Presence of capsular locus genes in immunochemically identified encapsulated and unencapsulated *Streptococcus pneumoniae* sputum isolates obtained from elderly patients with acute lower respiratory tract infection

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The principal virulence factor of *Streptococcus pneumoniae* is capsular polysaccharide, and encapsulated pneumococci are more common causes of disease than unencapsulated strains. This study analysed the presence of capsular genes in 59 pneumococcal isolates using two PCR methods targeted at the *cpsA* and *cpsB* genes of the capsular biosynthesis locus. The PCR method targeted at the *cpsB* gene, reported to be essential for encapsulation, was developed in this study. Of 59 pneumococcal isolates, 49 (83 %) were obtained from the sputum samples of elderly patients (≥ 65 years) with community-acquired pneumonia (CAP) and 10 (17 %) were from those with other acute lower respiratory tract infections (ARIs). Forty (82 %) of the CAP isolates and two (20 %) of the ARI isolates were encapsulated, as assessed by conventional immunochemical methods. Forty-one (98 %) of the 42 encapsulated strains had the *cpsB* gene present, and in 38 strains the *cpsA* gene was also detected. One of the unencapsulated isolates gave a positive result for the *cpsB* gene, and neither of the capsular locus genes were present in all the other unencapsulated strains. The distribution of encapsulated and unencapsulated isolates differed significantly between the two patient groups regardless of whether the presence of capsule was determined immunochemically ($P < 0.001$) or by *cpsB* PCR ($P = 0.002$). The *cpsB* PCR developed here was found to be a rapid and reliable method to detect the pneumococcal capsule locus and may have potential in sputum diagnostics when investigating the pneumococcal aetiology of CAP.

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INTRODUCTION

Streptococcus pneumoniae is a major human pathogen causing a wide variety of infections such as otitis media, sinusitis, bacteraemia, meningitis and pneumonia. Pneumococcus is the major bacterial agent in community-acquired pneumonia (CAP), which is a common disease with a high economic burden and is associated with significant morbidity and mortality, particularly among the elderly (File, 2003). Pneumococci are also common inhabitants of the upper respiratory tract of healthy people, especially in children under 2 years of age (Syrjänen *et al.*, 2001). However, the

prevalence of pneumococcal colonization seems to decrease with age (Cardozo, 2008).

Most *S. pneumoniae* strains are covered by a polysaccharide capsule, which makes them more virulent than unencapsulated strains (Kim & Weiser, 1998). At present, 92 different polysaccharide types have been identified by immunochemical methods (Henrichsen, 1995; Jin *et al.*, 2009; Park *et al.*, 2007b), and the sequences of all capsular loci have been determined (Bentley *et al.*, 2006; Park *et al.*, 2007a). For most serotypes, the capsule operon is flanked by the genes *dexB* and *aliA*. The first four capsule genes, *cpsABCD*, are conserved and the serotype-specific genes are located downstream of these (Bentley *et al.*, 2006; Park *et al.*, 2007a). It has been suggested that the product of the first gene of the capsular locus, *CpsA*, is a transcriptional

Abbreviations: ARI, acute lower respiratory tract infection; CAP, community-acquired pneumonia; CPS, capsule polysaccharide.

activator of capsule polysaccharide (CPS) production and influences the level of CPS produced, but is not necessary for encapsulation (Morona *et al.*, 2004). The products of the next three capsule genes, CpsB, -C and -D, however, have been shown to be essential for encapsulation and regulation of CPS production (Morona *et al.*, 2000, 2002). A conventional PCR assay targeted at the *cpsA* (*wzg*) gene has been published previously (Hanage *et al.*, 2006). In that study, it was shown that unencapsulated pneumococcal variants arise through both downregulation of the capsule and loss of the capsular biosynthesis locus.

When analysing *S. pneumoniae* isolates from sputum samples of elderly patients with acute CAP or other acute lower respiratory tract infections (ARIs), we found that almost one-third were unencapsulated, using conventional immunochemical methods, and most of the encapsulated strains were isolated from CAP patients. To confirm these immunochemical findings, we developed a new rapid real-time multiplex PCR targeted at the *cpsB* gene, which is necessary for encapsulation of pneumococci, and then studied the presence of the capsular biosynthesis locus in encapsulated and unencapsulated sputum isolates using this new PCR method and a previously published *cpsA* PCR method, and compared the distribution of encapsulated and unencapsulated pneumococci in the two patient groups.

METHODS

Bacterial isolates. The study material consisted of 59 pneumococcal strains isolated from 291 sputum samples collected from elderly non-institutionalized patients (≥ 65 years) with CAP or other ARIs (controls) (Fig. 1). The isolates were obtained in the 2-year Finnish Community-acquired Pneumonia Epidemiological Study conducted by the National Institute for Health and Welfare (THL, former National Public Health Institute) from May 2005 to May 2007 in Tampere, Finland. CAP cases were confirmed radiologically by at least two of the three independent reviewers. The sputum samples were obtained, processed with dithiothreitol (Sigma), plated on sheep blood agar plates, chocolate agar plates and blood agar plates with gentamicin, and incubated at 36–37 °C in a 5% CO₂ atmosphere. After at least overnight incubation, the plates were transported to the bacteriology laboratory. The plates were examined on arrival and α -haemolytic colonies suspected to be *S. pneumoniae* were identified as

described previously (Kaijalainen *et al.*, 2002). Briefly, α -haemolysis, optochin sensitivity and bile solubility were used to identify *S. pneumoniae*. For serotyping, the isolates were subcultured on blood agar plates and serotyped using counterimmunoelectrophoresis, latex agglutination (neutral serotypes/groups 7 and 14) and the Quellung reaction, as described previously (Kilpi *et al.*, 2001). The isolates were stored in 10% skimmed milk (Difco) at –75 °C.

The test panel for accuracy testing of the multiplex real-time PCR consisted of 86 isolates representing 52 different serotypes or serogroups of *S. pneumoniae* (1, 2, 3, 4, 5, 6A, 6B, 7F, 7B, 7C, 8, 9A, 9N, 9V, 10, 11A, 11B, 12F, 14, 15F, 15A, 15B, 15C, 16, 17, 18F, 18B, 18C, 19F, 19A, 19B, 19C, 20, 22F, 22A, 23F, 23A, 23B, 24, 25, 31, 33, 34, 35F, 35A, 35B, 37, 38, 39, 43, 45 and 47), 37 unencapsulated pneumococcal isolates and nine non-pneumococcal strains, including commercial strains of *Streptococcus pseudopneumoniae* (CCUG 49455 and CCUG 48465) and *Streptococcus bovis* (ATCC 9809), and blood isolates of *Streptococcus mitis*, *Streptococcus sanguinis* and *Streptococcus agalactiae*.

DNA extraction. DNA was extracted with a Magna Pure LC instrument (Roche Diagnostics) using a Magna Pure LC DNA Isolation kit III (Bacteria, Fungi). Before DNA isolation, external lysis was performed as follows. Bacterial colonies were collected from a fresh culture with a sterile loop in 200 μ l PBS and either stored at –20 °C or used immediately. The tubes were centrifuged for 10 min at 8000 *g* and most of the supernatant was discarded, leaving a final volume of 100 μ l bacterial suspension. Next, 130 μ l bacterial lysis buffer and 20 μ l proteinase K were added (both included in the kit). The samples were incubated overnight at 56 °C and inactivated for 10 min at 95 °C. The bacterial lysates were then subjected to DNA isolation according to the manufacturer's protocol.

PCR methods. The presence of a capsular locus was analysed by two PCR methods, each targeted at a different gene (*cpsA* or *cpsB*) of the capsular biosynthesis locus. The *cpsA* PCR method has been published previously (Hanage *et al.*, 2006); however, the correct reverse primer yielding the reported product of 481 bp is 5'-ACACCGAACTAATAGGACCA-3' as described by W. Hanage (Imperial College London, UK).

Six primers were designed with Primer3 software (<http://frodo.wi.mit.edu/>) using the *cpsB* gene of *S. pneumoniae* as a target gene (GenBank accession nos CR931632–CR931722). The oligonucleotides included two forward primers and four reverse primers and were all used in the same reaction mixture. Primer pair wzh2_F (forward) and wzh2_R (reverse) (Table 1) annealed to serotypes 25F and 38. The rest of the serotypes were amplified with a primer set including one forward primer (wzh_F) and three reverse primers (wzh_R, wzh_R2 and wzh_R3; Table 1). The amplicon product sizes were 117–169 bp and all the pneumococcus serotypes were expected to amplify based on

Fig. 1. Flowchart outlining the source of pneumococcal isolates used in this study.

Table 1. Oligonucleotides used in the multiplex real-time *cpsB* PCR

Primer	Sequence (5'→3')	<i>T_m</i> (°C)*
wzh_F	TAGATGACGGTCCCAAGTCA	54.4
wzh_R	GAGTTTCAAACATGCCCTTG	54.5
wzh_R2	ATTTCCCGAACCTGAAGAAAG	56.0
wzh_R3	TCTTTTGCAATTTCACGAACC	56.0
wzh2_F	GTTGGCTTGTCATGTCCTT	56.6
wzh2_R	ATTCGGTGAAACGACTCCTG	56.4

**T_m*, Melting temperature.

analysis with the *S. pneumoniae* CPS BLAST server (http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS/).

The *cpsB* PCR was performed using a LightCycler Instrument (Roche Diagnostics). The 20 µl reaction mixture contained 1 × LightCycler Fast Start DNA Master SYBR Green I mixture (Roche Diagnostics), 3 mM MgCl₂, 0.5 µM each primer and 8 µl extracted sample DNA. The protocol consisted of a pre-incubation step at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 63 °C for 5 s and extension at 72 °C for 8 s. After cycle 15, the annealing temperature was decreased by 1 °C at every cycle until the target temperature of 58 °C was reached. Fluorescence was measured in each cycle after an additional step where the temperature was raised to 80 °C. The temperature transition rate was 20 °C s⁻¹ in all steps. A melting-curve analysis was performed by heating at a rate of 20 °C s⁻¹ to 95 °C, cooling at 20 °C s⁻¹ to 60 °C, holding for 30 s and finally heating slowly at 0.1 °C s⁻¹ to 95 °C with continuous fluorescence measurement. Samples with a crossing point value below 40 and a melting peak above 80 °C were considered positive. The real-time PCR method targeted at the autolysin (*lytA*) gene of pneumococcus was performed as described previously (Sheppard *et al.*, 2004).

Statistical methods. Statistical analyses were carried out using SPSS version 16.0. Statistical significance was assessed using Fisher's exact test.

RESULTS AND DISCUSSION

A real-time multiplex PCR assay targeted at the *cpsB* gene was first developed. The *cpsB* gene has been found to be essential for encapsulation (Morona *et al.*, 2000). The first gene of the capsular locus, *cpsA*, was used as a target in a previously published conventional PCR method for detecting the presence of the capsular locus (Hanage *et al.*, 2006). In the study by Morona *et al.* (2004), a deletion in *cpsB* prevented CPS formation, whilst strains with a deletion in the *cpsA* gene were still able to express the capsule, although the level of CPS was reduced.

The assay developed in this study was optimized using 86 encapsulated pneumococcal isolates representing 52 different serotypes or serogroups. All these were amplified by the multiplex PCR. Two isolates representing serotypes 38 and 25 could not be detected by the previously published *cpsA* PCR, although they amplified well using the *cpsB* PCR described in this study. This is, however, probably not due

to mutations in or loss of the *cpsA* gene but rather due to the sequences of the primers used in the *cpsA* PCR, which were not able to detect serotypes 38 and 25F when analysed against the *cps* sequences available on the *S. pneumoniae* CPS BLAST server. Of the 37 unencapsulated pneumococcal isolates analysed in the test panel, two (5%) were positive by *cpsB* PCR. One of these *cpsB* PCR-positive unencapsulated isolates was also positive by *cpsA* PCR. This suggests that these two isolates had downregulated their capsule synthesis, whilst the other unencapsulated isolates of the test panel clearly had a defective capsule locus. The nine non-pneumococcal isolates, which represented five different species, remained negative by *cpsB* PCR after 45 cycles of amplification.

Altogether 59 clinical pneumococcal isolates from 291 sputum samples were analysed for the presence of capsule by immunochemical methods and two capsular PCR methods. In addition, *lytA* PCR was performed to confirm that the isolates were pneumococci. Forty-nine (83%) of the isolates were obtained from patients with CAP and ten (17%) isolates were from patients with other ARIs. Distribution of encapsulated and unencapsulated pneumococci was significantly different between the patient groups ($P<0.001$). Among the CAP patients, 40 (82%) of the 49 isolates were encapsulated by immunochemical methods, whilst only two (20%) of the ten isolates from ARI controls were encapsulated (Fig. 1). Different distribution of isolates between patient groups was also observed by *cpsA* PCR ($P=0.002$) and *cpsB* PCR ($P=0.002$). PCR amplification of the *cpsA* gene was positive in 36 (73%) of the 49 pneumococci isolated from CAP patients, whilst a positive PCR result was observed only in two isolates (20%) from the ARI controls. For the *cpsB* gene, a positive PCR amplification was observed in 40 isolates (82%) from CAP patients and three (30%) isolates from the ARI controls (Table 2).

There were some discrepancies between immunochemical methods and PCR assays in the demonstration of the presence of capsule. Among the CAP patients, three isolates (6%) were encapsulated by immunochemical methods and by *cpsB* PCR, but remained negative by *cpsA* PCR. These three isolates represented serotypes 1, 14 and 19C and were also negative by *lytA* PCR. However, as demonstrated previously, the *cpsA* gene is not essential for encapsulation (Morona *et al.*, 2004) and therefore deletions or mutations in this gene would not prevent CPS expression. Thus, pneumococcal isolates possessing the *cpsB* gene but no *cpsA* gene could exist; however, the negative result by *lytA* PCR raises the question of whether these isolates are true pneumococci.

One isolate (2%) from the CAP patients and one isolate from the ARI controls were positive by *cpsB* PCR alone (Table 3). It is possible that the *cpsA* gene was either deleted or mutated in these two unencapsulated isolates and that the *cpsB* gene was simply not expressed. On the other hand, these isolates were also negative by *lytA* PCR,

Table 2. Source of the 59 pneumococcal isolates analysed in this study and presence of capsule

Patient group	No. of isolates from sputum samples studied	Presence of capsule (<i>n</i>) by method used		
		Immunochemical methods (%)	<i>cpsB</i> PCR (%)	<i>cpsA</i> PCR (%)
CAP*	49/226	40 (82)	40 (82)	36 (73)
ARI controls	10/65	2 (20)	3 (30)	2 (20)

*CAP defined as any new opacity in a chest X-ray compatible with pneumonia in a symptomatic patient and confirmed radiologically by at least two out of three independent reviewers.

and the possibility that they were other streptococci and not pneumococci cannot be excluded. One isolate appeared to be serotype 21 by immunochemical methods, but none of the *cpsA*, *cpsB* or *lytA* genes could be amplified by PCR. As this encapsulated isolate was negative by *lytA* PCR and seemed to carry a polysaccharide capsule even in the absence of capsule genes *cpsA* and *cpsB*, it is reasonable to consider that this isolate might be a streptococcus other than pneumococcus and that a false-positive result was obtained by the immunochemical serotyping methods. In fact, it has been shown that members of the *Streptococcus mitis* group, in which *S. pneumoniae* and *Streptococcus oralis* are included, possess a C-polysaccharide-like antigen (Gillespie *et al.*, 1993) and the antigens from α -haemolytic streptococci can react not only with a polyvalent antipneumococcal serum (Omniserum; States Serum Institute) but also with the typing sera (Holmberg *et al.*, 1985; Sottile & Rytel, 1975).

The interesting finding in the present study was that encapsulated pneumococci seemed to be associated with CAP, whereas most strains isolated from sputa from patients

with other ARIs (suspected but not radiologically confirmed CAP) were mainly unencapsulated, pointing to the possibility that the demonstration of capsule in pneumococcal isolates is important for the aetiological diagnosis of CAP. In addition, the detection of capsular genes might be even more important than immunochemical detection of CPS. It has been shown that downregulation of capsule expression occurs during the contact phase between pneumococci and host cells (Hammerschmidt *et al.*, 2005). Therefore, an immunochemically unencapsulated pneumococcus may harbour the capacity to express capsule during the course of infection, and the detection of capsular genes might be a way to detect pneumococci capable of expressing capsule and causing CAP. The use of sputum samples in CAP diagnosis is a matter of controversy (García-Vázquez *et al.*, 2004; Kuijper *et al.*, 2003; Rosón *et al.*, 2000; Theerthakari *et al.*, 2001), partly due to possible contamination with microbes from the upper respiratory tract. Unfortunately, very little is known about the carriage of pneumococci in the elderly, and it is possible that unencapsulated pneumococci are more common in this group than in children. However, our results suggest that the finding of encapsulated

Table 3. Serotype distribution and PCR results in different patient groups

Patient group	Serotype	<i>n</i> (%)	No. <i>cpsB</i> PCR-positive (% of serotype)	No. <i>lytA</i> PCR-positive (% of serotype)
CAP (<i>n</i> =49)	19F	9 (18)	9 (100)	9 (100)
	3	3 (6)	3 (100)	3 (100)
	6B	3 (6)	3 (100)	3 (100)
	14	3 (6)	3 (100)	2 (67)
	4	2 (4)	2 (100)	2 (100)
	6A	2 (4)	2 (100)	2 (100)
	9N	2 (4)	2 (100)	2 (100)
	23F	2 (4)	2 (100)	2 (100)
	35F	2 (4)	2 (100)	2 (100)
	Other*	12 (24)	11 (92)	9 (75)
	NC	9 (18)	1 (11)	0 (0)
ARI controls (<i>n</i> =10)	11A	1 (10)	1 (100)	1 (100)
	19F	1 (10)	1 (100)	1 (100)
	NC	8 (80)	1 (13)	0 (0)

*Other included one isolate representing each of the following serotypes: 1, 7C, 7F, 9V, 11A, 15B, 15C, 19A, 19C, 21, 22F and 23A.

pneumococci in sputum samples of elderly patients with ARIs indicates pneumococcal CAP.

Several PCR-based techniques for detecting the pneumococcal capsular locus have been published in the last few years (Kong & Gilbert, 2003; Lawrence *et al.*, 2000; Pai *et al.*, 2006; Tarragó *et al.*, 2008). For instance, Pai *et al.* (2006) described a conventional PCR method for pneumococcal serotyping and used primers targeting the *cpsA* gene as an internal control. Moreover, a recently published real-time PCR method combined a pneumolysin gene (*ply*) PCR with serotype- or serogroup-specific PCR. This method was able to differentiate over 20 serotypes (Tarragó *et al.*, 2008). However, when screening pneumococci capable of causing diseases such as CAP, the real-time *cpsB* PCR developed here could offer diagnostic value with respect to time savings and cost. The advantages of real-time PCR over conventional PCR and culture are its speed and generally a lower limit of detection. With respect to expenses, the SYBR Green technology used in our PCR eliminates the need for expensive fluorescent probes and makes this method available at a lower cost than most real-time PCR applications described previously.

In addition, the suitability of widely used *ply* PCRs in CAP diagnosis has been questioned (Abdeldaim *et al.*, 2009; Smith *et al.*, 2009) and there is a need for new methods. Screening for pneumococci capable of forming the CPS and therefore potential pathogens of CAP could be the method of choice and, according to these preliminary studies, our newly developed *cpsB* PCR could have potential in this. However, the *cpsB* PCR should also be tested directly on sputum samples, and the specificity could be assessed further with larger numbers of strains from non-pneumococcal species.

In conclusion, we have shown that conventional immunochemical detection of CPS agrees well with the demonstration of the capsular locus genes in both encapsulated and unencapsulated pneumococcal isolates. The finding of encapsulated pneumococci in sputum samples appeared to be associated with CAP, and the real-time *cpsB* PCR detecting pneumococci capable of forming a capsule could have potential in sputum diagnostics when investigating the pneumococcal aetiology of this disease.

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