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ORIGINAL ARTICLE

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ANALYSIS DIVERSITY OF BACTERIA ISOLATED FROM PULMONARY TB PATIENTS WITH 16S rRNA GENE SEQUENCING TECHNIQUE AND TEST RESISTANCES OF ANTIBIOTICS

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ABSTRACT

Pulmonary infections are the case that frequently happends. Based on the initial survey that conducted at several hospitals in Ambons city, it is found that the presentation of patients who treated with pulmonary TB case, hence, it is needed to take as basis in executing "educated gues therapy". Isolated and identifications of bacteria is done by morphological observation and molecular technique using genetic information in the small subunit if ribosom 16S Rrna. Furthermore, resistance antimicroba testing by using " Disc Diffusion Method" according to the National Committee for Clinical Laboratory Standards (NCCLS). Based on the identification result, pathogen bacterial that obtained is " Streptococcus mitis, which those there kind of bacteries had resisted toward the antibiotics varience of Sulfamethoxaxole, Trimethoprim and Ceftazidime.

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I. INTRODUCTION

Acute respiratory tract infections are the latter case of tenoccurs. Acute respiratory infections include upper respiratory tract and the lower respiratory tract, the respiratory tract is defined as organs from nose to lung bubbles, along with the surrounding

organs such as the sinuses, middle ear space and the lining of the lung. Major cause of both cases of the disease can be caused by a variant of bacteria and resistance to antibiotics.

Each bacterium has a different genome profile, even a kind of bacteria that also have a different total genome. 16S rRNA technique is one of the best technology that is able to identify the species of bacteria, which is carried out by analyzing the structure or composition of bases of RNA contained in the 16S rRNA. DNA sequencing techniques ribosomal (rRNA sequencing) is used to determine the diversity and phylogenetic relationships of microorganisms according Radji (2010). Direct comparison of the DNA sequence is the most powerful tool for tracking the phylogenetic relationships between bacteria from various sources (Drancourt et al., 2000). The method can help to show patterns of genes from bacteria that infect the respiratory tract, so that medical action can be done right on target. Respiratory tract infections have a very broad spectrum antibiotics, there fore, ideally wait for the results of antibiotic treatment and isolation of the causative agent of antibiotic resistance testing (WHO Geneve, 1991). However, this approach is not very practical for people with bacterial infections would be too late to get treatment. Antibiotics will lead to the selection of bacterial populations and consequently the sensitivity of bacteria to antibiotics is likely to be reduced in line with the intensity and duration of use in society (Kumala, 2010). In harmony with its use, many pathogenic bacteria to adapt to its environment and become resistant to antibiotics. Required periodic review of patterns and pattern sensitivity causes the bacteria to antibiotics as a corner stone in therapy (Sjahrurachman in Kumala, 2010). If the infection treatment is done in a proper way it can reduce morbidity, prevent worsening of the patient's condition, prevent arise and spread of resistant pathogens.

Most strains of Staphylococcus resistant to antibiotics such as penicillin, metisillin, cephalosporins, erythromycin, lincomycin, vancomycin and rifampin (Kusuma, 2009). Penicillin-resistant Streptococcus pyogenes, Streptococcus pneumoniae resistant to trimethoprim/sulfametoksasol and penicillin, Escherichia coli, Klebsiella spp., Serratia spp., C.freundii, Morganella spp (gram negative bacteria) is resistant to beta-lactams. Proteus mirabilis resistant to nitrofuran to in and tetracycline, Pseudomonas aeruginosa resistant to trimethoprim sulfametoksasol, tetracyclines, and cephalosporins. Acinetobacter spp. Resistant to trimethoprim sulfametoksasol, ampicillin, and cephalosporins. Haemophilus in fluenzae resistant to cephalosporins and macrolide class (Mardiastuty, 2009). It would require periodic testing patterns of germs or bacteria causing respiratory infections and sensitivity to antibiotics (Widyaningsih, 2012). Making the genetic patterns of microorganisms or bacteria in each hospital will assistin the formulation of rational antibiotic policy.

METHODS

Sampling sputum/mucus conducted at Hospital Dr. M. Haulussy Ambon in patients infected with ARD, then isolated the bacteria from sputum/mucus and grown on blood agar and BHI media. Furthermore, the observation of morphological and cytological bacteria. The following stage is Optochin testing to prove true or not ten samples of bacteria isolated from the sputum of patients TB is a kind of bacterial pneumonia Streptoccocus and to analyze the diversity of pathogenic bacteria that infect the respiratory tract using 16S rRNA gene sequencing techniques. After wards, Sequences DNA we comparison with based data in BLAST program ini Gene Bank by (http: // blast.ncbi.nlm.nih.gov /Blast.cgi) website. And than with software Mega6 program, we make phylogenetic tree from Results of bacterial analysis . The last stage of this research is antimicrobial resistance test using the Disc Diffussion Method according the National Committee for Clinical Laboratory Standards (NCCLS). The antimicrobial is Sulfamethoxaxole, Trimethoprim andCeftazidime.

RESULTS

This study begins with a sample of sputum/mucus from five patients with ARD splaced in a closed container. Samples of sputum/mucus then taken to the microbiology

laboratory Fak. MIPA for isolation ofbacteria from a sample of sputum/mucus. Starting from the dilution10^{-1} until10^{-7} be fore grown on bloodagarmedia.

A. Characteristics Identification Results Morphology and Cytology of Bacteria

Table 1. The Characteristic Morphology and Cytology Colony of Bacteria.

Colonies code	Characteristics Morphological Isolates of Bacteria					
	Colony color	Shape colonies	Form colonies	Elevatio n colonies	Shiny/ Dreary	Thick colonies
P1 10^{-3}a	White	Round	Slippery	Arise	Shiny	Thick
P1 10^{-3}b	White	Round	Slippery	Arise	Shiny	Thick
P3 10^{-5}	White	Round	Slippery	Flat	Dreary	Thick
P3 10^{-7}a	Beige	Round	Slippery	Flat	Dreary	Thick
P3 10^{-7}b	Beige	Round	Slippery	Flat	Dreary	Thick
P4 10^{-7}	Green	Round	Wavy	Flat	Dreary	-
P4 10^{-7}a	White	Round	Slippery	Flat	Dreary	-
P4 10^{-7}b	White	Round	Slippery	Flat	Dreary	-
P5 10^{-7}a	White	Round	Slippery	Flat	Dreary	-
P5 10^{-7}b	White	Round	Slippery	Flat	Dreary	-

Note : P (patient), $10^{-\cdots}$(dilution rate), a & b (size of bacteria colonies : a (big colonies), b (small colonies)). Thick (the growth of bacteria that cause large colonies).

Table 2. Character Cytology of Bacteria Grow On Media Growth.

Colony Code	**Cytology Characteristics Isolates of Bacteria**	
	Gram	Shapes
P1 10^{-3}a	Positive	Coccus
P1 10^{-3}b	Positive	Coccus
P3 10^{-5}	Positive	Basil
P3 10^{-7}a	Positive	Coccus
P3 10^{-7}b	Positive	Coccus

P4 10^{-7}	Positive	Basil
P4 10^{-7}a	Positive	Coccus
P4 10^{-7}b	Positive	Coccus
P5 10^{-7}a	Positive	Elliptical
P5 10^{-7}b	Negative	Coccus

P (patient), 10^{-}...(dilution rate), a & b (size of the colony of bacteria: a (large colony), b(colony small)). Concentrated: the growth of bacteria that cause large colonies.

B. Results Identification of Bacteria with 16S rRNA Gene Techniques

Isolates were identified bacterial isolates with the code P1 10^{-3}b, P3, P4 10^{-7} and P5 10^{-7}a. Fourth bacteria have been considered to be representad from ten isolates were obtained based on morphological characteristics, cytology include cell shape and gram bacteria and based on the results of resistance testing. Be sides, the four bacteria were chosen based on their ability blood hemolitic.

Bacteria P1 10^{-3} Bacteria P4 10^{-7} Bacteria P5 10^{-7}

Figure 1. Blood hemolitic Bacteria Caption : P (patients), 10-..(dilution rate).

The identification results are as follows.

1. Resultsof Gel Photo

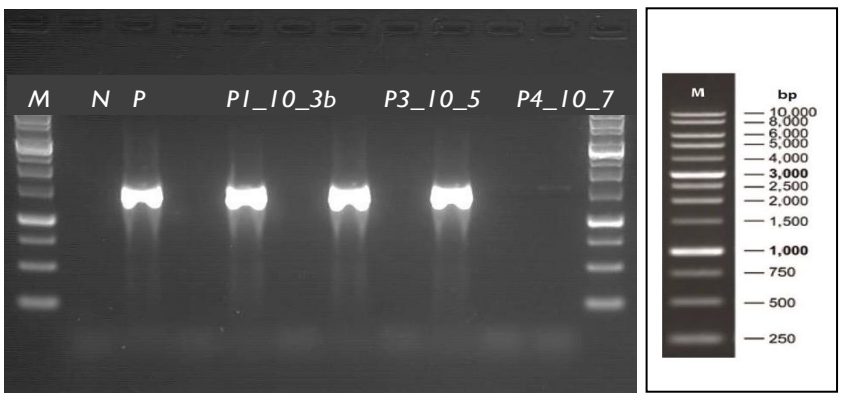

Figure 2. Photo 16S rRNA gene Gel Electrophoresison 1% agarose with 60-70 volts running conditions, pH buffer(TAE) 8, for 45 minutes.

The images on agarose gel electrophoresis showed that all isolates P110^{-3}b, P3 10^{-5}, P4 10^{-7} and P5 10^{-7}a a like are at 1500 bp band. The PCR results further disekunsingto get the base sequence of16S rRNA.

2. The results of 16S rRNA sequence comparisons with the data base at NCBI Gene Bank.

Table 3. Results Identification of Bacteria Sample.

No.	Origin Samples	Code Samples	Results Identification Based on16S rRNA sequences	Similarity
1.		P1 10^{-3}b	Streptococcus mitis	98%-100%
2.	Sputum/Muc	P3 10^{-5}	Bacillus cereus	99%
3.	Patients usus Pulmonary	P4 10^{-7}	Bacillu cereus	99%-100%
4.		P5 10^{-7}a	Bacillus anthracis	98%-99%

From Table 3. Results of sample identification code P110^{-3}b bacteria are bacteria Streptococcus mitis, P3 10^{-5} is Bacillus cereus, Bacillus cereusis P410^{-7} and P510^{-7}a is Bacillus anthracis. With rRNA sequence similarity compared to the Bank NCBI Gene data baset hat is up to 98% - 100%

1. Phylogenetic Analysis

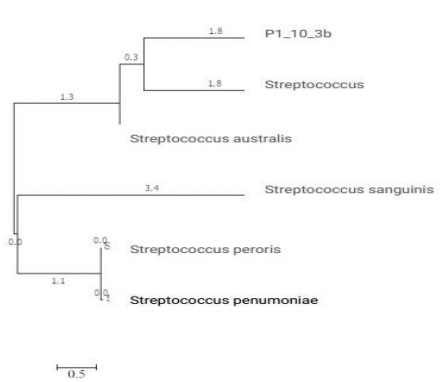

Figure 3. Phylogenetic tree Kinship Bacterial Isolates P110^{-3}b, based Method Using Neighbor Joining.

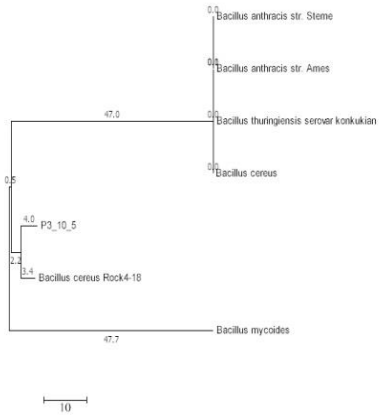

Figure 4. Phylogenetic tree Kinship Bacterial Isolates P310^{-5}, based Method Using Neighbor Joining tree software Mega.

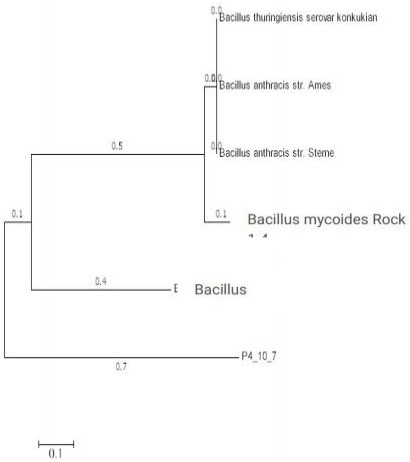

Figure 5. Phylogenetic tree Kinship Bacterial Isolates P310^{-5}, based Method Using Neighbor Joining Tree Software Mega6.

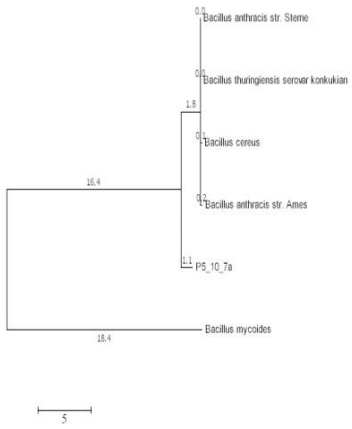

Figure 6. Phylogenetic tree Kinship Bacterial Isolates P510^{-7}a, based Method Using Neighbor Joining Tree Software.

The phylogenetic tree in Figure 3. Describes the closeness of kinship orgenetic

data bacteria 16S rRNA sequence alignment results at NCBI Gene Bank. Isolates with the code $P110^{-5}$b closely related to Streptococcus mitis, $P310^{-5}$ and $P410^{-7}$ closely related to Bacillus cereus and $P510^{-7}$a closely related to Bacillus anthracis.

C. Observations Test 3 Antibiotic Resistance Against Bacteria

An organism is said to be highly susceptible (sensitive) of a type of antibiotic if the diameter of the inhibition zone formed more than 19 mm, medium if the diameter size of 15-18 mm and resistant if diamter less than13 mm (Devi et al., 2011).

Table 4. Results of Antibiotic Resistance Testing Sulfamethoxazole, Trimethoprim and ceftazidime.

Isolates code	Antibiotics Zone Diameter (mm)			Resistant
	Sulfamethoxaxole	Trimethoprim	Ceftazidime	
P1 10^{-3}a	4 mm	3 mm	7 mm	√
P1 10^{-3}b	6 mm	6 mm	8 m	√
P3 10^{-5}	-	-	2 mm	√
P3 10^{-7}a	-	-	-	√
P3 10^{-7}b	-	-	-	√
P4 10^{-7}	-	-	7 mm	√
P4 10^{-7}a	-	-	2 mm	√
P4 10^{-7}b	-	-	9 mm	√
P5 10^{-7}a	-	-	-	√
P5 10^{-7}b	-	-	12 mm	√

Note: √(resistant). Clear zone (<13mm) antibiotic-resistant and (>19 mm) are sensitive to antibiotics (Devi et al., 2011).

D. Results of Optochin Testing

Table 5. Results of Optochin Testing.

Isolates code	Optochin
P1 10^{-3}a	R
P1 10^{-3}b	R
P3 10^{-5}	R
P3 10^{-7}a	R
P3 10^{-7}b	R
P4 10^{-7}	R
P4 10^{-7}a	R
P4 10^{-7}b	R
P5 10^{-7}a	R
P5 10^{-7}b	R

Description: R(resistance)

To determine that the isolated bacteria are bacteria Sterptococcus including pneumonia or not, performed testing using optochin. Based on the table above, it is known that all isolates were resistant to optochin result. It can be concluded that all these isolates are not Sterptococcus pneumonia.

DISCUSSION

A. Pathogens Bacterial Identified from Sputum

Based on the results of the base sequence alignment 16S rRNA gene of bacteria with the data base at NCBI Gene Bank, it is known that the bacterial isolates $P110^{-3}$b is Streptococcus mitis with a percentage of 98%-100% similarity. Bacterial isolates $P310^{-5}$ and $P410^{-7}$ bacterium is Bacillus cereus with the percentage of similarity is 99%-100% and $P510^{-7}$a bacterium is Bacillus anthracis with percentage of similarity is 99%-100%.

The results showed that, not only the

cause of pulmonary TB can be caused by the bacterium Streptococcus pneumoniae. Especially for cases in Ambon apparently there is a combination of other bacteria of the genus Bacillus origin of soil bacteria that can infect and cause pulmonary TB.

Based on the results of molecular identification, known bacterium Streptococcus mitis has a kinship with Streptococcus pneumoniae that is able to infect humans and cause pulmonary TB. Bacillus anthracis produces a toxin anthraxas the etiologic agent of inhalation, skin and gastrointestinal anthrax (Wilson et al., 2011). Humans can become infected with anthrax by exposure to infected animals or products from these animals. Humans infected with anthrax can experience death if not treated on time. The clinical forms of anthrax is the inhalation, skin, and gastrointestinal. In some cases, Bacillus antrachis can cause pneumonia. Although relatively rare, but the mortality rate is higher (Mericet al., 2008).

Bacillus aereus is mainly associated with diseases of food origin. This case usually arises because the preparation and improper food storage. The bacterium is a pathogenic bacterium that is aportunistik who has been involved in wound infections, endocarditis, osteomyelitis, endophathalmitis and urinary tract infections in humans. Cereus strain is also associated with respiratory anthrax infection in humans. This means that Bacillus cereus bacteria capable of producing anthrax toxins and the capsules are highly virulent.

Bacillus cereus can also cause pneumonia, meningitis and acute leukemia. The bacteria areable to produce exotoxin can cause blood hemolysis, tissue necrosis and affinity fo blood vessels, followed by hemorrhage (funadaet al., 1991). Thus, bacteria that become the main agent causing pulmonary TB is Streptococcus, but Bacillus bacteria can also serve as the bacteria tha tinduce. Bacillus bacteria can act as bacteria that can petrify but can also serve as pulmonary TB bacteria. This is evidenced by the above explanation. Coupled with the ability of bacteria in the blood hemolitics so as to reduce imunity of patitent and make another bacteria can infected.

B. Antibiotic Resistance Testing

Based on test results of bacterial resistance to antibiotics, it is known that all the bacterial isolates were isolated from the sputum pulmonary TB patients have experienced resistance to the three antibiotics used in testing. The third antibiotics is Sulfamethoxaxole, Trimethropim and ceftazidime.

Although there is a clear zone formed for somebacteria when antibiotic resistance testingis done, but the size of the diameter of the clear zone formed under 13mm. This suggests that, all isolates have under gone antibiotic resistance to all three kinds of antibiotics (Devi et al., 2011).

Therefore, it is necessary to review periodically the patterns of causes and patterns of sensitivity of the bacterial isolates against antibioitk as a corner stone in therapy (Sjahrurachman in Kumala, 2010). So the treatment is targeted.

CONCLUSION

Based on identification, it is known that:

1. Bacteria isolated from sputum pulmonary TB patients is Streptococcus mitis, Bacillus cereus and Bacillus anthrax. These results indicate that the cause of pulmonary TB infection humans are not only caused by the bacterium Streptococcus penumoniae, but can also be caused by other bacteria of the Bacillus genus.
2. All bacteria isolated from sputum pulmonary TB patients have under gone antibiotic resistance to antibiotics Sulfamethoxaxole, Trimethropim and Ceftazidime are of ten used in the treatment of cases of pulmonary TB.

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