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Evaluation of immunogenicity and protective efficacy of combination heat-killed immunogens from three entero-invasive bacteria in rabbit model

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ABSTRACT

Diarrhea is a very common health problem in both developing and developed countries. Among the major entero-invasive bacteria, *Shigella*, *Salmonella* and *Campylobacter* cause serious problems in different geographic regions. Recently we have shown immunogenicity and protective efficacy of heat killed multi-serotype *Shigella* immunogen in different animal models. In our present study, we have advanced our research by preparing a combination heat-killed immunogen of three different entero-invasive bacteria, *Shigella, Salmonella and Campylobacter*. After three doses on 0th, 14th and 28th day of oral immunization with tri-valent heat-killed (TVHK) immunogen in rabbit model, the immunogenicity was determined by differential count of white blood cells and immunoglobulin assay at various time points. During oral immunization differential count of lymphocytes increased where as polymorphonuclear leucocytes (PMN) count decreased. Serum IgG and IgA showed significant elevation during oral immunization and remained at a detectable value upto 120 days. Protection study was performed in both, *in vitro* and *in vivo* conditions, using bacteriocidal assay and rabbit ligated ileal loop model, respectively, which conferred protection against homologous bacteria. Moreover, immunoblot assay against whole cell lysate and lipopolysaccharide exhibited significant amount of antigen-specific immunoglobulins raised against three different bacteria which proved that proteins along with lipopolysaccharides played a pivotal role in immunogenicity and protective efficacy. This trivalent heat-killed immunogen could be a low-cost, simple, oral, non-living vaccine candidate for future use against invasive diarrhea.

1. Introduction

Enteric bacterial infection is one of the major causes of diarrhea worldwide especially in the developing world (Mathan, 1998 Mathan VI., 1998; Roy et al., 2010) Globally, there are nearly 1.7 billion cases of diarrhea every year, most of which is due to lack of sanitation and access to safe drinking water. In developing countries children experience on an average three episodes of diarrhea and malnutrition due to this each year (WHO, 2013). Among bacterial entero-pathogens the three most commonly circulating entero-invasive bacteria are *Shigella, Campylobacter* and non-typhoidal *Salmonella* (DuPont, 2009). The pathophysiology of these three pathogens is quite similar. Through uptake of contaminated food or water these pathogens pass the stomach of the host and colonize in their primary target site of host intestine. They modulate the host system for invasion and gradually establish infection.

Shigella is a gram-negative, facultative anaerobic and intracellular human pathogen (Phalipon and Sansonetti, 2007; Schnupf and Sansonetti, 2012), and about 160 million infections annually are caused by *Shigella* species in the developing countries. Among the children of age 12–59 months *Shigella* remains as the main cause of child morbidity (Global enteric multicenter study, 2014). Invasive *Shigella* can cross intestinal mucosa with the help of M-cells and is subsequently phagocytosed by macrophages and dendritic cells. It can also spread to neighbouring cells by actin based motility causing intestinal epithelial destruction and bloody diarrhea (Schroeder and Hilbi, 2008).

Similarly *Campylobacter* is prevalent in adults but the peak isolation rates are found in children less than 2 years old. While infection may remain asymptomatic, it may also range from watery diarrhea to bloody dysentery. Cattle and poultry are the sources of infection in developing as well as developed countries. Rarely, it may lead to

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Guillain-Barre syndrome (1 in 1000 people) with residual paralysis (Janssen et al., 2008; Riazi et al., 2013).

On the other hand gastroenteritis due to non-typhoidal *Salmonella* is now a global burden all year round. It is associated with acute onset of nausea, vomiting and diarrhea that may be watery or bloody. The organisms colonize the ileum and colon, invade the epithelium with the help of certain virulence factors and then proliferate intracellularly within the epithelium and lymphoid follicles. Preventive measures include improvement of sanitation, rehydration therapy (if dehydration occurs) and most essentially, antibiotic therapy (Thielman and Guerrant, 2004). These invasive enteric bacteria are showing signs of becoming untreatable disease due to the global emergence of multidrug resistance (MDR) (Hoge et al., 1998) which is a major challenge to global drug discovery programs. Improvement of sanitation is a major challenge in many Asian and African countries due to lack of resources. Therefore, developing vaccines is currently the only foreseeable way of preventing these diseases. Scientists, academicians, and public health experts are working to develop suitable vaccines. Although some trials are going on all over the world but no promising vaccine is available for public use.

Though a number of licensed vaccines against various enteric pathogens like cholera, rotavirus and typhoid fever are now available in the market, these vaccines are little used for routine control of disease in developing countries due to various financial constraints. It is well documented that live oral vaccines may fail to confer high levels of protection in poor populations living in impoverished settings in the developing countries (Clemens, 2011). Safety is also a major concern with live, attenuated vaccines depending on the degree of attenuation and the immunocompetence of the host (Kuehl et al., 2014). Therefore, in the present investigation we chose to use heat-killed whole cell bacteria for immunizing the animals.

Although single agent vaccines are currently available in the market, combination vaccines against the diarrheal agents are still in the initial stages of development. If different antigens from different bacteria can be merged into a single combination vaccine, that will protect against multiple strains of those infectious diarrheagenic bacteria. Such combination vaccines will be most beneficial to the pediatric population since they continue to be the worst affected. Studies are currently underway to test potential combinations against the most common pathogens including rotavirus, ETEC, *Shigella, Salmonella, Campylobacter* and *Vibrio cholerae* (Venkatesan and VandeVerg, 2015). A recent study showed high immunogenicity and protection against two noninvasive pathogens *V. cholerae* and ETEC when immunized with a combination vaccine of bacterial outer membrane vesicles of *V. cholerae* and ETEC (Leitner et al., 2015).

Thus from these studies and the tremendous impact of the above mentioned three organisms on the social milieu, a novel approach has been taken to study the combination of three common invasive enteric bacteria *Shigella, Salmonella* and *Campylobacter*. In our study we used heat killed immunogen of *Shigella flexneri* 2a 2457T, *Campylobacter jejuni* BCH 2594 and *Salmonella enterica* serovar Typhimurium SL-1344 and mixed them in equal volume to prepare a novel combination vaccine against invasive bacteria. We evaluated different immunological parameters, as well as a protection study in rabbit ligated ileal loop model using homologus strains, after three doses of immunization.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Invasive strains of *S. flexneri* 2a 2457T (Kuehl et al., 2014), *C. jejuni* BCH 2594 (used in this study) and *S. enterica* serovar Typhimurium SL-1344 (Gruzdev et al., 2012), were selected for our present experiments. All strains were stored in 15% glycerol with brain heart infusion broth (Difco, USA) at −80 °C. *S. flexneri* and *S.* Typhimurium were grown in Tryptic Soy Broth (TSB; Difco, USA) and Tryptic Soy Agar respectively (TSA; Difco, NJ, USA) at 37 °C. CCDA and Muller Hinton Broth (MHA; Difco, USA) were used for growth of *C. jejuni* at 37 °C in microaerobic conditions.

2.2. Animals

New Zealand white rabbits of either sex weighing about 2–2.5 kg were taken from the animal resource department of the National Institute of Cholera and Enteric Diseases, Kolkata and were divided into two groups of three each-control and immunized group. The control and experimental rabbits were kept in separate cages at 25 °C with 75% humidity and fed sterile food and water.

2.3. Ethics statement

All the animal experiments were conducted following the standard procedures as outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. The animal experimental protocol was approved by the Institutional Animal Ethical Committee of National Institute of Cholera and Enteric Diseases (PRO/117/June 2015–June 2018).

2.4. Preparation of heat killed immunogen

Overnight plate cultures of *S. flexneri* 2a 2457T, *C. jejuni* BCH 2594 and *S.* Typhimurium SL-1344 were suspended in phosphate-buffered saline (PBS) (pH 7.4) and centrifuged at 8000*g* for 10 min. The bacterial pellet so obtained was washed twice and resuspended in PBS. The suspension was adjusted to 10^9 cfu/ml by measuring OD at 600 nm to prepare heat-killed bacterial immunogen (65 °C for 2 h) (Nag et al., 2015) and used as a monovalent immunogen. Bacterial viability was further tested by streaking on selective media. No growth was observed on any of the plates, thus confirming the absence of viable bacteria. Equal volumes (1:1:1 ratio) of each heat killed bacteria (1×109 cfu/ml in PBS) was mixed together to prepare a TVHK immunogen and used for oral immunization.

2.5. Immunization protocol

The rabbits were immunized orally at 0th, 14th, and 28th days with cocktail immunogen for immunized group and with PBS for control group following a modified version of the protocol described by Roy et al. (2010). The rabbits were kept in a fasting state for 24 h, but water was given to them *ad libitum*. Thirty minutes before immunization, each rabbit was anaesthetized by intramuscular injection of a mixture of ketamine (35 mg kg^{-1} body weight; Sterfil Laboratories Pvt., Ltd., India) and xylazine (5 mg kg^{-1} body weight, Astra Zeneca Pharma India Ltd., India). Two boluses of sodium bicarbonate (15 ml of a 5% solution; SRL, India) at 15-min intervals were administered directly into the stomach via a feeding tube (Romsons Sci. and Surg. Pvt., Ltd., India). The second bolus was immediately followed by oral administration of the cocktail antigen (1 ml of 1:1:1 mixture) to the experimental rabbits and the same volume of PBS to the non-immunized control group. The rabbits were then returned to their cages and fed limited amounts of sterile food and water.

2.6. Collection of blood samples

Blood was collected from the ear veins of immunized and non-immunized rabbits at days 0, 7, 14, 21, 28, 35, 56, 63, 75, 90 and 120 after first immunization. The blood was collected in separate vials which either contained 0.1 μl heparin for anticoagulation, or were