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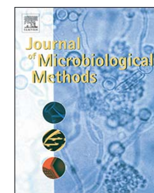

PCR-based discrimination of emerging *Streptococcus pneumoniae* serotypes 22F and 33F

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ABSTRACT

Serotyping of *Streptococcus pneumoniae* is important to monitor disease epidemiology and assess the impact of pneumococcal vaccines. Traditionally, the Quellung reaction used serotype-specific antibodies to classify *S. pneumoniae* based on differences in capsular antigens. More recently, PCR-based serotype deduction relying on serotype-specific capsule biosynthesis genes has been broadly applied for pneumococcal surveillance. However, PCR-based serotyping lacks discrimination for certain *S. pneumoniae* serotypes, including the differentiation of serotype 22F from 22A, and serotype 33F from 33A and 37. Serotypes 22F and 33F are emerging serotypes that are absent in the currently licensed 13-valent pneumococcal conjugate vaccine, but present in the new candidate 15-valent formulation. This study validated novel PCR reactions to detect and discriminate *S. pneumoniae* serotypes 22F and 33F. In order to differentiate *S. pneumoniae* serotypes 22F or 33F from genetically similar serotypes, two novel PCR reactions were designed and validated. The specificity of all PCR targets was evaluated using all 92 different *S. pneumoniae* serotypes, as well as 32 other streptococci. Reproducibility was evaluated using geographically and genetically diverse strains of *S. pneumoniae* serotypes 22F and 22A, or serotypes 33F, 33A, and 37 that were previously characterized by reputable reference laboratories. Overall, *S. pneumoniae* serotypes 22F and 33F could be accurately and reproducibly be detected and discriminated using PCR alone. Such a molecular serotyping approach provides a valuable diagnostic tool that is feasible in any molecular laboratory, to enable pneumococcal serotype surveillance and subsequent assessment of the impact of the new 15-valent candidate pneumococcal vaccine.

1. Introduction

S. pneumoniae (or pneumococcus) is a bacterium that causes significant morbidity and mortality worldwide (Le Blanc et al., 2017; Jain et al., 2015; McNeil et al., 2016; O'Brien et al., 2009; World Health Organization (WHO), 2016). Virulence in *S. pneumoniae* is primarily linked to its polysaccharide capsule, which helps subvert human immune defenses (AlonsoDeVelasco et al., 1995). Differences in capsule sugar composition, linkages, or modifications have been exploited by laboratory methods to characterize *S. pneumoniae* into different serotypes (Bentley et al., 2006; Mavroidi et al., 2007; Geno et al., 2015; Austrian, 1976). Serotype-specific capsular polysaccharides have also been successful vaccine targets. Currently licensed pneumococcal vaccines provide protection against a select number of *S. pneumoniae* serotypes, and have been shown to be effective against pneumococcal diseases; however over time, non-vaccine serotypes have emerged through serotype replacement (Le Blanc et al., 2017; Bonten et al., 2015; Tomcyzk et al., 2014; Centers for Disease Control and Prevention (CDC), 2005; Pilishvili et al., 2010; Demczuk et al., 2013; Hays et al., 2017). Recently, non-vaccine serotypes 22F and 33F have become predominant causes of pneumococcal disease, and coverage for these two serotypes has been added in a new 15-valent pneumococcal conjugate vaccine in clinical trials (Le Blanc et al., 2017; Demczuk et al., 2017; Golden et al., 2016; Horácio et al., 2016; McFetridge et al., 2015; Metcalf et al., 2016; Skinner et al., 2011). Assessing the burden associated with the serotypes 22F and 33F, as well as the impact of this novel pneumococcal vaccine, will require disease surveillance programs with accurate methods for serotyping.

The Quellung reaction has long been the reference method for serotyping, using an algorithm of specific antibodies targeting capsular

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antigens to classify *S. pneumoniae* into distinct serotypes or serogroups (Austrian, 1976). To date, over 92 serologically distinct serotypes of *S. pneumoniae* have been described, which fall into 46 serogroups (Geno et al., 2015). While the Quellung reaction remains a valid method for serotyping *S. pneumoniae* isolates, recent surveillance studies have transitioned to serotype deduction using conventional multiplex PCR (cmPCR), which relies on detection of serotype-specific genes within the capsule biosynthesis (*cps*) loci (Bentley et al., 2006; Mavroidi et al., 2007; Geno et al., 2015; Brito et al., 2003; Dias et al., 2007; Gillis et al., 2017; Jourdain et al., 2011; Lang et al., 2015; Lang et al., 2017; Morais et al., 2007; Pai et al., 2006; Varghese et al., 2017; Tanmoy et al., 2016). Unlike the Quellung reaction, cmPCR is ideal for large surveillance studies given high throughput and automated platforms, and cmPCR does not require the use of microscopy or live organism producing capsule (Brito et al., 2003; Dias et al., 2007; Gillis et al., 2017; Jourdain et al., 2011; Lang et al., 2015; Lang et al., 2017; Morais et al., 2007; Pai et al., 2006; Varghese et al., 2017; Tanmoy et al., 2016). This could be of significant for patients with pneumococcal disease in whom antibiotics had already been administered, or for direct culture-independent processing of clinical specimens (Lang et al., 2015; Morais et al., 2007; Tanmoy et al., 2016). On the other hand, cmPCR can lack discrimination for certain genetically similar serotypes (Brito et al., 2003; Dias et al., 2007; Gillis et al., 2017; Jourdain et al., 2011; Lang et al., 2015; Lang et al., 2017; Morais et al., 2007; Pai et al., 2006; Varghese et al., 2017; Tanmoy et al., 2016). For example, serotype 22F is classified as 22F/A due to the assay's inability to discriminate it from the closely related serotype 22A. Similarly, serotype 33F is classified as 33F/A/37 since cmPCR cannot discriminate it from serotypes 33A and 37. This lack of serotype discrimination poses challenges for epidemiology studies using cmPCR, and this limitation also applies to other molecular methods such as real-time multiplex PCR (rmPCR), nanofluidic rmPCR, Taqman array cards (TAC), microarray technology, and reverse line blot hybridization assay (Dhoubhadel et al., 2014; Jauneikatie et al., 2015; Pimenta et al., 2013; Tomita et al., 2011; Zhou et al., 2007). Next generation sequencing (NGS) pipelines like PneumoCaT have recently been used to assign *S. pneumoniae* serotypes based on comparisons of large genomic DNA sequences to databases including the *cps* loci for all 92 serotypes; however, such analyses requires sophisticated instrumentation and a high level of bioinformatics expertise (Kapatai et al., 2016; Epping et al., 2017; Metcalf et al., 2016a). With serotypes 22F and 33F being included in the new candidate 15-valent pneumococcal conjugate vaccine, this study designed and validated simple cmPCR reactions for accurate detection and discrimination of these serotypes using a methods that are amenable to any molecular laboratory.

2. Materials and methods

2.1. Primer design and bioinformatic analyses

Using accession numbers described previously (Bentley et al., 2006), the *cps* loci of serotypes 22F (CR931682.1), 22A (CR931681.1), 33F (CR931702.1), 33A (CR931698.1), and 37 (AJ131984.1) were retrieved from the National Center for Biotechnology Information (NCBI) Genbank database (https://www.ncbi.nlm.nih.gov/), and queried for *cps* loci targets described in the capsular type variant (CTV) database of PneumoCat (https://github.com/phe-bioinformatics/PneumoCaT), which is used to distinguish serotypes within a serogroup/genogroup (Kapatai et al., 2016). The corresponding target sequences for serotype 22F and 22A, or serotypes 33F, 33A and 37, were compared by multiple sequence alignments performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Desired target sequences were exported form NCBI, and forward and reverse primers were designed using default parameters of Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Each *S. pneumoniae* serotype target was subjected to a nucleotide Basic Local Alignment Search Tool (BLASTn) analysis (https://blast.ncbi.nlm.nih.gov) to ensure specificity *in silico*.

To ensure specificity with currently circulating strains of *S. pneumoniae*, primers and gene targets were compared to available genomes and different lineages of *S. pneumoniae* serotypes 22F and 33F by the National Microbiology Laboratory (NML) (Winnipeg, MB). For serotype 22F, comparisons were made to the 137 genomes described by Demczuk et al. (2017) and 21 genomes from Croucher et al. (2013), which spanned Canadian isolates from 2005 to 2015 and US isolates from 2001 to 2007, respectively. Similarly, genomes sequences for 31 strains of serotype 33F, one strain of serotype 33A, and four strains of serotype 37, collected between 2011 and 2016 were analyzed by the NML (Golden et al., 2016).

2.2. Bacterial culture

S. pneumoniae and other streptococci isolates (Tables S1, S2, and S3) were provided by the freezer strain biorepository in the Division of Microbiology, Nova Scotia Health Authority (NSHA), the Streptococcus and STI Unit at the NML (Winnipeg, MB), the Canadian Immunization Research Network (CIRN) (Halifax, NS), the American Type Culture Collection (ATCC) (Manassas, VA), the Global Pneumococcal Strain Bank at the Centers for Disease Control and Prevention (CDC) (Atlanta, GA) (https://www.cdc.gov/streplab/global-pneumo-strain-bank.html), or the Statens Serum Institute (SSI) (Copenhagen, Denmark). All bacteria were provided by providers as anonymous coded stock cultures, in accordance with local research ethic committees. All *S. pneumoniae* strains were characterized by traditional cmPCR reactions as previously described by Lang et al. (2017) and Quellung serotyping at the NML, CDC, or SSI using commercial omni, pool, group, type, and factor antisera (Statens Serum Institute, Copenhagen, Denmark) as recommended by the manufacturer (Austrian, 1976). All bacteria were maintained at -80 °C in skim milk and grown at 35 °C in 5% CO_2 on trypticase soy agar (TSA) plates with 5% sheep blood (Becton Dickinson and Company, Sparks, MD), with the exception of *Abiotrophia defectiva* and *Granulicatella adjacens* which were cultured onto enriched Chocolate agar (Oxoid, Nepean, ON). Bacteria were harvested from overnight growth, suspended in PCR-grade water (Invitrogen, Grand Island, NY) to a McFarland value of approximately 2.0, and 200 µl was subjected to a nucleic acid extraction.

2.3. Nucleic acid extraction

Total nucleic acids (TNA) were extracted from a 200 µl bacterial suspension using a MagNAPure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics Gmbh, Manheim, Germany) on a Roche MagNAPure 1.0 instrument, according to manufacturer instructions. TNA was eluted in a final volume of 100 µl, and 5 µl was used as template for all PCR reactions. TNA was stored at 4 °C for a maximum of 24 h, or at -20 °C for long-term storage. PCR-grade water (Invitrogen, Grand Island, NY) was used as a negative control for all nucleic acid extractions and PCR reactions.

2.4. Quantitative real-time PCR

To assess the quantity of pneumococcal DNA obtained from TNA extraction, quantitative *lytA* real-time PCR was performed as previously described by Lang et al. (2015). Briefly, real-time PCR reactions were performed using a Taqman Universal PCR Master Mix kit (Applied Biosystems, Branchburg, NJ) in 25 µl reactions as follows: 1 × Master Mix, 200 nM of primers LytA-F (ACG CAA TCT AGC AGA TGA AGC A) and LytA-R (TCG TGC GTT TTA ATT CCA GCT), and 200 nM of probe (LytA-pb) (FAM-TGC CGA AAA CGC TTG ATA CAG GGA G-BHQ1). Amplification was performed using an Applied BioSciences (ABI) 7500 Fast instrument under the following thermocycling conditions: initial activation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s and a combined annealing/extension step at 60 °C for

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