RESEARCH LETTER

Evaluation of P1 adhesin epitopes for the serodiagnosis of *Mycoplasma pneumoniae* infections

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Abstract

Most glycolipid antigens used for serological tests of *Mycoplasma pneumoniae* are not *M. pneumonia*-specific, and can cross-react with other microorganism antigens and body tissues, resulting in false positives. It is important to identify *M. pneumonia*-specific antigen(s) for serological testing and correct diagnosis. Two epitopes, rP1-534 and rP1-513, of P1 adhesin predicted by bioinformatics were successfully expressed and purified, and could be recognized by serum samples from *M. pneumoniae*-infected patients and His tag antibodies by Western blot. There was no cross-reactivity between the anti-recombinant proteins serum and other respiratory antigens. A total of 400 patients were investigated, their respiratory specimens tested by PCR, and sera tested by a commercial test kit; 56 with positive sera and positive respiratory specimens were designated as standard positive serum and 63 patients were designated as standard negative serum. The purified recombinant proteins were used as a combination of antigens or separately to test the serum. Serological test demonstrated that rP1-513 of the C terminal of P1 adhesin is a new candidate antigen with greater sensitivity and specificity for IgG and IgM serodiagnosis of *M. pneumoniae*-infected patients. The results confirmed that rP1-513 could be a useful new antigen for the immunodiagnosis of *M. pneumoniae* infection.

Introduction

Mycoplasma pneumoniae is a major cause of human respiratory tract infectious disease, resulting in pharyngitis, bronchitis, atypical pneumonia, and sometimes death. It accounts for up to 20% of community-acquired pneumonia and is very common among school-aged children and young adults. Approximately 10–30% of pneumonia cases in children and up to 50% of all cases that occur during an epidemic period are caused by *M. pneumoniae* (Vervloet *et al.*, 2007; Walter *et al.*, 2008; Thurman *et al.*, 2009). As this pathogen lacks a cell well, it is intrinsically resistant to beta-lactam antibiotics, and macrolides are usually considered to be first-choice agents for treatment (Pereyre *et al.*, 2007). Therefore, an early and specific diagnosis is necessary for clinicians to prescribe the correct antibiotic treatment.

The standard method for the diagnosis of *M. pneumoniae* is bacterial culture. However, *M. pneumoniae* grows slowly and poorly, and thus is difficult to isolate (She *et al.*, 2010). Serological testing and PCR are commonly used methods for the laboratory diagnosis of *M. pneumoniae* (Kim *et al.*, 2007). However, PCR, especially real-time PCR, requires strict quality control (Nilsson *et al.*, 2008; Al-Marzooq *et al.*, 2011). Serological tests, such as complement fixation and enzyme-linked immunosorbent assays (ELISAs) are other common laboratory tests (Talkington *et al.*, 2004). These two serological methods use membrane preparations, glycolipid extracts or whole cell lysate glycolipids as antigens, which are not *M. pneumonia*-specific (Morrison-Plummer *et al.*, 1986). These glycolipids may cross-react with antigens from other microorganisms and body tissues, resulting in false positives. Therefore, it is important to identify *M. pneumoniae*-specific antigens for correct diagnosis.

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Adherence is the first step for *M. pneumoniae* in the colonization of respiratory epithelium and subsequent infection. Studies have demonstrated that adherence is mediated by a tip terminal structure composed of several proteins (Hu *et al.*, 1977, 1982), including major surface adhesins P1 and P30, the subservient proteins P116, HMW1–HMW5, as well as proteins P40 and P90 (Baseman *et al.*, 1996; Layh-Schmitt *et al.*, 1997; Willby & Krause, 2002; Waldo *et al.*, 2005; Waldo & Krause, 2006). Of them, the P1 protein is a major adhesin that induces a strong humoral immune response during *M. pneumoniae* infection in humans and experimental animals, and so is a potential candidate as a new antigen for serodiagnosis. The presence of high numbers of UGA codons (stop codon in *Escherichia coli*) in *M. pneumoniae* genes makes expression of P1 protein in *E. coli* difficult.

Based on the considerations described above, the aims of this study were to predict the epitopes of P1 proteins using bioinformatics tools, construct and expression recombinant proteins, avoiding the stop codon in *E. coli*, and to investigate the effect of the predicted epitopes separately or in combination as the antigen for an ELISA-detecting system.

Materials and methods

Study group

From March 2010 to May 2011, a total of 400 patients between 5 and 17 years of age, who had been diagnosed previously as having pneumonia or a respiratory infection according to clinical symptoms at the Capital Institute of Pediatrics from the Affiliated Children's Hospital, China, were investigated. The study was approved by the Ethical Committee, and consent from the patients' parents was obtained before the children were included in the study. Basic information of these cases and antibiotic use during this period was collected. Blood was collected intravenously and placed in a sterile tube for 30 min, and the serum was separated by centrifugation at 900 ***g*** for 10 min, and stored at −20 °C for further analysis. Respiratory specimens were collected and tested by Real time PCR for *M. pneumoniae* infection. A total of 119 patient sera samples (56 positive and 63 negative patient sera samples) were tested for the presence of *M. pneumoniae* using a commercial kit (Serodia-Myco II Diagnostic Kit, Fujirebio Inc., Japan) and their respiratory specimens were tested for positive (56) or negative (63) reaction by real time PCR (Dumke, *et al.*, 2007); these sera were designated as positive and negative sera, respectively.

Antigenicity prediction

Antigenicity prediction was performed using the ANTIGENIC program of the Universidad Complutense, Madrid, Spain (http://imed.med.ucm.es/Tools/antigenic.pl). This program predicts potential antigenic regions of a protein, using the method of Kolaskar & Tongaonkar (1990).

Bacteria and growth conditions

The *M. pneumoniae* reference strain FH (ATCC 15531) was grown in pleuro-pneumonia-like organisms (PPLO) medium (Difco, Becton Dickinson, Sparks, MD) at 37 °C. After a slight color change of the medium from red to orange, *Mycoplasma* attached to the bottom was washed twice in room temperature phosphate-buffered saline (PBS) and scraped off the flasks into PBS. Bacteria were pelleted by centrifugation at 3000 ***g*** for 15 min and resuspended in 1 mL of PBS.

Cloning and plasmid construction

The DNA template was released with proteinase K (Invitrogen, Carlsbad, CA) in lysis buffer (10 mM Tris-HC1, pH 8, 1 mM EDTA). Bacterial pellets were digested at 55 °C for 1 h, then at 100 °C for 10 min. Selected parts of the gene, containing the epitopes of the P1 protein as determined by the bioinformatics tools, were amplified by PCR from genomic DNA of *M. pneumoniae*. The PCR products were purified, sequenced and blasted in NCBI (http://blast.ncbi.nlm.nih.gov/Blast), then double-digested with BamHI/XhoI according to the cloned region. The double digestion products were purified and cloned into the pET28c expression vector (Novagen, San Diego, CA) and cleaved with the same enzymes.

Expression and purification of recombinant proteins

Recombinant proteins were produced in *E. coli* BL21 (DE3 pLysS) from the pET28c expression vector containing the selected gene fused to an N-terminal polyhistidine tag. High levels of expression were obtained upon induction with isopropyl-β-D-thiogalactoside (IPTG). Selected proteins were purified by passage of the cell lysate through a nickel affinity column. Eluted proteins was checked for integrity by SDS-PAGE and stained with Coomassie brilliant blue R-250.

Animal immunization

Six-week-old female BALB/c mice were obtained from the Laboratory Animal Center of The Academy of Military Medical Sciences (AMMS, Beijing, China). They were maintained under specific pathogen-free conditions and provided food and water *ad libitum*. The experimental

protocol was approved by the Ethics Board of the Institute of Laboratory Animal Sciences of the Capital Medical University of China. The female BALB/c mice (10 mice per group) were each immunized with 25 μg purified recombinant protein mixed with Freund's adjuvant (Sigma, Shanghai, China) intraperitoneally. Control mice received PBS with FUSHI adjuvant. All mice were immunized with three doses at intervals of 12–14 days. Blood samples were obtained from each mouse by bleeding of the tail vein before immunization and 7 days after the last immunization. The serum was separated by centrifugation of clotted blood and was stored at −20 °C for further analysis. The titers of antibodies against the recombinant protein were determined by ELISA.

Cross-reactivity test of recombinant proteins

The following reference strains were used to assess the cross-reactivity of the recombinant proteins: *M. pneumoniae* FH (ATCC 15531), *Mycoplasma salivarium* (ATCC 23064), *Mycoplasma orale* (ATCC 23714), *Mycoplasma genitalium* (ATCC 33530), *Ureaplasma parvum* (ATCC 27813), *Mycoplasma fermentans* (ATCC 19989), *Mycoplasma hominis* (ATCC 23114), *Chlamydophila pneumoniae* (ATCC 53592), *Mycoplasma penetrans* (ATCC 55252), *Chlamydia trachomatis* (ATCC VR-348B), *Streptococcus pneumoniae* (ATCC 49619), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 35984), *E. coli* (ATCC 11229), *Pseudomonas aeruginosa* (ATCC 27853), *Haemophilus influenzae* (ATCC 49247), *Moraxella catarrhalis* (ATCC 25238), *Mycobacterium tuberculosis* (ATCC 27294), *Candida albicans* (ATCC 10231), Influenza viruses A, B (Flu A, Flu B), Respiratory syncytial virus (RSV) subgroup A and B, Parainfluenza virus 1–3 (PIV1, PIV2, PIV3), Adenovirus (AdV), Rhinovirus (RV), and Coronavirus (CoV) (stored at the Capital Institute of Pediatrics, Beijing, China). These strains were broken by ultrasonic wave and the supernates were then used for ELISA test. Flat-bottomed microplates with 96 wells (Costar) were coated with 100 μL of the above antigens (10 μg mL^{-1}) and left at 4 °C overnight, after blocking with 100 μL of 1% bovine serum albumin (BSA) for 2 h at 37 °C. Anti-recombinant protein mouse serum was applied at 1 : 100 dilution in PBS-Tween for 1 h at 37 °C. Every plate carried replicate wells of a negative control (1 : 50 diluted pre-immune serum). Plates were incubated for 2 h after the addition of the appropriate anti-mouse horseradish peroxidase (HRP)-conjugated IgG (Jackson Immunoresearch, West Grove, PA) at 37 °C, and then developed with o-phenylenediamine (OPD). Reactions were stopped using 2 M H_2SO_4. The optical density at 492 nm was measured with an ELISA plate reader.

Western blot analysis

The selected proteins were transferred to polyvinylidene difluoride membranes after SDS-PAGE, and the membranes were then blocked with blocking buffer (PBS supplemented with 5% dried milk powder) and incubated with human sera or anti-His tag antibody as primary antibodies diluted at 1 : 100 in blocking buffer for an additional hour at 37 °C. The secondary antibody HRP-conjugated goat anti-human IgG was diluted 1 : 2000 and incubated with the strips for 1 h at 37 °C. The membranes were washed three times with washing buffer (Tris-buffered saline, TBS, 0.05% Tween 20) after each of these incubations. Finally, the membranes were washed twice with TBS and detected by chemiluminescence using ECL detection liquid (Amersham, Shanghai, China).

Performance of the recombinant protein ELISA test in human serum

Taking the Serodia-Myco II Diagnostic Kit as the standard, which has a sensitivity of 100% and a specificity of 96.5%, the selected positive/negative human serum were tested with recombinant proteins. Flat-bottomed microplates with 96 wells (Costar) were coated with 100 μL of the recombinant proteins (5 μg mL^{-1}) alone or mixed in PBS and incubated overnight at 4 °C. After blocking with 200 μL of 1% BSA for 1 h at 37 °C, 100 μL human serum samples diluted 1: 100 in PBS were added in duplicate and incubated for 1 h at 37 °C. Then 50 μL per well of the secondary antibody HRP-conjugated anti-human IgG and IgM (Jackson ImmunoResearch), diluted 1 : 10 000, was added and incubated for an additional 1 h at 37 °C. The reaction was visualized with 50 μL OPD substrate incubated for 30 min at 37 °C in the dark and stopped by adding 100 μL of 1 M HCl solution. The optical density at 492 nm was measured with an ELISA plate reader.

Statistical analysis

Data management was performed using an EXCEL spreadsheet. All entries were checked for any keyboard errors. The sensitivity and the specificity of the ELISA were calculated by using the chi-squared test. P values of < 0.05 were considered statistically significant.

Results

Antigenicity prediction research

Results of the immunogenicity analysis using the ANTIGENIC program showed that the epitopes of the P1 adhesin protein are distributed throughout the protein. The

C-terminal region is usually considered more immunogenic than the N-terminal region. Two fragments (P1-513 and P1-534) in the C-terminal region were selected that contained seven epitopes according to their scores in the full-length P1 adhesin protein antigenicity prediction. The fragment P1-513 was amplified from the *M. pneumoniae* strain ATCC15531 chromosome by PCR using the forward primer 5′ CGGGATCCATAAGGGGGTGTGGGC GGA 3′ (BamHI restriction sites are underlined) and reverse primer 5′ TCCGCTCGAGGGTGGAGGAGGTG TTTC 3′ (XhoI restriction sites are underlined). The fragment P1-534 was amplified using the forward primer 5′ CGGGATCCATAGCACGAGTGACGGAAA 3′ (BamHI restriction sites are underlined) and reverse primer 5′ TCCGCTCGAGAGGTCACTGGTTAAACGGA 3′ (XhoI restriction sites are underlined).

Expression and purification of recombinant proteins

The two fragments of the P1 adhesin gene, P1-513 and P1-534, were amplified by PCR and then expressed in *E. coli* BL21 using a pET-28 vector. Both recombinant protein fragments were successfully expressed in *E. coli*. The apparent molecular mass was 26 kDa for both rP1-513 and rP1-534. The two proteins were expressed in the soluble fraction and then purified by Ni-NTA affinity chromatography to apparent homogeneity (Fig. 1). The purified proteins were recognized by anti-*M. pneumoniae* antibodies and anti-His antibody (Fig. 1).

Western blot analysis results with human sera

The Western blot results for the 12 representative positive patient serum samples and are shown in Fig. 1. *Mycoplamsa pneumoniae*-infected patient sera reacted with the recombinant P1 adhesin protein by Western blotting, whereas sera from uninfected patients did not show reactivity. These results indicate that proteins rP1-513 and rP1-534 are immunogenic (eight negative patient serum samples not shown).

Immune responses to rP1-513 and rP1-534

After intraperitoneal immunization with the two recombinant epitopes in mice, the specific serum IgG antibody titers for rP1-513 and rP1-534 were 1 : 1600 and 1 : 2000, respectively, which is significantly higher than for controls ($P < 0.01$). There was no significant difference in serum IgG antibody titers between the two immunization groups.

Cross-reactivity of recombinant proteins

Specificity analysis demonstrated that the serum from mice immunized with rP1-513 and rP1-534 had a strong reactivity with *M. pneumoniae* FH (ATCC 15531), but no cross-reactivity with other antigens tested (see Materials and methods section).

Performance of the recombinant protein ELISA test with human serum

The cut-off value was calculated with the following formula: sera from the healthy blood donors mean optical density value + two standard deviations. After the preexperiment, this value was found to be 0.304 for IgG and 0.216 for IgM. Taking the Serodia-Myco II Diagnostic Kit as the standard, the ELISA results showed that when the rP1-513 antigen was used alone or combined with rP1-534, 46 of 56 IgG-positive sera samples were found to be reactive to this protein, whereas 59 of 63 patient sera samples were found to be negative. Similarly, when rP1-534 was used as an antigen alone, 43 of 56 IgG-positive sera samples were found to be reactive to this protein, whereas 55 of 63 patient sera samples were found to be negative.

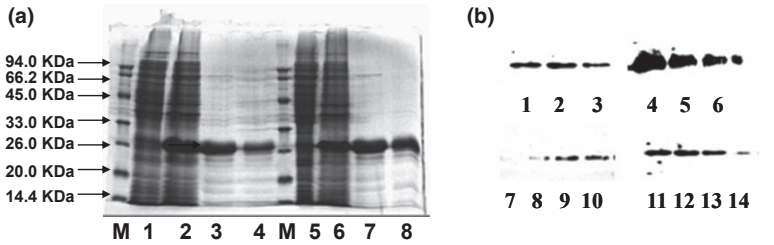

Fig. 1. (a) Expression and purification of the recombinant proteins. M: molecular weight marker, lane 1: rP1-534 protein before induction, lane 2: rP1-534 protein after induction, lane 3 and lane 4: elution proteins, lane 5: rP1-513 protein before induction, lane 6: rP1-513 protein after induction, lane 7 and lane 8: elution proteins. (b) Western blot analysis. Lane 1, 2: rP1-513 with human positive sera, lane 3: rP1-513 with anti-His antibody, lane 4, 6: rP1-534 with human positive sera, lane 5: rP1-534 with anti-His antibody. Lane 7-10: rP1-513 with human positive sera, lane 11-14: rP1-534 with human positive sera.

For the detection of the IgM antibodies in patient sera, use of rP1-513 alone had a better result, as 40 of 56 positive sera samples were found to be reactive to this protein, compared with 56 of 63 patient sera samples, which were negative. When the rP1-534 antigen was used alone or combined with rP1-513, 33 of 56 positive sera samples were found to be reactive to the this protein, whereas 57 of 63 patient sera samples were found to be negative (Table 1).

Discussion

Respiratory disease due to *M. pneumoniae* can be assessed by serological methods; of these, the complement fixation test and ELISA are the most widely used. To identify a new *M. pneumoniae*-specific antigen for serodiagnosis, adhesin proteins have been analyzed (Schurwanz *et al.*, 2009; Tabassum *et al.*, 2010). Besides their cytoadherence functions, they are also strongly immunogenic. In the current study, the N-terminal (P1-N1) and C-terminal (P1-C1) regions of P1 adhesin were analyzed as new antigens to detect *M. pneumoniae* in human serum. The C-terminal region of P1 adhesin (rP1-C) was shown to have better sensitivity for immunodiagnosis (Chaudhry *et al.*, 2005). Varshney *et al.* (2008) reported that the P30 antigen could also be used for *M. pneumoniae* detection. Drasbek *et al.* (2004) used a recombinant protein derived from the P116 protein and the P1 protein in two ELISA tests, demonstrating that both proteins were suitable candidate antigens, although the rP1-ELISA showed the best discrimination between positive and negative samples (Drasbek *et al.*, 2004). Nuyttens *et al.* (2010) identified the *M. pneumoniae* ATP synthase beta-subunit (AtpD) by serologic proteome analysis, and their results suggest that AtpD can be used as an antigen for the immunodiagnosis of early and acute *M. pneumoniae* infection in association with adhesin P1. The above research indicated P1 proteins as a promising specific antigen candidate for the diagnosis of *M. pneumoniae* infection.

Table 1. Performance of the rP1-513 and rP1-534 ELISAs in children's serum

Ig class	Antigen	Positive (*n*)	Negative (*n*)	Sensitivity (%)	Specificity (%)
IgG	rP1-513	46	59	82.14	93.65
IgG	rP1-513+ rP1-534	46	59	82.14	93.65
IgG	rP1-534	43	55	78.18	87.3
IgM	rP1-513	40	56	71.42	88.88
IgM	rP1-513+ rP1-534	33	57	58.92	90.48
IgM	rP1-534	33	57	58.92	90.48
	Standard serum	56	63		

As 21 UGA codons are present in the P1 gene, it is difficult to express the whole length of the P1 protein in *E. coli*. Bioinformatic tools are useful when identifying specific and immunogenic epitopes (Frikha-Gargouri *et al.*, 2008). In this study, we used this method for the prediction of specific and immunogenic regions in the P1 protein of *M. pneumoniae*. This program allowed us to detect several regions distributed throughout the P1 protein. A previous study indicated that the P1 C-terminal region appears more immunogenic than the N-terminal region, and thus two epitopes of the P1 C-terminal region were used for serodiagnosis of *M. pneumoniae* infection. After induction with IPTG, the two proteins were well expressed with a His tag from the pET-28a system and could be identified by the anti-His antibody and human positive serum, further demonstrating that the two proteins are the target proteins.

Serological methods are at present based on the total cell lysate, which sometimes shows cross-reactivity with other mycoplasma and microorganism antigens (Beersma *et al.*, 2005; Yamazaki *et al.*, 2006). The use of new specific antigens should avoid this weakness. In this study, we used the two P1 adhesin proteins to immunize mice and induce high antibody levels. The specific antibodies showed no cross-reactivity with other mycoplasmas and microorganisms that commonly exist in the respiratory tract, and these results indicated the two proteins could be used as new antigens for serodiagnosis and as vaccine candidates.With regard to standard positive and negative sera, previous studies only used one commercial ELISA test kit (Chaudhry *et al.*, 2005; Varshney *et al.*, 2008). However, single ELISA kit analyses can sometimes display false positive results (Beersma *et al.*, 2005). In our study, we used the Serodia-Myco II ELISA kit combined with real-time PCR. All the standard positive serum samples were detected as positive by serodiagnosis, along with a positive diagnosis in sputum or bronchoalveolar lavage fluid by PCR, which further ensures the reliability of the reference serum.

Several research workers have analyzed serodiagnosis using the P1 protein. Chaudhry *et al.* (2005) indicated that the C-terminal (P1-C1) region was a good antigen, as the sensitivity and specificity were 72.7 and 100%, respectively, compared with the Serion ELISA Classic kit (Chaudhry *et al.*, 2005). Drasbek *et al.* (2004) tested 125 atypical pneumonia patients using the C-terminal part of P1 protein and identified 55 positive samples by the complement fixation test and 57 by rP1-IgG (Drasbek *et al.*, 2004). Nuyttens *et al.* (2010) reported the sensitivity and specificity of the C-terminal part of P1 protein were 69 and 90%, respectively, when tested for IgG antibodies in

54 patients and 86 controls (Nuyttens *et al.*, 2010). In our study, two epitopes were used for serodiagnosis, with rP1-513 having a higher sensitivity than rP1-534 for IgG and IgM antibodies. The use of the two antigens in combination did not show greater sensitivity compared with rP1-513 alone. These results suggest that rP1-513 could be a novel antigen candidate for the serodiagnosis of *M. pneumoniae*. Although several studies have recently reported that a combination of selected antigens provided higher sensitivity than single antigens, we did not observe this phenomenon. This may be due to the epitope rP1-513 having greater combination ability than rP1-534, and the proteins just simply mixed with the antigen but did not bind covalently.

In summary, the results of our study demonstrated that two epitopes of the C-terminal of P1 adhesin could be used for the serodiagnosis of *M. pneumoniae*-infected patients, although rP1-513 had a better sensitivity and specificity.

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