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Original Article

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

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Abstract

Cholera, a life-threatening disease caused by the Gram-negative bacterium *Vibrio cholera*, remains a concern in developing countries. The present study investigated the immunogenicity and protective immunity of outer membrane vesicles (OMVs) and combination of OMV and killed whole cells (WC) of a local strain isolated from the last outbreak in Iran in addition to reference and local strains of *V. cholerae* El Tor O1 in comparison to Dukoral vaccine in mice model. The protein content, morphology, and size of extracted OMVs were evaluated by electrophoresis and microscopic analyses, respectively. The serum titers of total immunoglobulin G (IgG), IgG1, IgG2a, and immunoglobulin A (IgA) in addition to secretory IgA and total IgG in different mice groups were determined by enzyme-linked immunosorbent assay (ELISA). In addition, fluid accumulation (FA) assay regarding the resistance to live strain of *V. cholerae* in ligated ileal loops was carried out to determine immunogenicity by OMV or combination of OMV and WC in comparison to that reported for Dukoral vaccine. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified OMVs indicated protein profiles within the range of 34-52 kDa. Furthermore, transmission electron microscopy demonstrated the spherical shaped vesicles of 50-200 nm. The results of ELISA showed significant titers of systemic and mucosal immune anti-OMV IgGs in immunized BALB/c mice with different vaccine regimens. Additionally, a notable increase in the FA ratio was demonstrated in this study. The obtained results of the present study revealed that the WC-OMV combination of local strain can induce a high level of antibody response indicating more protection than OMV or WC separately. Moreover, it can be considered an effective immunogen against *V. cholerae*.

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

Évaluation de la protection de l'anse iléale de la souris contre les vésicules membranaires externes de *Vibrio cholerae* cliniquement isolées en tant que vaccin candidat

Résumé: Le choléra, une maladie potentiellement mortelle causée par la bactérie Gram-négatif *Vibrio cholera*, reste une préoccupation dans les pays en développement. Dans cette étude, l'immunogénicité et l'immunité protectrice des vésicules de membrane externe (VME) ainsi que la combinaison des VME et de cellules entières tuées (ET) ont été examinées. A cet effet, une souche locale isolée de la dernière épidémie en Iran a été utilisée en plus des souches de référence et locales de V. *cholerae. El Tor O1,* et leur efficacité a été comparé auvaccin Dukoral chez des souris modèles. La teneur en protéines, la morphologie et la taille des VME extraits ont été

évaluées par électrophorèse et analyses microscopiques, respectivement. Les titres sériques d'immunoglobuline totale G (IgG), IgG1, IgG2a et immunoglobuline A (IgA) ainsi que des IgA sécrétoires et des IgG totales ont été déterminés par dosage immuno-enzymatique (ELISA) dans les différents groupes de souris. De plus, un test d'accumulation de liquide (AF) concernant la résistance à la souche vivante de *V. cholerae* dans les anses iléales ligaturées a été réalisé pour comparer l'immunogénicité causée par les VME ou une combinaison des VME et des ET avec celle rapportée pour le vaccin Dukoral. L'électrophorèse sur gel de polyacrylamide-dodécyl sulfate de sodium des VME purifiés montrait des bandes de protéines dans la plage de 34 à 52 kDa. De plus, la microscopie électronique à transmission a mis en évidence des vésicules sphériques de 50 à 200 nm. Les résultats de l'ELISA ont montré des titres significatifs d'IgG anti- VME, indiquant une réponse immunitaire systémique et mucosale chez les souris BALB/c immunisées avec différents schémas de vaccination. De plus, une augmentation notable du ratio AF a été démontrée dans cette étude. Les résultats de cette étude ont révélé que la combinaison ET- VME d'une souche locale peut induire un niveau plus élevé d'anticorps comparée à l'utilisation des VME ou ET séparément. Cette combinaison peut être considéré comme un immunogène efficace contre *V. cholerae*.
Mots-clés: ELISA, Anse iléale, Vésicule de membrane externe, *Vibrio cholerae*, Vaccin

Introduction

Diarrhea infection caused by *V. cholerae* as a Gram-negative bacterium is always considered 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 loss of electrolytes may result in death within a few hours. Cholera has caused regressive pandemics in the world since 1871 (Arakawa et al., 1998). The World Health Organization (WHO) estimates 3-5 million cholera cases and 100,000-120,000 mortalities every year (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 cholera toxin B subunit (rCTB), and Shanchol as a combination 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 2 years of age, buffer solution requirement, trained personnel for administration, and costly transport chain (Holmgren and Czerkinsky, 2005). Therefore, there is a need for the 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 from 20 to 250 nm (Chatterjee and Chaudhuri, 2013). The OMV acts as a carrier for bacterial antigens, delivering toxins, enzymes, and deoxyribonucleic acid to eukaryotic cells (Kim et al., 2009) and containing lipopolysaccharide, peptidoglycan, and flagellin. The OMVs are effective in the activation of the innate immune system (Chatterjee and Chaudhuri, 2013). The results of 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 on meningococcal OMVs vaccines were performed within 1987 to 1991 for the first time (Acevedo et al., 2014). The OMVs are stable at room temperature and do not require a cold chain or buffer solution; therefore, they are considered cost-effective (Harder et al., 2017). In 1967, the shapes of OMVs were indicated in the 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 immunoglobulin G

(IgG), isotypes of IgG1 of IgG2a, and immunoglobulin A (IgA) in the serum. In addition, secretory IgA and IgG in the fecal pellets of mice were identified. The present study investigated the fluid accumulation (FA) ratio induced by *V. cholerae* infection as a challenge study in the inoculated mice. The aim of the present study was the assessment of the 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 the patients during the 2005 outbreak in Iran (Pourshafie et al., 2007) were used in this study. 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 for 8 h 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 0.1 M of Tris-HCl buffer containing 10 mM of ethylenediaminetetraacetic acid (EDTA) at 7.5 volume of primary wet weight. The extraction of OMVs was performed by adding 1/20 v/v of 0.1 M Tris, 10 mM of EDTA, and 100 g/L of deoxycholate sodium (DOC) buffer. The 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 and DOC (5 g/L) buffer and centrifuged for 2 h at 60,000× g, 4°C for the second time. The supernatant was consecutively filtered through 0.45 µm and 0.22 µm 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 cells. The bacterial suspension of *V. cholerae* strains with an optical density of 1 at 600 nm in phosphate-buffered saline (PBS) 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 24 h (Lebens et al., 2011).

Protein content of OMVs. The protein content of OMV was measured using a spectrophotometer at 280 nm (Thermo Scientific, USA) and Bradford assay using bovine serum albumin (BSA) as a standard (Bradford, 1976). Vesicle-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 of negative stain. Grids were floated in the OMV solution for 1 min and then washed with 2% acidic uranyl acetate. The blots became dried and imaged via electron microscopy (Hitachi S4160, Korea) (Wang et al., 2017).

Experimental animals. The BALB/c female mice with 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 the approved conditions of the Ethics Committee of Pasteur Institute of Iran (IR.PII.REC.1394.81). The mice were then caged separately and kept under controlled temperature and humidity (23-24°C; %50). All the immunized and non-immunized groups of mice were given food and water ad libitum within 56 days after immunization. The mice were habituated to the laboratory animal environment for at least 1 week before the initiation of the experiments.

Immunization. The mice were divided into seven groups (n=7 for each group). Before immunization, an equal volume of a 6% $NaHCO_3$ solution was directly injected into the mice's stomach through a disposable feeding needle (Austin, USA). Afterward, they were orally immunized with three doses (at days 0, 14, and

28) of seven vaccine regimens of *V. cholerae.* As the control groups, non-immunized groups of mice received PBS. The vaccine regimens were 1) a 25 µg dose of extracted OMVs/200 µl of PBS, 2) 5×10^8 WC of *V. cholerae* and Dukoral vaccine (S-10521, Sweden), and 3) complex of WC-OMV (Borde et al., 2011). The groups were all housed in similar conditions.

Blood sample collection and intestinal lavage. The venous tail blood of the control and immunized mice were collected on days 0, 7, 14, 21, 28, 35, 42, 49, and 56. The serum was then collected by the centrifugation of clotted blood at 6,000×g, 4°C for 10 min and stored at -20°C. Intestinal lavage was collected from the mice as previously described (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 a rapid dissection of spinal cords, and then the abdomen was opened. Subsequently, the small intestine was removed from each mouse, and 2 ml of PBS containing protease inhibitor cocktail (Sigma-Aldrich Chemie Gmbh Munich, Germany) was injected into the intestine. The mixture was centrifuged at 10,000×g for 10 min to remove intestinal debris, and the supernatant was stored at -20°C until the investigation.

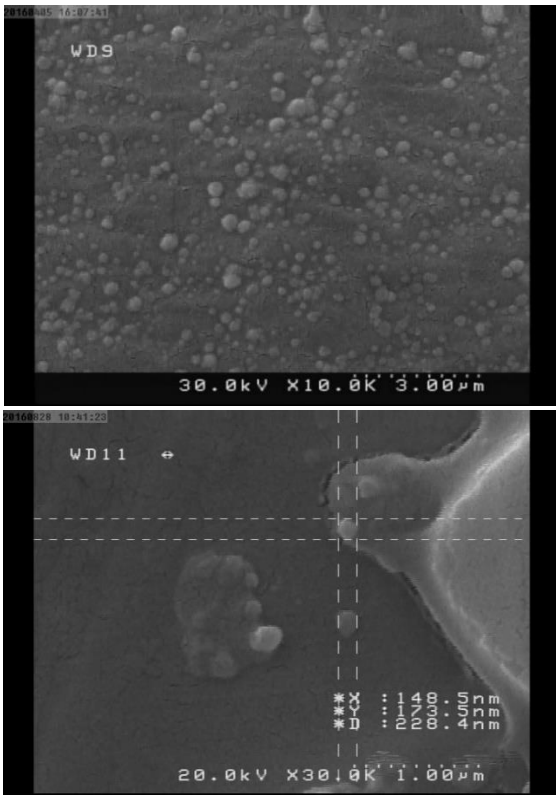
Antibody analysis by ELISA. Antibody titers in the serum and fecal pellet supernatant were analyzed by enzyme-linked immunosorbent assay (ELISA) as previously described (Vindurampulle and Attridge, 2003; Sinha et al., 2015). The titers of total IgG and IgG isotypes (i.e., IgG1 and IgG2a) and secretory IgA and IgG were determined. 96-Microtiter plate (Merck, Germany) was coated by 100 µL of viable *V. cholerae* of reference strain 14033 (10^9 cells/mL) and incubated for 16 h at 4°C. The wells were subsequently washed three times with PBS (pH 7.4) and blocked with 200 µl of 5% BSA (BSA, Merck, Germany) at 37°C for 2 h. The wells were washed three times with PBS-T (PBS with 0.5% Tween-20; Sigma-Aldrich Chemie Gmbh Munich, Germany) and incubated with a serial dilution of serum of the immunized and non-immunized mice at 37°C for 1 h. Following washing, 100 µl of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin total IgG (1:100000), IgG1 (1:10000), IgG2a (1:25000), and IgA (1:25000) (Abcam, UK) were separately added to each well in triplicate experiments. The plate was incubated at 37°C for 1 h. Then, 100 µL of tetramethylbenzidine 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 an ELISA plate reader (Biotek ELX 800, BioTek's World headquarters, USA).

FA ratio determination. In order to conduct in vivo bacterial assay, the ligated intestinal loop test was performed based on published methods (Roier et al., 2012; Sinha et al., 2015) with minor modifications. For this experiment, after three doses of oral immunizations on the 56^{th} day, four mice from each group of immunized and non-immunized were starved for 24 h. The mice were anesthetized with an intraperitoneal injection of a mixture of ketamine 100 mg/kg body weight and xylazine 5 mg/kg body weight (Alfasan, Woerden, Netherlands) 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. The 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 the assessment of the length and volume of accumulated fluids of each one. The extent of FA was expressed as a ratio of the volume (gr) of accumulated fluid per length (cm) of the loop (g/cm).

Statistical Analysis. The Student's t-test and analysis of variance were used for all statistical analyses using GraphPad Prism (version 6). Differences were considered statistically significant with a p-value of less than 0.05.

Results

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



A)

B)

Figure 1. Characterization of extracted *V. cholerae* outer membrane vesicles (OMVs); **A)** electron microscopy image of *V. cholerae* OMVs from clinical isolate; **B)** formation of *V. cholerae* OMV from clinical isolate on the cell surface (shown by arrow); absence of bacteria and its remains (e.g., pili) and flagella indicating purity of extraction of OMVs

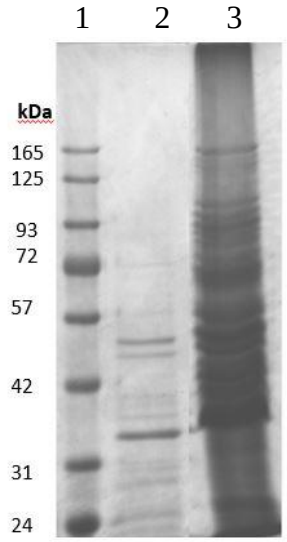


Figure 2. Extraction of outer membrane vesicles (OMVs) using procedure described in Methods; separation of analyzed OMVs by 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie blue; molecular weight marker in kDa (lane 1); OMVs of clinical *V. cholerae* (lane 2); whole cell of clinical *V. cholerae* (lane 3)

Humoral and mucosal antibody responses after oral immunization. Using ELISA, serum antibody responses were determined against seven regimens of *V. cholerae* vaccines for the sera collected on days 0, 7, 14, 21, 28, 35, 42, 49, and 56. The findings of the present study showed that the levels of total IgG, IgG1, IgG2a, and IgA were significantly higher than those of the controls after oral immunization ($P<0.05$). The level of IgGs was persistently increasing up to the end of the study (8 weeks; Figure 3). The ratio of IgG1 over IgG2a antibody responses on day 42 increased from 1.1 to 2.15 indicating the prevalence of IgG1 immune response as depicted in Figure 4. An increased IgG1 immune response was observed in the clinical *V. cholerae* (i.e., WC-OMV) regimen higher than those of other groups. Mucosal immune responses in the intestine of mice showed significantly higher levels of secretory IgA and IgG antibodies until day 35, compared to those reported for the non-immunized mice (Figure 5).

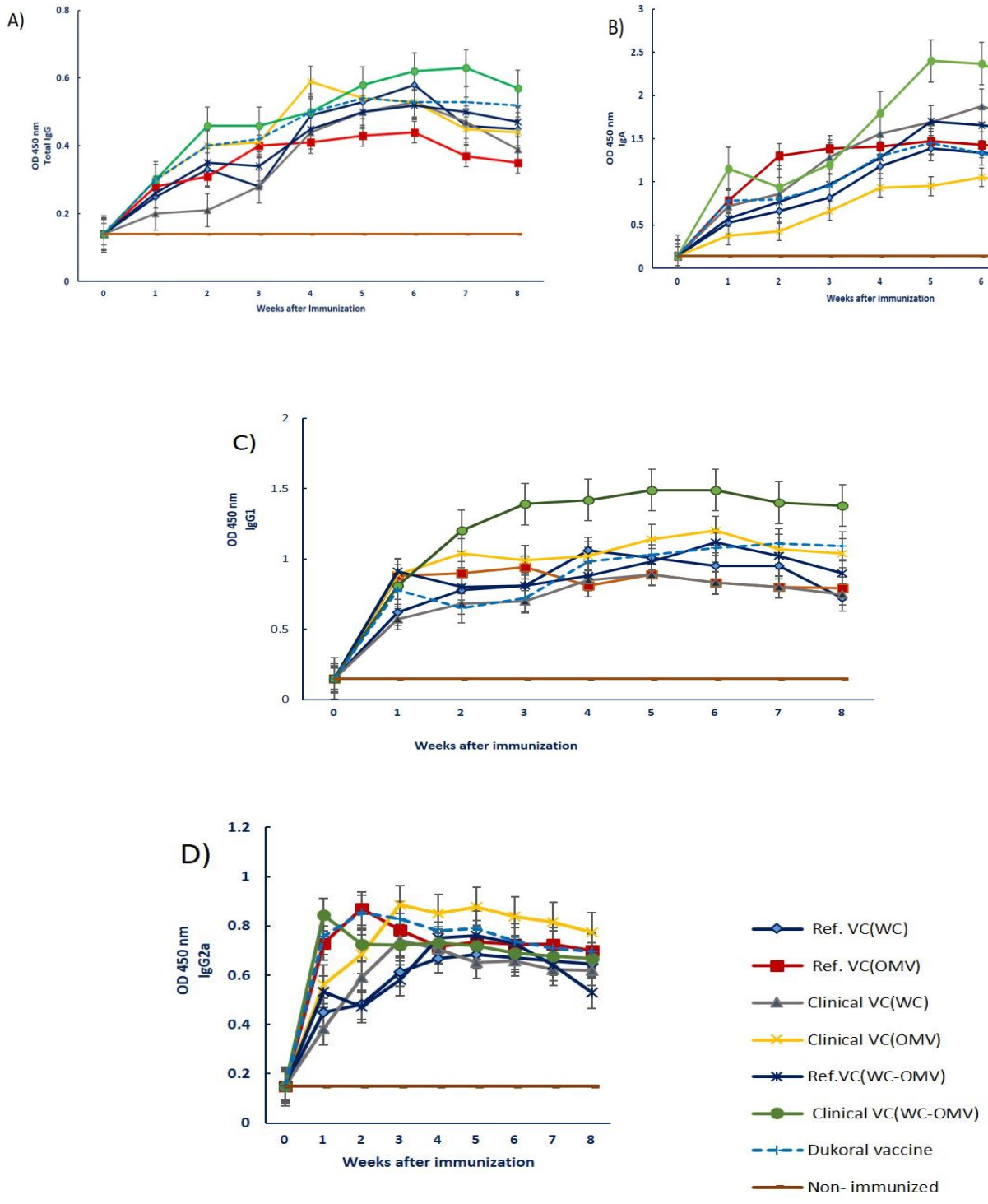


Figure 3. Separate measurement of serum titers immunoglobulin in immunized and non-immunized sera against live cells; **A)** serum total immunoglobulin G (IgG); **B)** serum immunoglobulin A; **C)** serum IgG1; **D)** serum IgG2a; blood collection weeks indicating along horizontal axis; observation of statistically significant difference in antibody response in seven immunized regimens against controls ($P<0.005$); significant difference in optical absorption in recipient group (i.e., whole cell-outer membrane vesicles) of clinical *V. cholerae* in comparison to other regimens; $P<0.05$, except in D panel (Reference, *V. cholerae*)

Intestinal fluid secretion in mouse model. *V. cholerae* intestinal colonization has an important role in infection and pathogenesis; therefore, FA was carried out. After three oral immunizations with seven vaccine regimens, on day 56, the mice were challenged by reference strain 14033 (~10^8 CFU). After 8 to 12 h of administration, protective immunity was detected in the immunized and non-immunized mice ileal loop upon evaluating the FA ratio of weight to length of the ligated intestine. The non-immunized group showed significant amounts of FA with intestinal swelling; however, in the immunized groups, the induction and swelling of intestinal fluid secretion were not significant (figures 6 and 7).

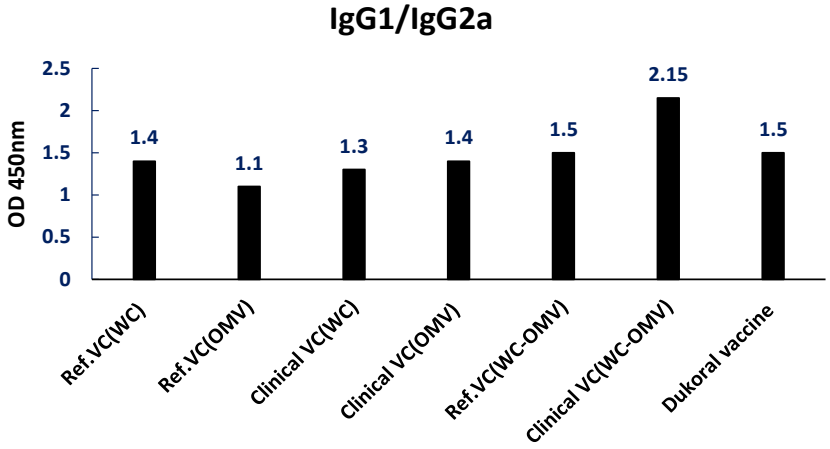


Figure 4 Ratio of immunoglobulin G1/immunoglobulin G2a against *V. cholerae* El Tor O1 (14033) in immunized mice serum on day 42, 2 weeks after last administration dose with vaccine regimens

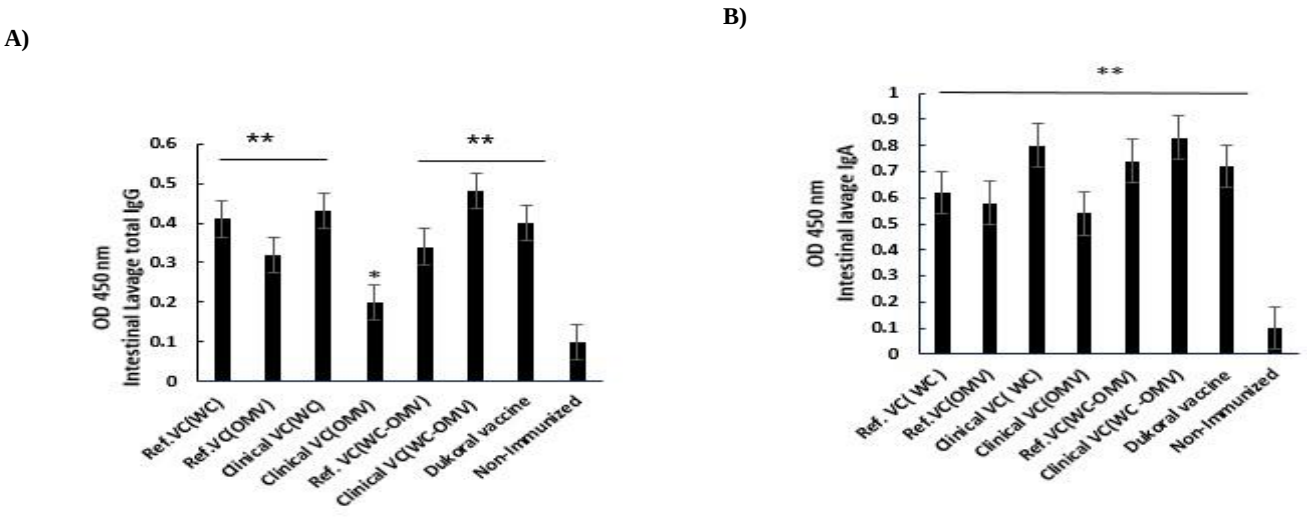


Figure 5. Collection of intestinal lavages on day 35 from three mice/per groups and measurement of immune responses against vaccine regimens by enzyme-linked immunosorbent assay; intestinal lavage total immunoglobulin G (panel A) and intestinal lavage immunoglobulin A (panel B); statistically significant difference in antibody responses between immunized mice and non-mmunized mice (*P<0.05; **P<0.001); mean values±standard deviation of three independent experiments (Ref. *V. cholerae*)

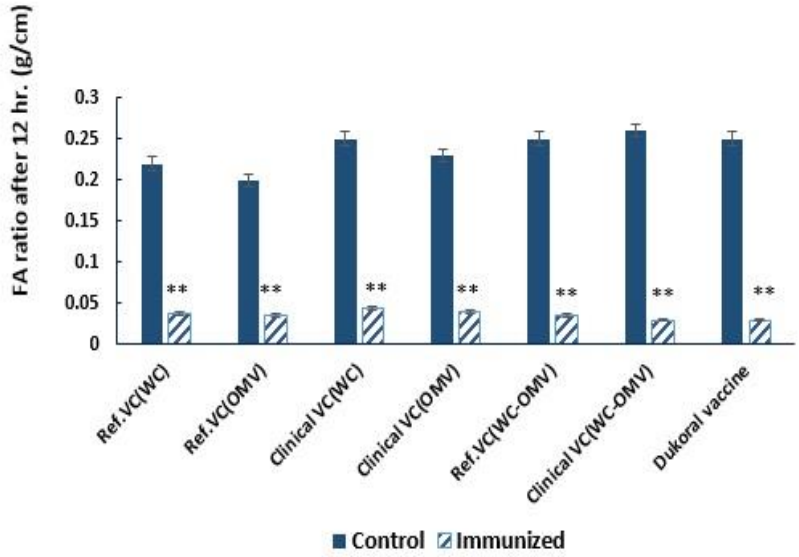


Figure 6. Protection study using ligated ileal loops with a dose of 10^8 CFU/loop of live *V. cholerae* cells (14033 strain) inoculated into ileal loops of immunized and non-immunized mice; measurement of fluid accumulation (FA) from the ratio of intestinal FA (weight/length, g/cm) 12 h after inoculation; presentation of data as mean ± standard error (four mice per group); ** *P<0.001*, compared to control groups

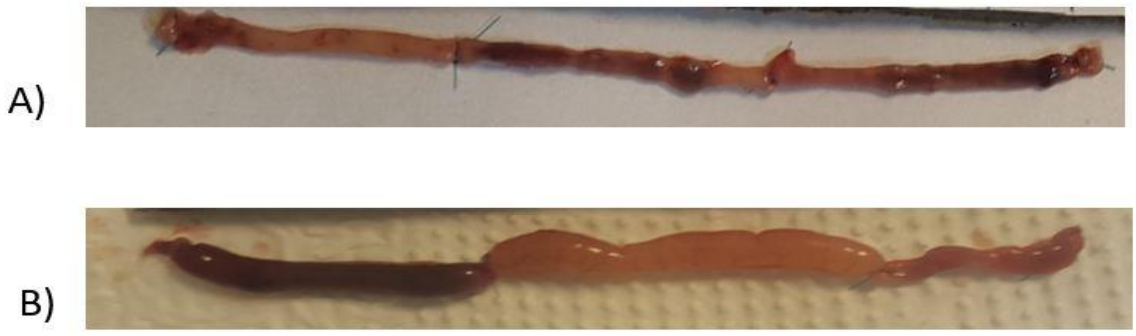


Figure 7. Photographs of ileal loops after inoculation with 108 CFU/loop of V. cholerae cells (Reference strain 14033) following a period of 12 h; **A)** photograph of ileal loops in treated mice; **B)** photograph of ileal loops in controlled mice (i.e., untreated mice)

Discussion

Iran is considered to be at risk of cholera resurgence 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 and Czerkinsky, 2005). Therefore, numerous efforts have been made to develop more effective, low-cost, and easily applicable vaccines (Schild et al., 2009; von Seidlein et al., 2013). The results of studies revealed that the OMVs of Gram-negative bacteria have the desired result as non-living vaccine candidates against pathogenic diseases (WHO, 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 demonstrated that the clinically killed WC of *V. cholerae* in combination with the purified rCTB enhances antibody titer and protection in the rabbit animal model (Boustanshenas et al., 2013). In the present study, a local strain with a predominant pattern of *V. cholerae* O1 El Tor serotype Inaba collected from patients through the 2005 outbreak in Iran was utilized as a source of OMVs and WC. Ribotyping, pulsed-field gel electrophoresis, and PhenePlate techniques revealed the clonal dissemination of a single *V. cholerae* strain during the aforementioned outbreak (Pourshafie et al., 2007). In the current study, the modified OMVs extraction method resulted in OMV formation within the size range of 50-300 nm with the preservation of the physical structure of OMVs. The immunogenicity evaluation of seven vaccine regimens of *V. cholerae* by analyzing humoral and mucosal antibody titer revealed a significant increase in the total IgG, IgG1, IgG2a, and IgA up to 56 days after immunization in addition to significantly high levels of secretory IgA and IgG antibodies up to 35 days. Previous studies reported that the isotype of serum antibodies can be used as an indicator of lymphocyte dominance (Mountford et al., 1994). The present study investigated the titer of IgG1 and IgG2a isotypes as markers for T helper 1 (Th1) and T helper 2 cytokines. The ratio of IgG1 to IgG2a was reported as 1.1 to 2.15 in all vaccine regimens, and this finding was the most noticeable in the WC-OMV combination of clinical strain regimen, indicating a Th1 tendency with an increase in humoral immunity, compared to that of cellular. However, other experiments, such as cytokine assays, are required to confirm this finding. According to the obtained results of the present study, it can be stated that WC-OMV regimens resulted in more immune responses and protection 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 titers on 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 titers of both sIgA and total IgG were higher in immunized mice receiving the WC-OMV combination of clinical strain against some other regimens (Figure 5). Based on the evidence, it was shown that OMV may be a factor for the inhibition of *V. cholerae* motility in the intestine. This feature of OMV prevents intestinal mucin penetration which is essential for the adherence of *V. cholerae* to epithelial cells (Bishop et al., 2010). The results of the current study confirmed the role of *V. cholerae*-induced intestinal fluid secretion measured by the FA ratio. In this study, the ileal loops of non-immunized mice were notably swelled due to the fluids secretion; however, a lower intestinal FA was observed in the immunized mice (Figure 7). Therefore, it was demonstrated that the high quantity of secretion associated with mucosal infections was due to cholera toxin-induced fluid secretion (Bishop et al., 2010). In the present study, the oral vaccination regimen of mice with OMV alone or in WC-OMV combination showed their protective immunity against live *V. cholerae* reference strains 14033 (~10^8 CFU). Furthermore, previous studies have indicated that the inoculation of 5 × 10^7 CFU/loop of *V. cholerae* into 10-cm loops of the

mice intestine resulted in no secretion in the animal intestine (Basu and Pickett, 1969). In the present study, the antibody responses to the 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 need for administration buffer are some of the shortcomings of Dukoral vaccine (Borde et al., 2011), leading to an increase in vaccine costs and fewer opportunities for purchasing in developing countries. One bacterial serogroup and one method for inactivating bacteria were correspondingly used in this study. Moreover, in Dukoral vaccine, a bacterial component of rCTB is used, which has a high cost; nevertheless, self-bacterial components were utilized in the current study. The obtained data showed that the stability of increasing IgGs by the 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. It is suggested to carry out further genetic studies, including proteomics, on this issue. In addition, the obtained data suggested that the combination of WC-OMVs of a single strain has potential protection against cholera; therefore, it may provide a new approach for the development of a simple and inexpensive vaccine of a single-strain oral cholera vaccine.

Authors' Contribution

Investigation and evaluation of reports: M. S.
Contributing in writing the manuscript and analysis: M. S., F. Sh., E. M. A. and M. K.
Major contributors in writing and editing: F. V. and S. A. N.
Design and supervision of the study: S. D. S.

Ethics

The authors declare that all the animal experiments were performed according to ethical codes.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

All experiments were performed in accordance with guidelines approved by Pasteur Institute International networks.

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