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# Whole gene pneumolysin PCR can be used as a diagnostic assay but cannot predict serotype

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*Streptococcus pneumoniae* is responsible for infections such as pneumonia, bacteraemia and meningitis. Despite antimicrobial therapy, invasive pneumococcal disease (IPD) remains a leading cause of morbidity and mortality worldwide, especially in the young and old.[1-3]

*S. pneumoniae* produces a number of virulence factors, one of which is pneumolysin. This is a 53 kDa polypeptide comprising 470 amino acids, encoded by the pneumolysin (*ply*) gene. Pneumolysin interferes with phagocyte function *in vitro* by exerting haemolytic activity and suppressing immune function.[4] This multifunctional virulence factor is produced by almost all clinical *S. pneumoniae* isolates and could be a suitable candidate for use in the detection of invasive *S. pneumoniae* infection.

Isolates of *S. pneumoniae* are serotyped according to their capsular type and can be characterised using multilocus sequence typing (MLST). The sequence type (ST) is derived from the nucleotide sequencing of housekeeping genes, which are unrelated to capsular type and often show a correlation between serotype and sequence type (unpublished data).

Polymerase chain reaction (PCR) methods have been described for the non-culture confirmation of *S. pneumoniae*.[5-10] Sequence variation has also been reported within the *ply* gene of serotypes 7 and 8.[11] Hence, sequence variation within the *ply* gene may provide the basis of a new typing method for pneumococci, based on the relationship between sequence variation of the *ply* gene and serotype.

Here, the development and evaluation of a whole gene *ply* PCR for the confirmation of IPD from body fluids is described. Also sequenced are the *ply* genes from the 23 serotypes (included in the 23-valent polysaccharide vaccine) that cause 96% of IPD to evaluate their suitability for use as a sequence-based typing method for the identification of different serotypes.

Ten strains each from the 23 *S. pneumoniae* serotypes were taken from the collection held by the Scottish Meningococcus and Pneumococcus Reference Laboratory (SMPRL). Five hundred clinical samples (whole blood and cerebrospinal fluid [CSF]) previously tested for pneumococcal antigen also were used to evaluate the assay. Genomic DNA was extracted from blood samples using the Nucleospin blood kit (Abgene, Epsom, UK), as described previously.[12] CSF samples did not require extraction. DNA was extracted from pneumococcal isolates by adding 10 colonies to 200 µL distilled water. The suspension was heated to 80°C for 4 min, centrifuged for 3 min at 13000 x*g* and the supernatant frozen at –70°C for a minimum of 1 h and then thawed before addition of the PCR mix.

PCR primers were designed from the published pneumolysin gene sequence (Genbank accession number M17717; www.ncbi.n/m.nih.gov/BLAST/) using Gene Fisher (http:/bibiserv.techfak.uni-bielefeld.de/genefisher/) (Table 1). The PCR reaction mix contained 21 µL Reddymix (Abgene), 1 µL each primer (2 pmol stock) and 3 µL DNA. PCR cycling conditions used were as follows: 95°C for 2 min, 35 loops of 95°C for 1 min, 51°C for 1 min 30 sec and 72°C for 2 min, followed by 5 min at 72°C.

Owing to the low DNA concentration in the clinical samples, a nested PCR step was used with the initial PCR product, which was purified using the Millipore Multiscreen 384-PCR plate (Millipore, Watford, UK), as described previously.[13] Nested primers were designed and produced as above (Table 1). For the nested PCR, 20 pmol each primer was prepared and used in 1 µL volumes. The thermocycling conditions were as follows: 95°C for 2 min, 35 cycles of 95°C for 1 min, 50°C for 1 min 30 sec and 72°C for 2 min, finishing with 72°C for 5 min. The amplified nested product was then

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**Table 1.** Primers used in the study.

| Name | Primer type | Sequence (5'-3') |
|---|---|---|
| plyPCR F | Amplification ply PCR (forward) | gga tca ctt agt cca acc a |
| plyPCR R | Amplification ply PCR (reverse) | caa gca ttc tcc tct cct a |
| plyPCR FN | Nested ply (forward) | tcg ctg aac aag tct gag |
| plyPCR RN | Nested ply (reverse) | aga aat cgt ccg ctt acg |
| plyPCR FSM | Sequence MegaBACE (forward) | agc act tct cgt cgt gga |
| plyPCR RSM | Sequence MegaBACE (reverse) | gct atc gct act tgc caa |
| plyPCR FSL | Sequence Licor (forward) 700 nm label | agg agg aga aga tgg caa |
| plyPCR RSL | Sequence Licor (reverse) 800 nm label | agg cac cac tat gat cca |

visualised on 1% agarose gel stained with ethidium bromide under ultraviolet transillumination.

The sensitivity of the PCR assay was evaluated using a standard 10-fold dilution series for each of the common 23 serotypes contained in the pneumococcal polysaccharide vaccine. Specificity was assessed using the organisms listed in Table 2. Automated DNA sequencers were used for nucleotide sequencing, as described previously,[14,15] using specifically designed primers (Table 1) for 10 strains of each serotype ($n$=230).

Nucleotide sequence data were read automatically from the DNA sequencers using the integrated image analysis and data collection software, which was then aligned using Li-Cor Align IR (MWG-Biotech, Milton Keynes, UK). Nucleotide polymorphisms were checked and a consensus sequence gained for each serotype.

Of the organisms tested for specificity ($n$=51), six were positive (*S. parasanguis, S. oralis, Escherichia coli, Neisseria meningitidis* and two of the eight *N. lactamica* isolates). Specificity of the *ply* gene PCR therefore was 88%. The first stage of the PCR only detected confluent growth; however, sensitivity was increased 10-fold (158 colony-forming units detected) by adding the nested stage.

A total of 211 pneumococcal isolates were recovered from the SMPRL culture collection. In addition, 520 clinical samples were used to evaluate the nested PCR method (208 CSF, 247 serum, 59 EDTA and six serum samples), including 17 that were confirmed positive for pneumococcal antigen. Of the 17 antigen-positive samples, 12 were positive using this PCR test (seven CSFs and five sera). The remaining five antigen-positive samples that were negative by PCR were serotype 12 (one CSF and one serum), serotype 3 (two CSFs) and serotype 1 (one serum). Of the remaining 503 clinical samples, 15 reported negative by previous testing methods were positive by PCR (five CSFs, 10 sera). Thus, 3% of the clinical samples tested (not including previously positive samples) were found to be positive.

Specificity of the PCR was high but false positives were found with *S. parasanguis* and *S. oralis*, which are usually non-invasive species that may be carried in the throat and mouth, and are known to be potential carriers of the pneumolysin gene.[16] False positives may arise when transient bacteraemia occurs, which may be falsely diagnosed as pneumococcal infection.

**Table 2.** Organisms used to assess specificity.

| Organism | Total number tested | PCR result | |
|---|---|---|---|
| | | Negative | Positive |
| *Neisseria meningitidis* | 10 | 9 | 1 |
| *N. mucosa* | 1 | 1 | 0 |
| *N. gonorrhoeae* | 8 | 8 | 0 |
| *N. lactamica* | 8 | 6 | 2 |
| *Escherichia coli* | 5 | 4 | 1 |
| *Haemophilus influenzae* | 3 | 3 | 0 |
| *Staphylococcus aureus* | 6 | 6 | 0 |
| *Streptococcus parasanguis* | 1 | 0 | 1 |
| *S. constellatus* | 1 | 1 | 0 |
| *S. gordonii* | 1 | 1 | 0 |
| *S. mitis* | 1 | 1 | 0 |
| *S. oralis* | 1 | 0 | 1 |
| Group B streptococci | 4 | 1 | 0 |
| Human genomic DNA | 1 | 1 | 0 |

The sensitivity of the PCR test was low but should prove sufficient to detect most cases of IPD, as seen in this study. Although the whole gene *ply* PCR did not appear to be an efficient method of identification, the initial aim of this study was to identify and subsequently sequence the whole gene PCR product with a view to typing the organism. More rapid, specific and sensitive PCR methods (e.g., fluorescence-based PCR) could be used with alternative primers to produce a shorter segment of the *ply* gene.

Small differences in pneumolysin nucleotide sequence were seen in individual strains in all serotypes. For the purpose of this study, a consensus sequence for each serotype was produced and single nucleotide polymorphisms for individual strains were not considered relevant; therefore, individual strain sequence data are not presented here. Consequently, it was not possible to design oligonucleotide primers that would amplify individual *S. pneumoniae* serotypes.

It has been reported previously[11] that serotypes 7 and 8 have a six-nucleotide deletion, resulting in the absence of Val270 and Lys271, and three amino acid substitutions (Thr172 → Ile, Lys224 → Arg, Ala265 →Ser). It was also found that isolates of *S. pneumoniae* serotype 8 appeared to produce very small amounts of pneumolysin *in vitro*, as judged by haemolytic activity. This may explain the difficulties experienced in amplifying the pneumolysin gene in serotype 8.

This study aimed to devise a typing system based on sequence variations that depended on serotype but the deletions and substitutions found were not serotype-specific and therefore this proved impossible. It would appear that use of the *ply* gene alone as a discriminating subtyping target is inadequate and that multiple targets such as the housekeeping genes used in MLST are more suitable for typing *S. pneumoniae*. □

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# *Moraxella catarrhalis* bacteraemia in an immunocompetent patient in Lahore, Pakistan

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*Moraxella catarrhalis* was once regarded as a common, essentially harmless inhabitant of the pharynx but is now recognised as an important pathogen in respiratory tract infections in both children and adults, particularly in immunodeficient and hospitalised patients.[1,2] However, bacteraemia caused by *M. catarrhalis* is less well understood and, although only reported infrequently over the last decade, the number of invasive infections has been increasing.[3-6] Bacteraemia can be subdivided broadly on the basis of the host's medical history, which commonly shows an underlying respiratory disorder or can be essentially normal; however, the major predisposing factor is neutropenia.[7,8]

In the immunocompetent patient the origin of infection is usually from the respiratory tract or ears, while the immunocompromised patient may have no defined portal of entry. If affected by *M. catarrhalis* bacteraemia, a patient's symptoms can range from those associated with a self-limiting febrile illness to life-threatening sepsis.[9,10] The report presented here details a case of *M. catarrhalis* bacteraemia in a previously healthy patient.

A 39-year-old women presented to the Shaukat Khanum Memorial Cancer Hospital out-patient department complaining of headache, dry cough with chest pains, fever and chills. On initial examination, the only remarkable feature was a temperature of 40°C. Chest X-ray showed no abnormality and there were no other visual signs. The patient was admitted for observation. Routine samples of blood and urine were taken, and two sets of blood cultures were drawn.

Chemical analysis on the urine sample showed no evidence of protein or glucose but a slightly increased number of white blood cells (WBCs) (35/mm$^3$; normal range: 0–25/mm$^3$) on microscopy. Blood smears for malaria were negative. Although blood WBC count was normal (4.83 x 10$^3$/μL), there was a slightly raised neutrophil count (3.81 x 10$^3$/μL; normal range: 1.53–3.62 x 10$^3$/μL). Erythrocyte sedimentation rate (ESR) was raised at 37 mm/hr (normal range: 0–15 mm). The patient was started on intravenous ceftazidime and gentamicin, as outlined by hospital policy for cases of pyrexia.[11]

After overnight incubation at 37°C, all blood culture bottles were tested and small Gram-negative cocci were seen on microscopy. The bottles were subcultured onto blood and chocolate agar plates and incubated at 37°C in 5% $CO_2$. The following day a β-lactamase-positive *M. catarrhalis* was isolated from all bottles and identity was confirmed using the API NH system (bioMérieux, France). An antibiotic sensitivity test was set up following NCCLS guidelines and the plates were incubated at 37°C in 5% $CO_2$.[12,13]

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