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Detection of biofilm formation in clinical isolates of *Streptococcus pneumoniae* in Sanglah General Hospital, Bali, Indonesia

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ABSTRACT

Background: *Streptococcus pneumoniae* causes broad-spectrum infections from mild to severe with high morbidity and mortality rates in almost all of the world, namely pneumonia and meningitis. This bacterium has virulence factors that help their survival, one of which is biofilms. Biofilms help *Streptococcus pneumoniae* become resistant to antibiotics; thus, treating infections caused by these bacteria is difficult to treat. This study aims to determine the biofilm production ability of *Streptococcus pneumoniae* isolated from the Clinical Microbiology Laboratory of Sanglah General Hospital, Denpasar, Bali, Indonesia using the tissue culture plate method.

Methods: The research design used was a descriptive observational study with cross sectional type. The clinical isolate of *Streptococcus pneumoniae* was isolated from the Clinical Microbiology Laboratory of Sanglah General Hospital. Biofilm formation was measured by the tissue culture plate method and carried out at the Microbiology Laboratory of the Faculty of Medicine, Udayana University. Data were analyzed using SPSS version 20 for Windows.

Results: Most of the specimens were collected from blood (59.37%), followed by sputum (31.25%), and others (9.38%). It was found that 1 of 32 (3.10%) clinical isolates could form a biofilm with a strong formation category (the optical density value> 0.38). In contrast, the rest did not form biofilms with an optical density value of ≤0.095.

Conclusions: Not all clinical isolates of *Streptococcus pneumoniae* isolated from the Clinical Microbiology Laboratory of Sanglah General Hospital Denpasar were able to form biofilms, suggesting that other virulence factors also play a role in pneumococcal infection. However, a molecular approach is necessary for the detection of genes encoding biofilm-producing isolates in future studies.

Keywords: *Streptococcus pneumoniae*, Biofilm, Crystal Violet, Tissue Culture Plate, Virulence Factors.
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INTRODUCTION

Streptococcus pneumoniae is a diplococcal gram-positive bacterium that is round arranged in a chain. It comes from the genus *Streptococcus* and is alpha-hemolytic (expresses a green zone around the colony).[1] This bacterium can cause life-threatening diseases such as pneumonia and meningitis.[1] Pneumonia is a disease with high morbidity and mortality rates worldwide, especially in developing countries. According to the previous study, The high mortality rate is often found in children under 5 years old, which reached 808,694.[2] It was reported that the bacteria that causes the most common pneumonia in children under 5 years old is *Streptococcus pneumoniae*.[2] The Bali Provincial Health Office notes that pneumonia is the 3rd of the ten significant disease patterns in hospitalized patients in public hospitals, with a number of 2,683.[3] This bacterium is also one of the causes of another deadly disease, namely meningitis. The incidence rate of bacterial meningitis is 2.6-6 per 100,000 adult population per year in developing countries and has a mortality rate of 13% -27%.[4]

Streptococcus pneumoniae is a normal flora in the mouth and pharynx that is resident or temporary.[5] This bacterium is more often found in children's nasopharynx than in adults depending on its geographic distribution.[5] There were more than 90 serotypes in *Streptococcus pneumoniae* whose virulent levels differ from one another. In some cases, these bacteria are usually harmless and only become a reservoir of pathogens involved in respiratory infections and other invasive diseases.[6] *Streptococcus pneumoniae* has virulence factors that help it evade the body's defense system, lowering the immune system. One of the virulence factors is biofilm.[7]

A biofilm is a group of bacterial cells attached to the surface encased in an extracellular matrix of protein and

carbohydrate secretions that creates a different phenotype from planktonic cells.[8] This biofilm formation is a bacterial response to cellular recognition of the attachment site, deficiency of nutrient cells, and exposure of planktonic cells to antibiotics.[8] In general, bacteria are found in 2 conditions, namely, planktonics and biofilms.[9] This biofilm tends to provide better protection and is able to provide 1000-1500x better antibiotic resistance capabilities than in the planktonic phase.[9]

The implication of this biofilm's ability, especially its ability to be resistant to biofilms, results in difficulties in handling infectious diseases caused by biofilm-forming bacteria, one of which is *Streptococcus pneumoniae*. The ineffectiveness of therapy and administration of antibiotics is an implication of biofilms for clinical management given to health services.[10] Because of these implications, it is important to know the formation of biofilms and their category of formation, especially in the bacterium *Streptococcus pneumoniae,* which has high morbidity and mortality rates. However, there is still little research in Indonesia that discusses the formation of biofilms of *Streptococcus pneumoniae*. Therefore, this study was aimed to determine the formation of biofilms in clinical isolates of *Streptococcus pneumoniae* at Sanglah General Hospital, Denpasar, Bali using the tissue culture plate method.

METHODS

Streptococcus pneumoniae was obtained from clinical specimens isolated in the Clinical Microbiology Laboratory of Sanglah General Hospital Denpasar in 2015-2019.

The biofilm detection method used is the tissue culture plate method used in Christensen GD et al., with slight modification.[11] Bacterial culture was inoculated on TSB media with a glucose concentration of 1% then incubated for 24 hours at 37 ° C. Each Corning® 96-well EIA / RIA Clear Flat Bottom Polystyrene Microplates was inserted with 200 µl of bacterial suspension, which was then sealed and incubated again for 48 hours. After the incubation process, each microplate's content was removed by gently patting it and washed with 0.2 ml of phosphate buffer saline (pH 7.2) twice. Furthermore, the microplate was fixed using 2% acetic acid and stained using 200 µl crystal violet with a concentration of 0.1%. To prevent contamination and carry-over between *Streptococcus pneumoniae* isolates, we ensure that we experimented in a biological safety cabinet (BSC) to avoid any contamination from the environment. Furthermore, we conducted the assay using sterile 96-well microplates, sterile barrier-tips (aerosol prevention tips) and ensured that micropipettes and related equipment were adequately decontaminated. During incubation, all microplates were properly sealed using sterile-microplate lids and then were covered with aluminum foil.

Any excess staining was removed using deionized water and then allowed to dry at room temperature. After the microplate was dry, 200 µl of 96% ethanol was added to each well in the microplate. Then, do the reading using a microplate reader with a wavelength of 620nm. This experiment was done in triplicate and repeated three times. The reading result is the Optical Density value that determines the category of biofilm formation that is adjusted to the Wróblewska criteria, which are classified into 4, namely non produce (OD≤0.095), weak (0.095OD), moderate (0.19OD), and strong (0.38> OD).[12] Data were analyzed using SPSS version 20 for Windows.

Table 1. The clinical specimens collected in this study

Source	Sample Count (N=32)	Percentage (%)
Blood	19	59.37
Sputum	10	31.25
Others	3	9.38

RESULTS

The samples used came from clinical specimens of blood, sputum, and others which can be seen in Table 1. Most of the specimens were collected from blood (59.37%), followed by sputum (31.25%) and others (9.38%) (Table 1).

It was found that one (3.10%) of 32 clinical isolates of *Streptococcus pneumoniae* was able to form a biofilm in the strong formation category with an OD value of 0.428 (Strong). In contrast, the other 31 isolates formed an OD value of ≤0.095 to be included in the non-produce category. Isolates that form biofilms are obtained from clinical specimens of blood. (Figure 1).

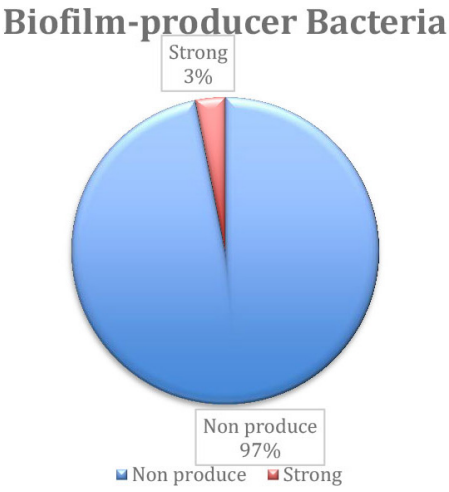

Figure 1. Distribution of *Streptococcus pneumoniae* that produced biofilm detected using tissue culture plate method.

DISCUSSION

Biofilm-forming bacteria cause difficult clinical management of infectious diseases, especially those with high morbidity and mortality rates almost worldwide, one of which is the disease caused by *Streptococcus pneumoniae* bacteria. Thirty-two *Streptococcus pneumoniae* clinical isolates from the Clinical Microbiology Laboratory of Sanglah General Hospital were used in this study. Among the study, one isolate (3,1%) of *Streptococcus pneumoniae* bacteria showed that only one (3.1%) isolate from blood could form a biofilm with strong capacity,

A similar study conducted by Wróblewska J et al., in 2016 used the tissue culture plate method, the isolates of which were derived from nasal swabs and bronchial alveolar lavage.[12] The study found that all samples formed strong

biofilms seen from the microplate assay reading of 570 nm wavelength with an OD value more significant than> 0.38.[12] Researchers used the same method with several modifications to the procedure, and the sources of the isolates used were different, and each source was blood, sputum, and others. In the research, there was no use of isolates whose source was the same as the research used as references, so that the possibility of finding strong biofilms was few.[12]

Previous research by Yadav MK et al., in 2012 demonstrated using variations in glucose and its incubation time.[13] The glucose concentrations used in this study were 1% and 2%. The incubation times were 6 hours, 12 hours, 18 hours, and 24 hours.[13] In this study, the samples formed a strong and optimal biofilm using a glucose concentration of 1% and an incubation time of 12-18 hours. This study is using the same glucose concentration of 1% and an incubation time of 48 hours. This difference in incubation time probably causes the small number of samples to form biofilms.[13]

Related to the source of clinical isolates, the researchers found that forming a strong biofilm was from blood. The results of this study are different from the results of research conducted by Garcia-Castillo M in 2007.[14] The study found that clinical isolates derived from sputum form more biofilms than clinical isolates taken from blood.[14] As many as 16 out of 2,035 isolates taken from sputum in cystic fibrosis sufferers can form biofilms.[14] Meanwhile, 11 out of 22 isolates from non-cystic fibrosis patients were able to form biofilms. Unlike previous studies, in this study, none of the *Streptococcus pneumoniae* isolated from sputum produced biofilm, maybe due to differences in patient characteristics.[14]

The formation of biofilms is strongly influenced by several things, such as the availability of nutrients in which glucose is used in this study and the anaerobic environment.[15] *Streptococcus pneumoniae* grown under various nutritional conditions showed different abilities to form biofilms according to their transformability. Limited (less) nutrition is important for optimal biofilm formation because rich and complex media results in poor biofilm formation with low transformation efficiency.[8] Another study also showed that the biofilm formation in *Streptococcus pneumoniae* bacteria was better in an atmosphere enriched with CO2.[16] The growth rate in an anaerobic atmosphere is higher than at the ambient temperature. This confirms that the anaerobic environment fosters the growth of a good biofilm in *Streptococcus pneumoniae*.[16]

CONCLUSIONS

Not all clinical isolates of *Streptococcus pneumoniae* bacteria isolated from the Clinical Microbiology Laboratory of Sanglah General Hospital were able to form biofilms. Biofilm is indeed one of the virulence factors possessed, but perhaps the role of other virulence factors plays a greater role in pneumococcal infection. Further study about the molecular characterization of *Streptococcus pneumoniae* biofilm production is necessary to be elucidated.

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CONFLICT OF INTEREST

There is no competing interest regarding the manuscript.

ETHICAL APPROVAL

Research Ethics Committee, Faculty of Medicine, Udayana University, Denpasar, Bali, Indonesia, permitted this study with number: 323/UN14.2.2.VII.14/LP/2020.

DISCLOSURE

The authors declare no conflict of interest.

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