Phenotypic and genotypic detection of carbapenemase enzymes producing gram-negative bacilli isolated from patients in Khartoum State [version 1; peer review: awaiting peer review]

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Abstract

Background: Carbapenems are used as antibiotics of last resort for treating infections due to multidrug-resistant Gram-negative bacilli, but emergence of Carbapenem resistant Gram-negative bacilli have been reported due to the production of Carbapenemase enzymes that significantly limits treatment options for life-threatening infections.

Objective: This study aimed to detect Carbapenem resistant Gram-negative bacilli from patients attended to different hospitals in Khartoum state and to detect Carbapenemase enzymes production by phenotypic and genotypic methods.

Methods: A hospital based cross sectional study was conducted in Khartoum state in the period from February to August 2016. Hundred and forty nine Gram-negative bacilli bacteria were isolated from different clinical specimens. Blood agar, Chromogenic agar media, MacConkey agar, XLD media and standard biochemical tests were used for isolation and identification of Gram-negative bacilli from different samples. Standard antimicrobial susceptibility testing to Carbapenem antibiotic was performed for all isolates, then detection of Carbapenemase enzymes production for the resistant isolates was performed using Modified Hodge Test and PCR.

Results: Hundred and forty nine Gram-negative bacilli were isolated from 147 different clinical specimens. The most predominant Gram-negative bacilli isolates was E.coli (54.4%), followed by Klebsiella species (29.5%). More than fifty percent of the isolates were Carbapenem resistant. Fifty six percent of the resistant isolates were positive by Modified Hodge Test. By using PCR, 17.3% of resistant organisms were harbored blaOXA48 gene, and 6.7% harbored blaIMP gene. E.coli was the most bacteria that harbored the blaOXA48 followed by Klebsiella species. blaIMP gene was harbored only by E.coli.

Conclusion: The percentage of resistance to Carbapenems due to production of Carbapenemase enzymes is very high in Sudan. BlaOXA48 gene is more predominant than blaIMP in this study.
Keywords
carbapenem resistance, carbapenemase enzymes, gram-negative bacilli,
Modified Hodge Test, PCR, bla IMP gene, bla OXA48

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Introduction

Carbapenems are beta-lactam antibiotics often used as last resort antibiotics for treating infections caused by multidrug resistant Gram-negative bacilli, since they have the broadest spectra among all beta-lactams. Carbapenemases (also known as carbapenem hydrolyzing enzymes) represent the most versatile family of beta-lactamases. Beta-lactamases can be classified according to their functional and molecular properties. Functional classification divides beta-lactamases into four major functional groups (A–D) based on amino acid sequence. In addition, based on the active site of the enzyme, they classified beta-lactamases into serine-beta-lactamases (class A, C and D) and metallo-beta-lactamases (class B).

Oxacillinas (OXAs) (class D), so called because of their ability to hydrolyze Oxacillin, are classified according to their hydrolysis spectrum. Broad-spectrum OXA enzymes are able to hydrolyze carbapenems. This family is plasmid encoded and they have been identified mainly in Enterobacteriaceae and Pseudomonas aeruginosa. Currently, there are 239 OXA enzymes, of which at least 37 are carbapenemases and at least 9 are extended spectrum beta-lactamases. The first OXA enzyme with carbapenemase activity was detected in 1985 in an Acinetobacter baumannii isolate from Scotland, and OXA-48 cluster is the most important among class D carbapenemases. IMP (for “active on Imipenem”) a metallo-beta-lactamase (class B), was first detected in 1990 in P. aeruginosa isolate from Japan, and then reported in Serratia marcescens isolate in Japan in 1991. IMP-2 was observed in Italy in A. baumannii. Currently there are 37 known IMP types which are most commonly seen in P. aeruginosa and A. baumannii isolates, but have also been reported in most Enterobacteriaceae members. One of the risk factors of acquiring carbapenem resistant bacteria is the increased consumption of carbapenems. Many patients are most likely affected by carbapenem resistant bacteria, including those who have poor immunity, prolonged hospitalization, admission to ICU, an indwelling medical device, and multiple exposures to different antibiotics. Also the increased frequency of international travel for work, leisure and migration contributes to their spread, and most of the carbapenemases are found on transferable plasmids, which are highly transmissible. The prevalence of carbapenemase producing gram negative bacteria is increasingly reported in Sudan and Africa. Manenzhe et al. (2014) reported about 83 studies conducted in Africa which showed that the prevalence of carbapenemase producer isolates in hospital settings ranged from 2.3% to 67.7% in North Africa and from 9% to 60% in sub-Saharan Africa. Oxacillinas especially blaOXA48 was the most predominant in the whole country.

In Sudan there are limited reports of carbapenemase producers, therefore the present study was performed. This study aimed to detect carbapenem resistant Gram-negative bacilli (using conventional biochemical tests and chromogenic agar media), determine the antimicrobial susceptibility pattern of the isolates against carbapenems, detect carbapenemase enzyme production for the resistant isolates (using phenotypic methods, Modified Hodge Test), and detect blaOXA48 and blaIMP genes for carbapenem resistant isolates (using PCR, genotypic methods).

Methods

This was cross sectional laboratory based study, conducted in Khartoum state in the period from February to August 2016. Ethical clearance was obtained from SUMASRI (University of Medical Sciences and Technology Sudanese Institute of Medical and Scientific Research) Institutional Review Board (SIRB); (IRB No: 00008867), which ensures that all ethical considerations for conducting the research in a way that protects patient’s confidentiality and privacy are followed. Informed consent was obtained from the hospital laboratories (laboratory manager) after providing them with the ethical clearance to collect samples during routine procedures from the microbiology laboratories. Participants’ privacy and confidentially was protected for all samples; personal information was not of great value in the current study and was thus not taken.

One hundred and forty nine Gram-negative bacilli were isolated from 147 different clinical specimens that were collected from patients attending different hospitals in Khartoum state. Blood agar, MacConkey agar, CLED media, and XLD media were used for primary plating depending on the type of clinical specimens, cultures were examined macroscopically for colonial morphology, and Gram stain was performed from suspected colonies. All Gram-negative bacilli isolates were selected then subcultured on MacConkey agar media for purity and further identification tests, and incubated at 37°C overnight. Chromogenic agar media and different standard biochemical reactions, including oxidase, Kligler Iron Agar (KIA), urease production, citrate utilization, indole production, and motility tests were performed for their identification (all media mentioned above were obtained from LAB M, UK, except chromogenic media which was obtained from Laboratories Flow Media, Sudan).

Standard antimicrobial susceptibility testing

Antimicrobial susceptibility testing to carbapenem antibiotic was performed for all Gram-negative bacilli isolates using the disc diffusion method, according to the Clinical Laboratory Standards Institute Guidelines. Bacterial colonies were suspended in sterile normal saline and compared with McFarland standard and cultured in Muller-Hinton agar media (media were obtained from Pronadisa Laboratories Conda., Spain) using a sterile cotton swab. After overnight incubation at 37°C, zone of inhibition was measured and the reading was compared to the sheet provided by manufacturer.

Gram-negative isolates showing resistant zones to Meropenem were tested for carbapenemase enzyme production using MHT and PCR.
Modified Hodge Test (MHT)

MHT was used to detect carbapenemase enzyme production. McFarland 0.5 dilution of *E.coli* (ATCC 25922) was prepared in 5 ml of sterile saline. The suspension was diluted to 1:10 by adding 0.5 ml of the 0.5 McFarland (Oxoid, UK) to 4.5 ml of sterile saline. A lawn of the 1:10 dilution of *E.coli* was streaked onto a Mueller Hinton agar plate using a sterile cotton swab. A 10 μg Meropenem susceptibility disk (Hi media, India) was placed on the center of the test area. The tested bacilli was streaked in a straight line from the edge of the disk to the edge of the plate. The plate was incubated overnight at 37°C in ambient air (Figure 1)\(^1\).

Positive MHT: Shown by clover leaf-like indentation of *E. coli* susceptible strains growing along the tested organism growth streak within the disk diffusion zone (Figure 1)\(^2\).

Negative MHT: No growth of *E. coli* along the tested organism growth streak within the disc diffusion zone\(^3\).

Molecular detection of *bla*\(_{\text{IMP}}\) and *bla*\(_{\text{OXA-48}}\) genes

**DNA extraction.** DNA extraction for all resistant isolates was done by boiling method from fresh bacterial cultures within 24 hours, in which bacteria are in the logarithmic growth phase, which is the most suitable phase for bacterial DNA extraction according to the following protocol: Cells of interest were suspended in sterile normal saline and pelleted (10\(^6\) to 10\(^7\)) by centrifugation at 4,000 rpm for 5 minutes using a labelled 1.5 ml safe-lock tube. The pellet was resuspended in 100 μl of phosphate buffer saline. The tubes were placed at 95°C for 15 minutes then centrifuged at >10,000 rpm for 5 minutes to pellet the cellular debris. Then the supernatant (lysate) was transferred into a labelled 1.5 ml safe-lock. The lysate was stored at -20 to 4°C. [https://www.dkfz.de/gpcf/fileadmin/ccontrol/lysate_Protocol_DKFZ.pdf](https://www.dkfz.de/gpcf/fileadmin/ccontrol/lysate_Protocol_DKFZ.pdf)

**PCR.** A multiplex conventional PCR was designed to detect *bla*\(_{\text{IMP}}\) and *bla*\(_{\text{OXA-48}}\) using specific primers for each. The primers were obtained from Macrogen Company, Korea (Table 1).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ - 3’</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BlaOXA-48-F</td>
<td>GCTTGATCGCCTCGATT</td>
<td>238</td>
</tr>
<tr>
<td>BlaOXA-48-R</td>
<td>GATTTGCTCCGTGGCCGAAA</td>
<td></td>
</tr>
<tr>
<td>BlaIMP-F</td>
<td>TCGTTTGAGAAGTAACCGG</td>
<td>568</td>
</tr>
<tr>
<td>BlaIMP-R</td>
<td>ATGTAAGTTTCAAGAGTGATCC</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Modified Hodge Test. Clover leaf like indentation from *E. coli* ATCC showing positive result.

The amplification of DNA was performed using a TC-312 PCR machine (TECHNE, UK). For amplification, 17 μl of distilled water were added to ready manufactured premix solution (Intron biotechnology, Korea) and mixed well. Then 0.5 μl from forward primer and 0.5 μl from reverse primer of both *bla*\(_{\text{IMP}}\) and *bla*\(_{\text{OXA-48}}\) genes were added. Then 2 μl of DNA was added to the mixture. For both genes, the cycling conditions were: initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 53°C for 1 minute, and elongation at 72°C for 1 minute. The cycles were repeated 35 times, with a final extension step at 72°C for 5 minutes\(^4\). PCR product (5μl) were analyzed by gel electrophoresis in 1.0% agarose stained with ethidium bromide. The results were photographed under ultraviolet light machine (Transilluminator; Uvite, UK) to detect the specific amplified product by comparing it with 100 base pairs standard DNA ladder (Figure 2)\(^4\).

Data analysis

Data obtained were analysed using descriptive statistics and chi-square tests performed using SPSS version 20.0, to check the statistical significance and Excel 2013. The p-value that considered significant was < 0.05.

Results

Clinical Gram-negative bacilli isolates

One hundred forty seven different clinical specimens were collected from patients attending different hospitals in Khartoum state. One hundred forty nine Gram-negative bacilli were isolated. Urine specimens were the most frequent specimen, comprising 104 out of 147 clinical specimens; 106 Gram negative bacilli were isolated from the 104 urine specimens.

![Figure 2. Amplified DNA under ultra violet light machine showing *bla*\(_{\text{OXA-48}}\) positive result. Lane 1, DNA ladder 100bp; Lane 2, negative control; Lane 3, typical band size of 238 bp corresponding to the molecular size of *bla*\(_{\text{OXA-48}}\) gene; Lanes 4–7, negative samples.](https://www.dkfz.de/gpcf/fileadmin/ccontrol/lysate_Protocol_DKFZ.pdf)
The isolated Gram-negative bacilli comprised of 81 (54.4%) E. coli, 44 (29.5%) Klebsiella species, 17 (11.4%) Proteus species, 6 (4.0%) Pseudomonas species and 1 (0.7%) Enterobacter species (Table 2).

**Carbapenem susceptibility testing**

Carbapenem (Meropenem) susceptibility testing using disc diffusion method showed that 75 (50.3%) of Gram-negative rods isolates were Meropenem resistant, 57 (38.3%) were Meropenem sensitive, and 17 (11.4%) were Meropenem intermediate (Table 3).

Regarding carbapenem (Meropenem) resistance, the most resistant organism was *E. coli*, which constituted 45 out of 75 resistant bacteria (30.2%), followed by *Klebsiella* species 15.4% (23 isolates), *Proteus* species 2.7% (4 isolates), *Pseudomonas* species 1.3% (2 isolates), and *Enterobacter* species 0.7% (1 isolate) (Table 3).

**Detection of carbapenemase enzymes production using MHT and PCR**

Carbapenemase production by both MHT and PCR constituted 42 isolates (56%) of the total carbapenem resistant isolates.

MHT was performed for the 75 resistant isolates, 42 isolates (56%) were positive and the other 33 (44%) were negative for carbapenemase enzyme production by MHT (Table 4).

Conventional PCR assay for detection of bla\(_{OXA-48}\) and bla\(_{IMP}\) genes was performed for all 75 resistant isolates. 17.3% of the resistant Gram-negative isolates were positive for bla\(_{OXA-48}\) gene, while 6.7% of them were positive for bla\(_{IMP}\) gene; both constituted 24% carbapenemase producing bacteria using PCR (Table 5).

Carbapenemase production constituted 42 isolates (56%) of all resistant isolates by both MHT and PCR; some were positive by both methods, some were positive by MHT only, and others were positive by PCR only.

Regarding MHT, *E. coli* was the most carbapenemase producer among the resistant isolates (it constituted 36% of the 56% resistant isolates) followed by *Klebsiella* species (it constituted 16% of the 56% resistant isolates). *Pseudomonas* species and *Enterobacter* species constituted 2.7% and 1.3%, respectively (Table 4).

Regarding bla\(_{OXA-48}\) gene production, *E. coli* was the organism that harboured the bla\(_{OXA-48}\) gene the most, which constituted 12.0%
Table 4. Cross tabulation between the isolated Gram-negative bacilli and Modified Hodge Test (MHT).

<table>
<thead>
<tr>
<th>Isolated Gram-negative bacilli</th>
<th>MHT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>E. coli</td>
<td>27(36%)</td>
<td>18(24%)</td>
</tr>
<tr>
<td>Klebsiella species</td>
<td>12(16%)</td>
<td>11(14.6%)</td>
</tr>
<tr>
<td>Proteus species</td>
<td>2(2.7%)</td>
<td>2(2.7%)</td>
</tr>
<tr>
<td>Pseudomonas species</td>
<td>0</td>
<td>2(2.7%)</td>
</tr>
<tr>
<td>Enterobacter species</td>
<td>1(1.3%)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>42(56%)</td>
<td>33(44%)</td>
</tr>
</tbody>
</table>

Table 5. Frequency of the resistant Gram-negative bacilli isolates that harboured bla\textsubscript{OXA48} and bla\textsubscript{IMP} genes, as obtained by PCR.

<table>
<thead>
<tr>
<th>Isolated Gram-negative bacilli</th>
<th>Bla\textsubscript{OXA48} Gene</th>
<th>Bla\textsubscript{IMP} Gene</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>E. coli</td>
<td>9(12%)</td>
<td>36(48%)</td>
<td>5(6.7%)</td>
</tr>
<tr>
<td>Klebsiella species</td>
<td>3(4.0%)</td>
<td>20(26.7%)</td>
<td>0</td>
</tr>
<tr>
<td>Proteus species</td>
<td>0</td>
<td>4(5.4%)</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas species</td>
<td>1(1.3%)</td>
<td>1(1.3%)</td>
<td>0</td>
</tr>
<tr>
<td>Enterobacter species</td>
<td>0</td>
<td>1(1.3%)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>13(17.3%)</td>
<td>62(82.7%)</td>
<td>5(6.7%)</td>
</tr>
</tbody>
</table>

Discussion

In this study, E. coli was the most predominant organism among the isolated Gram-negative bacteria followed by Klebsiella species. This result agreed with the study done by Hayajneh, et al. in Jordan 2011–2013, and disagreed with the study done by Mataseje, et al. in Canada 2009–2010, in which Pseudomonas species was the commonest isolate\textsuperscript{14,15}. This may be due to the difference in sample size and study area.

The resistance to carbapenem antibiotics in this study was 50.3%, which is not in agreement with a previous study conducted in India by Henkhoneng Mate, et al., the study conducted by Hayajneh, et al. in Jordan, and the study conducted by Gladstone et al., which showed that resistance to carbapenem antibiotics was 30%, 1.6%, and 12.2% respectively\textsuperscript{2,13,16}. This may be due to the uncontrolled and misuse of antibiotics including broad spectrum antibiotics in our study area (Sudan)\textsuperscript{17}.

Urine samples had the maximum number of carbapenem resistant isolates, which was similar to the study conducted by Henkhoneng Mate, et al. in India\textsuperscript{1}.

Carbapenemase production constituted 42 isolates (56%) of the total carbapenem resistant isolates, which disagreed with the study conducted in Tanzania 2013. That study showed that 35% of the resistant isolates were carbapenemase producers; this may be to the difference in the methods used for enzyme detection, since the previous study used only genotypic methods, while the present study used both phenotypic and genotypic methods\textsuperscript{14}.
A study conducted in India 2015 by Panduragan, et al reported that 62% of isolates produced carbapenemases, found using both phenotypic and genotypic methods, which is agreed with the current study, which reported a near percentage (56%)\textsuperscript{12}.

In this study, the most carbapenemase producing organism was E. coli, which disagrees with a previous study done by Mushli et al, who reported that K. pneumoniae was the most predominant carbapenemase producer\textsuperscript{14}.

Regarding MHT, 50.6% of the total carbapenem resistant isolates were positive for carbapenem production. This was not in agreement with a study conducted in India 2015 by Pandaragan, et al, who reported lower percentage (30.5%), and also disagreed with the study conducted by Henkhoneng Mate, et al in the same country, which reported a higher percentage (60.4%)\textsuperscript{12,14}. This may be due to the difference in study area, which may affect the types and percentage of carbapenemase enzymes according to their spread.

Regarding bla\textsubscript{OXA48} gene, 17.3% of the isolates harbour this gene, which disagreed with a previous study done by Memish, et al in Saudi Arabia 2015. This previous study showed a higher percentage (61.3%) of isolates with the bla\textsubscript{OXA48} gene, which may be due to the different study area\textsuperscript{14}.

bla\textsubscript{IMP} gene was harboured by 6.7% of the resistant isolates, which also disagreed with the study done in Tanzania (2013) and Thailand (2008). These studies showed that 21.6% and 15.4% of the resistant isolates, respectively, harbour the bla\textsubscript{IMP} gene\textsuperscript{14,16}. In addition, a study performed by Nasr El-din in Egypt 2014 revealed that the bla\textsubscript{IMP} gene was absent from isolates\textsuperscript{19}. This may be due to the different study area, the difference in sample size and the five different PCR assays that were performed in the study done in Tanzania, thus increasing the sensitivity and specificity compared to the present study.

In this study, bla\textsubscript{OXA48} gene was the most predominant carbapenemase harbouring by the resistant isolates. This is in agreement with the study performed by Memish et al in Saudi Arabia 2015\textsuperscript{15}. However, our results disagreed with the study performed in Tanzania 2013, which showed that bla\textsubscript{IMP} gene was the most predominant\textsuperscript{14}. This may be due to the difference in sample size and the five different PCR assays that were performed in this study.

Conclusion
This study concludes that the percentage of resistance to carbapenem in Khartoum state, Sudan, is very high and must be taken in consideration by the Sudanese government, and any large organization that can help in the control and prevention of carbapenemases, such as the World Health Organisation. E. coli, followed by Klebsiella species, were the most carbapenem resistant organisms and the largest carbapenemase producers. MHT is a simple method that can detect many types of carbapenemases enzymes, but it cannot detect some types of and does not specify the type of enzymes produced. In contrast, PCR is more sensitive, rapid and specific method for detection of specific types of carbapenemase enzymes. Additionally in the present study, the percentage of Gram-negative bacilli that produce bla\textsubscript{OXA48} gene was more than those producing bla\textsubscript{IMP}; however, one of the isolates harboured both bla\textsubscript{OXA48} and bla\textsubscript{IMP} genes. In this study, some Gram-negative bacilli were positive for carbapenemase enzyme production by both MHT and PCR, some were negative by MHT and positive by PCR, and some of them were positive by MHT and negative by PCR (this study did not detect all carbapenemase genes using PCR; thus those positive by MHT but negative by PCR may process other type of carbapenemase enzymes rather than OXA48 and IMP. Although this may also be because the gene is not expressed).

Recommendations
- A larger sample size should be tested to cover a wider range of isolates.
- Other specific tests for detection of carbapenemases enzymes should be used, such as EDTA disc synergy test, MDI, RDS, MBL E test, more primers should be used to detect most types of carbapenemase enzymes using PCR, and different types of PCR assays should be coupled with each other to increase the sensitivity for enzyme detection.
- Detection of carbapenemase producers should be introduced as routine tests in microbiology labs for rapid detection of resistant isolates and to control their spread, especially for newly admitted patients to the hospitals.
- Prevention and control programs of carbapenem resistant Gram-negative bacteria should be performed to prevent the spread of carbapenemase producers, which includes appropriate use of antimicrobials and facility-level prevention strategies, as recommended by the CDC.

Data availability
Dataset 1: Raw data for the 147 specimens analysed showing the results of the genotypic and phenotypic tests. The file contains the same data in Excel and SPSS formats. DOI, 10.5256/f1000research.12432.d17637\textsuperscript{20}.

Dataset 2: Pictures related to the methods used in the current study (in zipped file): ‘Standard antimicrobial susceptibility testing’, ‘Modified Hodge Test (MHT)’ and ‘Molecular detection of blaIMPs and blaOXA 48 genes’. DOI, 10.5256/f1000research.12432.d17637\textsuperscript{18}.

Competing interests
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References

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