COMPARISON OF PHENOTYPIC AND GENOTYPIC PROFILE OF CARBAPENEMASE PRODUCING Escherichia coli

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INTRODUCTION

In the last 15 years, specific concerns have been addressed to bacterial resistance to antibiotics, particularly broad spectrum antibiotics. The use of carbapenem, which is the drug of choice to eradicate pathogenic Extended Spectrum β-Lactamase (ESBL) producer...
Gram-negative bacteria, was quickly followed by evolution of carbapenemase-producer bacteria strains (Tängdén 2012).

*Escherichia coli* (*E. coli*) is the most frequent bacteria found in urine specimens. Over 50% of *E. coli* isolates obtained from patients in Dr. Soetomo Hospital, Surabaya, produced ESBL. Whereas, carbapenemase-producer *E. coli* has not been confirmed according to procedure recommended by Clinical and Laboratory Standards Institute (CLSI) 2016 (Sutandhio et al 2015, CLSI 2016).

Carbapenemase screening in clinical microbiology laboratory uses semi-automatic BD Phoenix system, which gives inaccurate results. This may lead to confusion and excessive cautions (Woodford et al 2010, CLSI 2016). Phenotypic carbapenemase confirmatory test is a combination of Modified Hodge Test (MHT), Sodium Mercaptoacetic Acid (SMA), and 3-Aminophenylboronic Acid (PBA). Phenotypic confirmatory test is expected to detect carbapenemases better than BD Phoenix system, and to classify the enzymes based on Ambler classification (Kim et al 2007, EUCAST 2013, CLSI 2016).

Ambler classified carbapenemases into three classes; A, B, and D. In class A, carbapenemases are mostly produced by Enterobacteriaceae. In class B, carbapenemases are metallo-β-lactamase (MBL) which are mostly produced by *Pseudomonas aeruginosa*, Enterobacteriaceae, and *Acinetobacter baumannii*. Whereas, in class D, carbapenemases are produced by *Acinetobacter spp.*, and in some cases, by *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *E. coli* (Queenan & Bush 2007, Thomson 2010).

**MATERIALS AND METHODS**

*E. coli* isolates from urine specimens that were potential carbapenemase-producers according to BD Phoenix semi-automatic system during six weeks period (23 August 2016 - 4 October 2016) in Dr. Soetomo Hospital, Surabaya, were examined by phenotypic confirmatory test; i.e., MHT, SMA, and PBA. The results of phenotypic confirmatory test were then compared to genotypic confirmatory test, i.e., Polymerase Chain Reaction (PCR).

**Modified Hodge Test (MHT)**

MHT was done by streaking each loopful of bacteria colony of: isolate tested, carbapenemase positive control, and carbapenemase negative control, on Mueller Hinton Agar straight from near the meropenem 10 µg disk (Oxoid) on the center to the side of petri dish. The entire agar surface had been previously inoculated with *E. coli* ATCC®25922 of density 1x107 to 2x107 CFU/ml which functions as indicator of carbapenemase production. Agar was then incubated for 18-20 hours in 37°C. MHT result were positive if indentations of *Escherichia coli* ATCC® 25922 growth near tested isolate were seen (Fig. 1).

![Positive Modified Hodge Test](image1)

**Fig. 1.** Positive Modified Hodge Test, marked by indentation of *Escherichia coli* ATCC® 25922 growth near tested isolate.

**Synergy test with Sodium Mercaptoacetic Acid (SMA)**

Suspension of tested isolate with 0.5 McFarland turbidity standard was inoculated on Mueller Hinton Agar surface. SMA 3 mg disk (Eiken Chemical Co., Ltd.) were put between meropenem 10 µg disk (Oxoid) and ceftazidime 30 µg disk (Oxoid) disk with 10-15 mm range of each disk. Agar was then incubated 18-20 hours in 37°C. SMA tested positive if there were enlargement or bulging of one or both antibiotic inhibition zones toward SMA disk (Fig. 2).

![Positive Sodium Mercaptoacetic Acid (SMA)](image2)

**Fig. 2.** Positive Sodium Mercaptoacetic Acid (SMA) test, marked by enlargement of meropenem zone of inhibition or bulging toward SMA disk.

**Synergy test with 3-Aminophenylboronic Acid (PBA)**

Suspension of tested isolate with 0.5 McFarland turbidity standard was inoculated on Mueller Hinton Agar surface. Two disks of meropenem 10 µg (Oxoid) were put in 40 mm distance on agar. 300 µg (6 µl) PBA was then added on one of meropenem disk. Agar were
incubated 18-20 hours in 37°C. PBA tested positive if there was enlargement of antibiotic inhibition zone diameter = 5 mm on antibiotic that was added with PBA compared with antibiotic disk that was not added with PBA (Fig.3).

Polymerase Chain Reaction (PCR)

Multiplex PCR procedure was done using PCR kit (Thermo Fisher Scientific), primers for 7 carbapenemase genes (OXA-48, IMP1, IMP2, GES, VIM, NDM, KPC) for multiplex PCR (Sigma-Aldrich) (Fig. 4).

Table 1. Classification of carbapenemase based on Ambler by Phenotypic Confirmatory Test

<table>
<thead>
<tr>
<th>Carbapenemase class</th>
<th>MHT</th>
<th>SMA</th>
<th>PBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Not carbapenemase</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

RESULTS

Out of 30 tested isolates, 6 isolates (20.0%) were positive for MHT, and 25 isolates (83.3%) were positive for SMA, which indicated that most isolates produced MBL. There was no positive result for PBA test. Genotypic confirmatory test by PCR revealed that 12 isolates (40.0%) had Verona Intergron-encoded Metallo-β-lactamase (VIM) gene, which is a member of carbapenemase class B (Table 3).

Table 2. Results of MHT, SMA, and PBA

<table>
<thead>
<tr>
<th></th>
<th>SMA</th>
<th>PBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHT +</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>MHT -</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3. Percentage of results of Phenotypic Confirmatory Test and Genotypic Confirmatory Test

<table>
<thead>
<tr>
<th>Trait</th>
<th>Test</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotypic</td>
<td>MHT</td>
<td>20.00%</td>
<td>80.00%</td>
</tr>
<tr>
<td>SMA</td>
<td>83.33%</td>
<td>16.67%</td>
<td></td>
</tr>
<tr>
<td>PBA</td>
<td>0.00%</td>
<td>100.00%</td>
<td></td>
</tr>
<tr>
<td>Genotypic</td>
<td>PCR</td>
<td>40.00%</td>
<td>60.00%</td>
</tr>
</tbody>
</table>

Analysis of Modified Hodge Test (MHT) results

Out of 12 isolates that had carbapenemase genes, there were 6 isolates that gave positive results for MHT (Table 4). There were 50.0% sensitivity and 100.0% specificity. The results of McNemar test on SPSS program were significant with p=0.031; which means a significant difference between MHT and PCR. Symmetric measures, which comprises of Phi measurement with value of 0.612 (p=0.001); Cramer’s V (p=0.001); and Kappa (p=0.001), revealed a match between MHT and PCR (p<0.05).

Table 4. Comparison of MHT to PCR results

<table>
<thead>
<tr>
<th>PCR</th>
<th>VIM +</th>
<th>VIM -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHT +</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>MHT -</td>
<td>6</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>18</td>
<td>30</td>
</tr>
</tbody>
</table>
Analysis of Sodium Mercaptoacetic Acid (SMA) results

Out of 12 isolates with carbapenemase genes, 11 isolates gave positive results for SMA test (Table 5). There were 91.7% sensitivity and 22.2% specificity. The results of McNemar test on SPSS program were significant with p=0.01, indicating significant difference between SMA and PCR. Symmetric measures found no match between SMA and PCR (p=0.317).

Table 5. Comparison of SMA to PCR results

<table>
<thead>
<tr>
<th></th>
<th>VIM +</th>
<th>VIM -</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMA +</td>
<td>11</td>
<td>14</td>
<td>25</td>
</tr>
<tr>
<td>SMA -</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>18</td>
<td>30</td>
</tr>
</tbody>
</table>

Analysis of MHT, SMA, and PBA combination results

According to phenotypic confirmatory test, 26 isolates produced carbapenemase, consisting of 25 isolates that produce carbapenemase Ambler B (MHT+/-, SMA+, PBA-), and 1 isolate carbapenemase Ambler D (MHT+, SMA-, PBA-) producers. PCR results showed only 12 isolates had carbapenemase class B genes (Table 6). Comparison of carbapenemase classification by phenotypic confirmatory test and PCR were 91.7%.

DISCUSSION

BD Phoenix semi-automatic system gave alert no. 399 for E. coli isolates that are phenotypically intermediate or resistant to carbapenems; i.e., imipenem, ertapenem, or meropenem. Isolates with alert no. 399 are considered as potential carbapenemase producers (BD 2011). The method is inaccurate for carbapenemase detection, thus it can only be used as screening method.

According to this study, MHT was a specific detection method (100%), but not sensitive (50%). Whereas, SMA was sensitive (91.7%), but not specific (22.2%). Combination of MHT, SMA, and PBA had 100% sensitivity and 22.2% specificity when compared to PCR.

Out of all isolates tested (n=30), only 6 isolates (20.0%) gave positive MHT results, with 50.0% sensitivity and 100.0% specificity. MHT had few weaknesses, i.e., problem in interpretation, low specificity due to false positive results on isolates producing extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase, and low sensitivity due to isolates producing Ambler B carbapenemase, such as New Delhi Metallo-β-lactamase (Thomson 2010, EUCAST 2013, Shenoy et al 2014, Bajaj et al 2016, CLSI 2016).

SMA is a cation chelator substance. SMA difference from other chelators, such as EDTA and dipicolinic acid is that SMA can bind to MBL active site specifically and render its function. EDTA inhibits MBL indirectly and unspecifically by reducing free zinc concentration on Mueller Hinton agar (Hattori et al 2013). The use of SMA with ceftazidime and meropenem is proven better in detecting MBL-producing isolates. Ceftazidime is used because all MBL-producing isolates has high resistance to ceftazidime. Meropenem is used because it has better sensitivity in detecting carbapenemase (Hattori et al 2013). In this study, SMA gave positive results for 83.3% isolates, with sensitivity of 91.7% and specificity of 22.2%. Low specificity were caused by inappropriately use of gold standard in diagnostic test.

Boronic acid is the only non-β-lactam substance that can inhibit active site of serin-type carbapenemases, which are members of Ambler class A. 3-Aminophenylboronic Acid (PBA) is one of boronic acid substrate that is used in laboratory. The test result is positive if zone of inhibition of disk meropenem+PBA is enlarged ≥5 mm compared with meropenem disk without PBA (Doi et al 2008, Pournaras et al 2010, Tsakris et al 2011). In this study, there were no positive result for PBA test, which indicated no producer of carbapenemase class Ambler A had been isolated. It matches with the dominance of Ambler class B carbapenemases in South and South East Asia regions (Hsu et al 2017). False negative results may also happen if quality of reagents were below standards. In this study, quality control for PBA was not done. Although confirmation with PCR showed there was no gene encoding class A carbapenemase in the isolates. PCR method is the gold standard to confirm and classify carbapenemases. PCR gives quick results, has best sensitivity (100%) and specificity (100%). PCR is not a routine procedure and usually done for research and epidemiology purposes only (Galani et al 2008, Ribeiro et al 2014).

Combination of MHT, SMA, and PBA to classify carbapenemase based on Ambler had 91.7% match with PCR. Out of 12 isolates which had VIM genes, one was phenotypically classified as Ambler class D carbapenemase producer.

PCR is not used as routine procedure to confirm carbapenemase because of vast diversity of the enzymes. Genetic variations of carbapenemases keep expanding overtime, thus carbapenemase detection by genetic
approach would take repeated procedures and a lot of resources (Diene & Rolain 2014).

Studies which attempt to detect carbapenemase genes in phenotypically carbapenem-resistant bacteria isolates cannot always identify carbapenemase genes in all isolates (Stuart & Leverstein-Van Hall 2010, Karunia-wati et al 2013). This is line with this study in which not all carbapenemase genes in phenotypically carbapenemase-producing organisms were identified.

Mismatch between phenotypic and genotypic confirmatory test in detecting carbapenemase can be caused by (1) carbapenemase genes in bacteria are different from the primers we used, in isolates which were confirmed as carbapenemase producers phenotypically but not genetically, or (2) another resistance mechanisms toward carbapenems, in isolates which were resistant to carbapenems but not confirmed as carbapenemase-producers, phenotypically and genotypically.

CONCLUSION

MHT, SMA, and PBA should be interpreted as union because process of carbapenemase detection in these tests are different, thus the tests complement each other. In this study, phenotypic confirmatory test detected more MBL-producers compared with PCR. It may be caused by (1) carbapenemase genes in bacteria were different from primers used for testing, or (2) another resistance mechanisms to carbapenems. The result of this study, although should be confirmed with larger scale of research, suggests that the use of phenotypic confirmatory test to detect carbapenemase may facilitate effectiveness of antibiotic therapy in patients infected with carbapenemase-producing bacteria, thus increase survival rate and reduce length of stay in hospitals.

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Karuniawati A, Saharman YR, Lestari DC. 2013. Detection of carbapenemase encoding genes in Enterobacteriaceae, Pseudomonas aeruginosa, and Acinetobacter baumannii isolated from patients at Intensive Care Unit Cipto Mangunkusumo Hospital in 2011.

Table 6. Comparison of phenotypic confirmatory tests (MHT, SMA, and PBA) and genotypic confirmatory test (PCR) interpretation

<table>
<thead>
<tr>
<th>Interpretation of Phenotypic Test</th>
<th>PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbapenemase +</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Carbapenemase -</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>18</td>
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</tbody>
</table>
Comparation of Phenotypic and Genotypic Profile of Carbapenemase Producing *Escherichia coli* (Silvia Sutandhio et al)

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