

Full Length Research Paper

High-level expression, purification, polyclonal antibody preparation against recombinant OprD from *Pseudomonas aeruginosa*

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OprD is a specific porin which can binds imipenem and carbapenems in *Pseudomonas aeruginosa*. OprD loss plays a central role in mediating carbapenem resistance. Therefore, purification of *oprD* protein lays a pavement for the study *in vivo* and *in vitro*. In our study, the *oprD* gene was cloned into pQE30 expression vector, in frame with a sequence coding an N-terminal hexahistidine tag to allow purification by Ni²⁺ column. The recombinant OprD-6His was overproduced in inclusion form in *Escherichia coli* M15. OprD-6His was purified under denatured conditions using Ni-NTA conjugates. Antiserum against this recombinant OprD-6His protein was prepared in rabbit. Western blot analysis and enzyme linked immunosorbent assay (ELISA) were carried out to identify the reaction abilities and sensitivity of anti-OprD-6His polyclonal antibody to purified OprD-6His. Our results indicated that it was induction of *E. coli* M15 cells with 0.5 mM of isopropylthio-d-galactoside (IPTG) at 33°C for 4 h that the predicted 48 kDa OprD-6His fusion protein was expressed as the form of inclusion bodies with about 55-65 mg of OprD-6His per liter of culture. Western blot showed that recombinant OprD-6His protein could be identified by self-developed anti-OprD-6His polyclonal antibody. Anti-OprD-6His polyclonal antibody was detectable with 1000 times dilution of the original polyclonal antibody solution. In conclusion, higher level expression of OprD was available in the pQE30 expression system at optimal condition. Self-prepared polyclone antibody can effectively detect difference of porin expression in *P. aeruginosa* with higher sensitivity and specificity.

Key words: *Pseudomonas aeruginosa*; OprD; polyclonal antibody.

INTRODUCTION

Pseudomonas aeruginosa is a clinically formidable nosocomial pathogen with both acquired and intrinsic mechanisms of antibiotic resistance (Livermore, 2001). Carbapenem antibiotics remain important agents for the therapy of serious infections due to multidrug-resistant of

P. aeruginosa. However, the development of carbapenem resistance severely compromises effective therapeutic options. In the absence of carbapenem-hydrolyzing enzymes, the mechanism leading to carbapenem resistance is mostly mediated by OprD loss, which primarily confers resistance to imipenem but also confers low grade resistance to meropenem (Köhler et al., 1999; Livermore, 1992). Mutational loss of OprD is frequent during imipenem therapy in a variety of clinical studies, imipenem resistance due to lack of OprD has emerged

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during treatment of *P. aeruginosa* infections in 25% of patients (Zanetti et al., 2003; Carmeli et al., 1999; Jaccard et al., 1998; Cometta et al. 1994).

OprD is the closest *P. aeruginosa* homolog of the *Escherichia coli* nonspecific porin OmpF (Huang et al., 1995). X-ray crystal structure reveals a monomeric 18-stranded beta-barrel characterized by a very narrow pore constriction, with a positively charged basic ladder on one side and an electronegative pocket on the other side (Biswas et al., 2007). It is a specific porin that binds basic amino acids, dipeptides containing a basic residue, imipenem and related zwitterionic carbapenems (Trias and Nikaido, 1990). Investigation of OprD mutants with deletions in specific loops demonstrated that both loop 2 and 3 deletions lose the ability to bind imipenem and mediate imipenem susceptibility (Huang and Hancock, 1996; Ochs et al., 2000). Thus, OprD differs from other specific porins (Koebnik et al., 2000) because loop 2 has also a role in substrate binding to the channel. Also loops 5, 7, or 8 deletion variants of OprD have increased susceptibility of *P. aeruginosa* to multiple antibiotics, and they correspondingly produce larger channels (that still bind imipenem), which indicates that these loops constrict the channel entrance to limit nonspecific movement of molecules through OprD channels (Huang et al., 1995). OprD is found as a moderately expressed outer membrane protein but is regulated by multiple systems. It is repressed by MexT (which also induces the MexEF-OprN efflux system), salicylate and catabolite repression (Köhler et al., 1997; Ochs et al., 1999b), and it is activated by arginine/ArgR and a variety of other amino acids as carbon and nitrogen sources (Ochs et al., 1999a).

In the present study, we first report the expression of the *oprD* protein in *P. aeruginosa* by the expression vector pQE30 (Amp^r, QIAGEN), and the preparation of polyclone antibody against *oprD*.

MATERIALS AND METHODS

Bacteria strain and growth conditions

P. aeruginosa PAO1 as quality control strain was used to amplify *oprD* gene. Bacteria were grown in Mueller-Hinton (MH) medium.

Extraction of genomic DNA and amplification

The genomic DNA of *P. aeruginosa* was extracted with Genomic DNA Extraction Kit (Qiagen, USA). According to the sequence submitted in GenBank database under Accession number X63152, primers were designed as shown below to amplify *oprD* from genomic DNA of PAO1. *Bam*HI and *Hind*III restriction sites were incorporated in the forward and reverse primers, respectively, to facilitate cloning in pQE30 expression vector. The amplification product was analyzed by electrophoresis on 1% agarose gel stained with ethidium bromide (0.5 µg/ml). Forward primer: 5' GCGGGATCCATGAAAGTGATGAAGTGGAG 3' (*Bam*HI restriction site underlined). Reverse primer: 5' ACAAGCTTTTACAGGATCGACAGCGGATAG 3' (*Hind*III restriction

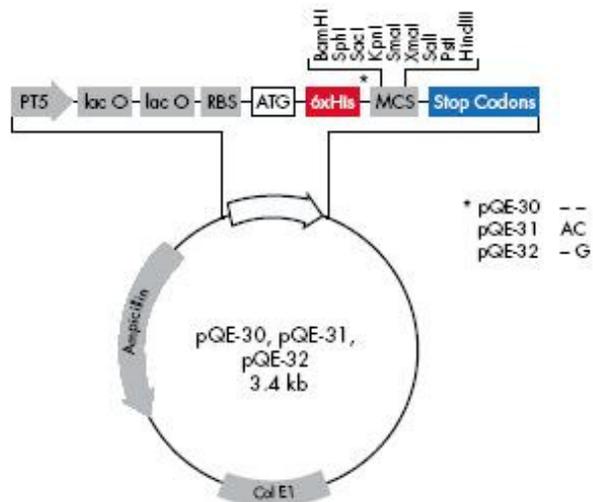


Figure 1. Schematic representation of expression vector, pQE30, with a fusion frame of 6His and ampicillin resistance.

site underlined).

pGEM-T easy vector subcloning and sequence analysis

The polymerase chain reaction (PCR) product was purified using PCR purification kit (Qiagen, USA) and inserted into pGEM-T Easy vector system (Promega Co., Germany). *E. coli* DH5α was transformed with the ligated plasmid and the transformed cells were cultured using LB broth containing ampicillin (100 mg/ml). The plasmid DNA was extracted with Plasmid Purification Kit (Qiagen, USA). Both strands of the cloned DNA fragments inserted into the recombinant plasmids were sequenced with an Applied Biosystems sequencer (ABI 3730). Nucleotide and predicted amino acid sequences were analyzed using DNAssist 2.0 and Basic Local Alignment Search Tool (BLAST) programs in non-redundant databases of National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/BLAST/>).

ubcloning in expression vector and protein expression

oprD cloned in pGEM-T Easy vector was digested with *Bam*HI and *Hind*III restriction enzymes and run on 1% agarose gel. A 1332 bp *Bam*HI-*Hind*III insert fragment was cut and purified from agarose gel by using gel extraction and purification kit (Qiagen, USA) and cloned into *Bam*HI-*Hind*III digested pQE30 vector (Figure 1). The recombinant plasmid named as pQE30-OprD (Figure 2) was transformed to the host *E. coli* M15 (Qiagen, USA), then selected on L-Broth agar containing 100 µg/ml ampicillin. The positive clones were confirmed by PCR and sequencing.

A fresh clone of *E. coli* M15, harboring the pQE30-OprD vector, was grown in MH medium containing 50 µg/ml ampicillin. When the cells had been cultured (37°C, 250 rpm) to an optical density (OD600) of 0.4, the expression of the fusion gene was induced with different concentration of isopropylthio-d-galactoside (IPTG) (Sangon, Shanghai, China) at different time. After induction, cells were lysed in 2xsample buffer (0.1 M Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 0.1 M DTT). Samples were loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gels (10% polyacrylamide, 0.1% SDS) as previously described (Laemmli, 1970). The protein profile was determined after

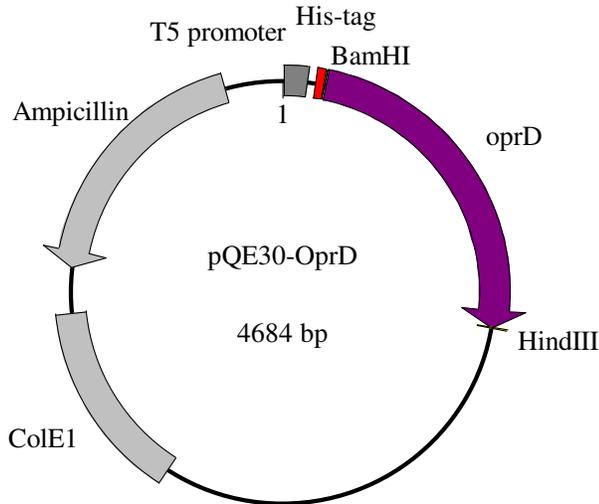


Figure 2. Schematic representation of the recombinant plasmid pQE30-OprD.

Coomassie brilliant blue staining of the gel. The uninduced control culture and the vector control culture were analyzed in parallel.

To identify the OprD-6His fusion protein, Western blot was done according to standard protocol (Sambrook et al., 1989). The proteins on the polyacrylamide gel were electrotransfer onto nitrocellulose membrane (Millipore, France) at 200 mA for 2 h using a mini Trans-blot electrophoretic transfer cell (Biorad). The blotted membrane was rinsed with TBST (0.02 M Tris-HCl, 0.5 M NaCl, 1% Tween-20, pH 7.5) three times for 10 min and then blocked in TBS (0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5) containing 1% (v/v) BSA for 2 h at room temperature. Then, the membrane was rinsed with TBST three times for 10 min and the membrane was incubated with the anti-6His monoclonal antibody (Novagen, German) diluted 1:1000 in TBST for 2 h at 4°C. After rinsing of the membrane three times for each 15 min in TBST, the membrane was treated for 1 h with alkaline phosphatase-labelled goat anti-mouse IgG (Novagen, German). Then the membrane was washed three times for 10 min in TBST and developed by BCIP/NBT (Sigma, German) for 1-3min.

To optimize the solubility of the recombinant protein, different induction temperatures (25, 28, and 30°C) were tried. Cells were harvested by centrifugation at 10,000×g for 10 min. The cell pellet was resuspended in Bugbuster Protein Extraction Reagent (Novagen, German) containing 1 kU/ml lysosyme and 1 µl/ml Benzonase (Novagen, German), incubated for 15 min. The obtained cell lysate was fractionated as soluble and insoluble fraction after being centrifuged at 16,000×g for 30 min. Soluble and insoluble fractions were then analyzed on 10% SDS-polyacrylamide gel electrophoresis (PAGE).

Protein purification

The insoluble fractions were washed with 1:10 diluted BugBuster Protein Extraction Reagent and centrifuged at 16,000×g for 15 min, repeatedly. The bodies were resuspended in binding buffer containing 6 M carbamide for 1 h on ice and centrifuged at 16,000 g for 30 min. The supernatant filtered through a 0.45 µm filter (Millipore, France) and applied to a His-bind resin column (Novagen, German), which was previously equilibrated with three column volumes of sterile deionized water, five column volumes of Charge buffer (50 mM NiSO₄) and three column volumes of 6 M carbamide binding buffer, in that order. The column was then

washed with ten column volumes of 6 M carbamide binding buffer and six column volumes of 6 M carbamide wash buffer with 20 mM imidazole. Proteins were eluted with a line gradient of imidazole (60-500 mM) in elute buffer. The target fraction was collected, ultrafiltered to desalt and then lyophilized for future use. The presence of the recombinant protein in the lyophilized powder was confirmed by SDS-PAGE analysis and western blot.

Production of polyclonal antibodies against the recombinant protein

Two groups of New Zealand rabbit were prepared for non-immunization group and recombinant OprD-6His vaccine group. The antigen was mixed with equal volume of Freund's complete adjuvant (Sigma Chemical Co., USA). The emulsion was originally injected intradermally at a dose of 0.3 mg/kg into male rabbit. Booster containing antigen at a dose of 0.15 mg/kg mixed with Freund's incomplete adjuvant (Sigma Chemical Co., USA) were injected into rabbit at 4th weeks, 6th weeks, 8th weeks, respectively. After 10 days of the last injection, the serum containing the polyclonal antibody against OprD-6His was collected.

Western blot analysis

To determine the specificity of the OprD polyclonal antiserum, expressed recombinant proteins were separated on SDS-polyacrylamide gels (10% polyacrylamide, 0.1% SDS) and electrotransfer onto nitrocellulose membrane following standard protocol. The membrane was incubated overnight at 4°C with the OprD polyclonal antiserum (1:200). A horse-radish peroxidase-conjugated anti-rabbit secondary antibody (DAKO, Denmark) and chemiluminescence substrates (Pierce) were used to determine the immuno-labeled bands by exposure to the X-ray film.

Enzyme linked immunosorbent assay (ELISA)

Well plate (96 well) was coated with 3 µg OprD-6His and incubated at 4°C overnight. The plate was then rinsed three times with phosphate buffered saline-Tween 20 (PBS-T) and incubated with blocking solution (PBS containing 5% (w/v) skimmed milk) at 37°C for 2 h. After washing with PBS-T, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600 or 1:3200 dilution of blocking solution containing anti-OprD-6His antisera. After 2 h incubation at 37°C, plates were rinsed three times with PBS-T, and 1:2000-diluted alkaline phosphatase-labelled horse anti-rabbit IgG was added to each well and incubated for 1 h at 37°C. After washing 5 times, the enzyme activity was determined by adding 100 µl of 3, 3', 5'-tetramethyl benzidine substrate solution. Optical density was read at 630 nm with an ELISA plate reader after incubating for 30 min.

RESULTS AND DISCUSSION

Cloning and sequencing of *oprD*

The PCR product was produced as a DNA fragment of 1332 bp. The *oprD* gene cloned in the pGEM-T easy vector system was confirmed by sequencing. The cloned sequence was entirely identical to the published sequence in the GenBank database (Accession number X63152).

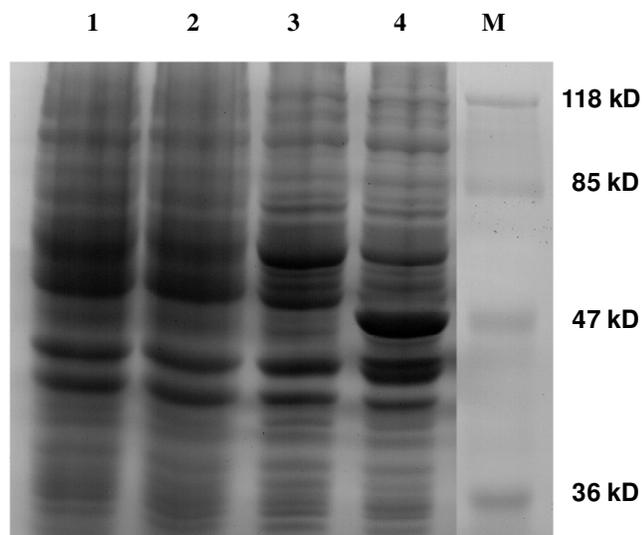


Figure 3. Expression of fusion OprD-6His protein in *E. coli* M15. M, Low molecular marker; lane 1, total protein of non-induced pQE30 in *E. coli* M15; lane 2, total protein of induced pQE30 in *E. coli* M15; lane 3: total protein of non-induced pQE30-OprD in *E. coli* M15; lane 4: total protein of induced pQE30-*oprD* in *E. coli* M15.

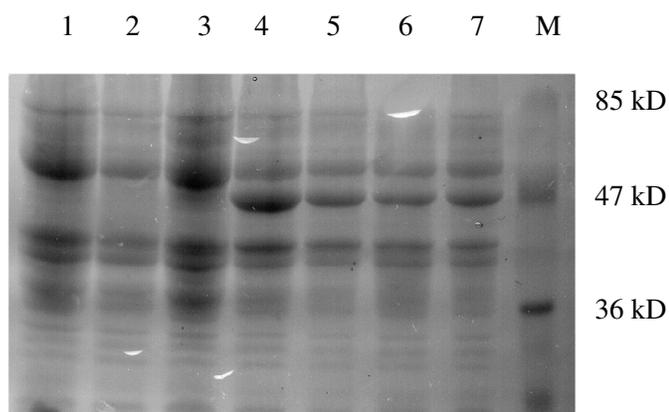


Figure 4: SDS analysis of expression fusion protein with different concentration IPTG. M, Low molecular marker; lane 1, total protein of non-induced pQE30; lane 2, total protein of induced pQE30; lane 3, total protein of non-induced pQE30-OprD; lane 4-7, protein induced with 0.5, 1.0, 1.5, 2.0 mM IPTG for 5 h.

Construction of the recombinant plasmid pQE30-*oprD*

*Bam*HI and *Hind*III sites were designed in the primers to facilitate cloning into the pQE30 vector. The 6His tag was located at the N-terminus of the recombinant plasmid pQE30-*OprD*. The 1332 bp *Bam*HI-*Hind*III insert fragment was cloned into the pQE30 vector. The pQE30-*OprD* plasmid was confirmed to refuse 6His tag to *oprD* N-terminal by sequence.

Expression of the OprD-6His fusion protein

The predicted 48 kDa OprD-6His fusion protein was expressed as the form of inclusion bodies in *E. coli* M15 cells at 33°C with IPTG induction. However, no protein production was observed in the negative control, *E. coli* M15 cells transformed with the pQE30 (induction and without induction) and *E. coli* M15 cells transformed with the pQE30-OprD (without induction) (Figure 3). The highest amount of fusion protein was produced after 5 h at 0.5 mM IPTG induction compared with at 1.0, 1.5, 2.0 mM IPTG concentration (Figure 4). Different cultivation temperatures were tried, but no soluble protein was found. The commonest reason is that the high-level expression protein in inclusion bodies affect normal protein fold. Inclusion bodies can be an advantage for purification because they are easily isolated by centrifugation to yield highly concentrated and relatively pure protein, and inclusion body formation protects the protein from proteolytic attack. In addition, toxic proteins may not inhibit cell growth when present in inactive form as inclusion bodies. Although OprD-6His fusion protein is insoluble fraction, it can be used directly as antigens for the preparation of antibodies against the protein.

Purification of the recombinant OprD-6His protein

The OprD-6His fusion protein was purified using the His-Bind purification kit (Novagen, German) as described in above. Before purification by column chromatography, we examined the optimal concentration of imidazole for purification. During the batch purification, the target proteins were eluted with a gradient of 60-500 mM imidazole, whereas, the concentration of the proteins eluted from the column with 60 mM imidazole was much denser than that with other concentration of imidazole. At this point, the OprD-6His protein was at least 93% pure (BandScan software). Western blot reconfirmed the purity of the OprD-6His fusion protein (Figure 5). This protocol yielded 55–65 mg of 93% purified OprD-6His protein per 1 L induced bacterial cell culture.

Production of OprD-specific polyclonal antibody

New Zealand rabbits were immunized by a subcutaneous injection with purified recombinant OprD-6His emulsified with an equal volume of Freund's complete or incomplete adjuvant. Western blot was carried out to identify the reaction abilities of anti-OprD-6His polyclonal antibody against recombinant OprD-6His. Recombinant OprD-6His protein could be detected by anti-OprD-6His polyclonal antibody. Also, ELISA was performed to determine the proper dilution of antiserum against OprD-6His. Anti-OprD-6His polyclonal antibody was detectable with 1000 times dilution of the original polyclonal antibody solution (Figure 6). However, 200 times dilution was used for the

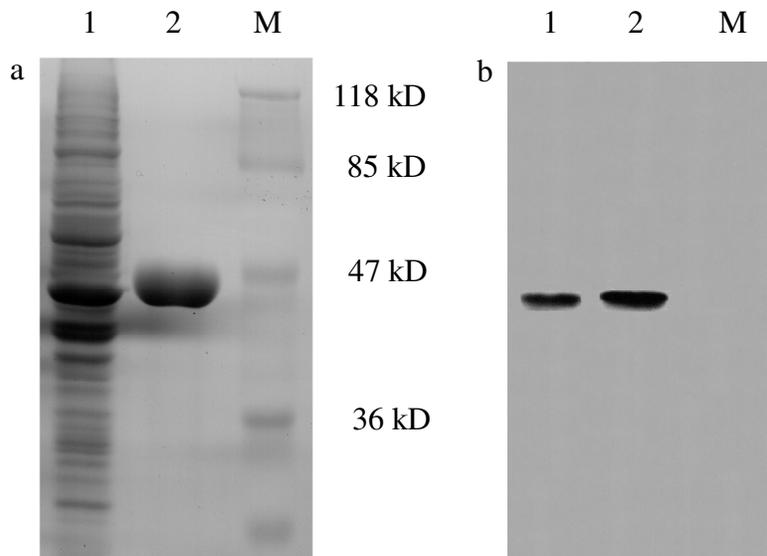


Figure 5. Purification of OprD-6His by column chromatography. (a) Protein were separated by SDS-PAGE and stained with Coomassie brilliant blue. (b) Western blot analysis using the anti-6His monoclonal antibody. M, Low molecular marker; lane 1, total protein of the recombinant bacteria after induction; lane 2, purified proteins.

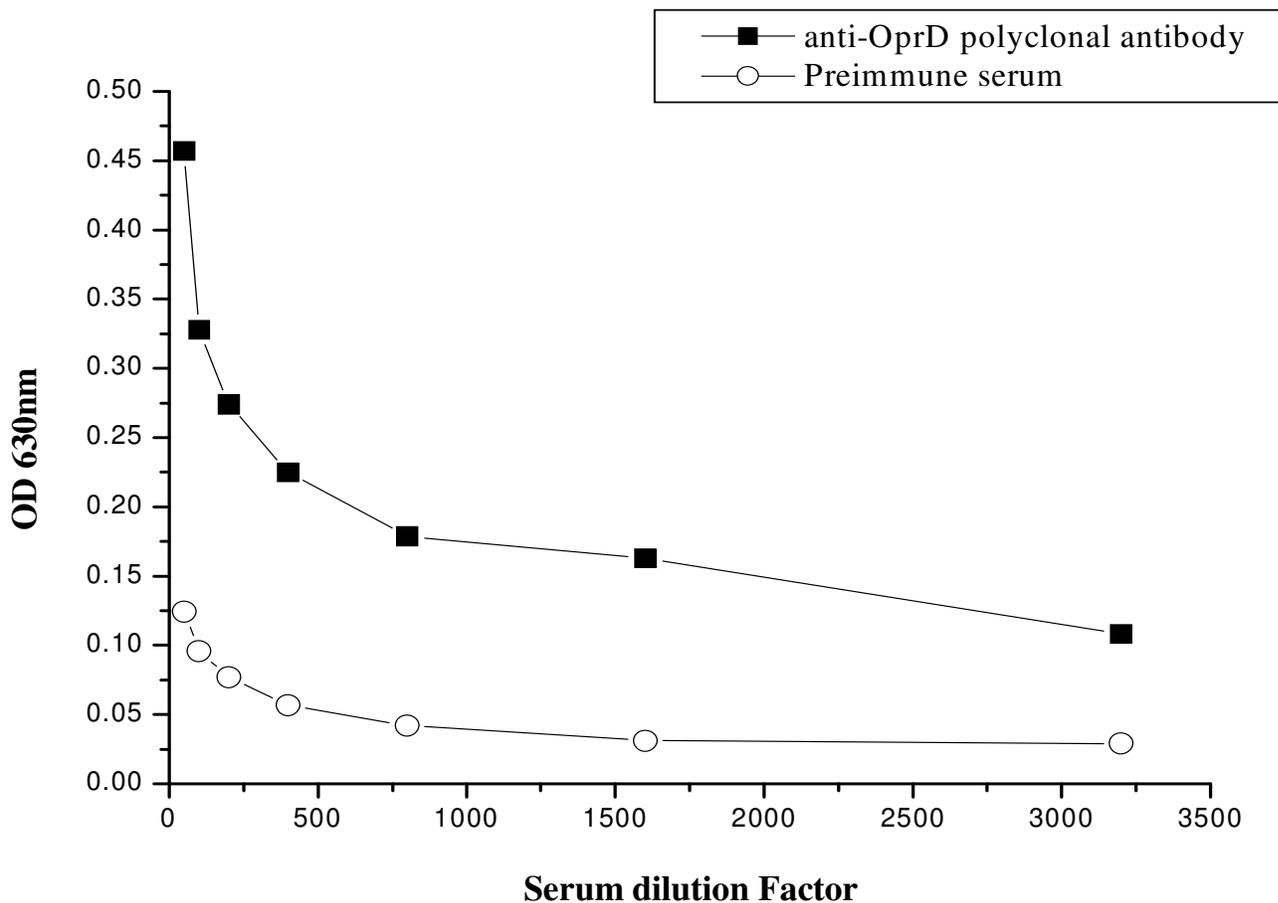


Figure 6. Determination of anti-OprD serum dilution concentration by indirect ELISA.

detection of OprD for safety from the error. These results indicated that the antiserum had a high level of specificity and could be used to detect OprD in *P. aeruginosa*.

The OprD-specific polyclonal antibody was produced and characterized in this study. This paper is the first to report the over expression and purification of the specific protein OprD of *P. aeruginosa*. The pQE30 expression system was used for overproduction of OprD. The OprD specific polyclonal antibody was produced using recombinant OprD-6His protein in rabbit. Western blot was carried out to evaluate the reaction abilities of anti-OprD-6His polyclonal antibody to OprD-6His. Indirect ELISA detected sensitivity and specificity of the anti-OprD-6His polyclonal antibody. Our results showed that anti-OprD-6His antiserum prepared with denatured inclusion body has high sensitivity and specificity. In China, imipenem resistance of *P. aeruginosa* was mainly due to lack or loss of OprD expression. The anti-OprD-6His polyclonal antibody will facilitate specific detection of difference of porin expression in *P. aeruginosa*.

Abbreviations

PCR, Polymerase chain reaction; **BLAST**, Basic Local Alignment Search Tool; **NCBI**, National Center for Biotechnology Information; **IPTG**, isopropylthio-d-galactoside; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis; **ELISA**, enzyme linked immunosorbent assay; **PBS-T**, phosphate buffered saline-Tween 20.

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