

Porin protein could be a vaccine candidate against *Riemerella anatipestifer* for ducks

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Abstract

such as domestic ducks and geese. Young birds have a high mortality rate after infection. The resistance caused by the abuse of antibiotics is also getting worse. Since there are 25 serotypes of *RA*, and the cross-immunization between serotypes is weak, it is necessary to find a vaccine that has cross-immunization against multiple serotypes of *RA*. In this article, the bioinformatics analysis of *RA* Porin protein was conducted, and it was speculated that it has the potential of a subunit vaccine. The protein was recombinantly expressed and purified, and immunized with Cherry Valley Duck. The results show that the serum antibodies of the Porin protein immunized group were positive at 1:24300 for the porin protein and *RA* strains CH1. The serum could improve the killing effect of complement and phagocytic cells on *RA*. After the challenge, the survival rate of Cherry Valley Duck can be increased by 80%.

Key Points

- There are 21 serotypes of *RA*, but the cross immunoprotective effect between each serotype is extremely weak. The development of subunit vaccines is a powerful way to prevent the infection of multiple serotypes of *RA*.
- Through bioinformatics analysis, it was found that Porin protein is conserved in *RA* and also has abundant epitopes. It is speculated that this protein has the potential to develop a subunit vaccine.
- The recombinant Porin protein was used to immunize Cherry Valley Duck. The serum produced had a cross-immunization effect on serum type 1 and serum type 3 *RA*, and enhanced the clearance of pathogens by complement and phagocytes. After challenge, the survival rate of Cherry Valley was improved.

Introduction

RA is a contact infectious bacterium that infects domestic ducks, geese(Chen et al.; Shu-Chuan et al., 1999), turkeys(Rubbenstroth et al.) and a variety of birds(Hinz et al., 1998). Its infection symptoms are fibrinous pericarditis, fibrinous perihepatitis, fibrinous air sacs, encephalitis, Arthritis, etc. The disease was first reported in Long Island, New York, USA in 1932, and subsequently in Europe, South Asia, and Oceania. At present, the occurrence and prevalence of this disease is extremely extensive, and the disease is prevalent in almost all places where ducks are raised. All kinds of ducks in poultry can be infected. Under natural conditions, ducks of all ages are susceptible, and the incidence rate is about 90%(Zhu et al., 2017).

Drug treatment is one of the important measures to prevent and treat the disease, mainly antibiotics and horizontal amines. The disease can occur in the whole stage of duck breeding, and birds can be infected repeatedly, resulting in higher cost of medication. Clinically, drug abuse, poor therapeutic effects, and even treatment failures often occur. Although *RA* is sensitive to many antibiotics, it is highly susceptible to drug resistance(Sun et al., 2019; Xing et al., 2015). Different regions have different susceptibility to antibacterial drugs in different regions, which brings difficulties to the actual treatment work. Therefore,

immunization is an effective way to prevent and control this disease. The vaccines currently used to prevent this disease mainly include inactivated vaccines, attenuated vaccines, and subunit vaccines. Due to the complexity of *RA* serotypes and the lack of cross-protection between different serotypes, both attenuated and inactivated vaccines are effective against specific serotypes but are not protective against other serotypes of *RA*.

Gram-negative outer membrane proteins (Omps) not only play an important role in maintaining the integrity of the cell envelope and transmembrane transport of the substances, but also in the the adhesion of bacteria to invading cells and bacteria to serum resistance. Outer membrane proteins can be divided into major proteins and microproteins according to their copy number in cells. Among them, Porin protein, also known as channel-forming protein, has a transmembrane domain with only 10 to 12 amino acid residues, forming a β -sheet structure, and antiparallel β -sheets interact to form a non-specific transmembrane. The channel allows small molecules with a relative molecular mass of less than 10,000 D to pass freely and is associated with bacterial resistance. Studies have shown that porin protein has good immunoprotective effects against strains such as *E. coli*(Fox et al., 2009), *Salmonella*(Dodd et al., 2011) and *Pseudomonas aeruginosa*(Bahey-El-Din et al., 2020). According to the genome sequencing results of *RA* (Wang, et al., 2012), it was found that porin was also present in *RA*. At present, the research of *RA* porin is still in the prediction stage, and there are no reports of expression and immune protection analysis.

In this paper, the conservation, structural properties and immunogenicity of porin protein were analyzed by bioinformatics, and the duck was immunized by recombinant expression of the protein to verify the cross-immunoprotective effect and immune mechanism of the protein.

Materials And Methods

Bacterial strains and culture conditions

RA strain ATCC 11845 were purchased from Shanghai yiyuan biological technology Co. Ltd (Shanghai, China). Serotype 1 *RA* strain CH-1 (RA-CH1) was isolated from sick ducks in HaiKou, China. *RA* strains were grown in tryptic soy broth medium (TSB) and at 37°C. *E. coli* strain DH5 α and BL21 (DE3) strains were purchased from TransGen Biotech, Inc. (Beijing, China). *E. coli* strains were grown on Luria-Bertani (LB) agar.

Animals

30 One-day-old Cherry Valley ducklings obtained from the Hainan Tianyuan Industrial Co., Ltd.(Haikou, China). The ducks were housed in cages, and provided water and food ad libitum during the study. The animal experiments were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee guidelines set by HanNan University.

Signal peptide prediction

Signal peptide cleavage site was predicted by SignalP 5.0 (<http://www.cbs.dtu.dk/services/SignalP/>).

Homology analysis and phylogenetic tree construction

The *RA* Porin protein (Uniprot NO. E4TDA8) was blasted by UniProtKB database with parameters including E-Threshold 10, Matrix BLOSUM-62 and no filtering in Uniprot (<https://www.uniprot.org/blast/>). The number of returned alignments was 50. The blasted data was aligned with ClustalW and phylogeny tree was constructed with maximum likelihood statistical method by MEGAX.

Transmembrane structure prediction

Mature peptide sequence of *RA* Porin protein was pasted into PRED-TMBB web server (<http://biophysics.biol.uoa.gr/PRED-TMBB/input.jsp>) (Bagos et al., 2004), which is for predicting the topology of beta-barrel outer membrane proteins. Viterbi (VI), N-best (NB) and Posterior Decoding (DY) method were applied and the common part of the three analyses was as prediction results.

Tertiary structure prediction

The tertiary structure of the Porin protein sequence from which the signal peptide was removed was predicted by the swissmodel online server (<https://swissmodel.expasy.org/>).

Antigenic prediction

The B cell epitopes were predicted by ElliPro web server (<http://tools.iedb.org/ellipro/>) based based upon structural protrusion. The predicted results were screened to retain only epitopes outside the membrane.

Expression and purification of poring protein of *RA*

The genome of *RA* strain ATCC 11845 is extracted by the genome extraction kit (TIANGEN Biotech(Beijing)Co.,Ltd.,BeiJing, China), and the porin gene (sequence accession number in genebank:NC_017045.1, from 1940759 to 1942012) is amplified by primer pairs porin F: 5'-GGATCCGTACCAGATACTATTATCGTTC-3' and porin R: 5'-GCGGCCGCAATTCCTATTTCTACTTGAAATCT-3', with Restriction site *Bam*H 1 and *Not*I. The gene was then ligated into the pET 28a vector, and the recombined vectors were transformed into BL21 competent cells. These successfully transformed BL21 *E. coli* cells were induced to express the target protein by auto-inducing medium (Studier, 2014). The expressed product was subjected to cleavage, separation, denaturation, renaturation, dialysis, and finally purification through a nickel column based on the previous protocol (Saleem et al., 2012). All proteins were determined by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein was then lyophilized with the ALPHA 1-2 LD plus freeze dryer (Christ, Germany) and kept in -20 °C.

Immunization protocols

The Cherry Valley ducks were randomly divided into 3 groups of 10 animals each injected with PBS (PBS group), PBS + adjuvant (PBS + adjuvant group), PBS + adjuvant + Porin protein (Porin group). The ducks were first immunized at 7 days of age. The Porin protein was mixed with Freund's complete adjuvant in a 1:1 oily form and injected subcutaneously. For the second immunization 7 day post the first immunization, Porin protein was mixed with Freund's incomplete adjuvant by 1:1 to form an oil emulsion. Both immunization doses were 0.2 mg/duck. The days of blood collection was 1, 7, 14, 21 and 28 days post the first immunization.

Indirect enzyme-linked immunosorbent assay (iELISA) for Porin protein and RA cells detection

96-well plates were coated at a concentration of 2 µg/ml protein antigen with 100 µl/well, overnight at 4 °C, and washed 4 times with 100 µl PBST wash for 5 min each. The plates were added with 200 µl/well blocking solution and incubated at 37 °C for 2 hours. Then the plates were washed 4 times with each time 5 min. The first well was diluted 1:100, diluted 1:3 in a gradient, 100 µl/well. The plates were then incubated at 37 °C for 1.5 h, and washed 4 times for 5 min each time. Horseradish-labeled mouse anti-duck IgG (baiaolaibo, Beijing) was diluted 1:5000, 100 µl/well, incubated at 37 °C for 20 to 30 min, and washed 4 times for 5 min each time. The plates were added with 1×TMB at 100 ul/well, and then incubated for 15-30 min at 37 °C. Finally, stop solution (2M H₂SO₄) was added into the plates at 50 µl/well.

The OD value of each well was measured at a single wavelength of 450 nm, and the ratio (Positive/Negative, P/N) of the OD value to the negative control well was greater than 2.1 as a critical point judged to be positive or negative.

96 well plates were added with 150 µl 0.1 M NaHCO₃ plus 2.5 % glutaraldehyde, incubated for 1 h at 37 °C, and washed four times with sterile water. The plates were then coated with 10⁷ CFU/100 µl RA cells per well and incubated at 37 °C until dry. Subsequent steps from antigen blocking were carried out in accordance with the above iELISA procedure.

Serum bactericidal assay (SBA)

The serum 7 days after the second immunization was used for SBA. A single colony of the purely cultured strain was picked, inoculated into TSB medium, and cultured to the early stage of growth (OD 600 = 1.5 or so). After washing with PBS, it was diluted to 10⁸ cfu/ml. Duck serum was diluted to a range of concentrations of 100%, 50%, 25%, 12.5%, 5% with PBS. Inactivated 100% duck serum and PBS were used as controls. 10 uL of the prepared bacterial solution was mixed with 190 uL of different concentrations of duck serum and incubated at 37 °C for 30 min. The number of bacteria survived was counted by plate counting.

Opsonophagocytosis assay

The serum 7 days after the second immunization was used for Opsonophagocytosis assay. *RA* single colonies were picked, inoculated into TSB liquid medium, and cultured to the early stage of growth (OD 600 = 1.5 or so). After washing with PBS, it was diluted to 5×10^4 CFU/ml. 20 μ l of the test serum was added to the 96-well cell plate and incubate for 20 min at 37 °C. 10 μ l of duck complement and 40 μ l of duck blood was add into the plates and incubate for 1 hour at 37 °C with shaking. The number of bacteria survived was counted by plate counting.

Challenge assay

30 one-day-old cherry valley ducks were randomly divided into three groups. Porin protein with adjuvant, PBS with adjuvant and PBS were separately immunized according to the previous method. After 14 days of secondary immunization, 10 times and a half lethal dose (LD50) *RA* CH1 was intraperitoneally injected for 1 week, and survival rate after challenge was recorded.

Statistical analysis

The SigmaPlot software (version 12) and graphpad prism 8.0.2 were used for all statistical analyses. One-way repeated analysis of variance (ANOVA) and the Mann–Whitney rank test were used to evaluate differences between groups. Differences were considered significant at $p < 0.05$.

Results

Signal peptide prediction

Cleavage site of Porin protein was between pos. 18 and 19: LKS-QE, and probability of prediction was 0.6038.

Homology analysis

The top 50 sequences with the highest similarity to the Porin protein in the Uniprot database were extracted for comparison. The identity of Porin protein in *RA* was above 94.2%, blew 68% with other strains. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The result was shown in Figure 1. The tree with the highest log likelihood (-11905.39) is shown. The analysis involved 50 amino acid sequences. There were a total of 451 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The Porin protein was conservative in *RA*.

Transmembrane structure prediction

Transmembrane structure of Porin protein was predicted in figure 2A. The predicted results of VI and NB were consistent and slightly different from the DY prediction results. The same parts of the three prediction structures were plotted and the results are shown in Figure 3. There were 10 extramembranous fragments, 20 transmembrane fragments, and 2B fragments located in the cytoplasmic space.

Tertiary structure prediction

50 templates were found and descended by GMQE score. The template name 2o4v.1.A was used for modeling. Structure assessment of the model performed by Ramachandran Plots was shown in Figure 3A. Ramachandran Favoured rate was 89.04%. Modeling results was downloaded and named PorinM, then viewed by Swiss-PdbViewer 4.1, which was shown in Figure 3B. The homo-trimer consisted of alpha helix, a beta sheet and some irregularities, forming a barrel-like protein dominated by beta sheet, which is a typical structure of the outer membrane protein of Gram-negative bacteria.

Antigenic prediction

The PDB file PorinM was used to analyze the linear and spatial epitopes of the porin protein. There were 14 linear epitopes and 6 spatial epitopes were initially predicted. After screening non-membrane epitopes, only 9 linear epitopes (Table 1) and 2 spatial epitopes (Table 2) remained.

Expression and purification of poring protein of *RA*

Expression and purification of *RA* was shown in Figure 4. Porin gene was cloned, as shown in figure 4A. Agarose gel electrophoresis showed that the gene was located between 1000bp and 2000bp, which was in line with the expected electrophoretic band position. The recombinant protein contains his tag, which is recombinantly expressed, cleaved, denatured and renatured, and then purified by a nickel column. SDS-PAGE showed that the molecular weight of the protein was between 43kd and 55kd (Figure 4B), which was in line with the expected results. The purity of the purified protein is about 90%.

Strong antibody induced by immunization with Porin protein.

After the first immunization and the second immunization, the serum antibodies of the Porin protein immunized group were positive at 1:24300 for the porin protein and *RA* strains CH1 (P/N values were higher than 2.1), while PBS and PBS+ adjuvant group serum were negative (Figure 5).

Bactericidal activity and phagocytosis of the anti-porin sera against *RA*

Bactericidal activity was shown in Fig.6. Compared with 0% serum concentration, when the serum concentrations were 12.5%, 25%, 50%, and 100%, the colonies of the Porin group decreased by 6.43%, 14.46%, 16.25%, and 22.95%, respectively, while the other two groups had no significant difference. This indicated that the serum immunized with Porin protein may activate complement sterilization in vitro.

Opsonophagocytosis assay was shown in Fig.7. Compared with the PBS control group, the number of viable bacteria in the PBS + adjuvant group and the Porin group decreased by 7% (the difference was not significant) and 58% (the difference was extremely significant), as shown in Fig.7. The results show that serum antibodies can effectively enhance the phagocytosis of phagocytes after Porin protein immunized ducks.

Protective efficacy of the Porin sera against *RA* in vivo

The survival rates of the Porin group and the PBS group after challenge were shown in Fig. 8. Porin group ducks died on the second day after challenge, and the survival rate remained at 80% as of the seventh day. Ducks in the PBS group died on the first day after challenge, and all died on the fourth day.

Discussion

RA mainly infects ducks, geese and turkeys, et al, and young animals have a higher mortality rate after infection. Antibiotic treatment has led to increasing resistance to *RA*. The current vaccines against *RA* are mainly inactivated vaccines(Liu et al.) and subunit vaccines(Chu et al., 2015). However, there are many *RA* serotypes, and the cross-immunoprotective effect between each serotype is weak. Currently reported serotypes with cross-immunoprotection are serotypes 1 and 2(Zhai et al., 2013). So, it is particularly important to develop subunit vaccines that have immune protection against multiple serotypes of *RA*.

The Porin protein has been reported to be immunoprotective in other strains. However, the protein was only presumed to be present in *RA* by sequencing, and has not been analyzed by recombinant expression, nor has it been verified whether it has immune protection against *RA*. The protein was firstly analyzed using bioinformatics tools. By blast comparison and Phylogenetic tree analysis, it can be seen that the protein is highly conserved in *RA* and has low similarity to Porin protein of other strains. It could be speculated that if the protein has immunoprotective effect on serotype 1 *RA*, then it may also have immunoprotective effect on other serotype *RA*. Our previous research also showed that even if the conserved antigen exists in different bacterial species, the antigen will provide cross-immunity protection(Guan et al., 2015; Guan et al., 2016). The transmembrane structure and epitope of Porin protein in *RA* were also predicted. Only epitopes located outside the membrane have the potential to develop vaccines. After screening, 9 linear epitopes and 2 spatial epitopes were considered to be effective. According to the analysis, this protein is conserved in *RA* and has abundant epitopes, which may have the potential to develop vaccines.

Bioinformatics analysis also showed that the protein may be expressed as inclusion bodies in *E. coli*. Therefore, the expressed protein needs to be refolded and renatured. The membrane protein contains a transmembrane portion and a hydrophilic portion. In order to keep the protein as natural as possible, a mild detergent needs to be added to the refolding buffer.

Serum antibody affinity was analyzed by iELISA. 28 days after the initial immunization, duck serum antibodies still have a strong affinity for Porin protein and serotype 1 *RA* strains. SBA and opsonization phagocytosis also prove that serum antibodies can improve the elimination of *RA* by the immune system. Compared with the control group, the survival rate of Porin protein immunized group after challenge increased, indicating that the protein has the potential to be developed into a vaccine.

Declarations

The article is original, has been written by the stated authors who are all aware of its content and approve its submission, and has not been published previously. It is not under consideration for publication elsewhere, no conflict of interest exists. If accepted, the article will not be published elsewhere in the same form, in any language, without the written consent of the publisher.

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of Hainan University Institutional Animal Use and Care Committee and was approved by the international bioethics committee. Written informed consent was obtained from individual or guardian participants.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

QG and QH conceived and designed research. HY and HZ conducted experiments. KW contributed new reagents or analytical tools. CL, JZ and JW analyzed data. QG wrote the manuscript. All authors read and approved the manuscript.

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Tables

Table 1 linear epitopes outside of outer membrane in *RA*.

No.	Start	End	Peptide	Number of residues
1	52	67	YYNFGKGVGITSPDSLF	16
2	86	94	NRSTEYKAA	9
3	125	133	GEIKEGNPL	9
4	196	202	NNTKANE	7
5	235	246	YPLGAFKNNGEF	12
6	278	287	RGKELLESRD	10
7	314	329	SNPLTHNTDFSKVIPV	16
8	359	368	NKSIQNLIPK	10
9	392	406	SKNNFLYIDGRKDNN	15

Table 2 spatial epitopes outside of outer membrane in *RA*

No.	Residues	Number of residues
1	A:S271, A:N272, A:F273, A:Q275, A:G276, A:G279, A:K280, A:E281, A:L282, A:L283, A:E284, A:S285, A:R286, A:D287, A:S314, A:N315, A:P316, A:L317, A:T318, A:H319, A:N320, A:T321, A:D322, A:F323, A:S324, A:K325, A:V326, A:I327, A:P328, A:V329, A:K360, A:S361, A:I362, A:Q363, A:N364, A:L365, A:I366, A:P367, A:K368, A:L397, A:Y398	41
2	A:N77, A:L79, A:E80, A:A81, A:D82, A:F83, A:N84, A:D85, A:N86, A:S88, A:T89, A:E90, A:Y91, A:K92, A:A93, A:A94, A:P236, A:L237, A:G238, A:A239, A:F240, A:K241, A:N242, A:N243, A:G244, A:E245, A:F246, A:K393, A:N395, A:F396, A:I399, A:D400, A:G401, A:R402, A:K403, A:D404, A:N405, A:N406	38

Figures

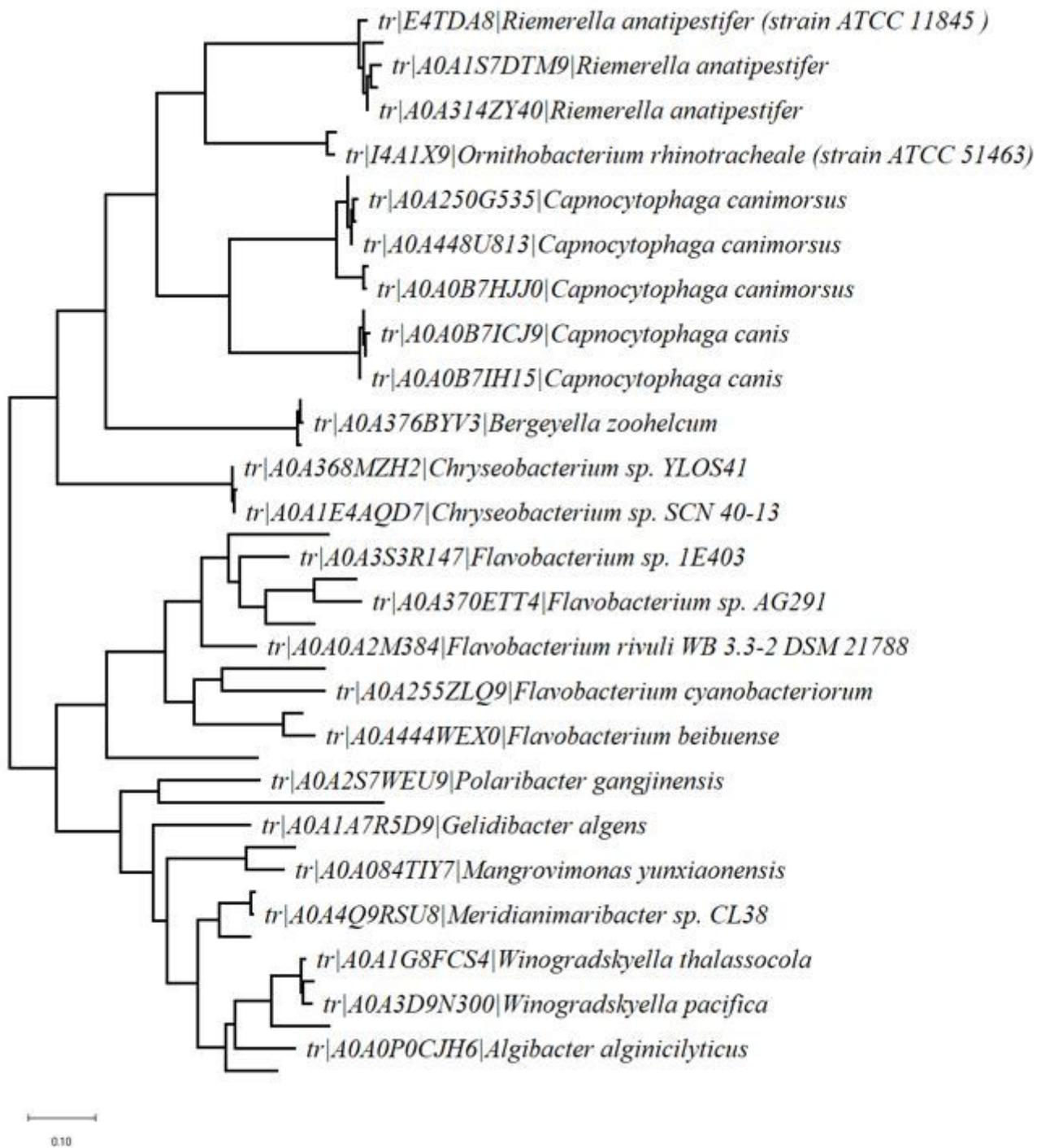
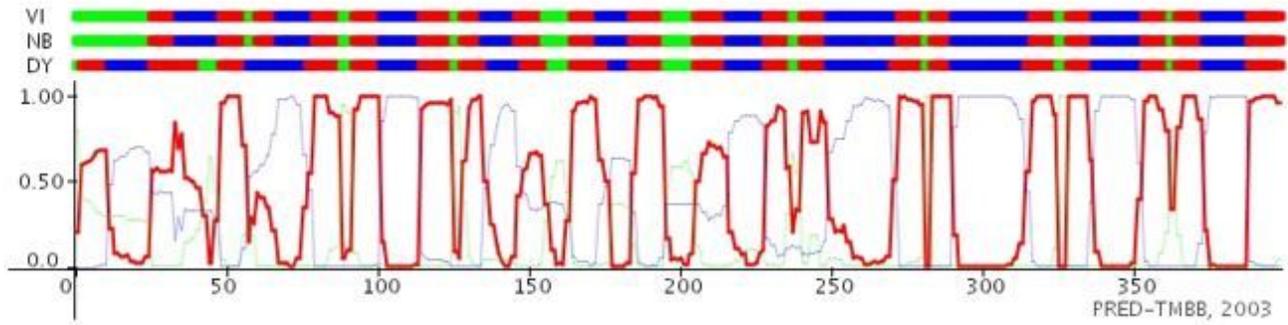
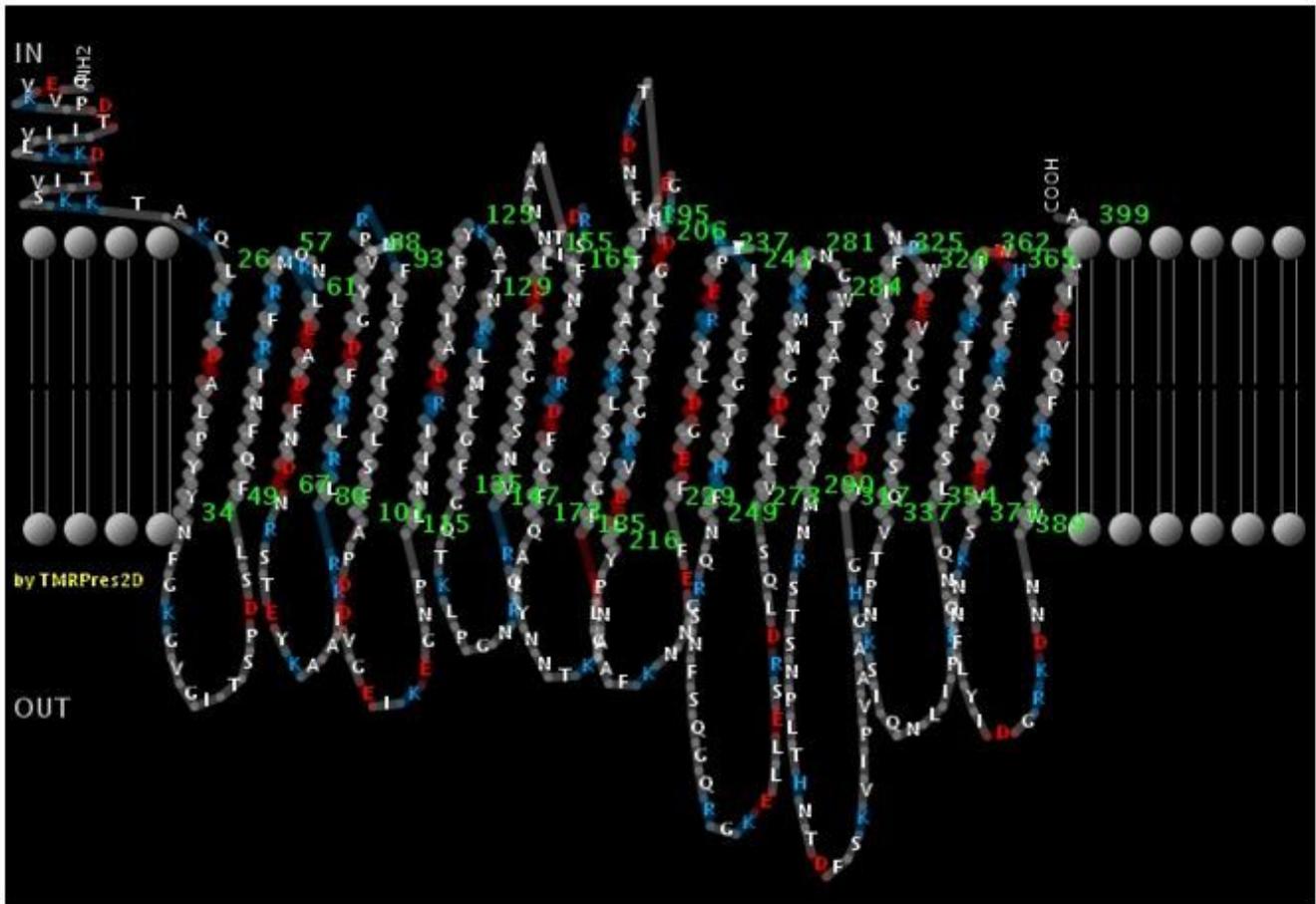


Figure 1

Molecular Phylogenetic analysis by Maximum Likelihood method



A



B

Figure 2

Transmembrane structure prediction of Porin protein. A: Prediction by VI, NB and DY methods. B: common elements of the prediction in three methods.

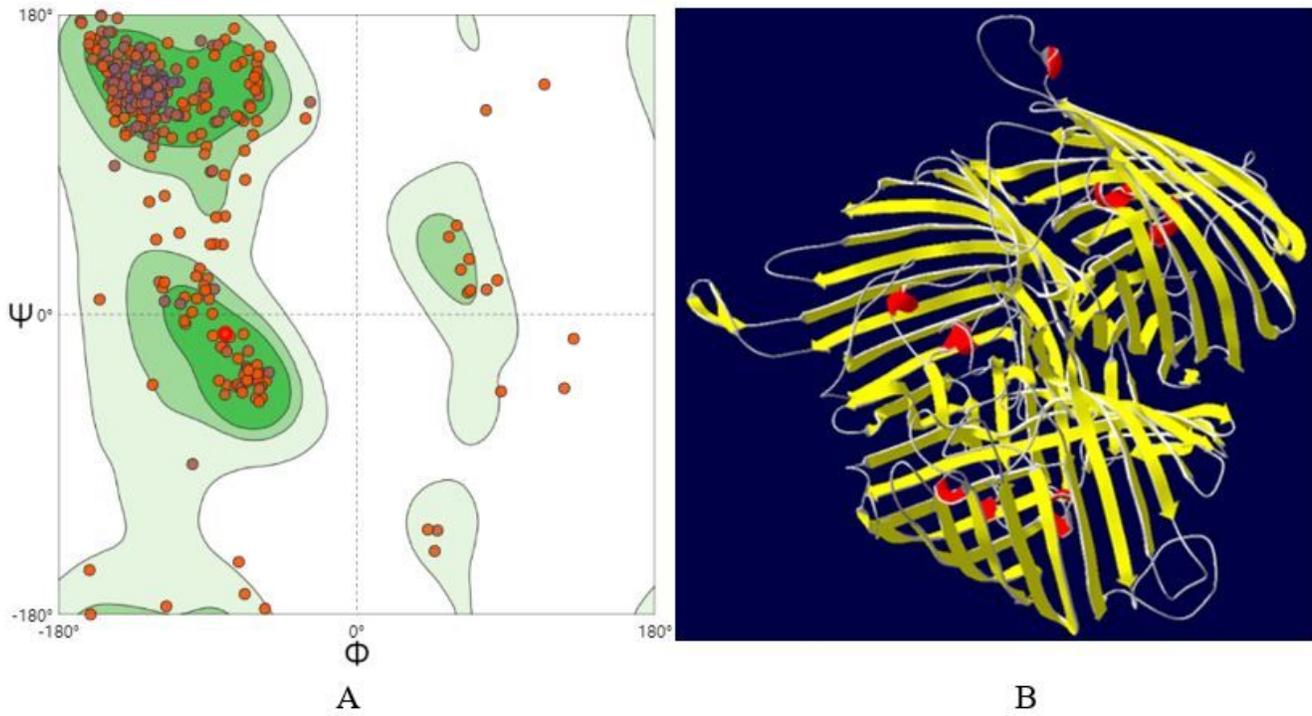


Figure 3

Tertiary structure prediction of Proin protein. A: Structure assessment of the model by Ramachandran Plots. B: 3D structure of PorinM.

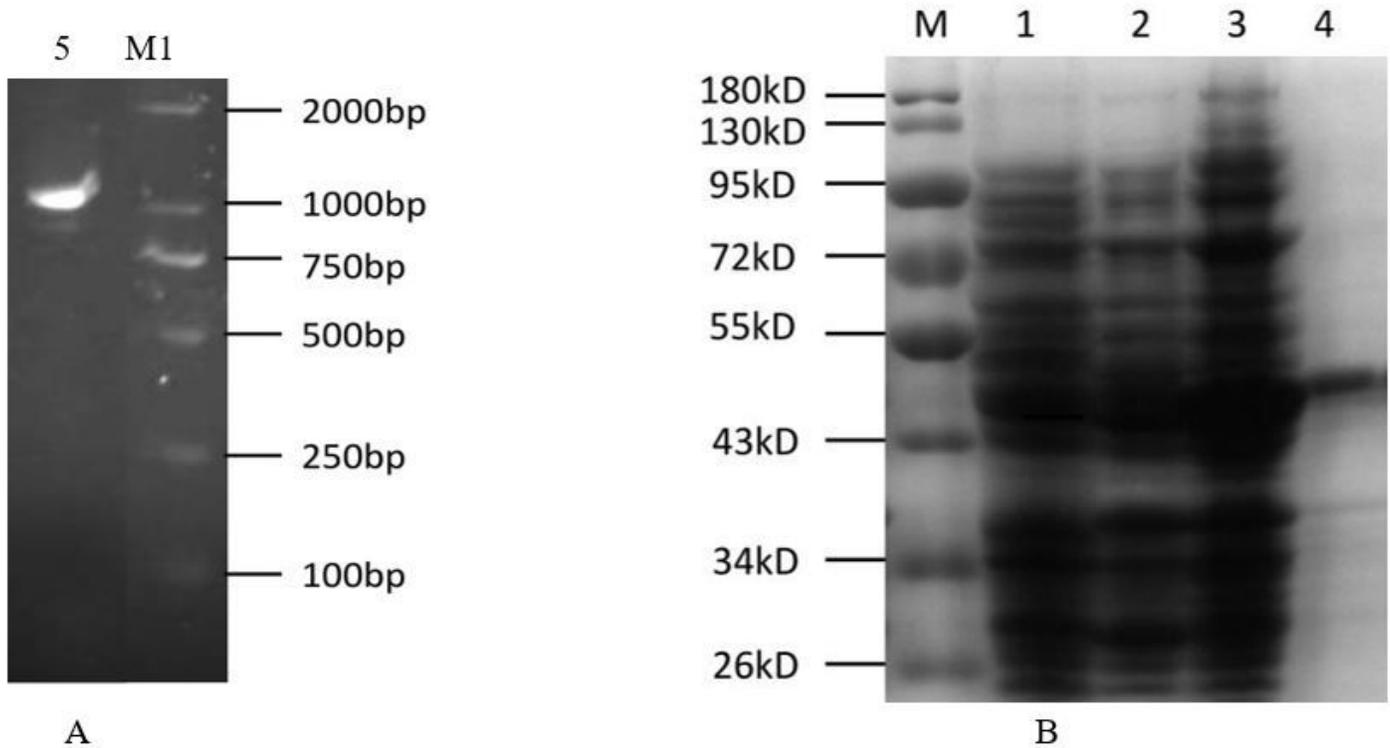


Figure 4

Electrophoresis of Porin gene cloning and protein purification. 4A: Agarose gel electrophoresis of Porin gene. 4B: SDS-PAGE of Porin protein. M: 180 kd protein ruler. M1: Trans 2K DNA marker. 1: BL21 strain. 2: BL21 strain with pET 28a vector. 3: BL21 strain with Porin gene. 4: Purified Porin. 5: Porin gene.

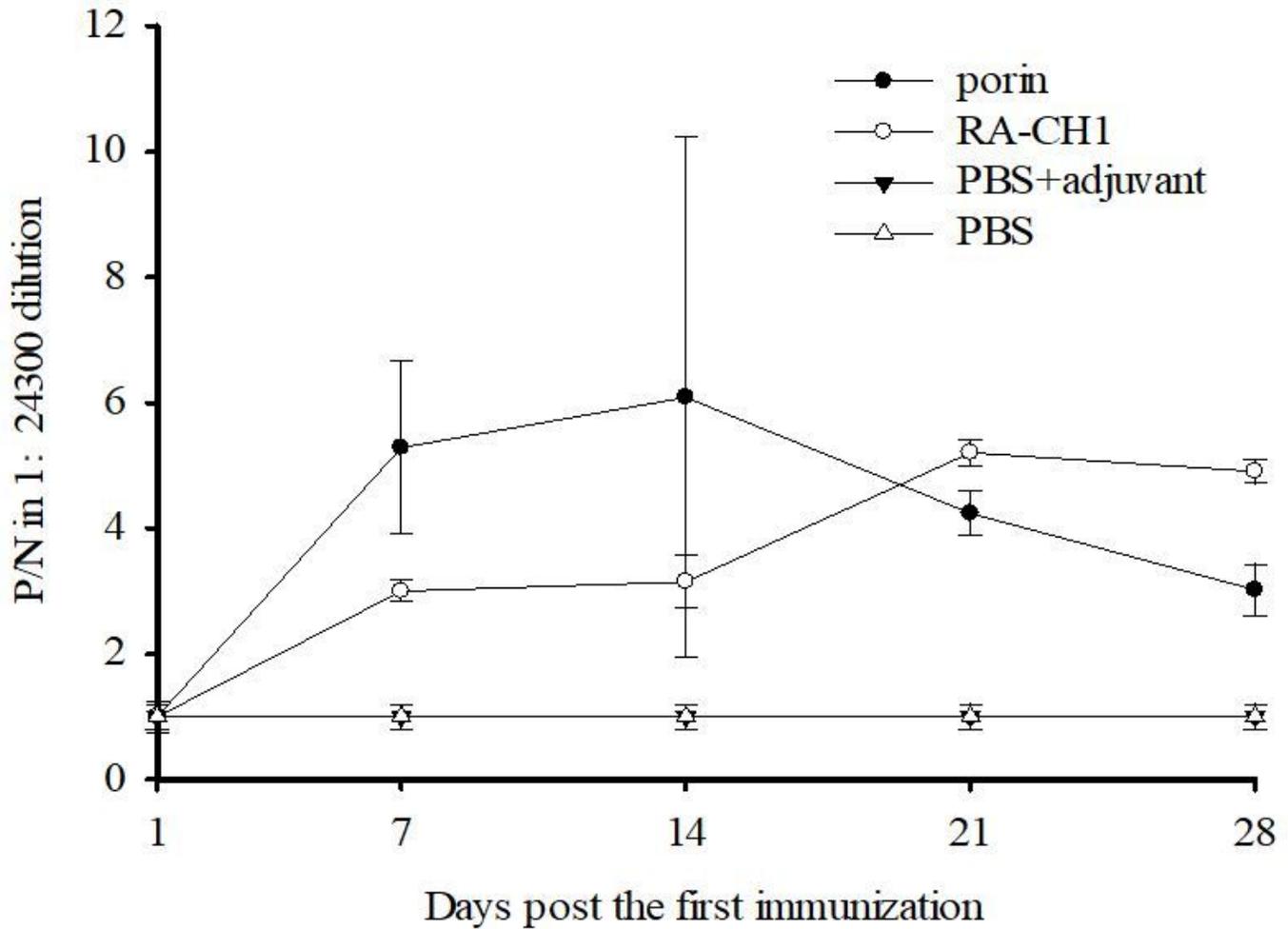


Figure 5

IELISA for Porin immunization serum.

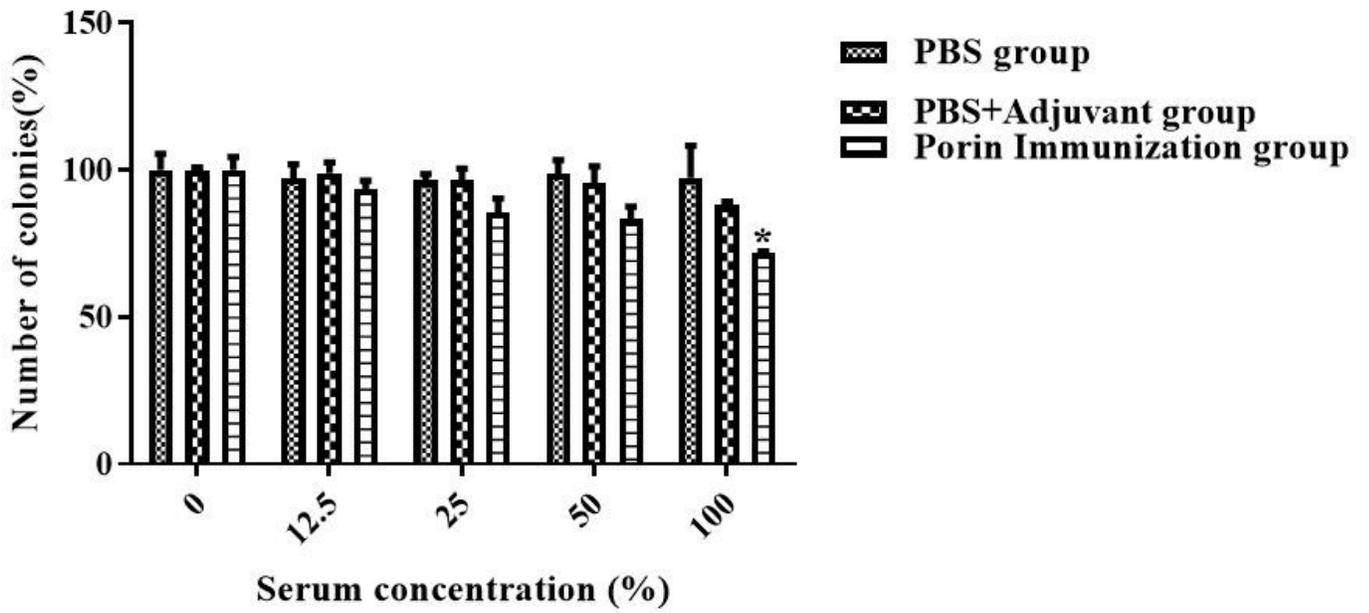


Figure 6

Colonies change in different dilution of surum.

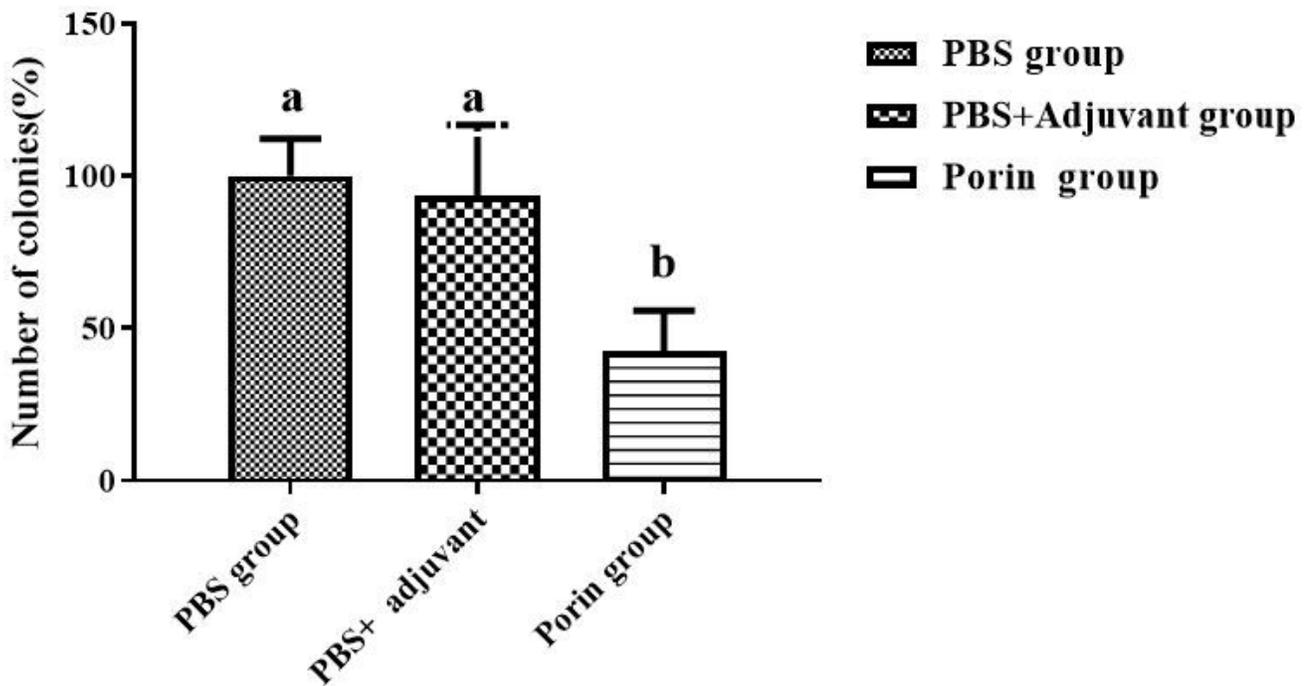


Figure 7

Phagocytosis of the rBamA sera in vitro.

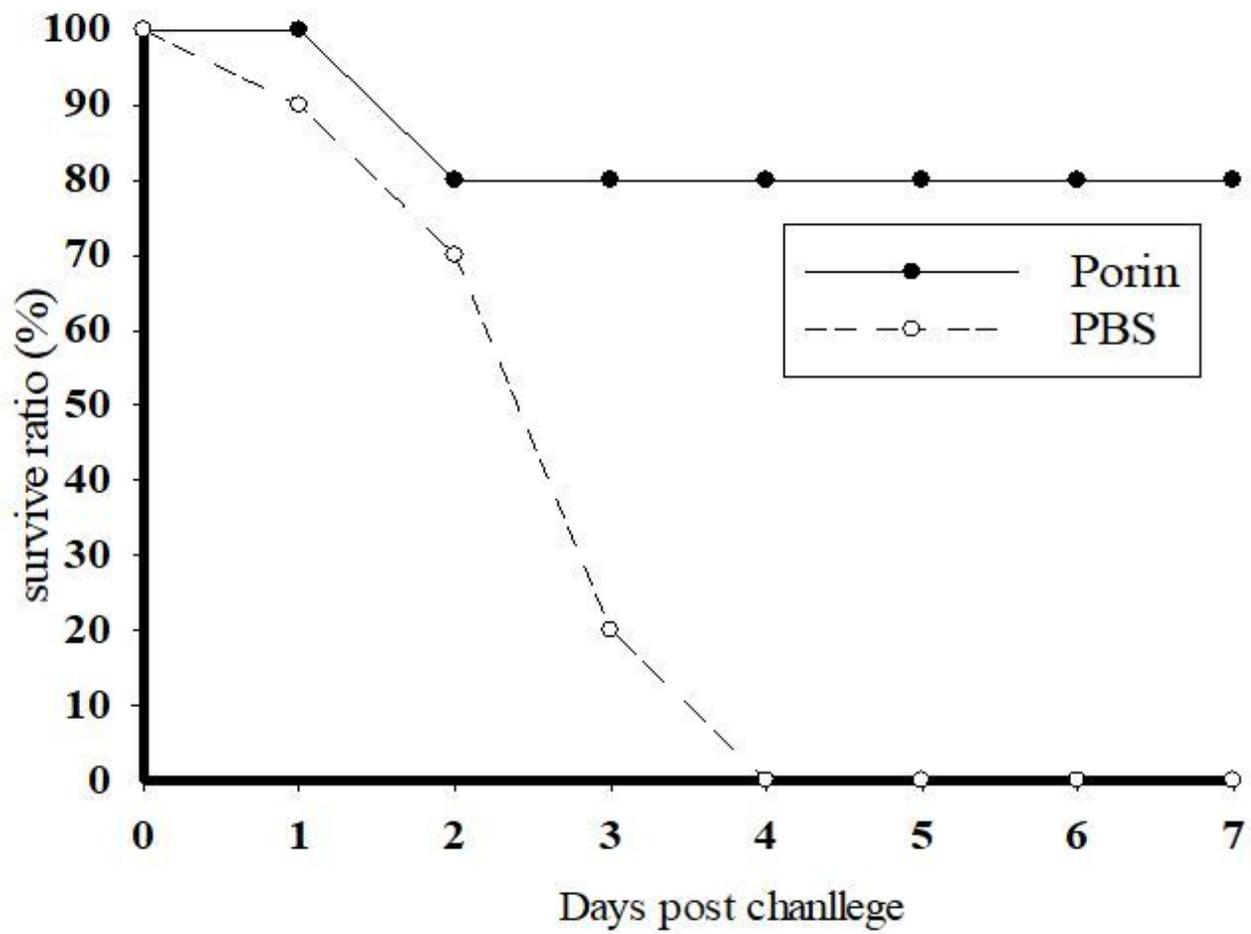


Figure 8

Survival percent of the mice immunized with Porin or PBS.