Genotypic identification of presumptive *Streptococcus pneumoniae* by PCR using four genes highly specific for *S. pneumoniae*

Nao Suzuki,¹ Mayumi Yuyama,² Sinsaku Maeda,² Haruhiko Ogawa,³ Kazuyuki Mashiko² and Yusuke Kiyoura¹

Correspondence

Yusuke Kiyoura

kiyusu@s5.dion.ne.jp

¹Division of Oral Bacteriology, Department of Oral Medical Science, Ohu University School of Dentistry, 31-1 Misumido, Tomitamachi, Koriyama 963-8611, Japan

²Jusendo General Hospital, 1-8-16 Ekimae, Koriyama 963-8585, Japan

³Division of Pulmonary Medicine, Saiseikai Kanazawa Hospital, 2-13-6 Akatsuchimachi, Kanazawa 920-0353, Japan

It was previously reported that two oligonucleotide primer sets (spn9802 and spn9828) for discriminating *Streptococcus pneumoniae* from pneumococcus-like oral streptococcal isolates using PCR had been developed. In this study, PCR amplification of the *lytA*, *ply*, spn9802 and spn9828 genes was used to identify presumptive *S. pneumoniae*. Two genetic groups were identified by analysing sputum samples from 28 patients with community-acquired pneumonia: the *lytA*-positive, *ply*-positive, spn9802-positive and spn9828-negative group, and the *lytA*-positive, *ply*-positive, spn9802-positive and spn9828-positive group. Isolates of the former group were resistant to optochin, while those of the latter group showed susceptibility to optochin. The *lytA*-positive, *ply*-positive, spn9802-negative and spn9828-negative isolates, and *lytA*-positive, *ply*-positive, spn9802-negative and spn9828-positive isolates, were not detected in sputum from patients with pneumonia. Subsequently, a total of 92 saliva samples from healthy individuals was screened by PCR using these primer sets. The *lytA*-positive, *ply*-positive, spn9802-positive and spn9828-negative group was identified more frequently in saliva from healthy children than in saliva from older healthy individuals and patients with pneumonia. The *lytA*-positive, *ply*-positive, spn9802-positive and spn9828-positive group was found frequently in saliva from healthy children, and in saliva and sputum from patients with pneumonia. This study demonstrates a rapid, optimal screening method for the genotypic identification of presumptive *S. pneumoniae* by PCR using four genes highly specific for *S. pneumoniae*.

Received 16 August 2005

Accepted 9 February 2006

INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is a common major human pathogen associated with community-acquired pneumonia, septicaemia, meningitis and otitis media (Brown & Lerner, 1998). Recently, various molecular assays, including a DNA probe test (Davis & Fuller, 1991; Denys & Carey, 1992), a loop-mediated isothermal amplification method (Seki *et al.*, 2005), and a TaqMan real-time PCR assay (Corless *et al.*, 2001), in addition to conventional PCR, have been developed and employed to identify pneumococcal strains and to detect pneumococci directly in clinical samples. PCR-based assays for identifying *S. pneumoniae* have frequently targeted genes encoding pneumococcal virulence factors, including autolysin (McAvin *et al.*, 2001), pneumolysin (Corless *et al.*, 2001), pneumococcal surface antigen A (Morrison *et al.*, 2000), manganese-dependent superoxide dismutase (Kawamura *et al.*, 1999), and penicillin-binding protein (O'Neill *et al.*, 1999). The autolysin (*lytA*) and

pneumolysin (*ply*) genes have been used for screening *S. pneumoniae* (Gillespie *et al.*, 1994; Ubukata *et al.*, 1996). Ideally, the amplification of these target genes should be specific for *S. pneumoniae* isolates only. However, the application of this strategy is complicated by recent reports that organisms that are genotypically and phenotypically related to oral streptococci, notably *Streptococcus mitis* and *Streptococcus oralis*, harbour the genes encoding the *S. pneumoniae* virulence factors autolysin and pneumolysin (Seki *et al.*, 2005; Verhelst *et al.*, 2003; Whatmore *et al.*, 2000).

In a previous study, we developed two primer sets, spn9802 and spn9828, that were highly specific for *S. pneumoniae*, in order to discriminate *S. pneumoniae* from pneumococcus-like oral streptococci harbouring the *lytA* and *ply* genes, by genomic subtractive hybridization between *S. pneumoniae* WU2 and *S. mitis* 903 (Suzuki *et al.*, 2005). The ORFs of *S. pneumoniae* R6 (GenBank accession no. AE007317) corresponding to the spn9802 and spn9828 amplicons encode

proteins of unknown function and show no marked homology with those of any organisms other than *S. pneumoniae*. The ORF of *S. pneumoniae* R6 corresponding to spn9802 has not been deposited in the GenBank database, while the gene of spn9828 corresponds to spr1523 identified by Hoskins *et al.* (2001). In this study, we tested the applicability of PCR amplification of four different genes, *lytA*, *ply*, spn9802 and spn9828, for identifying *S. pneumoniae* in 28 sputum samples from patients with pneumonia, and we classified the strains harbouring these pneumococcal genes into two genotypes. Subsequently, we examined the distribution of the genotypes in the saliva of healthy Japanese individuals. We found that PCR amplification with spn9802- and spn9828-specific primers, in combination with *lytA*- and *ply*-specific primers, was useful for identifying the genotypes of presumptive *S. pneumoniae*.

METHODS

Study population. To estimate infection of the oral cavity with *S. pneumoniae* and other pneumococcus-like alpha-haemolytic streptococci in Japanese individuals, a total of 92 saliva samples from healthy subjects, including 30 preschool children (mean age 4.1 ± 0.9 years, range 3–6 years), 32 young adults (mean age 29.9 ± 7.2 years, range 22–50 years) and 30 older people (mean age 69.5 ± 5.36 years, range 62–79 years), and 20 saliva and 28 sputum samples from patients with community-acquired pneumonia (mean age 67.3 ± 18.3 years, range 29–92 years) were examined by PCR using *lytA*-, *ply*-, spn9802- and spn9828-specific primers. All subjects and parents of preschool children who participated in this study understood the nature of the research project and gave informed consent.

Bacterial isolation and identification. All sputum samples were homogenized with 2% (w/v) *N*-acetyl-cysteine (Mucofilin; Eisai) and inoculated onto 5% (v/v) blood agar (Becton Dickinson) and 5% (v/v) heated blood agar (Becton Dickinson). Both plates were incubated at 37 °C in an atmosphere of 5% CO₂. To distinguish between *S. pneumoniae* and other alpha-haemolytic streptococci, the optochin-susceptibility test was performed using a 6.5 mm diameter disc containing 5 µg optochin (Eiken Chemical) in an atmosphere of 5% CO₂. The absence of an inhibition zone of at least 13 mm diameter was interpreted as a negative result, whereas a zone of inhibition of at least 13 mm diameter constituted a positive result.

Primer design. The oligonucleotide sequences of PCR primers used in this study are listed in Table 1. The oligonucleotide primers for the *lytA* and *ply* genes were designed as previously described by Nagai *et al.* (2001) and Salo *et al.* (1995), respectively. We have

previously used genomic subtractive hybridization to develop *S. pneumoniae*-specific spn9802 and spn9828 primers for discriminating pneumococci from other pneumococcus-like oral streptococci (Suzuki *et al.*, 2005).

Preparation of template DNA for PCR assay. Saliva and sputum samples were prepared for PCR amplification as follows. Briefly, 500 µl saliva and the same amount of PBS (0.12 M NaCl, 0.01 M Na₂HPO₄, 5 mM KH₂PO₄, pH 7.5) were mixed and centrifuged at 12 000 g for 10 min. Sputum and viscous saliva were mixed with 1 ml PBS using a vortexer for at least 30 s, and 500 µl supernatant was centrifuged at 12 000 g for 10 min. Then, 250 µl cell lysis buffer (1% Triton X-100, 20 mM Tris/HCl, 2 mM EDTA, pH 8.0) was added to the pellet, and the bacterial chromosomal DNA was extracted by boiling at 100 °C for 10 min. After the lysed cells were centrifuged, the supernatant containing the bacterial DNA was removed and frozen at –30 °C until use.

PCR assay. Amplification reactions were conducted using a TaKaRa PCR Thermal Cycler (TaKaRa Bio) with the following temperature profile: initial denaturation at 94 °C for 2 min, then 25 cycles consisting of 94 °C for 10 s, 58 °C for 15 s, and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. The amplification products were loaded onto 1.8% (w/v) agarose gels, separated by electrophoresis, stained with ethidium bromide (0.5 µl ml^{–1}), and photographed under UV light.

Statistical analysis. The statistical analysis of the difference in the frequency of the pneumococcal genes among clinical specimens was performed using the chi square and Fisher’s probability tests.

RESULTS AND DISCUSSION

Relationship between genetic type and optochin susceptibility

Optochin susceptibility is the phenotypic characteristic most frequently used to differentiate between *S. pneumoniae* and other alpha-haemolytic streptococci. However, optochin-resistant *S. pneumoniae* strains and optochin-sensitive oral streptococci are being isolated more frequently (Pikis *et al.*, 2001; Whatmore *et al.*, 2000), and may therefore be overlooked or misidentified, respectively, as many clinical microbiology laboratories today depend on the optochin-susceptibility test (Chandler *et al.*, 2000). In the present study, the optochin-susceptibility test, and PCR amplification of the pneumococcal genes encoding the putative virulence factors autolysin (*lytA*) and pneumolysin (*ply*) and

Table 1. Sequences of oligonucleotide primers

Primer	Sequence	Amplicon size (bp)	Reference
<i>lytA</i> -sense	5'-CAA CCG TAC AGA ATG AAG CGG-3'	308	Nagai <i>et al.</i> (2001)
<i>lytA</i> -antisense	5'-TTA TTC GTG CAA TAC TCG TGC G-3'		
<i>ply</i> -sense	5'-ATT TCT GTA ACA GCT ACC AAC GA-3'	329	Salo <i>et al.</i> (1995)
<i>ply</i> -antisense	5'-GAA TTC CCT GTC TTT TCA AAG TC-3'		
spn9802-143F	5'-CAA GTC GTT CCA AGG TAA CAA GTC T-3'	162	Suzuki <i>et al.</i> (2005)
spn9802-304R	5'-CTA AAC CAA CTC GAC CAC CTC TTT-3'		
spn9828-19F	5'-GGC ATT GTG AAT GGA TTG ATT G-3'	227	Suzuki <i>et al.</i> (2005)
spn9828-245R	5'-TCA TGT GCA TCC CAA GCT ACA-3'		

Table 2. Detection of pneumococcal genes by PCR from sputum of patients with pneumonia

ND, Not done.

Patient	Age (years)	Optochin test	Pneumococcal gene			
			<i>lytA</i>	<i>ply</i>	spn9802	spn9828
1	70	+	+	+	+	+
2	67	+	+	+	+	+
3	66	ND	+	+	+	+
4	65	ND	+	+	+	+
5	76	ND	+	+	+	+
6	29	—	+	+	+	—
7	85	—	+	+	+	—
8	85	—	+	+	+	—
9	54	ND	+	+	+	—
10	89	ND	+	+	+	—
11	29	—	—	+	—	—
12	34	—	—	+	—	—
13	84	—	—	+	+	—
14	53	ND	—	+	+	+
15	78	ND	—	—	+	—

the highly specific pneumococcal genes spn9802 and spn9828, were performed on sputum samples from 28 patients with community-acquired pneumonia. Table 2 shows the distribution of pneumococcal genes in 15 sputum samples amplified with all primers by PCR. Ten specimens (35.7%) gave amplification with both the *lytA*- and *ply*-specific primers, which encode typical pneumococcal virulence factors, and were classified into two genetic types: the *lytA*-positive, *ply*-positive, spn9802-positive and spn9828-positive genetic type, and the *lytA*-positive, *ply*-positive, spn9802-positive and spn9828-negative genetic type. Optochin-sensitive strains were isolated from samples positive for all four primer sets. One of them was identified as penicillin-resistant *S. pneumoniae* (patient 2). These isolates were typical *S. pneumoniae* in both the genotype and phenotype analyses. The clinical isolates from the *lytA*-positive, *ply*-positive, spn9802-positive and spn9828-negative samples showed resistance to optochin. Therefore, these isolates were identified as pneumococci by the genotype analysis, but would be considered to be other alpha-haemolytic streptococci by the optochin-susceptibility test. In a previous study, four pneumococcal isolates and 20 pneumococcus-like oral streptococcal isolates were tested to evaluate spn9802- and spn9828-specific primers (Suzuki *et al.*, 2005). All four pneumococcal isolates were positive with the *lytA*-, *ply*-, spn9802- and spn9828-specific primers and sensitive to optochin, while all 20 pneumococcus-like oral streptococcal isolates were resistant to optochin. It would be of interest to study whether the organisms of the genetic type that are positive with the *lytA*-, *ply*-, and spn9802-specific primers are optochin-resistant *S. pneumoniae* or another alpha-haemolytic streptococcus harbouring three pneumococcal genes. It would also be of interest to

investigate the difference in virulence and pathology between the two genetic types. The *lytA*-positive, *ply*-positive, spn9802-negative and spn9828-negative genetic type, and the *lytA*-positive, *ply*-positive, spn9802-negative and spn9828-positive genetic type were not identified. The other five specimens containing pneumococcal genes showed various amplification patterns, and their isolates were resistant to optochin.

Genetic analysis for healthy individuals and patients with pneumonia

Subsequently, a total of 92 saliva samples from healthy individuals were examined by PCR using the *lytA*-, *ply*-, spn9802- and spn9828-specific primers, and compared with the results of saliva and sputum samples from the same 20 patients with community-acquired pneumonia (Table 3). The frequency of the *ply* gene was significantly higher than that of the other genes specific for *S. pneumoniae* at $P < 0.0001$ in saliva from healthy individuals and $P < 0.05$ in saliva and sputum from patients with pneumonia. There was a significant difference between the frequency of *lytA* and spn9802 or spn9828 in saliva from healthy individuals ($P < 0.01$), while there was no significant difference in saliva and sputum from patients from pneumonia. Furthermore, the spn9802 gene was detected frequently in saliva from healthy children and sputum from patients with pneumonia ($P < 0.05$), while the spn9828 gene was detected more frequently in sputum from patients with pneumonia than that from healthy patients. This suggests that many oral bacteria harbouring *lytA* or *ply* genes inhabit the human oral cavity, and that the spn9802 and spn9828 genes are highly specific for *S. pneumoniae*.

Both *lytA*- and *ply*-positive specimens were detected significantly more often in saliva from children than in saliva from young people ($P < 0.01$) or from patients with pneumonia ($P < 0.05$; Table 4). This genotypic pattern, harbouring both genes that encode putative essential pneumococcal virulence factors, is used for the presumptive identification of *S. pneumoniae*. The mean age of patients with pneumonia tested in this study was 65 years (range 29–92 years). These findings suggest that the distribution of *S. pneumoniae* has not been increasing rapidly in patients with community-acquired pneumonia.

Next, we classified the specimens harbouring both the *lytA* and *ply* genes into three genetic types, using the results of the amplification with the spn9802- and spn9828-specific primers in addition to the *lytA*- and *ply*-specific primers (Table 4). Interestingly, no *lytA*-positive, *ply*-positive, spn9802-negative and spn9828-negative specimens were detected in any saliva or sputum samples from patients with pneumonia. In contrast, *lytA*-positive, *ply*-positive, spn9802-positive and spn9828-positive specimens, which represent the most typical pneumococcal genotype, were identified frequently in saliva and sputum from patients with pneumonia; *lytA*-positive, *ply*-positive, spn9802-positive and spn9828-negative specimens were identified more frequently in saliva from healthy children than in

Table 3. Discrimination of pneumococcal genes in healthy individuals and patients with pneumonia

Subjects	Number (%) with pneumococcal gene			
	<i>lytA</i>	<i>ply</i>	<i>spn9802</i>	<i>spn9828</i>
Saliva				
Healthy children	18* (60)	27† (90)	13‡ (43.3)	4 (13.3)
Healthy young people	13 (40.6)	20† (62.5)	5†‡ (15.6)	4 (12.5)
Healthy older people	11 (36.6)	22 (73.3)	4†‡ (13.3)	2‡ (6.6)
Total	42 (45.6)	69§ (75)	22 (23.9)	10 (10.8)
Patients with pneumonia	6* (30)	10† (50)	7 (35)	4 (20)
Sputum				
Patients with pneumonia	9 (45)	12† (60)	11‡ (55)	5‡ (25)

*Differences in frequency of *lytA* between groups by chi square test at $P < 0.05$.

†Differences in frequency of primers between healthy children and the other group by Fisher's probability test at $P < 0.05$.

‡Differences in frequency of primers between sputum from patients with pneumonia and the other group by Fisher's probability test at $P < 0.05$.

§Significant difference between *ply* and the other genes specific for *S. pneumoniae* by chi square test at $P < 0.0001$.

saliva from older healthy individuals or patients with pneumonia ($P < 0.05$). Although the functional analysis of the proteins encoded by *spn9802* and *spn9828* has not been performed, their association with the virulence of *S. pneumoniae* has been suggested.

Assessment of the PCR amplification of four pneumococcal genes

The analysis of sputum from patients with community-acquired pneumonia suggests that the *lytA* primers are the all-powerful primers for detecting *S. pneumoniae* (Table 2). The *lytA* gene has been frequently used as a reliable target

for the identification of *S. pneumoniae* (Gillespie *et al.*, 1994; Kawamura *et al.*, 1999; Ubukata *et al.*, 1996), and it has been reported that the *lytA* gene has higher specificity than the *ply* gene for *S. pneumoniae* (Neeleman *et al.*, 2004). Recently, however, the existence of organisms that appear to be genotypically and phenotypically related to *S. mitis*, but which harbour the *lytA* gene normally associated with pneumococci, has been reported (Seki *et al.*, 2005). We detected six saliva samples from healthy young adults (18.7%) and one saliva sample from a healthy child (3.33%) that harboured only the *lytA* gene (data not shown). Saliva samples from 26.6% of healthy children, 25% of healthy young adults, 36.6% of healthy older people, and 25% of

Table 4. Distribution of genetic types of presumptive *S. pneumoniae*

Subjects	Number (%) of the genetic type			Number (%) of presumptive pneumococci	Total number
	<i>lytA</i> +, <i>ply</i> +, <i>spn9802</i> −, <i>spn9828</i> −	<i>lytA</i> +, <i>ply</i> +, <i>spn9802</i> +, <i>spn9828</i> −	<i>lytA</i> +, <i>ply</i> +, <i>spn9802</i> +, <i>spn9828</i> +		
Saliva					
Healthy children	6* (20)	8† (26.6)	3 (10)	17†‡ (56.6)	30
Healthy young people	4 (12.5)	2† (6.25)	1* (3.12)	7‡ (21.8)	32
Healthy older people	7* (23.3)	2† (6.66)	2 (6.66)	11 (36.6)	30
Patients with pneumonia	0*§ (0)	1† (5)	4*§ (20)	5† (25)	20
Sputum					
Patients with pneumonia	0*§ (0)	5§ (25)	4*§ (20)	9 (45)	20

*Significant difference between patients with pneumonia and the other group by Fisher's probability test at $P < 0.05$.

†Significant difference between healthy children and the other group by Fisher's probability test at $P < 0.05$.

‡Significant difference between subject groups by chi square test at $P < 0.01$.

§Significant difference between genetic types by Fisher's probability test at $P < 0.05$.

patients with pneumonia, as well as 10 % of sputum samples from patients with pneumonia, were amplified using only the *ply* primers (data not shown). Since the spn9802 and spn9828 primer sets amplified a few chromosomal DNA extracts from sputum samples from which *S. pneumoniae* was not isolated, they are not yet regarded as complete primer sets for the detection of *S. pneumoniae* (Table 2). Amplification by both the *lytA*- and *ply*-specific primers was detected in 56.6 % of saliva samples from healthy children and in 45 % of sputum samples from patients with community-acquired pneumonia. The frequencies were consistent with those of genes from the teeth and tongue of young children detected by DNA–DNA checkerboard with digoxigenin-labelled, whole-chromosomal DNA probes for *S. pneumoniae* (Tanner *et al.*, 2002). An identification assay using DNA probes has been reported to show 100 % specificity and sensitivity for pneumococci (Davis & Fuller, 1991; Denys & Carey, 1992), but is prohibitively expensive for routine diagnostic use. PCR techniques using the *lytA* and *ply* genes may be adequate to screen for presumptive *S. pneumoniae* and may reduce costs; however, non-pneumococcal species harbouring both *lytA* and *ply* genes have been identified (Whatmore *et al.*, 2000). The *lytA*-positive, *ply*-positive, spn9802-negative and spn9828-negative specimens were detected in 17 saliva samples from 92 healthy individuals (18.4 %), but were not detected in saliva or sputum from patients with community-acquired pneumonia. In contrast, *lytA*-positive, *ply*-positive, spn9802-positive and spn9828-negative specimens, and specimens positive for all four primer sets were frequently detected in healthy children and in patients with pneumonia. The risk groups for pneumococcal infection are young children, older people and immune-deficient patients. The genetic analysis of clinical samples, including PCR of saliva and sputum using these four primer sets, will be useful in screening for *S. pneumoniae* and other alpha-haemolytic streptococci, and will contribute to the study of the pathology and classification of these organisms.

ACKNOWLEDGEMENTS

This investigation was supported primarily by a research grant from the Fukushima Society for the Promotion of Medicine (N.S.). We thank Masayoshi Kamata and Akinori Seino, Department of Clinical Science, Ohu University School of Dentistry, Yayoi Shirai, Department of Prosthetic Dentistry, Ohu University School of Dentistry, and Chiyoza Miyazawa and Fumio Kurumada, Department of Oral Hygiene, Ohu University School of Dentistry, for their assistance.

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