Iranian Journal of Microbiology

Volume 17 Number 1 (February 2025) 106-112
DOI: http://doi.org/10.18502/ijm.v17i1.17807

ORIGINAL ARTICLE

Immunological efficiency of *Haemophilus influenzae* type b polyribosyl ribitol phosphate combined with detoxified lipooligosaccharide in a rabbit model

Amin Arsang[1], Seyed Davar Siadat[1*], Masoumeh Saberpour[2], Farshad Nojoomi[3*]

[1]Department of Mycobacteriology & Pulmonary Research, Pasteur Institute of Iran, Tehran, Iran
[2]Department of Influenza and Other Respiratory Viruses, Pasteur Institute of Iran, Tehran, Iran
[3]Infectious Diseases Research Center, AJA University of Medical Sciences, Tehran, Iran

Received: July 2022**, Accepted:** December 2024

ABSTRACT

Background and Objectives: *Haemophilus influenzae* type b *(Hib)* could cause severe life-threatening infections in children. Combine vaccines have reduced invasive diseases, but disease management is still necessary. The aim of this research was to evaluate the immunological efficiency of polyribosyl-ribitol-phosphate combined with detoxified lipooligosaccharide (PRP-dLOS) in a rabbit model.

Materials and Methods: PRP purification, LOS extraction, and endotoxin evaluation were performed using modified CY medium, hot phenol, and limulus amebocyte lysate methods, respectively. Rabbit groups were immunized with PRP (10 µg), dLOS (20 µg), and PRP-dLOS combine (10 µg+20 µg) three times on days 0, 14, and 28. Serum samples were acquired on days 0, 14, and 28 post-immunization, then IgM and IgG levels were assayed by enzyme-linked immunosorbent assay.

Results: The concentrations of PRP, dLOS, and endotoxin were 1160 mg/L, 440 µg/mL, and 1450 EU/mL, respectively. PRP-dLOS combine led to a significant increase in IgG and IgM levels on days 14 and 28 post-immunization. After immunization with PRP-dLOS combine, serum levels of IgM and IgG increased from 16.8 to 29.3 µg/mL and 29.8 to 61.4 µg/mL, respectively from day 14 to day 28.

Conclusion: PRP-dLOS combine is a promising approach for Hib management without the fear of delay in immune responses and interference with other vaccines.

Keywords: *Haemophilus influenzae* type b; Immunological; Lipooligosaccharide; Meningitis

INTRODUCTION

Haemophilus influenzae (H. influenzae) is one of the major reasons of meningitis and invasive infections (1). *H. influenzae* strains are typically classified into six serotypes (a-f) according to their capsular antigens. *H. influenzae* Type B (Hib) accounts for more than 95% of invasive infections in infants and young children. Invasive diseases occur after microorganisms enter sterile body fluids including blood or cerebrospinal fluid, manifesting as pneumonia or meningitis (2). Outer membrane proteins (OMPs), capsule, O-deacylated lipooligosaccharide (LOS), adhesive proteins, pili, and IgA1 protease are

*Corresponding authors: Seyed Davar Siadat, Ph.D, Department of Mycobacteriology & Pulmonary Research, Pasteur Institute of Iran, Tehran, Iran. Tel: +98-21-66969291 Fax: +98-21-66465132 Email: d.siadat@gmail.com

Farshad Nojoomi, Ph.D, Infectious Diseases Research Center, AJA University of Medical Sciences, Tehran, Iran. Tel: +98-21-43822277 Fax: +98-21-43282073 Email: fnojoomi2@gmail.com

involved in Hib pathogenesis (3). The mechanisms of action of these factors are different; for instance, OMPs adhere to carcinoembryonic antigen-associated cell surface adhesion molecules (CEACAMs) on the upper respiratory epithelium, facilitating the entry of microorganisms into eukaryotic cells (4), and LOS leads to the induction of the immune system by enhancing the expression of ICAM-1 (intercellular adhesion molecule 1) and the production of cytokines, including interleukin 6, interleukin 8, and tumor necrosis factor alpha (5). As an adjuvant, LOS has the ability to enhance Th1-mediated cellular immune responses. However, its application is significantly restricted due to its endotoxic effects. There are three methods to reduce the toxicity of LOS, including chemical modification, artificial assembly, and genetic modification of bacteria (6).

Moreover, the capsule is a key factor that plays an important role in pathogenesis. It leads to bacterial escape from part of the immune system such as neutrophils and complement. Briefly, it inhibits phagocytosis by blocking antigen binding sites and preventing IgG attachment to the surface of bacteria, ultimately leading to inhibition of phagocytosis (7). Thus, the capsule ability to evade phagocytosis enhances bacterial survival, and the absence of antibodies against the capsule contributes to bacterial expansion (8). The complement system activation is initiated by the C3b molecule attachment to the surface of bacteria, followed by the activation of the protein cascade, ultimately leading to bacterial destruction. Capsule is a physical barrier that prevents the activation and deposition of complement proteins (9). Previous studies have revealed that some Hib virulence factors are potential candidates for vaccine development, and several factors have been used in the preparation of existing vaccines. The capsule contains polyribosyl-ribitol-phosphate (PRP) units. Recently, PRP has been commonly used in the formulation of Hib vaccines. It is a T-independent antigen presented by B cell receptors (IgM) similar to other polysaccharide antigens. The use of this antigen in vaccine formulation plays a crucial role in preventing Hib pathogenesis by inducing the production of specific antibodies that confer protection against Hib infections. The first Hib vaccine was prepared using purified PRP, although it was not sufficiently effective against Hib infection due to reduced protective antibody titers in the long term (10). Subsequent research confirmed that immunization with PRP combined to outer membrane protein of *Neisseria meningitidis* group B (PRP-OMP) induced the generation of specific antibodies against Hib and provided protection against both invasive and mucosal diseases caused by Hib. This vaccine also did exhibit good immunologic responses after the final immunization (11).

Recently, the innovative approach of common vaccines is to conjugate PRP with various proteins as carriers to enhance immune responses. Some conjugate vaccines include PRP-D (carrier: diphtheria toxoid), HbOC (carrier: CRM197), PRP-T (carrier: tetanus toxoid), and PRP-OMP (carrier: OMP) (12). Moreover, conjugated vaccines have appropriate efficiency against Hib, especially vaccination with a booster dose, leading to a significant reduction in invasive diseases in children (13). However, these vaccines also have some disadvantages. Studies have revealed that there is a delay in immune responses to conjugate vaccines in infants due to i) lack of natural immunity, ii) interference with other vaccines, and iii) improper vaccination schedule (14). Therefore, evaluation of immunological properties of other virulence factors is necessary. Virulence factors that continuously stimulate the immune system could be used as promising vaccine candidates. Considering the insufficient immunogenicity of PRP in stimulating immune responses and immunological properties of LOS as a positive regulator of intrinsic and acquired immune responses (5), this study was designed to investigate the hypothesis that PRP-dLOS combine could be considered as an appropriate combination to stimulate humoral and cellular immune responses against Hib infections.

MATERIALS AND METHODS

Growth conditions of *H. influenzae* serotype b. In this study, a standard strain of Hib (ATCC12022) was acquired from the bacterial archives of the Pasteur Institute of Iran. In brief, a lyophilized vial containing Hib strain inoculated in 0.5 mL of BHI (brain heart infusion) broth (Merck, Germany) was placed in an incubator at 37°C for 12 h. Afterward, 100 µL of the bacterial suspension was cultured on a chocolate agar plate (Merck, Germany) enriched with hemin (1%) (Sigma-Aldrich, Germany) and NAD (0.01%) (nicotinamide adenine dinucleotide) (Mast, Bootle, UK) and incubated at 37°C with 5% CO_2 for

24 h. Hib strain was confirmed using Gram staining, biochemical experiments (urease, indole production, ornithine decarboxylase), and slide agglutination test with a specific antiserum (15).

PRP purification. PRP purification was done according to the method used in our previous study (16), and optimal growth conditions were selected. The bacterial suspension in the exponential growth phase was used in all tests. A standard Hib strain (ATCC 10211) in the exponential growth phase was inoculated in 40 L of modified CY medium supplemented with glucose (6 g/L), yeast extract (2.5 g/L), hemin (0.03 g/L), and NAD (0.015 g/L). PRP concentration was evaluated by ribose assay. In brief, 1.5 mL the bacterial suspension was centrifuged at 6000 × g for 10 min, and the supernatant was mixed with 50 µL of cetavlon (100 g/L) and then precipitated. Then the pellet was re-suspended in NaCl (0.25 M). After dissolving 25 µL of the resulting mixture in 1 mL of deionized water, 100 mL of orcinol solution (100 g/L) and 1 mL of $FeCl_3$ (0.5 g/L) in HCl (12 N) were added, and then the resulting mixture was heated at 100°C for 40 min. The absorbance was measured at 670 nm, and the final ribose concentration was assayed by multiplying the ribose concentration by 2.55. In our previous study, the properties of PRP were evaluated using nuclear magnetic resonance (NMR) and Fourier-transform infrared spectroscopy (FTIR) methods at Faculty of Pharmacy of Tehran University of Medical Sciences (16).

Cell biomass production. Cell biomass was prepared according to the reference procedure. In brief, a standard strain of Hib (ATCC12022) was cultured on a BHI agar plate enriched with hemin (1%) and NAD (0.01%) and incubated at 36.6°C for 24 h. Following incubation time, fresh colonies were inoculated into a flask containing CY medium (5 L) along with NAD (0.02 g/L) and hemin (0.04 g/L) and incubated at 37°C within 24 h. Then this culture was inoculated into an industrial fermenter containing 35 L of CY medium and incubated at 37°C for 12 h. Then 10% phenol was added to the culture to inhibit the fermentation process. In the final step, cell biomass was extracted using centrifugation at 4000 rpm and 4°C for 45 min (17).

LOS extraction. LOS extraction was performed using hot phenol (Westphal and John) method with some modifications. The cell biomass was inoculated into 170 mL of sterile distilled water. Next, 190 mL of hot phenol (90%) (66°C) was transferred to the mixture. The resulting solution was then stirred at 66°C for 14 min, cooled to 0°C, and centrifuged at 8500 g (4°C) within 15 min. To extract LOS, 25% cold ethanol (4°C) was transferred to the liquid phase, and 100% cold ethanol was added to phenol. Then 100 mL of 75% cold ethanol (4°C) was introduced into the aqueous phase supernatant, and then the solution was stored at 4°C for 6 h and centrifuged at 8500 g for 15 min. Finally, the resulting sediment was dissolved in sterile distilled water and centrifuged at 8500 g for 1 h. In the final step, the supernatant containing LOS was saved for further purification. To eliminate potential contaminants including nucleic acid and protein, RNase, DNase, and proteinase K in a final concentration of 40 , 20, and 100 µg/mL, respectively (all material from Roche, Germany) were added to the solution (18). In the final purification step, to precipitate LOS, 1 g of trichloroacetic acid (TCA) was introduced into each 20 mL of the extracted sample, and the mixture was placed at 4°C for a duration time of 3 h. Then the mixture was centrifuged at 2000 g and 4°C for 10 min and dialyzed by deionized water for 72 h to achieve further purification (17). Two standard curves of LOS and protein (Bradford assay) were plotted based on concentration and wavelength to assess LOS and protein concentrations in the extracted sample. Moreover, endotoxin concentration was determined using a LAL (Limulus Amebocyte Lysate) assay kit (Xiamen Bio-endo Technology, China) based on the manufacturer's procedure. The extracted samples were assayed by a microplate reader at 405 nm (19).

LOS detoxification using alkaline method. LOS detoxification was performed using alkaline method. In the first step, an equal volume of NaOH (0.2 M) was mixed with purified LOS and heated at 65°C for 2 h. Then 1 mL of HCl (1 M) was transferred to the solution. Dialysis was done on the resulting mixture against distilled water to eliminate free fatty acids. Next, the mixture pH was set by to 7 using acetic acid (1 M), which was then precipitated by adding ethanol (4 vol). Finally, the product containing dLOS was lyophilized (20).

Animals. All the experiments were performed based on the Guideline for the Care and Use of Laboratory Animals in Iran. Female rabbits aged 6 to 8

weeks (KBL, NZW) were sourced from the Pasteur Institute of Iran. The average weight of rabbits was between 4 and 6 kg, and they were free from specific pathogens. Rabbits were placed in separate cages under normal conditions with a 12-h light-dark cycle at 22°C for 12 h prior to the experiments. Environmental conditions were controlled in terms of temperature, dryness, and darkness to ensure optimal conditions. All animals received suitable food and water. Food and water equipment were continuously cleaned and disinfected to avoid disease transmission (21).

Rabbit immunization and serum sample collection. Rabbits were anesthetized with IM (intramuscular) injection of xylazine (5 mg/kg) and ketamine (35 mg/kg) pre-immunization (22). This study was performed on four treatment groups (n=10), dLOS, PRP, dLOS-PRP, and sterile phosphate-buffered saline (PBS 1X) as a negative control. Rabbit groups were immunized with specific doses selected according to the reference with minor modification (dLOS: 20 µg, PRP: 10 µg, PRP-dLOS: 10 µg+20 µg, and PBS) three times on days 0, 14, and 28 (Table 1) (5, 23). Then blood samples were acquired from rabbits' hearts on days 0, 14, and 28 post-immunization. Blood samples were maintained at 4°C within 60 min, and then centrifuged at 10,000 rpm within 10 min. Serum samples were subsequently isolated and maintained at -20°C for further use. The general condition of rabbits was evaluated for 24 days, including fever, photophobia, stiff neck, vomiting, coma, vomiting, and mortality rate.

Assessment of antibody responses. ELISA (enzyme-linked immunosorbent assay) (Novus kit, USA) technique was employed to evaluate the levels of both IgM and IgG in the sera of rabbit groups using 96-well ELISA microplates. Each well received 100 µL of the sera in 1/10 through 1/10000 dilutions. To determine the best dilution of serum that will react with the antibody. The plates were placed in an incubator at 37°C for 2 h. Then 50 µL of HRP (horseradish peroxidase)-conjugate anti-rabbit IgG (Sigma-Aldrich) was immediately transferred to each well (not to the blank well). The microplates were incubated in at 37°C for 30 min. Following this time, the plates were rinsed four times with 200 µL of wash buffer for 2 min. In the next step, 90 µL of TMB (tetramethylbenzidine) was transferred to each well and maintained in a dark room for 20 min. Finally, the stop solution was added, and the absorbance was read at 450 nm via an ELISA reader (Labsystems, model no. 352). To calculate the antibody titer, the absorbance at 450 nm was multiplied by a dilution factor of 100.

Table 1. Formulation of suspension for each group of rabbits

Groups	Suspension received
PRP	2 mL PBS containing PRP (10 µg)
dLOS	2 mL PBS containing dLOS (20 µg)
PRP-dLOS	2 mL PBS containing PRP-dLOS combine (10 µg + 20 µg)
Negative control	2 mL PBS

Statistical analysis. Results were analyzed via GraphPad Prism 8 using one-way ANOVA. Moreover, p-value of less than 0.05 was considered as significance results. All tests were done in triplicate, and data were shown as the mean of tests ± standard deviation.

Ethical approval. This project was reviewed and approved by Medical Ethics Committee Pasteur Institute of Iran.

RESULTS

Characterization and purification of PRP. The results demonstrated that the highest cell mass and PRP concentrations were 5.8 g/L and 1160 mg/L, respectively. In our previous study, PRP was purified by a similar method and characterized using NMR and FTIR methods. The NMR analysis results revealed various peaks and signals associated with hydrogen and carbon atoms in ribose and hydrogen atoms in ribitol. Our pervious findings have revealed that the purification method successfully preserved the chemical composition and structure of PRP (16).

Evaluation of LOS and protein concentrations in extracted samples. As shown in Fig. 1, the concentration of LOS with OD (optical density) ~ 0.66 was equal to 440 µg/mL according to the standard curve. To determine the sample contamination, a standard curve was plotted according to the Bradford method (Fig. 2). The protein concentration was assayed with a spectrophotometer. The results exhibited that the concentration of protein with OD_{595} ~ 1.8 was equal to 75 µg/mL. The concentration of endotoxin in the extracted sample was 1450 EU/mL.

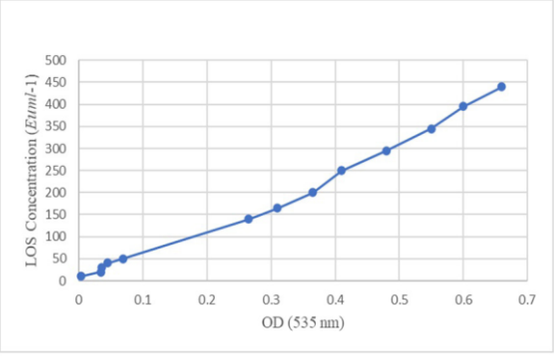

Fig. 1. Standard curve representing OD vs. LOS concentration. LOS concentration in extracted sample was 440 µg/mL.

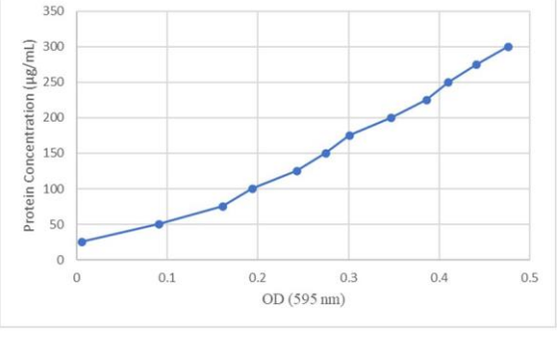

Fig. 2. Standard curve representing OD vs. protein concentration. Protein contamination in extracted sample was 75 µg/mL.

Symptoms and signs of Hib infection. The body temperature of rabbit groups was monitored for 24 days. No signs of fever were detected in any of them. Additionally, no other symptoms were observed.

IgM and IgG responses. ELISA was performed on the serum samples of rabbit groups immunized with dLOS, PRP, PRP-dLOS, and PBS on days 0, 14, and 28 post-immunization. The maximum levels of both IgM and IgG were recorded on day 28 (at a dilution of 1:100). As shown in Figs. 3 and 4, IgM and IgG levels in the serum samples of rabbits immunized with PRP-dLOS combine increased from 16.8 to 29.3 µg/mL and 29.8 to 61.4 µg/mL, respectively from day 14 to 28. The findings indicated that the levels of IgM and IgG in the treated groups with PRP and PRP-dLOS increased twofold from day 14 to day 28.

There was a significant difference in IgM and IgG levels between the PRP and PRP-dLOS groups compared to the PBS group (as a negative control group) on days 14 and 28 ($p< 0.0001$), while no significant difference in the levels of IgM (Fig. 3) and IgG (Fig. 4) was observed between the dLOS and control groups on days 0, 14, and 28. IgM and IgG levels in the group receiving PRP-dLOS combine were higher than in the PRP group on days 14 and 28, while the levels of both antibodies in these groups were significantly higher than in the control group ($p< 0.0001$). Therefore, the highest humoral immune responses including IgM and IgG production were found in rabbits vaccinated with dLOS-PRP combine. dLOS-PRP combine induced a significant protective effect against Hib infection compared to PRP and dLOS.

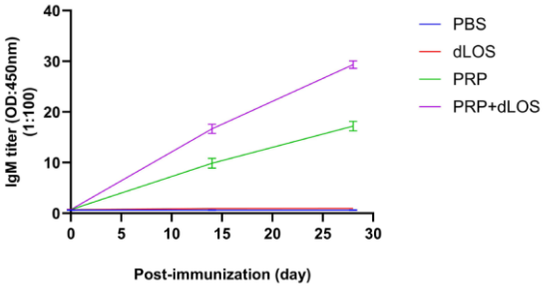

Fig. 3. The total IgM evaluated in sera from the control and experimental groups. Values are presented as mean ± SD based on 10 rabbits in each group.
P < <0.0001 indicate the groups that were significantly different.

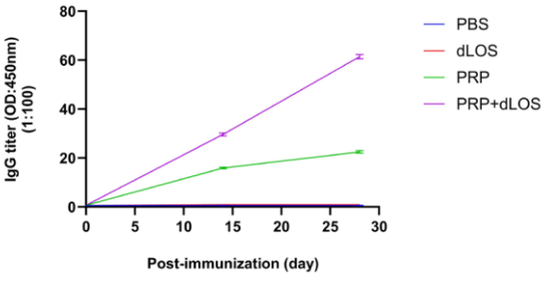

Fig. 4. The total IgG evaluated in sera from the control and experimental groups. Values are presented as mean ± SD based on 10 rabbits in each group.
$p< 0.0001$ indicate the groups that were significantly different.

DISCUSSION

Hib is a significant global health issue in infants and young children, and vaccination is an effective method to combat Hib disease. The Hib vaccine is one of the main health achievements of the past three decades

worldwide (24). The first Hib vaccine was prepared using purified PRP, which stimulated T-independent immune responses. Sufficient immunogenicity and appropriate immune responses were unfortunately not induced by PRP vaccine. Subsequently, PRP was combined with various compounds such as proteins and adjuvants to induce T-dependent immune responses, which ensure long-term immunity and enhance vaccine efficacy (12). Combine vaccines have successfully eradicated invasive diseases in industrialized societies. While challenges remain in vaccination programs, such as lack of booster doses and interference with other vaccines, leading to diminished immune responses. Therefore, the disease remains a health challenge in developing countries, and developing low-cost vaccines against Hib is essential.

This study findings revealed the effectiveness of PRP-dLOS combine in enhancing humoral immune responses. The findings revealed that antibody production against Hib was time-dependent. A significant increase in IgM and IgG levels was observed on day 14, which doubled on day 28 post-immunization. Previous studies have revealed that combine vaccines are more successful than PRP vaccine in inducing immune responses. In a study by He et al. (2023), the mouse groups were immunized with two types of vaccines: PRP alone and PRP combined with aluminum hydroxide adjuvant (PRP-Al). Then humoral and cellular immune responses were assessed, including antibody production and cytokine secretion. The results demonstrated that PRP-Al was more effective than PRP in stimulating both humoral and cellular immune responses (25). These data are consistent with the present study findings, which demonstrated that PRP-dLOS combine significantly increased specific antibody production compared to PRP. These findings show the importance of an adjuvant in the Hib vaccine formulation to induce antibody-mediated immune responses.

On the other hand, studies have shown that administration of multiple boosters with an appropriate schedule could enhance the effectiveness of combine vaccines. Perrett et al. (2014) demonstrated that specific levels of IgG were lower in children receiving three doses of combine vaccine compared to those receiving four doses. Therefore, the booster dose has an crucial role in the stability of protective antibodies (26). In a research, after immunization of children with combine vaccines, humoral immune responses were evaluated. It was manifested that immune responses to the combine vaccines were delayed in children aged 6 to 11 months, although this delay was not observed in children older than 11 months. Delayed immune responses to combine vaccines appear to be due to lack of booster doses, interference with other vaccines, and combinations with improper carriers (14).

The findings show that dLOS is a suitable adjuvant to enhance antibody-mediated immune responses with only three doses. Moreover, a booster dose may be able to enhance the effectiveness of PRP-dLOS combine. The significant production of specific antibodies against PRP-dLOS combine is attributed to three main factors, including the use of dLOS as an adjuvant in the formulation of PRP-dLOS combine, the use of optimal concentrations of PRP and dLOS in immunization, and a suitable vaccination schedule. This study evaluated the effect of PRP-dLOS combine on humoral immunity, while its effect on other immune responses is unknown. It is suggested to evaluate its effect on cellular immune system in future studies. This research has several limitations: 1) due to financial restrictions, we were not able to examine the effect of PRP-dLOS combine on other immune responses, 2) LOS application is significantly limited due to its endotoxic effects, and we used dLOS.

CONCLUSION

In conclusion, dLOS could be considered as a suitable adjuvant in the formulation of PRP-dLOS combine, considering the induction of specific antibody production against Hib only after the administration of three doses. Therefore, it could be used as a new strategy to combat Hib without fear of delay in immune responses and interference with other vaccines.

ACKNOWLEDGEMENTS

We wish to thank the Research Council of Pasteur Institute of Iran, Teheran.

REFERENCES

1. Kostyanev TS, Sechanova LP. Virulence factors and mechanisms of antibiotic resistance of *Haemophilus*

influenzae. *Folia Med (Plovdiv)* 2012; 54: 19-23.
2. Park JJ, Narayanan S, Tiefenbach J, Lukšić I, Ale BM, Adeloye D, et al. Estimating the global and regional burden of meningitis in children caused by *Haemophilus influenzae* type b: A systematic review and meta-analysis. *J Glob Health* 2022; 12: 04014.
3. Harrison A, Mason KM (2015). Pathogenesis of *Haemophilus influenzae* in humans. in: Human Emerging and Re-emerging Infections: Viral and Parasitic Infections, Volume 1. pp. 517-533.
4. Tchoupa AK, Lichtenegger S, Reidl J, Hauck CR. Outer membrane protein P1 is the CEACAM-binding adhesin of *Haemophilus influenzae*. *Mol Microbiol* 2015; 98: 440-455.
5. Choi J, Cox AD, Li J, McCready W, Ulanova M. Activation of innate immune responses by *Haemophilus influenzae* lipooligosaccharide. *Clin Vaccine Immunol* 2014; 21: 769-776.
6. Zariri A, van der Ley P. Biosynthetically engineered lipopolysaccharide as vaccine adjuvant. *Expert Rev Vaccines* 2015; 14: 861-876.
7. Gao S, Jin W, Quan Y, Li Y, Shen Y, Yuan S, et al. Bacterial capsules: Occurrence, mechanism, and function. *NPJ Biofilms Microbiomes* 2024; 10: 21.
8. Khattak ZE, Anjum F (2023). *Haemophilus influenzae* Infection. In: StatPearls. Treasure Island (FL): StatPearls Publishing.
9. Hallström T, Riesbeck K. *Haemophilus influenzae* and the complement system. *Trends Microbiol* 2010; 18: 258-265.
10. Rodriguez RS, Mascarenas C, Conde-Glez CJ, Inostroza J, Villanueva S, Velázquez ME, et al. Serological protection induced by *Haemophilus influenzae* type B conjugate vaccine in Mexican children: is a booster dose of the vaccine needed? *Clin Vaccine Immunol* 2010; 17: 1639-1641.
11. Kim KH, Lee H, Chung EH, Kang JH, Kim JH, Kim JS, et al. Immunogenicity and safety of two different *Haemophilus influenzae* type b conjugate vaccines in Korean infants. *J Korean Med Sci* 2008; 23: 929-936.
12. Gilsdorf JR. Hib vaccines: their impact on *Haemophilus influenzae* type b disease. *J Infect Dis* 2021; 224(12 Suppl 2): S321-S330.
13. Khatuntseva EA, Nifantiev NE. Glycoconjugate vaccines for prevention of *Haemophilus influenzae* type b diseases. *Russ J Bioorg Chem* 2021; 47: 26-52.
14. Schouls L, Schot C, de Voer RM, van der Klis F, Knol M, Tcherniaeva I, et al. Lagging immune response to *Haemophilus influenzae* serotype b (Hib) conjugate vaccine after the primary vaccination with Hib of Infants in The Netherlands. *Vaccines (Basel)* 2020; 8: 347.
15. Potts CC, Rodriguez-Rivera LD, Retchless AC, Buono SA, Chen AT, Marjuki H, et al. Antimicrobial susceptibility survey of invasive *Haemophilus influenzae* in the United States in 2016. *Microbiol Spectr* 2022; 10(3): e0257921.
16. Arsang A, Tabatabaie A, Vaziri F, Nejati M, Zolfaghari MR, Fateh A, et al. Optimization of large scale production of *Haemophilus influenzae* type b polyribosyl-ribitol phosphate. *Minerva Biotecnol* 2017; 29: 17-23.
17. Arsang A, Yari S, Masoumi M, Neamatollahi AN, Vaziri F, Nejati M, et al. Extraction and purification of haemophilus influenzae type b lipooligosaccharide by Modified Phenol method. *Vac Res* 2014; 1: 28-30.
18. Rezania S, Amirmozaffari N, Tabarraei B, Jeddi-Tehrani M, Zarei O, Alizadeh R, et al. Extraction, purification and characterization of lipopolysaccharide from *Escherichia coli* and *Salmonella typhi*. *Avicenna J Med Biotechnol* 2011; 3: 3-9.
19. Long J, Zhao X, Liang F, Zeng Y, Liu N, Sun Y, et al. An innovative lab-scale production for a novel therapeutic DNA vaccine candidate against rheumatoid arthritis. *J Biol Eng* 2024; 18: 19.
20. Cross AS, Opal SM, Palardy JE, Drabick JJ, Warren HS, Huber C, et al. Phase I study of detoxified *Escherichia coli* J5 lipopolysaccharide (J5dLPS)/group B meningococcal outer membrane protein (OMP) complex vaccine in human subjects. *Vaccine* 2003; 21: 4576-4587.
21. Ahmadi-Noorbakhsh S, Mirabzadeh Ardakani E, Sadighi J, Aldavood SJ, Farajli Abbasi M, Farzad-Mohajeri S, et al. Guideline for the care and use of laboratory animals in Iran. *Lab Anim (NY)* 2021; 50: 303-305.
22. Hassan MA, Abd Elkawi M, Saber M, Senosy W, Nassif M, ElSherif M. Evaluation of xylazine ketamine anesthesia in rabbits undergoing tendon surgery: A prospective randomized controlled study. *New Valley Vet J* 2024; 4: 10.21608/nvvj.2023.247402.1038.
23. Hutter J, Pasetti MF, Sanogo D, Tapia MD, Sow SO, Levine MM. Naturally acquired and conjugate vaccine-induced antibody to *Haemophilus influenzae* type b (Hib) polysaccharide in Malian children: serological assessment of the Hib immunization program in Mali. *Am J Trop Med Hyg* 2012; 86: 1026-1031.
24. Howie SR. Global Hib vaccination: reasons to cheer and fear. *Lancet Glob Health* 2016; 4(3): e142-e143.
25. He Y, Zhao Y, Liang H, Wang X, Lan H, Tian D, et al. Systematic evaluation of the distribution of immune cells following subcutaneous administration of *Haemophilus influenzae* type B vaccine to mice. *Diseases* 2023; 11: 139.
26. Perrett KP, John TM, Jin C, Kibwana E, Yu L-M, Curtis N, et al. Long-term persistence of immunity and B-cell memory following *Haemophilus influenzae* type B conjugate vaccination in early childhood and response to booster. *Clin Infect Dis* 2014; 58: 949-959.