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Original Article

Polysaccharide Capsule Serotype and Antibiotic Susceptibility Pattern of *Streptococcus pneumoniae* Clinical Isolates in Bali

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ABSTRACT

Streptococcus pneumoniae (*S. pneumoniae*) is a causative agent of pneumonia that can spread progressively, cause invasive disease, and increase mortality in humans. Pneumococcal or polysaccharide conjugate vaccination reduces pneumonia rates by vaccine-covered serotypes, but increases infection by non-vaccine serotypes. To determine the polysaccharide capsule serotype of *S. pneumoniae* isolates that cause infection at Prof. Dr. I.G.N.G. Ngoerah General Hospital and patterns of *S. pneumoniae* susceptibility to antibiotics from April 2017 to March 2022. All *S. pneumoniae* isolates from April 2017 and April 2022 were stored in STGG media in a freezer at -80 °C then subcultured on sheep blood agar. Polymerase Chain Reaction (PCR) was performed to determine pneumolysin and capsular polysaccharide serotypes of *S. pneumoniae*. Of the 22 isolates studied, the order of the number of serotypes from the highest was serotype 19F, 3, 6A/B, 33F, 15B/C, 4, and 6V. Seven isolates were untypeable. Antibiotic sensitivity pattern *S. pneumoniae* was found to be sensitive to linezolid 91%, vancomycin 86%, levofloxacin and benzylpenicillin 82%, ceftriaxone and clindamycin 73%, erythromycin 55%, and chloramphenicol 45%. Serotype 19F was identified as the most dominant capsular serotype; however, serotypes 33F and 15B/C were also found. Interestingly, the 33F serotype is not covered in the 13-valent pneumococcal conjugate vaccine (PCV13) but is covered in pneumococcal polysaccharide vaccine 23 (PPSV23), and the 15B/C serotype is not included in either PCV13 or PPSV23. The antimicrobial susceptibility patterns revealed that *S. pneumoniae* was susceptible to linezolid, vancomycin, benzylpenicillin, and levofloxacin.

Keywords: : Antibiotic Susceptibility, Polysaccharide Capsule Serotype, Pneumococcal Vaccine, Polymerase Chain Reaction, dan *Streptococcus pneumoniae*.

Highlights: A serotype of *S. pneumoniae* was found outside the vaccine candidate and is helpful for the development of a vaccine for *S. pneumoniae* infection in the future.

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INTRODUCTION

Pneumonia, meningitis, sepsis, and otitis media are among the most prevalent invasive and non-invasive illnesses caused by *Streptococcus pneumoniae*, especially in younger and older individuals.^{1,2} The invasive disease of *S. pneumoniae* is caused by colonization of these bacteria in the respiratory tract, which progressively infect and spread to other cells.³ Approximately one million children under five die annually because of these bacteria.²

A polysaccharide capsule on the surface of *S. pneumoniae* is considered a significant virulence factor. It was discovered and published by Pasteur in 1880. A study carried out during the first three decades of the 20th century showed the presence of several capsule serotypes of *S. pneumoniae*. The polysaccharide capsule of *S. pneumoniae* was first isolated by Dochez and Avery in 1917. They found that the capsule of *S. pneumoniae* is a soluble pneumococcal substance consisting of proteins. In 1925, Avery et al. discovered that the capsule of *S. pneumoniae* consisted of polysaccharides, the first known non-protein antigen. The thickness of the capsule was approximately 400 nm, accounting for more than half of the volume of *S. pneumoniae*.⁴ These non-protein antigens protect bacteria from phagocytosis. Capsules are powerful antioxidants against oxidative stress. They can evade immune responses, such as protection from endosome killing by the host and increasing the translocation rate into organs during bacteremia.¹ There are 100 types of *S. pneumoniae* capsules, yet only 20–30 types of capsules are capable of causing invasive disease. This serotype indicates that each capsule has different specific properties.¹ Various capsule types are linked to the disease, with serotypes 14 and 1 causing most childhood pneumonia cases, and serotypes 3, 6A, 6 B, 9N, and 19F, which exhibit higher mortality rates.³ A study in Central Lombok revealed that the

predominant capsule types of *S. pneumoniae* as 6A/B, 19F, 23F, and 15B/C.⁵ Meanwhile, in a study in Jakarta, the capsule serotypes found were 3,6A/B, 15B/C and 35F.⁶ Research conducted in Semarang found that capsular serotypes in children were 6A/B, 15B/C, 11A, 23F, 19F, and 23A, and 6A/B, 15B/C, and 15A in adults.⁷ According to a prior investigation carried out at Prof. Dr. I.G.N.G. Ngoerah General Hospital, the primary serotypes identified were 19F (7 isolates), 23F (2 isolates), 6A/B (2 isolates), 7F (1 isolate), and 15B/C (1 isolate).⁸

Bacterial pneumonia can be prevented using polysaccharide and pneumococcal conjugate vaccines. Four types of conjugate vaccines for pneumonia have been developed: PCV7, PCV9, PCV10, and PCV13. Each type of PCV is associated with a different serotype. Consequently, the efficacy of a given vaccine is contingent on its ability to effectively target its serotypes.⁹ PCV7 has been granted an official licensure by the United States and European Union governments. This vaccine is composed of serotypes 23F, 19F, 18C, 14, 9V, 6 B, and 4. PCV13 and PCV10 vaccines were released in 2000 and 2001, respectively. PCV7 vaccination was improved by adding serotypes 7F, 1, and 5, which were already in use for PCV10, and by adding serotypes 19A, 7F, 6A, 5, 3, and 1 for PCV13.^{10,11} The UK has sold PPSV23 since 2003. This vaccine contains PCV13 serotypes, except 6A and 11 others: 9N, 10A, 2, 8, 11A, 17F, 20, 22F, 12F, 15 B, and 33F. The advice is for those aged ≥ 65 years and in clinical risk categories.^{12,13}

S. pneumoniae has shown increased resistance to antimicrobials that were once efficacious. Therefore, the antimicrobial susceptibility pattern of *S. pneumoniae* helps determine empirical therapy while waiting for culture results to establish definitive therapy.³ In a retrospective, multicenter study conducted in 14 institutions from 13 provinces in China, Chen et al. discovered that antibiotic regimens for the definitive treatment of invasive pneumococcal disease

were frequently inappropriate, with excessive prescriptions of carbapenems, vancomycin, and linezolid.¹⁴ Additionally, a descriptive study conducted by Mohanty et al. in the United States demonstrated increased levels of antimicrobial resistance (AMR) in *S. pneumoniae* isolates acquired from adults diagnosed with either invasive or noninvasive pneumococcal disease. Furthermore, this research revealed a tendency toward heightened macrolide resistance.¹⁵

The researchers aimed to analyze the distribution of serotype polysaccharide capsules of *S. pneumoniae* isolates and their antibiotic susceptibility profile at Prof. Dr. I.G.N.G. Ngoerah General Hospital between April 2017 and March 2022 based on the provided data.

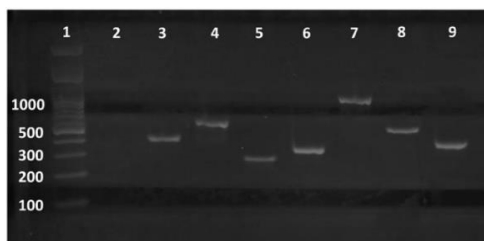


Figure 1. Gel Electrophoresis Results of PCR Serotype *Streptococcus pneumoniae*. Line (1) Marker, (2) Negative Control, (3) Serotype 3 (371bp), (4) Serotype 15B/C (496bp), (5) Serotype 6A/B (250bp), (6) Serotype 19F (304bp), (7) Serotype 9V (753bp), (8) Serotype 4 (430bp), (9) Serotype 33F (338bp).

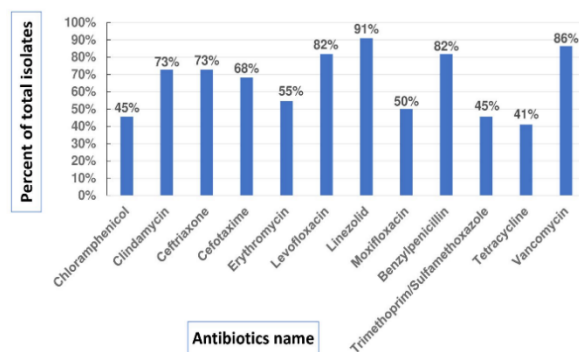


Figure 2. Antibiotic Susceptibility Pattern 22 Isolates of *Streptococcus pneumoniae*.

MATERIALS AND METHODS

Bacterial strain, medium, and growth conditions

A total of 22 isolates of *S. pneumoniae* were stored in STGG media at a temperature of -80 °C and were subjected to cultivation on 5% sheep blood agar. The process of incubation was incubated in an aerobic environment at a temperature of 37 °C for a duration of 24 h. Microbial isolates were obtained from blood (18%, n=4), sputum (36%, n=8), and other clinical specimens (45%, n=10) between April 2017 and April 2022. Identification of *S. pneumoniae* was carried out presumptively by evaluating colony characteristics, type of hemolysis, and susceptibility using 5 g optochin discs (Oxoid, Thermo Fisher Diagnostics B.V., The Netherlands). If the Optochin test results were sensitive, the bacterial colonies were subjected to identification tests (GP ID card) and AST (AST-ST03 card) using the VITEK-2 Compact machine (bioMérieux®). Antibiotics tested for sensitivity were linezolid, vancomycin, benzylpenicillin, levofloxacin, ceftriaxone, clindamycin, cefotaxime, erythromycin, moxifloxacin, chloramphenicol, trimethoprim/sulfamethoxazole, and tetracycline.

Bacterial DNA Isolation

S. pneumoniae was extracted using a Roche High Pure PCR Isolation Kit Template (Roche Life Science, Indianapolis, USA). *S. pneumoniae* colonies were suspended in 200 µL of pH 7.3 phosphate-buffered saline. DNA isolation from the bacterial suspensions was performed according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR) for the pneumolysin gene (ply)

PCR was used to detect the presence of the pneumolysin gene (ply). Amplification of the PCR mixture was performed using GoTaq Green Master Mix (Promega, Madison, WI, USA). The present study employed a specific primer pair targeting ply, with the forward primer sequence being 5'-

ATTTCTGTAACAGCTACCAACGA-3' and the reverse primer sequence being 5'-GAATTCCTGTCTTTTCAAAGTC-3'.¹⁶

The PCR process consisted of an initial pre-denaturation step at a temperature of 94 °C for 2 min. This was followed by 35 cycles of denaturation at 94 °C for 30 seconds, primary annealing at 54 °C for 30 seconds, and extension at 72 °C for 1 min. The final step involved a final extension at 72 °C for 5 min. An iCycler, a Bio-Rad thermal cycler, was used for this process. The amplicons were subjected to electrophoresis on 1.5% agarose gel in TBE buffer at 100 volts for 35 min. DNA was visualized using GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA 94545) and subsequently captured using Gel Doc (Bio-Rad). Amplification of a 348 bp band size has yielded positive results.

Polymerase Chain Reaction (PCR) for Serotyping Capsular Polysaccharides (CPS)

PCR used serotype primers 4, 5, 18, 19A, 7C, 15A, 9V, 17F, 1, 14, 23A, 3, 19F, 12, 33F, 15B/C, 23F, 6A/B, and 7F for capsular serotyping. PCR was performed using two distinct techniques. First, multiplex PCR was used to simultaneously determine CPS type. Multiplex PCR was performed using the Kapa 2G Fast Ready Mix PCR Kit with Dye (Kapa Biosystems, Promega, Madison, WI, USA) as its master mix. Modifications to the multiplex PCR protocol were described in a previous study.¹⁷ The cycle consisted of 2 min of pre-denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing primer at 54 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min (iCycler, Bio-Rad thermal cycler). PCR for CPS serotyping was performed using primers detailed in previous studies.^{8,17} Uniplex PCR was performed with Go Taq® Green Master Mix (Promega, Madison, USA) to ascertain the type of CPS that multiplex PCR had not typed. For CPS serotyping using

uniplex PCR, primer concentrations of 0.3 M were utilized. The PCR cycle was initiated with a 2-minute pre-denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, primary annealing at 54 °C for 1 min, extension at 72 °C for 1 min, and a 5-minute final extension at 72 °C (iCycler, Bio-Rad thermal cycler). The amplicons were then electrophoresed for 35 min on a 1% agarose gel in TBE buffer at 50 volts. DNA was visualized using GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA 94545) and then documented using Gel Doc (Bio-Rad).

The positive control used in this study was obtained from a previous study conducted by Fatmawati et al. ; however⁸, the positive controls for newly discovered serotypes 3, 4, 6V, and 33F were not included.

Examination of the sensitivity of *S. pneumoniae* to antibiotics

Antibiotic sensitivity information was obtained from the VITEK-2 Compact (bioMérieux®) results using a VITEK® 2 AST-ST03 card. Then, using Microsoft Excel 2019, the antibiotic susceptibility patterns of the clinical isolates of *S. pneumoniae* in Bali were determined.

RESULTS AND DISCUSSION

All isolates were found to be *S. pneumoniae* based on sensitivity to optochin, presence of a pneumolysin-specific gene, and phenotypic matching to the colony description. Of the 22 isolates studied, the most common serotype was 19F (32%; n=7), followed by serotype 3 (9%; n=2) and serotype 6A/B (9%; n=2), followed by serotype 33F, 15B/C, 4, and 6V which were obtained for each serotype. The positive controls for capsule *S. pneumoniae* serotype detection used in this study were obtained from a previous study conducted by Fatmawati et al. ; however⁸, the positive controls for newly discovered serotypes 3, 4, 6V and 33F were not included. The remaining

isolates were confirmed to be untypable strains (32%; n=7). The types of serotypes found can be seen in the PCR results shown in Figure 1. In this study, 15 isolates were serotyped, accounting for 87% (n=13/15) of the total serotyped isolates covered by PCV 13. Interestingly, in this study, two isolates were not included in PCV 13, namely serotypes 33F and 15B/C.

This study obtained seven untypable isolates because not all capsule pneumonia serotypes were designed for primary sequence detection by PCR. Therefore, an untypable isolate may represent another serotype outside the designed primary sequence.

The antibiotic susceptibility pattern from these 22 isolates is sensitive to linezolid (91%; n=20), vancomycin (86%; n=19), benzylpenicillin (82%; n=18), levofloxacin (82%; n=18), ceftriaxone (73%; n=16), clindamycin (73%; n=16), cefotaxime (68%; n=15), erythromycin (55%; n=12), moxifloxacin (50%; n=11), chloramphenicol (45%; n=10), trimethoprim/sulfamethoxazole (45%; n=10), and tetracycline (41%; n=9), as shown in Figure 2.

In this study, serotype 19F was dominant among the isolates, followed by serotype 3, 6A/B, 33F, 15B/C, 4, and 6V. This result supports the findings of Zhao et al., who discovered that serotype 19F was the largest five-serotype capsule of *S. pneumoniae* found in China.¹⁸ The five serotypes (19F, 6A/B, 3, 4, and 6V) found in our study included the PCV13 serotype, two serotypes non-PCV13 were serotype 33F and 15B/C, and one serotype that excluded both PCV13 and PPSV23 was 15B/C.

Although the efficacy of the current pneumococcal conjugate vaccination against the targeted serotype has significantly decreased the disease burden produced by this serotype, several countries have reported an increase in infectious pneumococcal illnesses caused by non-vaccine strains. The

increased prevalence of infection with serotypes not covered by PCV13 in children and adults, specifically due to serotypes 22F and 33F, has been documented in multiple nations.¹⁹ Several studies have also identified non-vaccine strains, namely serotype 15B/C, in France, Colombia, Thailand, and Indonesia (Lombok, Jakarta, and Semarang).^{5-7,20-22} Interestingly, serotype 15B/C is not covered by PCV13 or PPSV23. Serotype 15B/C has potential as a vaccine candidate, considering that cases of invasive pneumonia caused by this serotype, including serotype 15B/C, are increasing in several countries such as France, Colombia, Thailand, and Indonesia.^{5-7,20-22}

The introduction and use of unconjugated versus conjugated pneumococcal polysaccharide vaccines have reduced the proportion of pneumococcal infections caused by *S. pneumoniae*. Those two years of age or older are protected against invasive illness caused by the 23 capsular serotypes included in the PPSV23 vaccination because of the development of a vaccine called PPSV23. Children under two years old do not have an immune response that may be considered protective after receiving the PPSV23 vaccination. The PCV7 vaccine was developed to target seven PPSV23 serotypes responsible for causing invasive pediatric diseases. It was specifically designed and recommended for administration to children under two years of age. In addition, the development of vaccines aligns with the *S. pneumoniae* serotype responsible for pneumonia in humans, specifically PCV10, with the most recent iteration being PCV13. The PCV13 vaccine comprises seven PCV7 serotypes, five supplementary PPSV23 serotypes, and an additional serotype. Novel pneumococcal serotypes that are absent in PPSV23 and PCV7 have been identified.²³ Multiple systematic reviews and meta-analyses have presented empirical data supporting the efficacy and safety of PCV13 and PPSV23 vaccines in preventing pneumococcal

disease. Studies have shown that these vaccines are safe for both children and older people.²⁴ Thromp et al. showed that PCV13 protects fewer serotypes than PPSV23; however, PCV13 is recommended for pneumococcal vaccines because of its higher immunogenic potential than PPSV23. This study also demonstrated that PCV13 could be administered before PPSV23 to achieve a more robust response. To date, no studies have compared the optimal interval between PCV13 and PPSV23 vaccinations.²⁵

Currently, PCV is effective in reducing the incidence of IPD caused by vaccine-covered serotypes. However, difficulties remain, primarily related to the emergence of non-vaccine serotypes as a cause of IPD. A pneumococcal protein ubiquitously expressed in all serotypes is an intriguing alternative for the development of pneumococcal vaccines, independent of serotypes. However, additional assessment of this approach is necessary. Preclinical studies have assessed protein fragments and peptides with antigenic properties as potential vaccine antigens. These antigens have been studied independently and in conjunction with complete pneumococcal proteins as vaccine candidates, and have shown potential as viable alternatives. The antigens above possess the beneficial characteristics of protein antigens, such as the ability to elicit an immune response in neonates, the capacity to establish immune memory, and the capability of "priming/boosting" independent of serotype. Peptide-based vaccines, which are both low-cost and stable, can combine multiple antigens that are highly conserved. These vaccines can potentially activate both cellular and humoral immune responses, thereby preventing various stages of pneumococcal disease. In addition, they provide a more comprehensive range of serotype coverage and offer improved protection. The vaccines in question will utilize innovative delivery system methodologies, including conjugation to Toll-like receptors (TLRs) and the

encapsulation of peptide antigens within nanoparticles, to substantially enhance their immunogenicity.²⁶

In this study, most *S. pneumoniae* isolates remained sensitive (> 80%) to benzylpenicillin, levofloxacin, vancomycin, and linezolid. The sensitivity to chloramphenicol, clindamycin, ceftriaxone, cefotaxime, erythromycin, moxifloxacin, trimethoprim, sulfamethoxazole, and tetracycline started to decrease below 80%. This resembled the findings of Sander et al. However, in a study by Sander et al., the MDR and XDR sensitivity patterns of *S. pneumoniae* were specifically explained.²⁷ In contrast to a study by Assefa et al., the sensitivity of *S. pneumoniae* to chloramphenicol was 45%. In contrast, the study by Assefa et al. in Northwest Ethiopia found 100% of isolates sensitive to chloramphenicol, with all populations from the study being adults.²⁸ In the present study, the population of stored isolates varied with age. In addition, it is different from the research of Temesgen et al., who found that the sensitivity of *S. pneumoniae* to erythromycin was 96.7%, whereas the sensitivity of isolates in this study was 55%.²⁹ In most outpatient cases of CAP, the Infectious Disease Society of America (IDSA) recommends the use of doxycycline, macrolides, or fluoroquinolones as first-line antibiotic treatment. In this study, *S. pneumoniae* was 82% sensitive to levofloxacin.³⁰

Serotype 19F had the highest antibiotic resistance, which was resistant to trimethoprim/sulfamethoxazole (86%; n=6/7), followed by erythromycin (71%, n=5/7). Apart from being resistant to trimethoprim/sulfamethoxazole and erythromycin, the possibility of resistance to other antibiotics cannot be ruled out. These results are similar to those of a study by Nagaraj et al., who showed 100% serotype 19F resistance to erythromycin (n=2/2).³¹

STRENGTH AND LIMITATION



The limitation of this study pertains to the restricted number of isolates that were subjected to testing, as only 22 isolates were retained. However, positive controls for serotypes 3, 4, 6V and 33F were not included in this study because sequencing was planned for further studies. In addition, future studies are necessary to improve the serotype test by designing primer sequences to test other serotypes that were not present in this study, as seven isolates remained non-typeable.

CONCLUSIONS

Serotype 19F is the predominant capsule serotype of *S. pneumoniae*. Serotype 33F, which is not part of PCV13 but part of PPSV23, was also found. Serotypes 15B/C were also identified in this study. This serotype is not a part of PCV13 or PPSV23.

S. pneumoniae was found to be sensitive to linezolid in 91% of the cases, vancomycin in 86% of the cases, levofloxacin and benzylpenicillin in 82%, ceftriaxone and clindamycin in 73%, erythromycin in 55%, and chloramphenicol in 45%.

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ETHICAL CLEARANCE

The research protocol was approved by Faculty of Medicine Research Ethics Commission Udayana University, ETHICAL CLEARANCE No: 1078/UN 14.2.2.VII.14/LT/2022.

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CONFLICT OF INTEREST

All authors who contributed to this work confirmed that there were no conflicts of interest.

AUTHOR CONTRIBUTION

INMM performed the writing, original draft, investigation, and visualization. IPBM performed the writing – review and editing, methodology, software, and data curation. NMAT performed the writing – review and editing, supervision, validation, and conceptualization.

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