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Multiplex PCR for Identification of Seven *Streptococcus pneumoniae* Serotypes Targeted by a 7-Valent Conjugate Vaccine

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Here we describe a *Streptococcus pneumoniae* one-step multiplex PCR assay which identifies by amplicon size the seven capsular polysaccharide serotypes targeted by the 7-valent conjugate vaccine. The multiplex PCR assay was used to blindly assay clinical isolates recovered during 1999 in the Republic of Ireland from cases of invasive disease whose serotypes were previously determined by classical methods.

Pneumococcal disease is a major public health problem throughout the world. At least 1 million children die of pneumococcal disease every year, most of these being young children in developing countries (15). In the developed world, elderly persons carry the major disease burden. The etiological agent, *Streptococcus pneumoniae* (the pneumococcus), is surrounded by a polysaccharide capsule. Differences in the composition of this capsule permit serological differentiation between about 90 capsular types. However, despite the large variety of capsular types, only a small fraction of these cause most cases of invasive disease. Invasive pneumococcal infections include pneumonia, meningitis, and febrile bacteremia; among the common noninvasive manifestations are otitis media, sinusitis, and bronchitis.

Immunity following pneumococcal disease is directed primarily against the capsular serotype involved. The currently licensed pneumococcal polysaccharide vaccine is based on the 23 most common serotypes, against which the vaccine has an overall protective efficacy of about 60 to 70% (7). Children under two years of age and persons suffering from various states of immunodeficiency, for example, human immunodeficiency virus infection, do not consistently develop immunity following vaccination, thereby reducing the protective value of the vaccine in some major target groups for pneumococcal disease (5). However, in a healthy elderly population, the polysaccharide vaccine provides relatively efficient protection against invasive pneumococcal disease (14). To overcome the problems with polysaccharide vaccines, extensive clinical trials with a new generation of pneumococcal vaccines are now under way. These protein-polysaccharide combinations, known as conjugate vaccines, contain 7 to 11 selected polysaccharides bound to a protein carrier and induce a T-cell-dependent immune response (11). Conjugate vaccines are immunogenic even in children down to two months of age and may reduce pneumococcal carriage and transmission through herd immunity.

A 7-valent antipneumococcal vaccine (Prevnar; Wyeth Pharmaceuticals) is already licensed in several countries and has shown positive results (3, 18). Pneumococcal conjugate vaccines have been shown to reduce the acquisition of vaccine serotypes in the nasopharynx, thereby reducing infection caused by those serotypes (16). Surveillance from the United States since the introduction of the 7-valent antipneumococcal vaccine has estimated 32%, 8%, and 18% reductions in invasive pneumococcal disease due to herd immunity in the age groups from 20 to 39 years, 40 to 69 years, and over 70 years, respectively (18). Studies have also revealed that since the introduction of the 7-valent vaccine in the United States, the rate of disease caused by penicillin-nonsusceptible pneumococci has decreased by 35% (18).

As the 7-valent conjugate vaccine emerges as the leading form of pneumococcal protection, there is a need for a fast and simple means of identifying isolates with the serotypes targeted by this vaccine. In order to evaluate the efficacies of both current and future conjugate vaccines within new populations, it is necessary to understand the serogroup-specific epidemiology of pneumococci and their associated disease types.

Here we describe a one-step multiplex PCR that facilitates the determination of the following seven pneumococcal capsule types targeted by the currently licensed 7-valent conjugate vaccine: 14, 4, 9V, 6B, 18C, 19F, and 23F. The capsular polysaccharide loci were used as targets for PCR because they represent the genetic loci of pneumococcal capsular serotypes (Table 1). All primers had previously been reported in separate studies (6, 12), except for 9VF and 9VR, which were designed by aligning the nucleotide sequence of the seven capsular-type alleles by using Clustal X (17) and selecting primer sequences specific to the 9V capsular polysaccharide synthesis gene locus. The amplicon produced from each primer pair was sequenced to confirm their specificity. PCR products were purified using the High Pure PCR product purification kit (Roche). Approximately 100 ng of purified DNA was sent to MWG (Germany) for sequencing. The sequences obtained were subjected to BLASTN and BLASTX (1) searches of the GenBank database (National Center for Biotechnology Information). Database matches for all seven amplicons sequenced corresponded to the appropriate capsular polysaccharide loci at an identity level of ≥98%, confirming the specificity of each product.

The efficacy of the multiplex PCR was then blindly assayed

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TABLE 1. Nucleotide primer sequences and predicted amplicon sizes for each *S. pneumoniae* capsular polysaccharide gene detailed within this study

Serotype	Primer	Sequence (5′–3′)	Amplicon size (bp)	Reference or source
9V[a]	9VR	CATGAACAAGAACGATATCAGGC	507	This study
	9VF	GATATCCCCGGAATAAATGAAG		
4	4F	CTGTTACTTGTTCTGGACTCTCGTTAATTGG	430	6
	4R	GCCCACTCCTGTTAAAATCCTACCCGCATTG		
18C	18CF	GCATCTGTACAGTGTGCTAATTGGATTGAAG	354	6
	18CR	CTTTAACATCTGACTTTTTCTGTTCCCAAC		
14	14F	GTCTGTTTATTCTATATACAAAGAGGCTCC	268	6
	14R	GCATTGCTACAATCGCTATACTAGATATGC		
6B	6BF	CGACGTAACAAAGAACTAGGTGCTGAAAC	220	6
	6BR	AAGTATATAACCACGCTGTAAAACTCTGAC		
23F	23FFor	TGGTAGTGACAGCAACGA	177	12
	23Frev	CAAAGGCTAATTCAGCATC		
19F	19FFor	GTTCAACGACTAGGACGC	130	12
	19Frev	TAGGCACCAATGTTTCACT		

[a] GenBank accession number for the 9V capsular locus targeted by 9VR and 9VF is AF402095.

against all 144 consecutive *S. pneumoniae* isolates recovered from cases of invasive disease during 1999 in the Republic of Ireland. The serogroups of all pneumococcal clinical isolates screened had been previously determined by coagglutination and/or by direct slide agglutination (2). There was a bimodal distribution in the 144 cases according to patient age; 9.7% of the cases were in patients that were ≤1 year old, and 49.3% of the cases were in patients that were >60 years old. A total of 72.9% of the isolates were recovered from individuals that were ≥40 years old. For each isolate, separate PCRs were performed in 50-μl volumes comprising 1 U of *Taq* polymerase (Gibco-BRL/Life Technologies), 3.2 mM $MgCl_2$, 10 μl of 10 × buffer (1× buffer contains 10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100), 0.8 mM deoxynucleotide triphosphates (dATP, dGTP, dCTP, and dTTP), 1 mM of primers 9VR and 9VF, 0.1 mM of primers 14F and 14R, 0.1 mM of primers 19FFor and 19Frev, 0.3 mM of primers 18CF and 18CR, 0.3 mM of primers 4F and 4R, 1.2 mM of primers 23FFor and 23FRev, and 0.28 mM of primers 6BF and 6BR, to which 1 μl of purified *S. pneumoniae* genomic DNA (~200 ng) (Puregene DNA isolation kit; Gentra Systems, Minneapolis, Minn.) from each of the 144 isolates was added. PCR amplifications were carried out on a PTC 200 DNA Engine (MJ Research) thermal cycler using the following cycling conditions: 95°C for 5 min and 40 cycles of 94°C for 1 min, 54°C for 1.5 min, and 68°C for 3 min. Following amplification, all reactions were extended at 72°C for 10 min and held at 4°C. Five microliters of each PCR mix was analyzed on a 2% (wt/vol) agarose (Sigma) gel containing 0.5 μg of ethidium bromide per ml and viewed by using UV light.

Amplification products of 507 bp were observed for serotype 9V, amplicons of 430 bp were observed for serotype 4, 354-bp amplification products were observed for serotype 18C, 268-bp amplification products were observed for serotype 14, 220-bp amplification products were observed for serotype 6B, 177-bp amplification products were observed for serotype 23F, and 130-bp amplification products were observed for serotype 19F (Fig. 1). The multiplex PCR assay correctly detected 30 isolates of serotype 14, 22 isolates of serotype 9V, 18 isolates of serotype 4, 5 isolates of serotype 23F, 4 isolates of serotype 19F, 3 isolates of serotype 18C, and 3 isolates of serotype 6B. These results correlate exactly with the numbers of each of the seven serotypes identified by using coagulation and/or slide agglutination, which revealed that 59% of the invasive pneumococcal isolates recovered in the Republic of Ireland during 1999 was of one of the seven serotypes targeted by the 7-valent conjugate vaccine.

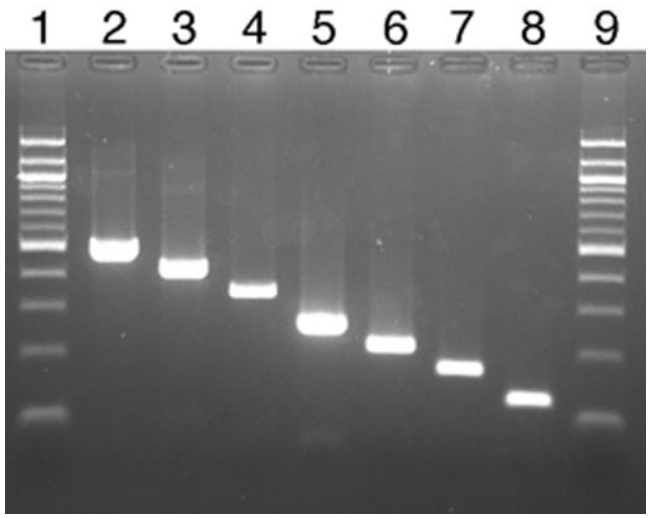

FIG. 1. Gel electrophoresis of purified multiplex PCR amplification products. Lanes 1 and 9, 100-bp DNA ladder (New England Biolabs); lane 2, serotype 9V; lane 3, serotype 4; lane 4, serotype 18C; lane 5, serotype 14; lane 6, serotype 6B; lane 7, serotype 23F; lane 8, serotype 19F.

The specificity of the multiplex PCR assay was evaluated by using purified genomic DNA from the remaining 59 isolates which were not targets of the multiplex PCR assay, out of the 144 invasive isolates recovered in the Republic of Ireland during 1999. These isolates included the following 18 serotypes: 1, 3, 5, 6A, 7F, 8, 10A, 12F, 12B, 15A, 16F, 19A, 20, 22F, 23A, 29, 31, and 34. The primer pairs targeting the serotype 9V was shown to be specific, as there was no cross-reactivity with the other 18 types screened. The primer pairs targeting serogroups 14 and 4 were also shown to be serogroup specific in both our study and those by previous researchers (6). Concerning the primer pairs targeting serotypes 6B and 18C, previous authors demonstrated that these primer pairs are serogroup specific only. This observation was also seen from our results, as the primer pair 6BF and 6BR identified serotype 6A isolates contained within our sample population. The primer pairs targeting the serotypes 19F and 23F did not cross-amplify with any other serotypes tested, were previously evaluated with type strains of the 23 most prevalent serotypes, corresponding to those contained in current polysaccharide vaccines (serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9A, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F), revealed no cross-reactivity (12).

This study demonstrates a very robust and reliable assay for identifying seven of the most prevalent serotypes of *S. pneumoniae*. There are many advantages in using the method described here to type pneumococci. The technique is straightforward and easily established in laboratories which are equipped for DNA template preparation and PCR. The multiplex PCR is optimized for purified genomic DNA templates, which can reduce potential false negatives. Unlike immunological methods used for typing pneumococci, the multiplex PCR assay described is more reliable, as results are very obvious through analysis of the amplicons by agarose gel electrophoresis. The method presented also has advantages over other PCR assays which have been described for typing pneumococci and involve several PCR steps, as the method detailed here involves only one PCR step. Finally, the multiplex PCR assay presented is a fast and far more cost-effective alternative to traditional immunological methods of typing pneumococci. The time taken to perform the PCR assay we describe for up to 100 samples would take approximately 6 h, which includes genomic DNA template preparations and multiplex PCR assays. This is approximately the same length of time required to perform the capsular reaction on up to 100 samples. We calculated the cost of typing each isolate in our laboratory using our multiplex PCR assay to be ~€1 ($1.30), whereas to serotype each isolate by the capsular reaction method in our laboratory costs ~€19.69 ($25.66).

Serotypes identified within the multiplex PCR assay described here accounted for 60 to 70% of all pneumococcal isolates in the 6- to 59-month-old range in a data set comprising strains from Finland, France, Greece, Israel, several East European countries, the United States, and Argentina (9) and 60% global distribution coverage among pediatric patients of noninvasive disease (8). A recent study in France (4) predicted the theoretical coverage of the Prevnar heptavalent vaccine towards pneumococcal invasive disease to be around 80%, which represents one of the best serotype coverage estimates in Europe. The annual reported incidence rates for laboratory-confirmed severe pneumococcal disease in the Republic of Ireland during 1999 may underestimate the morbidity and mortality of disease by 21% and 28%, respectively (13). There is, therefore, a great need for more efficient tests for detecting the most prevalent disease-causing serotypes of *S. pneumoniae*. If more-sensitive diagnostic tests had been available and applied in cases of "unspecified" disease in the Republic of Ireland during 1999, an additional 8 cases of meningitis, 90 cases of septicemia, and 1,348 cases of pneumonia may have been assigned pneumococcal etiology (13). The serotype of the capsule expressed has a major effect on virulence (10), and as the multiplex PCR assay in this study amplifies regions within the capsular loci, it provides a very efficient and important means of studying the epidemiological distribution of the predominant pneumococcal serotypes. We would therefore recommend the use of the multiplex PCR assay described here as an invaluable tool for pneumococcal surveillance and serotype identification to accompany routine detection.

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