ORIGINAL ARTICLE 10.1111/j.1469-0691.2009.02702.x

Diagnostic detection of *Streptococcus pneumoniae* PpmA in urine

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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
Original Submission: 7 June 2008; **Revised Submission:** 6 August 2008; **Accepted:** 7 August 2008
Editor: F. Allerberger
Clin Microbiol Infect 2009; **15:** 443–453

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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).