Identification of carbapenemases enterobacteriaceae producing gene blaVIM in clinical isolates

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Abstract. Carbapenemase enzymes play the most important role in the existence of CRE (Carbapenem-resistant Enterobacteriaceae). VIM is one of the carbapenemase enzymes encoded by the blaVIM gene. The genes which are partially located in genetic mobile elements may facilitate the spread of the resistance to other bacteria. The aim of this study was to identify the blaVIM gene in CRE isolated from infected patients in dr. Mohammad Hoesin Palembang. This descriptive observational laboratory study using 709 samples of Enterobacteriaceae isolated from various specimens of infected patients at dr. Mohammad Hoesin Palembang during the September-November period which would be identified as CRE using Vitek 2 Compact. 21 isolates which have been identified by CRE was taken by purposive sampling method to detect blaVIM gene with conventional polymerase chain reaction (PCR). The PCR product was electrophoresed and visualized to see a single 390bp DNA band. Out of 709 isolates of Enterobacteriaceae, 88 (12.4%) isolates were identified as CRE. Only 21 isolates of CRE performed PCR. Three (14.3%) isolates carrying the blaVIM gene which found in Enterobacter sp. (N=2; 66.7%) and Klebsiella pneumoniae (n=1; 33.3%). In this study 14.3%, Enterobacteriaceae with CRE phenotype has blaVIM gene genotype.

1. Introduction

Enterobacteriaceae is family gram-negative bacteria which has a rod-shaped and anaerobic facultative bacteria. This group of bacteria consists of Salmonella sp., Shigella sp., Klebsiella pneumoniae, Proteus sp., Salmonella sp., Enterobacter sp., and Escherichia coli. Some of these bacteria are normal flora of human and animal intestines and are responsible for some diseases such as neonatal meningitis, sepsisemia, pneumonia, urinary tract infections and various infections of the gastrointestinal tract. In addition, some species such as Escherichia coli, Klebsiella sp., and Enterobacter sp., is also an opportunistic pathogen that plays a role in the pathogenesis of nosocomial infection [1,2].

Beta-lactam group antibiotics such as the penicillin group, and cephalosporins often used for infection caused by Enterobacteriaceae [3]. However, since ESBL (extended-spectrum β-lactamase),
an enzymes that capable for hydrolyzing beta-lactam ring in the penicillin, cephalosporin and aztreonam classes (except cephemine or carbapenem) [4] was found, the management of infectious diseases caused by Enterobacteriacea be more complicated. The incidence of ESBL has also been reported in many regions of the world, including in North America, South America, Europe, Africa, and even Asia [5]. A large number of ESBL incident reports raised the numbers of morbidity and mortality [6].

The wide distribution of ESBL-producing Enterobacteriacea encourages some health centers to make carbapenem antibiotics as the last choice in the treatment of MDR (multi-drug-resistant) Enterobacteriacea [7]. However, the irrational use of antibiotic was also triggered by the emergence of Carbapenem-Resistant-Enterobacteriacea (CRE) [8].

CRE is a term used to represent Enterobacteriacea that is resistant to carbapenem antibiotics. CRE was first reported in 1993 with the discovery of the NMC-A (Not Metalloenzyme Carbapenemase-A) enzyme, the first carbapenemase enzyme identified in Enterobacteriacea [9]. The incidence of CRE has been reported in many countries. In 2008 at 15 health centers in the United States, the incidence of Klebsiella pneumonia resistance to meropenem was reported at 5.6% [10]. In Asia, based on research in 2009-2012, the incidence of Enterobacteriacea resistance to imipenem was reported at 1.2%, where the top three countries with the highest rates of resistance in Asia were Indonesia (5.8%), Vietnam (3.0%) and Philippines (3.7%) [7]. The incidence rate of Klebsiella pneumoniae resistance to meropenem in dr. Mohammad Hoesin Palembang taken in January-June 2015 was reported at 22% [11].

The emergence of Enterobacteriacea resistance to carbapenem antibiotics generally occurs through several mechanisms, (1) antibiotic inactivation by ESBL enzyme, Ampe β-lactamase and carbapenemase; (2) drug penetration disorder to target penicillin-binding protein (PBPs); (3) excessive activity of the efflux pump, and (4) the absence or change of porin form [3,12,13].

Among all of the resistance mechanisms, carbapenemase plays the most important role in the mechanism of resistance to carbapenem [14]. This is related to the hydrolysis activity of carbapenemase in most beta-lactam antibiotics, including carbapenem (imipenem, ertapenem, meropenem, and doripenem) [15].

Carbapenemase is an enzyme belonging to the β-lactamase group distinguished by the classification of Ambler to A, B and D. The samples of each carbapenemase in classes A, B and D are KPC (Klebsiella pneumoniae carbapenemase), VIM (Verona Integron-encoded Metallo-β-lactamase) and OXA-48 (oxacillinase-48) [16]. Each of these enzymes classes encoded by different gen, such as VIM that encoded by blaVIM (β-lactamase VIM) [17].

The blaVIM gene is mostly located on genetic mobile elements such as plasmids. The gene’s location in genetic mobile elements such as plasmid may facilitate the spread of the resistance to other bacteria [18,19]. Other than that, VIM which is one of the most widely reported class B carbapenemases is also known to be responsible for treatment failure, length of therapy, higher relapse rate and worse outcome in infectious patients due to Enterobacteriacea [20].

This research aim to an identification of the blaVIM genes on CRE at dr. Mohammad Hoesin Hospital Palembang. This study also expected to assist in the CRE infection control program and the prevention of the spread of carbapenem resistance through the use of antibiotics.

2. Methods

This study is a laboratory observational descriptive research to identify the blaVIM genes in CRE that isolated from infected patients in dr. Mohammad Hoesin Palembang by using the PCR method.

The study was conducted for 4 months, starting from September 2017 until December 2017. Samples taken from dr. Mohammad Hoesin Hospital Palembang. DNA isolation and PCR was done at the Molecular Biology Laboratory of Medical Faculty of Sriwijaya University Palembang.

Collecting data was performed by taking Enterobacteriacea to isolate from infected patients collected in the Department of Microbiology dr. Mohammad Hoesin Hospital Palembang with criteria: Enterobacteriacea consisting of Klebsiella pneumonia, Escherichia coli, Proteus mirabilis, Enterobacter sp., and Citrobacter sp. that proved to be CRE by Vitek 2 Compact (bioMerieux, USA).
3. Results
Total of 709 Enterobacteriaceae isolates collected from September to November 2017 was examined using Vitek 2 Compact (bioMerieux, USA) to detect CRE. Bacteria detected as CRE are 88 isolates. Further identification of bla\textsubscript{VIM} genes performed to 21 isolates by PCR.

3.1. Identify CRE Phenotype by Vitek 2 Compact
Enterobacteriaceae was CRE positive if MIC $\geq 4$ in antibiotic meropenem and/or $\geq 2$ in antibiotic ertapenem [21], when examined using Vitek 2 Compact (bioMerieux, USA). Among 709 isolates of Enterobacteriaceae, 88 (12.4%) isolates were CRE positive whereas 621 (87.6%) isolates were not CRE. Table 1 present the distribution of Enterobacteriaceae identified as CRE with the Vitek 2 Compact (bioMerieux, USA).

| Phenotype       | N  | %   |
|-----------------|----|-----|
| Positive CRE    | 88 | 12.4|
| Negative CRE    | 621| 87.6|
| Total           | 709| 100 |

In this study, CRE mostly found in Klebsiella pneumonia (55.7%), Escherichia coli (19.3%), and Enterobacter sp. (18.2%). Table 2 presents the distribution of CRE according to the type of bacteria.

| Specimen                  | N  | %   |
|---------------------------|----|-----|
| Klebsiella pneumonia      | 49 | 55.7|
| Escherichia coli          | 17 | 19.3|
| Enterobacter sp.          | 16 | 18.2|
| Proteus mirabilis         | 4  | 4.5 |
| Citrobacter sp.           | 2  | 2.3 |
| Total                     | 88 | 100 |

CRE show similar patterns sensitivity to the antibiotics ertapenem and meropenem. Detailed data on CRE sensitivity patterns can be seen in table 4.

| Antibiotic Sensitivity Patterns | N  | %   |
|---------------------------------|----|-----|
| Ertapenem (S) + meropenem (R)   | 4  | 4.6 |
| Ertapenem (R) + meropenem (S)   | 9  | 10.2|
| Ertapenem (R) + meropenem (R)   | 73 | 83  |
| Ertapenem (R) + meropenem (I)   | 1  | 1.1 |
| Ertapenem (I) + meropenem (R)   | 1  | 1.1 |
| Total                           | 88 | 100 |

Description: S = sensitive, R = resistant, I = intermediates

As for the type of specimen, bacterial isolates are derived from various specimens of infected patients in dr. Mohammad Hoesin Palembang. CRE mostly found in sputum (36.4%).

3.2. Identification of \textit{bla}_{VIM} genes with Polymerase Chain Reaction (PCR)
Identification of \textit{bla}_{VIM} genes using PCR technique was performed on 21 samples of CRE. 21 CRE samples were taken based on the percentage of each Enterobacteriaceae species that mentioned in table 2. Thus, PCR was done by taking 12 isolates of Klebsiella pneumonia, 5 Escherichia coli isolates, and 4 isolates of Enterobacter sp. PCR was performed using a reverse primer (5'-CGAATTGCCAGCACCCAG-3') and a forward primer (5'GATGAGTGGTTGTCGCT-3') results from 1 band of 390 bp.
Table 4. CRE distribution by specimen type.

| Specimen          | N | %  |
|-------------------|---|----|
| Sputum            | 32| 36.4|
| Urine             | 28| 31.8|
| Pus               | 13| 14.8|
| Blood             | 3 | 3.4 |
| Feces             | 2 | 2.3 |
| Pleural fluid     | 2 | 2.3 |
| Vaginal Swab      | 2 | 2.3 |
| Tongue Swab       | 1 | 1.1 |
| Wound Swab        | 1 | 1.1 |
| Oropharynx        | 1 | 1.1 |
| Swab              | 1 | 1.1 |
| Gastric rinse      | 1 | 1.1 |
| Drainage fluid    | 1 | 1.1 |
| Tissue            | 1 | 1.1 |
| Total             | 88| 100|

At this visualization stage, the positive amplicons containing the bla\textsubscript{VIM} gene will form 390 bp bands as seen in samples 18, 19, and 20. Positive visualization of the amplicons containing bla\textsubscript{VIM} genes can be seen in figure 1.

![Figure 1](image)

Figure 1. A result of visualization of positive genetic amplicon bla\textsubscript{VIM} gene: K\textsubscript{-} = Negative Control, M = DNA marker, 18-20 = sample number

From the research, we obtained 3 of the 21 samples of CRE (14.3%) expressed positively bla\textsubscript{VIM} gene genotype. Among 3 CRE samples positively containing the bla\textsubscript{VIM} genes, 2 (66.7%) samples were from Enterobacter \textit{sp.}, and 1 (33.3%) sample was from Klebsiella \textit{pneumoniae}. The interpretation data from visualization result are presented in table 5.

Table 5. Sensitivity patterns bla\textsubscript{VIM} gene positive CRE against ertapenem and meropenem.

| Antibiotics         | bla\textsubscript{VIM} Gene Positive (N = 3) | bla\textsubscript{VIM} Gene Negative (N = 18) |
|---------------------|----------------------------------------------|---------------------------------------------|
|                     | n (%)                         | n (%)                        |
| Ertapenem           | 0 0 3 (100) 2 (11.1) 0 16 (88.9) 0 16 (88.9) |
| Meropenem           | 0 0 3 (100) 0 0 18 (100) 0 18 (100) |

Description: S = sensitive, R = resistant, I = intermediates

As for the bla\textsubscript{VIM} genes positive CRE sensitivity pattern, none of the isolates were sensitive to ertapenem and meropenem or otherwise resistant to both antibiotics.

4. Discussions

4.1. Distribution of CRE by Number of Enterobacteriaceae

In this study, 88 of 709 isolates of Enterobacteriaceae identified as CRE, whereas 621 isolates were non-CRE. The incidence of CRE in this study was quite low at 12.4% if compared with the study at
Cipto Mangunkusumo Hospital Jakarta with the discovery of 27.6% CRE [22]. The low incidence rates in this study may be due to a higher variation of Enterobacteriaceae species in the study at Cipto Mangunkusumo Hospital. However, if the results of this study were compared with the study at Universiti Sains Hospital Malaysia that is only 4.05% [23], the incidence rate of CRE in hospitals dr. Mohammad Hoesin Palembang is quite high. It also proves that in Asia, Indonesia is still the country with the highest CRE incidence compared to other Asian countries [7].

4.2. Distribution of CRE by Type of Bacteria and Specimen
In this study, from 88 Enterobacteriaceae CRE positive isolates, Klebsiella pneumoniae (55.7%), Escherichia coli (19.3%), and Enterobacter sp. (18.2%) were found. While Proteus mirabilis and Citrobacter sp. found less than 5%. This is similar to a study conducted in 2009-2011 at a hospital of one of the universities in Thailand, where Klebsiella pneumoniae is the most common Enterobacteriaceae (38.7%) identified as CRE followed by Escherichia coli (37.2%) and Enterobacter sp. (6%) [24]. In a study conducted by Kazi et al in 2012, Klebsiella pneumoniae (40.7%) also ranks most of the CRE [25].

These findings are also reinforced by similar studies in 38 countries in Europe which states that carbapenemase was most generated by Enterobacteriaceae especially Klebsiella pneumoniae and Escherichia [26].

CRE isolates in this study mostly came from sputum (36.4%), urine (31.8%), and pus (14.8%). This may be related to the type of infectious diseases that the patient suffered, like urinary tract and respiratory tract infections that can be caused by five types of Enterobacteriaceae taken in this study, there are Klebsiella pneumonia, Escherichia coli, Enterobacter sp., Proteus mirabilis and Citrobacter sp [27].

4.3. The proportion of blaVIM on CRE
From this research, we found that 3 of 21 CRE were the positive blaVIM gene (14.3%). These results indicate that the distribution of blaVIM on CRE in dr. Mohammad Hoesin hospitals Palembang is quite high when compared with research that conducted by Karuniawati, Saharman and Lestari (2011) at Cipto Mangunkusumo Hospital (RSCM) Jakarta who did not find any CRE with blaVIM genes. The increased distribution of blaVIM gene in this study was influenced by the time of the study, samples that only taken from ICU patients at RSCM, and also showed that the transmission of the gene in the CRE isolate occurred very easily and rapidly due to the location of genes in mobile genetic elements such as plasmid [18,19].

In the study of Kazi et al (2012), the distribution of blaVIM gene is quite low compared to this study [25] because blaNDM (carbapenemase encoding gene NDM) is the most dominant carbapenemase in India [28]. However, when compared with the research of Rajabnia et al (2015) [29] and Barbarini et al (2012) [30], the blaVIM distribution in this study is smaller. This is due to the dominant blaVIM gene that found in Europe Albiger et al (2015) [26].

4.4. Sensitivity Pattern CRE
CDC defines CRE as Enterobacteriaceae that is resistant to imipenem, ertapenem, doripenem, meropenem or Enterobacteriaceae which produced carbapenemase [14]. The results using the Vitek 2 Compact (bioMerieux, USA) showed that from 88 CRE isolates, 9 (10.2%) isolates were sensitive to meropenem and 4 (4.6%) were sensitive to ertapenem. This finding is similar to Heller, Grif, and Orth (2012) studies which showed that the sensitivity to meropenem is higher than ertapenem antibiotic.

In addition, all of the 3 CRE isolates positive for the blaVIM genes showed resistance to meropenem and ertapenem.

The important role in causing carbapenem antibiotic resistance due to its hydrolysis capability [15]. In this study also found that from 21 CRE, as much as 85.7% that didn't contain blaVIM gene also remain resistant to ertapenem or meropenem. This indicated that CRE that does not have blaVIM genes can occur through another resistant mechanism through the expression of other carbapenemase.
genes that produce other resistance enzymes as well or through non-enzyme mechanisms such as porin changes, drug penetration disorders to PBPs, or excess pump activity efflux [3,12,13].

| Table 6. Research of bla\text{VIM} gene distribution on CRE in different countries. |
|---------------------------------|---------------------------------|-----------------|----------------|
| Researcher (year)               | Population                      | Number of Samples | bla\text{VIM} Gene |
| This study (2017)               | Palembang, Indonesia            | 21               | 14.3%           |
| Karuniawati, Saharman and Lestari (2011) | Jakarta, Indonesia              | 27               | 0%              |
| Kazi et al (2012)               | Mumbai, India                   | 113              | 3.5%            |
| Rajabnia et al (2015)           | Iran                            | 26               | 57.7%           |
| Barbarini et al (2011-2015)     | Spanish                         | 463              | 29.2%           |

As mentioned previously, carbapenem is the last choice in treating patients with ESBL-producing Enterobacteriacea [7] With the discovery of the bla\text{VIM} genes in CRE, the antibiotic choices become more limited [31]. Several studies have shown that the use of cholestyrene, tigecycline, phosphomycin, polymyxin B, aminoglycoside or combination may be considered to treat patients with CRE infection [32]. However, the CRE infection control program, the policy of antibiotic use in hospitals and the regular reporting of bacterial sensitivity tests should be a top priority to prevent this emergence in the future.

5. Conclusions

Of 709 Enterobacteriacea bacteria population in clinical microbiology laboratories RSMH during the period September-November, 2017, identified 88 samples (12.4%) bacteria phenotype CRE. Only 21 isolates continue with PCR procedure. PCR identified 3 samples (14.3%) bacterial genes phenotype bla\text{VIM}. Isolates carrying bla\text{VIM} genotype gene found in Enterobacter sp and Klebsiella pneumonia.

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**Acknowledgments**

This research was supported by the University of Sriwijaya which has held Sateks grant so this research can run well. We thank our colleagues from Microbiology Department University of Sriwijaya who provide insight and expertise for the best progress of this research.