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Determination of *bla$_{VIM}$* and *bla$_{IMP}$* Resistant Genes Againts Meropenem of *Pseudomonas aeruginosa* Isolated from HCU Bronkopneumonia Inpatients at Internal Medicine RSUP Dr. M. Djamil Padang

RINGGA NOVELNI[1]*, MARLINA[2], AND RAVEINAL[2]

[1]*Biomedical Laboratory Faculty of Medicines, Universitas Andalas, Jalan Perintis Kemerdekaan, Padang 25127, Indonesia;*
[2]*Faculty of Pharmacy, Universitas Andalas, Limau Manis Campus, Padang 25163, West Sumatera, Indonesia*

The aim of this research is to detect *bla$_{VIM}$* and *bla$_{IMP}$* the resistant genes against Meropenem in *Pseudomonas aeruginosa* from sample of hospitalization patients at the Internal Medicine HCU of RSUP DR. M. Djamil Padang. Firstly, bacterial isolate of *P. aeruginosa* were isolated from the sputum samples of patients who suffered bronkopneumonia. The isolation were started with samples cultivation to the Cetrimide Agar media which was a selective media for *P. aeruginosa*. To determine the species of the bacteria, the identification using Gram staining, Triple Sugar Iron Agar (TSIA) test, citric test, urease test, Methyl Red/Voges-Proskauer (MR/VP) test, and molecular marker of 16S rRNA genes have been conducted. The isolation and identification result showed that from 20 sputum samples of the patients there were just 10 (50%) samples were positively containing *P. aeruginosa*. From the *P. aeruginosa* isolates, the resistant genes against meropenem *bla$_{VIM}$* and *bla$_{IMP}$* were amplified using PCR. The result showed that all these *P. aeruginosa* isolates have positively genes encoding for Metallo-β-Lactamase (MBLs).

Key words: *bla$_{VIM}$* gene, *bla$_{IMP}$* gene, meropenem, Metallo-β-Lactamase (MBLs) enzyme, *Pseudomonas aeruginosa*, RSUP Dr. M. Djamil Padang

Penelitian bertujuan untuk mendeteksi gen *bla$_{VIM}$* and *bla$_{IMP}$* yang resisten terhadap meropenem pada *Pseudomonas aeruginosa dari* sampel pasien rawat inap di HCU penyakit dalam RSUP DR M. Djamil Padang. Pertama, isolat bakteri *P. Aeruginosa* diisolasi dari sampel sputum pasien yang menderita bronkopneumonia. Isolasi diawali dengan penanaman sampel pada media Cetrimide Agar yang merupakan media selektif untuk bakteri *P. aeruginosa*. Untuk menentukan spesies bakteri, dilakukan identifikasi dengan pewarnaan Gram, uji TSIA, uji sitrat, uji urease, uji MR/VP, dan deteksi gen penanda 16S rRNA. Hasil isolasi dan identifikasi menunjukkan dari 20 sampel sputum pasien hanya 10 (50%) sampel positif mengandung bakteri *P. aeruginosa*. Dari isolat yang positif *P. aeruginosa* dilanjutkan dengan pendeteksian gen *bla$_{VIM}$* dan *bla$_{IMP}$* penyebab resisten terhadap meropenem dengan menggunakan PCR. Hasil penelitian menunjukkan bahwa semua isolat *P. aeruginosa* positif memiliki gen yang mengkode enzim Metallo-β-laktamase (MBLs).

Kata kunci: enzim Metallo-β-Lactamase (MBLs), gen *bla$_{VIM}$*, gen *bla$_{IMP}$*, meropenem, *Pseudomonas aeruginosa*, RSUP Dr. M. Djamil Padang

Infectious disease is one of the major problem in the world. Most countries, especially developing ones are facing this problems. Infectious disease is caused by microbial pathogens that so dinamic (Darmadi 2008). One of the infectious diseases that cause increasing of the number of morbidity, mortality, and care cost at the hospital is diseases caused by the microbial pathogens that produce Metallo-β-Lactamase (MBLs) enzyme such as *Pseudomonas aeruginosa* (Amudhan *et al*. 2011).

Strain of *P. aeruginosa* who produced the Metallo-β-Lactamase (MBLs) enzyme was firstly reported in Japan in 1991 and since then, they has been found in Asia, Eropa, Australia, South America, and North America (Kaleem *et al*. 2010).

P. aeruginosa that able to produce Metallo-β-Lactamase (MBLs) enzyme is a threat to the infection in the hospital, especially in the Intensive Care Unit (ICU). This negative gram bacteria has the high level of resistance to the β-lactam antibiotics because MBLs they produced can hydrolyze the ring of β-lactam from the antibiotics of β-lactam group including carbapenem. ICU Inpatients commonly suffer the severe disease and are in the immunocompromised condition (Adisasmito *et al*. 2006).

The reasearch conducted by Jamshidi *et al.* from 2005 to 2006 on ICU inpatients of Iran hospital, detected some bacteria like *P. aeruginosa* (43.2%), *Klebsiella* spp. (33.7%), and *Staphylococcus aureus* (39.2%). These pathogens were isolated from sputum sample, blood, urine, foley catheters, nasogastric

*Corresponding author; Phone: +62-823-8576-1188; Email: ringga.novelni@gmail.com

tubes, and endotracheal tubes (Adisasmito *et al.* 2006, Jamshidi *et al*. 2009).

In surgical wards of DR. M. Djamil Padang hospital the prevalence of *P. aeruginosa* as the cause of quiet high infectious around 7.32% in 2010 (Lestari *et al*. 2010) and 6.7% in 2011 (Raihanna *et al*. 2011). Besides, based on the resistance test result, those bacterial isolates have resistance to the various of antibiotics like ceftriaxon, ceftazidime, ciprofloxacin, and meropenem (Lestari 2010; Raihana 2011).

Imipenem and meropenem are the antibiotics that often used to medicate the infection that caused by the multiresistance bacteria in ICU and wards inpatients who need the intensive care. Furthermore, karbapenem is selective antibiotics to cure the infection that caused by *P. aeruginosa*. However, resistance prevalence of *P. aeruginosa* to carbapenems increase in all around the world (Kali *et al*. 2013). The number of meropenem and imipenem resistance prevalence to *P. aeruginosa* were 22.16% and 17.32% respectively (Gupta *et al*. 2006).

Based on mentioned back ground, therefore in this research we tried to determine the resistant genes against resistance to meropenem from *P. aeruginosa* isolated from the inpatients at Internal Medicine HCU of RSUP Dr. M. Djamil Padang with PCR method. This research is useful for the health workers in order to be able to handle and prevent the resistance in the future with using wisely antibiotics to the patient so the patients will get the best and effective medication.

MATERIALS AND METHODS

Sampling. Sampling was conducted prospectively to the in patients at Internal Medicine HCU of RSUP DR. M. Djamil Padang. *P. Aeruginosa* cultures were isolated from sputum sample of 20 bronkopneumonia inpatients. Other inpatients with incomplete medical records and those unable to take his/her sputum because of their condition were excluded.

Bacterial Isolation. The isolation was started with the sample cultivation to the Cetrimide Agar media which the selective media to *P. aeruginosa* bacteria. To determine the bacteria species, the following methods Gram colouring, Triple Sugar Iron Agar (TSIA) test, citric test, urease test, Methyl Red/Voges-Proskauer (MR/VP) test, and detection of 16S rRNA gene have been done.

DNA Extraction. DNA extraction was performed based on protocol of vivantis® kit. Several colonies of *P. aeruginosa* were obtained from cultures which were prepared in cetrimide media, then added to eppendorf tubes which contained 500 µL PBS solution. The samples were centrifuged for 2 min at 13 000 rpm. The precipitate were added with 100 µL buffer R1 and 10 µL lysozyme, then homogenized and incubated for 20 min at 37 °C. After the incubation, the samples were centrifuged again for 1 min at 13 000 rpm. Then, 180 µL buffer R2 and 20 µL proteinase K were added. The samples then homogenized and incubated at 65 °C for 20 min. After the incubation, 20 µL RNAse was added. Then, the samples were homogenized and incubated at 37 °C for 5 min. The samples then added with 400 µL buffer RG, then homogenized and incubated at 65 °C for 10 min (repeated 2 times). After the incubation, 200 µL cold ethanol 96%, then homogenized (repeated 2 times). After the homogenization, DNA binding was performed. The samples then centrifuged at 12 000 rpm for 1 min (repeated 2 times). The supernatant was removed, then DNA pellets were added with 650 µL wash buffer, then centrifuged at 12 000 rpm for 1 min. The precipitate was obtained, then centrifuged at 12 000 ×g for 1 min. The precipitate was added with 100 µL elution buffer, then leaved for 15 min. The samples were centrifuged at 13 000 rpm at 3 min. The supernatant was obtained, then stored at -20 °C.

PCR Amplification for 16S rRNA. The primers were used to amplify 16S rRNA fragment specific for *P. aeruginosa* are 16S rRNA-F (5'-GACGGGTGAG TAATGCCT-3'A) and 16S rRNA-R (5'-CACTGGTG TTCCTTCCTATA-3'), which amplifies 617 bp. The running condition per cycle are 95 °C for 2 min for hot start followed by 30 cycles (92 °C for 60 sec, 57 °C for 1 min or 30 sec depends on the primer, and 72 °C for 1 min), the last extension is 72 °C for 10 min. The PCR products were analyzed by electrophoresis on agarose gel 1.5% and red gel.

Determination of Antibiotics Resistance. As much as 2 ose of test bacteria was suspended into physiological NaCl in the sterile test tube and homogenized with vortex. Then it was compared of its muddiness from the suspension with McFarland standard. Bacterial suspension was taken with sterile cotton swab and cultivated to Mueller Hinton Agar media with spreading evenly on the media, then meropenem antibiotics disc (10 µg) was placed carefully on the media and pressed slowly with sterile tweezer so it would contact to the bacteria on the media. Space between disc and the petri dish edge was 15 mm and space among discs was 24 mm. The culture was incubated at temperatures 37 °C for 24 h. Characterization was done with measuring and

comparing the obstacle area diameter against the standard table. Sensitive (S) and Resisten (R) against the antibiotics was concluded based on the obstacle clear area diameter around the antibiotics disc.

Phenotype Detection of MBLs. The bacteria suspension was spreaded evenly on the Mueller Hinton Agar media. Then, the meropenem disc (10 µg) and meropenem disc (10 µg) + 10 µL EDTA 0,5 M placed carefully on the bacteria culture. The culture was incubated at temperature 37 °C for 24 h. There is MBLs activity if the deviation of meropenem disc obstacle diameter (10 µg) + 10 µL EDTA 0.5M ≥ 7 mm than meropenem disc obstacle diameter (10 µg).

MBLs Genotype Detection (bla_{vim}, bla_{imp}) Using Polymerase Chain Reaction (PCR) Method. In one eppendorf tube containing a mixture of mixture 2 µL template DNA, 12.5 µL go taq mastermix, 1 µL forward and reverse primers. The steril water was added to make 25 µL volume. The sequence of the primers used are bla_{VIM}-F (5'-GTCCGTGATGGTG ATGAGT-3') and bla_{VIM} -R (5'-ATTCAGCCAGATC AGCATC-3') for bla_{VIM} gene, and bla_{IMP} -F CATGGTTTGGTGGTTCTTGT and bla_{IMP} -R ATAATTTGGCGGACTTTGGC for bla_{IMP} gene, which amplifies 437 bp and 448 bp. The running condition per cycle are 94 °C for 4 min for hot start followed by 30 cycles (94 °C for 30 sec, 52 °C for 1 min or 30 sec depends on the primer, and 72 °C for 1 min), the last extension is 72 °C for 5 min. The PCR products were analyzed by electrophoresis on agarose gel 1.5% and red gel.

RESULTS

From the sputum sample isolated from 20 inpatients who suffered bronkopneumonia at internal medicin HCU of RSUP Dr. M. Djamil Padang, 10 samples (50%) were positively contained isolates *P. aeruginosa*. The existence of these bacteria are also shown by the results of 16S rRNA gene identification using the PCR method. The positive result of 16S rRNA gene were obtained, which is spesific for the identification of Gram negative bacteria, one of which is *P. Aeruginosa* (Fig 1).

Based on the resistance test result of 10 bacterial cultures against the meropenem antibiotics showed that all *P. Aeruginosa* isolates were resistant to meropenem (Table 1). The *P. aeruginosa* of isolates were able to produce Metallo-β-Lactamase (MBLs) enzyme, as shown from MBL phenotype detection result, where the deviation of meropenem disc obstacle diameter + 10 µL EDTA 0.5 M larges than 7 mm, bigger compared to meropenem disc obstacle diameter. Meropenem disc obstacle diameter was about 5-6 mm and meropenem disc obstacle diameter + 10 µL EDTA 0.5 M was about 20-26 mm (Table 2). By the detection result of isolates resistance cause gene of *P. aeruginosa* against the meropenem antibiotics with PCR, there were obtained 10 positive cultures of bla_{VIM} and bla_{IMP} gene (Fig 2).

DISCUSSION

The samples that were used in this research were

Table 1. Resistance test data of *Pseudomonas aeruginosa* against meropenem antibiotics

Samples	*P. aeruginosa* Culture Obstacle Area Diameter (mm)	Obstacle Area Diameter Standard (mm)		
		Resistance ≤	intermediate	Sensitive ≥
Sample 1	R	13	14-15	16
Sample 2	E	13	14-15	16
Sample 3	S	13	14-15	16
Sample 4	I	13	14-15	16
Sample 5	S	13	14-15	16
Sample 6	T	13	14-15	16
Sample 7	A	13	14-15	16
Sample 8	N	13	14-15	16
Sample 9	C	13	14-15	16
Sample 10	E	13	14-15	16

Table 2. Test result of *Pseudomonas aeruginosa* phenotype bacteria

Sample	Meropenem disc obstacle diameter (mm) (A)	Meropenem disc obstacle diameter + EDTA (mm) (B)	MBL positive if B ≥ 7, ≥ A
Sample 1	6	20	+
Sample 2	5	24	+
Sa mple 3	5	21	+
Sample 4	5	22	+
Sample 5	5	23	+
Sample 6	5	21	+
Sample 7	5	20	+
Sample 8	5	22	+
Sample 9	5	22	+
Sample 10	5	26	+

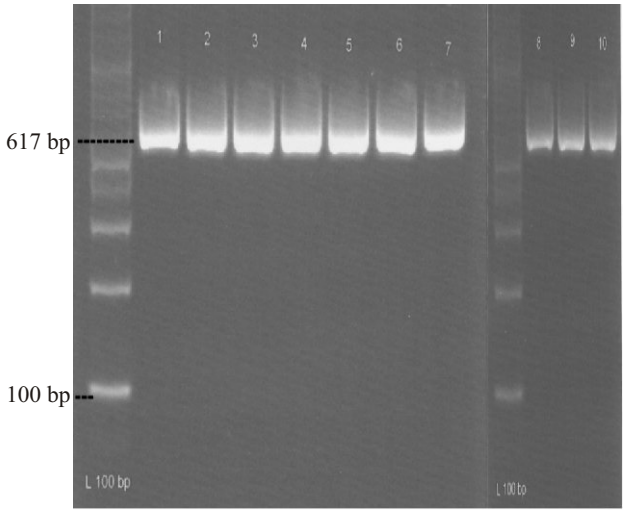

Fig 1 617 bp PCR products of 16S rRNA which was specific for *Pseudomonas Aeruginosa* were identified in all samples in 1.5% agarose gel electrophoresis.

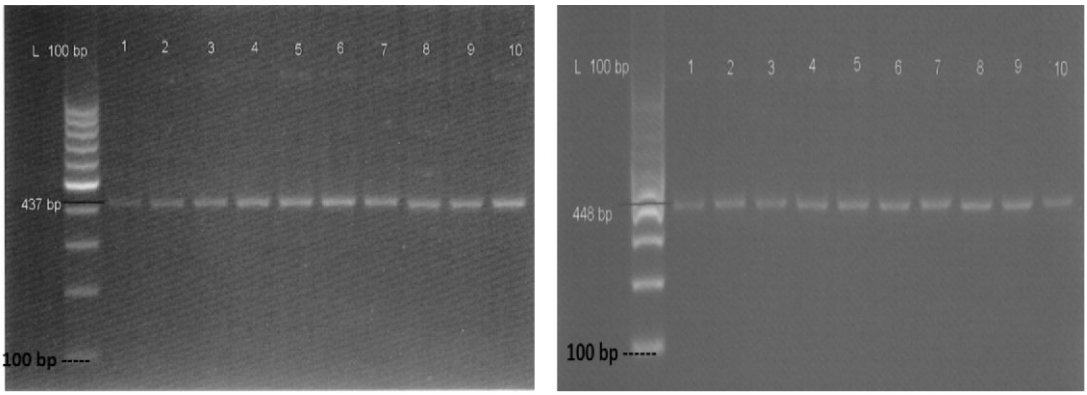

Fig 2 Photo of agarose gel that showed bla_{VIM} gene (left) and bla_{IMP} gene (right) that were MBLs marker gene of *Pseudomonas aeruginosa* bacteria which was isolated from sputum sample.

inpatient's sputum who suffered bronkopneumonia hospitalized at internal medicine HCU wards of RSUP DR. M. Djamil Padang. The isolation was started with sample cultivating to the Cetrimide Agar media as the selective media for *P. aeruginosa* bacteria. Cetrimide was the quarter ammonium salt that could hamper another bacteria growth with denaturation its bacteria cell protein (King 1954). *P. aeruginosa* produced piosianin pigment, so there would be green colour on the Cetrimide Agar media.

The isolates which were grown from the selective media, were confirmed with identification test, such as Gram colouring and biochemistry test. The gram colouring was used to determine the type of Gram positive and negative bacteria. *P. Aeruginosa* bacteria was Gram negative bacteria. Gram negative bacteria would become red because of lipid on its cell wall dissolved in time of abstersion with alcohol so the pores and cell wall permeability would be bigger and cause release of violet crystal complex which was absorbed previously, and Gram negative bacteria was red after it has been given safranin. From observation result under microscope of Gram colouring, there were negative red gram bacteria that rod shaped or bacilli, those ends were spherical or oval, pairs or sometimes chain-shaped that suspected as *P. aeruginosa*.

The biochemistry test was used to determine the species of bacteria (Lay 1994). The first test was Triple Sugar Iron Agar (TSIA) test. Reaction in the TSI Agar media was used to differentiate enteric organisms based on the ablity of fermentation of glucose, sucrose, and lactose. The media which was used contained glucose, sucrose, and lactose as sugar that would be fermented, phenol red as pH indicator, sodium thiosulfate as sulfur source, and ferrous sulfate as H_2S indicator (Duncan 2005). The bacteria which was able to ferment glucose would form yellow on the base part of media, while the bacteria which was able to ferment lactose and/or sucrose would formed yellow on the slant part (Brown 2001). *P. aeruginosa* was only able to ferment glucose to acid in the medium so there was a colour changing of phenol red indicator to be yellow on the base part and red on the slant part because of alkaline reaction.

Citric test was used to see the ability of microorganisms used citric as its one and only carbon source. Citric utilization based on the activity of citrase enzyme that was produced by bacteria. The positive result was shown with the existence of bacteria growth and colour changing of media from green to blue. *P. aeruginosa* bacteria provided the positive result to citric test, because of this bacteria was able to use citric as its only one carbon source.

On the urease test of *P. aeruginosa* bacteria showed the positive result and that meant that this bacteria could produce the urease enzyme which could decompose the urea micromolecule ($(NH_2)_2CO$) to be carbondioxyde (CO_2) and ammonium (NH_3). Ammonium was the nitrogen source that used to biosynthesize amino acids and another molecule that contained N atom. Ammonium that was produced would increase media pH to over 6.8 so there would be media changing from pink to yellow.

Methyl Red (MR) test was used to determine the existence of mix acid product from glucose fermentation through the mix acid fermentation track that were generally lactic acid, acetic acid, formic acid, and succinic acid. On the other hand, Voges-Proskauer (VP) test was to determine the existence of acetoin that was neutral metabolites from glucose fermentation through the butanediol track (Brown 2001). In methyl red (MR) test, the positive result was shown with red ring formation to the media by adding the red menthyl indicator as the effect of media pH decreasing because the acid products were produced in a big number from glucose fermentation. The negative result would be obtained if there was no red ring formation and also it indicated that there was a little or none of organic acid remained in the media. This thing was happened because those acids has been changed to be the neutral products that could be detected with Voges-Proskauer (VP) test (Brown 2001).

In the glucose fermentation through the butanediol track, there was resulted that next was changed to be acetolactate (acetylmethylcarbinol) and carbondioxide. Acetolactate was enzymatically changed to be acetoin or first oxidized to be diacetyl, then reduced to be acetoin. Neutral acetoin was reduced to be butanediol next (Brown 2001). In Voges-Proskauer (VP) test, there was added the reagents of Barritt's A (α-naphtol) and Barritt's B (potassium hidroxide). The positive result was shown by the red complex which formed from the reaction between alkaline diacetyl and creatinine (Brown 2001). Acetoin would react with α-naphtol reagent and potassium hydroxide to form the red ring in the media. *P. aeruginosa* provided the negative result in Voges-Proskauer (VP) test and positive result in methyl red test.

After the Gram colouring and biochemistry test, then inspected the genetic characterization with PCR method. This aims to make sure that growth isolates in Cemitride Agar media was *P. aeruginosa* bacteria. This

PCR method was used to detect 16S rRNA gene sensitively and specifically. This gene was one of the genetic regulatory factors and specific gene of negative Gram bacteria (Hasan 2012).

From the detection of gram colouring result and biochemistry test, it can be concluded that 10 of the growth samples in Cetrimide Agar media were *P. aeruginosa.*

P. aeruginosa was opportunistic bacteria that utilizing the damage of host defense mechanism to begin the infection, so this was a threat to the immunocompromised patients, especially in intensive care unit (HCU) that commonly with the severe disease and immunocompromised. Infection by *P. aeruginosa* associated significantly with bad prognosis from patient who was in treatment in the hospital, where this could increase the probability of another infection and aggravating the exist infection (Adisasmito *et al*. 2006).

Patients, especially in intensive care unit (HCU) that commonly with the severe disease and immunocompromised. Infection by *P. aeruginosa* associated significantly with bad prognosis from patient who was in treatment in the hospital, where this could increase the probability of another infection and aggravating the exist infection (Adisasmito *et al*. 2006).

HCU inpatients generally get the atibiotics therapy in a long time. The longer patients get the antibiotics therapy, there will be easier to onset the colonization with microbes that have antibiotics resistance. Besides, usually patients get the antibiotics therapy including ceftriaxone, ciprofloxacin, azithromycin, and meropenem.

Antibiotics exposure especially the third generation of cephalosporins like ceftriaxone, would spur the production of β-lactamase enzyme (Waterer *et al*. 2001). One of the β-lactamase enzyme was Metallo-β-Lactamase (MBLs) enzyme that was produced by *P. aeruginosa*. Under the selective antibiotics pressure, *P. aeruginosa* was proven to get the resistance properties that carried by both plasmid and chromosomal. R factor in plasmid which bring the resistance cause genes against one or more antibiotics. Gene in the plasmid which cause the resistance against antibiotics were often to produce enzymes that destructive power of drug action. One of those enzymes was β-lactamase enzymes. These resistance genes could move from the resistance bacteria to the sensitive bacteria through the conjugation, so the sensitive bacteria would become resistant against the same antibiotics (Brooks *et al*. 2010; Adisasmito *et al*. 2006). This was one of the high prevalence cause of bacteria which multi Drug Resistance (MDR) in the hospital, especially in the Intensive Care Unit like HCU.

One of the third generation of antibiotics from the carbapenem class that potential empirically and definitively fight against serious infection that caused by Multi Drug Resistance (MDR) was meropenem. Moreover, this antibiotics was the selective antibiotics to medicate the infection by *P. aeruginosa*. However, the bacteria resistance prevalence of *P. aeruginosa* against the carbapenem has increased in the whole world (Kali *et al*. 2013). Metallo-β-Lactamase (MBLs) enzyme that was produced by *P. aeruginosa* was one of the main cause of resistance to the meropenem antibiotics. This enzyme was able to hydrolize β-lactam ring from the antibiotics class of β-lactam including meropenem, so this cause disfunction of antibiotics (Amudhan *et al.* 2011).

To know whether *P. aeruginosa* produce the MBLs enzyme or not the phenotype detection has been done. This process was started with making bacteria suspension then it was spreaded evenly on the Mueller Hinton Agar media. Thereafter, they were placed carefully on the bacteria culture, meropenem disc and meropenem disc + 10 µL EDTA 0.5M. Then it was incubated at temperature 37 °C for 24 h. If the deviation of meropenem disc obstacle diameter + 10 µL EDTA 0.5M ≥ 7 mm than meropenem disc obstacle diameter (Erfani 2013), then there is MBLs activity based on the MBLs phenotype detection that has been done, there was result that all cultures of *P. aeruginosa* bacteria that has been isolated from positive sputum samples produced MBLs enzyme. This event was shown with the deviation of meropenem disc obstacle diameter + 10 µL EDTA 0.5M ≥ 7 mm than meropenem disk obstacle diameter. MBLs enzyme was one of the cause *P. aeruginosa* resistance against meropenem antibiotics. Encoding gene of MBLs enzyme that cause resistance against meropenem mostly detected from *P. Aeruginosa* were bla_{VIM} dan bla_{IMP} gene (Doosti *et al.* 2013). For the correct and effective medication to the infectious diseases patients who caused by *P. Aeruginosa* which could result the MBLs enzyme, we have to know the resistance gene type from that *P. aeruginosa* isolates.

Resistance gene type from *P. aeruginosa* isolates was detected with PCR method. This ampilification process was done at the temperature and reduplication cycle that has been determined and could be set on the PCR machine. The PCR result showed that 10 pure

cultures of *P. aeruginosa* bacteria contained positively bla_{VIM} and bla_{IMP} genes which encoding gene of MBLs enzyme.

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