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The Molecular Approaches and Challenges of *Streptococcus pneumoniae* Serotyping for Epidemiological Surveillance in the Vaccine Era

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Abstract

Streptococcus pneumoniae (pneumococcus) belongs to the Gram-positive cocci. This bacterium typically colonizes the nasopharyngeal region of healthy individuals. It has a distinct polysaccharide capsule – a virulence factor allowing the bacteria to elude the immune defense mechanisms. Consequently, it might trigger aggressive conditions like septicemia and meningitis in immunocompromised or older individuals. Moreover, children below five years of age are at risk of morbidity and mortality. Studies have found 101 *S. pneumoniae* capsular serotypes, of which several correlate with clinical and carriage isolates with distinct disease aggressiveness. Introducing pneumococcal conjugate vaccines (PCV) targets the most common disease-associated serotypes.

Nevertheless, vaccine selection pressure leads to replacing the formerly dominant vaccine serotypes (VTs) by non-vaccine types (NVTs). Therefore, serotyping must be conducted for epidemiological surveillance and vaccine assessment. Serotyping can be performed using numerous techniques, either by the conventional antisera-based (Quellung and latex agglutination) or molecular-based approaches (sequetyping, multiplex PCR, real-time PCR, and PCR-RFLP). A cost-effective and practical approach must be used to enhance serotyping accuracy to monitor the prevalence of VTs and NVTs. Therefore, dependable pneumococcal serotyping techniques are essential to precisely monitor virulent lineages, NVT emergence, and genetic associations of isolates. This review discusses the principles, associated benefits, and drawbacks of the respective available conventional and molecular approaches, and potentially the whole genome sequencing (WGS) to be directed for future exploration.

Keywords: *Streptococcus pneumoniae*, serotyping, PCR, sequetyping, PCR-RFLP, WGS

Introduction

Streptococcus pneumoniae (pneumococcus) is a Gram-positive bacterium colonizing the human nasopharyngeal region. *S. pneumoniae* causes a significant global disease burden in the unvaccinated population. Globally, over one million annual fatalities are attributed to invasive and deadly pneumococcal diseases (Wahl et al. 2018; Butters et al. 2019). Young children and the elderly are the most susceptible to pneumococcal infections like septicaemia, meningitis, and otitis media (Castiglia 2014).

The global case count for invasive pneumococcal disease (IPD) is estimated at 14.5 million for the year 2000, prior to the use of pneumococcal conjugate vaccines (PCVs) (Balsells et al. 2017). The pneumococcal polysaccharide vaccine (PPV) was developed in 1983 to offer protection irrespective of age by enhancing T cell-specific immunity (Westerink et al. 2012). The older Pneumovax® 23 (Merck & Co., Inc., USA) is a PPV effective

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at protecting adolescents but not efficacious for children below two years of age (Falkenhorst et al. 2017).

In the year 2000, the United States introduced the polysaccharide-protein conjugate vaccine (PCV7) for immunising children. The vaccine comprises antigens that defend the body against seven serotypes: 4, 6B, 9V, 14, 18C, 19F, and 23F. PCV7 is predicted to offer some degree of cross-protection concerning vaccine-related serotypes (VRTs) having subgroups identical to the seven specific targets. Nevertheless, "serotype replacement" takes place where vaccine serotypes (VTs) are reduced while non-vaccine serotypes (NVTs) increase due to the extensive implementation of vaccines (Weinberger et al. 2011). An example is the evolution of serotype 19A, which becomes the primary circulating serotype not covered in PCV7 exhibiting multiple drug resistance (Muñoz-Almagro et al. 2008). Consequently, the infection likelihood from highly antibiotic-resistant NVT pneumococci is a new significant concern in societies and medical fraternities (Arushothy et al. 2020).

Later, two additional PCVs were introduced in response to the emergence of NVTs. In 2009, Synflorix® (PCV10; GlaxoSmithKline plc., UK) was approved; it is effective against ten pneumococcal serotypes (additional serotypes 1, 5, and 7F), followed by Prevnar 13® (PCV13; Wyeth Pharmaceuticals LLC, a subsidiary of Pfizer Inc., USA), introduced in 2010, which covers against three additional serotypes 3, 6A, and 19A. All 13 serotypes are conjugated with CRM197 carrier protein. Recently in 2021, two more additional PCVs were approved; Vaxneuvance™ (PCV15; Merck Sharp & Dohme Corp., a subsidiary of Merck & Co., Inc., USA), which includes all the PCV13 serotypes with the addition of serotypes 22F and 33F, and PCV20 (Wyeth Pharmaceuticals LLC, a subsidiary of Pfizer Inc., USA), known as Apexxna® in the United Kingdom, and Prevnar 20® (Wyeth Pharmaceuticals LLC, a subsidiary of Pfizer Inc., USA) in the United States, offering additional protection against serotypes 8, 10A, 11A, 12F, and 15B (Kobayashi et al. 2022).

Although PCVs offer protection against colonization by VTs; however, they do not inhibit pneumococcal carriage in the nasopharyngeal region. Hence, risk of NVT evolution and the emergence of the other various serotypes over the time span still exists (Weinberger et al. 2011). Generally, low-virulence pneumococci colonization is required for vaccine effectiveness in the long term but repeated horizontal gene transfers in the bacterial population might produce new variants with newer high-virulence strains (Sabharwal et al. 2014). Therefore, extensive PCV's use will induce pneumococci to evolve and emerge as new strains in responding to the pattern of vaccine use. NVTs might occupy the former predominant vaccine serotypes, which are suppressed due to the vaccine selective pressure. Hence, VT to NVT replacement in the bacterial population is of great concern because presently available pneumococcal vaccines offer protection only against the 24 pneumococcal serotypes, while there are 101 serotypes that have been already known (Ganaie et al. 2021).

Policymakers must have complete serotype prevalence and distribution data in monitoring the circulating strains to foresee vaccine efficacy and subsequent serotype replacement (John 2015). Presently used pneumococcal vaccines target the organism's capsular polysaccharides (CPS), which play important roles in colonization and virulence. It is associated with the polysaccharide capsular abilities to protect the organism against the host's defense mechanisms (Briles et al. 1992; Hyams et al. 2010; Larson and Yother 2017). The capsular characteristics with antigen variety differ across the 101 known pneumococcal serotypes (Tomita et al. 2011; Ashu et al. 2016; Ganaie et al. 2021), indicating a different extent of capability on various serotypes to dodge off host immune mechanisms. Several serotypes were known to have a stronger correlation with clinical and carrier isolates, resulting in the invasive outcome of diseases (Jauneikaite et al. 2012; Ziane et al. 2016). Studies concerning pneumococcal serotypes and associated invasive diseases in 51 nations indicated that serotype 14 was widespread in Northern America, Latin America, Europe, Asia, Oceania, and Africa (Johnston et al. 2010). Childhood invasive pneumococcal disease is frequently associated with serotype 19A concerning early PCV7 vaccinations (Beall et al. 2011; Balsells et al. 2017). Nevertheless, implementing PCV10 and PCV13 resulted in a higher emergence of non-PCV13 serotypes in several countries. Non-PCV13 serotypes have the following descending prevalence sequence: 22F, 12F, 33F, 24F, 15C, 15B, 23B, 10A, and 38 (Balsells et al. 2017). On the other hand, data from South East Asian countries revealed that the most common disease-causing serotypes were 19F, 23F,14, 6B, 1, 19A, and 3. Meanwhile, the serotypes of *S. pneumoniae* in healthy children younger than five years were 6A/B, 23F, and 19F (Jauneikaite et al. 2012; Daningrat et al. 2022). Studies conducted in various regions have reported the emergence of non-PCV serotypes following the implementation of the PCV. In Southwest Sweden, Bergman et al. (2021) observed an increase in non-PCV13 serotypes, particularly among elderly individuals aged ≥ 65 years. In China, the post-vaccination era has been characterized by the predominance of non-PCV7 *S. pneumoniae* serotypes 19A and 3 (Li et al. 2021). Similarly, in Brazil and Ethiopia, the introduction of PCV10 in 2010–2011 was associated with increased infection rates of non-PCV10 serotypes and decreased infection rates of PCV10 serotypes (Berezin et al. 2020; Sharew et al. 2021). Conversely, in South Korea, the introduction of PCV13 in 2014 led to an increase in non-PCV13

serotypes, such as 10A, 34, 23A, 22F, 35b, and 11A among invasive *S. pneumoniae* strains responsible for invasive pneumococcal disease (Kim et al. 2023).

In Malaysia, the common and widespread serotypes associated with diseases are 19F, 23F, 14, 6B, 19A, and 6A (Arushothy et al. 2019; Dzaraly et al. 2021; Lister et al. 2021). PCV10 was only recently included in the national immunisation program in late 2020. Therefore, within the context of vaccination, Synflorix® (PCV10; GlaxoSmithKline plc., UK) is stipulated to be effective against the four most prevalent serotypes in Malaysia, whereas Prevnar 13® (PCV13; Wyeth Pharmaceuticals LLC, a subsidiary of Pfizer Inc., USA) should provide a higher degree of coverage concerning both reduction of disease burden and net cost savings for the healthcare system (Shafie et al. 2020).

However, serotypes involved in vaccine development do not always coincide with the those causing the disease. It is because a proportion of infections is also caused by serotypes not included in the vaccine. The coverage rates of PCV13 were previously reported to range from 73% to 88% (Dinleyici and Yargic 2009). Notably, France showed high coverage rates with a rate of >91%, while Canada had a coverage rate of 86%, followed by Malawi exhibited a coverage rate at 80.7%, and China had a rate of 80.5% (Deceuninck et al. 2015; Zhao et al. 2019; Levy et al. 2020; Bar-Zeev et al. 2021). It is expected as serotype distribution, availability, and accessibility of pneumococcal vaccination vary across different countries (Levy et al. 2019). For the latter, some countries included pneumococcal vaccination as a part of their routine national immunisation program (NIP), specifically for children under the age of two. In contrast, in some other countries, there is no legal obligation to vaccinate against pneumococcal diseases.

The genome of *S. pneumoniae* is exceptionally complex, consisting of capsular polysaccharide (*cps*) loci polymorphisms and mutational activities that may also allow "switching" event from one serotype to another in a single isolate (Coffey et al. 1998; Jiang et al. 2001). The *cpsA*, *cpsB*, *cpsC*, and *cpsD* (*wzg*, *wzh*, *wzd*, and *wze*) genes are highly conserved and located on the 5'-end of the *cps* locus followed by central areas for serotype-specific genes, and lastly, by genes responsible for sugar production (e.g., rhamnose genes), which are critical for capsule development (Bentley et al. 2006). Analysis of the *cps* loci implies several mechanisms that create antigenic diversity in CPS. These include the divergence of a single serotype through a point mutation or the insertion or deletion of a single gene; it results in two related serotypes with slightly different CPS structures or nonencapsulated strains (Mavroidi et al. 2004). The constant evolution of the capsular genes, likely due to the pressure of immunisation and cell-to-cell competition, justifies the need for practical methods for distinguishing the serotypes and monitoring their transmission. Hence, this review highlights several pneumococcal serotyping techniques and their associated benefits and challenges.

Serotyping

S. pneumoniae serotyping is needed to assess the epidemiological aspect of vaccine efficacy during the post-PCV implementation; therefore, swift, precise, and economic typing approaches are vital (Jauneikaite et al. 2015). Before the implementation of pneumococcal vaccines PCV13, a fraction of invasive pneumococcal disease attributed to non-PCV13 serotypes was slowly rising (Yoo et al. 2019). The pneumococcal serotype replacement observed through current extensive vaccination is then getting a higher momentum posing a significant challenge (Hanquet et al. 2022). Hence, serotyping is critical to elucidate the VT-NVT serotype transition (Tocheva et al. 2011). Even though data concerning pneumococcal serotypes does not directly affect laboratory diagnosis or patient treatment regime, information concerning disease and carriage-based pneumococcal serotypes is vital for managing vaccination strategy given the currently reported NVT's clonal expansion.

Presently, there are two primary approaches to determining the pneumococcal capsular types: the serological and genetic methods (Neves and Pinto 2022). In the serological method, Quellung or co-agglutination techniques use antisera (Statens Serum Institut, Denmark) for serotyping the pneumococcal cultures. This method is considered a gold standard; however, due to the high cost and technical expertise, the technique is usually available in specialized reference laboratories (Veeraraghavan et al. 2016). The conventional Quellung technique is labor-intensive, relying on self-visual assessment, and the results sometimes need to be understood and correctly interpreted. On the other hand, DNA-based techniques provide a more evidence-based specificity of detection (Lawrence et al. 2000; Leung et al. 2012).

PCR-based serotyping approaches like PCR-RFLP, multiplex PCR, real-time PCR, and sequetyping were devised as substitutes to agglutination approach for amplifying specific capsular DNA elements to offer an economical, sensitive, and relatively straightforward serotyping than conventional methods (Lawrence et al. 2003; Leung et al. 2012; García-Suárez et al. 2019). Employing a PCR-specific reaction in serotyping is advantageous because it facilitates serotype detection, specifically when conventional techniques are challenged with the cost of antisera and constraints in getting good bacterial culture due to the fastidious nature

of certain serotypes (Gonzales-Siles et al. 2019). Nevertheless, PCR approaches (e.g., PCR-RFLP) relying on extended *cps* elements and several restriction enzymes are still associated with a different scope of challenges, such as reproducibility and availability of molecular devices, as well as expertise (Lawrence et al. 2000; Batt et al. 2005).

The recent advancement in whole genome sequencing (WGS) has shown another option considering the massive DNA data for genetic mining targeting the capsular genes. Its current limitations in terms of cost and time consumption hinder its widespread adoption. However, the prospects of bioinformatic pipeline automation and the declining reagent costs make WGS an attractive choice in the future for monitoring and studying pneumococcal serotypes (Mauffrey et al. 2017).

Table I lists the currently available methods with their associated benefits and challenges. The specific scopes of discussion for the respective agglutination- and genetic-based serotyping are presented below.

Quellung approach and Latex Agglutination (LA). In 1902, Neufeld described the Quellung reaction, also called the capsular reaction or the Neufeld test, as the "gold standard" for conducting pneumococcal serotyping. Since then, this reaction has been extensively employed using pneumococcal antisera produced by the Statens Serum Institute (SSI) in Copenhagen (Henrichsen 1979). The Quellung reaction involved

Table I
Serotyping methods with associated benefits and challenges.

Technique	Target areas	Benefits	Drawbacks	References
Quellung	Pneumococcal capsular polysaccharides	• Straightforward • High sensitivity and specificity • Rapid outcomes	• Time, cost, and labor intensive • Cannot detect multiple serotypes using one sample • Inconvenient for larger samples • Extensive skills are required	• Henrichsen 1979 • Sørenson 1993 • Lalitha et al. 1999 • Jauneikaite et al. 2015 • Jin et al. 2016
Latex agglutination	Pneumococcal capsular polysaccharides	• Straightforward • Easy • Rapid outcomes • Appropriate for resource-poor conditions • Needs the least expertise	• Cost-intensive • Questionable accuracy • Culture-negative isolate might be missed	• Slotved et al. 2004 • Ortika et al. 2013 • Porter et al. 2014 • Swarthout et al. 2021
Multiplex PCR	Glycosyltransferase gene	• Economical • Rapid outcomes • Versatile • Accurate • Sensitive	• Several primers • Several steps • Limited serotypes supported • Lack of internal control for specific serotypes • Emerging NVTs remain unexploited	• Lawrence et al. 2003 • Carvalho et al. 2010 • Jin et al. 2016 • Zhou et al. 2021
Sequetyping	CpsB gene	• Straightforward and economical • Single PCR amplification • Single-step PCR • Needs one primer set	• Restricted to single isolate detection • Some isolates exhibit high homology of the cpsB gene across different serotypes • Needs sequencing facilities or services	• Leung et al. 2012 • Jin et al. 2016 • Nagaraj et al. 2017 • Zhou et al. 2021
Real-time PCR	Pneumococcal capsular polysaccharides	• High sensitivity • Able to replicate DNA in low copy number • Serotyping from culture-negative samples • No sample manipulation is needed after the amplification	• Need specialised equipment • Costly PCR probes • Emerging NVTs remain unexploited	• Tarragó et al. 2008 • Azzari et al. 2010 • Pimenta et al. 2013 • Pernica et al. 2014 • Kralik et al. 2017
PCR-RFLP	cps genes	• Straightforward • Fast • Cost-effective • Versatile • Reproducible	• Needs targeted restriction enzymes • Needs several restriction enzymes • Cannot assess mutation type • Time-intensive	• Batt et al. 2005 • Camargo et al. 2015 • Dai and Long 2015 • Garcia Suarez et al. 2019
Whole genome sequencing (WGS)	Pneumococcal capsular polysaccharides	• High accuracy • Greater resolution • Comprehensive analysis • Reduced turnaround time	• Cost-intensive • Needs an advanced bioinformatic tool • Requires technical expertise and skill	• Kapatai et al. 2016 • Mauffrey et al. 2017 • Epping et al. 2018 • Knight et al. 2021 • Sheppard et al. 2022

rabbit antiserum reacting with pneumococcal suspension, resulting in capsular reaction visibility due to in situ immunoprecipitation (Sørensen 1993). According to Henrichsen (1999), four types of typing antisera are available for diagnostic purposes. The first type is Omniserum, which consists of nine pooled sera labeled with A through I. The second type is the chessboard panel, which comprises up to 12 pools. Each pool contains antibodies that are specific to groups of serogroups and serotypes. The third type is monospecific sera, which can replace factor sera specific to a particular serotype. For serogroups that contain multiple serotypes, group sera are cross-reactive with serotypes within the serogroup. Finally, the fourth type is the pneumococcal monoclonal antisera, which is used to differentiate serotypes within a serogroup.

Sørensen (1993) improved the serotyping approach using a chessboard system. This chessboard system consists of seven existing pooled antisera and five new pools to identify 23 vaccine serotypes. About 90–95% of blood and cerebrospinal fluid (CSF) based pneumococcal strains could belong to various serogroups or serotypes (Sørensen 1993). This method needs a panel of expensive sera (Lalitha et al. 1999). In 2004, the Statens Serum Institute (SSI) formulated Pneumotest-Latex based on a chessboard system that utilizes polystyrene latex particles with the antisera to provide simplicity and rapidity (Slotved et al. 2004). The Pneumotest-Latex kit comprises latex particles to which 14 specific pooled pneumococcal antisera are applied, including pools A to I and P to T. The chessboard system guided the step to-step of antisera panels for more systematic and coordinated testing, starting from serogroups to the possible specific serotypes.

Kirkman et al. (1970) were the first to introduce microtiter plate agglutination typing, which involved testing a microtiter plate against 46 type-specific sera for agglutination using a single antiserum. Before testing, the culture was pre-treated with formalin to facilitate apparent agglutination, which can be visualized with the naked eye. Using a microtiter plate confers several advantages, such as the ability to compare reactions simultaneously and the ease of performing the assay. Initially, latex agglutination (LA) was used to detect ß-streptococci. Subsequently, LA was adapted for use in the Pneumoslide test, which utilizes latex beads coated with specialized Omniserum to identify the presence of 83 pneumococcal capsular polysaccharide antigens. The resulting latex particle aggregation was of enough size to enable rapid visualization of positive agglutination (Browne et al. 1984). In 1988, Lafong and Crother (1988) improved the LA technique by introducing a simple slide agglutination method that employed specific antibody-coated latex particles, requiring minimal antiserum. The visualization of agglutinations indicated positive results, while a milky suspension suggested a negative result (Lafong and Crother 1988).

Singhal et al. (1996) modified the LA technique for rapid detection. To achieve this, latex particles were sensitized with the Omniserum reagent, allowing for direct serotyping of cerebrospinal fluid samples without prior treatment. The sensitivity of this method was found to be 100% for both pneumococci and *Haemophilus influenzae* type B, with specificities of 97.1% and 99.6%, respectively. The Pneumotest LA assay was performed to ascertain the serotype linked with the observed agglutination reactions. The kit enabled the differential identification of all the PCV13 serotypes. Additionally, the kit allowed for the differentiation of a limited number of serogroups; however, it did not provide further differential identification of NVT. This Pneumotest LA exhibited precision concerning vaccine serotype identification for practical use and assessment (Swarthout et al. 2021).

The results obtained from LA were mostly found to be consistent with those obtained from the Quellung reaction. As a result, LA has been recommended as an appropriate substitute for the Quellung reaction by World Health Organization (WHO) due to its simplicity and minimal technical expertise requirements, making it easy to use (O'Neill et al. 1989; Lalitha et al. 1996; Porter et al. 2014).

Quellung requires an isolate cultured from a sample (Azzari et al. 2008), and a specific isolate must be assessed against several antisera, impeding detection speed. It requires significant expertise and labor for acceptable performance (Lafong and Crother 1988; Arai et al. 2001). Inconsistencies arise due to laboratory errors and the high cost of antisera panels for both Quellung and LA. In another study, a substitute was devised for commercial LA reagents through modification in the dilution of antisera and polystyrene latex beads, the inclusion of centrifugation and washing steps, and utilization of a higher concentration of bovine serum albumin (Ortika et al. 2013). It used in-house reagents composed of glycine-buffered saline, 0.2% bovine serum albumin, polystyrene latex particles, and sodium azide as a preservative. These reagents are relatively inexpensive, easy to produce, and have a long shelf life, making them suitable for use in low-income nations (Ortika et al. 2013).

Multiplex Polymerase Chain Reaction (PCR). Multiplex PCR in pneumococcal serotyping targets the operon's central region (e.g., glycosyltransferases (GTs)), where serotype-specific genes are located (Brito et al. 2003; Kong et al. 2003; Lawrence et al. 2003; Kong et al. 2005; Billal et al. 2006). GTs catalyze glycosidic bond development to connect a nucleotide-activated sugar (donor) with a lipid-linked glycan precursor (acceptor). Consequently, GTs initiate the component order for the

repeated polysaccharide elements that form pneumococcal capsules. Though multiplex PCR techniques enhance sensitivity and accuracy, numerous serotype-specific primers are required to ensure comprehensive serotype coverage (Lawrence et al. 2003). Therefore, this method becomes more challenging when the discoverable serotype count increases in the population. Notwithstanding the need for several primers, this technique is regarded as straightforward and economical for evaluating DNA amplification of the targeted genes using agarose gel electrophoresis (Brito et al. 2003).

Pai et al. (2006) augmented seven reactions of sequential multiplex PCR to differentiate 17 serotypes from invasive clinical specimens with a higher specificity than serological culture approaches. The multiplex PCR can identify all six serogroups of isolates, but due to the high sequence homology between 6A and 6B *cps* loci, it cannot distinguish between serotypes 6A and 6B. Thus, pyrosequencing was developed to overcome this limitation (Pai et al. 2005). Billal et al. (2006) reported their inability to identify and differentiate pneumococcal serotypes within serogroup 6 using multiplex PCR on patients with acute otitis media samples. While in 2010, the Centers for Disease Control and Prevention (CDC; USA) employed eight reactions in a sequential multiplex PCR for detecting 40 serotypes (da Gloria Carvalho et al. 2010). Jourdain et al. (2011) developed assays to rapidly detect the carriage-associated serotypes of *S. pneumoniae* in Belgian preschool children. Seven sequential reactions were employed using the CDC primer sequence, and discovered that three PCR reactions were adequate for typing 75% of the isolate collection.

Shakrin et al. (2013) formulated five sets of primers targeting the predominant serotypes in Malaysia and Asia (Set A-E) in a sequential multiplex PCR and tested them against 41 pneumococcal strains. When the sequential multiplex testing is performed using the set A to E order on isolates of unknown serotypes, amplification probability rises because the high dominance of serotype-associated primer in the earlier set is targeted in the first few PCR reactions, followed by the fewer ones (Shakrin et al. 2013). The initial three processes used several primer sets for performing sequential multiplex PCR assessment to distinguish the six most-common types in Asia (19F, 19A, 14, 23F, 15, 6). If the specimen's initial three reactions were negative, it was subjected to eight sequential multiplex reactions as specified by CDC (Jin et al. 2016). In another study, a multiplex system with eight reactions successfully identified 73.3% of *S. pneumoniae* serotypes in the study collection; nevertheless, identifying the serotypes 17A, 24F, 28A, 28F, 29, and 33B was unsuccessful primarily because of the limited target primers in the multiplex PCR (Zhou et al. 2021).

CDC recommends the multiplex PCR technique because of its ease of use, relatively low labor requirement, and fast results, facilitating a quick screening of about 40 serotypes (da Gloria Carvalho et al. 2010). Therefore, more serotype-specific primer sets are needed to facilitate a more comprehensive serotype assessment, at least explicitly covering all the VTs, including those newly added in PCV15 and 20 (Brito et al. 2003; Lawrence et al. 2003). Potential NVT emergence will require multiplex PCR to include those as well. However, priorities need to be made for which NVT to be included as there are many more of them, and that combination should also be workable in the multiplex system. Moreover, the internal regulation employed for multiplex PCR, *cpsA*, is not ubiquitously present in all serotypes; some variants like 25F and 38, and the seldom 14 might be designated as non-typeable (da Gloria Carvalho et al. 2010; Veeraraghavan et al. 2016). The lack of *cpsA* amplification might be attributed to pneumococcal isolates having different or altered genes or a lack of *cps* locus (Brito et al. 2003). Serotype-specific variations, lack of distinction concerning serotypes within serogroups, specifically concerning serogroups 6, 9, and 18, and the likelihood of absence of an intrinsic positive control are the drawbacks of the multiplex PCR approach (Pai et al. 2005; Jourdain et al. 2011; Veeraraghavan et al. 2016).

Sequetyping technique targeting *cpsB*. All serotypes, except for serotypes 3 and 37, exhibit extensively preserved 5'-end *cps* locus (*cpsA*, *cpsB*, *cpsC*, and *cpsD*) regulatory genes, suggesting the perseverance of these genes for the production of the *S. pneumoniae* capsule. Regulatory genes, specifically *cpsB*, have higher conservation levels in serotypes than in other *cps* locus areas (McEllistrem et al. 2004; Leung et al. 2012). Therefore, the *cpsB* is employed as the ubiquitous target concerning the novel single primary sequetyping (Leung et al. 2012; Nagaraj et al. 2017). Lately, PCR sequetyping based on the single-CpsB gene has been formulated as an alternative for serotyping. This technique produces a 1,061 bp sequence representing the complete *cpsB* area, enhancing resolution and the probability of routine discernment of common serotypes (Leung et al. 2012; Nagaraj et al. 2017; Gonzales-Siles et al. 2019).

Data on the *cpsB* sequence indicated that about 95% of the pneumococcal collection in a study had a correct serotype mapping (McEllistrem et al. 2004). This technique simultaneously distinguished 46 serotypes (from a total of 93) during its development (Leung et al. 2012). Sequetyping was associated with other challenges concerning the amplification and differentiation of 25A and 38 into the precise serogroups; at the same time, other strains provided ambiguous outcomes concerning serotypes 17F/33C, 11A/D/1818F, and 13/20 (Dube et al. 2015). It was found that *cpsB* could not be amplified for

serotypes 25F, 37, 38, 39, and 43 (Mauffrey et al. 2017). There is still significant homology concerning *cpsB* from some distinct serotypes, which can only be identified as a combined form of serotypes or serogroup, e.g., 6C-6D or 13-20A-20B (Zhou et al. 2021).

Jin et al. (2016) developed a detailed *cpsB* sequetyping database, complemented by the selective and modified CDC's sequential multiplex PCR; 191 isolates were successfully "serotyped" from a 193 isolates collection. A comparison of the Jin et al. (2016) *cpsB* and GenBank's *cpsB* databases indicated mildly better outcomes for the former database, specifically for inconsistent outcomes, because the database was more detailed in terms of greater identification of serotypes at the serotype level and fewer serotype misidentification (Mauffrey et al. 2017).

Nagaraj et al. (2017) modified the sequetyping technique by performing PCR amplification in two steps, called PCRseqTyping, and the results correlated with the Pneumotest results. A set comprising 91 pneumococcal serotypes (without serotypes 3 and 37) was split into homologous (32 serotypes) and non-homologous (59 serotypes) classes. The first amplification sequencing step comprised 59 serotypes, while the second PCR iteration precisely attributed 32 serotypes to the corresponding serotypes based on group-specific primers and sequencing (Nagaraj et al. 2017). Gonzales-Siles et al. (2019) employed newly developed internal primers *wzh*-mid-F and *wzh*-mid1-R to improve the sequetyping assessment, producing two moderately overlapping sequences that created two amplification elements for further sequencing. Since amplicon size is typically larger than Sanger sequencing capabilities, the new technique helps identify with appreciable quality the 1,061 bp part comprising the entire *cpsB* sequence (Gonzales-Siles et al. 2019). Nevertheless, this technique significantly benefits the extensive range of theoretically identifiable serotypes using sequetyping.

Sequetyping using a single primer set is an alternative for multiplex PCR, where the latter requires several primer steps and pairs; these steps can likely be time-intensive when labor is accounted for. Sequetyping offers a cost-effective, efficacious, and relatively straightforward serotyping approach with appreciable coverage, including NVT and the ability to use one amplification reaction to determine the pneumococcal serotype (Leung et al. 2012; Nagaraj et al. 2017; Gonzales-Siles et al. 2019). This approach offers an economical substitute for traditional serotyping and multiplex PCR. It is feasibly applicable to laboratories offering sequencing and PCR options for serotyping most of pneumococcal strains. Sequetyping will benefit from the increasing availability of genomic information using public databases. Nevertheless, the *cpsB* sequence information in public datasets must be appropriately managed and updated regularly. This aspect creates challenges concerning partial data, wrong serotype designations, and assignments (Zhou et al. 2021).

Despite the rising use of molecular approaches for pneumococcal typing, phenotypic approaches, together with LA and the Quellung method, offer higher reliability for identifying and verifying probable false-positive PCR outcomes since several serotypes are not completely differentiable using the *cps* sequence (Mauffrey et al. 2017; Jin et al. 2016). Moreover, sequetyping is constrained to single isolates due to difficulties in differentiating serotypes during sequence chromatogram evaluation (Gonzales-Siles et al. 2019). In contrast, multiplex PCR is an efficacious approach because one reaction facilitates concurrently identifying one or more alleles or genes. Nevertheless, the current approach presents serotypes within serogroups through an identical signal because of the higher degree of genetic similarity, specifically for primer sets for serogroup 6 (6A, 6B, 6C, and 6D) (Mauffrey et al. 2017). Several serotypes cannot be discriminated and are only assigned in combinations, e.g., 6C-6D or 13-20A-20B (Zhou et al. 2021).

Real-Time PCR. Real-time PCR is a highly efficient diagnostic tool for microbiological infections, offering superior sensitivity in detecting serotype-specific genes in clinical samples compared to conventional multiplex PCR. While conventional multiplex PCR is practical and cost-effective for laboratory use, it may be inadequate to identify serotypes in low DNA copy numbers (Azzari et al. 2010). To enhance the precision of pneumococcal capsular typing from culture-negative clinical specimens, TaqMan MGB probes were designed with a high sensitivity to target DNA sequences within the capsular polysaccharide gene cluster. This specific design enables the identification of 24 serotypes through a real-time PCR assay, as Tarragó et al. (2008) reported.

Tavares et al. (2019) emphasize the value of a real-time PCR assay that targets SP2020 (putative transcriptional regulator gene) and the *lytA* gene to identify pneumococci from culture-independent samples accurately. While the detection of these genes individually may lead to the identification of non-pneumococcal strains, the combination of both assays can avoid misidentification. In the context of pediatric community-acquired pneumonia (CAP) complicated by effusion/empyema, the application of multiple monoplex real-time PCR for detecting *S. pneumoniae* in parapneumonic effusion samples has been found to be more effective than culture-based methods. Specifically, while culture-based methods identified the bacterium in only 14% of samples, real-time PCR yielded positive results in 62% of cases, indicating its superior performance in the microbiological diagnosis of CAP and the potential to more accurately identify the microbiological cause of the condition (Pernica et al. 2014).

In the same study, 35 *S. pneumoniae* PCR-positive samples underwent PCR serotyping. The results of real-time PCR testing revealed that the majority (97%) of *S. pneumoniae* parapneumonic effusions were caused by serotypes included in the 13-serotype vaccine (Slinger et al. 2014). Additionally, Kralik and Ricchi (2017) demonstrated that real-time PCR is a safer method to avoid cross-contamination since there is no need for additional sample manipulation following amplification.

According to Dube et al. (2015), real-time multiplex PCR effectively detects the 21 serotypes/groups targeted by the assay, although it is limited by serotype coverage, resulting in the inability to detect a significant proportion of serotypes. Consequently, this limitation restricts the usefulness of the assay in regions where pneumococcal conjugate vaccines have been introduced, as serotype replacement may occur due to either serotype unmasking or capsular switching. Therefore, additional assays are required to target emerging NVTs to enhance the current capability of multiplex real-time PCR serotyping (Pimenta et al. 2013). A newly expanded sequential real-time PCR scheme of 14 quadruplex reactions has been developed (Velusamy et al. 2020). The PCR assay identifies 64 individual serotypes/serogroups, antibiotic resistance, and pili genes. Furthermore, Velusamy et al. (2020) have demonstrated the capability of this expanded sequential real-time PCR to differentiate between all individual serotypes in serogroup 6 accurately.

PCR Restriction Fragment Length Polymorphism (PCR-RFLP). Botstein et al. (1980) first described RFLP that produced a genetic association map for humans; it was the first DNA profiling approach used extensively for gene description and genome mapping (Botstein et al. 1980; Dai and Long 2015). The PCR-RFLP approach is based on the amplification of a particular DNA target areas and digestion based on restriction enzymes to produce several distinctly-sized DNA fragments to enable distinguishing between distinct serotypes (García-Suárez et al. 2019; Lawrence et al. 2000). Restriction enzymes used in the PCR-RFLP technique make use of the *cps* polymorphisms in pneumococci for serotype detection (Camargo et al. 2015).

Numerous studies employed the PCR-RFLP technique; however, the *cpsA-cpsB* and *dexB-aliA* regions contain extensive fragments, requiring the selection of a sequence corresponding to the *cps* gene and the specific restriction enzyme set for generating fragments (García-Suárez et al. 2019). Moreover, PCR amplification of a regulatory area between the *cpsA* and *cpsB* locus based on three distinct restriction enzymes (*AluI*, *HinfI*, and *RsaI*) facilitates distinguishing the serogroup level, while the specific serotype cannot be distinguished (Lawrence et al. 2000). Targeting the *cpsA* and *cpsB* regions indicated a mild correlation between serotypes and restriction patterns, except for serotype 6A strains with identical banding patterns with several serotype 6B.

According to Batt et al. (2015), the disadvantages of this approach are that it requires two or three restriction enzymes to generate distinguishable patterns and the increased time required for strain assessment. Therefore, PCR-RFLP was improved by amplifying the complete *cps* locus for every serotype using a single digestion enzyme (*HinfI*), which is capable of discriminating at least 46 types of *S. pneumoniae* to serogroup or serotype level; the band profiles observed from the restriction digest protocol were robust and reproducible (Batt et al. 2005). While endonuclease *XhoII* was able to generate 76% and 100% serotyping and serogrouping outcomes, respectively (Camargo et al. 2015). Hence, the *XhoII*-augmented cps-RFLP technique produced a superior outcome than antisera and other molecular serotyping approaches. The most recent PCR-RFLP version specifies a serotyping approach based on the same PCR primers for amplifying comparatively minor regulatory areas between *cpsA-cpsD* and an RFLP process using *Sse9I* for resolving serogroup and serotype (García-Suárez et al. 2019). *Sse9I* digestion-based *cps* analysis offered a potent tool for precise serotyping of the most prevalent *S. pneumoniae* serotypes (1, 3, 6A, 14, and 19A, including non-PCV13 serotypes such as 24F, 23B, and 10A) (García-Suárez et al. 2019).

Whole Genome Sequencing (WGS). Whole genome sequencing has become a valuable tool in epidemiology, particularly for identifying antimicrobial resistance, diversity of clones, and capsular serotyping. Additionally, WGS enables accurate and efficient serotyping of bacterial isolates, which is crucial for public health measures such as vaccine development and outbreak investigations (Kim et al. 2016). The first fully automated pipeline bioinformatic tool is the Pneumococcal Capsular Typing (PneumoCaT), which uses the Capsular Typing Variant (CTV) database to predict capsular type from WGS data and may potentially identify novel serotypes (Kapatai et al. 2016). However, PneumoCaT is slow and computationally intensive, taking several minutes per serotype determination. SeroBA is a faster and high-throughput serotyping tool that uses a *k*-mer approach, although it cannot automatically recognize mosaic sequences associated with serotypes (Epping et al. 2018). Mixed serotype detection remains challenging for both PneumoCaT and SeroBA, but the development of SeroCall and PneumoKITy has improved the accuracy and speed of mixed serotype detection (Knight et al. 2021; Sheppard et al. 2022).

WGS is costly that requires specialized bioinformatic platform but provides a precise assessment of a population change over time, which was previously difficult and time-consuming (Everett et al. 2012).

The emergence of new clones and variations can affect the accuracy of serotyping techniques, highlighting the importance of using WGS (Chang et al. 2018). As seen with the new variant of serotype 14 streptococci, serotyping results can differ depending on the method employed, and in some cases, testing with antisera is necessary to achieve accurate serotyping. For example, while SeroBA classified three isolates as serotype 14, PneumoCaT identified them as non-typeable, and SeroCall categorized two isolates as serotype 14 and one as non-typeable (Manna et al. 2022). Similarly, Cao et al. (2021) reported that while SeroBA predicted an isolate belonged to serogroup 24, subsequent testing with antisera specific to the group revealed that the isolate only reacted with 24D, not 24C or 24E, leading to its classification as serotype 24F. In a study by Manna et al. (2018), the 33F capsule locus sequence was analyzed using PneumoCaT, which identified a new clone of serotype 33F that contained a frameshift mutation, lacked *wcjE*, and had a *wcyO* pseudogene. In 2018, Kjeldsen et al. (2018) employed a comprehensive approach comprising NMR spectroscopy, production of antisera, and serotyping using PneumoCaT, which enabled the identification of a novel serotype, 7D. When compared to sequential multiplex PCR and sequetyping, WGS was found to be more accurate in determining the serotype for *S. pneumoniae* (Mauffrey et al. 2017).

In the coming years, WGS is anticipated to become more widely used as a tool for serotype inference due to its various advantages over conventional methods. With WGS, additional information, such as antimicrobial resistance and multi-locus sequence type, can be inferred from the same dataset without additional testing. Moreover, serotypes can be definitively assigned using this technique. As sequencing costs continue to decline and bioinformatic pipelines become increasingly automated, the use of WGS is expected to replace conventional pneumococcal typing tools, even in low-resource settings (Dube et al. 2015)

Conclusion

The emergence of NVTs and their clonal proliferation are expected to change future vaccination approaches. Therefore, dependable pneumococcal genotyping and serotyping are necessary to precisely detect virulent lineages, comprehend the genetic associations between isolates, and understand how NVTs emerge. Additional comparative genomic assessment of outbreak-related strains can provide details concerning virulence and emphasize the significance of evaluating serotype substitution and capsular switching after a childhood immunization schedule is updated to include the pneumococcal vaccine. The emerging technology in whole genome sequencing may offer a better comprehensive genomic scale for validation.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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