Vaccine xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Cholera toxin-B (ctxB) antigen expressing *Salmonella* Typhimurium polyvalent vaccine exerts protective immune response against *Vibrio cholerae* infection

Vikalp Vishwakarma[a], Sushree Sangita Sahoo[a], Susmita Das[a], Shilpa Ray[a], Wolf-Dietrich Hardt[b], Mrutyunjay Suar[a,*]

[a] School of Biotechnology, KIIT University, Bhubaneswar 751024, Odisha, India
[b] Institute of Microbiology, ETH Zurich, Zurich, Switzerland

ARTICLE INFO

Article history:
Received 4 August 2014
Received in revised form 25 January 2015
Accepted 4 February 2015
Available online xxx

Keywords:
Live attenuated vaccine: LAV
Z234-pMS101 vaccine
Polyvalent vaccine
ctxB
Cholera
Salmonellosis
Immunocompromised host

ABSTRACT

Live attenuated vaccines are cost effective approach for preventing a broad range of infectious diseases, and thus are of great interest. However, immune-defects can predispose the patient to infections by the vaccine candidate itself. So far, few live vaccine candidates have been designed specifically for immune compromised individuals. Recently, we reported a new *Salmonella* Typhimurium Z234-vaccine strain (Periaswamy et al., PLoS ONE 2012;7:e45433), which was specifically attenuated in the NADPH-oxidase deficient host. In the present study, the Z234-vaccine strain was further engineered to express heterologous antigen (*Vibrio cholerae* toxin antigen subunit-B, *i.e.* CtxB) with the intention of creating a vector for simultaneous protection against Cholera and Salmonellosis. The primary aim of this study was to ensure the expression of CtxB antigen by the recombinant vaccine strain Z234-pMS101. The antigen CtxB was expressed through Z234 as a fusion protein with N-terminal signal sequence of *Salmonella* outer protein (SopE), an effector protein from *Salmonella* under the control of SopE promoter. The CtxB-expressing plasmid construct pMS101 (pM968-pSopE-ctxB) was found to be stable both *in vitro* and *in vivo*. In an oral mouse infection model, the vaccine strain Z234-pMS101 efficiently colonized the host gut. The extent of protection was confirmed after challenging the immunized hosts with live *V. cholerae*. Vaccinated mice showed reduced gut colonization by *V. cholerae*. Further assessment of immunological parameters supported the possibility of conferring effective immune response by Z234-pMS101 vaccine strain. Overall, the Z234-pMS101 vaccine strain showed potential as a promising polyvalent vaccine candidate to protect against *S.* Typhimurium and *V. cholerae* infection simultaneously.

1. Introduction

Infections by enteric pathogens such as *Salmonella enterica* and *Vibrio cholerae* are one of the major causes of mortality and morbidity in most developing countries. [1–4]. *S. enterica* serovar Typhimurium, a Gram negative enteric pathogen, has the ability to cause diverse disease profiles ranging from mild self-limiting gastroenteritis to severe systemic infection [5,6]. More than two million people in the world die annually from foodborne illness. Typhimurium and Enteritidis serovars of *Salmonella* are two major infectious agents causing human gastroenteritis [1,7]. The disease, cholera, has been prevailing in the Indian subcontinent since the 5th century BC, but has been vastly under-reported in the present context [2,8]. Both *S. enterica* and *V. cholerae* afflict in the same epidemiologic context [9,10]. According to the WHO report, about three million cases occur predominantly in Asia and Africa every year [11]. The unmet need to develop an affordable and effective vaccine for these pathogens is highly essential [12–14]. Hence, a polyvalent live attenuated vaccine to simultaneously target both of the pathogens might represent an effective solution to problems like Salmonellosis and Cholera.

Salmonella Typhimurium causes a self-limiting gut infection by colonizing in the gut lumen, gut tissues, and the organized structures of the gut-associated immune system. It can establish systemic infections in complicated cases and in immunocompromised hosts [15–17]. The type-III secretion systems (T3SS) encoded by SPI1 (T3SS1) and SPI2 (T3SS2) islands, majorly contributes to the virulence, and enable *Salmonella* to deliver its effector proteins

* Corresponding author. Tel.: +91 9437011465.
E-mail address: msbiotek@yahoo.com (M. Suar).

http://dx.doi.org/10.1016/j.vaccine.2015.02.014
0264-410X/

into host cells, inducing cytoskeletal rearrangement and establishing different phases of infection [6,16,17]. In this context, the *Salmonella* outer protein (SopE) is a crucial effector virulence protein that triggers different sets of RhoGTPase signaling cascades, thus playing a very important role in establishing infection [18]. The *Salmonella* strains, lacking specific virulence protein, offers the opportunity to design attenuated vaccine strains. For typhoid fever, there are certified safe Live-attenuated vaccines (LAV) available for human. For non-typhoidal strains of *Salmonella* strains, there are commonly used LAV for veterinary use but not for humans, specifically for *Salmonella* serovars Typhimurium and Enteriditis [19,20]. Researchers have attempted to develop live-attenuated vaccine strains like WTo5 (aroC, ssaV), LH1160 (phoP, phoQ, purB), VNP20009 (*S.* Typhimurium *ΔmsbB ΔpurI*), CVD1941 (*S.* Enteritidis R11 Δ*guaBA* Δ*clpP*) [21–23]. Although these vaccine strains elicit efficient immune responses but incidents of vaccine strain inflicted disease development have also been reported [24,25]. These reactions were observed in normal healthy hosts and in immune-compromised individuals [26–28]. Hence, researchers are now focusing on constructing LAV against the non-typhoid *Salmonella* (NTS) strains which would be safe for immunocompromised individuals while maintaining the required potency to elicit effective immune responses in normal individuals as well.

V. cholerae, the causative agent of cholera, is classified into more than 200 serogroups by the O-antigen of the lipopolysaccharide [3,29]. Of all of the serotypes, O1 and O139 serogroups cause epidemic cholera [29]. Early isolates of *V. cholera* O1 are susceptible to most antibiotics; however, *V. cholerae* O139 and some isolates of *V. cholera* O1 E1 Tor, have acquired an SXT element that mediates resistance to co-trimoxazole, streptomycin, and other broad-spectrum antibiotics [30,31]. The prevalence of these multi-resistant strains has made cholera a prime target for vaccine development [2,32,33]. Oral vaccines of cholera, Dukoral [34] and Shanchol [35] have showed excellent safety profiles and mucosal immunity. Several live attenuated oral cholera vaccines have also been developed, including CVD 103-HgR and Peru-15 [36,37]. These genetically modified organisms shared the ability to express a detoxified form of cholera toxin. These strains have been shown to be immunogenic in volunteer studies [38–40], but they failed to show their protective efficacy. Also, the safety profile of these vaccines in immune-compromised subjects was not validated [41–49].

Previously, we designed the *S.* Typhimurium Z234 vaccine strain (Δ*ssa*V SL_1344_3093::*aphT*) and reported its protective efficacy against *S.* Typhimurium infection in the vaccinated hosts. We also demonstrated the safety aspects of Z234 in immunocompromised hosts [50]. Further, we were interested to engineer Z234 to mount protective response against additional mucosal infections caused by bacteria, such as *V. cholerae*. The present study addresses the design of a polyvalent vaccine against cholera and salmonellosis by exploiting the previously published vaccine strain Z234 and T3SS1-encoded effector protein (SopE) of *Salmonella* [51–53]. In this study, we demonstrated the immunogenicity and efficacy of the engineered vaccine strain Z234-pMS101 using a *V. cholerae* colonization model.

2. Materials and methods

2.1. Bacterial strains, plasmids and primers

All the bacterial strains, plasmids and primers used in this study are listed in Table 1. LB broth supplemented with 0.3 M NaCl was used to culture the bacterial strains and to stimulate the expression of the components associated with T3SS1. The development of plasmid pMS101 and strain Z234-pMS101 is described in supplementary file (Fig. S1).

2.2. Plasmid stability assay

The experimental test strain (Z234-pMS101) was tested for its plasmid (pMS101) stability both *in vitro* and *in vivo* conditions. For *in vitro* assessment, the test strain was grown overnight in LB medium (0.3 M NaCl) supplemented with ampicillin (5 μg/ml); further, it was repeatedly subcultured daily for 12 days in LB (0.3 M NaCl) without antibiotic. Daily cultures were suitably diluted and plated on MacConkey agar plates (supplemented with 100 μg/ml ampicillin; to get counts of plasmid pMS101-harboring bacteria) and plates without any antibiotic (to get total count of the total bacteria in the culture). Finally the cumulative data obtained from two sets was compared. Similarly, for *in vivo* analysis of plasmid stability, streptomycin pretreated C57BL/6 mice ($n = 4$) were infected with 5×10^5 CFU of Z234-pMS101 test strain and their feces were collected at different time intervals (day 1, 4, 8 and 12). The feces were homogenized in PBS and grown on MacConkey agar plates supplemented with or without ampicillin (5 μg/ml). Finally, obtained CFUs were compared and the cumulative data was plotted.

2.3. Evaluation of CtxB expression by Z234-pMS101

Bacteria from *Salmonella* Typhimurium SL1344 (wild-type), parental vaccine strain Z234 and the test vaccine strain Z234-pMS101 cultures were harvested. The proteins of supernatant fraction was precipitated by chilled acetone (protocol described in Supplementary file S3) and stored at -80^{0}C for detection of CtxB in supernatant fraction by ELISA. The cellular pellet fraction was washed with PBS, resuspended in 1 ml of fresh PBS and processed further for assessment of CtxB expression. The

Table 1
Strains, plasmids and primers used in this study.

Strains	Derived, relevant phenotype or characteristics	Reference
SL_1344	*Salmonella* Typhimurium (wild type) (Sm^R)	[50]
Z234	Δ*ssa*V SL_1344_3093::*aphT* (Sm^R, Km^R)	[50]
Z234-pMS101	Δ*ssa*V SL_1344_3093::*aphT* with P^{sopE}–$SopE_{100}$-ctxB in pMS101 (Sm^R, Km^R)	This study
V. cholerae	*Vibrio cholerae 569B* (wild type) (No antibiotic resistance)	Gift from Dr. D.V. Singh
Plasmids	Characteristics	Reference
pM968	Amp^R, f1 ori, GFP marker gene (Amp^R)	[62]
pM2155	pM968–ctxB (no GFP)	This study
pMS101	pM968–P^{sopE}–$SopE_{100}$–ctxB	This study
Primers	Sequence (5′–3′)	
Fw-PsopE-Not1	CGAAACAAGAGGCCGCTTCAATGCCAGAACGGCAAGG	
Rw-PsopE-Pst1	GAATTCCTGCAGCCGCACTACCTCTAATATCTATATCATTGAGCG	
Fw-ctxB-EcoRI	CCC GAA TTC GAA TT ATGATTAAATTAAAATTTGG	
Rw-ctxB-HindIII	CCC AAG CTT TTAATTTGCCATACTAATTGCGGC	

Please cite this article in press as: Vishwakarma V, et al. Cholera toxin-B (ctxB) antigen expressing *Salmonella* Typhimurium polyvalent vaccine exerts protective immune response against *Vibrio cholerae* infection. Vaccine (2015), http://dx.doi.org/10.1016/j.vaccine.2015.02.014