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A simple PCR assay for the identification of the novel *Streptococcus pneumoniae* serotype 7D

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Abstract

The identification of the novel pneumococcal serotype 7D by Neufeld quellung reaction requires significant expertise. To circumvent this, we developed a simple serotype-specific PCR method to discriminate serotype 7D from the closely related serotypes 7C, 7B and 40. The established PCR was validated with the strain collection of the German National Reference Center for Streptococci (GNRCS). However, no isolate initially assigned as serotype 7B, 7C or 40 was identified as serotype 7D.

INTRODUCTION

The opportunistic pathogen *Streptococcus pneumoniae* is a prominent cause of severe medical conditions such as pneumonia, sepsis and meningitis in young, immunosuppressed and elderly individuals [1]. Among other factors, the expression of the polysaccharide (PS) capsule with its anti-phagocytic properties enables pneumococci to evade the immune response of the host by antigenic diversity [2]. Currently, over 100 serotypes, most commonly discriminated by commercially available polyclonal rabbit antisera (factor sera), have been described [3, 4]. Each serotype has its unique capsular polysaccharide (CPS) structure, with some types presenting a PS combination of different serotypes [5]. Available vaccines use the pneumococcal CPS with or without protein conjugation for immunization against the most prevalent serotypes, frequently resulting in serotype replacement.

Until 2018, pneumococcal serogroup 7 consisted of the subtypes 7F, 7A, 7B and 7C. Despite being conflated in the same serogroup, the *cps* loci cluster in two syntenic pairs of amino acid sequence composition (7F/7A and 7B/7C) and show little similarity in CPS structure [6, 7]. In 2018, Kjeldsen *et al.* described a novel serotype, denoted 7D, displaying a combination of serotype 7C and 7B CPSs with an approximate proportion of 5:1 [6]. In consequence, it has been shown that 7D strongly reacts with factor serum 7 f specific for serotype 7C and rather weakly with factor serum 7e specific for serotype 7B. Furthermore, serotype 7D shows no reaction with the serotype 7F and 7A factor sera 7b and 7 c. To date, no other isolate with serotype 7D has been found.

Serotypes 7B and 7C CPSs have similar carbohydrate backbones consisting of the five residues [α-D-Gal*p*NAc-(1→2)-α-L-Rha*p*-(1→2)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*Nac-(1→O-P→] or [α-D-Gal*p*NAc-(1→2)-α-L-Rha*p*-(1→2)-β-L-Rha*p*-(1→4)-β-D-Glc*p*-(1→4)-α-D-Gal*p*Nac-(1→O-P→], respectively, differing only in the terminal sugar molecule. This variable terminal sugar residue (α-GlcNAc or α-Gal*p*NAc) is 3-substituted with a side chain [α-L-Rha*p*(4→1)-β-L-Rib*f*] to the 1-position of the α-rhamnose (α-L-Rha*p*). The *cps*-locus of the analysed 7D isolate (PHESPD0846) turned out to be identical to serotype 7B (CR931641.1), except for a single-nucleotide polymorphism (SNP T385C) in the glucosyltransferase gene *wcwK*, resulting in an amino acid exchange of leucin to phenylalanine. This mutation enables the enzyme to transfer both N-acetyl α-glucosamine (α-GlcNAc) and N-acetyl α-galactosamine (α-Gal*p*NAc) as terminal sugar residue to the carbohydrate backbone.

The current gold standard for pneumococcal serotype assignment is the Neufeld quellung reaction [8]. However, the reliable performance of this technique requires a great deal of expertise as well as a variety of expensive group and factor sera. Therefore, the aim of this study was the development of a simple and cost-effective PCR assay for the identification of the

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Abbreviations: CPS, capsular polysaccharide; GNRCS, German National Reference Center for Streptococci; IPD, invasive pneumococcal disease; PHE, Public Health England; PS, polysaccharide; SNP, single nucleotide polymorphism; UKHSA, UK Health Security Agency; α-GalpNAc, N-acetyl α-galactosamine; α-GlcNAc, N-acetyl α-glucosamine; α-l-Rhap, α-rhamnose.
One supplementary figure and one supplementary table are available with the online version of this article.

novel pneumococcal serotype 7D. The performance of this new method was verified by a screening approach using the strain collection of German National Reference Center for Streptococci (GNRCS). Because of the described similarities, all isolates previously identified as serotype 7B and 7C were included. Additionally, all serotype 40 isolates were analysed. Serotype 40 is a member of the same serotype subcluster with a high amino acid similarity of its *cps* loci to 7C and an even higher one to 7B, and shows cross-reactivity with group serum C and factor serum 7 h [6, 7].

METHODS

Bacterial samples

The strain collection of the GNRCS consists of more than 68000 pneumococcal isolates, covering all currently described serotypes. All pneumococcal isolates classified by Neufeld quellung reaction (*Pneumococcus* antisera for Neufeld, SSI Diagnostica) as serotypes 7B (*n*=110), 7C (*n*=239) or 40 (*n*=6) were analysed for the presence of serotype 7D attributes using the developed PCR method. The analysed sample cohort contains isolates from 1992 to 2022, most of them from German patients suffering from invasive pneumococcal disease (IPD) (Table S1, available in the online version of this article). The serotype 7D reference strain (PHESPD0846) was kindly provided by the UK Health Security Agency [UKHSA; formerly Public Health England (PHE)] and used as a positive control for the PCR reaction.

DNA preparation

Pneumococcal isolates were cultivated on tryptone soya agar with sheep blood (Oxoid, Thermo Scientific) for 18 h at 37 °C and 5% CO_2. DNA extraction was performed from a single colony sub-culture using a quick preparation procedure. Briefly, half an inoculation loop of bacterial material was resuspended in 200 µl 0.9% NaCl solution, incubated for 5 min in a water bath at 100 °C, followed by 5 min of chilling at −21 °C. The suspension was centrifuged for 7 min at 16.200 ***g*** and the supernatant containing the genomic DNA was transferred into a new reaction tube. DNA samples recurrently not generating PCR products were replaced by purified DNA using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's recommendations.

PCR method

The first PCR for the detection of serotype 7D contained 2.5 µl template DNA, 1.25 µl forward and reverse primer (0.5 µmol l^{-1} final concentration each), 0.5 µl forward and reverse primer for internal control (0.2 µmol l^{-1} final concentration each), 15 µl HotStar *Taq* Polymerase Master Mix (Qiagen), and 4 µl molecular biology-grade H_2O (Qiagen) in a 25 µl batch. The optimized PCR sequence used was: 95 °C for 15 min; 25 cycles of 94 °C for 30 s, 58 °C for 60 s and 72 °C for 40 s; 72 °C for 10 min.

The second PCR for verification of a positive result in the first PCR was composed equivalently with 0.5 µl forward and reverse primer (0.2 µmol l^{-1} final concentration each) and 6.5 µl molecular biology-grade H_2O (Qiagen), respectively. The sequence for the second PCR used was: 95 °C for 15 min; 25 cycles of 94 °C for 30 s, 50 °C for 60 s and 72 °C for 40 s; 72 °C for 10 min. Visualization of PCR products was performed using agarose gel electrophoresis on a 2% gel containing 0.18% ethidium bromide (Fig. S1).

Table 1. Oligonucleotide primers for identification of serotype 7D

Designation	Gene	Amplicon size (bp)	Sequence (5′–3′)
wcwK_385nt_f	*wcwK*	312	GAA ATT TGT TCT TTT TAA TGA TGA TC
wcwK_385nt_r			CAA TAA CTT ACT GGA ATA TGA T
wzh1_f	*wzh*	640	GAT GTA GAT GAC GGT CCC AAG TC
wzh1_r			TCG CTC CAT ATT TCT TAG CAA TG
wcwK_Seq_f	*wcwL*	1152	CAA CAT ACA AGA GAG ATT ATA AAT G
wcwK_Seq_r	*wcxU*		GAA CGT AGA ATT TCT AAA CAA C

Last letter of primer designation indicates orientation: f, forward, r, reverse.

RESULTS

Design and optimization of serotype 7D PCR

The identification of pneumococci with serotype 7D was addressed by the development of three different primer pairs (Table 1). Primer wcwK_385nt_f was designed to bind with the 3′ end to the serotype 7D-specific mutation (SNP T385C) in the *wcwK* gene to discriminate serotype 7D from the closely related serotypes 7B, 7C and 40. In combination with the antisense primer wcwK_385nt_r, an amplicon of 312 bp is generated (Fig. 1). Primer pair wzh1_f/wzh1_r produces an amplicon of 640 bp in all four serotypes and serves as internal control for template quality. It binds within the *wzh* gene, which is located 6087 bp upstream of *wcwK* to avoid the generation of amplicons by primer–pair mix. To improve the efficiency of the detection system, both primer pairs are used combined in a duplex reaction.

Positive reactions with primer pair wcwK_385nt and wzh_1 indicating a serotype 7D isolate should be further examined using primer pair wcwK_Seq, which binds 24 bp upstream and 73 bp downstream of the *wcwK* gene in *wcwL* and *wcxU,* respectively. This generates an amplicon of 1152 bp covering the complete *wcwK* gene for Sanger Sequencing based verification of the serotype 7D specific SNP.

The nucleotide sequences of all three primer pairs were verified by Sanger sequencing and checked for specificity using BLASTN seach against the nucleotide and the refseq database. To improve annealing specificity and to minimize the risk of non-specific amplicon generation, HotStar *Taq* DNA Polymerase (Qiagen) was used. The duplex reaction was optimized with respect to annealing temperature, amplification time, primer concentration and number of reaction cycles.

Screening for serotype 7D in GNRCS strain collection

The developed duplex PCR system was used to screen the *S. pneumoniae* strain collection of the German National Reference Center for Streptococci. Purified DNA of the serotype 7D reference strain PHESPD0846 was used as positive control, whereas molecular biology-grade H_2O worked as negative control. Three hundred and fifty-five isolates previously classified as serotype 7B (*n*=110), 7C (*n*=239) or 40 (*n*=6) were tested. The genomic DNA of all isolates generated a 640 bp amplicon with the wzh_1 primer pair. However, no serotype 7D-specific amplicon was detected in the analysed samples. This indicates that, so far, no isolate of the novel serotype 7D could be identified in the German national surveillance of invasive pneumococcal disease.

DISCUSSION

The pathogenic potential of *Streptococcus pneumoniae* is described to be mainly dependent on the antiphagocytic polysaccharide capsule, which is also the target of all vaccine approaches currently on the market [9, 10]. Therefore, surveillance of pneumococcal serotype distribution is of high importance for public health concerns. The constantly growing number of serotypes described for this opportunistic pathogen makes the adaptation and expansion of serotype detection methods mandatory. Our work offers an optimized, simple and cost-effective duplex PCR assay for the identification of the novel pneumococcal serotype 7D. The developed detection system was validated using the pneumococci strain collection of the German National Reference Centre for Streptococci. However, no *S. pneumoniae* isolate of this new serotype could be detected in the German national surveillance cohort.

This negative result might generate doubts regarding the robustness of the primer binding site selection. Indeed, the availability of only one serotype 7D *wcwK* sequence represents a major limitation of the developed PCR and even though the primer specificity was verified by comprehensive comparative analyses including serotype 7B, 7C and 40 sequences, the risk of false-negative results caused by unexpected mutations cannot be eliminated. Nevertheless, since its first detection in 2012 in a hospital in London [6], this variant seems not to have found its way to other countries, as no other serotype 7D isolates have been described so far.

The resulting question of whether this serotype will ever be isolated away from its place of origin or just represents a patho-evolutionary dead end is difficult to address. The increasing number of pneumococcal serotypes may serve as an immunological evasion mechanism that enables the bacterium to colonize and infect a large part of the human population, avoiding eradication by the resulting number of passively or actively immunized people [11–14]. However, this smart mechanism stands or falls with the serological uniqueness of a newly evolved serotype, allowing the pathogen to infect a sufficient number of host individuals already protected against established pneumococcal serotypes, to assert itself against the common players in this environment [15–17]. The newly described serotype 7D, even though equipped with a unique capsule composition, represents a serological hybrid of the well-established serotypes 7B and 7C. Consequently, as described by Kjelden *et al.*, this new variant shows binding of both factor sera, indicating a potential cross-protection of antibodies specific for 7B and 7C against 7D [6]. Therefore, the serological uniqueness and the resulting pathogenic success of this particular capsule type is questionable. However, ongoing surveillance of invasive pneumococcal disease in Germany and other countries might be able to prove this verdict wrong. Serotypes with hybrid PSs might even be a possible tool to include more serotypes within a vaccine formulation, without increasing the number of capsular PSs, as Park *et al.* pointed out in 2015 [18]. The main consequence of this admittedly daring argument is the question

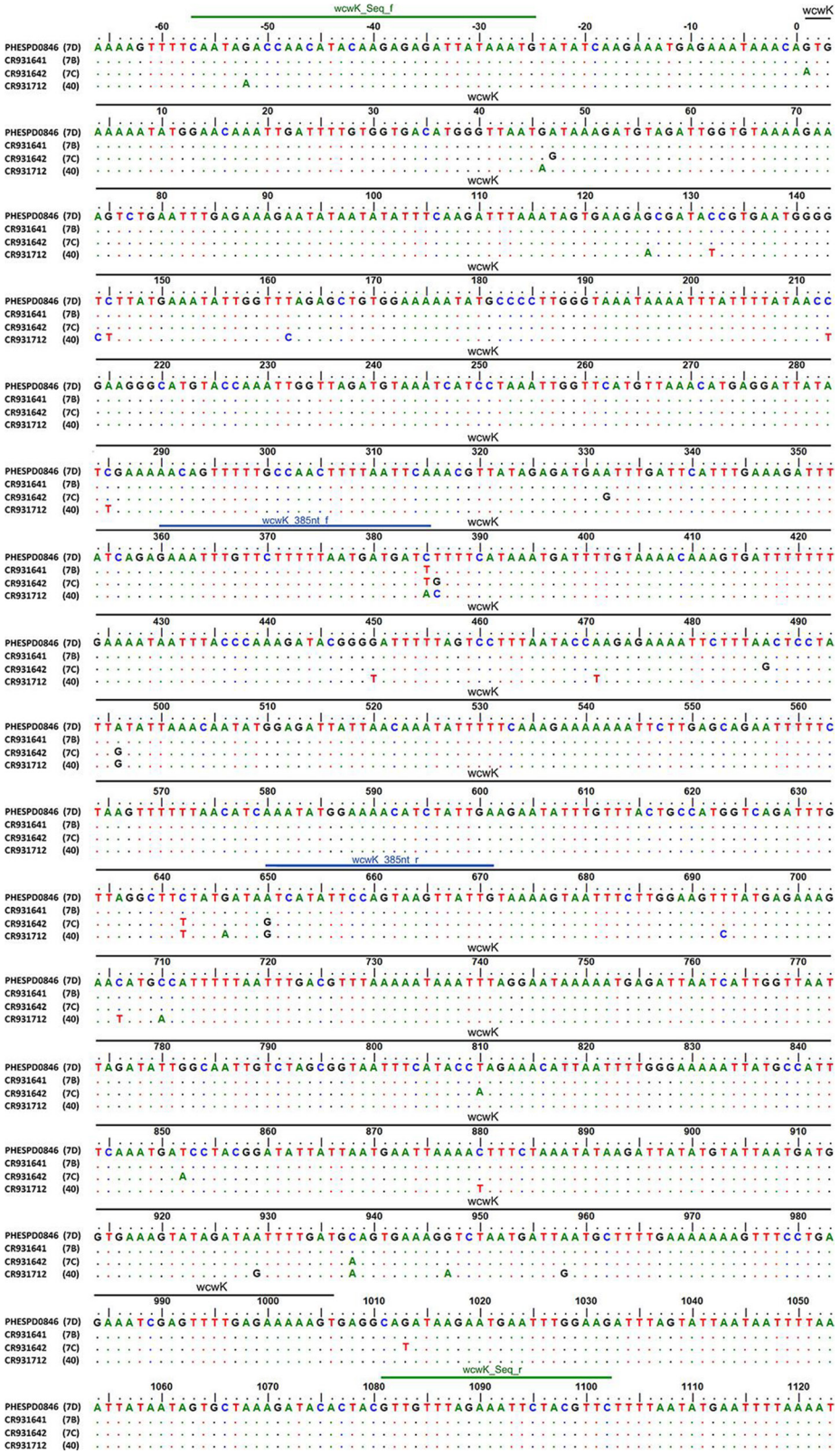

Fig. 1. Primer binding sites and amplicon localization of serotype 7D-specific duplex PCR. Nucleotide alignment of the *wcwK* gene (black line) with corresponding up- and downstream regions of the *cps*-locus of the serotype 7D strain PHESPD0846 in combination with the 7B strain CR931641, the 7C strain CR931642 and the serotype 40 strain CR931712. Primer binding sites for primer pairs wcwK_385nt (blue lines) and wcwK_Seq (green lines) are highlighted.

of whether the term serotype should be used in a different manner, not directly linked to the capsule type, but independently describing the uniqueness of the induced antibody-dependent immune response in the host.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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