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Immunogenicity and Protective Efficacy of an Acellular Pertussis Vaccine Candidate in in a Murine Model

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ABSTRACT

Acellular pertussis vaccines (aPVs) have been developed as an alternative to whole-cell pertussis vaccines (wPVs) because of their similar efficacy but reduced reactogenicity. The aPV contains 3 or more immunogenic components of *Bordetella pertussis*. We aimed to evaluate the immunogenicity and protective potency of an aPV vaccine produced in our laboratory consisting of pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin (PRN) in mice.

The aPV components were produced and purified from the supernatant and pellet of the bacterial culture. Two doses of the formulated vaccine, in parallel with two commercial vaccines, were administered intraperitoneally to mice at 3-week intervals. Antibody titers against aPV antigens were measured by enzyme-linked immunosorbent assay (ELISA) after primary and booster vaccinations. To assess the protective efficacy, an intranasal challenge with a live pathogenic *B pertussis* strain was conducted 2 weeks after the booster vaccination, and bacterial counts (colony-forming units [CFUs]) in the lungs were determined 2 hours and 10 days after the challenge.

The results demonstrated a significant increase in antibody titers against all pertussis antigens in the serum of the vaccinated groups compared with the negative control group, following both the primary and booster doses. No significant differences were observed between our formulation and the commercial vaccines. Furthermore, the CFU results after the challenge showed complete eradication of infection 10 days after the challenge in all immunized groups, in contrast to the control group.

Our aPV formulation, the first aPV candidate developed in Iran, exhibits immunogenicity and protective efficacy comparable to those of commercial vaccines. Further investigation of human subjects is warranted.

Keywords: Acellular pertussis vaccine; Animal model; Bacterial challenge; Immunogenicity; Protective potency

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INTRODUCTION

Bordetella pertussis (*B pertussis*) is a gram-negative aerobic coccobacillus that is responsible for pertussis infection, commonly known as whooping cough. This infectious disease is a significant contributor to child mortality worldwide, particularly in developing countries.[1,2] Vaccination plays a crucial role in preventing and controlling the spread of pertussis.[3] Since the introduction of the diphtheria-tetanus-whole cell pertussis (DTwP) vaccine in the 1950s, the incidence of pertussis has dropped significantly.[4,5] The DTwP vaccine, which includes the *B pertussis* bacteria along with diphtheria and tetanus toxoids, has been instrumental in this reduction. However, concerns regarding vaccine-associated reactogenicity and potential vaccine-induced encephalopathy have had a negative impact on public confidence and compliance.[6,7] Additionally, because of waning immunity against pertussis in many adults, booster doses are necessary for those who remain susceptible.[8] Unfortunately, administration of the whole-cell pertussis vaccine (wPV) as booster doses is hindered by noticeable side effects.[9,10] Consequently, in the late 1990s, acellular pertussis vaccines (aPVs) were introduced as a safer alternative in most developed countries.[11,12] These aPVs have demonstrated fewer adverse reactions. Pediatric aPVs typically contain 1 to 5 *B pertussis* antigens, such as pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin (PRN), adsorbed to alum. They are combined with diphtheria and tetanus toxoids (DTaP) and may also include polio, *Haemophilus influenzae* type b, and hepatitis B components.[13,14] Adolescent and adult booster vaccines (Tdap) contain lower concentrations of diphtheria-tetanus (DT) and *B pertussis* antigens.[15]

In Iran, similar to many developing countries, wPV is used in the vaccination program.[16] The number of pertussis cases in Iran has been steadily increasing since the late 2000s: 14 patients in 2017, 335 patients in 2018, and 242 patients in 2019.[17,18] Given the changing epidemiological landscape in Iran, it is now crucial for adolescents and adults to receive the Tdap vaccine. Therefore, it is imperative for Iran to prioritize the administration of Tdap booster shots to both adolescents and adults. However, there is currently no locally approved aPV in Iran.

A key feature of aPVs is their utilization of immunogenic components of *B pertussis* instead of the entire bacterium.[19] However, this particular approach has led to challenges in isolating these proteins to the necessary level of purity and recovery rate.[20] The conventional process for separating vaccine antigens typically relies on techniques based on ionic and hydrophobic interactions.[21-23] Nevertheless, in most of these traditional methods, because of the nonspecific binding of proteins to columns during purification, multiple purification steps are required to achieve the desired level of purity.[22-24] Therefore, an attempt was made in this study to purify 2 antigens, PRN and FHA, by modifying conventional methods that require fewer steps for antigen purification and also have a more acceptable recovery and purity rate.[25,26] Also, considering the economic perspective of the production of aPV and the low recovery rate of PT isolation in studies that are based on the traditional purification methods,[23,24] we used an alternative method that is based on immunoaffinity chromatography with the aim of achieving high antigen purity and recovery rates.

When it comes to newly developed vaccines incorporating aPV antigens, the World Health Organization (WHO) recommends conducting preclinical studies, particularly if the vaccine contains a novel antigen or if it has been manufactured using a new procedure, manufacturer, or strain.[27,28] The objective of our present study was to evaluate the immunogenicity, protective efficacy, and safety of our acellular pertussis vaccine candidate in a murine model. The outcomes of this investigation will contribute to the facilitation of future clinical studies on this vaccine formulation.

MATERIALS AND METHODS

B pertussis Culture

B pertussis was grown at 36°C on Bordet-Gengou blood agar (Quelab, London, UK) for 72 hours. For liquid culture, a modified cyclodextrin (CL) medium was used. The CL medium consisted of 0.75 g/L of methylcellulose and 0.75 g/L of yeast extract, as stated in a previous study.[29] A starter culture from one petri dish was added to 50 mL of CL medium in a 0.5-liter Erlenmeyer flask and cultured at 36°C for 24 hours on a shaker incubator. After the initial 24-hour culture, 12.5 mL of the starter culture was transferred into 250 mL of CL medium with an optical density of around 0.1 at 650 nm in a 1-liter Erlenmeyer flask. The culture was then shaken at a temperature of 36°C for a total of 72 hours, in accordance with previous protocols.[24]

Purification of the *B pertussis* Antigens

Purification of Pertactin

The PRN antigen is known to separate from the bacterial cell wall, with its concentration reaching its peak approximately 24 hours after the initiation of bacterial liquid culture.[30] To isolate the PRN antigen, the bacterial pellet was obtained after bacterial centrifugation (12,000*g* for 30 minutes). A modified version of the method described by Gould-Kostka et al was employed for the purification of PRN. In this process, cells were resuspended in a buffer containing 10 mM Tris-HCl, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) (Merck, Darmstadt, Germany), and pH 8.0 at a ratio of 5% (wt/vol). The suspension was then incubated at 60°C for 1.5 hours. Subsequently, the suspension was centrifuged at 10,000*g* for 30 minutes at 4°C to extract the PRN antigen and remove bacterial cells.

The heat extract was subjected to dialysis against 10 mM Tris-HCL, supplemented with 1 mM PMSF at pH 8.8 (referred to as buffer A). Subsequently, the dialyzed extract was applied to a diethylaminoethyl (DEAE)-Sepharose column that had been pre-equilibrated with 5 column volumes of buffer A. The column was washed with buffer A to remove impurities, followed by the use of a buffer A solution containing 0.02 M NaCl to further eliminate any remaining contaminants. Then, 0.07 M NaCl in buffer A was used to elute the column. Afterward, fractions containing PRN were dialyzed against 20 mM Tris-HCL, supplemented with 1 mM PMSF (pH 7.4) (buffer B), and applied to the Affi-gel blue column that had been pre-equilibrated with 5 column volumes of buffer B To remove any contaminating proteins, the column was initially washed with buffer B, followed by a wash with 0.5 M potassium phosphate (prepared in buffer B, pH 7.5). Finally, elution of the PRN protein was achieved using 1.5 M NaCl in buffer B.

Purification of Filamentous Hemagglutinin (FHA)

The FHA antigen was isolated using the method developed by Ozcengiz et al,[26] which has been previously described. In summary, the supernatant of the bacterial culture was centrifuged at 12,000 *g* for 30 minutes, 72 hours after culture initiation. Subsequently, solid ammonium sulfate (3 M) was slowly added to the bacterial supernatant and left on a mixer overnight at 4°C to precipitate the protein. The resulting mixture was then centrifuged at 4000*g* for 30 minutes at 4°C to remove the supernatant. The obtained precipitate was dissolved in 50 mM sodium phosphate buffer (pH 8.0) containing 1 M NaCl. After centrifugation at 4000*g* and 4°C for 30 minutes, the supernatant was discarded, and the crude extract was saved. To dialyze the extract, it was placed in 50 mM Na-phosphate buffer with 2 M urea (buffer C) at pH 8.0. The pH of the dialysate was later adjusted to pH 6.0 using the same buffer solution. The dialysate was then introduced into a carboxymethyl (CM)-Sepharose CL-6B column, which had been pre-equilibrated with buffer C at pH 6.0. The column was washed with buffer C at pH 7.2, and the FHA antigen was eluted using 350 mM NaCl in buffer C, at pH 7.2. The fractions containing the FHA antigen were stored at −20°C for further use.

Purification of Pertussis Toxin (PT)

As previously mentioned, after 72 hours of bacterial liquid culture initiation, the supernatant was separated by centrifugation of the culture at 12,000 *g* for 30 minutes. Subsequently, the separated supernatant was passed through a 0.22-µm filter. The filtered supernatant was passed through the rabbit anti-PT affinity column prepared in our laboratory. Next, the column was washed with a solution containing 50 mM Tris-HCl and 0.5 M NaCl (pH 8.0). To elute the PT, a solution consisting of 0.5 M Gly-HCl, 0.5 M NaCl, and 2 M urea (pH 3.5) was used, which was subsequently neutralized with 1 M Tris-HCl to prevent PT denaturation. The fractions containing PT were then stored at −20°C. To detoxify PT before administering it to mice, the purified sample was treated with glutaraldehyde and formaldehyde consecutively, as previously described.[30]

Quantification of *B pertussis* Antigens by ELISA

For quantification of *B pertussis* antigens, we developed antigen-specific sandwich ELISAs using polyclonal and monoclonal antibodies (MAbs) developed in our laboratory. Several assays have been performed to assess the validity and specificity of the antibodies against PRN,[31] FHA (Journal of Chromatography B, 2024, accepted for publication), and PT (manuscript in progress).

Initially, 50 µL/well of anti-PT (1C6), anti-PRN (2B9), and anti-FHA (2C10) MAbs (2-5 µg/mL) in phosphate-buffered saline (PBS) were coated onto a 96-well ELISA microplate (Nunc, Thermofisher, USA) and incubated at 37°C for 1 hour. After washing the plates with PBS-Tween 0.05% (PBS-T) and blocking with 3%

nonfat skim milk (Merck, Darmstadt, Germany) for 1 hour, the plates were incubated with serial concentrations of standard antigen (PT; NIBSC, 90/518, UK) (PRN; NIBSC, 18/154, UK) (FHA; NIBSC, 90/520, UK) for 1 hour at 37°C. Following another round of washing, appropriate dilutions of detector antibodies (horseradish peroxidase-conjugated sheep anti-FHA, rabbit anti-PRN, and rabbit anti-PT polyclonal antibodies) were added to the wells and incubated at 37°C for 1 hour. Subsequently, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sina biotech, Tehran, Iran) was added to the plates, and the reaction was stopped using 1 N HCl. Finally, the optical density (OD) was measured at 450 nm using an ELISA reader (Biotek 800TS, Biotek, USA). The concentration of antigens was extrapolated from the corresponding standard curve using the GraphPad Prism program.

Western Blot Analysis

Antigens, 1.2 µg of purified proteins were electrophoresed in 8%, 10%, and 12% gels (for FHA, PRN, and PT, respectively) under reducing and nonreducing conditions. Subsequently, the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) at 100 V and 4°C for 100 minutes. The membranes were blocked with PBS containing 0.05% skim milk, washed with PBS-T, and incubated with a 1:1000 dilution of commercial sheep anti-PT (NIBSC, 97/564), sheep anti-FHA (NIBSC, 97/564), and sheep anti-PRN (NIBSC, 97/564) To determine the purity of antibodies, followed by horseradish peroxidase (HRP)–conjugated rabbit anti-sheep antibody (1:1500, Sina biotech, Tehran, Iran) at room temperature for 45 minutes on a shaker. Finally, the bands were detected using a chemiluminescence (ECL) prime solution (Amersham Pharmacia Biotech, Chalfont, UK).

Antigen, Adjuvant, Animals, and Immunization Protocols

Four-week-old female BALB/c mice (Royan Institute, Tehran, Iran) were used and placed in an animal house with a standard light/dark cycle (12/12 hours) and temperature (25°C). The mice were divided into 5 groups (10 mice in each group) according to the administered primary and booster vaccines. Mice were immunized IP with ¼ of a human dose of formulated vaccine (purified antigens adsorbed with alum adjuvant) at intervals of 3 weeks, concurrently with 2 doses of commercial vaccines (Table 1) (Figure 1).

As a primary and booster vaccination, group 1 mice received the commercial DTaP vaccine (Infanrix, GlaxoSmithKline, Middlesex, UK), and group 2 mice were administered the commercial Tdap vaccine (Boostrix, GlaxoSmithKline, Middlesex, UK). It is important to note that the Tdap vaccine has lower concentrations of pertussis, diphtheria, and tetanus antigens compared with the DTaP vaccine. Group 3, known as Infanrix, and group 1 both received the same amount of pertussis antigens in their doses. Specifically, they each contained 6.25 µg of PT, 6.25 µg of FHA, and 2 µg of PRN. Similarly, group 4, which received Boostrix, and group 2 also had identical amounts of pertussis antigens in their doses. Both groups contained 2 µg of PT, 2 µg of FHA, and 1.25 µg of PRN (Table 1). Group 5 mice were injected with DT emulsified with an alum adjuvant (Razi Vaccine and Serum Research Institute, Karaj, Iran).

Table 1. Type of vaccines and their antigen components

Groups	Pertussis antigen content (µg)			DT (Lf)		Alum (Al (OH)3) (mg)
	PT	FHA	PRN	Diphtheria	Tetanus	
Infanrix (DTaP)	6.25	6.25	2	7.5	10.0	0.125
Boostrix (TdaP)	2	2	0.625	0.625	1.25	0.075
aPV1 (DTaP) *	6.25	6.25	2	3.75	1.25	0.125
aPV2 (TdaP) *	2	2	0.625	0.50	1.25	0.075
Negative control (DT)	-	-	-	3.75	1.25	

DTaP: diphtheria-tetanus-acellular pertussis, Tdap: tetanus toxoid, reduced diphtheria toxoid, and acellular pertussis; PT: pertussis toxin, FHA: filamentous hemagglutinin, PRN: pertactin.

* acellular pertussis vaccine candidate with different concentrations of antigens

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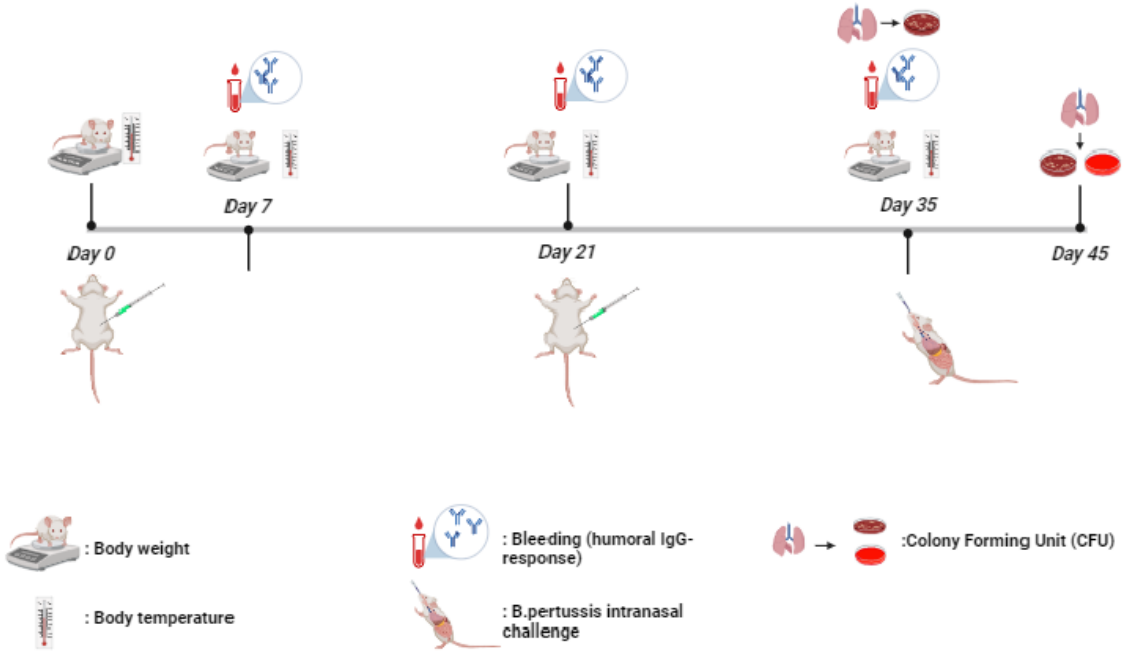

Figure 1. Schemes for vaccination, challenge, and assays of study

Assessment of the Antigen-Specific Antibody Response

To evaluate the humoral immune response, blood samples were collected 1 and 3 weeks after the first dose of the primary vaccination and 2 weeks after the booster vaccination. An indirect ELISA was used to measure the concentration of antibodies against 3 pertussis antigens (PT, FHA, and PRN). Briefly, the 96-well plates were coated with PT (2 µg/mL), FHA (2 µg/mL), and PRN (2 µg/mL) in PBS. Following overnight incubation at 4°C, the plates were washed with PBS-T and subsequently blocked for 2 hours using PBS-T containing 3% skim milk. After incubation and further washing, serum from all mice was added to the wells at dilutions of 1:100, 1:400, 1:800, and 1:1600 and incubated for 1 hour at 37°C. Then, polyclonal sheep anti-mouse immunoglobulin conjugated with HRP (Sina biotech, Tehran, Iran) was introduced to the wells. Finally, an ELISA reader measured the ODs at 450 nm after adding TMB substrate (Sina biotec).

Quantitation of Anti-PT, Anti-FHA, and Anti-PRN IgG by ELISA

Principles of the ELISA procedure, including sample preparation, coating, blocking, incubation, secondary antibody, and substrate incubation, follow the same scheme as for the above-referred ELISA assays. Plates are coated with 2 µg/mL pertussis antigens (PT, FHA, and PRN) in a well and incubated at 4°C overnight. The coated plates are washed and blocked, and the standard pool as well as sample dilutions are prepared on a separate plate. A pool of positive serum (Infanrix-immunized group; 2 weeks after booster dose) was used to construct a standard curve and diluted in an 8-step serial dilution (from 1:50 to 1:6400, in 2.0-fold steps) in triplicate. The final arbitrary units (AU)/mL for each sample was found by taking the median AU/mL from all the dilutions in the linear range of the curve. The lowest AU dilution in the linear range of the curve is thought to be equal to 100 AU/mL, and as the dilution factor goes up, the AU amount goes down by ½. To determine the final AU, multiply the results of this procedure by the dilution factor for the serum sample.[32]

Bacterial Intranasal Challenge

The protective efficacy against *B pertussis* infection was assessed using an intranasal clearance test. The mice were challenged with *B pertussis* strain Tohama-I 2 weeks after bacterial cultivation on a Bordet-Gengou agar plate containing 15% defibrinated sheep blood dissolved in a solution of 0.9% saline and 0.1% casein. All mice were challenged with 1010 CFUs of *B pertussis* using an

aerosol nebulizer (NE-U17E; Omron, China). Two hours after intranasal infection, 3 mice from each group were euthanized, while the remaining mice (7 mice in each group) were euthanized 10 days after the challenge, and their lungs were extracted (Figure 1). Bronchoalveolar lavage (BAL) was isolated immediately from their lungs in a sterile condition under the hood. This was carried out by injecting 1 mL of sterile PBS into the lung with an insulin syringe. The lavage was diluted to 10^{-1} and 10^{-3}. Each diluted sample was cultured on Bordet-Gengou agar medium and incubated at 37°C for 5 days. The CFUs on each medium were determined, and mean CFUs were compared between the groups at each time point.

Assessment of Safety

To assess the safety of the aPV, the mouse weight gain test (MWGT) and the body temperature test (BTT) were conducted. The weight of the mice was measured on days 0, 7, 21, and 35 after immunization (Figure 1). The mean body weights of the 5 groups were compared at each time point, and the vaccine was considered nontoxic if the mean body weight of the vaccinated group exceeded 60% of that of the negative control group 7 days after vaccination.[33] Rectal thermometers were used to measure body temperature for the BTT. Body temperatures were measured before vaccination as well as 3 and 24 hours later. Because the exact pass criteria for the BTT have not been established, body temperatures were simply compared between groups, and test group values were assessed to determine whether they consistently fell within the range of mean±SEM for assessing the assay validity.[33]

Bradford Assay

The Bradford assay was used to determine total protein concentration as described elsewhere.[34]

Statistical Analysis

The study used Prism 7.0 GraphPad software for statistical analysis. To evaluate the immune response differences among groups, antibody titers pre- and post-booster shots were compared using a one-way analysis of variance (ANOVA) test followed by a Tukey multiple comparison test when more than 2 groups of mice were tested. Additionally, for the intranasal challenge test, the CFU values of *B pertussis* in the lungs of the mice in the vaccine candidate group and the positive and negative control groups were compared at different time points using a one-way ANOVA test and Tukey multiple comparison test. Statistical significance was determined for p values less than 0.05. Results are reported as mean±SEM.

RESULTS

Production and Purification of Native PT, FHA, and PRN Antigens

Following the production of *B pertussis* antigens in modified CL medium, purification of these proteins was conducted as described in Materials and Methods. The results of the production and purification of *B pertussis* antigens showed that the proteins were successfully produced and recovered at an appropriate rate (Table 2).

Assessment of the purified PT by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting demonstrates that the eluted fractions are enriched with PT protein and have 3 major bands of 25, 13, and 10 kDa (Figure 2A). Moreover, an appropriate recovery rate for the FHA was obtained by purifying this antigen using a CM-Sepharose column. SDS-PAGE analysis confirmed that the purified FHA protein was pure and consisted of 3 bands with molecular weights of 220, 120, and 100 kDa (Figure 2B). Similarly, the 2-step purification of the PRN antigen resulted in a pure antigen with an appropriate recovery rate (Figure 2C).

Polyclonal sheep anti-PT, anti-FHA, and anti-PRN were used to identify the purified antigens by immunoblotting assay (Figure 2). The similarity between the pattern and the position of the bands in these 2 assays suggests that the bands revealed by SDS-PAGE are specifically related to the purified antigens.

Assessment of the Antigen-Specific Antibody Response in Immunized Mice

The humoral immune response was evaluated 1 and 3 weeks after administration of the first dose and 2 weeks after the booster vaccination. The levels of anti-PT, anti-FHA, and anti-PRN antibodies in all vaccinated groups significantly increased 3 weeks after the primary vaccination. As expected, these antibody levels further increased 2 weeks after the second dose of vaccination in all groups (Figure 3).

The mean titer of anti-PT IgG peaked at 23,760 AU/mL and 5368 AU/mL in the control groups vaccinated with the commercial Infanrix and Boostrix vaccines (groups 1 and 2), respectively, and at 25,760

AU/mL and 5576 AU/mL in the groups vaccinated with our counterpart candidate vaccines (groups 3 and 4), respectively, at 2 weeks after the booster dose. Similarly, the mean titer against the FHA antigen increased after booster vaccination, reaching 23,904 AU/mL and 11,832 AU/mL in groups 1 and 2, respectively, and 24 344 AU/mL and 12,004 AU/mL in groups 3 and 4, respectively. The anti-PRN IgG titer also reached its peak 2 weeks after boosting:[35] 904 AU/mL and 24,818 AU/mL in groups 1 and 2, respectively, and 37,672 AU/mL and 24,000 AU/mL in groups 3 and 4, respectively. Furthermore, there were no significant differences in the titers of anti-PT IgG, anti-FHA IgG, and anti-PRN IgG between groups 1 vs 3 and groups 2 vs 4 at each time point (Figure 3) (Supplementary Table 1).

Protection Efficacy of Our Vaccine Candidate Against *B pertussis* in the Intranasal Infection Model

Two hours after the intranasal bacterial challenge, all mice were successfully infected, and the average CFU in the lungs ranged from 1.98×105 to 2.3×105 CFU/lung (Figure 4). Ten days after challenge, however, *B pertussis* was eliminated from all 4 vaccinated groups, while the negative control group showed a significant number of CFUs in the lung (2.06×10^5 CFU/lung) (Figure 4) (Supplementary Table 2).

Table 2. Purification Process and Recovery Rate of the Purified *B pertussis* Antigens

Antigen	Purification method	Concentration in supernatant (µg/mL)[a]	Concentration in pellet (µg/mL)[a]	Recovery rate (yield) after purification (%)[b]	Purity (%)[c]
PT	Affinity chromatography (rabbit anti-PT)	3.1 ± 0.20	—	74 ± 4.9	>95
FHA	Cation exchange chromatography (CM-Sepharose)	27 ± 4.35	—	71 ± 4.24	>95
PRN	Anion exchange chromatography (DEAE-Sepharose) and hydrophobic interaction (Affi-gel Blue)	—	21 ± 3.73	84 ± 4.0	>95

CM: carboxymethyl, DEAE: diethylaminoethyl, FHA: filamentous hemagglutinin, PT: pertussis toxin, PRN: pertactin

[a] Protein concentration in supernatant and pellet of bacteria was determined by ELISA. [b] Recovery rate of purified antigens was determined by ELISA and Bradford assay. [c] Purity of purified antigen was determined by ImageJ software. Data are presented as mean±SEM from 2 independent assays.

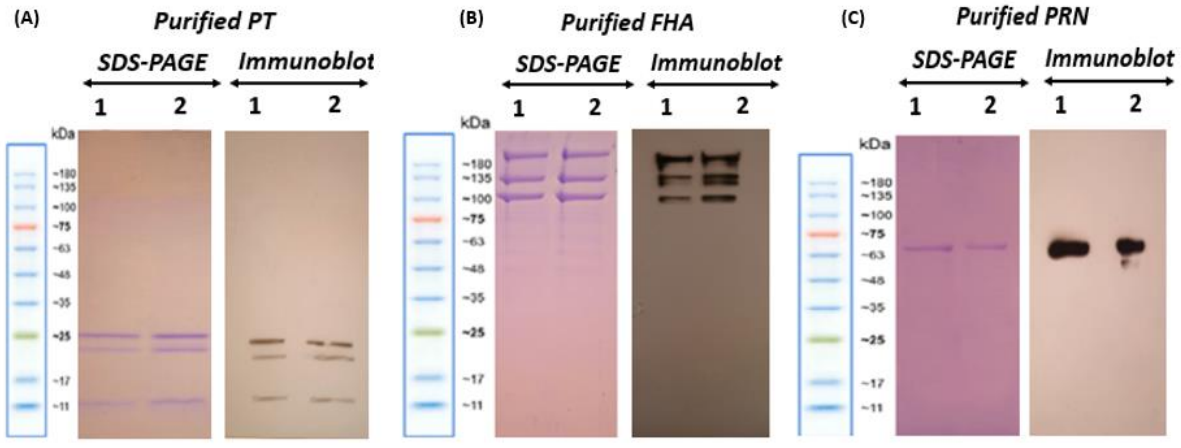

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot of purified *BP* proteins. Approximately 2 µg of PT and PRN, and 5 µg of FHA were loaded in each line for SDS-PAGE and 1.2 µg of each protein for immunoblotting. A) PT SDS-PAGE and immunoblot (1: NR, 2: R). B) FHA SDS-PAGE and immunoblot (1: NR, 2: R). C) PRN purified from cell pellet extracts SDS-PAGE and immunoblot (1: NR, 2: R). NR: Non-reduced, R: Reduced

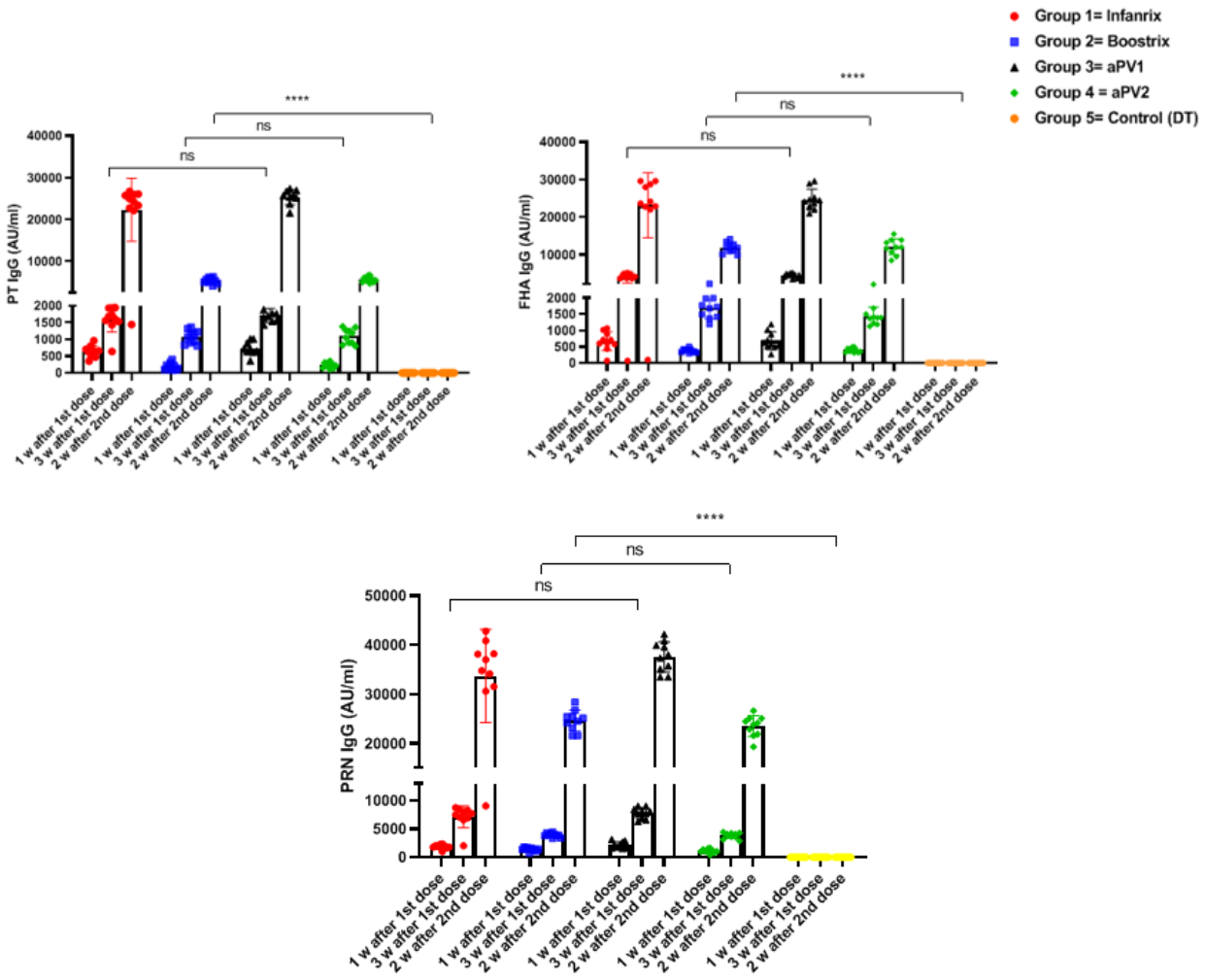

Figure 3. Antibody response against vaccine components in all groups of immunized mice

Antibody levels were assessed 1 and 3 weeks after primary vaccination and 2 weeks after booster vaccination. In all conditions, experiments were performed on 10 mice per group. Statistical differences were tested with unpaired *t*-test and one-way ANOVA comparison test. A sandwich ELISA was used to measure the levels of anti-PT, anti-FHA, and anti-PRN IgG. (PT: pertussis toxin, FHA: filamentous haemagglutinin, PRN: pertactin) (**p<0.0001). ns: not significant. The data are presented as mean±SEM.**

Assessment of the Safety of the Vaccine Formulations

The safety of the vaccines was partly evaluated by determining the body weight and temperature of the vaccinated mice at different time points post-vaccination. There were no significant differences in the average body weight of all groups (groups 1–5) pre- and post-vaccination. Following vaccination, there were no significant differences in the mean body weight at 0, 7, 21, and 35 days (Figure 5A) (Supplementary Table 3). Moreover, there were no significant variations in the mean body temperature of all groups vaccinated with aPV (groups 1-4) at 3 and 24 hours post-vaccination compared with the control group vaccinated with DT plus adjuvant (group 5) (Figure 5B) (Supplementary Table 3).

Altogether, in this study, PT, FHA, and PRN antigens were prepared with appropriate purity and recovery rates. Using a murine model, the candidate aPV showed comparable immunogenicity and protective efficacy to the current commercial *B pertussis* vaccines. Our candidate vaccine can be tested in future clinical trials to confirm its efficacy and safety.

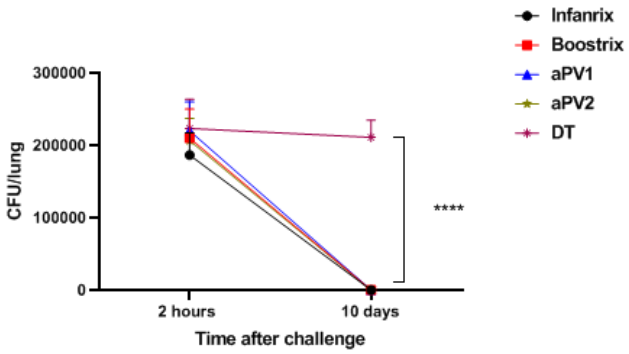

Figure 4. Kinetics of *Bordetella pertussis* clearance from the lungs after respiratory challenge of mice immunized with the aPVs. Following intranasal infection, the mean colony-forming units (CFUs) of all groups ranged from 1.98 × 10^5 to 2.35 × 10^5 (CFU/lung), with no significant difference across the groups. In groups 1 to 4, *B pertussis* was completely eliminated 10 days after the intranasal challenge. (**, *p*<0.0001). The data is presented as mean±SEM.**

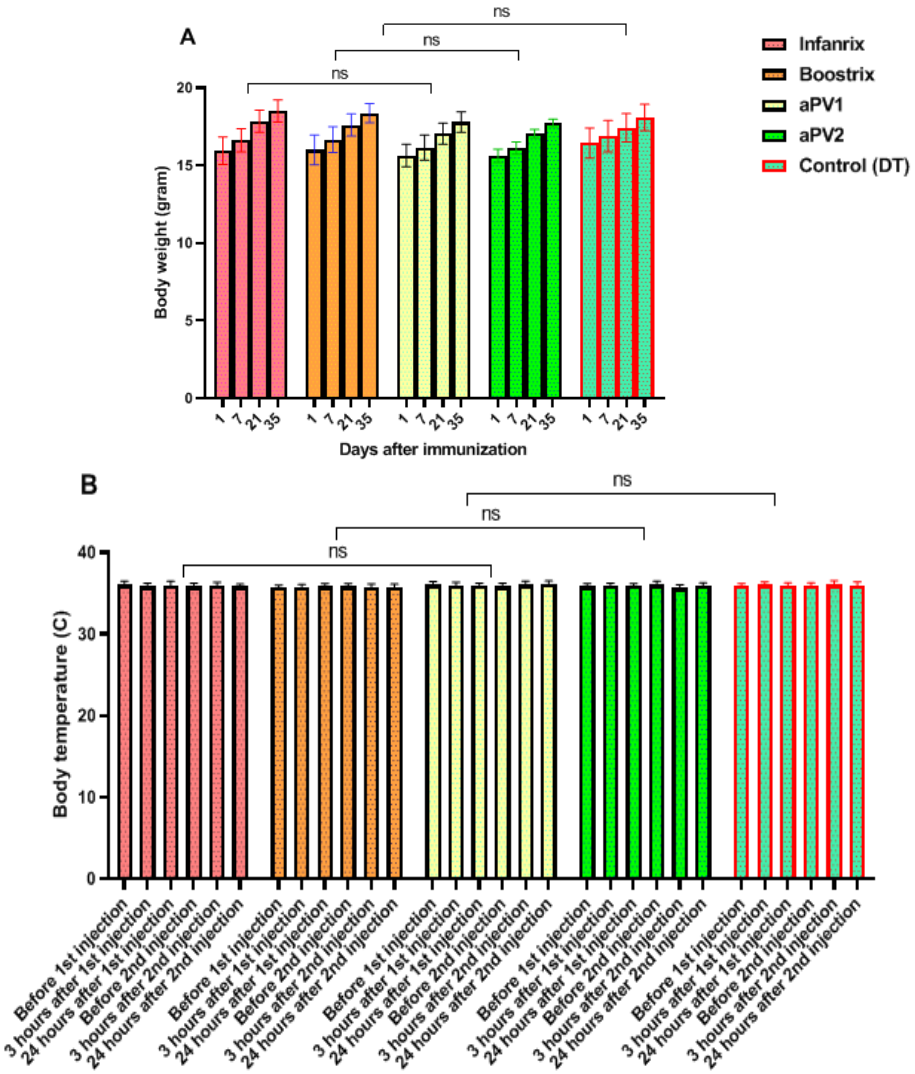

Figure 5. Vaccine safety assessed by mouse weight gain and body temperature tests. A) Mean body weight and, B) Mean body temperature in immunized mice. Values are presented as mean±SEM. ns: not significant

DISCUSSION

Pertussis, commonly referred to as whooping cough, is a highly contagious respiratory ailment that predominantly affects newborns and young children.[35] In the early 20th century, immunization efforts against pertussis involved the development and assessment of whole-cell preparations. These vaccines demonstrated significant success in preventing the disease, especially in children.[36] However, wPVs often induce localized adverse reactions at the injection site, such as redness, swelling, and soreness.[37] Additionally, they may lead to moderate systemic side effects like fever, fatigue, anxiety, and loss of appetite.[38] Despite high vaccination rates in some communities, pertussis can still circulate, with adults acting as a reservoir for severe and potentially fatal infections in newborns.[39]

Infants who have not completed all necessary doses of wPVs face an increased risk of contracting the disease. Furthermore, asymptomatic adults or those with mild symptoms contribute to disease transmission.[39,40] Moreover, wPVs cannot serve as booster doses for adolescents, and immunity diminishes with age, leaving individuals susceptible to becoming carriers.[41] To address these issues, many countries have shifted to using aPVs instead of whole-cell ones. This transition is driven by concerns about the side effects of wPVs and their limited effectiveness as booster doses in individuals with low immunity levels.[42,43]

Numerous surveillance studies and larger clinical trials consistently conclude that aPVs are both effective and safe.[44,45] Despite this, Iran continues to employ whole-cell pertussis vaccines in its vaccination program, witnessing a steady increase in pertussis cases since the late 2000s.[39] Given changes in the epidemiological landscape, adolescents, and adults with weakened immunity in Iran are now advised to receive aPVs. Additionally, aPVs present a viable alternative because of the side effects associated with wPVs and the established effectiveness and safety of acellular alternatives.[37]

One of the primary obstacles to preparing antigens for aPVs lies in their relatively low production and the intricate, time-consuming purification process.[22,46] This is a major contributor to the high manufacturing costs associated with these vaccines. Presently, aPVs are predominantly used in developed countries.[47] Because of financial constraints, the majority of impoverished and third-world nations continue to implement routine vaccination programs using wPVs.[48,49] Despite substantial global advancements in aPV production and the development of vaccines with diverse components, Iran has yet to produce any aPVs and lacks the necessary technologies for their manufacturing.

In this research, we used immunoaffinity chromatography (for purifying PT) and modified traditional techniques (for purifying FHA and PRN) to enhance the purity and recovery rates during the purification process. These methods streamline the process, resulting in fewer steps, improved purity, and higher recovery rates. To accomplish this, aPV antigens underwent purification using various chromatographic techniques following bacterial *B pertussis* culture in the modified CL medium. Through optimization, we managed to produce a reasonable quantity of aPV antigens, taking into account findings from other studies (Table 2).[25,26,43] These enhancements simplify the purification process, ensuring that the antigens attain the desired levels of purity and recovery rate. The purification methods employed in this study facilitated the recovery of antigens at a rate ranging from 70% to 85%, which is quite satisfactory when compared with currently available methods (Table 2). The results obtained from SDS-PAGE and immunoblot assays, along with the comparison of band patterns for each antigen with those from other studies, affirm the purity of all 3 purified antigens.[22,26]

After assessing purity and antigen concentration, diverse antigen concentrations were combined with an alum adjuvant for comparison with commercial vaccines like Infanrix and Boostrix. It is important to note that DT vaccines were administered to mice in the produced aPV groups and the negative control group. This was done to ensure that the vaccines developed in this experiment closely resembled commercial vaccines in terms of antigen content.

Following WHO recommendations, both a nonclinical research study and a clinical study are imperative before the approval and implementation of a new vaccine containing aPV antigens.[33] Consequently, numerous nonclinical studies have been published on the immunogenicity and protective effectiveness of aPVs.[27,28]

Humoral immune responses have been shown to play a complementary role in guarding against pertussis, with the assessment of humoral immune responses against *B*

pertussis infection frequently used to evaluate DTaP and Tdap vaccinations.[50,51] Additionally, as maternal antibodies can reach the fetus, the humoral immunity developed by pregnant women receiving aPVs could protect newborns from contracting pertussis.[21] To compare the newly developed aPVs with existing commercial DTaP and Tdap vaccines, this study investigated their immunogenicity, protective efficacy, and safety. The novel vaccine demonstrated comparable immunogenicity, protective efficacy, and safety compared with the currently available aPVs on the market.

In this study, antibody titers against pertussis antigens notably increased 3 weeks after the first dose and booster vaccination, even in groups 2 and 4, where a lower dose of antigens was administered. Although the anti-PT, anti-FHA, and anti-PRN IgG titers against pertussis antigens in groups 2 and 4 were lower than those in groups 1 and 3, this could be attributed to differences in the concentration of received antigens in these groups. Therefore, using a higher dose of antigen can induce a stronger humoral immune response. Furthermore, no significant difference was observed before and after booster vaccination between the new aPVs and commercial vaccines (Figure 3) (Supplementary Table 1).

According to the guidelines of both the WHO and the European Medicines Agency (EMA), it is imperative that there be no significant difference in the antibody response against pertussis antigens between the commercial vaccines and the new pertussis vaccines.[52,53] Fortunately, the vaccines used in this study demonstrated this crucial capability. As previously mentioned, numerous studies have been conducted to generate and evaluate the immunogenicity of aPVs.

Our study's humoral immune response pattern aligned with that of Han et al,[54] who developed the Tdap vaccine (Green Cross Corporation, Yan-gin, Korea). Their study showcased significant increases in antibody titers against pertussis antigens (anti-PT, anti-FHA, and anti-PRN) following booster vaccination. Kang et al,[55] also demonstrated the effectiveness of a novel aPV against pertussis, revealing elevated levels of anti-PT, anti-FHA, and anti-PRN IgG even before booster vaccination, with a continuous rise thereafter. Furthermore, Kwon et al[33] reported significantly higher antibody titers of anti-PT and anti-FHA IgG after booster vaccination with a new reduced-dose tetanus-diphtheria-acellular pertussis vaccine compared with before the booster vaccination.

In addition to the humoral immune response, we also investigated the protective efficacy of our aPVs. In this test, infected bacterial numbers showed no significant difference among the 4 groups, and bacterial pertussis was completely eliminated 10 days after intranasal infection in all 4 groups receiving aPVs. It is noteworthy that in unvaccinated mice, the bacterial count in the lungs increased 10 days after intranasal infection, followed by a subsequent decrease (Figure 4).

Several studies, akin to ours, have been conducted to evaluate the protective efficacy of newly produced aPVs. Han et al,[54] reported in 2015 that aPV-vaccinated mouse groups were able to eradicate the infection from their lungs by the fifth day after the challenge, comparable to the commercial Boostrix vaccine. In contrast, the negative control group, which did not receive any vaccine, did not exhibit a significant difference in the bacterial count in their lungs until the tenth day after the challenge. It is crucial to note that their study assessed CFUs in the lungs 2 hours, 5 days, and 10 days after the challenge.

In another study conducted by Kwon and colleagues,[33] it was revealed that the groups of mice receiving the aPVs effectively cleared the lung infection on the seventh day after the challenge, similar to the commercial vaccine. The assessment of bacterial presence in the lungs was conducted at various time points, including 2 hours, 2 days, 7 days, and 9 days after exposure.

Another study by Kang et al,[55] demonstrated that the groups of mice receiving the aPV cleared the lung infection on the fifth day following the challenge, comparable to the commercial vaccine. Conversely, the negative control group exhibited no significant difference in bacterial levels in the lungs compared with the initial exposure, even until the tenth day.

While the WHO guidelines for nonclinical research specifically focus on the humoral immune response and protection efficacy of aPVs, our study went beyond that to assess the safety of aPVs. In our study, the results of the MWGT and BTT were similar to those of vaccines already on the market. Additionally, no significant difference was observed between the 4 groups receiving aPVs and the negative control (group 5) that received Razi Institute's DT vaccine, indicating the absence of toxic effects from the vaccines (Figure 5) (eTable 3 in the Supplement). As a result, the mean body weight change of groups 2 and 4 that received the aPVs was greater than 60% of that of the positive and negative control groups, suggesting the safety of aPVs.[33]

While the safety of biosimilar aPVs is typically not assessed, the study by Kwon and colleagues[33] evaluated mean body weight changes in mice before and 7 days after each vaccine dose. Similar to our results, no significant difference was found between the groups receiving the vaccine and the negative control group.

Considering the early eradication of infected *B pertussis* in intranasal infection and the lack of significance in different antibody titers against pertussis antigens after booster vaccination in groups receiving new aPVs and commercial vaccines in the present study, the novel aPV may be successfully used in clinical settings. It should be noted that according to the guidelines of the WHO regarding the production and evaluation of aPVs, when vaccines are used that are similar to commercial vaccines in terms of antigenic content, the investigation of immunogenicity and immunity is emphasized.[52] Also, this study has been conducted on a laboratory scale, and many additional tests (sterility, toxicity, and safety) will be performed at the same time as the test is repeated under good manufacturing practice conditions. Moreover, it is essential to note that these findings are based on a murine model, and further clinical trials are necessary to confirm the vaccine's efficacy and safety in humans.

In conclusion, we have successfully generated and purified 3 pertussis antigens (PT, FHA, and PRN) used in aPV. Additionally, we have established various types of ELISA tests with optimal sensitivity to accurately determine the concentration of *B pertussis* antigens throughout the production and purification processes. Furthermore, through experimentation on a murine model, we have demonstrated that the newly developed aPV exhibits comparable immunogenicity and protective efficacy to the currently available vaccine for *B pertussis*. In forthcoming clinical trials, the innovative aPV can be used to further validate its effectiveness and safety.

STATEMENT OF ETHICS

All animal studies were performed under the protocols authorized by Tehran University of Medical Sciences (TUMS). The Ethics Committee in Biomedical Research of TUMS reviewed and approved this study (Approval ID: IR.TUMS. SPH.REC.1400.283).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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