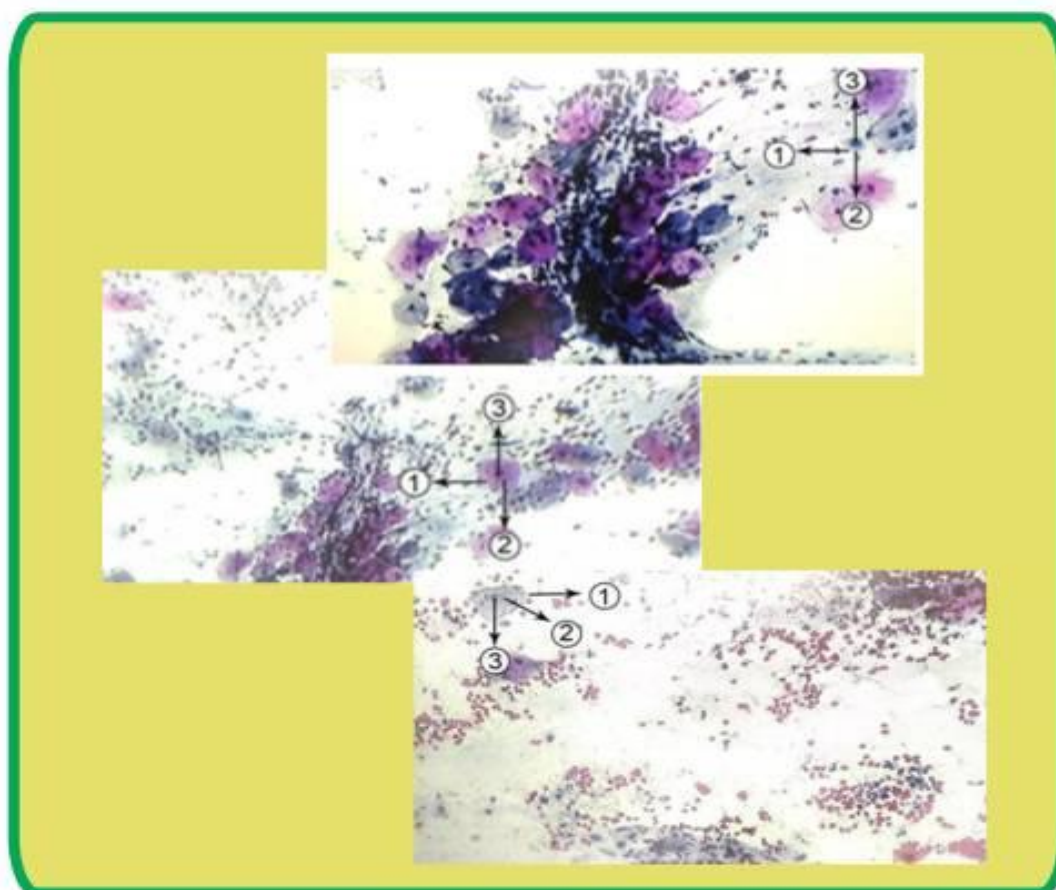


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
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
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
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Detection of LytA Genes in *Streptococcus pneumoniae* Isolated from sputum pneumonia patients

Mustika Sari Hutabarat¹, Firdaus Hamid², Irawaty Djaharuddin², Alfian Zainuddin²,
Rossana Agus³, dan Muhammad Nassrum Massi²

¹DIV Medical Laboratory Technology, Faculty of Health, Musi Charitas Catholic University Palembang, Indonesia

²Department of Microbiology, Faculty of Medicine, Hasanuddin University, Indonesia

³Faculty of Mathematics and Natural Sciences, Hassanudin University, Indonesia

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ABSTRACT

Streptococcus pneumoniae (pneumococcus) is a Gram-positive facultative anaerobic bacterium that is a major cause of morbidity and mortality worldwide. But the lack of reporting of disease by this bacterium in Indonesia, one of the causes is because the diagnosis of pneumococcal infection is often clinically not typical and conventional methods which are still the standard gold method often give false-negative results. So the purpose of this study was to evaluate the performance of culture and molecular diagnostic methods using the Polymerase Chain Reaction (PCR) technique in detecting *Streptococcus pneumoniae* in sputum clinical samples using the Autolysin (LytA) gene which is a virulence factor of this bacterium. 57 isolates from 60 samples were confirmed as *Streptococcus sp* through microscopic identification, culture, and biochemical tests. Then the sensitivity test with an optochin test of 9 (9%) compared the results descriptively with the PCR technique using the Autolysin A (LytA) gene which was obtained more sensitive by 15 (25%).

*Corresponding author:

Mustika Sari Hutabarat

DIV Medical Laboratory Technology, Faculty of Health, Musi Charitas Catholic University Palembang, Indonesia

Email: mustikasarihutabarat33@gmail.com



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INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is a Gram-positive facultative anaerobic bacterium that is a major cause of morbidity and mortality worldwide. Pneumococcal often causes bacterial pneumonia, otitis media, meningitis, and septicemia. People who are at high risk of using pneumococcal pneumonia are those who have a weak immune system or have problems with the immune system of the elderly, infants, cancer patients, AIDS patients, and postoperative patients. Infection caused by bacteria occurs through the respiratory tract and then colonization occurs in the nasopharynx and the bacteria will penetrate the barrier defense system and enter the bloodstream which then persists in the lining of the brain and Cerebro Spinal Fluid (CSF) which can ultimately be used meningitis (Maestro and Sanz, 2016).

Streptococcus pneumoniae produces a large number of virulence factors that are associated with various bacterial structures which are mostly present on the surface of bacterial cells such as polysaccharides, surface proteins, excreted proteins, and cytoplasmic proteins. Where the polysaccharide virulence factors include Capsular Polysaccharides (CPS), Cell Wall Polysaccharide (CWPS), and Cell Wall Polysaccharide (CWPS). Whereas the virulence protein factor in *Streptococcus pneumoniae* consists of Pneumococcal surface protein A (PspA), Pneumococcal surface protein C (PspC), Autolysin (LytA), Pneumolysin (Ply), Neuraminidase, Pneumococcal surface adhesion A (PsaA), and IgA (Scholz *et al.*, 2012).

Among the proteins associated with the surface protein *Streptococcus pneumoniae* LytA (N-acetylmuramoyl-L-alanine-amidase) which is also a major autolytic enzyme of pneumococci. LytA is responsible for the occurrence of cell lysis induced by deoxycholate and penicillin in the stationary phase of great clinical importance. The main autolysin LytA enzyme from *Streptococcus pneumoniae* is a member of a group of cell wall degrading enzymes that has a role in various physiological functions and helps in diagnosing bacteria both culturally or molecularly (Simões *et al.*, 2016).

In Indonesia, there is no data on the prevalence of pneumococcal as a cause of IPD, but the Indonesian Health Profile (2017) said Pneumonia is the cause of 16% of under-five deaths, which is estimated as many as 920,136 under-fives in 2015. And the lack of reporting of disease by this bacterium is one of them caused by a diagnosis of pneumococcal infection clinically often not typical and conventional methods that are still the gold standard method often give false-negative results, this can be due to the nature of fastidious bacteria (Varghese, Jayaraman and Veeraraghavan, 2017).

Therefore, molecular methods that are promising can detect various proteins that encode *Streptococcus pneumoniae*, namely the Polymerase Chain Reaction (PCR) which is more sensitive and accurate for detecting *Streptococcus pneumoniae* (Haas *et al.*, 2011).

Among the proteins associated with surface proteins as described above, Autolysin is a cell-degrading enzyme that forms a pneumococcal capsule that has a role in sharing the physiological functions of bacteria and is said to help identify bacteria both conventionally and molecularly (Gholamhosseini-Moghaddam *et al.*, 2015).

So this research is based on the aim of detecting *Streptococcus pneumoniae* in specimens that are more sensitive and accurate for detecting *Streptococcus pneumoniae*. sputum pneumonia patients using the Autolysin (LytA) gene as a target of detection in sputum samples.

MATERIALS AND METHODS

Materials

This study uses a descriptive method which is a laboratory exploratory study that is a study that provides an overview of the characteristics of the type of DNA virulence factors of *Streptococcus pneumoniae* isolated from sputum using the PCR method of LytA genes with 320bp fragments of the LytA gene amplified and detected by electrophoresis and read by document gel, a sample DNA volume used in each 5 μ reaction mixture (Gholamhosseini-Moghaddam *et al.*, 2015).

Sputum Sampling

The material used in this study was a clinical sample of the patient is sputum which was produced spontaneously or by being induced with a NaCl nebulizer. The quality of sputum is assessed microscopically with a ratio of leukocytes and epithelium > 1 / Field of view, but all sputum samples are processed and tested according to the procedure regardless of the quality of the sputum.

Phenotype identification of *S. pneumoniae*

Microscopic identification

Identification is done by making preparations directly from sputum before culture and from sputum colonies that have been cultured and then colored with gram staining. The staining procedure is done by making sample preparations on clean, fat-free glass objects. Then it is stained by using a gentian violet, Lugol, alcohol 90%, and safranin reagent solution then observe under a 100x magnification microscope with additional oil immersion (Balsells *et al.*, 2017).

Macroscopic identification

Sputum samples that have been collected are inoculated on Blood Agar Plate (BAP) and Chocolate Agar Plate (CAP) media for 24 hours with an incubation temperature of 24 hours with 5% CO₂ addition. Inoculation of the media results in the growth of bacterial colonies that have α -hemolytic clear zones, the surface of the

colony is flat and in the middle of the colony looks concave (draughtsman) during the incubation period > 24 hours and the growth of more fertile bacteria in isolates that are inoculated on the CAP media (Abdeldaim *et al.*, 2010).

Catalase Test

After that, the catalase test was continued using a bacterial colony that had been isolated on BAP media using a disposable loop placed on a glass object which was then dripped with a 30% H₂O₂ solution. Results show positive if there are bubbles (Mousavi *et al.*, 2013).

Sensitivity Test

The identification of *Streptococcus pneumoniae* was confirmed by susceptibility test using optochin. This examination generally uses optochin discs with a diameter of 6 mm containing 5 μ g optochin and is assessed by looking at the diameter of the resistance zone > 14mm, which is incubated in an atmospheric environment with 5% CO₂ levels and temperature of 35-37°C for 24 hours (Zweifel *et al.*, 2016).

Genotype identification of *Streptococcus pneumoniae*

In the research of Lung and Rello, (2014), it can be shown that the detection of the *LytA* gene is important in molecular pneumococcal identification. This study uses the *LytA* gene with the following primers:

Table 1. Oligonucleotides used as a primary in detecting *S. pneumoniae*

Gene	Primary	Size amplicon	Reference
LytA	F: 5'CAA CCG TAC AGA ATG AAG CGG-3' R: 5'TTA TTC GTG CAA TAC TCG TGC G-3'	320bp	(Gholamhosseini-Moghaddam <i>et al.</i> , 2015)

DNA extraction

The clinical isolate DNA extraction was isolated from the patient's sputum in the hospital using BAP media, then the growing colonies were extracted using the boiling method. The colony was placed in an effendor tube containing 200 μ l Phosphate buffer Saline (PBS), then coiled with a temperature of 90°C for 30 minutes (Bogaert, De Groot and Hermans, 2004).

DNA Amplification

DNA is performed to detect the presence of specific genes coding for virulence factors. Amplification reactions were carried out using the gene primers listed in Table 1. The PCR core system (Toyobo) reagent mix is 20 μ l, ddH₂O 23 μ l, Forward Primer 1 μ l, Reverse Primer 1 μ l, Template 5 μ l, in total the volume becomes 50 μ l. The mixing is done in a cooler box so that the

DNA and enzymes used are not damaged (Bandettini and Melioli, 2012). The amplification conditions for the LytA gene are: using the LytA gene primer to detect *Streptococcus pneumoniae* with the primary nucleotide sequence used is; F: 5'CAA CCG TAC AGA ATG AAG CGG-3 'R: 5' TTA TTC GTG CAA TAC TCG TGC G-3 'with amplicon size 320 bp². The mixture of PCR core system (Toyobo) reagent 7µl, ddH₂O 2 µl, primary Reverse 0.5, Reverse primer 0.5 µl, template 5 µl total volume of 15 µl. The LytA test with PCR was performed using the Bio-Rad T100™ Thermal Cycler instrument. PCR conditions in this amplification are: Pre denaturation temperature of 950C for 10 minutes, each cycle consists of: Denaturation (DNA separation) 950C for 30 seconds, Annealing (primary attachment) 52 0C for 30 seconds, Elongation / Extension (DNA elongation) 72 0C for 2 minutes (lasts until 35 cycle) the last stage of the cycle is extended by 1 step elongation 720C for 8 minutes to allow the elongation process to run perfectly and 1 step 12 0C overwait maintaining the amplicon remains stable even if left behind 24 hours (Gholamhosseini-Moghaddam *et al.*, 2015). PCR products (amplification results) were analyzed using 2% agarose gel electrophoresis using an

ethidium bromide 2.5 ul / 100 ml dilator and read under ultraviolet light (Chen *et al.*, 2013).

RESULT AND DISCUSSION

Sputum samples were 60 of the patients treated at Wahidin General Hospital originating from inpatients at Wahidin Sudirohusodo Hospital with an average stay of > 5 days. The average age of patients is over 45 years (range 16-82 years), and 43 (75%) patients are male. A total of 53 (88.3%) of the 60 patient samples that were examined microbiologically from microscopic starts were by gram staining as many as 60 samples were identified as gram-positive bacteria diplococcus, culture as many as 53 samples growing with characteristics of alpha hemolysis, and biochemical tests with catalase test as many as 50 negative samples with catalase test where these results point to the bacteria *Streptococcus sp.* Then, from the 50 samples tested with sensitivity with Opthocin, the results of Streptococcus spp were 93%, then confirmed by susceptibility test using Optochin disk 5µg obtained 9(10%) samples identified as *Streptococcus pneumoniae*, while the identification results can be seen as follows:

Table 2. Results Microscopic identification of bacteria

No	Gram Staining Results	n= 60	%
1	Gram (+) diplococcus	3	7,0
2	Gram (+) diplococcus lancet	57	93,0
Total		60	100

Tabel 3. Characteristics of the colony on the media

No	Macroscopic Observation Results	N	%
1	α- hemolysis, rounded colonies, gray, mucoid, waterish	53	86,0
2	β- hemolysis, convex surface, rounded colonies	7	10,0
Total		60	100

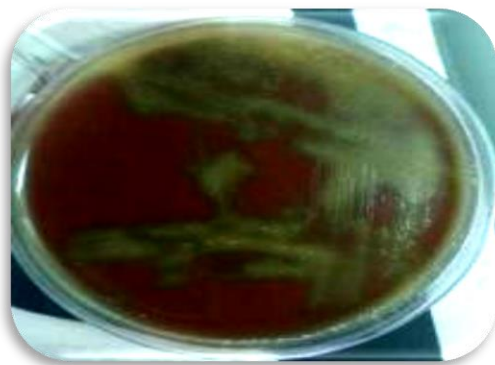


Figure 1. *Streptococcus pneumoniae* colonies on BAP media form α -hemolysis zones

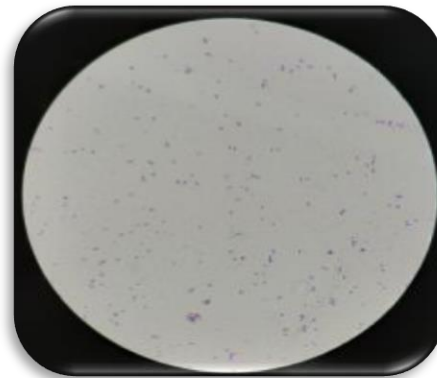


Figure 2. Morphology of Gram-positive *Streptococcus pneumoniae* lancet lancet

Tabel 4. Catalase Test Result

No	Hasil Pengamatan	N	%
1	Positive / (+)	10	0
2	Negative /(-)	50	100
Total		60	100

Description: (+): There is a bubble; (-): No gas bubbles

Tabel 5. Optochin Sensitivity Test Results

No	Hasil Uji	N	%
1	Resistant	41	82,0
2	Sensitive	9	18,0
Total		50	100

Macroscopic Observation Result 9 (8.0%) isolates were confirmed as *Streptococcus pneumoniae* out of 50 (82%) isolates of *Streptococcus* spp. The study sample came from inpatients who were not only specifically diagnosed with pneumonia, where the results of isolation of sputum were *Streptococcus* spp bacteria, where 9 (18%) samples from identified as *Streptococcus pneumoniae* were characterized by the results of the formation of bacterial sensitivity to Ophochin by forming a clear zone > 14 mm (Fischetti and Ryan, 2015). Although pneumococci are generally sensitive to optochin, a pneumococcal strain that is resistant to optochin

can also be found (Zweifel *et al.*, 2016). Therefore, all samples identified by *Streptococcus* spp were examined by PCR test using the *Streptococcus pneumoniae* virulence factor coding gene LytA (Sampson *et al.*, 1997). Distribution of LytA genes among *Streptococcus* sp isolates confirmed by microscopy, culture, and biochemical testing of 9 samples, then by PCR technique using LytA genes as many as 15 (30%) positive samples. Clinical samples showing bands on electrophoretic visualization that are suitable for base-pair size (bp) expected to use LytA can be seen in Figure 3, electrophoresis results from PCR products as follows:



Figure 3 . Visualization of PCR Products

Tabel 6. Distribution of positive LytA samples by the diagnosis of sputum samples

Diagnosis	Pasien	Hasil Positif LytA
Pneumonia as the main diagnosis	13 (24,5%)	7(53,8%)
Pneumonia as the secondary diagnosis	16 (30,2%)	8(50%)
Other Diagnosis	24 (45,3%)	0
Total sample	53	15

The Optochin sensitivity test is the most common test used to differentiate *Streptococcus pneumoniae* from other oral Streptococcus. Optochin sensitivity tests are carried out to measure the ability of *Streptococcus pneumoniae* to form inhibition zones > 14 mm or larger. The results of this study indicate that 15 (30%) LytA genes were detected in the sample, this result is in line with Moghaddam's study (2015) which states that LytA is generally a virulence factor that is almost present in all serotypes (90 serotypes) which greatly contribute to pathogenicity *S. pneumoniae* in conducting colonization of target cells, this is slightly different from the *psaA* gene that is not shared by all *Streptococcus pneumoniae*, so it is quite possible if there are several isolates *Streptococcus pneumoniae* has the LytA gene but not the *psaA* gene and this is evidenced by various reports related to the role of LytA in the pathogenesis of *Streptococcus pneumoniae* which also shows that this gene can not only be used as a method in detecting *Streptococcus pneumoniae* but also more precisely as a vaccine antigen against *Streptococcus pneumoniae* (Prendki *et al.*, 2019).

Streptococcus pneumoniae were identified as many as 9 samples from 50 *Streptococcus sp* isolates through the Optochin sensitivity test and 41 other

samples were *Streptococcus sp* which were resistant to Optochin. Although pneumococci are generally sensitive to optochin, based on the results of the study of Zweifel *et al.*, (2016) which states that the strain of *S. pneumoniae* has also been found to be resistant to optochin. This statement is also in line with the results of sequencing which show that samples that are resistant to Optochin but detected with the LytA gene indicate that the isolate is *Streptococcus pneumoniae* through sequencing results conducted on one positive LytA sample. Therefore some groups use the LytA probe to identify and characterize *S. pneumoniae* and the results show a good correlation between the LytA probe and bile solubility. Autolysin has also been used successfully to distinguish *Streptococcus pneumoniae* from *Streptococcus viridians* and it is said that LytA is present in all capsulated *Streptococcus pneumoniae* (Zweifel *et al.*, 2016).

Streptococcus pneumoniae which is a normal flora in the nasopharynx turns out to be opportunistic and pathogenic in certain circumstances and infects target cells where one of the targets is the lungs so that it can cause pneumonia. Clinical samples that show the presence of bands on the visualization of electrophoresis at what size of base pair is LytA 320 bp for the 7/15 (45.8%) LytA

gene were detected in samples from patients with a primary diagnosis of pneumonia while 8/15 (53%) detected from samples originating from samples with a secondary diagnosis of pneumonia. Autolysin which can be found in all *S. pneumoniae* serotypes is the advantage of this gene compared to other genes so that this gene can be used as a target gene in detecting *Streptococcus pneumoniae* molecularly in detecting the causes of pneumococcal pneumonia and this is evident from the results of the study in table 6. it was seen that where the LytA gene can detect *S. pneumoniae* in a sample of pneumonia patients. And the LytA gene has also been used successfully to distinguish *Streptococcus pneumoniae* from *S. viridans* (Wu *et al.*, 2015)

Even so, molecular examination still has a weakness in detecting genes if the extracted sample is contaminated so that it can cause false positives or false negatives. Therefore, each check must be accompanied by positive and negative controls so that it is easier for us to interpret the correct and reliable results.

CONCLUSION

We conclude that the Autolysin gene (LytA) can be used in detecting *Streptococcus pneumoniae* in clinical samples of sputum which is a bacterium that causes pneumonia in the Wahidin Sudirohusodo General Hospital Makassar.

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