RESEARCH ARTICLE

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

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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 *bla_{OXA48}* gene, and 6.7% harbored *bla_{IMP}* gene. *E.coli* was the most bacteria that harbored the *bla_{OXA48}* followed by *Klebsiella* species. *bla_{IMP}* gene was harbored only by *E.coli*.

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Any reports and responses or comments on the article can be found at the end of the article.		

Conclusion: The percentage of resistance to Carbapenems due to production of Carbapenemase enzymes is very high in Sudan. $\text{bla}_{\text{OXA48}}$ gene is more predominant than bla_{IMP} in this study.

Keywords

carbapenem resistance, carbapenemase enzymes, gram-negative bacilli, Modified Hodge Test, PCR, bla_{IMP} gene, $\text{bla}_{\text{OXA48}}$

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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^{1,2}. carbapenemases (also known as carbapenem hydrolyzing enzymes) represent the most versatile family of beta-lactamases³. Beta-lactamases can be classified according to their functional and molecular properties. Functional classification divides beta-lactamases into four major functional groups (1, 2, 3, and 4), whereas in molecular classification Ambler and others have classified beta-lactamases into four classes (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)³. <https://www.microrao.com/micronotes/pg/carbapenemases.pdf>

Oxacillinases (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^{3,4}. <https://www.microrao.com/micronotes/pg/carbapenemases.pdf>

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* members⁵. <https://www.microrao.com/micronotes/pg/carbapenemases.pdf>

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 transmissible⁶. <https://www.safetyandquality.gov.au/wp-content/uploads/2013/12/MRGN-Guide-Enterobacteriaceae-PDF-1.89MB.pdf>

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. Oxacillinases especially bla_{OXA48} was the most predominant in the whole country⁷.

In Sudan there are limited reports of carbapenemase producers, therefore the present study was performed⁸. 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 bla_{OXA 48} and bla_{IMP} 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 confidentiality 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° 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)⁹.

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 Guidelines⁹. 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 manufacturer⁹.

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 production¹⁰. 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)¹¹.

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)¹².

Negative MHT: No growth of *E. coli* along the tested organism growth streak within the disc diffusion zone¹².

Molecular detection of bla_{IMP} and bla_{OXA48} 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 (10⁶ to 10⁷) 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

PCR. A multiplex conventional PCR was designed to detect bla_{IMP} and bla_{OXA48} using specific primers for each. The primers were obtained from Macrogen Company, Korea (Table 1).

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_{IMP} and bla_{OXA48} 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 minutes¹³. PCR product (5µl) were analyzed by gel electrophoresis in 1.0% agarose stained with ethidium bromide. The results were photographed under ultra-violet light machine (Transilluminator; Uvite, UK) to detect the specific amplified product by comparing it with 100 base pairs standard DNA ladder (Figure 2)¹⁴.

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.

Table 1. Sequences and length of bla_{OXA} and bla_{IMP} primers.

Primer	Sequence 5' - 3'	Length (bp)
BlaOXA-48-F	GCTTGATCGCCCTCGATT	238
BlaOXA-48-R	GATTTGCTCCGTGGCCGAAA	
BlaIMP-F	TCGTTTGAAGAAGTTAACGG	568
BlaIMP-R	ATGTAAGTTTCAAGAGTGATCC

Figure 2. Amplified DNA under ultra violet light machine showing bla_{OXA48} 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_{OXA48} gene; Lanes 4–7, negative samples.

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_{OXA48} and bla_{IMP} genes was performed for all 75 resistant isolates. 17.3% of the resistant Gram-negative isolates were positive for bla_{OXA48} 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_{OXA48} gene production, *E.coli* was the organism that harboured the bla_{OXA48} gene the most, which constituted 12.0%

Table 2. Cross tabulation between the type of specimens and the isolated Gram-negative bacilli.

Type of specimens	The isolated Gram-negative bacilli					Total
	<i>E.coli</i>	<i>Klebsiella</i> species	<i>Proteus</i> species	<i>Pseudomonas</i> species	<i>Enterobacter</i> species	
Urine	67	24	12	2	1	106
Wound swab	12	6	4	3	0	25
Blood	0	10	0	0	0	10
Sputum	0	3	0	0	0	3
Semen	1	1	0	0	0	2
Tissue	0	0	1	1	0	2
Eye swab	1	0	0	0	0	1
Total	81	44	17	6	1	149

Table 3. Cross tabulation between isolated Gram-negative bacilli and carbapenem susceptibility testing by disc diffusion method.

Isolated Gram-negative bacilli	Carbapenem (Meropenem) susceptibility testing			Total
	Sensitive	Intermediate	Resistant	
<i>E.coli</i>	28(18.8%)	8(5.4%)	45(30.2%)	81(54.4%)
<i>Klebsiella</i> species	16(10.8%)	5(3.3%)	23(15.4%)	44(29.5)
<i>Proteus</i> species	9(6.0%)	4(2.7%)	4(2.7%)	17(11.4)
<i>Pseudomonas</i> species	4(2.7%)	0	2(1.3%)	6(4.0%)
<i>Enterobacter</i> species	0	0	1(0.7)	1(0.7)%
Total	57(38.3%)	17(11.4%)	75(50.3%)	149(100%)

Table 4. Cross tabulation between the isolated Gram-negative bacilli and Modified Hodge Test (MHT).

Isolated Gram-negative bacilli	MHT		Total
	Positive	Negative	
<i>E.coli</i>	27(36%)	18(24%)	45(60%)
<i>Klebsiella</i> species	12(16%)	11(14.6)	23(30.7%)
<i>Proteus</i> species	2(2.7%)	2(2.7%)	4(5.3%)
<i>Pseudomonas</i> species	0	2(2.7%)	2(2.7%)
<i>Enterobacter</i> species	1(1.3%)	0	1(1.3%)
Total	42(56%)	33(44%)	75(100%)

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

Isolated Gram-negative bacilli	Bla _{OXA48} gene		Bla _{IMP} gene		Total
	Positive	Negative	Positive	Negative	
<i>E.coli</i>	9(12%)	36(48%)	5(6.7%)	40(53.3%)	45(60%)
<i>Klebsiella</i> species	3(4.0%)	20(26.7%)	0	23(30.7%)	23(30.7%)
<i>Proteus</i> species	0	4(5.4%)	0	4(5.3%)	4(5.3%)
<i>Pseudomonas</i> species	1(1.3%)	1(1.3%)	0	2(2.7%)	2(2.7%)
<i>Enterobacter</i> species	0	1(1.3%)	0	1(1.3%)	1(1.3%)
Total	13(17.3%)	62(82.7%)	5(6.7%)	70(93.3%)	75(100%)

of the 17.3% bla_{OXA48} gene positive result, followed by *Klebsiella* species (4%), and *Pseudomonas* species (1.3%). *Proteus* species and *Enterobacter* species were negative for bla_{OXA48} gene. bla_{IMP} gene was positive only for *E.coli* (constituted the whole percentage of resistant bacteria that harbored bla_{IMP} gene). None of the other types of resistant Gram-negative isolates harboured the bla_{IMP} gene (Table 5).

Dataset 1. Raw data for the 147 specimens analysed showing the results of the genotypic and phenotypic tests

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The file contains the same data in Excel and SPSS formats.

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 blaIMP and blaOXA 48 genes'

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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^{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^{2,13,16}. This may be due to the uncontrolled and misuse of antibiotics including broad spectrum antibiotics in our study area (Sudan)¹⁷.

Urine samples had the maximum number of carbapenem resistant isolates, which was similar to the study conducted by Henkhoneng Mate, *et al* in India².

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 methods¹⁴.

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%)¹².

In this study, the most carbapenemase producing organism was *E. coli*, which disagrees with a previous study done by Mushi *et al*, who reported that *K. pneumoniae* was the most predominant carbapenemase producer¹⁴.

Regarding MHT, 50.6% of the total carbapenem resistant isolates were positive for carbapenemase 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%)^{2,12}. 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_{OXA48} gene, 17.3% of the isolates harboured 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_{OXA48} gene, which may be due to the different study area¹⁸.

bla_{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, harboured the bla_{IMP} gene^{14,19}. In addition, a study performed by Nasr El-din in Egypt 2014 revealed that the bla_{IMP} gene was absent from isolates²⁰. 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_{OXA48} gene was the most predominant carbapenemase harboured by the resistant isolates. This is in agreement with the study performed by Memish *et al* in Saudi Arabia 2015¹⁸. However, our results disagreed with the study performed in Tanzania 2013, which showed that bla_{IMP} gene was the most predominant¹⁴. 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 carbapenems 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 carbapenemase 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_{OXA48} gene was more than those producing bla_{IMP}; however, one of the isolates harboured both bla_{OXA48} and bla_{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 carbapenemase 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.d176377](https://doi.org/10.5256/f1000research.12432.d176377)²¹.

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 bla_{IMP} and bla_{OXA48} genes'. DOI, [10.5256/f1000research.12432.d176378](https://doi.org/10.5256/f1000research.12432.d176378)²².

Competing interests

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Tanzina Nusrat

Department of Microbiology, Chattogram Medical College, Chattogram, Bangladesh

- What is the study population?
- How many gram positive bacteria did you find, how did you separate them? Why is there no method for that?
- There is no odds ratio, please add the odds ratio?
- Why is the infection rate so high? Briefly describe the study limitation.
- Why didn't you choose the other genes?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 17 Dec 2021

Reem Dahab, University of Medical Sciences and Technology, Khartoum, Sudan

Greetings,

I am Reem Dahab the author of this paper. I am very glad that you reviewed my paper.

I would like to answer your questions.

1. What is the study population?

The study population are the patients attended to different hospitals in Khartoum state having bacterial infections in the period from February to August 2016.

2. How many gram positive bacteria did you find, how did you separate them? Why is there no method for that?

I didn't mention them because my research concerns gram negatives, the method mentioned above in detail is for isolation of gram negative bacilli from gram positive ones. Culture media were used to identify gram negative from gram positive ones according to the clinical specimen type for primary culture then gram staining was performed to differentiate between gram negatives and gram positives.

Chromogenic agar culture was performed. Subculturing to purify gram negative isolates were done in blood agar and MacConkey agar media, then , biochemical tests were used for differentiation. At this time of submission I was limited to a word count for this paper, but there is a whole thesis written in detail especially methods and results, from it I wrote this small paper.

3. There is no odds ratio, please add the odds ratio?

Can you clarify this questions because the statistics were done by specialist.

4. Why is the infection rate so high? Briefly describe the study limitation.

The sample size is small, due to the budget limitations because as the research was self-funded and I was a masters degree student. This paper is taken from a complimentary thesis. The infection rate is very high in our country Sudan as no preventive methods are followed as well as the over the counter use of antibiotics. There are many causes included here, but unfortunately this is the fact, regarding a large amount of research in Sudan.

5. Why didn't you chose the other genes?

I wanted to cover all genes to find if there is an increased rate from the previous studies in our country and Africa, but according to my budget it was very expensive so I reviewed the most abundant genes in Africa and in our region. I checked each carbapenemase family the most important for me as per previous studies.

I will improve my discussion in my new papers with my respect. There will be a new version soon, as the word count is no longer a matter this will include more details adhering to the journal policies.

With my respect, I am grateful.

Best Regards,

Reem Dahab

Competing Interests: No competing interests were disclosed.

Reviewer Report 16 February 2021

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Elsa De La Cadena

Grupo de Resistencia Antimicrobiana y Epidemiología Hospitalaria, Universidad El Bosque, Bogotá, Colombia

It is an interesting investigation, which provides relevant information. But it needs to have a better methodological design.

Enterobacteriaceae are currently called Enterobacterales. Nowadays, Enterobacteriaceae is a limited group of species within the Enterobacterales. It is suggested to change the term.

They use Modified Hodge Test. Currently, THM is not the recommended method to identify carbapenemases. It has very low sensitivity for metalloenzymes. Should not be performed on *Pseudomonas*

To identify resistance to carbapenems it is better to use ertapenem in *Enterobacterales*. It is the first carbapenem to rise when there is a resistance mechanism, and it could still be sensitive or intermediate to meropenem.

The ideal would have been to search for NDM, VIM and KPC that have been previously described in African countries and could be the cause of resistance. Due to this, it is wrong to speak of the

percentage of carbapenemases if the presence of other carbapenemases is not ruled out.

The discussion needs to be improved, it is a bit repetitive.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Bacterial resistance in Gram negative.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Dec 2021

Reem Dahab, University of Medical Sciences and Technology, Khartoum, Sudan

Greetings,

I am the author of this paper Reem Dahab. The research was done in 2016. The MHT was from the most useful method, easiest and cheapest one. So this was one of my goals which concluded that It is simple to detect many carbapenemases, but it can't detect all. This conclusion is written on the thesis.

I couldn't write detailed method and conclusion here and I couldn't write my recommendations as well because at the time of writing I had to adhere to a word count.

I know its poor sensitivity regarding some families, but my research is about just 2 genes according to my budget which were confirmed by PCR.

Regarding the ertapenem, I read many research articles that compare the best means to check the carbapenem resistance, all said meropenem and this is why I used it according to previous studies.

You said I should engage with research on VIM, NDM, KPC which are previously described in Africa. I already saw the previous studies before I chose the genes, I wish to do it all but lacked funding. So I chose the most abundant resistance genes in Africa at that time. I didn't include the KPC because I already tried it and I had a problem with the primer so I excluded it.

I said the percentage of carbapenemases because those were the most abundant ones in Africa, as well as any other percentage written were related to the 2 genes detected.

I will improve my discussion in my new papers with my respect. There will be a new version soon, as the word count is no longer a matter this will include more details adhering to the journal policies.

Best Regards,

Reem Dahad

Competing Interests: No competing interests were disclosed.

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