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Diagnostic detection of *Streptococcus pneumoniae* PpmA in urine

M. d. M. García-Suárez[1], L. E. Cron[2], B. Suárez-Álvarez[3], R. Villaverde[1], I. González-Rodríguez[1], F. Vázquez[1], P. W. M. Hermans[3] and F. J. Méndez[1]

1) *Área de Microbiología, Departamento de Biología Funcional, Universidad de Oviedo, Oviedo, Asturias, Spain,* 2) *Laboratory of Paediatric Infectious Diseases, Radboud University Nijmegen Medical Centre, 6500 HB Nijmegen, The Netherlands and* 3) *Área de Inmunología, Departamento de Biología Funcional, Universidad de Oviedo, Oviedo, Asturias, Spain*

Abstract

Streptococcus pneumoniae infections are often difficult to diagnose accurately, as it is not uncommon for clinical samples to be culture-negative, particularly after antibiotic administration. The rapid Binax NOW *S. pneumoniae* urinary antigen test lacks specificity in children, owing to pneumococcal antigen reactions in children who are nasopharyngeal carriers of *S. pneumoniae*. A western blot assay with a specific polyclonal antibody was developed for direct detection of the putative proteinase maturation protein A (PpmA) in urine samples from children with pneumococcal infections. The sensitivity and specificity of the assay were 66.7% and 100%, respectively. Previous antibiotic treatment or *S. pneumoniae* nasopharyngeal colonization did not affect PpmA antigenuria. Results also demonstrated the presence of PpmA cross-reactive epitopes in commensal bacteria that co-colonize the nasopharyngeal niche, although the non-pneumococcal cross-reactive protein(s) did not interfere with the detection assay. *S. pneumoniae* PpmA in the urine of children with pneumococcal infections may be a marker that has the potential to be used in the clinical diagnosis of pneumococcal infection.

Keywords: Diagnosis, pneumococcal infection, proteinase maturation protein A (PpmA), *Streptococcus pneumoniae*, urine
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Corresponding author and reprint requests: M. Del Mar García-Suárez, Área de Microbiología, Facultad de Medicina, C/Julián Clavería, 6, 33006 Oviedo, Asturias, Spain
E-mail: garciamar@uniovi.es

Introduction

Streptococcus pneumoniae is an important human pathogen that causes meningitis, otitis media, sepsis, and pneumonia. Accurate and rapid diagnosis of pneumococcal infections is critical, because they entail substantial morbidity and mortality worldwide [1,2]. The current reference standard for the diagnosis of *S. pneumoniae* infections is the isolation of the organism in culture from normally sterile body fluids. However, the sensitivity of diagnostic cultures has been shown to be low, and it takes at least 2 days to obtain a result [3]. C-polysaccharide is a cell wall component of *S. pneumoniae* that can be detected in the urine of patients suffering from pneumococcal infections. A rapid urinary pneumococcal antigen test (Binax NOW *S. pneumoniae* urinary antigen test; Binax Inc., Portland, ME, USA) is available for the detection of this antigen in patients; however, C-polysaccharide is also present in small amounts in the urine of healthy children [4,5]. *S. pneumoniae* present in the nasopharynx is the most likely antigen source in pneumococcal carriers testing positive when this assay is used [6]. Positive results in non-carriers might be due to undetected, low-level pneumococcal colonization, or colonization by *Streptococcus mitis*, which has been shown to harbour pneumococcal C-polysaccharide-like antigens [7]. Furthermore, urine samples can remain positive for weeks after a pneumococcal infection, and the test will also be affected if the patient has been vaccinated with a pneumococcal conjugate vaccine shortly before the test [6]. Hence, it is doubtful whether this assay is useful for discriminating between children with and without pneumococcal infections, particularly in developing countries, where nasopharyngeal colonization rates are high [8]. Molecular biology-based techniques, including PCR assays and DNA hybridization assays, have been reported for the rapid, specific, and sensitive detection of microorganisms in blood samples [9,10]. More recently, a sensitive and specific ELISA has been developed to detect pneumolysin in urine samples, thereby allowing differentiation between healthy carriers and patients [11].

The putative proteinase maturation protein A (PpmA) of *S. pneumoniae* is homologous to members of the family

of peptidyl-prolyl *cis–trans* isomerases (PPIases), which accelerate the rate-limiting *cis–trans* or *trans–cis* conformational changes at X-Pro bonds during protein folding [12]. There are three distinct families within the enzyme class of PPIases: the cyclophilins, the FK506-binding proteins, and the parvulins. PpmA is homologous to the members of the parvulin family. Although PPIase activity has never been demonstrated, the protein is considered to be involved in enzyme secretion and activation, and it is a surface-located immunogenic lipoprotein that contributes to bacterial virulence [13,14]. PpmA is conserved across serotypes, elicits protective immune responses, and is therefore a candidate for the development of new pneumococcal vaccines [15].

The present study is the first to provide evidence of the presence of *S. pneumoniae* PpmA in urine samples of children with pneumococcal infections, independently of previous antibiotic treatment, and of its absence in such samples from healthy children, even if their nasopharynx is colonized with *S. pneumoniae*. Furthermore, the development of a simple western blot assay for the detection of *S. pneumoniae* PpmA in urine samples is reported, which establishes the necessary basis for future randomized clinical trials of the assay with a larger number of individuals.

Materials and Methods

Bacterial strains

Reference strains were obtained from the American Type Culture Collection (ATCC, Manassas, USA), the National Collection of Type Cultures (NCTC, London, UK), and the Culture Collection University of Göteborg (CCUG, Göteborg, Sweden). The *S. pneumoniae* D39 *ppmA*$^-$ strain was kindly supplied by P. W. M. Hermans, Radboud University Nijmegen Medical Centre. Clinical isolates of *Streptococcus mutans*, *Streptococcus agalactiae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Neisseria meningitidis*, *Legionella pneumophila*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Proteus mirabilis*, *Providencia stuartii*, *Serratia marcescens* and *Candida albicans* were obtained from the Microbiology Laboratory of the Hospital Universitario Central de Asturias.

Expression and purification of PpmA

S. pneumoniae D39 (NCTC 7466) was grown in Todd–Hewitt broth (Oxoid, Basingstoke, UK), supplemented with 0.5% yeast extract, to logarithmic phase at 37°C in a 5% CO_2 atmosphere. Genomic DNA was extracted using the E.Z.N.A. DNA purification kit (Omega Bio-tek, Doraville, GA, USA), following the manufacturer's recommendations. The *ppmA* gene was amplified from *S. pneumoniae* genomic DNA, using PCR with the primers ppma-fd (5′-GGAGTACAT ATGAAGAAAAAATTATTGGCAG-3′) and ppma-r1 (5′-CT CATGGATCCGGACTATTCGTTTGATG-3′). The forward and reverse primers contain *Nde*I and *Bam*HI recognition sequences, respectively. The amplified *Nde*I–*Bam*HI-digested *ppmA* fragment was cloned into pET-15b (Invitrogen, Carlsbad, CA, USA), encoding a His(6)-tag followed by four amino acids and a thrombin cleavage site. DNA sequencing of the recombinant plasmid confirmed the insertion of the *ppmA* gene, previously described by Overweg *et al.* [13], and its sequence showed 99% identity with that of the serotype 4 strain TIGR4 (ORF SP0981; accession number ABJ55379) [16]. The *ppmA* recombinant sequence was different only at the 5′-end, due to the vector-borne extension. For the expression of recombinant PpmA protein, the recombinant pET-15b was transferred into electro-competent *Escherichia coli* BL21 (DE3) pLysS (Novagen Inc., Madison, WI, USA) by electroporation. Expression of recombinant PpmA was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 2 h. The His-tagged 36.6-kDa PpmA was purified to near-homogeneity from the soluble fraction of recombinant *E. coli* lysates, using immobilized metal affinity chromatography with a His-Bind purification kit (Novagen Inc.) under native conditions, following the manufacturer's recommendations. Fractions containing the purified protein were pooled, dialysed against phosphate-buffered saline (PBS), and stored at −20°C.

Production of hyperimmune rabbit sera

Male New Zealand White rabbits were intramuscularly immunized with purified 36.6-kDa PpmA (100 μg/immunization) and with 10^8 CFU of heat-killed whole *S. pneumoniae* type 14 (CCUG 1086) and *S. pneumoniae* R36A (a non-encapsulated strain; ATCC 12214), ten times at 2-week intervals. Immunizations were performed using Freund's incomplete adjuvant (Sigma Chemical Co., St Louis MO, USA). Pre-immune sera were obtained, and samples of immune serum were drawn weekly from the marginal ear veins of the rabbits. Pre-immune sera did not react with PpmA or pneumococcal lysates in western blot analysis, and were used as negative controls. The animals were bled 7 days after the last immunization. To remove antibodies that recognized soluble *E. coli* proteins, lyophilized lysates were incubated with polyclonal sera at 37°C for 3 h and overnight at 4°C; the sera were centrifuged at 10 000 ***g*** to remove bacterial debris, and stored at −20°C until used. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Oviedo (Spain).

SDS-PAGE and western blot

Prior to analysis, urine samples were thawed at room temperature, and an aliquot was centrifuged at 15 000 ***g*** for 1 min. The supernatants were diluted in Laemmli buffer [17], boiled for 5 min, and subjected to electrophoresis. SDS-PAGE was carried out in the Bio-Rad minigel system (Bio-Rad Laboratories, Richmond, CA, USA) with polyacrylamide (12%) gels. The proteins were transferred to poly(vinylidene difluoride) membranes (Inmobilon-P; Millipore, Bedford, MA, USA), using a mini-trans-blot system (Bio-Rad Laboratories), and the membranes were blocked in Tris-buffered saline (0.1 M Tris, 0.15 M NaCl, pH 7.5) (TBS) containing 1% blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany) for 1 h at room temperature or overnight at 4°C. Hyperimmune rabbit antisera or human sera were diluted 1 : 1000 in blocking solution (TBS containing 0.1% blocking reagent) and added to the membrane strips. After 1 h of incubation, membrane strips were washed three times with TBS–Tween-20 (0.05%) for 10 min. Goat anti-rabbit IgG (whole molecule) peroxidase conjugate (Sigma Chemical Co.) or anti-human γ-chain peroxidase conjugate (Sigma Chemical Co.) (each diluted 1 : 5000) was added, and the strips were incubated for 30 min. After washing of the strips, protein was detected using BM Chemiluminescent blotting substrate (POD) (Roche Diagnostics GmbH) and visualized on Kodak film.

Binax NOW *S. pneumoniae* test

The Binax NOW *S. pneumoniae* test (Binax Inc.) was performed with non-concentrated urine samples obtained from all enrolled patients according to the manufacturer's instructions. The results were read visually after 15 min. The test was interpreted according to the presence or absence of pink–purple-coloured lines.

PLY-ELISA

All urine samples were tested with pneumolysin (PLY)-ELISA, performed as previously described [11]. Briefly, Combiplate white breakable plates (Labsystems, Helsinki, Finland) were coated with anti-PLY mouse monoclonal PLY-7 (1 μg/well) [11] in PBS at 37°C for 3 h. Plates were blocked with blocking buffer (ELISA-Light chemiluminescence detection system; Tropix, Applied Biosystems, Bedford, MA, USA). Samples were then added to the wells and incubated at 37°C for 1 h with shaking. After this, plates were incubated with rabbit polyclonal anti-PLY IgG diluted 1 : 1000 in blocking buffer and incubated at 37°C for 30 min. Secondary anti-rabbit IgG–alkaline phosphatase antibody (Sigma Chemicals Co.) (1 : 5000) was added and incubated as above. Plates were loaded and read on a Luminoskan RS (Labsystems) luminometer after automatic filling of the wells with substrate/enhancer solution (0.4 mM CSPD with 1× Sapphire-II) and incubation at 37°C for 10 min.

Bacterial and yeast lysates

Gram-positive bacterial strains were grown overnight in Todd–Hewitt broth (Oxoid) supplemented with yeast extract, and Gram-negative bacteria were grown in 2xTY broth [18]. *L. pneumophila* and *H. influenzae* were grown on BCYE and chocolate agar–fetal bovine serum plates (Oxoid), respectively. *C. albicans* was grown on Sabouraud agar (Oxoid). Plates were incubated at 37°C under 5% CO_2 for 48–72 h. Cells were prepared in PBS, lysed by shaking with acid-washed glass beads (106 μm) (Sigma Chemical Co.), and centrifuged for 15 min at 3000 ***g***. Soluble proteins were quantified at $A_{280\ nm}$ and adjusted to 1 g/L. For SDS-PAGE, 20 μg of protein per lane was used. *Mycoplasma pneumoniae* FH and *Chlamydia pneumoniae* CWL-029 cell lysates were purchased from Microbix Biosystems Inc. (Toronto, ON, Canada). *Mycobacterium tuberculosis* and *Mycobacterium bovis* cell lysates were obtained from Unidad de Referencia Regional de Micobacterias, Hospital Universitario Central de Asturias (Oviedo, Spain).

Patients and samples

Urine samples were collected from children potentially infected with *S. pneumoniae* at Hospital Universitario Central de Asturias (Oviedo, Spain) and from healthy children at Centro de Salud de Lugones receiving routine clinical care, between January 2003 and December 2006. The study was performed as a retrospective, multicentre study; the samples were obtained with the approval of the Hospital Universitario Central de Asturias Research Ethics Committee, and informed consent was obtained from the childrens' parents or guardians. Ten-millilitre urine specimens were collected in sterile containers, using aseptic techniques, and frozen at −70°C until use.

The presence of PpmA was tested for in urine samples collected from 84 children (47 males and 37 females). The mean and median ages were 5.03 years (±4 years) and 4 years (newborn to 14 years), respectively. The group with culture-proven pneumococcal infections included 14 urine samples from patients with pneumonia (n = 9), septicaemia (n = 1), meningitis (n = 2), sinusitis (n = 1), and a finger infection (n = 1). The group with probable pneumococcal infections included 23 urine samples from patients with pneumonia (n = 15) and otitis (n = 8). The group with non-pneumococcal infections (n = 12) included: four samples from patients with pneumonia caused by *M. pneumoniae* (n = 1), *Chlamydia pneumoniae* (n = 1), *H. influenzae* (n = 1), and respiratory syncytial virus (n = 1); one from a patient

with gastroenteritis due to *Salmonella* spp.; one from a patient with tonsillitis due to *Streptococcus pyogenes*; three from patients with a common cold; two from patients with urinary tract infection caused by *E. coli*; and one from a patient with vaginitis caused by *Candida* spp.. The non-infectious disease group included samples from children with allergic rhinitis (n = 5). The group with unknown aetiology of disease was composed of 18 urine samples from patients with pneumonia (n = 14), otitis media (n = 2), sinusitis (n = 1), and conjunctivitis (n = 1), and all were discarded from test performance calculations. The group of healthy individuals included 12 without clinical or radiological signs of pneumonia or infection; they had not received any antibiotic treatment during the preceding week.

Microbiological analysis and diagnostic criteria

Nasopharyngeal swab samples were taken using a calcium alginate-tipped swab on a flexible aluminium wire (TRANSWAB; Medical Wire and Equipment Co., Ltd, Corsham, UK) and streaked onto horse blood (5%) agar supplemented with gentamicin (5 mg/L); the plates were incubated in 5% CO_2 for 24 h. All *S. pneumoniae* isolates were identified according to colony morphology, Gram stain, optochin susceptibility, and bile solubility.

All urine samples were cultured to control for any cross-reactivity. Cultures of blood (ESP system; Difco Laboratories, Detroit, MI, USA), cerebrospinal fluid, ascites, pleural fluid, tracheal aspirates, sputum, urine and pus, and of otic and vaginal samples, were performed according to standard microbiological methods [19]. Standard methodology was used for the microimmunofluorescence, virus culture and shell-vial assays [20]. Specific monoclonal fluorescein isothiocyanate-conjugated antibodies were obtained from Vircell S.L. (Granada, Spain). A microimmunofluorescence test was performed for detection of specific antibodies against *Chlamydia pneumoniae* in patient sera. Commercial ELISA tests were used for detection of IgM against *M. pneumoniae* and *Chlamydia pneumoniae* (Vircell S.L.). Commercial complement fixation tests were used for detection of IgM/IgG against adenovirus and respiratory syncytial virus (Virion; Institute Virion Ltd, Rüschlikon, Switzerland). All commercial tests were performed according to the manufacturers' instructions. The diagnosis of acute infection was considered when the IgM test result was positive or when a four-fold or greater rise in IgM and IgG titres was observed in sera taken at 3-week intervals.

The diagnosis of pneumococcal infection was established on the basis of standard radiological, clinical and laboratory criteria. Patients were classified as having pneumonia if they presented with an acute illness with the presence of a new or progressive infiltrate seen on a chest radiograph, in addition to at least two of the following symptoms: fever, cough, shortness of breath, and pleuritic chest pain. Patients were classified as having culture-proven pneumococcal infection when *S. pneumoniae* was isolated from blood (n = 9), cerebrospinal fluid (n = 2), pleural fluid (n = 1), ascitic fluid (n = 1), or pus (n = 1). Patients were classified as having probable pneumococcal pneumonia when *S. pneumoniae* was isolated from tracheal aspirates ($\geq 10^6$ CFU/mL) (n = 4) or sputum (polymorphonuclear neutrophil/squamous epithelial cell ratio ≥ 2 or Q (quality) score $\geq +1$ on Gram stain) (n = 1), or when both Binax NOW *S. pneumoniae* urinary antigen (Binax Inc.) and PLY-ELISA urine test results were positive (n = 12). Patients were classified as having probable pneumococcal otitis media when *S. pneumoniae* was isolated from otic exudates, and both Binax NOW *S. pneumoniae* urinary antigen (Binax Inc.) and PLY-ELISA urine test results were positive (n = 8). The diagnosis of urinary tract infection was considered if $>10^5$ CFU/mL of a single bacterium was found in urine cultures. Non-infectious diseases were diagnosed according to standard criteria.

Multiple alignments of protein sequences

The identification of putative PPIases was performed using the BLASTP program [21] at The National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and the protein sequences of *Streptococcus sanguinis* SK36, (YP1034736), *S. mutans* UA159 (NP721076), *S. agalactiae* A909 (YP329554), *S. pyogenes* MGAS10270 (YP598812), *Enterococcus faecalis* V583 (NP814435) and *Listeria monocytogenes* strain 4b F2365 (YP014061) were retrieved from the NCBI protein data base. The PpmA sequence of *S. mitis* was retrieved from the Institute for Genomic Research database (http://www.tigr.org). Protein sequences were aligned using the ClustalW programme (http://www.ebi.ac.uk/clustalw). Calculation of the percentage of amino acid identity was carried out using the LALIGN program (http://www.ch.embnet.org/software/LALIGN_form.html) [22]. The theoretical lipoprotein signal peptide-cleaving site was predicted using the Lipop 1.0 Server (http://www.cbs.dtu.dk/services/LipoP/) [23]. Calculation of percentage of similarity of the sequences was carried out using EMBOSS Pairwise Alignment Algorithms (http://www.ebi.ac.uk/Tools/emboss/align/index.html). The theoretical molecular masses of the predicted proteins were calculated using the Compute pI/Mw tool on the ExPASy server (http://www.expasy.ch/tools/pi_tool.html).

Statistical analysis

The data were analysed with GraphPad PRISM software (v.4.00 for Windows; GraphPad Software, San Diego, CA,

USA). CIs were calculated using Wilson's method. We used the chi-square test or, when appropriate, Fisher's exact test to compare proportions of qualitative variables. The limit of statistical significance was set at a p-value of 0.05.

Results

Detection of PpmA using western blot

In order to produce a hyperimmune anti-PpmA rabbit antiserum, expression and purification of recombinant PpmA was carried out. After induction of recombinant *E. coli* cultures, two main protein bands were observed at 32 and 36 kDa (Fig. 1a, lane 3). The theoretical molecular mass of PpmA (34.4 kDa) increased to 36.6 kDa in the recombinant protein, owing to fusion at the N-terminus with a His-tag followed by a thrombin cleavage site. The predicted theoretical molecular masses (Fig. 1d) of the protein with (36.6 kDa) and without (32.4 kDa) lipoprotein signal peptide were very similar to the molecular masses of proteins observed after induction. In order to confirm that both proteins were recombinant PpmA, lysates of *E. coli* were assayed using different sera. Proteins reacted with rabbit anti-heat-killed *S. pneumoniae* type 14 and anti-unencapsulated *S. pneumoniae* R36A antisera, and with two adult pneumococcal pneumonia patient sera (Fig. 1b). His-tagged PpmA was purified to near-homogeneity using immobilized metal affinity chromatography (Fig. 1a). Serum from rabbits immunized with 36.6-kDa purified protein showed reactivity against 32-kDa and 36-kDa PpmA in recombinant *E. coli* lysates (Fig. 1c, lane 1). Hyperimmune rabbit antiserum also reacted with a 34.4-kDa protein from *S. pneumoniae* D39 lysates, and no reactivity was observed against *S. pneumoniae* D39 *ppmA*$^-$ mutant lysates (Fig. 1c, lanes 2 and 3).

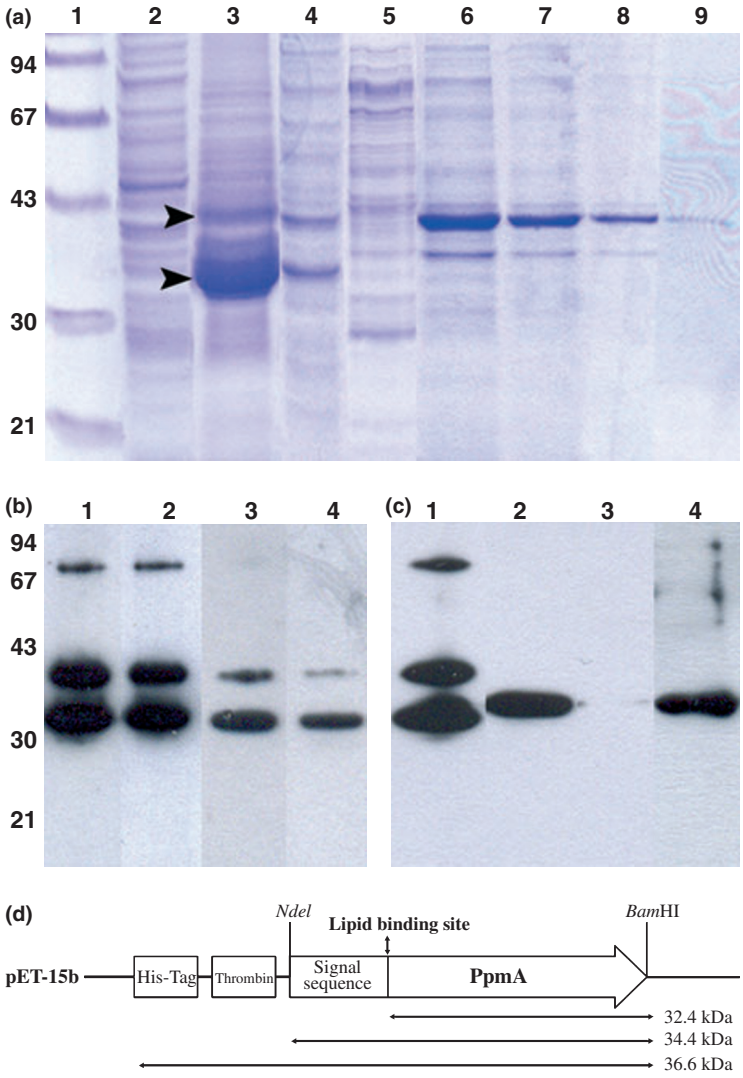

FIG. 1. Characterization of recombinant proteinase maturation protein A (PpmA) and hyperimmune rabbit antisera. (a) Expression and purification of recombinant PpmA. Lane 1: molecular mass markers. Lane 2: PpmA recombinant *Escherichia coli* BL21 (DE3) pLysS cultures before isopropyl thio-β-D-galactoside (IPTG) induction. Lane 3: PpmA recombinant *E. coli* BL21 (DE3) pLysS cultures after IPTG induction, showing main protein bands (arrows). Lane 4: soluble cell lysate. Lane 5: immobilized metal affinity chromatography (IMAC) unbound proteins. Lanes 6–9: IMAC eluted protein fractions. (b) PpmA recombinant *E. coli* lysates were incubated with rabbit anti-*Streptococcus pneumoniae* type 14 antiserum (lane 1), anti-*S. pneumoniae* R36A antiserum (lane 2), and pneumococcal pneumonia patient sera (lanes 3 and 4). (c) Anti-PpmA hyperimmune rabbit antiserum was incubated with PpmA recombinant *E. coli* lysate (lane 1), *S. pneumoniae* D39 wild-type lysate (lane 2), *S. pneumoniae* D39 *ppmA*$^-$ lysate (lane 3), and a urine sample from a paediatric patient with pneumococcal pneumonia (lane 4). (d) Schematic illustration of the recombinant PpmA full expression cassette, showing the theoretical molecular masses of the different expressed proteins.

Anti-PpmA hyperimmune rabbit antiserum

The specificity of the anti-PpmA hyperimmune rabbit antiserum was tested against lysates from nasopharyngeal commensal bacteria and microorganisms known to cause pneumonia, sinusitis, otitis and urinary tract infections in children (Table 1). In western blot analysis, anti-PpmA antiserum recognized proteins with relative molecular masses ranging between 30 and 40 kDa, in *Streptococcus* spp. and *Enterococcus faecalis* lysates. BlastP homology searches revealed that pneumococcal PpmA shares sequence identity/similarity with PPIases from other streptococci (Fig. 2). The protein sequences of *S. mitis*, *S. sanguinis*, *S. mutans*, *S. agalactiae*, *S. pyogenes* and *Enterococcus faecalis* showed percentages of identity with *S. pneumoniae* PpmA of 93.6%, 57.6%, 46.4%, 47.5%, 40.1%, and 37.8%, respectively. Percentages of similarity were 97.1%, 74.8%, 65.7%, 66.4%, 57.6%, and 56%, respectively. PpmA identity and similarity with that of other Gram-positive bacteria, e.g. *Listeria monocytogenes*, were 29.2% and 52.8%, respectively. The predicted molecular masses of these proteins were consistent with those in antibody-stained bands observed in western blot analysis (Table 1).

TABLE 1. Reactivity of hyperimmune anti- proteinase maturation protein A (PpmA) rabbit antiserum against pathogenic and non-pathogenic commensal bacterial lysates

Microorganism[a]	Reactivity of anti-PpmA antiserum (molecular mass, kDa)[b]
Streptococcus pneumoniae D39 (NCTC 7466)	+ (34)
Streptococcus mitis ATCC 33399	+ (34)
Streptococcus sanguinis ATCC 10556	+ (36)
Streptococcus mutans	+ (36)
Streptococcus agalactiae	+ (36)
Streptococcus pyogenes ATCC 19615	+ (38)
Streptococcus salivarius ATCC 7073	+ (38)
Streptococcus oralis ATCC 35037	+ (35)
Streptococcus anginosus ATCC 12395	+ (35)
Haemophilus influenzae	–
Moraxella catarrhalis	–
Neisseria meningitidis	–
Legionella pneumophila	–
Mycoplasma pneumoniae FH[c]	–
Chlamydia pneumoniae CWL-029[c]	–
Klebsiella pneumoniae ATCC 13883	–
Mycobacterium tuberculosis	–
Mycobacterium bovis	–
Pseudomonas aeruginosa ATCC 27853	–
Listeria monocytogenes	–
Enterococcus faecalis	+ (37)
Proteus mirabilis	–
Proteus vulgaris ATCC 13315	–
Providencia stuartii	–
Candida albicans	–
Enterobacter cloacae ATCC 23355	–
Staphylococcus aureus ATCC 25923	–
Escherichia coli ATCC 25922	–
Serratia marcescens	–

[a]Collection strains and clinical isolates were used. Clinical isolates were obtained from patients at the Microbiology Laboratory of the Hospital Universitario Central de Asturias.
[b]Relative molecular mass of the proteins recognized.
[c]*Mycoplasma pneumoniae* FH and *Chlamydia pneumoniae* CWL-029 cell lysates were purchased from Microbix Biosystems Inc., Toronto, Canada. Cells were lysed, and 20 μg of total protein per lane was used for antigen detection using western blots.

Detection of PpmA in urine samples

Hyperimmune rabbit antiserum reacted with a 34-kDa protein in urine samples from children with pneumococcal infection. A representative result of a western blot analysis is shown in Fig. 1c, lane 4. The results of PpmA-based western blot analyses of paediatric urine samples are shown in Table 2. The sensitivity, specificity, and positive and negative predictive values of the diagnostic test based on the detection of PpmA by western blot analysis were 57.1%, 100%, 100%, and 59.2%, respectively. Secreted PpmA was observed in eight of 12 children with life-threating pneumococcal infections, with sensitivity and negative predictive values of 66.7% and 87.9%, respectively. In contrast, 100% of the urine samples from the healthy, non-pneumococcal and non-infectious disease groups yielded negative PpmA results. PpmA was also detected in urine samples from adults with culture-proven pneumococcal pneumonia, and no reactivity was observed in urine samples from non-pneumococcal pneumonia patients (data not shown). The main characteristics of the paediatric patients with pneumococcal infections and the results obtained with other urinary antigen tests, as correlated with the PpmA test results, are shown in Table 3. No significant differences were found in terms of gender or nasopharyngeal pneumococcal carriage. Half of the patients in the present study had been treated with antibiotics before the time of collection of the clinical specimens. The western blot was positive for PpmA for six of 17 (35.3%) patients who had received prior treatment with antibiotics, and for nine of 17 (52.9%) who had not received prior antibiotic treatment; this difference was not statistically significant.

In the culture-proven pneumococcal infection group, 13 of 14 (92.9%) children were positive according to the low-specificity Binax NOW *S. pneumoniae* urinary antigen test (Binax Inc.), whereas 11 of 14 (78.6%) and eight of 14 (57.1%) were positive with the specific PLY-ELISA and PpmA western blot assay, respectively (Table 4). Therefore, 11 of 14 (78.6%) children were diagnosed as positive when one or both protein-specific antigen tests were used.

Discussion

This study found the presence of *S. pneumoniae* PpmA in urine, and examined its usefulness in the diagnosis of pneumococcal infections in children. Recombinant, full-length PpmA without lipoprotein signal peptide has been previously expressed in *E. coli*, in order to study its immunogenicity

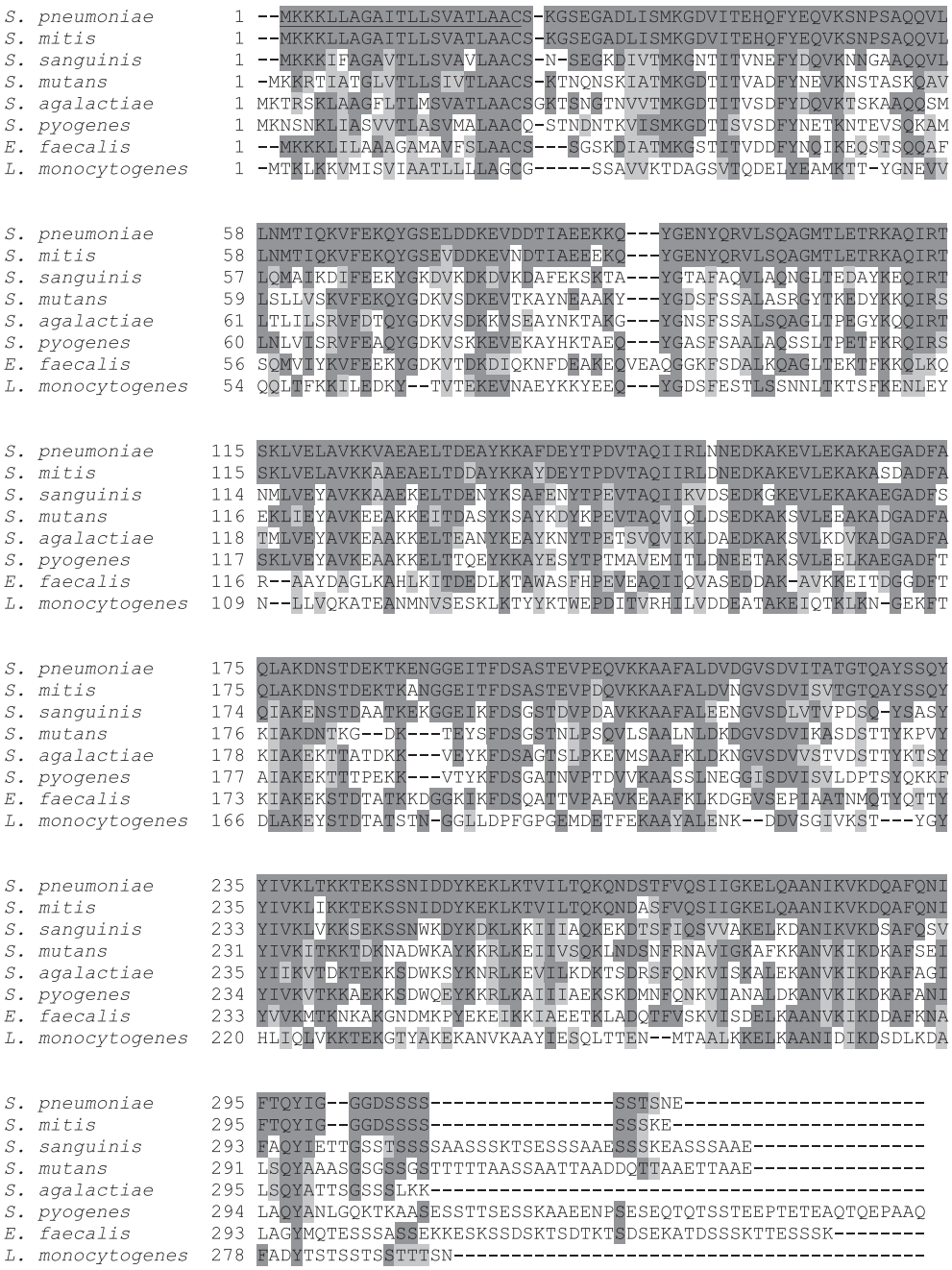

FIG. 2. Multiple alignment of parvulin-like peptidyl-prolyl *cis–trans* isomerases (PPIases) and proteinase maturation protein A (PpmA). Protein sequences were aligned using the ClustalW programme and manually coloured. The predicted signal sequence and lipid-binding site of PpmA is underlined. Regions of 100% identity with PpmA of *Streptococcus pneumoniae* are shown in dark grey. Light grey shading of residues indicates similarity. Numbers to the left indicate the location within the amino acid sequence.

[15,24–26], as the protein has a surface-exposed C-terminal domain, whereas the N-terminus is most probably covalently bound to the cell membrane [13]. We selected expression of full-length protein in *E. coli* and subsequent polyclonal antiserum production in rabbits. Two protein bands corresponding to PpmA migrated in SDS-PAGE gels, suggesting that they corresponded to PpmA with and without the lipoprotein signal peptide, as the molecular masses matched the molecular masses predicted for the processed and unprocessed protein. Furthermore, after purification, only the higher molecular mass protein was obtained, suggesting that the 32.4-kDa protein had lost the N-terminal His-tag. This

TABLE 2. Detection of proteinase maturation protein A in urine samples of paediatric patients in relation to patient condition group

Group (n = 84)	Patient no.	No. of positive samples (%)	95% CI
Culture-proven pneumococcal infections[a]	14	8 (57.1)	32.6–78.7
Pneumonia, meningitis, septicaemia	12	8 (66.7)	38.8–86.5
Others[b]	2	0 (0)	0.0–70.0
Probable pneumococcal infections	23	9 (39.1)	22.1–59.3
Pneumonia[c]	15	6 (40)	19.7–64.3
Otitis media[d]	8	3 (37.5)	13.5–69.6
Non-pneumococcal infections	12	0 (0)	0.0–28.2
Pneumonia	4	0 (0)	0.0–54.6
Others[e]	8	0 (0)	0.0–37.2
Non-infectious diseases	5	0 (0)	0.0–48.9
Healthy subjects	12	0 (0)	0.0–28.2
Unknown aetiology	18	2 (11.1)	1.9–34.1

[a]*Streptococcus pneumoniae* was isolated from blood (n = 9), cerebrospinal fluid (n = 2), pleural fluid (n = 1), ascitic fluid (n = 1), and pus (n = 1).
[b]Sinusitis (n = 1) and finger infection (n = 1).
[c]*S. pneumoniae* was isolated from tracheal aspirates (n = 5), sputum (n = 1), or both; Binax NOW *S. pneumoniae* urinary antigen (Binax Inc.) and PLY-ELISA urine test results were positive (n = 12).
[d]*S. pneumoniae* was isolated from otic exudates, and both Binax NOW *S. pneumoniae* urinary antigen (Binax Inc.) and PLY-ELISA urine test results were positive (n = 8).
[e]Gastroenteritis caused by *Salmonella* spp. (n = 1), tonsillitis caused by *Streptococcus pyogenes* (n = 1), common cold (n = 3), urinary infection caused by *Escherichia coli* (n = 2), and vaginitis caused by *Candida* spp. (n = 1).

TABLE 3. Main characteristics of patients with pneumococcal infections as correlated with positive urinary proteinase maturation protein A (PpmA) antigen test results

Characteristics (n = 37)	Patient no.	No. of positive samples (%)	95% CI	p
Gender				
Male	21	10 (47.6)	28.3–67.6	0.8150
Female	16	7 (43.8)	23.1–66.9	
Nasopharyngeal carriage				
Positive	10	4 (40.0)	16.7–68.8	0.7256
Negative	27	13 (48.2)	30.7–66.0	
Age				
≤2 years	10	8 (80.0)	47.9–95.4	0.0625
>2 years	27	11 (40.7)	24.5–59.3	
Streptococcus pneumoniae blood culture				
Positive	9	5 (55.6)	26.6–81.2	0.7034
Negative	28	12 (42.9)	26.5–61.0	
Binax NOW test				
Positive	35	17 (48.6)	33.0–64.4	0.4895
Negative	2	0 (0.0)	0.0–71.0	
PLY-ELISA test				
Positive	31	16 (51.6)	34.8–68.0	0.1886
Negative	6	1 (16.7)	1.1–58.2	
Diagnosis				
Pneumonia	24	13 (54.2)	35.1–72.1	0.1728
Others[a]	13	4 (30.8)	12.3–57.9	
Previous antibiotic treatment				
Yes	17	6 (35.3)	17.2–58.8	0.3001
No	17	9 (52.9)	31.0–73.8	
Undetermined	3	2 (66.7)	20.2–94.4	

[a]Including septicaemia (n = 1), meningitis (n = 2), otitis media (n = 8), sinusitis (n = 1), and a finger infection (n = 1) due to *Streptococcus pneumoniae*.

TABLE 4. Comparison of three urinary antigen test results in the same urine samples from children with proven pneumococcal infections

Patient no.	Diagnosis	*Streptococcus pneumoniae* culture-positive	Binax NOW	PLY-ELISA	PpmA western blot
1	Pneumonia	Blood	+	+	+
2	Pneumonia	Blood	+	+	+
3	Pneumonia	Blood	+	+	+
4	Pneumonia	Blood	+	+	+
5	Pneumonia	Blood	+	+	+
6	Pneumonia	Blood	+	+	–
7	Pneumonia	Blood	+	–	–
8	Pneumonia	Pleural fluid	+	+	+
9	Pneumonia	Ascitic fluid	+	+	+
19	Meningitis	Cerebrospinal fluid	+	+	+
11	Meningitis	Cerebrospinal fluid	+	–	–
12	Septicaemia	Blood	+	+	–
13	Sinusitis	Blood	–	–	–
14	Finger infection	Pus	+	+	–

PpmA, proteinase maturation protein A.

protein was the most prominent after induction, probably because the lipoprotein signal peptide of Gram-positive bacteria is recognized by *E. coli* signal peptidase II, even though lipoprotein signal peptides of Gram-positive bacteria differ from those of Gram-negative bacteria [23]. It was supposed that both proteins have diacylglycerol attached to the thiol of the conserved cysteine, which should reduce its solubility. However, most probably because of the high levels of expression after induction in *E. coli*, a low percentage of the proteins still remained in the soluble fraction. His-tagged protein was immunogenic in rabbits.

PpmA is homologous to the members of the ubiquitous family of parvulin-like peptidyl-prolyl isomerases, and is widely represented among bacteria [12,27]. One urine sample from a patient with *S. pyogenes* infection was tested, and no reactivity was observed, probably because of the low amounts of *S. pyogenes* PpmA homologue released into the urine. However, urine samples from patients with *Enterococcus faecalis* infection will need to be tested in order to assess potential cross-reactivity. These results are consistent with the low level of identity observed among parvulin-like PPIases retrieved from the protein databases. PpmA shares a high degree of amino acid sequence identity/similarity only with the proteinase maturation protein of *S. mitis*, and has a lower degree of identity/similarity with parvulin-like PPIases of *Streptococcus* spp. Interestingly, the present results confirm the presence of PpmA cross-reactive epitopes in commensal bacteria co-colonizing the nasopharyngeal niche, as previously suggested [24]. These findings explain why a correlation between anti-PpmA antibody titres and pneumococcal carriage or infection cannot be established, even though PpmA is an immunogenic protein [25,26]. Presumably, early in life, the very first acquisition of microflora on the nasopharyngeal mucosa, in particular of streptococci, leads to a systemic antibody response against PpmA.

Owing to the commensal nature of *S. pneumoniae*, especially in children, the key factor in the laboratory diagnosis of invasive pneumococcal infections is the differentiation between infection and colonization. The limitation to the application of the Binax NOW test for diagnosing pneumococcal infections in children is that false-positive results are obtained with samples from healthy children who are nasopharyngeal carriers of *S. pneumoniae*. Therefore, this test can give positive results in patients with non-pneumococcal infections who are *S. pneumoniae* carriers. In contrast, the test for PpmA was specific for pneumococcal infections and gave negative results for *S. pneumoniae* nasopharyngeal carriers. Previously, the detection of pneumolysin in urine samples for the differentiation between colonization and invasive pneumococcal infections in children has been reported by this group [11]. All urine samples from healthy carriers tested negative for the antigen, despite the abundance of PpmA in the transparent colony-type variants selected during nasopharyngeal colonization [27]. The presumed different antigenic composition of colonizing and invasive pneumococci has been investigated repeatedly in the context of pneumococcal virulence gene expression, and this has demonstrated that the mechanism of pneumococcal pathogenesis is highly complex and multifactorial and that it remains unclear [28–31]. The key for discriminating between healthy carriers and patients might be related to the nature of the antigens and their capacity to cross the renal barrier.

The diagnostic potential of PpmA detection in urine was studied using western blot analysis. The test used cannot be considered to be a rapid clinical diagnostic test; nevertheless, it is a helpful tool with which to evaluate the presence of new antigens in urine samples, and could be transformed into an ELISA or immunochromatographic assay subsequent to the preparation of anti-PpmA monoclonal antibodies. Although detection of PpmA in patients with pneumococcal infection was associated with very poor sensitivity and negative predictive values, detection of PpmA in patients in whom *S. pneumoniae* was isolated from blood or other sterile fluids notably increased the diagnostic validity. Interestingly, these results were very close to those obtained with pneumolysin detection in urine using an ELISA assay [11]. Several factors could be involved in the low sensitivity observed in the western blot assay. To our knowledge, there are no data regarding *ppmA* expression levels *in vivo* during the progress of pneumococcal infection, or regarding the stability of PpmA released into the urine. However, we speculate that protein levels in urine are correlated with the spread of pneumococci. On the other hand (and on the basis of results obtained with Binax NOW), it seems reasonable to think that the number of PpmA molecules per cell could be lower than that of C-polysaccharide.

The strategy of using the Binax NOW *S. pneumoniae* assay as a primary screen, and in addition the PLY-ELISA and PpmA assays with Binax NOW-positive samples, could improve the diagnostic accuracy for pneumococcal infections. Although protein antigen tests are of relatively low sensitivity, the specificity of the PLY and PpmA assays is higher than that of Binax NOW, and therefore positive results obtained with the two assays indicate true pneumococcal infections, increasing the accuracy of diagnosis of pneumococcal infections in children. The kinetics of PpmA release into the urine of patients with pneumococcal infection were not studied here. A larger, prospective study of paediatric populations with controlled immunization status to evaluate the relevance of the detection of this antigen in urine will be conducted in the future in this laboratory.

There was a near-significant (p 0.06) difference between patients under 2 years of age (80% positive) and patients over 2 years of age (40% positive). Although we have no reliable explanation for this finding, it could be due to differences in immunological status, as has been suggested for the C-polysaccharide [5]. Previous studies have shown that antibiotics could influence the production and release of bacterial virulence factors [32–35]. The diagnosis of *S. pneumoniae* infection in adults based on the Binax NOW urinary antigen test (Binax Inc.) has been established more often in patients who had not received antibiotics before the test [36]. In the present study (probably owing to the limited number of patients), no correlation between PpmA antigenuria and previous antibiotic treatment could be established.

This study shows for the first time that the kidney barrier in patients with pneumococcal infection is permeable to the *S. pneumoniae* protein PpmA, resulting in its excretion in urine, which can be detected using western blots. Despite the limited sensitivity of the PpmA detection assay, the availability of this simple immunoassay using samples obtained with non-invasive procedures, e.g. urine, is expected to trigger further research into the usefulness of pneumococcal protein antigens as differential biomarkers of pneumococcal carriage. Until now, conventional assays of antigenuria have not provided such markers.

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Transparency Declaration

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