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Original Article

Comparison of Two Different Methods for the Extraction of Outer Membrane Vesicles from the *Bordetella pertussis* as a Vaccine Candidate

Soltani, M. S[1], Eftekhar, F[1], Noofeli, M[2], Banihashemi, S. R[3], Shahcheraghi, F[4] *

1. Department of Microbiology and Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran
2. Department of Human Bacterial Vaccines Production and Research, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
3. Department of Immunology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
4. Department of Bacteriology, Pasteur Institute of Iran, Tehran, Iran

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Corresponding Author: shahcheraghifereshteh@yahoo.com

Abstract
Despite the availability of a vaccine, pertussis is still a worldwide health problem. Outer membrane vesicles (OMVs) in gram-negative bacteria can stimulate the immune system due to several outer membrane proteins and are very good candidates in vaccine development. OMVs obtained from *Bordetella pertussis* contain several antigens, which are considered immunogenic, and could make them a potential candidate for vaccine production. The current study aimed to compare the current OMV extraction method (with ultracentrifuge) and a modified extraction method (without ultracentrifuge) and to evaluate the physicochemical properties as well as the expression of their main virulence factors. Vaccinal strain BP134 grown on Bordet Gengo agar were inoculated in Modified Stainer-Scholte medium for mass cultivation. OMVs were prepared using two different methods. They were then stained and examined with a transmission electron microscope. Protein contents were measured by the Bradford method, and then the protein profile was evaluated by SDS-PAGE. The presence of immunogenic antigens was detected by Western blotting. The size and shape of the OMVs obtained from the modified method without the use of ultracentrifuge were similar to the current method and had a size between 40 and 200 nm. The total protein yields of the OMV isolated using the current and modified methods were 800 and 600 µg/ml, respectively. Evaluating the protein profile of extracted OMVs showed the presence of different proteins. Finally, the presence of PTX, PRN, and FHA was observed in OMVs extracted from both methods. Comparison of the two OMV extraction methods showed that the obtained vesicles have a suitable and similar shape and size as well as the expression of three important pathogenic factors as immunogens. Despite the relatively low reduction in protein yield as the modified method does not require ultracentrifuge, this extraction method can be used as a suitable alternative for extracting the outer membrane vesicles from *B. pertussis*, especially in developing countries. It should be noted that further experiments including immunogenicity determination of OMVs obtained as vaccine candidates in animal models are required.
Keywords: *B. pertussis*, outer membrane vesicle, virulence factors

Comparaison de Deux Méthodes Différentes pour l'extraction des Vésicules de la Membrane Externe de *Bordetella pertussis* en Tant Qu'un Candidat Vaccin

Résumé: Malgré la disponibilité d'un vaccin, la coqueluche reste un problème de santé mondial. Les vésicules de la membrane externe (VME) chez les bactéries gram-négatives peuvent stimuler le système immunitaire

grâce à plusieurs protéines de la membrane externe et sont de très bons candidats pour le développement de vaccins. Les VMEs obtenues à partir de *Bordetella pertussis* contiennent plusieurs antigènes, considérés comme immunogènes, et pourraient en faire un candidat potentiel pour la production de vaccins. La présente étude visait à comparer la méthode d'extraction actuelle de VME (avec ultracentrifugeuse) et une méthode d'extraction modifiée (sans ultracentrifugeuse) et à évaluer les propriétés physico-chimiques ainsi que l'expression de leurs principaux facteurs de virulence. La souche vaccinale BP134 cultivée sur gélose Bordet Gengo a été inoculée dans du milieu Stainer-Scholte modifié pour une culture de masse. Les VMEs ont été préparées en utilisant deux méthodes différentes. Elles ont ensuite été colorées et examinées au microscope électronique à transmission. Les teneurs en protéines ont été mesurées par la méthode de Bradford, puis le profil protéique a été évalué par SDS-PAGE. La présence d'antigènes immunogènes a été détectée par Western blot. La taille et la forme des VMEs obtenues à partir de la méthode modifiée sans l'utilisation d'ultracentrifugeuse étaient similaires à la méthode actuelle et avaient une taille comprise entre 40 et 200 nm. Les rendements protéiques totaux du VME isolé en utilisant les méthodes actuelles et modifiées étaient respectivement de 800 et 600 µg/ml. L'évaluation du profil protéique des VMEs extraites a montré la présence de différentes protéines. Enfin, la présence de PTX, PRN et FHA a été observée dans les VMEs extraites des deux méthodes. La comparaison des deux méthodes d'extraction de VME a montré que les vésicules obtenues ont une forme et une taille appropriées et similaires ainsi que l'expression de trois facteurs pathogènes importants en tant qu'immunogènes. Malgré la réduction relativement faible du rendement en protéines car la méthode modifiée ne nécessite pas d'ultracentrifugation, cette méthode d'extraction peut être utilisée comme une alternative appropriée pour extraire les vésicules de la membrane externe *de B. pertussis*, en particulier dans les pays en développement. Il convient de noter que d'autres expériences, y compris la détermination de l'immunogénicité des VMEs obtenues en tant que candidats vaccins dans des modèles animaux, sont nécessaires.
Mots-clés: *B. pertussis,* vésicule de membrane externe, facteurs de virulence

1. Introduction

Pertussis, or whooping cough, is a highly contagious bacterial respiratory tract infection caused by *Bordetella pertussis* (1). Some of the major virulence factors which play important roles in its pathogenesis are pertussis toxin (PTX), pertactin (PRN), and filamentous hemagglutinin (FHA). PTX induces protective immunity and plays an important role in the disease development (2). PRN is a surface protein involved in mediating adherence to the epithelium of the respiratory tract (3). FHA is also a major adhesion factor on the surface of *B. pertussis* (4).

This respiratory disease was a major cause of infant mortality worldwide before the vaccine was introduced in the 1940s. Widespread vaccination with the first generation of vaccines, whole cell pertussis (wP) vaccines that consisted of detoxified killed whole bacteria, significantly reduced morbidity attributed to the disease. However, in the 1970s, in some countries, concerns about the reactogenicity of wP vaccines led to rising rates of vaccine refusal and, consequently, to increased pertussis incidence (5, 6).

Since the 1980s, many countries have replaced wP vaccines with less reactogenic acellular pertussis (aP) vaccines. Acellular vaccines are composed of pertussis toxin (PTX) as a major protective antigen and other surface proteins such as bivalent, trivalent, and pentavalent pertussis vaccines. In general, five-component vaccines (pentavalent) (including pertussis toxin, filamentous hemagglutinin, pertactin, and fimbriae 2 and 3) are considered more effective than bivalent or trivalent vaccines (7-9).

Unexpectedly, despite the high vaccination rate, recent years have seen a large number of pertussis outbreaks that had not been seen since pre-vaccine days. Indeed, pertussis is now recognized as a reemergent disease and is still among the main causes of death in children worldwide (10-14).

This reemergence is due to short-term immunity induced by aP vaccines. While wP vaccines induce Th1/Th17 responses that lead to lung clearance and long-lasting immunity, aP vaccines mainly induce a Th2 response. Because *B. pertussis* is an intracellular bacterium, a Th1 response must be induced for lung clearance. To overcome these problems, a new

generation of vaccines needs to be developed, as the outer membrane vesicles (OMVs) of *B. pertussis* contain phospholipids, lipooligosaccharides, nucleic acid, and several immunogenic antigens. Moreover, they have shown a basal level of protection against *B. pertussis* that also induces a Th1 response, potentially making them an attractive alternative over the currently available vaccines (15, 16).

The current study aimed to extract outer membrane vesicles (OMVs) from *B. pertussis* using a modified procedure without ultracentrifuging at a very high speed in comparison with the current method which uses ultracentrifuge and evaluate the physicochemical properties and the expression of the main virulence factors, PTX, PRN, and FHA of the extracted OMVs as potential vaccine candidates.

2. Material and Methods

2.1. Bacterial Strains and Cultures

B. pertussis vaccinal strain BP134 was obtained from Razi Vaccine and Serum Research Institute (RVSRI). This strain was grown on Bordet-Gengou agar (BGA; Difco) supplemented with 10% defibrinated sheep blood at 37 °C for 72 h. Then colonies were confirmed with biochemical tests and slide agglutination using *B. pertussis* antiserum (Difco) for the final approval. Bacterial colonies were subcultured on the same medium for 48 h and then inoculated in liquid Modified Stainer-Scholte medium (MSS) with methyl-β-cyclodextrin at 200 rpm in a Beckman-Coulter shaker (BeckmanCoulter, Brea, CA) until decelerating phases (optical densities, OD_{600} between 0.7 and 1.0) were reached for the large-scale production of cultures (17, 18).

2.2. Isolation of OMVs

With the current method used in previous studies, sequential ultra-centrifugation at speeds above 100,000 g has been used to isolate OMVs as described before (15, 16, 18). In this modified method, however, simple steps with centrifuging at a lower speed was used as described in the following.

First, 600 ml of MSS broth was inoculated with 20 ml of a decelerating phase culture of *B. pertussis* for large-scale production. After about 30 h, the decelerating phase was reached at 36 °C with aeration at 200 rpm (Beckman-Coulter, Brea, CA). The cultures were pelleted by centrifugation at 8000 g for 30 min at 4 °C, and then the pellets were washed twice in phosphate-buffered saline (PBS) to eliminate cell debris. The pellet (1 g wet weight) was subsequently resuspended in 7.5 ml of TE buffer (Tris-HCl, EDTA, pH 8.5) and homogenized completely to make a uniform suspension. Next, the suspension was incubated at room temperature for 30 min, sonicated for 10 min (MSE sonicator, 4-5 pm amplitude, 20 kHz on ice with intervals of 30 s), and then centrifuged at 10,000 g for 20 min at 4 °C. The pellets were washed with TE, centrifuged, and the supernatants pelleted at 60,000 g for 2 h at 4 °C. Subsequently, the resulting pellets were resuspended in 0.1 M Tris, 10 mM EDTA, DOC (5 g/L) buffer, and mixed again several times by pipetting to make a homogenized suspension which was incubated for 10 min then centrifuged for 2 h at 60,000 g at 4 °C. Afterward, the supernatant was separated carefully in a new tube and treated with TE buffer and centrifuged again for 1 h at 60,000 g at 4 °C. The pellets were dissolved in 5 ml of 3% sucrose and passed through 0.22 μm pore size filters (PVDF, syringe filters, Germany). The filtered sample containing the OMVs was inactivated by heating in a water-bath at 56 °C for 30 min. The suspensions were then spread on blood agar and Bordet-Gengou agar plates and incubated at 37 °C for 48 h to confirm bacterial inactivation. Each extraction method was repeated four times, and in all repeats, the physiochemical properties of the OMVs extracted by the two methods, such as shape, size, SDS-PAGE, and Western profiles, were determined (15, 16, 18).

2.3. Protein Assay

The proteins in the outer membrane vesicles were quantified by Bradford assay with bovine serum albumin (BSA-Sigma) as standard using Nanodrop

(Thermo Scientific, Wilmington, DE, USA) (15, 16, 18).

2.4. Transmission Electron Microscopy (TEM)

The OMV preparations were suspended in 0.1 M ammonium acetate (pH 7.0), and a drop was placed on a grid coated with a carbon-reinforced formvar film. After 30 s evaporation, the excess fluid was removed by absorption with filter paper and the grids stained with 2% (w/v) phosphotungstic acid. The grids were examined on a Zeiss EM10C TEM (Germany) operating at an accelerating voltage of 100 kV (15, 18).

2.5. SDS-PAGE and Western Blotting

Protein profiles of the extracted OMVs were studied by SDS-PAGE followed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) used for the separation of protein molecules based on their molecular weight. After electrophoresis, proteins were stained by 0.1% (w/v) Coomassie blue (DNAbiotech, IRAN) with gentle shaking for 1 h. The gels were then washed three times with methanol 40% (v/v) and acetic acid 10% (v/v) in double-distilled water (ddw) for 20 min. Washed solution was discharged and gels were fixed with a solution containing 2% phosphoric acid (v/v), 18% ethanol (v/v), and 15% ammonium sulfate (w/v) in ddW for 30 min with minor modifications (15). Moreover, western immunoblotting analyses using specific antibodies against PTX, FHA, and PRN were employed to characterize and describe OMV properties.

The proteins from the polyacrylamide gel were transferred to the polyvinylidene difluoride (PVDF) membrane which was blocked overnight with 5% skimmed milk in PBS. Membranes were then washed three times with PBS in 0.05% Tween 20 (PBST) and next, exposed to mouse monoclonal immune sera directed against the PTX, PRN, and FHA (NIBSC No. 97/572, 97/558, and 97/564, respectively) for 1 h at 37 °C. Membranes were washed three times with PBST, followed by incubation with rabbit anti-sheep horseradish peroxidase-conjugated (HRP) antibody (PADZA Company, Iran) at a 1:1000 dilution for 1 h at room temperature. Finally, after washing three times with PBST, the color reaction was ultimately generated in the presence of metal-enhanced 3,3`-diaminobenzidines (DAB) substrate.

3. Results

OMVs were isolated from *B. pertussis* vaccinal strain BP134 using the current method and a modified method. Both obtained samples were negatively stained and examined with an electron microscope with a mean size of 70 nm and ranging from 40 to 200 nm (Figure 1A and 1B).

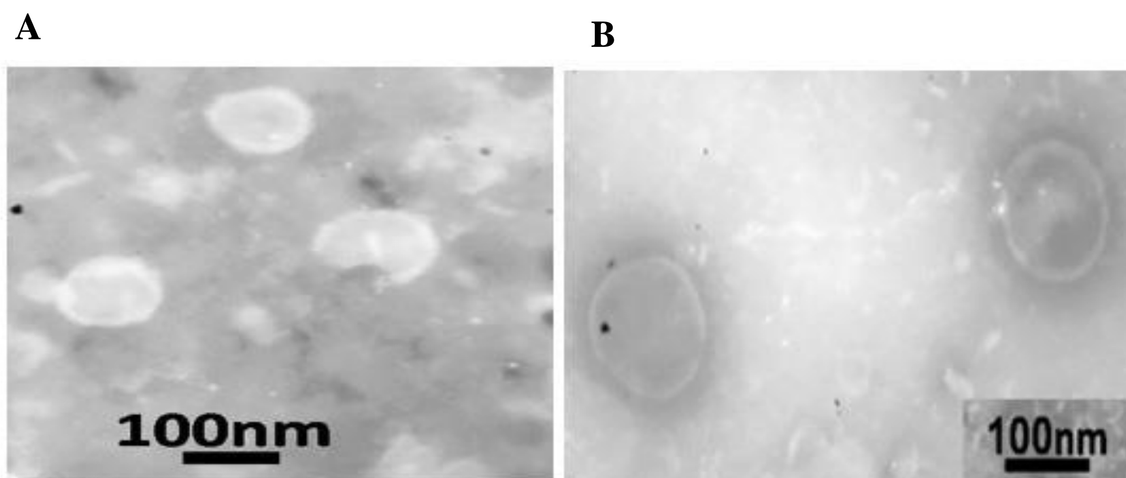

Figure 1. Negatively stained *Bordetella pertussis* OMVs examined with an electron microscope. **(A)** shows the OMV obtained from *B. pertussis* with the current method. **(B)** shows the OMV obtained from *B. pertussis* with the modified method. Vesicle sizes range from 40 to 200 nm.

At least four independent extractions of OMVs using the two methods and characterization were carried out. Similar morphologies were observed in all cases; the OMVs' size range was consistent from batch to batch and similar to previously described OMV preparations (15).

The amounts of the membrane vesicle's protein were 800 and 600 µg/ml, respectively.

To further characterize the OMV properties, including the virulence factors, the electrophoretic pattern was assessed by 12% SDS-PAGE. Bands similar to each other and to other studies, including 32, 69, and 140-180 kDa, were observed (Figure 2).

Western blotting was performed using specific monoclonal antibodies against PTX, PRN, and FHA to confirm the presence of known pertussis immunogens in OMVs which finally confirmed and verified the presence of these important antigens in extracted OMVs by two methods as also described by previous authors (18) (Figure 3).

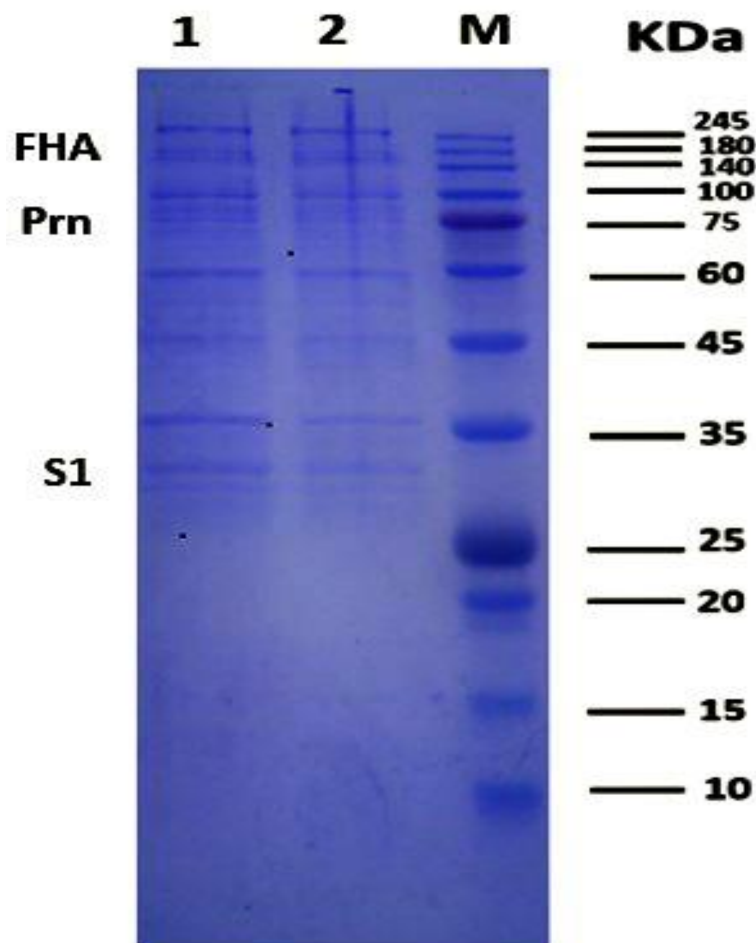

Figure 2. SDS-PAGE Pattern of OMV containing PTXS1, Prn, FHA. Lane 1 shows the OMV obtained from *B. pertussis* using the modified method, and lane 2 shows the OMV obtained with the current protocol. M: Marker. Molecular weights are indicated at the right.

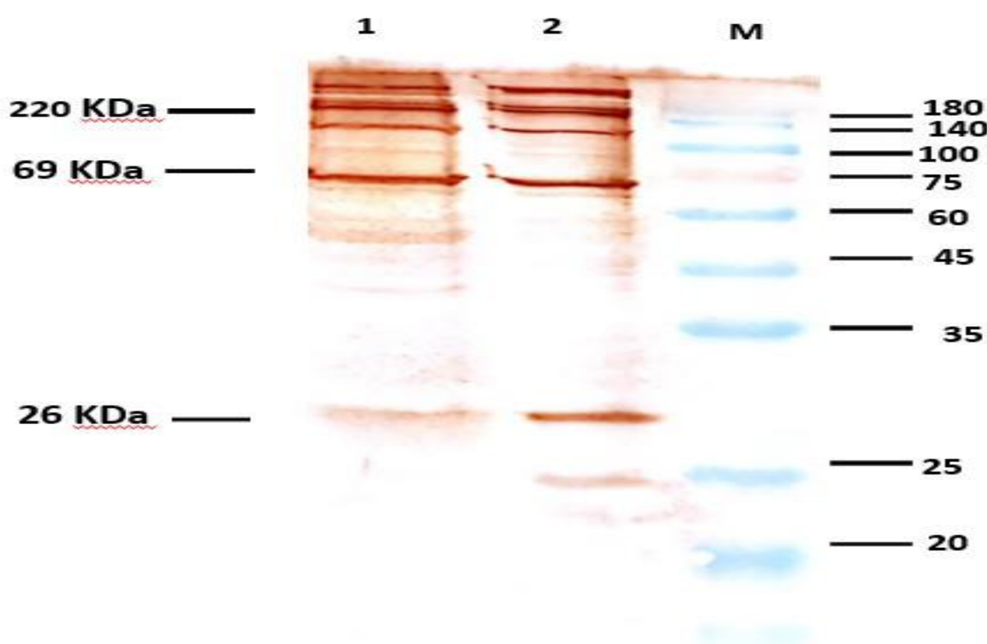

Figure 3. Western blot of OMVs using anti-PTX S1, anti-PRN, and anti-FHA. Lane 1 shows the OMV obtained from *B. pertussis* with the modified method, and lane 2 shows the OMV obtained from the current protocol. Molecular weights are indicated at the right.

4. Discussion

The resurgence of pertussis has been reported in several countries that have shifted from wP to aP vaccines. The side effects associated with whole-cell vaccines as well as their inadequacy to induce protective immunity led to the development of aP vaccines, but they, too, have some disadvantages, such as low efficacy, waning antibodies after immunization, providing immunity against a limited number of antigens, and inducing a humoral response. Because of these drawbacks, much research has focused on other parts of the bacterium (14, 19).

From the vaccine point of view, OMVs derived from *B. pertussis,* which contain main bacterial surface antigens, have been shown to successfully exhibit a basal level of protection and induce Th1, Th2, Th17, similar to wP vaccines. As OMVs are spherical nanoparticles, they are expected to exert an improved uptake of the antigen by antigen-presenting cells than the bacterial whole cell (16). Another attractive feature of OMVs is its advantage over the currently used aP vaccines, as they are capable of conferring both long-lasting immunity as well as protection against different strain genotypes that result in better protection. Therefore, OMVs could be considered as good vaccine candidates against *B. pertussis* (20).

Accordingly, several studies have revealed that because meningococcal OMVs express immunogenic antigens, they could be naturally taken advantage of for the important features required for a good vaccine. There are currently two licensed vaccines for serogroup B meningococcal disease based on OMVs. The efficacy and safety of these OMV vaccines have been proven (21-24). As OMVs are stable even at room temperature and they don't require cold chain or buffer solution, the possibility of employing OMVs as a good vaccine candidate for the prevention of diseases should be considered (25).

Roberts and Moreno (15) recently demonstrated that OMVs derived from *B. pertussis* can protect against an intranasal pertussis challenge when administered by either the intraperitoneal or intranasal route in a mouse model of infection. Moreover, the isolation of OMVs has important advantages over purified proteins, as OMV extraction based on simple steps eliminates the need for costly prior purification of each antigen, which must be done for the current acellular pertussis vaccines (26).

This research is a comparison study of the current OMV extraction method with the modified method. At least five independent OMV extractions were carried out using each method, and similar morphologies and size ranges (from 40 to 200 nm) were observed in all.

Analysis of the OMVs isolated in this study demonstrated that they were numerous nanoparticle vesicles, which were perfectly fitted with *B. pertussis* OMVs used in other studies and also with each other (15, 18, 26, 27).

The main protective bacterial antigens in the development of effective pertussis vaccines are virulence factors such as filamentous hemagglutinin, fimbriae, and pertactin, which allow *B. pertussis* to bind to the ciliated epithelial cells in the upper respiratory tract (1, 28). However, the presence of PTX, PRN, and FHA in OMVs is very important (29, 30).

Our findings show the expression of PTX, PRN, and FHA in OMVs isolated from the vaccinal strain with the current method and the modified method.

As OMVs reported herein contained several protective immunogens, they might be considered as a possible basic material for the development of a *B. pertussis* vaccine.

The current results have revealed no noticeable differences in their size or shape, nor in their protein profile; however, the modified product yield showed a slight difference. It is worth noting that this method was modified from previous methods to avoid the need for ultracentrifuging at high speed, which is an expensive and advanced technology not generally available in laboratories or research centers. Therefore, this modified method can be a valuable alternative method, especially in developing countries.

It is obvious that further studies and challenges are necessary to determine the potential immunogenic effect of OMVs in formulation with different adjuvants and the probability of producing immune responses and the efficiency of the OMV as a vaccine candidate to improve the current *B. pertussis* vaccines.

Authors' Contribution

Study concept and design: F. Sh. and M. N.
Acquisition of data: M. S. S.
Analysis and interpretation of data: M. S. S.
Drafting of the manuscript: M. S. S. and M. N.
Critical revision of the manuscript for important intellectual content: M. S. S., M. N., F. Sh. and F. E.
Statistical analysis: M. S. S., M. N., F. Sh. and F. E.
Administrative, technical, and material support: M. N., F. Sh. and S. R. B.

Ethics

All procedures performed in studies involving animals were in accordance with the ethical standards of the Razi Vaccine and Serum Research Institute.

Conflict of Interest

The authors declare that they have no conflict of interest.

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