

Short Communication

Determination of Pneumococcal Serotypes by Sequotyping and Sequential Conventional Multiplex PCR in the Vaccine Era

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ABSTRACT

Pneumococcal serotyping is required for epidemiological surveillance to guide vaccination strategy. DNA-based approaches are more affordable, but the combination of sequotyping and sequential conventional multiplex polymerase chain reaction (cmPCR) may complement one another. A total of 101 isolates were subjected to sequotyping and sequential cmPCR following previously published protocols, and the outputs were compared. The sequotyping method determined up to the serotype level for 99 isolates (98%). On the other hand, the sequential cmPCR technique identified 91 isolates (90.1%), with 63 of them (62.4%) up to the serotype level. Sequotyping generated discrete serotypes for 6A/B, 11A/D, 15A/F, and 15B/C as 6A (n = 11), 6B (n = 10), 11A (n = 5), 15C (n = 1), and 15A (n = 1). In conclusion, the *cpsB* gene sequotyping method produced a comparable output with sequential cmPCR, further discriminating some sub-serogroups among the isolate collection.

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INTRODUCTION

Streptococcus pneumoniae (pneumococci) is a normal microflora of the upper respiratory tract. It is also an opportunistic pathogen that may cause non-invasive and invasive pneumococcal disease in

children worldwide. Pneumococcal disease is a global health problem that causes an estimated one million mortalities annually, mainly in children younger than five (Wahl et al., 2018).

Polysaccharide capsules (CPSs) are the main factors that enable pneumococci to evade the host's immune system. The capsular structural variation and antigen differentiate *S. pneumoniae* into 101 serotypes (Ganaie et al., 2021). Some *S. pneumoniae* serotypes are more commonly associated with clinical or carrier isolates, or both, which differ in invasiveness (Ganaie et al., 2020). Data from Southeast Asia showed that the most prevalent pathogenic serotypes were 19F, 23F, 14, 6B, 1, 19A, and 3, while the prevalence of *S. pneumoniae* in healthy children under the age of five was 6A/B, 23F, and 19F (Daningrat, 2022; Jauneikaite, 2012).

The most frequent serotypes in Malaysia across all pneumococcal diseases are 14, 6A/B, 23F, 19A, and 19F (Dzaraly et al., 2021). With regards to vaccines, the pneumococcal conjugate vaccine (PCV) Synflorix (PCV10, GlaxoSmithKline) covers four of the most widely disseminated serotypes in Malaysia, but Prevnar (PCV13, Pfizer) offers a better serotype-coverage than PCV10. The serotypes covered by the PCVs are referred to as vaccine types (VT). Malaysia recently implemented a pneumococcal vaccination policy incorporating PCV into the National Immunisation Programme in December 2020. The Synflorix pneumococcal vaccine has been included for the first two years of

implementation and protects against ten serotypes: 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F. Thus, accurate determination of *S. pneumoniae* serotypes in clinical and community settings is critical, as vaccine development relies on serotype prevalence data.

The conventional agglutination based-serotyping method, the Quellung reaction test, has been used for several decades to conduct epidemiological research on pneumococcal diseases. Despite being the "gold standard," it requires significant cost and expertise; hence, its use has been limited in specialised reference laboratories. Then, the commercial latex agglutination test is appropriate for laboratories with a rapid turnaround and fewer resources. This technique has the primary benefit of not needing sophisticated equipment for testing (Swarthout et al., 2021). Moreover, latex agglutination is suggested as a substitute for the Quellung approach for pneumococcal serotyping. Latex agglutination is among the simplest laboratory techniques for serotype identification; nevertheless, it has several disadvantages, such as weak outcomes leading to challenging interpretations and self-agglutinations that make typing infeasible (Batt et al., 2005). Inconsistencies arise due to laboratory errors and the high cost of antisera panels for Quellung and latex agglutination, suggesting that the DNA-based approach using PCR is a more affordable serotyping approach (Batt et al., 2005; Leung et al., 2012; Park et al., 2019).

PCR-specific serotyping approaches such as sequotyping and sequential

conventional multiplex PCR (cmPCR) were devised as the alternatives for Quellung and latex agglutination by amplifying capsular type-based DNA elements; this offers an economical, sensitive, and relatively straightforward serotyping approach compared to the conventional methods (Leung et al., 2012). Hence, the Centers for Disease Control and Prevention (CDC) develops protocols through a series of multiplex PCR, which uses primer combinations targeting various serotypes or serogroups. Several sets of serotype-specific primers are needed to facilitate detailed serotype evaluation, and currently, 41 primer sets have been available covering 22 single serotypes and 18 single serogroups through eight sequential multiplex PCR as updated by the CDC *Streptococcus* Laboratory at <https://www.cdc.gov/streplab/pneumococcus/resources.html>. However, sequential cmPCR has some limitations, including the need to run sequential reactions until the serotyping for the sample is obtained, which is time-consuming. In addition, sequential cmPCR detection can be affected by mutations in the target sequences, which can lead to false-positive or false-negative results.

Sequotyping is another method that uses a single primer set that can be used for *S. pneumoniae* serotyping. Sequotyping is previously claimed as a cost-effective, efficacious, and relatively straightforward serotyping approach offering appreciable coverage, including the serotypes not covered by PCVs, referred to as non-vaccine serotypes (NVT) (Leung et al., 2012). This

technique uses only one amplification reaction followed by DNA sequencing to determine the pneumococcal serotypes (Leung et al., 2012). Therefore, it can be applied in laboratories that offer PCR and sequencing for determining a wider coverage of pneumococcal serotypes. The sequotyping method may provide a certain level of expediency over other previously reported serotyping methods. Thus, in this study, the performance between sequotyping and sequential multiplex PCR was evaluated in terms of serotype identification in our archived collection of local *S. pneumoniae* isolates.

MATERIALS AND METHODS

Ethical Clearance

This study was approved by the Ethics Committee for Research involving Human Subjects of Universiti Putra Malaysia (Reference: JKEUPM-2021-647). This study utilised old isolates collected from previous studies only for the objective of this study without any relation to the patient's background associated with the isolates (patients' data are restricted and not traceable).

Bacterial Isolates

A total of 101 hospital isolates from various isolation sites (sputum, pus, blood, tracheal aspirates, eye, and nasopharynx) collected in 2017–2019 were available in our archived culture collection at the Applied Microbiology Laboratory, Faculty of Medicine and Health Sciences, Universiti

Putra Malaysia. Isolates were re-identified for viability and purity following standard methods.

Identification of *S. pneumoniae* Using the Standard Bacteriological Method

A standard bacteriological method was used to identify all isolates as *S. pneumoniae*, including colonial morphology on Columbia agar with 5% sheep blood (Isolab Sdn Bhd, Malaysia), Gram-positive (Gram-positive diplococci in the chain), and a catalase test (negative reaction) (Sigma Aldrich, Switzerland). Furthermore, isolates were subjected to a standard confirmatory test such as a bile solubility (Hardy Diagnostics, USA) and susceptibility test with ethylhydrocupreine (Liofilchem, Italy) or optochin (Liofilchem, Italy).

DNA Extraction

Bacterial DNA was extracted from single colonies grown on an agar plate using the GeneAll ExgeneCell SV Mini genomic extraction kit (GeneAll, South Korea) according to the manufacturer's instructions.

Molecular Identification of *S. pneumoniae* Virulence Genes Using the *ply* and *lytA* Genes

The *S. pneumoniae* virulence genes *ply* and *lytA* were amplified using conventional PCR assays as a spp. verification following protocols by Seki et al. (2005) and Sheppard et al. (2004). The tube of PCR reactions mixed with a DNA template of *S. pneumoniae* ATCC 49619 served as a positive control, and the reaction mixture without the DNA

template served as a negative control. All suspensions of the PCR tube reaction mixture of 25 µl were thoroughly mixed and run in the BioRad MyCycler™ thermal cycler (BioRad, USA).

Sequencing Using *cpsB* Single Gene Primer

Sequencing was performed using primers designed by Leung et al. (2012). The reaction mixtures contained 12.5 µl of MyTaq™ Red Mix Bioline (Meridian Biosciences, USA), 0.8 µl forward primer (*cps1*), 0.8 µl reverse primer (*cps2*), and 3 µl of genomic DNA that made up the final volume of 25 µl with nuclease-free sterile distilled water. The reaction cycle consisted of an initial denaturation step at 95°C for 5 min, followed by 30 amplification cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30 s, extension at 72°C for 1 min, and the final extension at 72°C for 5 min. Amplified DNAs were sequenced at Apical Scientific Sdn. Bhd., (Malaysia) and searched for homology in the GenBank. All sequences were submitted to GenBank with accession numbers OP081815, OP136953, OP235918, OP235920-OP235037, OP270727-OP270740, and OP270742-OP270793.

Sequential Conventional Multiplex PCR (cmPCR)

The serotypes of the pneumococcal isolates were determined by sequential cmPCR using the primer sequences recommended by the CDC (2021). The primers targeted the *glycosyltransferase* genes (*wzg*, *wzx*, *wzy*,

wciP, *wciN* Beta, *wciL*, *wcwL*, and *wcwV*). This study's combination of the CDC's recommended primers was based on the multiplex target sets formulated by Shakrin et al. (2013, 2014). The sequential reactions were based on six sets labelled as A, B, C, D, E, and F in the sequence of the serotype prevalence in Asia and Malaysia: the most prevalent serotypes/serogroups were targeted first, followed by the less prevalent serotypes/serogroups. Each set of primer pairs contained four serotypes/serogroups and an internal positive control pair of primers that targeted a conserved region of the pneumococcal capsular polysaccharide synthesis gene (*cpsA*). The reaction mixtures contained 12.5 µl of MyTaq™ Red Mix Bioline (Meridien Biosciences, USA), 2.0 µl of serotype-specific forward primer pairs, 2.0 µl of serotype-specific reverse primer pairs, 0.5 µl of *cpsA* forward and reverse primers, and 3 µl of genomic DNA, which made up the final volume of 25 µl with nuclease-free sterile distilled water. The reaction cycle consisted of an initial denaturation step at 95°C for 1 min, followed by 30 amplification cycles of denaturation at 95°C for 15 s, annealing at 54°C for 15 s, extension at 72°C for 10 s, and the final extension at 72°C for 5 min.

RESULTS

Characteristics and Identification of *S. pneumoniae*

All 101 isolates exhibited typical *S. pneumoniae* characteristics, including purplish, elongated diplococci under the microscope, alpha (α) hemolysis, sensitivity

to 5 µg optochin disk (Liofilchem, Italy), and a solubility test with 2% sodium deoxycholate (Hardy Diagnostics, USA). Furthermore, the presence of *lytA* and *ply* genes in the 101 isolates further confirmed the identity of *S. pneumoniae*.

Sequencing Employing *cpsB* Single Gene Primer for Serotype Identification

Ninety-nine of 101 (98%) pneumococcal isolates produced a positive signal with a 1,061 bp band. Two isolates (2%) were not amplified for the *cpsB* gene and were classified as non-typeable (NT). Upon sequencing and homology search, 16 different serotypes were determined. The most frequent was serotype 19F (n = 22; 21.8%) followed by 19A (n = 13; 12.9%); 14 (n = 12; 11.9%); 23F (n = 12; 11.9%); 6A (n = 11; 10.9%); 6B (n = 10; 9.9%); 11A (n = 5; 5%); 3 (n = 3; 3%); 1 (n = 2; 2%); 4 (n = 2; 2%); 35B (n = 2; 2%); 13 (n = 1; 1%); 15A (n = 1; 1%); 15C (n = 1; 1%); 23A (n = 1; 1%), and 34 (n = 1; 1%) (Table 1).

Sequential cmPCR for Pneumococcal Serotype and Serogroup Determination

All 101 samples were positive for amplifying the internal positive control *cpsA* gene. However, 10 isolates (9.9%) could not be identified for any molecular target by the sequential cmPCR and were designated as NT. These include two isolates that could not be detected by sequencing. Eleven serotypes and six serogroups were found in 91 (90.1%) isolates. The most frequent was serotype 19F (n = 22; 21.8%) followed by 6A/B (n = 21; 20.8%); 19A (n = 13;

Table 1
Serotype distribution by sequotyping and sequential conventional multiplex PCR

Serotypes	Sequotyping	Sequential cmPCR
1	2	2
3	3	(a)
4	2	2
6A/B	(b)	21
6A	11	(c)
6B	10	(c)
11A/D	(b)	5
11A	5	(d)
11D	0	(d)
13	1	(a)
14	13	12
15A/F	(b)	1
15A	1	(e)
15F	0	(e)
15B/C	(b)	1
15B	0	(f)
15C	2	(f)
19A	13	13
19F	22	22
23A	1	(g)
23F	12	12
34	1	(a)
35B	2	(a)
NT	2	10
Total	101	101

Note.

- (a) = Primer not included in our multiplex set
- (b) = Sequotyping able to discriminate to the serotype level/single serotype
- (c) = Determined as 6A/B
- (d) = Determined as 11A/D
- (e) = Determined as 15A/F
- (f) = Determined as 15B/C
- (g) = Determined as non-typeable (NT)

12.9%); 14 (n = 12; 11.9%); 23F (n = 12; 11.9%); 11A/D (n = 5; 5%); 1 (n = 2; 2%); 4 (n = 2; 2%); 15B/C (n = 1; 1%); 15A/F (n = 1; 1%) (Table 1). The initial sequential cmPCR reaction covered 67.3% of the total

isolates in Set A (serotypes 6A/B, 19A, 19F, and 23F), the most prevalent serotypes. The second reaction covered 14.9% in Set B (serotypes 1, 14, and 15B/C), and the third reaction covered 5% in Set C (serotype 11A/D) of the total isolates, respectively.

Comparison of *cpsB* Sequotyping and Sequential cmPCR

Table 1 compares the serotype distribution by sequotyping and sequential cmPCR. The result of sequotyping is in concordance with sequential cmPCR in most of the isolates. Sequotyping determined 99 isolates (98%) up to serotype level, while sequential cmPCR identified 91 isolates (90.3%) with 63 (62.4%) up to serotype level (Figure 1). Sequotyping could not amplify two isolates (2%), which also failed to be determined by the sequential cmPCR. Sequential cmPCR failed to amplify 10 isolates (9.9%) labelled NT, although all gave a positive result for the internal control *cpsA* gene. The highest serotype distribution was 19F (n = 22; 21.8%) when detected by sequotyping and sequential cmPCR. Meanwhile, the second most prevalent serotype was 19A (n = 13; 12.9%) by sequotyping and 6A/B (n = 21; 20.8%) by cmPCR. Furthermore, sequotyping identified four NVTs: 13, 23A, 34, and 35B, respectively. Serotype 3 is present in PCV13 but was not included in Shakira et al. (2013, 2014) formulation of the sequential multiplex sets of primers. Using sequotyping, serotype 23A was successfully amplified, but sequential cmPCR listed it as NT even though the primer was listed in our reaction sets. The

sequential cmPCR method identified some isolates as only 6A/B, 11A/D, 15A/F, and 15B/C (serogroup level) due to limited primers, while the sequotyping method discriminated the isolates as 6A (n = 11; 10.9%); 6B (n = 10; 9.9%); 11A (n = 5; 5%), and 15C (n = 1; 1%) (Figure 1).

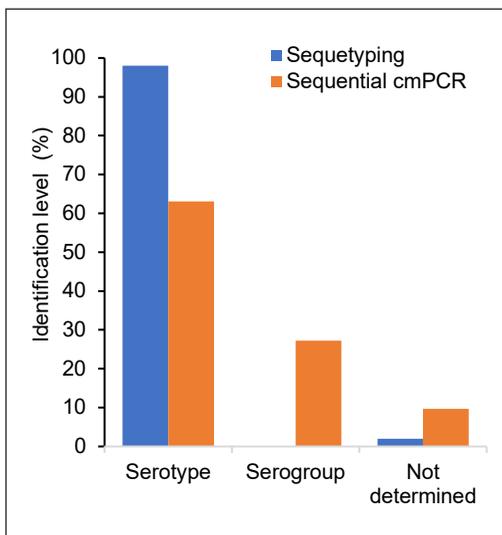


Figure 1. Comparison of serotyping outputs using sequotyping and sequential conventional multiplex PCR

Note. 'Not determined' refers to non-typeable (NT) isolates that cannot be detected using sequotyping and sequential cmPCR

DISCUSSION

Generally, DNA-based techniques allow the differentiation of a proportion of the pneumococcal serogroups and serotypes commonly associated with diseases. Although not all 101 serotypes can cause severe infections, accurately identifying *S. pneumoniae* serotypes is still necessary for prophylactic purposes. It is crucial to monitor the distribution of serotypes to manage strategy in disease control

and prevention. Increasing numbers of pathogenic and carriage pneumococci are being investigated as part of post-PCV surveillance studies; therefore, rapid, accurate, and economical typing approaches are essential. Additionally, the proportion of IPD attributable to NVT gradually increases after PCVs are implemented (Løchen et al., 2020). Therefore, serotyping is important in screening for both VTs and NVTs.

In the sequential cmPCR in this study, six sets of CDC's recommended primer sequences (Set A–Set F) targeting 10 serogroups and 14 serotypes were employed to include the prevalent VTs in the Asian region and Malaysia (Shakrin et al., 2013, 2014). The sequential multiplex PCR (A to F) successfully identified 91 (90.1%) from a total of 101 isolates, with 63 (62.4%) of the isolates detected at the serotype level. Taking the overall reactions (Sets A to C), the sequential cmPCR based on Shakrin et al. (2013, 2014) formulation in this study showed a better coverage at 87.2% of the total isolates for the cumulative reactions 1, 2, and 3, as compared to the results of Park et al. (2019) at 79.4% in the same reactions. For the later study, Park et al. (2019) developed a modified sequential multiplex of conventional PCR using 30 primer sets to cover the predominant serotypes in Asian countries. It reflects the dynamic of conventional multiplex reactions that can always be subjected to improvement, but a technically and chemically workable PCR reaction in the presence of multiple targets usually requires sound optimisations and is not easily achieved.

Meanwhile, sequotyping is a more straightforward approach involving only a single gene amplification. Nevertheless, the amplified gene needs to be sequenced, which may look intricate, but the process is feasible due to the wide availability of service providers to continue with the downstream analysis. In this study, it was able to serotype 99 (98%) among the 101 isolates and discriminated serogroups 6A/B, 11A/D, 15A/F, and 15B/C as previously reported in the sequential cmPCR. Serotypes 6A and 6B, including 6C and 6D, could not be distinguished with sequential cmPCR alone due to the higher degree of genetic similarity that the current primers are not specific for the four sub-serotypes (Mauffrey et al., 2017; Pai et al., 2005). Jin et al. (2009) developed a serotype-specific primer targeting the *wciP* gene to differentiate serotypes 6A and 6B and another primer targeting the *wciN* gene to further differentiate serotypes 6A and 6C. Nevertheless, this adds extra steps; thus, more time and labour are needed to discriminate the serogroup. The *cpsB* gene sequotyping, on the other hand, can detect the specific sequence polymorphism at the nucleotide level to discriminate into 6A and 6B without the need to perform additional PCR reactions (Lawrence et al., 2000).

Several serotypes (9.9%) were undetectable by the sequential cmPCR in this study because of the limited primer coverage in the current multiplex sets. Whereas for serotype 23A, it was successfully determined by sequotyping but could not be amplified using the

sequential cmPCR despite the primer being included in the current set of reactions. The misidentification might be due to the mutations in the *cps* locus that prevent amplification with the primers employed (Richter et al., 2013). There are 17 additional serotypes covered in the eight sequential sets as of the CDC's latest primer list, and the additional serotypes include the ones identified by the sequotyping in this study. It was observed that the sequotyping also failed to amplify the *cpsB* gene in two isolates (2%); this could be due to nucleotide variations or the absence of the targeted amplicons in the *cpsB* gene of these strains (Zhou et al., 2022). In the long run, sequotyping may benefit from the increasing availability of genomic information in public databases. Therefore, more studies are warranted to continuously update the database with the *cpsB* gene sequence of isolates from various geographical regions, particularly in view of the potential genetic diversity to establish the universality of the *cpsB* as the target for worldwide serotyping. This aspect has been a challenge regarding incomplete data, leading to incorrect serotype designations and assignments (Zhou et al., 2022).

Sequential cmPCR is an effective method as one reaction allows the simultaneous identification of one or more alleles or genes (Mauffrey et al., 2017). Nevertheless, due to the requirement of multiple PCR primers and reactions, the process may be time-consuming along the sequential steps, and unusual serotypes may remain undetected

even after a series of consecutive reactions (Leung et al., 2012; Zhou et al., 2022). Therefore, the sequotyping approach can be complementary as it is easy to perform and could detect a wide range of serotypes with a single amplification of the *cpsB* gene to fill up the gap left in the conventional multiplex approach (Leung et al., 2012). However, there are limitations to the *cpsB* gene amplifications in serotypes 25F, 37, 38, 39, and 43 as the *cpsB* gene is lacking, making these serotypes unidentifiable with this method (Leung et al., 2012; Mauffrey et al., 2017). To address this issue, additional measures, such as the Quellung and latex agglutination methods, need to be utilised to verify the likelihood of false-positive PCR outcomes since some serotypes cannot be entirely differentiated based solely on the *cps* sequence (Jin et al., 2016; Mauffrey et al., 2017).

Although sequotyping is easier and more convenient, it requires an additional step of sequencing, which incurs an additional cost but less PCR reaction compared to sequential cmPCR. Thus, in selective cases, a combination of *cpsB* sequotyping with sequential cmPCR can significantly increase the accuracy and effectiveness of serotyping and offer a financially viable alternative to the traditional agglutination-based serotyping for *S. pneumoniae* (Zhou et al., 2022). In addition, there has been a revolutionary advancement in whole genome sequencing (WGS) to generate comprehensive sequence data for a better-curated analysis of the possible serotype-related DNA sequences. With such data,

the need for validation with the antisera test (Quellung) for certain serotypes that cannot be definitively differentiated from the *cps* sequences (Mauffrey et al., 2017) would probably not be necessary anymore. Nonetheless, WGS is still associated with high costs and requires bioinformatic expertise and digital platforms, which are only available in specialised and well-funded facilities.

CONCLUSION

This study showed that the *cpsB* gene sequotyping method might complement sequential cmPCR to discriminate further a range of serogroups such as 6A/B, 11A/D, 15A/F, and 15B/C. The combination of both would best suit the current need for surveillance until WGS becomes more affordable. As for the limitations pertaining to this study, we only investigated using *cpsB* sequotyping and sequential cmPCR, where the latter only targets 24 serotypes/serogroups of Asian prevalence due to limited resources. For a more meaningful validation, results should be verified by the gold standard: the Quellung technique or latex agglutination, especially for the NTs. The cmPCR requires more updated and extended serotype coverage with feasible primers-combinations that are doable in the sequential multiplex system. Meanwhile, this study and the associated discussion were based on culture steps prior to serotyping, where the strains have been propagated for purity with likely sufficient DNA for the downstream serotyping assay. In the case of direct DNA extraction from host samples,

cmPCR may not have sufficient sensitivity to amplify low DNA copy numbers, and the subsequent sequential multiplex PCR process will be a lot more challenging.

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REFERENCES

- Batt, S. L., Charalambous, B. M., McHugh, T. D., Martin, S., & Gillespie, S. H. (2005). Novel PCR-restriction fragment length polymorphism method for determining serotypes or serogroups of *Streptococcus pneumoniae* isolates. *Journal of Clinical Microbiology*, 43(6), 2656–2661. <https://doi.org/10.1128/JCM.43.6.2656-2661.2005>
- Centers for Disease Control and Prevention. (2021). *PCR-based methods for identification of S. pneumoniae serotypes/serogroups*. CDC. <https://www.cdc.gov/streplab/pneumococcus/resources.html>
- Daningrat, W. O. D., Amalia, H., Ayu, I. M., Satzke, C., & Safari, D. (2022). Carriage of *Streptococcus pneumoniae* in children under five years of age prior to pneumococcal vaccine introduction in Southeast Asia: A systematic review and meta-analysis (2001–2019). *Journal of Microbiology, Immunology and Infection*, 55(1), 6–17. <https://doi.org/10.1016/j.jmii.2021.08.002>
- Dzaraly, N. D., Mohd Desa, M. N., Muthanna, A. R., Masri, S. N., Taib, N. M., Suhaili, Z., Sulaiman, N., Baharin, N. H. Z., Shuan, C. Y., Ariffin, Z., Rahman, N. I. A., Mohd Rani, F., Palanisamy, N. K., Soh, T. S. T., & Abdullah, F. H. (2021). Antimicrobial susceptibility, serotype distribution, virulence profile and molecular typing of piliated clinical isolates of pneumococci from east coast, Peninsular Malaysia. *Scientific Reports*, 11, 8220. <https://doi.org/10.1038/s41598-021-87428-z>
- Ganaie, F., Maruhn, K., Li, C., Porambo, R. J., Elverdal, P. L., Abeygunwardana, C., van der Linden, M., Duus, J. Ø., Sheppard, C. L., & Nahm, M. H. (2021). Structural, genetic, and serological elucidation of *Streptococcus pneumoniae* serogroup 24 serotypes: Discovery of a new serotype, 24C, with a variable capsule structure. *Journal of Clinical Microbiology*, 59(7), e00540-21. <https://doi.org/10.1128/JCM.00540-21>
- Ganaie, F., Saad, J. S., McGee, L., van Tonder, A. J., Bentley, S. D., Lo, S. W., Gladstone, R. A., Turner, P., Keenan, J. D., Breiman, R. F., & Nahm, M. H. (2020). A new pneumococcal capsule type, 10D, is the 100th serotype and has a large *cps* fragment from an oral streptococcus. *mBio*, 11(3), e00937-20. <https://doi.org/10.1128/mBio.00937-20>
- Jauneikaite, E., Jefferies, J. M., Hibberd, M. L., & Clarke, S. C. (2012). Prevalence of *Streptococcus pneumoniae* serotypes causing invasive and non-invasive disease in South East Asia: A review. *Vaccine*, 30(24), 3503–3514. <https://doi.org/10.1016/j.vaccine.2012.03.066>
- Jin, P., Wu, L., Oftadeh, S., Kudinha, T., Kong, F., & Zeng, Q. (2016). Using a practical molecular capsular serotype prediction strategy to investigate *Streptococcus pneumoniae* serotype distribution and antimicrobial resistance in Chinese local hospitalized children. *BMC Pediatrics*, 16, 53. <https://doi.org/10.1186/s12887-016-0589-7>
- Jin, P., Xiao, M., Kong, F., Oftadeh, S., Zhou, F., Liu, C., & Gilbert, G. L. (2009). Simple, accurate, serotype-specific PCR assay to differentiate *Streptococcus pneumoniae* serotypes 6A, 6B, and 6C. *Journal of Clinical Microbiology*,

- 47(8), 2470–2474. <https://doi.org/10.1128/JCM.00484-09>
- Lawrence, E. R., Arias, C. A., Duke, B., Beste, D., Broughton, K., Efstratiou, A., George, R. C., & Hall, L. M. C. (2000). Evaluation of serotype prediction by *cpsA-cpsB* gene polymorphism in *Streptococcus pneumoniae*. *Journal of Clinical Microbiology*, 38(4), 1319–1323. <https://doi.org/10.1128/jcm.38.4.1319-1323.2000>
- Leung, M. H., Bryson, K., Freystatter, K., Pichon, B., Edwards, G., Charalambous, B. M., & Gillespie, S. H. (2012). Sequotyping: Serotyping *Streptococcus pneumoniae* by a single PCR sequencing strategy. *Journal of Clinical Microbiology*, 50(7), 2419–2427. <https://doi.org/10.1128/JCM.06384-11>
- Løchen, A., Croucher, N. J., & Anderson, R. M. (2020). Divergent serotype replacement trends and increasing diversity in pneumococcal disease in high income settings reduce the benefit of expanding vaccine valency. *Scientific Reports*, 10, 18977. <https://doi.org/10.1038/s41598-020-75691-5>
- Mauffrey, F., Fournier, É., Demczuk, W., Martin, I., Mulvey, M., Martineau, C., Lévesque, S., Bekal, S., Domingo, M.-C. C., Doualla-Bell, F., Longtin, J., & Lefebvre, B. (2017). Comparison of sequential multiplex PCR, sequotyping and whole genome sequencing for serotyping of *Streptococcus pneumoniae*. *PLOS One*, 12(12), e0189163. <https://doi.org/10.1371/journal.pone.0189163>
- Pai, R., Limor, J., & Beall, B. (2005). Use of pyrosequencing to differentiate *Streptococcus pneumoniae* serotypes 6A and 6B. *Journal of Clinical Microbiology*, 43(9), 4820–4822. <https://doi.org/10.1128/JCM.43.9.4820-4822.2005>
- Park, D., Kim, S. H., Bae, I. K., Kim, N. Y., Kook, J.-K., Park, Y.-H., & Shin, J. H. (2019). Evaluation of modified sequential multiplex PCR for *Streptococcus pneumoniae* serotyping. *Japanese Journal of Infectious Diseases*, 72(4), 224–227. <https://doi.org/10.7883/yoken.JJID.2018.422>
- Richter, S. S., Heilmann, K. P., Dohrn, C. L., Riahi, F., Diekema, D. J., & Doern, G. V. (2013). Evaluation of pneumococcal serotyping by multiplex PCR and quellung reactions. *Journal of Clinical Microbiology*, 51(12), 4193–4195. <https://doi.org/10.1128/JCM.01876-13>
- Seki, M., Yamashita, Y., Torigoe, H., Tsuda, H., Sato, S., & Maeno, M. (2005). Loop-mediated isothermal amplification method targeting the *lytA* gene for detection of *Streptococcus pneumoniae*. *Journal of Clinical Microbiology*, 43(4), 1581–1586. <https://doi.org/10.1128/jcm.43.4.1581-1586.2005>
- Shakrin, N. N. S. M., Balasubramaniam, S. D., Yusof, H. A., Mastuki, M. F., Masri, S. N., Taib, N. M., Nordin, S. A., Jamal, F., Clarke, S. C., & Desa, M. N. M. (2013). Evaluation of PCR-based approach for serotype determination of *Streptococcus pneumoniae*. *Tropical Biomedicine*, 30(2), 338–344.
- Shakrin, N. N. S. M., Masri, S. N., Taib, N. M., Nordin, S. A., Jamal, F., & Desa, M. N. M. (2014). Genotypic characterization of Malaysian human isolates of *Streptococcus pneumoniae* from carriage and clinical sources. *Comparative Immunology, Microbiology and Infectious Diseases*, 37(5–6), 347–354. <https://doi.org/10.1016/j.cimid.2014.10.005>
- Sheppard, C. L., Harrison, T. G., Morris, R., Hogan, A., & George, R. C. (2004). Autolysin-targeted LightCycler assay including internal process control for detection of *Streptococcus pneumoniae* DNA in clinical samples. *Journal of Medical Microbiology*, 53(3), 189–195. <https://doi.org/10.1099/jmm.0.05460-0>
- Swarthout, T. D., Gori, A., Bar-Zeev, N., Kamng'ona, A. W., Mwalukomo, T. S., Bonomali, F., Nyirenda, R., Brown, C., Msefula, J., Everett, D., Mwansambo, C., Gould, K., Hinds, J.,

- Heyderman, R. S., & French, N. (2021). Evaluation of pneumococcal serotyping of nasopharyngeal-carriage isolates by latex agglutination, whole-genome sequencing (PneumoCaT), and DNA microarray in a high-pneumococcal-carriage-prevalence population in Malawi. *Journal of Clinical Microbiology*, 59(1), e02103-20. <https://doi.org/10.1128/JCM.02103-20>
- Wahl, B., O'Brien, K. L., Greenbaum, A., Majumder, A., Liu, L., Chu, Y., Lukšić, I., Nair, H., McAllister, D. A., Campbell, H., Rudan, I., Black, R., & Knoll, M. D. (2018). Burden of *Streptococcus pneumoniae* and *Haemophilus influenzae* type b disease in children in the era of conjugate vaccines: Global, regional, and national estimates for 2000–15. *The Lancet Global Health*, 6(7), e744–e757. [https://doi.org/10.1016/S2214-109X\(18\)30247-X](https://doi.org/10.1016/S2214-109X(18)30247-X)
- Zhou, M.-L., Wang, Z.-R., Li, Y.-B., Kudinha, T., Wang, J., Wang, Y., Xiao, M., Xu, Y.-C., Liu, Z.-Y., & Hsueh, P.-R. (2022). Rapid identification of *Streptococcus pneumoniae* serotypes by *cpsB* gene-based sequencing combined with multiplex PCR. *Journal of Microbiology, Immunology and Infection*, 55(5), 870–879. <https://doi.org/10.1016/j.jmii.2021.11.004>