IDENTIFICATION OF THE OXA-48 GENE CODING FOR CARBAPENEM RESISTANCE IN KLEBSIELLA PNEUMONIAE AND ESCHERICHIA COLI AT DR. MUHAMMAD HOESIN CENTRAL GENERAL HOSPITAL, PALEMBANG

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Introduction

Infection is disease which causes by infection agent namely bacteria, virus, fungi and protozoa. One of the causes of infection disease is bacteria. *Enterobacteriacea* is Gram-negative bacteria which the rod is located in the human intestine; one of the examples is *Escherichia* [1]. *Escherichia coli* are the pathogen bacteria as the cause of morbidity and mortality [2].

The interesting pathogen group with several anti-microbe agents which shows the improvement of resistance level is *E coli*. Resistance of *ciprofloxacin* only 4,1% in the first year and continue to increase become 31,8% after 10 years. Amino-glycosides is also shows the slower increase of resistance, but it still consistence, from 1999 (*tobramycin*, resistance on 1,5%) to 2007 (10,3%). The resistance of *cefepime* to *E. coli* strain, first time shows in 2004 (0,4) and faster increase become 5,1% only in 5 years (ESBL). Between *Klebsiella* spp. Isolate, carbapenem resistance is rarely observed before the year of 2003, but after the its existence, the resistance level is quickly increased to almost 8% in 2007 before falls to 4.3% in 2008 related with the intervention of local infection control. The increase of resistance on *fluoroquinolonei* and Amino-glycosides resistance is recorded in 2005 [3].

The usage of anti-bacteria which is not widely indicated, repeated and in the long current time can causes the existence of resistance on anti-bacteria, namely resistance on resistance to carbapenem class antibiotics. Resistance on class antibiotic occurs of the improvement and become a phenomenon to watch out for at this time. This phenomenon has proven by researcher of a hospital or by the analysis with using National database. According to the data from *European Centre for Disease Prevention and Control* (ECDC) in 2009 to 2012 occurs the increase of infection cases to the blood which causes by *Klebsiella pneumonia* that resistance of Carbapenem occurs the improvement to 5% in 5 different countries [3].

According to [4], OXA-48 is β-D-Glucopyranose which is not inhibited by clavulanic acid, tazobactam and sulbactam, because the activity is possibly inhibited in-vitro by NaCl. Some b-lactamase enzyme is hydrolyzing carbapenem and it defined as *carbapenem-hydrolysing*, as D class of β-lactamase (CHDLs). Isolate OXA-48 produced isolate which have the ability of resistance on many medicines and shows the higher resistance for the entire β-lactam, including broad spectrum *cephalosporins*, *cephamycins*, *monobacton* and *carbapenems*, gene OXA-48 found in plasmid.

Gene bla OXA-48 have the relationship on sequence insertion IS1999 to *K.pneumoniae*, then expressed β-lactamase [5]. Has proven that blaOXA-48 is the part of composite transposon called Tn1999 and made of two copies IS1999 which grouped by this gene.

Antibiotic resistance is phenomena which following by several where is the discovery of antibiotics found the ability of very good adaptation for bacteria which is in therapy with several of antibiotic. So, the bacteria have the ability to mutation as mechanism of life survival [6].

This research is prevalence study to identify the existence of gen OXA as Carbapenem Resistance Encoders to *Escherichia coli* and *Klebsiellapneumoniae* with the method of *Polymerase Chain Reaction* (PCR) genotypically confirm the resistance cases in the hospital, especially in Dr. Mohammad Hoesin Palembang. The data is very important as the first information for the next research related with distribution of Gene blaOXA Resistance Encoders to *Escherichia coli* and *Klebsiella pneumoniae*.

Materials and methods

Methodology

Type of research

This research used the descriptive observational research with laboratory research design to identify gene OXA-48 with using PCR to *Escherichia coli* and *Klebsiella Pneumoniae*, Carbapenem resistance which isolate from the infection patient in RSUP Dr. Mohammad Hoesin Palembang.

Time and place of research

This research is implemented in the Molecular Biology Laboratorium of Medicine Faculty, Sriwijaya University Palembang and Microbiology Laboratorium of RSUP Dr. Mohammad Hoesin Palembang.

Population and Sample of Research

The population is the entire bacteria isolate *Escherichia coli* and *Klebsiella pneumonia* from the entire specimen types of carbapenem resistant infection patients which found in Microbiology Laboratorium of RSUP Dr. Mohammad Hoesin Palembang period of September-November 2017.

The data collection technique used *purposive sampling* Technique referred from the previous research implemented by Radhiyatul Husna, Deasy Nataliani and Rahmat M Usman of 2017. The isolated bacteria from infection patient which collected in the Department of Microbiology RSUP Dr. Mohammad Hosein Palembang and have identification as *Escherichia coli* and *Klebsiella*

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pneumonia of Carbapenem resistance with using Vitek 2 Compact which taken during period of 2017.

Inclusion Criteria

1. Isolate Bacteria of *Escherichia coli* and *Klebsiella pneumonia* which comes from the infection patient.
2. Isolate Bacteria proven as *Escherichia coli* and *Klebsiella pneumonia* through the examination of culture and gram stain, and identified from Vitek 2 Compact.

Procedure

Identification of Carbapenem Resistant Escherichia Coli and Klebsiella Pneuomonia

Identification of resistance Carabapenem *Escherichia coli* dan *Klebsiella pneuomonia* implements using Vitek 2 Compact (bioMerieux, USA). This steps implemented by the previous research of 2017, there are the procedures as it explained below:

1. Bacteria isolate is previously implemented the Gram Stain to determine the card will used. The test is implements to bacteria isolate which proven negative Gram.
2. Added 3 mL of 0.45-0.5% sterile saline, pH 4.5-7.0 into a tube measuring 12 x 75 mm.
3. Bacteria isolate added into a tube which contains saline solution with swab for making bacteria suspense. Suspension then homogenized with feculent 0,50-0,63 Mc Farland (for negative Gram Bacteria) with using VITEK 2 DensiCHEK.
4. Added to tube of bacteria suspense and VITEK 2 GN special crust card (*cassette*).
5. Those rack then added into vacuum chamber station manually. Bacteria suspension removed into well by the tool and transfer tube cut automatically.
6. The card is removed into the incubator room after 15 minutes, then will analyze automatically.

Sources: [7]

DNA Isolate

Bacteria isolate have confirmed CRE then isolate by DNA. This step have implemented by the previous research of 2017. The way this is done is as follows:

A. Tools and Materials

1. Eppendorf tube 1,5 mL
2. Pipettor with various size (10-100µL, and 100-1000 µL)
3. Pipet tip volume 100 µL
4. Vortex
5. ddH_2O
6. Phosphate Buffered Saline (PBS)
7. Chelex 20% in ddH_2O
8. Micro centrifugal
9. Water bath
10. Freezer -20ºC
11. Saponins in 0,5% PBS
12. Bacteria colony of *Escherichia coli* and *Klebsiella pneumoniae* are carbapenem resistant

B. Procedures

1. Provide sample to be tested, because sample is stored at cold temperature (-20ºC), The sample must be left at room temperature before opening
2. Gives the label eppendorf tube 1,5 mL, then added to the bacterial colony whose DNA will be isolated.
3. Bacteria washed with 1mL PBS pH 7,4 then incubated during 10 minutes.
4. Bacteria centrifugations with the speed of 5000 rpm during 5 minutes, remove the supernatant with a pipettor, this steps is repeated 3 times.
5. Added 500 µL of 0,5% saponins in PBS, added in vortex in order to blended well.
6. The mixture was incubated overnight at temperature -20ºC.
7. In the next morning, the mixture was centrifuged at speed 12.000 rpm during 10 minutes, the supernatant is removed.
8. Supernatant removed then added with 50 µLChelex 20% in ddH_2O pH 10,5 and added 100 µL ddH_2O.
9. Added 50 µL Chelex 20% into ddH_2O pH 10,5 and added 100 µLddH_2O.
10. The mixture was incubated in the boiled water during 10 minutes.
11. The centrifuged mixture with speed 12.000 rpm during 10 minutes.
12. Supernatant DNA moved into sterile eppendorf tube 1,5 mL and stored in the temperature -20ºC.

Primary design

The primary design used to detect the OXA-48 gene coding for Carbapenem resistance on *Klebsiella pneuomoniae* dan *Escherichia coli* which found in the study of [8]. It found two primary designs that used in the multiplex PCR research is forward sequent which used are 5′-GCGTGGTTAAGGATGAACAC-3′*reverse* sequent which used 5′-CATCAAGTTCAACCCAACCG-3′.

PCR gene OXA-48

The DNA isolation results are stored later in PCR to identify the presence or absence of the desired gene. In this research implemented PCR with using every primary gene OXA. Both result then compared to find whether or not there are bacteria that have these two genes together.

A. Tools and Materials

a. Eppendorf tube 0,2 and 1,5 Ml
b. Pipettor with various size (10-100 µL, and 100-1000 µL)
c. Pipet tip volume 100 µL and 1000 µL
d. Taq polymerase
e. ddH_2O
f. transcription DNA from the DNA isolate result
g. primer *forward* and *reverse*

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Table 2 PCR Mixture Composition

Materials	Volume (µL)
ddH_2O	12
Taq polymerase	8
Primer *forward*	1
Primer *reverse*	1
DNA	3
Total	25

Tabel 3 Base Sequences, Primary Lengths, and PCR and Primary Products

Primary	Base Sequence	Primary Length (nt)	PCR Product (bp)
Forward	5′-GCGTGGTTAAGGATGAACAC-3′	20	477
Reverse	5′-CATCAAGTTCAACCCAACCG-3′	20	

Sources: (8)

B. Procedures

1. Go *Taq polymerase*, ddH_2O, Transcription DNA and Primary is poured with each volume multiplied by the number of samples poured in the eppendorf tube 1,5 mL used pipettor sized 100-1000 µL and pipet tip 1000 µL. It implemented the single PCR for gene OXA, it added so poured one by one primer for the gene to be tested.
2. Moved the mixture into eppendorf tube 0,2 mL as much as the total sample with volume 25 µL every tube used pipettor sized 100-1000 µL and pipet tip 1000 µL.
3. Added all into eppendorf tube 0,2 mL into PCR machine.
4. Implements the thermal temperature adjustment during PCR, with the temperature as follows:

Table 4 Thermal Temperature Adjustment For Amplifications Process Gene OXA-48

Gene	Process	Temperature	Length	Total cycles
OXA-48	Initiation of cell lysis	94°C	10 minutes	
	Denaturation	94°C	30 second	36x
	Annealing	71°C	40 second	
	Extension	72°C	50 second	
	Final Extension	72°C	5 minutes	

Sources: (8)

Electrophoresis Gel Agarose

The PCR results then visualized by Agarose Gel Electroforesis. The procedure is implements as follows:

A. Tools and Materials

a. One set of electrophoresis tools
b. Micropipette and tip
c. Gel Doc
d. Power Supply
e. DNA Marker
f. DNA Product
g. Ethidium Bromide (EtBr)
h. ddH_2O
i. Agarose Gel
j. Loading Buffer (Loading Dye)
k. Buffer Tri-acetate EDTA (TAE)

B. Procedures

Gel Agarose Making:

1. Dissolve 0,4 gram of agarose powder into 50 ml buffer TAE by warming it up, after dissolve then let it sit in the temperature ±50°C.
2. Gel Comb installed into electrophoresis tanks.
3. The solution agarose poured into electroforensis tanks and cooled until firm.
4. After the gel is firm, gel comb can be picked and ready to use.

Electrophoresis

1. Agrose gel soaked with TAE buffer.
2. 1 µL *loading dye* mixed with 3 µL DNA product on parafilm that has been available with the help of a micropipette.
3. The mixture is poured into well on the agarose gel.

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4. While for 3µL DNA marker 1kb poured into well which it at the top and bottom edge.
5. Electrophoresis tanks then closed and connected into power supply. *Power supply* (400mA, 90V) turned on for 30 minutes.

Data Processing and Analysis Plan

The data that obtained next will organized to simplify analysis. The data will be processed with using *Microsoft Excel 2010* programs. The next analysis implemented with the simply statistic calculation in the form of percentage. The result from the data analysis will described in a narrative and presented in tabular form.

Results and Discussion

Result

The research related identification of the OXA-48 gene encoding carbapenem resistance into *Klebsiella pneumoniae* and *Escherichia coli* will implements in the Laboratory of Biotechnology, Faculty of Medicine, Sriwijaya University and Microbiology Laboratory of Dr. Moh. Hoesin Palembang in 2017, total is 23 samples.

Detection of the phenotype of *Klebsiella pneumoniae* and *Escherichia coli* bacteria in the Microbiology Laboratory of Dr. Mohammad Hoesin Palembang using the Vitex 2 Compact bioMerieux, USA with assess the resistance antimicrobial MIC carbapenem on the *Enterobacteriaceae* Bacteria. After it implements the test to know the presence or absence of the OXA-48 gene in bacteria *Klebsiella pneumoniae* and *Escherichia coli* with PCR method in Biotechnology Laboratorium, Faculty of Medicine, Sriwijaya University.

Data Sample of Klebsiella Pneumoniae and Escherichia Coli

The sample which identification as *Klebsiella pneumoniae* and *Escherichia coli* with using Vitek 2 Compact tools, it implemented the distribution which based on bacteria species. The result, shows that *Klebsiella pneumonia* bacteria is more was found, namely 15 (65.2%) while for *Escherichia coli* only 8 (34.8%) were found.

In the table 5 explains several specimens were taken for the purpose of supporting data in this research and calculate the specimens contained in this study obtained sample number 3 from positive urine specimens.

Table 5 Distribution of OXA-48 on the Carbapenem Based On the Specimen Types

No	Specimen	N	Gen bla$_{OXA-48}$			
			Positive n	(%)	Negative n	(%)
1	Pus	4	0	0	4	100
2	Sputum	9	0	0	8	100
3	Urine	7	1	14,3	6	85,7
4	Tissue	1	0	0	1	100
5	Pleura fluid	1	0	0	1	100
6	Swap the wound	1	0	0	1	100
7	Feces	1	0	0	1	100
	Total	24				

In the table 6, identification the Positive OXA-48 Isolate from 24 tested isolate previously, after implementing electrophoresis and seen with UV light, identification based on *base pair* 477 bp obtained 1 isolate that was positive *Carbapenem* sensitive.

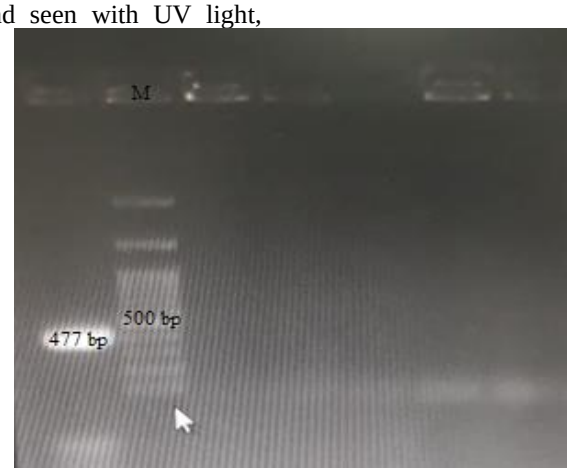

Figure 1 Visualizes OXA-48 gene

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Table 6 Distribution of OXA-48 Gen in the samples

Variable	OXA-48 (+)	OXA-48 (-)	Total
K. pneumoniae	1	14	15
E. coli	0	8	8
Total	1	22	23

The sample sensitivity pattern on Antimicrobial based on Genotype bla$_{OXA-48}$

The result of sample sensitivity based on Genotype bla$_{OXA-48}$ can be seen from the table 10 found the significant difference in positive *Klebsiella pneumoniae* OXA-48 bacteria that 13 antibiotics *Carbapenem* only obtain *gentamicin* (Intermediate). While to *Escherichia coli* bacteria only obtain negative OXA-48.

This is shows that *Gentamicin* is still can be said to be successful in treatment even though its effectiveness is only moderate.

Table 7 Sensitivity Pattern *Klebsiella Pneumoniae* on the Carbapenem of Various Antibiotics (N=15)

Antibiotics	Positive Gene OXA-48 n (%)						Negative Gene OXA-48 n (%)					
	R	(%)	I	(%)	S	(%)	R	(%)	I	(%)	S	(%)
Ampicillin	1	100	0	0	0	0	14	100	0	0	0	0
Aztreonam	1	100	0	0	0	0	12	85,7	0	0	2	14,3
Cefixime	1	100	0	0	0	0	12	85,7	0	0	2	14,3
Amikacin	1	100	0	0	0	0	7	50	1	7,1	6	43,9
Ceftriaxone	1	100	0	0	0	0	12	85,7	0	0	2	14,3
Cefazolin	1	100	0	0	0	0	12	85,7	0	0	2	14,3
Ciprofloxacin	1	100	0	0	0	0	8	57,2	3	21,4	3	21,4
Ceftazidime	1	100	0	0	0	0	12	85,7	0	0	2	14,3
Gentamicin	0	0	1	100	0	0	10	71,4	0	0	4	28,6
Nitrofurantoin	1	100	0	0	0	0	9	64,3	4	28,6	1	7,1
Ertapenem	1	100	0	0	0	0	14	100	0	0	0	0
Meropenem	1	100	0	0	0	0	14	100	0	0	0	0
Ampicillin-Sulbactam	1	100	0	0	0	0	13	92,9	0	0	1	7,1

Table 8 Sensitivity Pattern *Escherichia Coli Carbapenem* Resistant On the Several Antibiotic (N=8)

Antibiotics	Negative Gen OXA-48 n (%)					
	R	%	I	%	S	%
Ampicillin	8	100	0	0	0	0
Aztreonam	4	50	0	0	4	50
Cepefim	1	12,5	0	0	7	87,5
Amikacin	1	87,5	0	0	7	87,5
Ceftriaxone	3	37,5	0	0	5	62,5
Cefazolin	3	37,5	0	0	5	62,5
Ciprofloxacin	4	50	0	0	4	50
Ceftazidime	3	37,5	0	0	5	62,5
Gentamicin	3	37,5	0	0	5	62,5
Nitrofurantoin	0	0	3	37,5	5	62,5
Ertapenem	8	100	0	0	0	0
Meropenem	8	100	0	0	0	0
Ampicillin-Sulbactam	8	100	0	0	0	0

Information: R : Resistant; I : Intermediate; S: Sensitive

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Discussion

According to (4), since early 2000, *carbapenemase* Amble namely type KPC A or B class (enzyme IMP, VIM, and NDM) regarded as important *carbapenemase* in *Enterobacteriaceae*, because:

1. Hydrolytic activity not only including *carbapenem*, but also broad spectrum *cephalosporins*;
2. *Carbapenemase* activity is significant;
3. The gene already in accordance identification around the world.

Otherwise, OXA-48 not regarded as problem realizing:

1. Hydrolytic spectrum is not including abroad spectrum *cephalosporins*
2. *Carbapenamase* activity little low rather than with enzyme class A and B
3. The spreading blaOXA-48 should have been confined to Turkey for years

Despite the fact that blaOXA-48 is the part of functional transition, the spreading in accordance with plasmid supporting spreading and not from *transposons*. Another interesting observation in this connection with genetic context gene is similar with blaOXA-48 in accordance with the fact that all have identification in the relationship with insertion sequence, while most of class D gene *b-lactamase* is often identified. Although it closely related in the nucleotide sequence things, gene blaOXA-48 always identified in the different context genetic (a *transposon* which made IS1999 or ISEcpl), shows that both gene is evolved each other with the mobilization of the different gene from two strains Shewanella donor [9].

From the research result above it known that found 1 positive sample (4,2%) blaOXA-48 gene genotype, which the sample also have OXA-48 gene. Meanwhile in U.S. is very rare to find as causes the resistance *carbapenem* case to the *Enterobacteriaceae* (10), OXA-48 first to be found in Turkey at 2001 [5]. It known in the research by [11] that cases OXA-48 located in *Klebsiella pneumonia* because of *nosocomial* infections in Turkey have the highest epidemiology level is Level 5 with the status of an endemic situations. Meanwhile, in Spanish, France, Belgium and Romania the epidemiology level only reaches limited to level 4 with the spreading status is inter regional.

Clinical consequences related with OXA-48 type of producer OXA-48 is quite important, because many of these producers classified as susceptible on *Carbapenem* in accordance with EUCAST guideline or CLSI [12]. Nowadays, the recommendation suggested by the guidelines are reported vulnerabilities on Carbapenem as found, regardless of whether the isolate produces carbapenemase or not. Recently, reported that imipenem can success used to treat bacteremias because K.pneumoniae is susceptible on the imipenem but produced OXA-48 [13].

The burden of antimicrobial resistance among Gram-negative pathogens, in particular resistant Enterobacteriaceae on carbapenem, it is increasing rapidly around the world. The treatment option for CRE infection then nowadays is still currently very limited. Dosage optimally and combination therapy are might be the most appropriate treatment strategy for now. However, the next researcher is suggested, particularly randomized controlled trials, to determine the right treatment for serious CRE infection [14].

Conclusion

It has identified 1 sample is positive genotype blaOXA-48 from 24 samples comes from the patient infected patient in Muhammad Hoesin Hospital Palembang on September-November 2017 using PCR method.

Suggestion

1. The next research is needed for the clinical importance because still lacking the research related OXA-48 gene in Asian Part especially Indonesia on another *Enterobacteriaceae.*
2. The next research is needed related with the connection between the sensitivity level and the associated gene.
3. It needed deeper survey, broad and accurate from time related to antibiotics sensitivity pattern become as guidelines for determining the antibiotic of decision for doctor in the hospital.

Identification of the Oxa-48 Gene Coding For Carbapenem Resistance in Klebsiella pneumoniae and Escherichia Coli at Dr. Muhammad Hoesin Central General Hospital, Palembang

Muhammad Fitrizal

OXA-48 is a class D *β-lactamase* which is not inhibited by *clavulanic acid*, *tazobactam* and *sulbactam*, because their activity may be inhibited in vitro by NaCl. Some *b-lactamase* enzymes hydrolyze carbapenem and are therefore defined as hydrolysis of class D carbapenem *b-lactamase*. Carbapenem-resistant *Klebsiella pneumoniae* isolates were found in Istanbul, Turkey, in 2001. OXA-48 produces isolates that have the ability to fight drugs and cause very beneficial resistance to *β-lactams*, including widely available *cephalosporins, cephamycins*, and *monobactone* and carbapenem. The OXA-48 gene is found on plasmids. Antibiotic resistance is a phenomenon taken from the discovery of antibiotics where there is a very good adaptability for bacteria which are treated with various antibiotics. It has bacteria that have the ability to mutate as a protection for survival. This study is a case study involving the OXA gene as an encoder for carbapenem in *Escherichia coli* and *Klebsiella pneumoniae* using the Polymerase Chain Reaction (PCR) method to use genotypes used in hospitals that can be used in Dr. RSUP. Mohammad Hoesin Palembang. Bacterial isolates derived from patients with *Klebsiella pneumoniae* and *Escherichia coli* infections in the period September-November 2017 were identified using Vitek 2 Compact. bla_{OXA-48} gene was detected by PCR, followed

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by visualization through electrophoresis. The detection results were then analyzed by comparing the pattern of antibiotic resistance. From 24 samples tested only 1 (4.7%) positive samples were obtained from the *bacterium Klebsiellapneumoniae* and no positive gene was found in *Escherichia coli.* This study identified 1 (4.2%) samples that had a positive OXA-48 gene while 23 (95.8%) samples had a negative OXA-48 gene.
Keywords: *Klebsiella pneumoniae*, *Escherichia coli*, OXA-48, Carbapenem

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