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Cloning, Expression, and Immunological Characterization of the P30 Protein of *Mycoplasma pneumoniae*▽

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***Mycoplasma pneumoniae*, a self-replicating cell wall-deficient prokaryote, has a differentiated terminal organelle that is essential for cytadherence and gliding motility. P30, an important protein associated with the terminal organelle, is required for the cytadherence and virulence of *M. pneumoniae*. P30 is a transmembrane protein with an intracytoplasmic N terminus and an exposed C terminus. In the present study, we amplified and sequenced the full-length *p30* gene of *Mycoplasma pneumoniae* directly from 18 Indian asthmatic patients. Sequence diversity was observed in the *p30* genes from 16 clinical samples when the sequences were compared with the sequence of strain M-129. We also successfully expressed a fragment of the *p30* gene (P30B) that includes the complete C-terminal proline-rich amino acid sequences in different *Escherichia coli* expression systems. The maltose binding protein (MBP)-P30B fusion protein was recognized by *M. pneumoniae*-infected patient sera in immunoblots, and the protein was immunogenic in mice. We further analyzed the reactivity of the MBP-P30B fusion protein with patient sera in an enzyme-linked immunosorbent assay (ELISA) and compared it with the reactivity obtained with a commercial kit (the Serion ELISA Classic kit). The sensitivity and the specificity of the in-house ELISA were 78.57% and 89.47%, respectively. This study suggests that the P30 protein can be used as an antigen along with other adhesin proteins for the immunodiagnosis of *M. pneumoniae* infection.**

Mycoplasma pneumoniae is a unique cell wall-deficient bacterium that causes chronic respiratory infections, including bronchitis and primary atypical pneumonia, in humans. It accounts for up to 30% of all community-acquired cases of pneumonia and is very common among primary school children and their parents. *M. pneumoniae* is also responsible for other respiratory tract infections, such as tracheobronchitis, bronchiolitis, and less severe upper respiratory tract infections in older children and young adults (7, 14). A wide variety of extrapulmonary manifestations, such as myocarditis and acute disseminated encephalomyelitis, have also been associated with *M. pneumoniae* infection (22). *M. pneumoniae* infection sometimes results in chronic pulmonary lung damage, and a growing body of evidence supports a correlation of *M. pneumoniae* infection with the exacerbation and recurrence of asthma (5, 20).

M. pneumoniae has a complex cellular organization and possess a specialized tip organelle, a membrane-bound cell extension distinguished by an electron-dense core (2, 4). The well-defined apical organelle mediates adherence to host cells (cytadherence) and gliding motility (6). Analysis of an *M. pneumoniae* hemadsorption (HA)-negative mutant has resulted in the identification of a number of proteins associated with cytadherence, such as P1, P30, P116, and HMW 1 to 3 (3, 11, 16, 18). These proteins are localized on the apical organelle, which is the leading end in the gliding motility. Among these organelle-associated genes, P30 is required for cytadherence and is associated with proper cell development (19). *M. pneumoniae* mutant M7, which exhibited a truncated P30 protein, had lost the ability to adsorb to erythrocytes and to bind to epithelial cells (17). P30 has also been shown to be associated with gliding motility. *M. pneumoniae* HA mutant II-3, which lacks the P30 protein, has been shown to be nonmotile, while HA mutant II-7, which produces an altered P30 protein, showed 50-fold less motility than that of the wild type (12, 13).

The *p30* gene contains an open reading frame of 825 nucleotides that codes for a protein of 275 amino acids with a calculated molecular mass of 29.743 kDa. The gene has only one UGA codon (nucleotides 46 to 48), which codes for tryptophan in the case of *M. pneumoniae* (8). P30 is a membrane-bound protein that orients with the N terminus in the cytoplasm and the C terminus exposed on the cell surface. The protein contains three types of repeat sequences at its carboxy end. One stretch of Pro-Gly-Met-Ala-Pro-Arg occurs seven times, whereas two stretches of Pro-Gly-Met-Pro-Pro-His and Pro-Gly-Phe-Pro-Pro-Gln are repeated three times. On the basis of the orientation of the protein, it is envisaged that the C terminus of the P30 protein is exposed on the surface of *M. pneumoniae.* It is likely to generate an immune response in *M. pneumoniae*-infected patient sera and thus can be exploited for the immunodetection of *M. pneumoniae*-infected human sera.

In the present study, we sequenced the full-length *p30* gene of *M. pneumoniae* directly from 18 clinical samples (throat swabs) from asthma patients attending a pediatric asthma clinic at the All India Institute of Medical Sciences (AIIMS),

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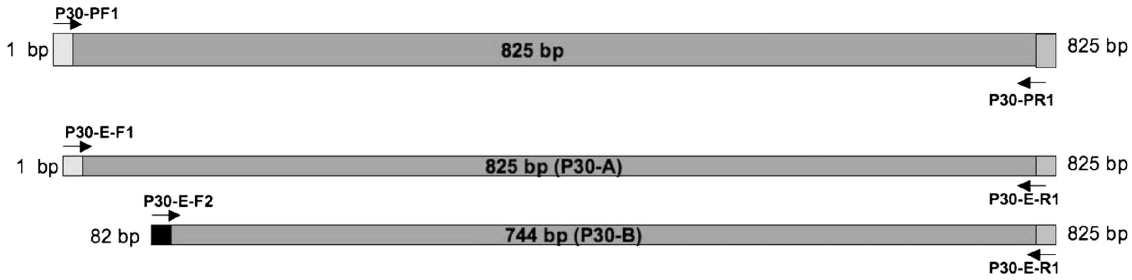

FIG. 1. Schematic diagram showing the primers used for amplification of the *p30* gene and fragments for expression.

New Delhi, India. We also analyzed the reactivity of P30 with *M. pneumoniae*-infected patient sera.

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MATERIALS AND METHODS

Growth of *M. pneumoniae*. A standard strain of *M. pneumoniae*, FH-Liu, was procured from the National Collection of Type Cultures (London, United Kingdom), reconstituted in PPLO broth, incubated aerobically at 37°C, and monitored at regular intervals (24 h) for a color change (red-yellow). After 5 to 7 days, the broth was subcultured on PPLO agar plates. The plates were incubated in 5% CO_2 at 37°C in candle jars with a sterile cotton ball soaked in water to maintain the humidity and were examined microscopically at 5-day intervals at ×10 magnification, without opening the plates, for the presence of typical round granular colonies. Suspected colonies were stained with Diene's stain and checked by light microscopy. Further confirmation of the isolates as *M. pneumoniae* was done by the growth inhibition test.

Study group. A total of 150 patients between 5 and 15 years of age who had previously been diagnosed with moderate or severe persistent asthma (1) and who were attending the Pediatric Chest Clinic, AIIMS, during the period from November 2004 to May 2006 were investigated. The study was approved by the AIIMS Ethical Committee for Human and Animal Ethical Clearance, and the consent of the patients' parents was obtained before the children were included in the study. Blood was collected intravenously and placed in a plain sterile tube. The serum was separated by centrifugation at 3,000 rpm for 10 min and was stored at −20°C until use. Throat swab specimens were collected from the study group and were transported in PPLO broth medium for *M. pneumoniae* culture and PCR.

PCR amplification of the *p30* gene of *M. pneumoniae*. Throat swabs from the infected patients were inoculated and incubated at 37°C in PPLO broth for 24 h, and DNA extraction was carried out by the method previously described by Stauffer et al. (21). Likewise, the standard strain was grown in PPLO broth for 72 h, and the DNA was extracted accordingly. The extracted DNA was stored at −20°C until use.

On the basis of the sequences available in the database, oligonucleotide primers were designed to amplify the *p30* gene from the *M. pneumoniae* genomic DNA (8). Primers P30-PF (forward primer; 5′-ATGAAGTTACCACCTCGAAGAAGC-3′) and P30-PR (reverse primer; 5′-TTAGCGTTTTGGTGGAAAACCGGGTTG-3′) were used for the amplification of the *p30* gene (Synthesized from Bio Basic, Canada). PCR was performed in a 50-μl reaction mixture containing 1 U of *Taq* polymerase, 1× PCR buffer, 200 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 10 pmol of each primer, and 6 μl of extracted DNA. The reaction conditions were standardized at an initial denaturation of 94°C for 10 min, followed by 35 cycles of 94°C for 30 s, 54°C for 45 s, and 72°C for 1 min 20 s. A final extension was done at 72°C for 10 min. The PCR products were analyzed in a 1.2% agarose gel and purified with a gel extraction kit (Qiagen, Hilden, Germany). The amplified product was further cloned in the pGEMT-E vector. For the expression of the *p30* gene and its fragment in heterologous expression systems (pQE-30), the following primer set was synthesized: forward primer P30-EF1 (5′-CCATGGGACCATGAAGTTACC ACCTCGAAGAAAGCTTAAACTGTTTTTATTAGCCTGGATG-3′) and reverse primer P30-ER1 (5′- GTCGACTGCAGCGTTTTGGTGGAAA ACCGGG TTG-3′). For the expression of the *p30* gene (P30B) fragment in a pMAL-p2x vector system, forward primer P30-EF2 (5′-CCATGGGATCCGCAACCTTAATTTTGG TACAGCAC-3′) and reverse P30-ER1 primer were used (Fig. 1). Note that the expression primer, P30-EF2, was designed to exclude the UGA codon region of the *p30* gene of *M. pneumoniae*.

Sequencing of *p30* gene from clinical samples. The PCR products amplified from 18 clinical samples were analyzed in a 1.2% agarose gel and purified with a QIAquick PCR purification kit (Qiagen). The purified *p30* gene was subsequently sequenced by automated DNA sequencing (ABI Prism 310 instrument; Applied Biosystems, Foster City, CA). For each clinical sample, three independent amplification and sequencing reactions were carried out.

The nucleotide sequences of the *p30* gene amplified from the clinical samples were compared with the *p30* sequence of *M. pneumoniae* strain M129 (GenBank accession no. M57245). Sequence analysis was performed with the Clustal W and Gene Doc tools, and protein translation was done with the EditSeq tool of DNAStar software (DNAStar Inc., Madison, WI) by using the *Mycoplasma* coding table.

Cloning and expression of *p30* gene and its fragment of *M. pneumoniae*. The amplified and purified DNA fragment was ligated into the pGEMT-E vector system (Promega, Madison, WI), according to the manufacturer's protocol. Briefly, a 10-μl reaction mixture was set up with 10 ng vector DNA, 5.0 μl of ligation buffer (2×), 1.0 μl T4 DNA ligase (1 U), and 3 μl of the PCR-amplified product (insert DNA, 300 ng). The ligation reaction mixture was incubated at 4°C for 16 h and was transformed into chemically competent DH5α cells.

Colonies were selected by blue-white selection screening on Luria-Bertani (LB) agar containing 100 μg/ml of ampicillin, 20 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and 200 mg/ml of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma). Individual white colonies from the plate were inoculated in 5 ml LB broth containing 100 μg/ml of ampicillin and were allowed to grow for 16 h at 37°C. Plasmids were extracted with a Qiagen mini-prep plasmid extraction kit and digested with EcoRI to ensure the presence of the insert, and sequencing was done to rule out any mutation in the sequence.

For expression, subcloning of the full-length P30 protein (P30A) and a fragment of the *p30* gene (P30B) was done in the pQE-30 vector and the pMAL-p2x fusion vector, according to the manufacturer's instructions, with some modifications (Fig. 1). Briefly, the pGEMT-E construct of the *p30* gene was digested with the BamHI and SalI restriction endonucleases, and the digestion mixture was separated on a 1% agarose gel. The full-length P30 protein (825 bp) and the fragment corresponding to the *p30* gene (744 bp) were excised, and DNA was eluted by using a gel extraction kit (Real Biotech Corporation, Germany). The pQE-30 and pMAL-p2x vectors were also digested with the same restriction endonucleases used to digest the insert. The digested vectors and insert were ligated at 16°C overnight. The ligation mixtures were transformed into *Escherichia coli* M-15 cells (for the pQE-30 vector) and *E. coli* TB-1 cells (for the pMAL-p2x vector). Transformants were selected on LB agar plates containing 100 μg/ml ampicillin and 25 μg/ml of kanamycin for the pQE-30 vector and 100 μg/ml ampicillin for the pMAL-p2x fusion vector. The recombinants were screened by restriction digestion and sequencing.

M-15 cells and TB-1 cells containing the recombinant plasmids were cultivated in 5 ml of LB broth at 37°C with shaking until the optical density (OD) reached 0.4 to 0.6. Expression was induced by treatment with 1 mM IPTG for 5 h at 37°C. Bacterial cells were collected by centrifugation and were further subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to analyze the expression of the recombinant proteins.

SDS-PAGE and Western blotting. The crude induced and uninduced lysates of the samples were dissolved in 30 μl of SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2.3% [wt/vol] SDS, 5% [vol/vol] β-mercaptoethanol, and 0.05% [wt/vol] bromophenol blue) and boiled for 10 min. The proteins were resolved on a 10% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250. For immunoblotting, the protein was separated on a 10% SDS-

polyacrylamide gel, and the fractionated proteins were transferred from the gel onto the nitrocellulose membrane (Amersham) in a Transblot apparatus (Mini Protein III; Bio-Rad). The membrane was blocked in blocking buffer (1× phosphate-buffered saline [PBS], 0.05% Tween 20, 5% milk) for 2 h. The blots were washed and incubated with primary antibody for 1 h: anti-His (1:5,000 dilution; Qiagen), anti-maltose binding protein (anti-MBP) antibody (1:5,000 dilution; New England Biolabs), anti-*M. pneumoniae* antibody (1:5,000 dilution; National Collection of Type Cultures), and patient sera (1:100 dilution). Later, the blots were washed and incubated for 1 h with secondary antibody (1:2,000 dilutions of anti-mouse, anti-rabbit, or anti-human antibody) conjugated to horseradish peroxidase. The bands were visualized with 3,3′-diaminobenzidine tetrahydrochloride–H_2O_2 (Sigma-Aldrich).

Purification of recombinant proteins. After confirmation of the recombinant protein by Western blotting, the rMPN30–pQE-30 recombinant protein was purified by using a nickel-nitriloacetic acid column (Qiagen) and the rMPN30–pMAL-p2x recombinant protein was purified on an amylose resin column (New England Biolabs). For large-scale production, cells expressing the P30 protein were grown in a 100-ml culture and were induced with 1 mM IPTG. The bacterial cells were then harvested by centrifugation at 5,000 × *g*, and the pellets were resuspended in a buffer consisting of 10 mM Tris (pH 7.5) and 50 mM NaCl and lysed by sonication with 1-min pulses at 1-min intervals 10 times. The soluble and insoluble fractions were separated by centrifugation at 20,000 × *g* and were analyzed by SDS-PAGE.

The recombinant P30B protein (rMPN30–pQE-30) was purified under denaturing conditions by metal affinity chromatography with nickel-nitriloacetic acid resin (Qiagen). Elutions were carried out with imidazole buffer (8 M urea, 20 mM Na_2HPO_4, 5 M NaCl, 500 mM imidazole). The protein was dialyzed in dialysis buffer containing 50 mM Tris and 1 mM EDTA for 48 h, with buffer changes every 12 h to remove the urea and imidazole. The purity of the recombinant protein was evaluated by SDS-PAGE.

Another recombinant P30B protein (rMPN30–pMAL-p2x) was purified under soluble conditions by using affinity chromatography with amylose resin (New England Biolabs), according to the manufacturer's protocol, with some modifications. Elutions were carried out with maltose buffer (10 mM maltose, 10 mM Tris, and 500 mM NaCl). Fractions containing the purified recombinant protein were pooled and dialyzed against PBS. The column eluent containing rMPN30–pMAL-p2x was concentrated by centrifugation with Centricon-30 concentrators (Amicon Inc., Beverly, MA). The concentrated rMPN30–pMAL-p2x protein was run on an SDS-polyacrylamide gel stained with Coomassie blue R-250, and the desired protein band was excised from the gel with a sterile razor blade and electroeluted by using a GeBAflex-tube kit (Gene Bio-Application Ltd., Israel). The protein concentration was determined by the Bradford method.

Immunization of mice. All animal experiments were carried out with the approval of the Animal Ethics Committee, in accordance with the rules and regulations set forth by the AIIMS Animal Ethics Committee. Immunization was carried out with 6- to 8-week-old female Swiss albino mice, which were maintained in the animal facility of AIIMS. The Swiss albino mice were administered 40 μg MBP-P30B fusion protein emulsified in 200 μl of complete Freund's adjuvant (CFA; Sigma) intraperitoneally. Subsequent booster injections were given on the 21st, 35th, and 49th days by injecting 40 μg of the MBP-P30B fusion protein emulsified in 200 μl of incomplete Freund's adjuvant (IFA; Sigma) intraperitoneally. Control mice were injected with CFA and IFA in PBS according to the immunization schedule. Blood samples were obtained from each mouse by bleeding of the tail vein at 14, 21, 35, 42, and 56 days. 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 enzyme-linked immunosorbent assay (ELISA).

Comparative ELISA with purified P30 fusion protein and commercial IgM assay. Serum was separated by centrifugation of clotted blood from patients at 3,000 rpm for 10 min and was subjected to an immunoglobulin M (IgM) ELISA (ELISA Classic; Serion, Germany) for the detection of anti-*M. pneumoniae* antibodies. A total of 47 patient serum samples (28 positive and 19 negative patient serum samples tested for the investigation of *M. pneumoniae* with the commercial Serion ELISA Classic kit) were included in this study. Due to inadequate sample volumes, the remaining 103 specimens could not be tested for P30 antibodies. MBP and the alpha subunit of the beta-galactosidase protein were used as negative controls in the ELISA experiments.

The antibody response to the recombinant fusion protein (rMPN30–pMAL-p2x) was evaluated by an ELISA for IgM antibodies. The experiment was done in duplicate. A total of 100 μl of coating buffer (0.1 M bicarbonate, pH 9.3) containing 100 ng of the P30 protein was added to 96-well microtiter plates, and the plates were incubated overnight at 4°C. The plates were then washed with PBS–0.05% Tween 20 (PBS-T) and blocked with 5% bovine serum albumin in PBS (pH 7.4) for 2 h. The plates were washed twice with PBS-T and once with PBS and were then incubated with the individual patient sera (1:100 dilution) at 37°C for 1 h. The wells were washed and then incubated with horseradish peroxidase-conjugated anti-human IgM (Sigma) diluted 1:3,000 in PBS-T for 1 h at 37°C. The plates were washed three times with PBS-T, and the color was developed by adding 100 μl of 3,3′,5,5′-tetramethylbenzidine–H_2O_2 substrate (Banglore Genei, India) and incubation in the dark for 20 min. The reaction was stopped by adding 100 μl of 2 N H_2SO_4, and the absorbance was read at a wavelength of 450 nm with an ELISA reader (Bio-Tek Microplate reader). The sample was considered positive if the OD at 450 nm was >0.5.

Statistical analysis. Data management was done on an Excel spreadsheet. All entries were checked for any keyboard errors. The sensitivity and the specificity of the in-house P30 ELISA were calculated by using Epi-6 software.

Nucleotide sequence accession number. The sequence presented in this study has been submitted to the NCBI GenBank database and can be found under accession no. EF 614306.

RESULTS

PCR amplification and analysis of *p30* gene from Indian clinical samples. The full-length *p30* gene (825 bp) was amplified by PCR from genomic DNA isolated from 18 clinical samples that were PCR positive for the *p1* gene (Fig. 1). Of the 18 *M. pneumoniae*-infected clinical samples, 2 samples showed sequences similar to that of the *p30* gene of strain M-129. Sixteen clinical samples showed four base substitutions at positions 239, 323, 583, and 696 that resulted into three amino acid changes at positions 80, 108, and 195 (valine-glycine, leucine-serine, and proline-serine, respectively). The last nucleotide substitution was a silent mutation that did not result in a change at amino acid position 232 (glycine-glycine). The sequences of 16 samples showed similarity to the sequence of the *p30* gene of strain FH-Liu, which was also sequenced in the present study (Fig. 2). Three independent sequencing reactions were carried out for each clinical sample.

Expression and purification of recombinant P30 protein fragment. The full-length *p30* gene, i.e., P30A (825 bp), and a smaller fragment of the *p30* gene, i.e., P30B (744 bp), were amplified by PCR and cloned into an *E. coli* expression vector, pQE-30 (Fig. 1). No significant expression was observed after IPTG induction for the full-length P30 protein. Nevertheless, a moderate level of expression was seen in the case of the P30B (rMPN30–pQE-30) protein, as indicated by Western blot analysis with anti-penta-His antibody (Fig. 3B). The apparent molecular mass of the protein was approximately 40 kDa. However, upon fractionation, the protein was found in the pellet fraction of *E. coli*. The protein was subsequently purified on a nickel-nitriloacetic acid affinity column under denaturing conditions (Fig. 3A), and the denatured protein was recognized by anti-*M. pneumoniae* antibody (Fig. 3C). Our attempts to purify and refold the protein under nondenaturing conditions repeatedly failed.

Next, we expressed the P30B protein as an MBP fusion protein (rMPN30–pMAL-p2x) in *E. coli* by cloning the gene fragment in the pMAL-p2x vector. The fusion protein was expressed as a soluble protein of approximately 87 kDa, as detected with anti-MBP antibody (Fig. 4A). The rMPN30–pMAL-p2x fusion protein was purified on an amylose column, and the protein was purified up to a level of ~80% (Fig. 4B). To further purify the protein to homogeneity, the rMPN30–pMAL-p2x fusion protein was run on a 10% SDS- polyacrylamide gel, and the gel was stained with Coomassie blue R-250. The major band pertaining to the MBP-P30 fusion protein was

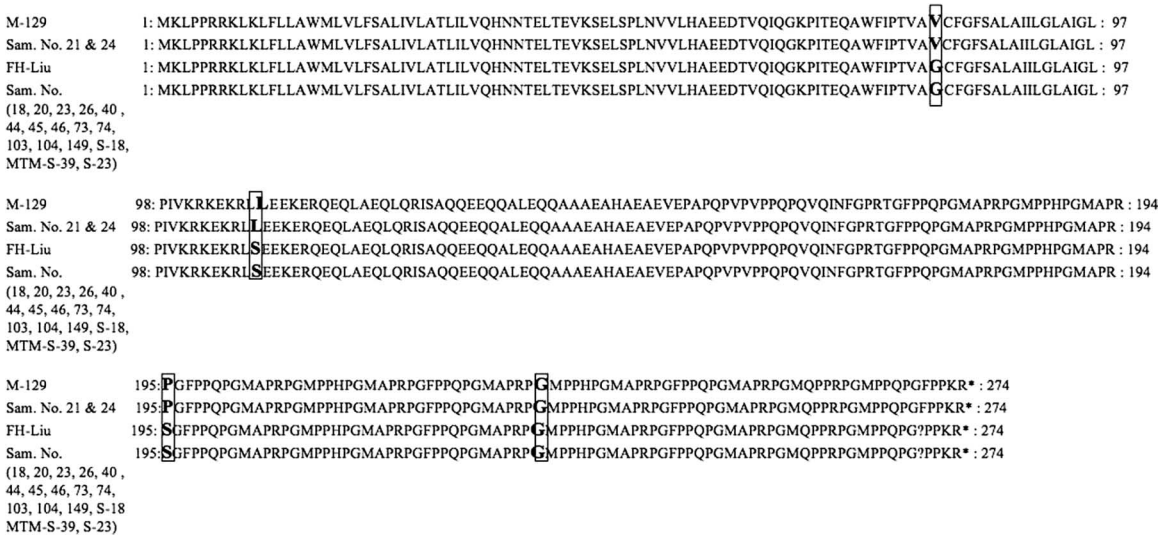

FIG. 2. Amino acid sequence variations among 18 clinical samples. Sam. No., sample number.

cut and electroeluted from the gel. Figure 4 shows the Coomassie blue-stained SDS-polyacrylamide gels of the eluted P30 protein. The purified protein was recognized well by anti-*M. pneumoniae* antibodies (Fig. 4D).

Immunoblot analysis of recombinant P30 protein with *M. pneumoniae*-infected patient sera. To know whether the P30B (rMPN30–pMAL-p2x) protein induced an immunological response, we analyzed the reactivity of the rMPN30–pMAL-p2x fusion protein with sera from 28 patients (18 positive and 10 negative sera). Immunoblot analyses of the eight representative patient serum samples are shown in Fig. 5. *M. pneumoniae*-infected patient sera recognized the protein very well on Western blots, while sera from uninfected patients did not show reactivity with the recombinant protein. These results indicate that *M. pneumoniae*-infected patients have circulating anti-P30 antibodies, thereby suggesting that the P30 protein is immunogenic.

Immunogenicity of rMPN30–pMAL-p2x fusion protein. To know whether the P30 protein generates an immune response in mice, the recombinant P30 protein was formulated in CFA and was injected into mice. The immune response against the MBP-P30B protein is shown in Fig. 6. High antibody responses were observed with the CFA or the IFA formulation. The time course of the immune response showed that the antibody titers gradually increased with the first boost and peaked after the second boost. The titers started to decrease at day 56, i.e., 1 week after the third boost. The endpoint titer was >256,000.

Comparative ELISA analysis. A comparative evaluation of the reactivity of the patient sera with the P30 protein and was carried out with the Serion ELISA Classic kit to determine whether the P30 protein can be used as an antigen for the development of an immunodiagnostic assay for *M. pneumoniae* infection. A total of 47 patient serum samples were analyzed for their reactivities to the rMPN30–pMAL-p2x fusion protein in an

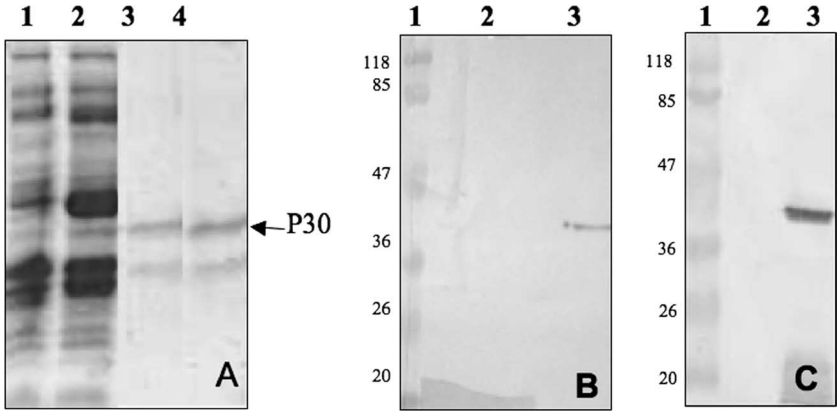

FIG. 3. (A) SDS-polyacrylamide gel showing the purification of the P30 protein from *E. coli* extracts. Lanes: 1, initial flowthrough; 2, wash 1; 3, elution 1; 4, elution 2. (B and C) Western blots of *E. coli* extracts showing the expression of the P30 protein with antihistidine antibody (B) and anti-*M. pneumoniae* antibodies (C). Lanes: 1, prestained protein marker; 2, uninduced P30 protein; 3, induced P30 protein. The numbers on the left are molecular masses (in kilodaltons).

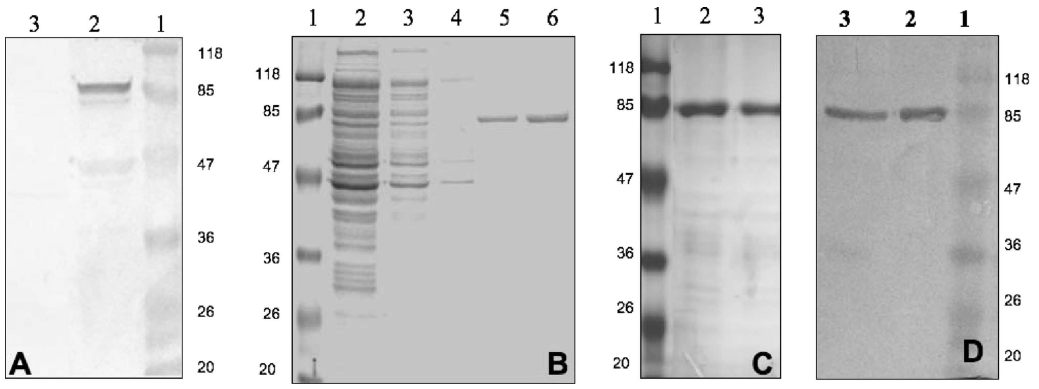

FIG. 4. (A) Western blot of *E. coli* extracts showing the expression of the P30 protein with anti-MBP antibody. Lanes: 1, prestained protein marker; 2, induced P30 protein; 3, uninduced P30 protein. (B) SDS-polyacrylamide gel showing the purification of the P30 fusion protein. Lanes: 1, marker; 2, initial flowthrough; 3, wash 1; 4, wash 2; 5, elution 1; 6, elution 2. (C) Electroelution of the P30 protein from the purified MBP fusion construct, Lanes: 1, standard protein marker; 2 and 3, eluted P30 fusion protein. (D) Western blot of *E. coli* extracts showing the expression of the P30 protein with anti-*M. pneumoniae* antibodies. Lanes: 1, prestained protein marker; 2 and 3, induced P30 protein. The numbers on the left are molecular masses (in kilodaltons).

ELISA. As shown in Fig. 7, a total of 22 of 28 positive serum samples (based on the reactivity of the Serion ELISA Classic kit) were found to be reactive to the P30 protein fusion, while 17 of 19 patient serum samples were found to be negative by our ELISA, and they also showed no reactivity by use of the commercial kit.

Statistical analysis. A comparative analysis of the P30 protein ELISA and the assay with the Serion IgM ELISA Classic kit, which was used as the standard, was done by applying the diagnostic test. The cutoff value was taken as an absorbance value of 0.5. At 0.5 absorbance, the cutoff points for the sensitivity and the specificity of P30 protein ELISA were determined to be 78.57% and 89.47%, respectively.

DISCUSSION

P30, an adhesin protein on the *M. pneumoniae* surface, localizes primarily to the terminal organelle. The protein has a C-terminal proline-rich repeat region that is exposed on the cell surface. The characterization of HA-negative mutants revealed that P30 is an important adhesin and is also involved in the gliding motility of the organism (12). In the present study, we amplified and sequenced the *p30* gene of *M. pneumoniae* directly from Indian clinical specimens. PCR amplification and sequence analysis of the *p30* gene revealed that there is a predominance of strain FH-Liu among Indian clinical isolates. Of the 18 samples studied, the *p30* gene sequence from 16 samples showed similarity to the strain FH-Liu *p30* gene sequence, while the *p30* gene sequences amplified from 2 clinical samples showed similarity with the *p30* gene sequence of strain M-129 (subtype 1). Compared with the sequence of strain M-129, four base substitutions in the *p30* gene that resulted in three amino acid variations were found in the *M. pneumoniae* genomes present in Indian patients. These substitutions were also present in the *p30* gene sequence of the standard FH-Liu strain of *M. pneumoniae*. Of these four differences, the last two nucleotide substitutions that resulted in a single amino acid change have also been reported in one of the clinical isolates studied by Dumke et al. (10). To the best of our knowledge, this is the first report of the *p30* gene sequence among Indian clinical samples. Among these sequences, we did not find any mutants of the *p30* gene with truncated sequences.

Since *M. pneumoniae* is an organism that is difficult to isolate and grow, standard methods for the detection of *M. pneumoniae* are serology and PCR. Serological methods are presently based on the total cell lysate, which sometimes shows cross-reactivity with other mycoplasmas and microorganisms. Our group has been interested in developing protocols to produce large amounts of *M. pneumoniae* surface antigens. In the

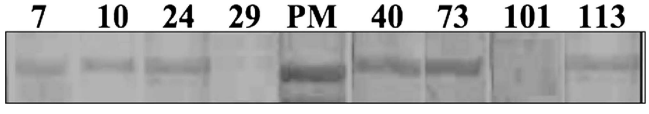

FIG. 5. Immunoblot of the purified P30 fusion protein with individual positive and negative patient serum samples and anti-MBP antibodies. Lanes 7, 10, 24, 40, 73, and 113, individual positive serum samples; lanes 29 and 101, negative controls (negative individual serum samples that tested negative with the commercial kit). Lane PM, P30 fusion protein reacted with anti-MBP antibodies.

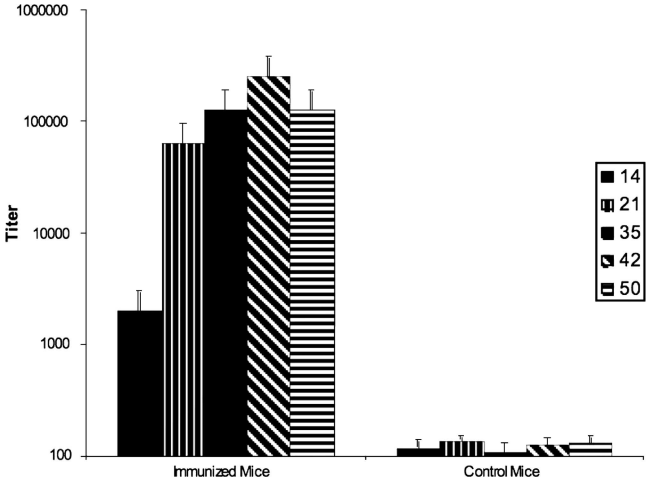

FIG. 6. Immune response in BALB/c mice immunized with the P30B-MBP fusion protein formulated in CFA or IFA.

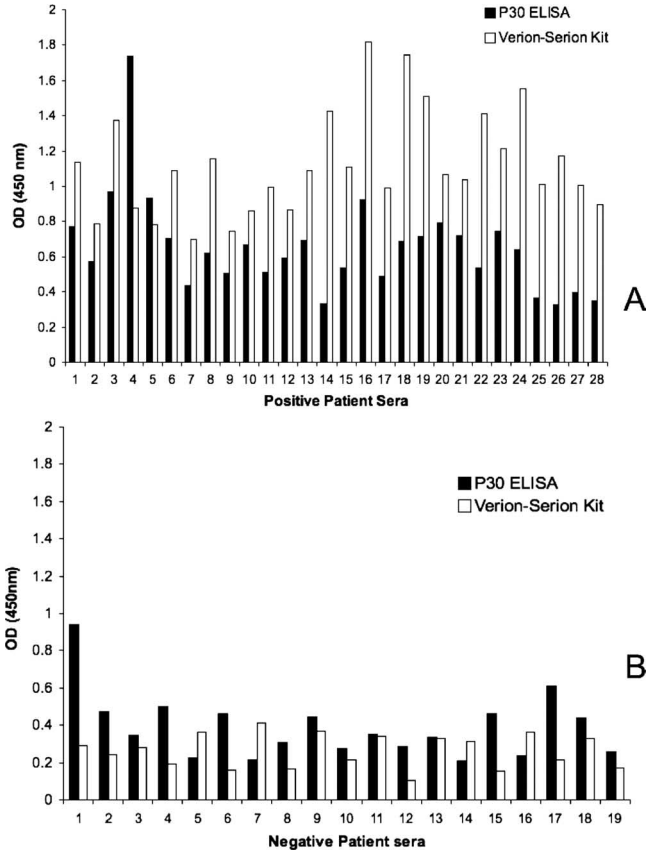

FIG. 7. Comparative ELISA analysis by the recombinant P30 ELISA and with the Serion Classic IgM ELISA kit with sera from 47 patients. (A) Positive patient sera. (B) Negative patient sera.

present study, we expressed a fragment of the *p30* gene that includes the complete C-terminal proline-rich amino acid sequences. The protein was expressed as a His-tagged and MBP-P30 fusion protein; however, we failed to obtain the His-tagged P30 protein in soluble form under nondenaturing conditions. Nonetheless, we were able to express and purify the MBP-P30 fusion protein in soluble form. The yield of purified protein was approximately 1 mg/liter. The purified protein was recognized by anti-*M. pneumoniae* antibodies. Although the P30 protein has previously been expressed in an *E. coli* expression vector as well as in *Bacillus subtilis*, it appears that the protein was expressed in very small amounts (15, 17).

Previous studies of the P30 protein have mainly been focused on understanding its role in cytadherence and gliding motility (9, 11, 16, 19). Its use as an immunodiagnostic molecule has not been explored. As the C-terminal proline-rich fragment of P30 is exposed on the surface of *M. pneumoniae*, we carried out a detailed immunological analysis of the rMPN30–pMAL-p2x fusion protein. The fusion protein was immunogenic, as it elicited a boostable immune response in mice. The protein was recognized by *M. pneumoniae*-infected patient sera as well. We next analyzed the reactivity of the rMPN30–pMAL-p2x fusion protein with patient sera in an ELISA analysis and compared the reactivity with that obtained with the Serion ELISA Classic kit. This kit has been reported to be 100% sensitive and 75% specific, as described in the manufacturer's instructions. The sensitivity and the specificity of our ELISA with the purified MBP-P30B fusion protein were found to be 78.57% and 89.47%, respectively.

In conclusion, the results from the present study indicate that the P30 protein is an important antigen and can be used along with two other adhesin molecules, P1 and P116, for the development of a sensitive assay for the diagnosis of *M. pneumoniae* infection. The use of this protein to study cytadherence may also provide insight into the pathophysiology of this disease.

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