Assessment of Mouse Ileal loop Protection against Clinically Isolated Vibrio cholerae Outer Membrane Vesicles as a Vaccine Candidate

Sedaghat 1, M., Siadat 2*, S. D., Shahcheraghi 1, F., Mirabzadeh Ardakani 3, E., Keramati 4, M., Vaziri 2, F., Nojoumi 2, S. A.

1. Department of Microbiology, Pasteur Institute of Tehran, Iran
2. Department of Mycobacteriology and Pulmonary Research, Pasteur Institute of Tehran, Iran
3. Department of Biotechnology Research, Pasteur Institute of Tehran, Iran
4. Department of Pilot of Nano-Biotechnology, Pasteur Institute of Tehran, Iran
Corresponding author email: d.siadat@gmail.com

ABSTRACT

Cholera, a life-threatening disease caused by the Gram-negative bacterium Vibrio cholerae, remains as a concern in developing countries. Present study reports the immunogenicity and protective immunity of outer membrane vesicles (OMVs) and combination of OMV and killed whole-cell (WC) of local strain isolated in the last outbreak in Iran as well as reference strain of V. cholerae El Tor O1 in comparison with Dukoral vaccine in mice model. The protein content, morphology and size of extracted OMVs were evaluated by electrophoresis and microscopic analyses, respectively. The serum titer of total IgG, IgG1, IgG2a, as well as secretory IgA and total IgG in different treatment of mice groups were determined by ELISA assay.

In addition, fluid accumulation assay in the context of resistance to live strain of V. cholerae in ligated ileal loops was carried out to determine of cholera infection by OMV or combination of OMV and WC in comparison with Dukoral vaccine.

SDS-PAGE analysis of purified OMVs indicated proteins profile ranging from 34 to 52 kDa and transmission electron microscopy (TEM) revealed the spherical shaped vesicles of 50-200 nm diameter. ELISA assay indicated significant titers of systemic and mucosal immune anti-OMV IgGs in immunized BALB/c mice with
different vaccine regimens; additionally, the fluid accumulation evolution demonstrated a notable increase in FA ratio. The results indicated that the combination of WC-OMV of local strain is able to induce a high level of antibody responses exhibiting more protection than OMV or WC, separately. Additionally, it can be considered as an effective immunogen against V. cholerae.

**Keywords:** Elisa, Ileal loop, Outer membrane vesicle, *Vibrio cholerae*, Vaccine

**INTRODUCTION**

Due to cholera infections of Gram-negative bacterium, diarrhea caused by *V. cholerae*, is always considered as one of the most important global health problems. *V. cholerae* is transmitted via the fecal-oral route. Gastric acid, intestinal movement and mucosal secretion are the general defenses against *V. cholerae*. However, the uncontrolled dehydration and electrolytes loss may result in death within a few hours. Cholera has caused regressive pandemics in the world since 1871 (Arakawa et al., 1998). World Health Organization (WHO) estimates 3-5 million cholera cases and 100,000-120,000 deaths annually (Ali et al., 2012).

Currently, the two WHO pre-qualified oral cholera vaccines are Dukoral including killed whole cells (WC) of *V. cholerae* O1 and recombinant form of cholera toxin B subunit and Shanchol a mix of WC of *V. cholerae* serotype O1 and O139. Despite the efficacy of Dukoral vaccine, there are several shortcomings such as short-term protection, limitation in administration to children younger than two years old, buffer solution requirement, trained personnel for administration, and costly transport chain (Holmgren et al., 2005). Therefore, there is a need for development of long-lasting protection and low-cost vaccines which can easily be prepared, distributed, and stored.
Outer Membrane Vesicles (OMVs) are usually formed by budding from the outer Gram-negative bacterial membrane with the size ranging 20 to 250 nm (Chatterjee and Chaudhuri, 2013). OMV acts as a carrier for bacterial antigens, delivering toxins, enzymes, and DNA to eukaryotic cells (Kim et al., 2009) and containing lipopolysaccharide (LPS), peptidoglycan, and flagellin. OMVs are effective in activating the innate immune system (Chatterjee and Chaudhuri, 2013). Studies revealed that the OMVs derived from Neisseria meningitidis, Helicobacter pylori and Acinetobacter baumannii may cause the induction of immune responses and protection in mice after immunization (Schild et al., 2008). Studies of meningococcal OMVs vaccines were performed between 1987 and 1991 for the first time (Acevedo et al., 2014).

OMVs are stable at the room temperature and do not require a cold chain or buffer solution. Therefore, it will be cost-effective (Harder et al., 2017). In 1967, shapes of OMVs were indicated in growing cells of V. cholerae (Chatterjee and Chaudhuri, 2011). In this study, oral immunizations were demonstrated with a single serotype OMVs of V. cholerae. The study detected total IgG, isotypes IgG1, IgG2a and IgA in serum. In addition, secretory immunoglobulins IgA, as well as IgG in fecal pellets of mice were identified. The present study characterizes the use of fluid accumulation (FA) ratio induced by V. cholerae infection as a challenge study in the inoculated mice. The aim of the project was the assessment of humoral and mucosal immunity and protection process of V. cholerae OMVs extracted from the local strain in an attempt to design an oral vaccine candidate.

**MATERIAL AND METHODS**

**Bacterial Strains.** V. cholerae O1 El Tor serotype Inaba (reference strain 14033) and V. cholerae O1 El Tor serotype Inaba isolated from patients during 2005 outbreak in Iran (Pourshafie et al., 2007) were used. All the strains were stored in
15% glycerol with brain heart infusion broth (Difco, USA) at -70°C until further use.

**Extraction of OMVs.** In order to achieve more OMVs preparation, both reference and local strains were cultured on Luria–Bertani broth (LB) for 8 hours at 37°C to the late exponential-phase (Claassen et al., 1996; Siadat et al., 2007). The suspension of bacteria was inactivated for 30 min at 56°C. Inactivated bacteria were centrifuged at 4500×g, 4°C for 1 h. The cell pellet was resuspended in Tris-HCl buffer 0.1 M containing 10 mM EDTA at 7.5 volume of primary wet weight. Extraction of OMVs was performed by adding 1/20 v/v of 0.1 M Tris, 10 mM EDTA, 100 g/L deoxycholate sodium (DOC) buffer. OMVs were subsequently purified by centrifugation at 60,000×g, 4°C for 2 h. The cell pellet was resuspended in 0.1 M Tris-10 mM EDTA, DOC (5g/l) buffer and was centrifuged for 2 h, 60,000×g, 4°C for the second time. The supernatant was consecutively filtered through 0.45µm and 0.22µm pore size filters (PVDF filters, Germany), respectively. Eventually, the extracted OMVs were suspended in 3% sucrose solution and stored at -70 °C until further use.

**Preparation of killed whole cell.** The bacterial suspension of *V. cholerae* strains with optical density of 1 at 600 nm in PBS buffer was inactivated at 56°C for 1 h. To ensure bacterial inactivation, a sample of inactivated bacteria was cultured onto blood agar, and incubated at 37 °C for 24h. (Lebens et al., 2011).

**The protein content of OMVs.** The protein content of OMV was measured using spectrophotometer at 280 nm (Thermo Scientific, USA) and Bradford assay using bovine serum albumin as a standard (Bradford. 1976). Vesicles-associated proteins were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.
Transmission electron microscopy. Purified OMVs images were obtained using transmission electron microscopy (TEM) of negative stain. Grids were floated in OMV solution for one minute and then washed with 2% acidic uranyl acetate. The blotted became dried and imaged via electron microscopy (Hitachi S4160, Korea) (Wang et al., 2017).

Experimental animals. BALB/c female mice, 8-10 weeks of age were taken from the Animal Laboratory Department of the Pasteur Institute of Iran. They were used for all immunization experiments under approved conditions of ethics committee of the Pasteur Institute of Iran (IR.PII.REC.1394.31). Mice were then caged separately and kept under controlled temperature and humidity (23-24°C, 65%). All immunized and non-immunized groups of mice were given food and water ad libitum within 56 days post immunization. Mice were habituated to the laboratory animal environment for at least 1 week before the beginning of experiments.

Immunization. Mice were divided into 7 groups (n=7 for each group). Before immunization, an equal volume of a solution of NaHCO3 6% was fed directly into the mice stomach through a disposable feeding needle (Austin, USA). Afterwards, they were orally immunized with 3 doses (days 0, 14 and 28) of 7 vaccine regimens of *V. cholerae*. As the control groups, non-immunized groups of mice received PBS. The vaccine regimens were, 1) a dose of 25 µg of extracted OMVs/200µl PBS, 2) 5×10⁸ WC of *V. cholerae* and Dukoral vaccine (S-10521, Sweden) and 3) the complex of (WC-OMV) (Borde et al., 2011). The groups were all housed in the same conditions.

Blood sample collection & intestinal lavage. Venous tail blood of control and immunized mice were collected on days 0, 7, 14, 21, 28, 35, 42, 49 and 56. The
serum was then collected by centrifugation of clotted blood at 6,000×g, 4°C for 10 min and stored at -20°C.

Intestinal lavage was collected from mice as described earlier (Elson et al., 1984) with some modifications. Intestinal lavage was collected 35 days after immunization.

Three mice of each group were euthanized with an overdose of ketamine and xylazine followed by rapid dissection of spinal cords, then the abdomen was opened. Afterwards, the small intestine was removed from each mouse and 2 ml of PBS containing protease inhibitor cocktail (Sigma) were injected into the intestine. The mixture was centrifuged at 10,000 ×g for 10 min to remove intestinal debris and supernatant was stored at -20°C until investigation.

**Antibody analysis by ELISA.** Antibody titers in serum and fecal pellet supernatant (FPS) were analyzed by enzyme-linked immunosorbent assay (ELISA) as described previously (Sinha et al., 2015; Vindurampoulle et al., 2003). Titers of total IgG and IgG isotypes (IgG1 & IgG2a) and secretory IgA and IgG were determined. 96-Microtiter plate (Merck, Germany) was coated by 100 µL of viable *V. cholerae* of reference strain 14033 (10⁹ cells/mL) and incubated for 16 h at 4°C. Wells were subsequently washed three times with PBS (pH 7.4) and blocked with 200 µl of 5% bovine serum albumin (BSA, Merck) for 2 h at 37°C. The wells were washed three times with PBS-T (PBS with 0.5% Tween-20, Sigma), and incubated with serial dilution of serum of immunized and non-immunized mice at 37°C for 1 h. Following washing, 100 µl HRP-conjugated goat anti-mouse immunoglobulin total IgG (1:100000), IgG1 (1:10000), IgG2a (1:25000), and IgA (1:25000) (Abcam, UK) were added to each well, separately in triplicate experiments. The plate was incubated at 37°C for 1 h. Then, 100 µL of Tetramethyl benzidine (TMB) as a substrate was added to each well. The reaction was stopped after 10
min by adding 100 µl of sulfuric acid 2 N. The optical density was determined at 450 nm by ELISA plate reader (Biotek ELX 800).

**FA ratio determination.** In order to conduct *in vivo* bacterial assay, the ligated intestinal loop test was performed based on published methods (Sinha et al., 2015, Roier et al., 2012) with minor modification. For this experiment, after three doses of oral immunizations on the 56th day, four mice from each group of immunized and non-immunized were starved for 24 h. The mice were anesthetized with intraperitoneal injection of a mixture of ketamine 100 mg/kg body weight and xylazine 5 mg/kg body weight (Alfasan, Woerden-Holland) while keeping the body temperature at 37°C by a heating pad. The mice were then prepared for laparotomy. The small intestine was removed and ligated at a distance of approximately 4 cm in length. A dose of $10^8$ CFU/loop of reference strain 14033 in 0.2 ml of PBS was injected into the mouse intestinal loops and their abdomen was closed again. Mice were housed under a sanitary condition for 12 h. The mice were subsequently euthanized, and the abdomen was reopened. The loops were pulled out for assessment of the length and the volume of accumulated fluids of each one. The extent of fluid accumulation was expressed as a ratio of the volume (in gr) of accumulated fluid per length (in cm) of the loop (g/cm).

**Statistical Analysis.** The Student’s t-test and ANOVA test were used for all statistical analyses using Graph Pad Prism 6. Differences were considered significant with $P <0.05$.

**RESULTS**

**Isolation and characterization of OMVs.** Minor modifications in extraction method led to an improved spherical nono-structure and productivity of OMVs. Electron microscopy analysis of OMVs indicated that the extracted vesicles were 50-300 nm in size (Figure 1). The amount of total protein OMVs were 1.26 and
1.29 mg/mL for clinical and reference strains, respectively. Protein profiling by SDS-PAGE analysis showed bands ranging in size 34- 52 kDa (Figure 2).

**Figure 1. Characterization of extracted *V. cholerae* outer membrane vesicles:**

A, Electron microscopy image of *V. cholerae* OMVs from clinical isolated.

B, The formation of *V. cholerae* OMV from clinical isolated on the cell surface (showed by arrow). Absence of bacteria and its remains, such as pili, flagella indicated the purity of the extraction of OMVs.

**Figure 2.** OMVs were extracted using the procedure described in Methods. Analyzed OMVs were separated via SDS-12% (w/v) polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue. Molecular weight marker in kDa (lane 1), OMVs of clinical *V. cholerae* (lane2), Whole cell of clinical *V. cholerae* (lane 3).

**Humoral and mucosal antibody responses after oral immunization.** Using Elisa assays, serum antibody responses were determined against 7 regimens of *V. cholerae* vaccines for sera collected on days 0, 7, 14, 21, 28, 35, 42, 49, 56.

Our finding showed that levels of total IgG, IgG1, IgG2a, and IgA were significantly higher than those of controls after oral immunization, P-value<0.05. The level of IgGs was persistently increasing and until the end of the study (8 weeks) (Figure 3). The ratio of IgG1 over IgG2a antibody responses on day 42, increased from 1.14 to 2.15 indicating the prevalence of IgG1 immune response as shown in (Figure 4). An increased IgG1 immune response was observed in the clinical VC(WC-OMV) regimen, more than other groups.

Mucosal immune responses in the intestine of mice showed the significantly higher levels of secretory IgA and IgG antibodies until day 35 compared to non-immunized mice (Figure 5).

**Figure 3.** Titers of serum immunoglobulin in immunized and non-immunized sera were separately measured against live cells. (A) Serum total IgG, (B) Serum IgA, (C) Serum IgG1 and (D) Serum IgG2a.
Blood collection weeks indicated along the horizontal axis. A statistically significant difference in antibody response was observed in 7 immunized regimens against controls (P-value < 0.005). The difference in optical absorption in the recipient group (WC-OMV) of clinical VC was significant compared to the other regimens, P-value < 0.05, except in D panel. [Ref. Reference., VC .*Vibrio cholerae*]

**Figure 4.** The ratio of IgG1/ IgG2a against *V. cholerae* El tor O1 (14033) in immunized mice serum, on the forty- second day, two weeks after the last administration dose with vaccine regimens.

**Figure 5.** Intestinal lavages were collected on day 35 from 3 mice/per group and immune responses against vaccine regimens were measured by ELISA. Intestinal lavage total IgG (Panel A) and intestinal lavage IgA (Panel B). The difference in antibody responses between immunized mice against Non-Immunized mice is statistically significant with (*P<0.05,* **P < 0.001). The mean values ± standard deviation (SD) of three independent experiments. Ref. (Reference), VC (*Vibrio cholerae*).

**Intestinal fluid secretion in mouse model.** *V. cholerae* intestinal colonization has an important role in infection and pathogenesis; hence, the fluid accumulation assay was carried out. After three oral immunizations with 7 vaccine regimens, on day 56, mice were challenged by reference strain 14033 (~10⁸ CFU). After 8 to 12 hours of administration, protective immunity was detected in immunized and non-immunized mice ileal loop upon evaluating fluid accumulation ratio of weight to length of the ligated intestine. The non-immunized group showed significant amounts of fluid accumulation with intestinal swelling while in immunized groups, the induction and swelling of intestinal fluid secretion was not significant (Figure 6, 7).

**Figure 6.** Protection study using ligated ileal loops with a dose of 10⁸ CFU/loop of live *V. cholerae* cells (14033 strain) which was inoculated into ileal loops of immunized and non-immunized mice. Fluid accumulation was measured from the ratio of intestinal FA (weight/length, g/cm) at 12h after inoculation. Data were stated as mean± S.E. (n=4 mice per group), **P < 0.001, compared with control groups.
Figure 7. Photographs of ileal loops after inoculated with $10^8$ CFU /loop of V. cholerae cells (Reference strain 14033) after 12h. 
(A) Photograph of ileal loops in treated mice. 
(B) Photograph of ileal loops in controlled mice (untreated mice).

DISCUSSION

Iran is considered to be at the risk of resurgence of cholera due to close relations with the neighboring countries such as Afghanistan, Iraq and Pakistan (Azizi and Azizi, 2010).

Various cholera vaccines have been prepared during the last 20 years. However, they were not all sufficiently effective (Holmgren et al., 2005). Therefore, numerous efforts are being made to develop more effective, low cost and easily applicable vaccines (Schild et al., 2009; Seidlein et al., 2013). Studies revealed that OMVs of Gram-negative bacteria have the desired result as non-living vaccine candidates against pathogenic diseases (Global task force, 2005). The combination of multi OMVs from some strains of V. cholerae as a cholera vaccine candidate showed convincible protective efficacy (Sinha et al., 2015). In addition, it was showed that the clinically killed whole cell of V. cholerae in combination with the purified rCTB enhances antibody titer and protection in rabbit animal model (Boustanshenas and Bakhshi, 2012).

In the present study, a local strain with a predominant pattern of V. cholerae O1 El Tor serotype Inaba collected from patients through 2005 outbreak in Iran was utilized as a source of OMVs and WC. Ribotyping, Pulsed-field gel electrophoresis (PFGE) and PhenePlate (PhP) techniques revealed the clonal dissemination of a single V. cholerae strain during that outbreak (Pourshafie et al., 2007).
In the current study, the modified OMVs extraction method resulted in OMV formation ranging in size between 50-300 nm with the preservation of the physical structure of OMVs. Immunogenicity evaluation of 7 vaccine regimens of *V. cholerae* by analyzing humoral and mucosal antibody titer revealed significant rise in the total IgG, IgG1, IgG2a, and IgA up to 56 days post immunization as well as significantly high levels of secretory IgA and IgG antibodies until 35 days. Prior studies reported that the isotype of serum antibodies can be used as an indicator of the lymphocyte dominance (Mountford et al., 1994).

In the present study, the titer of IgG1 and IgG2a isotypes as markers for Th1 and Th2 cytokines were investigated. The ratio of IgG1 to IgG2a was (1.1 to 2.15) in all vaccine regimens and this finding was the most noticeable in combination of WC-OMV of clinical strain regimen, indicating a Th1 tendency which demonstrated an increase in humoral immunity compared to cellular. However, other experiments such as cytokine assays are required to confirm this finding.

According to the results, it can be stated that WC-OMV regimens resulted in more immune responses and protection rather than the sole regimens which may be due to antigenic enhancement or adjuvant properties of OMVs.

In addition, the elevation of secretory IgA and total IgG titer on the day 35 after immunization in the fecal pellets represented an induced immune response at the mucosal surface in the gastrointestinal tract of mice, which is the site of colonization by *V. cholerae*. The titer of both sIgA and total IgG was higher in immunized mice that received combination of WC-OMV of clinical strain against some other regimens (Figure 5).

Studies have shown that OMV may be a factor for inhibiting *V. cholerae* motility in intestine. This feature of OMV prevents intestinal mucin penetration which is essential for adherence of *V. cholerae* to epithelial cells (Bishop et al., 2010).
This study confirmed the role of *V. cholerae*-induced intestinal fluid secretion measured by fluid accumulation (FA) ratio. In this research, the ileal loops of non-immunized mice were notably swelled due to the fluids secretion while in immunized mice lesser intestinal fluid accumulation was observed (Figure7). It was, therefore, demonstrated that the high quantity of secretion associated with mucosal infections was due to Cholera toxin-induced fluid secretion (Bishop et al., 2010). In our study, the oral vaccination regimen of mice with OMV alone or in combination of WC-OMV showed their protective immunity against live *V. cholerae* reference strains 14033 (~10^8 CFU). Further, previous studies have illustrated that inoculation of 5 ×10^7 CFU/loop of *V. cholerae* into 10-cm loops of the mice intestinal resulted in no secretion in animal intestine (Basu and pickett, 1969). In the present study, the antibody responses to combination of WC-OMV of clinical strain and Dukoral vaccine were similar to the case of protective immunity. The complex production process, using three different strains of *V. cholerae*, two different methods for bacterial inactivating, cold chain requirement for transportation and the need for administration buffer are some of shortcoming of Dukoral vaccine (Borde et al., 2011), leading to an increase in vaccine prices and less opportunities for purchasing in developing countries. In this study, one bacterial serogroup and one method for inactivating bacteria was correspondingly used. Furthermore, in Dukoral vaccine, a bacterial component of recombinant (rCTB) is used, which has a high price, while in this study, self-bacterial components were used.

Data showed that stability of increasing IgGs by oral administration of WC-OMV of clinical strain might be due to genetic differences including a greater number of copies of toxins or disparities in their promoters. More genetic studies including proteomics should be conducted on this topic in future.
The data obtained suggested that combination of WC-OMVs of a single strain has a potential protection against cholera. Therefore, it may provide the new approach for simple and inexpensive vaccine design of a single-strain oral cholera vaccine.

**Ethics**

Authors declare animal experiments were performed according to ethical codes.

**Conflicts of interest**

The authors declare that they have no conflict of interest.

**Acknowledgment**

This study was part of a Ph.D. project, funded by the Pasteur Institute of Iran.

**References**


Figure 1. Characterization of extracted *V. cholerae* outer membrane vesicles:

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(A) Photograph of ileal loops in treated mice.
(B) Photograph of ileal loops in controlled mice (untreated mice).