Pneumolysin Is a Key Factor in Misidentification of Macrolide-Resistant Streptococcus pneumoniae and Is a Putative Virulence Factor of S. mitis and Other Streptococci

Chris Neeleman,1 Corné H. W. Klaassen,2 Debbie M. Kломberg,2 Hanneke A. de Valk,2 and Johan W. Mouton2*

Department of Intensive Care Medicine, University Hospital St. Radboud,1 and Department of Medical Microbiology and Infectious Diseases, Canisius/Wilhelmina Hospital,2 Nijmegen, The Netherlands

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We evaluated the applicability of ply PCR for confirmation of the identification of Streptococcus pneumoniae. bytA PCR, 16S rRNA sequencing, and amplified-fragment length polymorphism were used as reference methods. In contrast to the bytA gene, the ply gene proved to be not specific for S. pneumoniae. The presence of the ply gene in other streptococci, in particular Streptococcus mitis, suggests that pneumolysin plays a pathogenic role.

In addition to conventional methods, such as methods for determining optochin susceptibility, bile solubility, and capsular reaction, new DNA-based methods have been developed for identification of pneumococci (7, 10). However, accumulating data indicate that identification of Streptococcus pneumoniae by molecular biological and conventional biochemical methods may lead to controversial results (2, 9). The ply gene, encoding the pneumococcal virulence factor pneumolysin, is an attractive target for PCR-based identification of S. pneumoniae that is considered to be highly specific (13, 15). However, the use of this target was not compared to other DNA-based techniques in a large study. In the present study we evaluate the applicability of ply PCR to confirmation of the identification of macrolide-resistant S. pneumoniae isolates.

During three recent surveys (2001 to 2003) in The Netherlands we collected 141 macrolide-resistant strains identified as S. pneumoniae from 38 clinical laboratories. Identification of strains was done initially by the participating laboratories using their own standard identification techniques: usually optochin susceptibility as the primary identification method and, if necessary, confirmation by another test (bile solubility, Accuprobe [GEN-PROBE, San Diego, Calif.], Pneumoslide [BBL Microbiology Systems, Cockeysville, Md.], or Api strep [rapid ID 32 Strep; BioMerieux]). Strains were stored in polypropylene vials at −70°C. DNA was isolated with an automated DNA extraction platform (MagNAPure; Roche, Almere, The Netherlands) after treatment with mutanolysin (Sigma, Zwijndrecht, The Netherlands). Detection of the pneumolysin gene was performed by a real-time PCR procedure targeting the ply gene with primers and probe sequences described by Viro- lainen et al. (15). Detection of the autolysin gene was performed by a real-time PCR procedure targeting the bytA gene with primers and probe sequences described by McAvin et al. (10). Minor adjustments to both original protocols were made to adapt these assays to a LightCycler format (Roche). Sequence analysis of part of the 16S rRNA gene was performed with broad-range PCR primers to amplify a variable region in the 5’ end of the 16S gene. The PCR primers used were 5’- CGGCGTGCTAATACATGC-3’ and 5’- CGGCGTGCTAATACATGC-3’. PCR conditions were as follows: 0.5 μM primers, 1.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphates, 1 U of HotGoldStar DNA polymerase (Eurogentec, Seraing, Belgium), 1× reaction buffer, and 2 μl of DNA in a total volume of 50 μl. Cycling conditions were as follows: 30 s of denaturation at 94°C, 30 s of annealing at 56°C, and 1 min of elongation at 72°C for 35 cycles. Cycling was preceded by a 10-min incubation at 94°C. An additional incubation for 10 min at 72°C was included before the reaction mixtures were cooled to room temperature. PCR products were purified by High-Pure chemistry (Roche). Purified PCR products were sequenced on a MegaBACE 500 automated DNA analysis platform under conditions recommended by the manufacturer (Amersham Biosciences, Roosendaal, The Netherlands). Obtained sequences were compared to sequences in the public DNA libraries by using the World Wide Web-based BLAST interface (1). Amplified-fragment length polymorphism ( AFLP) was performed as described earlier (12).

Identification of the 141 presumptive pneumococcal isolates by AFLP and 16S rRNA sequencing confirmed that 91 (65%) strains were indeed S. pneumoniae (Table 1). Thirty-two strains were identified as Streptococcus mitis. The remaining 18 strains could be identified only as streptococcal species because the identical 16S sequences in the public DNA libraries were designated streptococcus species without further specification to the species level. All bytA-negative isolates (n = 50) were non-pneumococcal strains, and all bytA-positive isolates (n = 91)
were *S. pneumoniae*, resulting in sensitivity and specificity of 100%. In contrast, almost all strains (*n* = 132; 94%), including 31 of 32 *S. mitis* strains, tested positive for the pneumolysin gene. These results indicate that the *ply* gene is not specific for *S. pneumoniae*.

An increasing number of reports have shown that identification of *S. pneumoniae* is a more complicated issue than previously assumed (2, 9, 14). In addition to classical phenotypic identification methods DNA probes have been developed for more-definitive identification of pneumococci (3, 7, 10). In the present study we evaluated *ply* PCR for confirmation of the identification of macrolide-resistant *S. pneumoniae* isolates. This gene amplification method is generally considered to be highly specific for *S. pneumoniae* (9). Remarkably, however, we identified a high number of nonpneumococcal strains among presumptive pneumococcal isolates, and most of these harbored the *ply* gene. Most of these nonpneumococcal isolates proved to be *S. mitis* strains. Thus, the observation of Whatmore et al. (17) that the *ply* gene was present in some nonpneumococci or atypical pneumococci in a collection of atypical strains extends to a significant proportion of strains isolated during routine clinical practice.

Pneumolysin is generally considered to be an important virulence factor specific for *S. pneumoniae*. This cytoplasmic protein is produced by all clinical pneumococcal isolates and has cytotoxic and proinflammatory activities facilitating adherence, invasion, and dissemination (4, 8, 11). Since most of the identified clinical *S. mitis* strains in this study (31 out of 32) harbored the *ply* gene, it is tempting to speculate that pneumolysin may be a virulence factor in *S. mitis* as well. Presence of a virulence factor of *S. pneumoniae* in phylogenetically related species, such as *S. mitis*, might imply interspecies recombination events. Putative exchange of virulence-encoding genes among *S. pneumoniae* and closely related species is supported by data of Whatmore et al., who identified pathogenic nonpneumococcal hemolytic streptococci harboring the *ply* and *lytA* genes (17).

Clearly, more work in vitro and in vivo is required to elucidate the putative pathogenic role of pneumolysin in *S. mitis*. Recombination events and gene transfer between *S. pneumoniae* and *S. mitis* may not only influence the evolution of virulence of these bacteria but may also change phenotypic behavior such as alpha-hemolysis or resistance to optochin (5, 17). This may play a role in misidentification of *S. pneumoniae* by conventional phenotypic characteristics.

Because *S. mitis* strains have patterns of reduced antimicrobial susceptibility compared to *S. pneumoniae*, failures to differentiate between these two species will increase pneumococcal resistance rates substantially (16). Overestimation of pneumococcal resistance may lead to incorrect antibiotic use (6). Alternatively, it could be argued that, because these *S. mitis* strains harbor the *ply* gene, they might be more pathogenic and comparable to *S. pneumoniae* in that respect, with identical requirements for treatment. This needs to be further investigated.

In conclusion, our results show that the *ply* gene is not specific for *S. pneumoniae* and is a key factor in misidentification of pneumococci. In contrast, the *lytA* gene was highly specific for *S. pneumoniae*, and this confirms the possible use of this gene for confirmation of the identification of *S. pneumoniae* (10, 12). The presence of the *ply* gene in most clinical *S. mitis* strains identified in this study suggests a pathogenic role for pneumolysin in *S. mitis*. The putative interspecies exchange of pneumococcal virulence factors, resulting in “pneumococcus-like” organisms, underscores the need for a “gold standard” or reference method for identification of *S. pneumoniae*.
