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Original Article

In Silico Studies of Outer Membrane of *Neisseria Meningitidis* Por A: Its Expression and Immunogenic Properties

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Neisseria meningitidis is a major causative agent of bacterial septicemia and meningitis in humans. Currently, there are no vaccines to prevent disease caused by strains of *N. meningitidis* serogroup B. The Class 1 Outer Membrane Protein (OMP) has been named *porA* which is a cation selective transmembrane protein of 45 KDa that forms trimeric pore in the meningococcal outer membrane. PorA from serogroup B *N. meningitidis* was cloned into prokaryotic expression vector pBAD-gIIIA. Recombinant protein was expressed with arabinose and affinity purified by Ni-NTA agarose, SDS-PAGE and western blotting were performed for protein determination and verification. BALB/c mice were immunized subcutaneously with purified rPorA together with alum adjuvant. Serum antibody responses to serogroups B *N. meningitidis* were determined by ELISA. Serum IgG response significantly increased in the group immunized with rPorA together with alum adjuvant in comparison with control groups. These results suggest that rPorA can be a potential vaccine candidate for serogroup B *N. meningitidis.*

Key words: *Neisseria meningitidis* , *por A*, vaccine

N. meningitidis is not only a common bacterial commensal of the human upper respiratory tract (nasopharynx) but also an important and devastating human pathogen (1–2). Meningococci are gram-negative diplococci, can be encapsulated or unencapsulated, and are the worldwide cause of epidemic meningitis and rapidly progressing fatal shock. Meningococcal strains are classified into 13 serogroups based on the chemical composition of the polysaccharide capsule (3). Serogroups A, B, C, Y and W-135 are associated with diseases worldwide (4). Nearly one-half of all cases of meningococcal disease in the United States are caused by capsular group B strains, for which there is no widely effective vaccine (5). A quadrivalent group A, C, W-135 and Y polysaccharide-protein conjugate vaccine was introduced in the United States and is recommended for routine use. Control of meningococcal disease, however, will not be achieved until a widely effective vaccine is available against group B strains (6).

Meningococci contain four classes of major membrane proteins (OMPs), i.e, class 1, 2/3, 4 and 5 proteins. The class 5 OMPs appear to be

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unattractive vaccine candidates, since they exhibit extreme antigenic heterogeneity (7). The function of the class1 OMP is unknown. The structural gene for this protein has recently been cloned and sequenced (8), and it appears that the protein is structurally related to the gonococcal porins and to class 2 OMP (9). Therefore, in addition class 1 OMP may have a pore function. The class 1 OMP has been named PorA and the corresponding gene has been designated *porA* (10).

Monoclonal antibodies have been raised against the class 1 and class 2/3 OMPs; in both cases, antibodies with bactericidal activity have been obtained. Thus making this protein the prime vaccine candidate for meningococcus group B (11). The two PorA variable regions (VR1 and VR2) that confer the subtypes are especially important because they elicit bactericidal antibodies in humans (12). Consequently, a number of meningococcal vaccines under development contain the PorA protein as a major component (13). In humans, the protein elicits a more protective immune response than any other meningococcal surface protein (14). Much of this evidence comes from the clinical evaluation of OMV vaccines in which PorA is the immuno-dominant antigen (15). The amino acid sequence of PorA does not vary within an isolate and its expression in a heterologous strain induces a full bactericidal response against the donor serosubtype in the recipient strain. Considering together, these results make the PorA protein an attractive vaccine candidate (16).

Therefore in the present study, we investigated serum antibody responses elicited in mice after immunization with PorA from a *N. meningitidis* expressed in *E. coli*.

Materials and Methods

Bacterial strains and pBAD gIIIA strain

N. meningitidis CSBPI, G-245 was obtained from the Pasteur Institute of Iran and was grown on chocolate agar. *Escherichia coli* Top10 and pBAD-gIIIA vector were obtained from Invitrogen. The pBAD-gIII plasmids are pBR322-derived expression vectors designed for the regulated, secreted recombinant protein expression and purification in *E. coli*. The *E. coli* cells harboring recombinant plasmids were grown aerobically at 37° C in Luria-Bertani broth with 50 µg/ ml ampicillin.

In silico analysis of protein

The following sites were used in this study:
http://www.expasy.org/tools/protparam.html.
http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html.
http://web.expasy.org/protscale.
http://www.cbs.dtu.dk/services/YinOYang.

DNA isolation

Plasmid DNA was prepared by using a Qiagen plasmid DNA kit (Qiagen GmbH, Dusseldorf, Germany) according to the manufacturer's instructions. Genomic DNA from *N. meningitidis* was prepared using a genomic DNA extraction kit.

Amplification of *porA*

The *porA* gene was amplified from *N. meningitidis* chromosomal DNA by the polymerase chain reaction (PCR) with a PCR kit from Fermentas in accordance with the manufacturer's instruction. The primers were designed on the basis of the published nucleotide sequence of *porA* from *N. meningitidis*.

The sequences of the primers were as follows

Forward: 5' CCGCTCGAGATGCGAAAA AAACTTACC 3'. Reverse: 5' GCATCTAGAGTG AATTTGTGGCGCAAAC 3'. Amplification of the DNA was achieved by using Prime STAR DNA polymerase: a 25 µl reaction mixture contained: 0.5 pM of each primer, 5 µl 5× prime STAR buffer, 0.2 mM concentration of each dNTP, 2.5 U of prime STAR DNA polymerase and 100 ng genomic DNA. Amplification was performed using 35 cycles of denaturation at 94 ^{o}C for 10 sec, annealing at 59 ^{o}C for 15 sec, extension at 72 ^{o}C for 90 s and 10 min at

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72 °C for final extension. The PCR product was recovered from the gel and purified using the PCR purification kit.

Cloning and sequence analysis

The PCR product was digested with XhoI and XbaI and ligated into pBAD digested with the same enzymes which provided six His residues at the N-terminus of the expressed protein. Ligation was performed with T4 DNA ligase. The ligation mixture was transformed into competent *E. coli* Top10 cells. Plasmid DNA was prepared from positive colonies by using a Quiagen plasmid DNA kit. Restriction endonuclease analysis and DNA sequencing of plasmids containing the *porA* gene were performed for further confirmation.

Protein expression from *porA* g

Since each recombinant protein has different characteristics that may affect optimum expression, it is helpful to vary the L-arabinose concentration to determine the best condition for optimal expression of a particular protein. For expression, the clones were grown in LB medium supplemented with ampicillin at 37 °C with shaking (250 rpm) to an A650 of 0.5 and then, arabinose was added to a final concentration of 2%, 0.2% and 0.02% and incubation was continued for 4 hours.

Purification of recombinant PorA

A pellet of TOP10 cells harboring rPorA was suspended in PBS Buffer 1X. (10 mM Na2HPO4, 12H2O, 2 mM KH2PO4, 2.7 mM KCl, 137 mM NaCl adjusted to pH 7.5). The suspension was subjected to sonication (15 cycles, 15 seconds each, with intervals of 15 seconds on ice) and then centrifuged for 15 minutes at 6000×g. After centrifugation, the supernatant and precipitate were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to verify the expressed recombinant protein. The crude rPorA was purified by affinity chromatography on a nickel-nitrilotriacetic acid (Ni-NTA) gel matrix (Qiagen, Crawley, United Kingdom) under denaturing conditions. A column containing 1.5 ml of Ni-NTA resin was equilibrated with 10 volumes of buffer containing 8 M urea, 0.1 M NaH2PO4, and 0.01 M Tris (pH 8.0), and the cleared cell lysate was loaded onto the column. The column was washed with 5 volumes of wash buffer (8 M urea, 0.1 M NaH2PO4, 0.01 M Tris (pH 8.0), followed by 5 volumes of the same buffer, but at pH 6.3. The rPorA protein was then eluted by elution buffer (8 M urea, 0.1 M NaH2PO4, 0.01 M Tris (pH 4.5). Fractions containing the recombinant protein were pooled and dialyzed against PBS, pH 7.4 to remove urea. Protein concentrations were determined by nanodrop analyzer (Biorad, Hercules, CA, USA) and bradford protein assay with bovine serum albumin as standard.

SDS-PAGE and western blotting

After purification, the expressed protein was characterized by 15% SDS PAGE. For western blotting, the proteins separated by SDS-PAGE were blotted onto polyvinylidene difluoride (PVDF) membrane (Hi-bond Amersham Biosciences, USA) by using a wet transfer unit. The membrane was blocked by 5% (w/v) nonfat milk in PBS according to standard procedures. Membranes were then incubated with peroxidase- conjugated rabbit anti mouse immunoglobulins at a dilution of 1/2000.

Mouse immunization

Groups of seven weeks-old BALB/c female mice were immunized subcutaneously with 50 µg of purified rPorA and immunized with 50 µg rPorA mixed with alum adjuvant for three doses at weeks 0, 2 and 4. Control groups consisted of mice receiving PBS alone. Sera were collected at weeks 0, 2, 4 and 6 to determine the antibody response. Aliquots of serum were stored at-20 °C for further use.

Determination of serum IgG and its subclasses

The IgG antibody responses to *N. meningitidis* serogroup B whole cells were determined by whole-cell ELISA as previously described on pooled sera collected two weeks after the last immunization. The IgG, IgG1, IgG2a and IgG2b antibody

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responses against purified rPorA were determined by ELISA. Flat-bottom, 96-well microtiter plates were coated overnight at 37 ^{o}C with 5 µg/ 100µl of purified rPorA in PBS. The coated plates were first blocked with 5% (w/v) bovine serum albumin (BSA) in PBS and then incubated with antisera (1:10, 1:100 and 1:1000 diluted in PBS/ BSA) for 2 h at 37 ^{o}C. Plates were washed three times with PBS and goat anti-mouse immunoglobulin G ,G1, G2a ,G2b –peroxidase conjugate diluted 1:6000 in PBS/ BSA were added. Plates were incubated for 1 h at 37 ^{o}C and washed three times in PBS, then ABTS Elisa HRP Substrate (KPL Company) was added to each well. Reaction was stopped after 15 min by adding 1% SDS and the absorbance was measured at 405 nm. All assays were performed in triplicate.

Statistical analyses

The statistical analyses were performed by Graphpad Prism 5, following normalization of the data. One-way ANOVA and t-tests were used to check for differences between data sets. P values (≤0.05) were considered statistically significant.

Results

Physical and chemical properties of PorA

The physical and chemical properties of PorA of *N. meningitidis* were analyzed utilizing online service (http://www.expasy.org/tools/protparam.html). The results indicated a molecular weight of 41885.7Da, a theoretical pI of 9.06, a formula corresponding to $C_{1860}H_{2897}N_{529}O_{572}S_2$ and a total number of atoms equal to 5860. The estimated half-life was >10 hours in *Escherichia coli, in vivo*. The instability index (II) was computed to be 30.75; this classifies the protein as stable. Aliphatic index was 76.49 and the grand average of hydropathicity (GRAVY) was - 0.402.

Secondary structure of PorA

The secondary structure of PorA was predicted utilizing online service (http://npsa-pbil.ibcp.fr/ . cgi-bin/npsa_automat.pl? page = / NPSA /npsa_sopm-a.html). The results showed that PorA consisted of 19.43% alpha helix, 25.13% extended strand, 51.81% random coil and 3.63% beta turn (Figure 1).

Hydrophilicity prediction of PorA

Hydrophilicity of PorA was predicted utilizing online service (http://web.expasy.org/protscale/). The results showed that the site of Glu is the most hydrophilic (Score:-3.500) and Phe the most hydrophobic (Score: 2.800) (Figure 2). We came to the conclusion that the PorA is a hydrophilic protein.

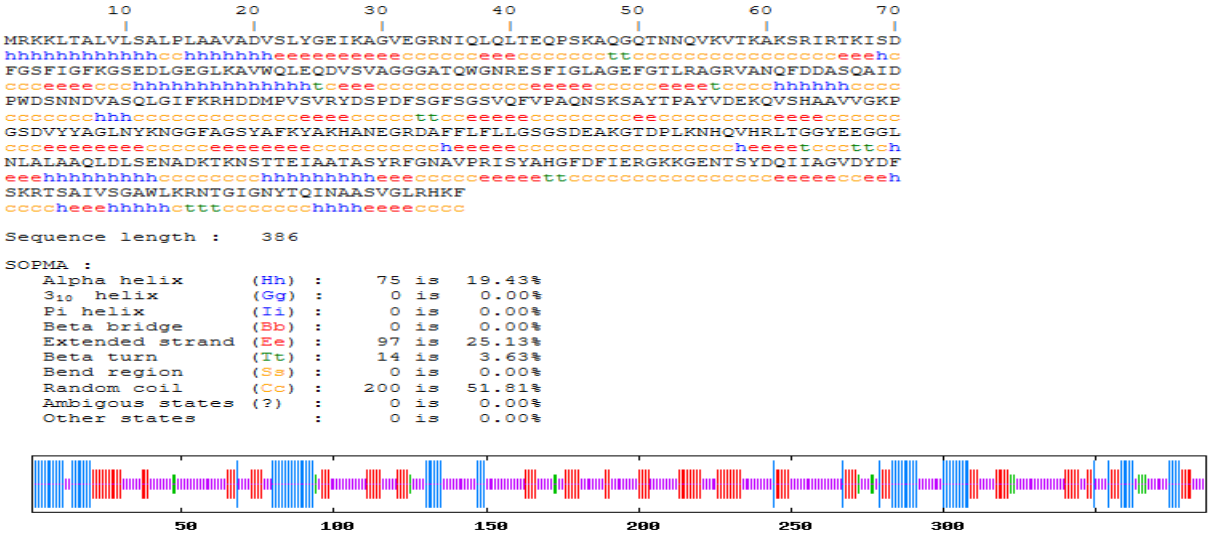

Fig. 1. The secondary structure of PorA.

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Amplification of *porA* and construction of pBAD *-porA*

Specific primers were designed to amplify *porA* from the *N. meningitidis* stain. The expected size of the *porA* PCR product, approximately 1167 bp, is shown in Figure 3. Double digested DNA isolated from *N. meningitidis* strain was ligated into digested pBAD. *E. coli* Top10 cells, transformed with the ligation mixture, were selected for resistance to ampicillin. Individual colonies were screened with colony PCR to determine positive clones. Restriction enzyme analysis of plasmid DNA from the positive clones showed that they carried the insert (Figure 4).

Analysis of the nucleotide sequence of *porA* gene

Identity and orientation of *porA* in the construct was confirmed by sequencing which revealed that the length of fragment was 1167 bp. Sequence comparison with the published sequence of *porA* gene in the Genbank showed that the nucleotide sequences of the cloned *porA* gene was 99% identical to the *porA* sequence published in the Genbank AF226339.

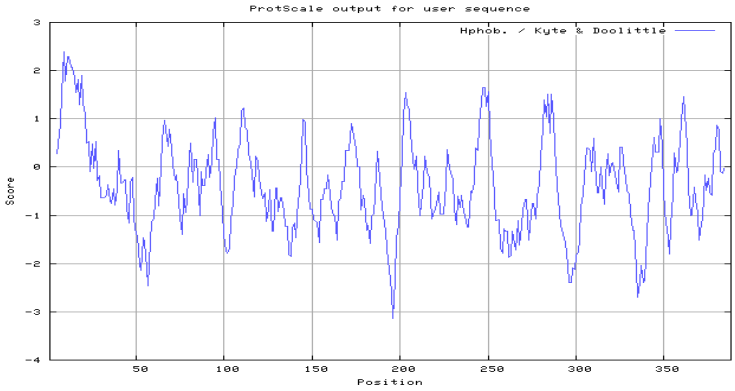

Fig. 2. Hydrophilicity profile of PorA.

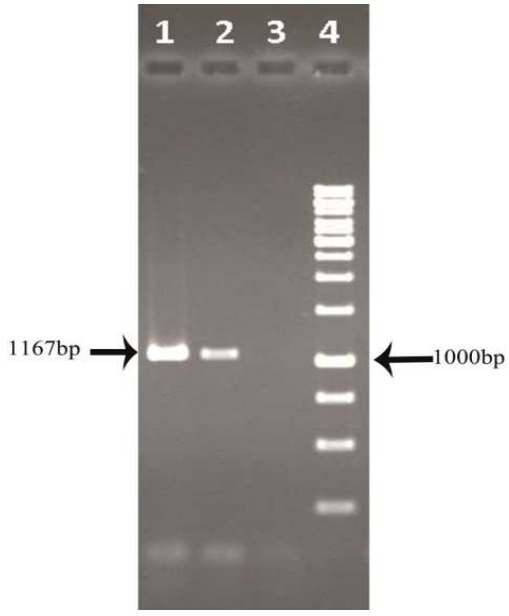

Fig. 3. Electrophoresis of PorA PCR product on agarose gel (1%).Lane1, 2: Single expected band of *porA* gene (1167 bp), Lane 4: 1kb DNA size marker.

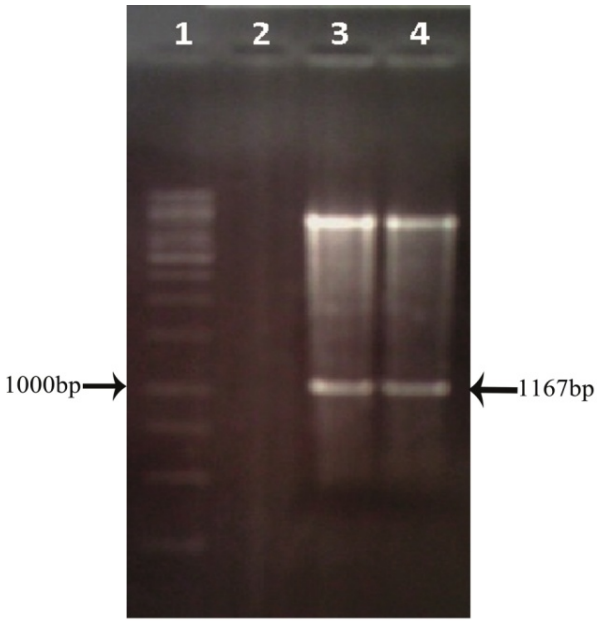

Fig. 4. Electrophoresis of recombinant p-BAD-PorA on agarose gel (1%). Lane 1: 1 kb DNA size marker, lane3,4: Double digestion of recombinant pBAD-PorA with *XhoI* and *XbaI* restriction enzymes (pBAD: 4145bp and *porA:* 1167bp).

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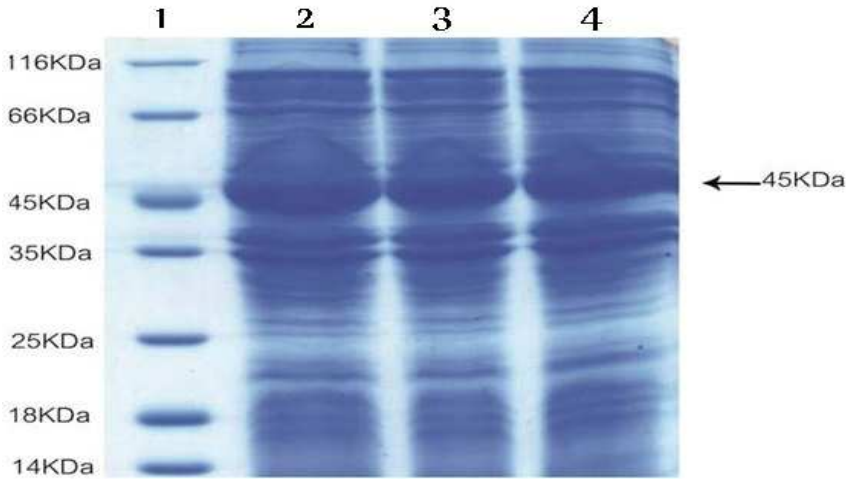

Fig. 5. SDS-PAGE analysis of recombinant PorA with Coomassie-stained. Expression of rPorA in Top10 cells induced with 2,0.2 and 0.02 Arabinose. Lane 1: Protein marker, Lane 2-4 Induced with arabinose (45 kDa).

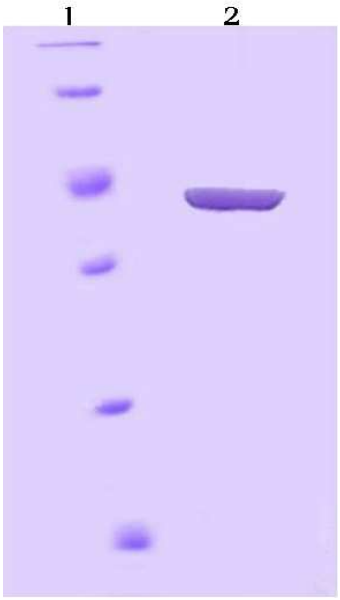

Fig. 6. SDS-PAGE analysis of purified recombinant PorA with Coomassie-stained. Lane1: protein marker, lane 2: recombinant PorA with molecular weight of 45 kDa that purified with Ni-NTA affinity chromatography.

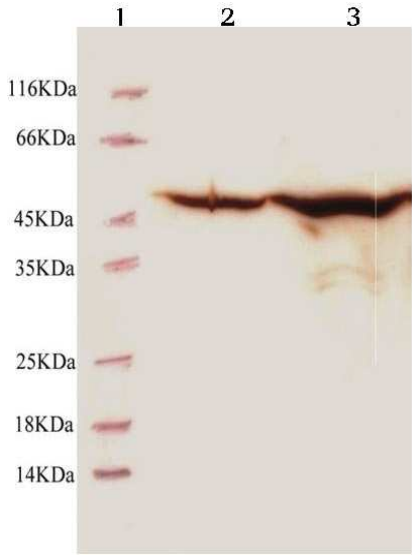

Fig. 7. Western blot analysis of recombinant PorA with Peroxidase-Conjugated rabbit anti mouse immunoglobulins(anti-His). Lane1: protein marker, lane 2,3: recombinant PorA with molecular weight of 45 kDa that purified with Ni-NTA affinity chromatography.

Expression and purification of rPorA

The Top10 cells harboring pBAD-*porA* plasmid were cultured at 37°C in the presence and absence of an inducer. Induction of the cells with arabinose (2 %) at 37°C for 4 hours was found to be optimal to achieve high-level expression of rPorA (Figure 5). The whole-cell lysates were analyzed by 15% SDS-PAGE. A major band appeared approximately at the 45 kDa position after arabinose induction. Recombinant protein was carefully purified with Ni-NTA affinity chromatography under denaturing conditions (Figure 6).

Western blot analysis

Western blot analysis was performed to confirm the expressed protein, the 45 kDa protein observed in SDS-PAGE, was confirmed as rPorA protein by western blot analysis using Anti-6XHis tag (Figure 7).

Serum responses after immunization with rPorA

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BALB/c mice immunized with recombinant protein mixed with Alum exhibited high level of specific antibody responses. Serum IgG responses were significantly increased in groups immunized with PorA in comparison with control group. The booster doses were effective to significantly increase the responses of anti- PorA IgG. Antisera produced against rPorA demonstrated strong surface reactivity to serogroups B *N. meningitidis* tested by whole- cell ELISA.

The IgG antibody response against rPorA was determined by ELISA of individual serum samples collected at weeks 2, 4 and 6. The bars represent the geometric mean of obtained ELISA signals for optimum dilution (1.1000). Optimum dilution was determined prior to the comparison by testing serially diluted sera against the coated antigen. Subcutaneous immunization with rPorA alone induced increased level of specific IgG titre, but the group immunized with rPorA together with Alum showed a higher antibody response than control groups ($P \leq 0.001$). This combination induced 1.9 fold higher IgG titre following the first injection than rPorA alone. The response to the second booster was statistically different from the first one ($P \leq 0.008$). All assays were performed in triplicate (Figure 8).

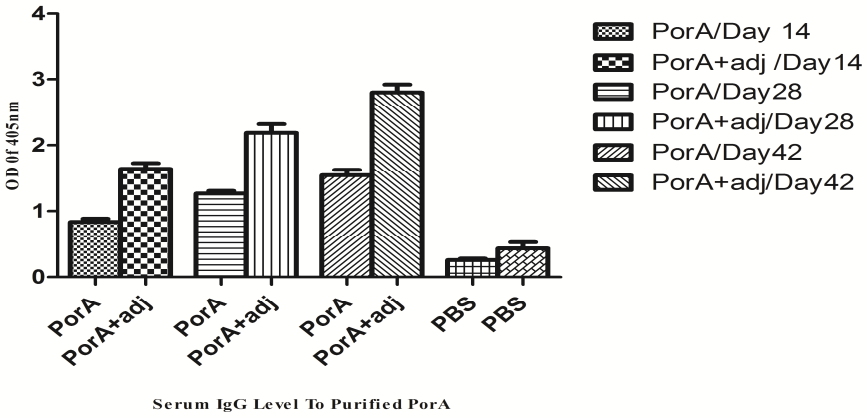

Fig. 8. Serum IgG Level to purified rPorA alone and with adjuvant.

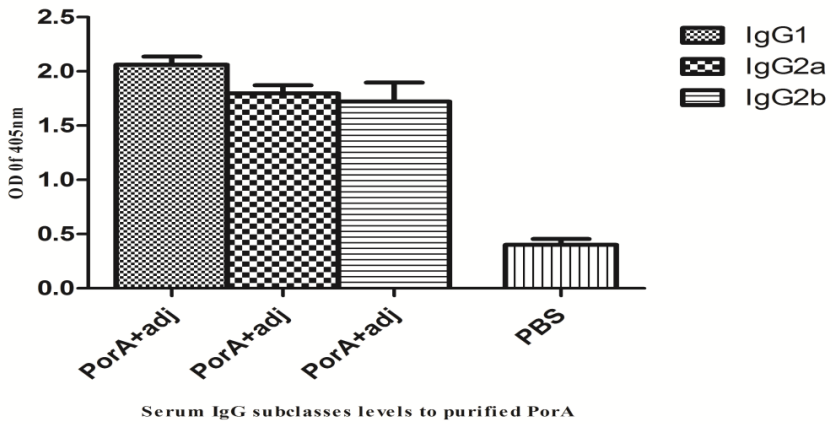

Fig. 9. Serum IgG subclasses levels to purified rPorA with adjuvant.

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The IgG subclasses antibody responses against *N.meningitidis* serogroup B were determined by ELISA on sera collected at week 6. The bars represent the geometric mean of obtained ELISA signals for optimum dilution (1/1000). Evaluation of subclasses showed that specific response of IgG1 was relatively higher than IgG2a and IgG2b (Figure 9). Infected mice developed increased IgG1 responsiveness to antigen, whereas IgG2a responses were lower. This confirms that Th2 responses are dominant in infected mice. In comparison, vaccinated mice developed lower levels of IgG1 and higher levels of IgG2a.

Discussion

Neisseria meningitidis is a major case of bacterial meningitis and septicemia worldwide. In the absence of a comprehensive vaccine against this organism, the characterization of its variable surface antigen is important for epidemiologic monitoring and vaccine development (17).

PorA is a cation selective transmembrane protein of 44 kDa that forms trimeric pores in the meningococcal outer membrane. The model predicts eight extended extracellular loops. Two of these surface exposed loops (loops 1 and 4) are highly immunogenic and evoke antibodies that induce complement mediated bacterial killing (18).

Bioinformatic is a rapidly developing field of science which uses the advantage of computer technology to analyze the molecular biology and helps us to obtain information concerning the identity and properties of the molecule under study.

The capacity of meningococcal PorA to evoke antibodies that induce complement-mediated bacterial killing has been incited to use this protein as a target in vaccine development. In clinical vaccination trials with outer membrane vesicles, it has been shown that the presence of antibodies correlates with protection against meningococcal disease (18-19).

Vander et al. showed that the strains devoid of PorA poorly induced bactericidal antibodies in mice. The transfer of one *porA* gene and its expression in a heterologous strain induced a full bactericidal response in the recipient strains (18). Considered together, these results make the PorA protein an attractive vaccine candidate.

Previous studies showed that PorA is immunogenic during infection and therefore a target for bactericidal antibodies, following immunization with experimental OMVs (20, 21). PorA protein is highly immunogenic, and specific antibodies induced by OMV vaccines are generally serosubtype specific and exhibit both bactericidal and opsonic functions as well (22-26). Single or multivalent vaccines with different composition of PorA epitopes have been developed and used in clinical trials (26).

The two PorA variable regions (VR1 andVR2) that confer the subtypes are especially important because they elicit bactericidal antibodies in humans (27). Consequently, a number of meningococcal vaccines under development contain the PorA protein as a major component (13).

Evaluation of subclasses antibody responses showed that specific response of IgG1 was higher than IgG2a. Therefore, it can be concluded that rPorA antigen induced exclusively IgG1 antibody, which is involved in a predominant Th2 response generation. Therefore, the capacity of rPorA to induce antibodies, together with its relatively conserved sequence makes it a potential candidate for vaccine against serogroup B meningococci.

Serogroup B outer membrane vesicles (OMV) with iron regulated proteins (IRP) from *Neisseria meningitidis* constitute the antigen for the vaccine against the disease caused by this bacterium and recombinant OMV- formulations with various PorA antigens have been developed in some studies (28). In a previous study, it was shown that polysaccharide from serogroup C (PSC) conjugated with OMV can be used as a serogroup B/C bivalent antigen (29). In an effort to establish efficient

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immunization in infants, a serogroup B/C vaccine consisting of PSC conjugated to OMV from one serogroup B serosubtype prevalent in Brazil, combined with OMV from another prevalent serosubtype, using adjuvants is in clinical trial for human use.

Nowadays, adjuvants are extensively used as immuno- stimulator and immuno-modulator compounds to design subunit vaccines. The adjuvants of microbial origins are more considered among the other currently used adjuvants. The outer membrane vesicle (OMV) of *Neisseria meningitidis* is among the newly studied components with microbial origin, which could be applied as an adjuvant. The potency of OMV as a carrier (conjugated to a hapten) is now proven (30). The adjuvant properties of OMV- derived particles have been demonstrated for potential cancer vaccines. Overall, previous studies showed that the predominant outer membrane proteins (OMPs) (PorA, PorB and rmpM) from *N. meningitidis* present in the Meningococci B Cuban vaccine had different capacities to prime the immune system (31).

As mentioned in our results, IgG titres exponentially and significantly increased in the immunized group compared to the control group. Thus, the immunogenicity of PorA and the significant difference ($p< 0.001$) of these titres could make rPorA a potential adjuvant.

Conflict of interest

The authors declared no conflicts of interest.

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