotyped using specific *V. cholerae* O1 polyvalent antiserum (Difco, Sparks, MD, USA). For challenging mice, bacteria were grown to an optical density at 540 nm of 1.0 (ca. 1×10^9) colony-forming units (CFU)/mL centrifuged at 8000 rpm for 10 min at 4 °C and the pellet was washed twice with 0.01 M phosphate-buffered saline (PBS) (pH 7.4) and ultimately suspended in PBS. Quantification of sustainable cell number was accomplished by plating on Luria agar and TCBS plates.

Bacteriophage preparation

This procedure is routinely followed at our laboratory (Sarkar et al., 1994). A plate lysis procedure was used to acquire a high titer of phages. Briefly, a fresh nutrient broth culture (10^7 cells per ml) of *V. cholerae* MAK 757 was mixed with phage at a multiplicity of infection (MOI) of 0.01 and plated on a soft agar (0.8%) overlay (3.5 ml) on nutrient agar and incubated at 37 °C. The soft agar layer was scraped off after complete lysis and suspended in 1 ml of Tris–$MgCl_2$ buffer (0.05 M Tris–HCl, pH 7.5, with 0.02 M $MgCl_2$) (Sigma, St. Louis, USA) and centrifuged at 10,000 × *g* for 20 min at 4 °C. Aqueous layer was filtered through a 0.22 μm membrane filter (Millipore, MA, USA) and the concentrated phage particles were preserved at 4 °C. A combination of five (ATCC 51352 – BI, B2, B3, B4, and B5) lytic *V. cholerae* O1 biotype El Tor phages diluted with PBS was used in this study. The five phages were mixed to prepare a cocktail of 1×10^8 plaque forming unit/ml (PFU/ml).

Pulse field gel electrophoresis (PFGE)

Pulse field gel electrophoresis was performed according to modification of the protocol defined by Shen et al. (2012). Concentrated phage suspension (ca. 1×10^{11} PFU ml) was mixed with an equal volume of molten 1% agarose (SeaKem Gold Agarose, Lonza), which was allowed to solidify in a mold. The solid block was lysed at 55 °C overnight by proteinase K (10 mg/ml) in cell lysis buffer (50 mmol/l Tris, 50 mmol/l EDTA at pH 8.0, 1% Sarcosine). After the proteinase K lysis, the agarose plug was washed in TE buffer three times for 20 min each. The plugs were loaded onto 1% agarose prepared with 0.5× TBE (pH 8.0) running buffer. The plugs were analyzed by PFGE (using the CHEF-MAPPER apparatus from Bio-Rad, Richmond, CA, USA) at 14 °C with a ramping time of 2–12 s for 8 h, at field strength of 6 V/cm.