Prevalence and Molecular Characterization of Carbapenem resistance Gram negative bacilli among hospitalized patients in Khartoum state

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Research article

Keywords: Gram negative bacilli, carbapenemase producing, carbapenem resistance gene

Posted Date: September 7th, 2019

DOI: https://doi.org/10.21203/rs.2.14050/v1

Abstract

Background

Carbapenems are broad-spectrum β-lactam antibiotics widely prescribed for the treatment of multidrug-resistant gram negative bacilli in systemic infections. In the past ten years the Carbapenem resistance among gram-negative bacilli is rapidly expanding across nosocomial infection isolates. This study was conducted to determine the prevalence and to characterize Carbapenem resistance genes among Gram negative bacilli (GNB) isolated from patients treated in hospitals in Khartoum state, Sudan.

Methods

A cross-sectional laboratory based study was conducted over six months at the microbiology department in Soba University Hospital and Institute of Endemic Diseases, University of Khartoum. A total of 206 GNB isolates from different clinical specimens were analyzed for carbapenem resistance genes using phenotypic tests and affirmed by genes detection. Multiplex PCR was performed for each strain to detect the carbapenemase genes, including those encoding the NDM-, VIM-, and IMP -type Metallo-beta-lactamases, the class A KPC-type carbapenemases, and the class D OXA-48 enzyme. In addition to CTXM, TEM and SHV. DNA sequencing and bioinformatics analysis were used to detect genes subtypes.

Results

Of 206 isolates, 171 (83%) positive phenotypically and 121 (70.7%) from 171 isolates were confirmed for present one or more carbapenemase gene. NDM-types were the most predominant genes, blaNDM 107(88.4%), followed by blaIMP 7 (5.7%), blaOXA-48 5(4.1%), blaVIM 2 (1.6%) and blaKPC 0 (0%). Co-resistance genes with NDM producing Gram-negative bacilli were detected in 87 (81.3%) of all NDM positive isolates. Correlation between phenotypic and genotypic resistance was observed (P- value < 0.001). Carbapenemase genes were mostly detected in the K. pneumoniae 70 (42.6%), followed by P. aeruginosa 33 (20%), A. baumannii 30 (18.2%) and E. coli with 18 strains (10.9%). NDM-1 was detected in 75 isolates (70%), other subtypes of NDM were identified by sequencing were NDM- 5 and NDM-6.

Conclusions

The prevalence of carbapenemase producing bacilli was fond to be high in Khartoum hospitals. NDM was found to be the most prevalent carbapenemase genes among clinical isolates and belong to Indian lineage. For prevention infection control and regular surveillance must be enhanced.

Introduction

Carbapenems are important broad-spectrum β-lactam antibiotics widely prescribed for the curing of multidrug-resistant gram negative bacilli in systemic infections. Carbapenems have been considered as a robust antibiotic to treat the extended spectrum β-lactamases (ESBLs) in the past ten years [1]. ESBLs are one of the most common resistant genes distributed among gram negative bacilli through plasmids and

transposons [2] and the novel β-lactamases with direct carbapenem-hydrolyzing activity has contributed to an increased prevalence of carbapenem resistant *Enterobacteriaceae* (CRE), which is causing therapeutic failure worldwide [3]. Carbapenemase enzymes including New Delhi Metallo-beta-lactamase (blaNDM), veron integron metallo-beta-lactamases (blaVIM), imipenemase (blaIMP), *Klebsiella pneumoniae* carbapenemases (blaKPC), and oxacillinase–48 (blaOXA–48) [4]. These enzymes are encoded by what is termed carbapenem resistance determining genes (CRDG), which hydrolyze β-lactam drugs including Carbapenems and other β-lactam agents [5]. Moreover resistant to carbapenem can occur by other mechanisms including overproduction of ESBL or AmpC enzyme in combination with porin mutations by reduced outer membrane permeability and activation of multidrug efflux pumps in response to antibiotic exposure [6]. Carbapenem resistance genes are enhancing mechanism of antibiotic resistance among the family *Enterobacteriaceae* and non-lactose fermenting gram-negative bacilliin consequence of the selective pressure assessed by inadequate use of carbapenem and third generation cephalosporins [7]. Moreover plasmids coding for carbapenemase enzymes may carry co-resistance genes for other β-lactam and non β-lactam antibiotics [5]. Detection of carbapenemase production by clinical microbiology laboratory is essential to guide the clinicians to provide appropriate therapy and update treatment guild lines furthermore provide evidence on clinically observed treatment failure using molecular analysis.

The major problem in Sudan is the lack of control over antimicrobial use and despite the lack of specific data about the incidence of AMR in Sudan. This study was designed to describe the current situation of carbapenemase producing Gram-negative bacteria and determine the resistance genes involved in Khartoum state, Sudan.

Material And Methods

Study design and clinical strains

A cross-sectional laboratory based study was conducted at the microbiology department in Soba University Hospital and institute of Endemic Diseases, University of Khartoum; involving gram negative clinical bacterial isolates, that suspected as carbapenemase producing strains based on carbapenem sensitivity testing zone inhibition (zone size less than 20mm). These were isolated from a variety of clinical specimens: blood, urine, swabs, sputum, a tip of catheters, and body fluids, between October 2016 and February 2017 from hospitalized patients in Soba University Hospital. Quality control strains used in antimicrobial susceptibility testing and the biochemical test had been used for primary identification [8]. Molecular identification (PCR) using species specific primers for *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumannii* and Universal primers (16SrRNA) were used for more confirmation Table.1. All these strains stored in 20% glycerol at –20 °C until use.

Subculturing and Disk Diffusion Susceptibility Testing

Isolates were subcultured on blood agar (BA) and then subjected to susceptibility testing to the following antibiotics: Amoxycillin clavulanate (AMC) (30µg); Cefuroxime (CXM)(30µg); Cephalexin (CL)(30µg); Ceftriaxone (CRO)(30µg); Ceftazidime (CAZ) (30µg); Cefotaxime (CTX)(30µg); Meropenem (MEM)(10µg) Imipenem (IPM) (10µg); Amikacin (AK)(30 µg); Gentamicin (Gen)(10 µg); Ciprofloxacin (CIP)(5 µg); Trimethoprim-sulfamethoxazole (SXT)(25 µg); Temocillin (TEM)(30 C); Azteroname (AZT)(30 µg). That was done using the Kirby Bauer disk diffusion; each isolate was swabbed on the Muller-Hinton agar and the antibiotic discs were placed on top and incubated at 37° C for 18–24 hours and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [9].

Phenotypic screening and confirmatory test for carbapenemase

The bacterial isolates screened for carbapenemase production according to CLSI guidelines (CLSI, 2017). In this method, carbapenem antibiotics, meropenem (MEM) and imipenem (IPM) discs (10 g, each) (Mast Diagnostic, UK) were used and Isolates that showed a zone of inhibition ≤ 21 mm in diameter for meropenem were considered as suspected carbapenemase producers. Phenotypic confirmatory test for carbapenemases production by Boronic acid synergy test for class A β-lactamases, the EDTA synergy for Metallo-β-lactamase and the Modified Hodges Test (MHT) for detecting KPC and OXA–48 producers [10].

Detection of Carbapenemase encoding genes

The PCR was carried out using thermal cycler (analytikjena® Biometra TADVANCED, Germany), by using the following primers (Macrogen, Korea), *bla* VIM, *bla IMP, bla NDM, bla NDM*-1, *bla KPC, bla OXA–48, bla*TEM, *bla* SHV and *bla* CTX-M genes Table 2. The reaction was carried out in a total reaction volume of 25 µl (5µl Master mix of Maxime RT premix kit (*iNtRON BIOTECHNOLOGY,* Seongnam, *Korea),*, 0.6 µl of forward primer, 0.6 µl of reverse primer, 2µl of DNA and 16.8 µl deionized sterile water). The reaction was conducted for 30 cycles [6]. The purity and integrity of each PCR product were evaluated electrophoresis in a 2% agarose gel in TBE 1X, that contain 2.5 µl of (20mg/ml) ethidium bromide at 100V for 40 min. The specific amplified product was detected by comparing with 100 base-pairs standard DNA ladder (*iNtRON BIOTECHNOLOGY, Seongnam, Korea)* Bands were visualized under U.V transilluminater (*analytikjena® Biometra BDAcompact, Germany)*..

DNA Sequencing

The PCR product of *bla* NDM genes and 16srRNA were purified and Sanger sequencing was performed by Macrogen Company (*Seoul, Korea)*..

Bioinformatics Analysis

First of all we ensure the ambiguous sites are correctly called and determined the overall quality of the sequences proofed the nucleotides chromatogram by using Finch TV software version 1.4.0 (http://www.geospiza.com/Products/finchtv.shtml). Then nucleotides sequences of the NDM genes achieved were searched for sequence similarity using nucleotide BLAST [11] (http: //blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment for highly similar sequences, which retrieved from NCBI using the MEGA version 7 software [12]. Phylogenetic tree of *bla* NDM genes and their evolutionary relationship with those obtained from database were conducted using MEGA version 7 [12].

Statistical analysis

Data were analyzed using SPSS software version 20.0. Cross tabulation was used to present the relations between data, qualitative data were performed through χ2test, and significance was set at $p \leq 0.05$.

Results

Antimicrobial susceptibility

Antibiotic resistance pattern is shown in (figure 1). Out of 206 isolates tested by disk diffusion test, the highest percentage of resistance 98%, 93.5% were found in ampicillin and cephalexin respectively, followed by amoxicillin clavulanic acid 90%, cefotaxime 89.7%, ceftriaxone 88.4%, ceftazidime 84.2% and aztreonam 66%, temocillin 64%, co-trimoxazole 78.4% and nitrofurantoin 75.2%. Resistance rate also higher in ciprofloxacin 83.1%, gentamicin 85% and amikacin 70%. Meropenem and imipenem were the most effective antibiotic tested and resistant were observed in high rates with 63.1% and 61.6% respectively.

Prevalence of carbapenemase producing gram-negative bacilli based on phenotypic tests

Carbapenemase activity was detected in 171 (83%) of the 206 clinical isolates were positive for the production of one or more carbapenemase enzymes by phenotypic methods as the following 24 (14%) by MHT method and Boronic acid screen, 105 (61.5%) by the EDTA test and 42 (24.5%) of the isolates were positive for both EDTA and Boronic acid methods. Details of the carbapenemase activity among different strains by phenotypic tests are shown in Table 3. That mean the MBL type is the most prevalent type of carbapenemase hydrolysis enzyme among Gram-negative bacilli in Khartoum state, OXA and KPC types are present at a low level.

Prevalence and distribution of Carbapenemase genes among gram negative bacilli

Carbapenemase genes were detected in 121 (70.7%) from 171 positive carbapenemase producing isolates using PCR, one or more carbapenemase genes were detected in the strains. *bla*NDM was detected in the highest rate among the isolates mainly in *K. pneumonia,* which was the species with the highest number of these genes. *bla*NDM was also detected higher in other strains *A. baumannii, P. aeruginosa* and *E. coli.* The most prevalent gene was *blaNDM* 107(88.4%), followed by *blaIMP* 7 (5.7%), *blaOXA–48* 5(4.1%), *blaVIM* 2 (1.6%) and *blaKPC* 0 (0%). And ESBL were detected among these strains, it showed a high prevalence in 183 isolates (88.8%) as the following CTXM 127(61.6%), SHV 84(40.7%) and TEM 80(38.8). The genes were unevenly distributed among the different study isolates for more details table 4.

Co resistance genes carried with NDM gene among gram-negative bacilli

Many strains were herbed more than one gene with NDM gene. Co-resistance carbapenemase genes were observed in few isolates, NDM + OXA–48 were detected in three strains, while NDM+ VIM and NDM+IMP were detected in two strains one for each other. On the other hand, ESBL were observed in high prevalence with NDM in 87 (81.3%) of NDM positive isolates (107). Most of the strains carried NDM with one ESBL gene in (43.5%) as the following; NDM+ CTXM in (24 isolates, 27.6%), NDM+ TEM (8 isolates, 9.1%), and NDM+ SHV (6 isolates, 6.8%). Strains carried NDM with two ESBL genes in (39.2%) as the following:NDM+ CTXM+ SHV10 isolates, 11.5%),NDM+ CTXM+ TEM10 isolates, 11.5%),NDM+ SHV+ TEM14 isolates, 16.2%). Strains carried NDM with three ESBL genes, NDM+ CTXM+ SHV +TEM (15 isolates, 17.3%). The destitution of co resistance genes among different gram negative bacilli is shown in table 5.

The *frequency of carbapenemase producer Gram-negative bacilli by type of specimens and hospital units*

Carbapenemase producers were more frequent distributed among clinical isolates from blood (36%) and then followed by wounds (24%), urine (21%), body fluids (7%) and catheter tips and sputum (6%) for both.

With regard to the distribution of Carbapenemase producer among hospital units, the most carbapenemase producer isolated from neonatal intensive care unit NICU 32(26%), followed by medicine wards 26(22%) and pediatric wards 22 (18%), Surgery 18(15%), ICU 15(12%) and Renal Unit 8(7%).

Molecular characterization of NDM genes

Out of 107 NDM genes detected 75 (70 %) were NDM–1, other subtypes of NDM were identified by sequencing including NDM- 5, and NDM–6. Figure 2

Bioinformatics analysis of NDM genes

Fourteen samples were successfully sequenced by Macrogen Company. All sample showed 97–100% similarity with NDM genes from the NCBI database with accession number MF379688 and MG764089.

Multiple sequence alignment

The Nucleotide sequences of NDM Deoxyribonucleic acid (DNA) sequences were compared against DNA databank using BLASTp. Fourteen different NDM beta-lactamases genes were compared against the NDM in the database, (http://blast.ncbi.nlm.nih.gov/Blast.cgi), they have produced a significant alignment to NDM–1 beta-lactamase of Klebsiella pneumoniae (gb|MK425054|), and the strains were shown 97% to 100% identity.

Multiple sequence alignment of NDM proteins done by MEGA7 software version 7.0.9.0 against similar proteins that obtained from BLASTp, NDM–1 from Sudan were similar to |KX100583.1| Escherichia coli NDM–1 (blaNDM) gene from India and | MH891562| Klebsiella pneumoniae NDM–1 from Bangladesh. NDM–5 from Sudan were similar to | MH991817 |, Escherichia coli NDM–5fromIndia and | MH168510| Klebsiella pneumoniae NDM–5 from Bangladesh while NDM–6 from Sudan were similar to | MH683607 | Escherichia coli from India, | JN967644 | Escherichia coli from the United States and | JQ235754 | Escherichia coli from New Zealand.

Nucleotide sequence accession number

The sequence of the 14 NDM genes have been deposited in the GenBank database under accession numbers MK033562, MK033563, MK033564, MK363705, MK363706, MK363707, MK363708, MK363709, MK363710, MK371542, MK371543, MK371544, MK37154 5, and MK371546.

Phylogenetic tree

The phylogenetic analysis of the NDM proteins sequences revealed that the NDM–1 and NDM–5 were related to the same NDM lineage as the Indian and Bangladeshi isolates. The NDM–6 gene was found as closed to NDM–6 from India, New Zealand, and the United States as shown in Figure 2.

Discussion

Carbapenems have become the drugs of choice for the treatment of severe nosocomial infections caused by Gram-negative bacilli; however, carbapenemase producing Gram-negative bacilli have been reported worldwide. CRE is a considerable health problem worldwide and associated with increased mortality. The rapid detection of carbapenem resistance and adequate treatment of such cases is therefore mandatory. This study was therefore undertaken, to determine the prevalence of different types of carbapenemase

producing bacteria among Gram-negative bacilli isolated from various hospitalized patients in Khartoum State. Accurate detection of carbapenemase producing microorganisms is a challenge for the laboratories, requiring not only phenotypic tests but also genotypic tests for all genes associated with carbapenemase production. In the present study, among 206 isolates 171(83%) were positive by phenotypic analysis including strains with resistance to carbapenem. Furthermore, genotypic analysis detected 121 (70.7%) positive strains. This finding indicates that the studied resistance is not only associated with enzyme encoding genes but also due to other resistance mechanisms such as overproduction of ESBLs, porin loss or mutations [13,14].

The current situation according to this study, show that the prevalence of carbapenemase producing among different gram negative isolates is increasing up to (83%). This finding is higher than the incidence in a previous study conducted in Khartoum state in 2017 which showed the prevalence was 56% by phenotypic tests [15] and other done in 2013 by Ali reported the MBL was 37.7% among Pseudomonas spp. isolates in Khartoum state [16]. This high frequency of MBL in Khartoum state is a result of excessive use of third generation cephalosporins, in addition to selective of ESBL with prevalence 88%, make the treatment option of those patients was meropenem and excessive use of it lead to release of carbapenem resistance genes. This finding agrees with a study in Egypt reported carbapenem resistance rate was 62.7% among Enterobacteriaceae [17]. As well, carbapenem resistance has been observed in Africa in high rate study conducted by Okoche et a.,l in 2015 in Uganda. He found 28.6% of strains were carbapenemase producer [18]. In Tanzania the prevalence of carbapenemase producer was 35% [19] and higher incidence 68% In South Africa [20]. Low prevalence was observed in Nigeria 11.9% [21]. This finding in the poor populations in Africa may be as a result of unrestricted use of antibiotics in these countries where most people consume the antibiotics without prescription by a clinician [22].

Carbapenemase genes have been recognized during the past ten years, and these genes are associated with mobile genetic elements that allow their rapid circulation among bacterial strains, for instance, NDM type have a potential for rapid spread within the country and to other countries [23]. In this study, carbapenemase genes were detected by using PCR in 121 (70.7%) of the resistant isolates. The most prevalent gene among the isolates was blaNDM (88.4%) mainly in K. pneumonia and other gram negative bacilli including A. baumannii, P. aeruginosa and E. coli, this agrees with studies in India reported the NDM gene was observed between 31% and 55% of Carbapenemase resistance Enterobacteriaceae [24,25], and study in South Africa published the most carbapenemase gene was NDM among K. pneumonia (20). As well, NDM–1 was reported as the most common carbapenemase gene in Saudi Arabia and other Middle Eastern countries [26].

Carbapenemase genes are reported to be more frequent in some regions. For example KPC genes are dominant in some countries such as Greece, Israel, and USA, while NDM genes are prevalent in isolates reported from the Far East, India, and Pakistan [14]. Carbapenemase production in Turkey mostly occurs in OXA type genes (23). OXA–48 was reported first from Turkey, subsequently followed by reports from Middle Asia and Europe as well [27]. In this current study the genes were unevenly distributed among the

different study isolates. In comparison with other carbapenem resistant genes were detected in low prevalence comparing with NDM gene blaIMP (5.7%), blaOXA–48 (4.1%), blaVIM (1.6%) and blaKPC (0%). This finding disagrees with many studies, in Okoche study, the most common gene was blaVIM 1(0.7%), and blaNDM–1 (2.6%) was the lowest gene [18], while Mushi reported IMP types were the most predominant at (21.6%) in his study (19). Other studies reported blaOXA–48 was the most prevalence gene [15,28]. In our study KPC wasn't detected among the isolates that disagree with international reports of high prevalence of KPC genes [14,29].

The blaNDM–1 was first identified in a clinical isolate of K. pneumoniae in New Delhi, India, and suddenly got disseminated around the world [30]. NDM variants have been described, differing by several amino acid changes. A first variant, NDM–2, has been described in an A. baumannii clinical isolate from Egyptian patient in Germany, NDM–4, NDM–5, NDM–6 have been detected from E. coli in India and NDM–7 from E.coli in France [30]. In this study, 107 NDM producer strains had been identified using PCR, the most subtype 75 (70 %) were NDM–1other subtypes of NDM were detected by sequencing including NDM- 5, and NDM- 6 among different Gram negative bacilli including K. pneumoniae, E. coli, A. baumannii, P. aeruginosa and Enterobacter spp.

Carbapenemase genes are becoming largely distributed among Enterobacteriaceae, A. baumannii, P. aeruginosa and other Gram-negative bacilli. The prevalence of carbapenemase producer in each species in this study, higher frequency in A. baumannii (37.3 %) followed by K. pneumoniae (27.3%), P. aeruginosa (24.8 %) and E. coli (21.1%), which agree with many studies that reported A. baumannii and K. pneumonia were the most predominant carbapenemase producer strains [31,32]. The prevalence of carbapenemase producer varies from area to area. This variation could be attributed to differences in time of collection of isolates and differences in study populations and designs. A study in Turkey showed the most carbapenemase strains were K. pneumoniae (13.6%), Pseudomonas spp. (17.8%), A. baumannii (13.8%), S. maltophilia 7.5% and E. coli 2.8% [33]. In Nigeria the highest prevalence of carbapenemase producers was in P. mirabilis (16.0%), then P. aeruginosa, K. pneumoniae (13.3% each) and E. coli (11.5%) [21], while in Tanzania, E. coli was the most prevalent species with carbapenemase producing (14%), followed by K. pneumoniae (10.57%), P. aeruginosa (10.13%), K. oxytoca (1.76%) and A. baumannii (1.3%) [19].

Carbapenemase-encoding genes had been commonly associated with bacteria isolated from blood, urine, wound swabs, and sputum as reported in many studies in Uganda [19], Tanzania [34], Nigeria [21], and India [35]. In this study Carbapenem producer were more frequently isolated from blood (39%) followed by wound (25%) and urine (22%) this is in line with study in South Africa which reported blood was the most common specimen type (25%), followed by urine (22%) [20].

Many studies considered young age as a risk factor for CRE infection which agrees with current finding, that carbapenemase-producing Gram negative bacilli were most frequent in neonate age group isolated from nursery and pediatric wards (26% and 18% respectively). Besides, carbapenemase producers were

observed in high rate among elderly patients from medicine (22%) and ICU (12%), which agrees with another study that found that CRE to be more frequently isolated in the elderly [36].

Carbapenem resistance gram negative bacilli are usually resistant to other routinely used antimicrobial agents [37–39]. The Plasmids carrying carbapenemase genes like NDM–1 are diverse and can harbor a high number of additional resistance genes (e.g., ESBL-alleles) as well as other carbapenemase genes like Oxa–48 types, VIM types, and so forth, as the source of multidrug resistance in one single bacterium [25,40]. Moreover mechanisms of resistance to β-lactam by producing ESBL, AmpC and carbapenemase were also noticed as some of the isolates produce different combinations of the enzymes. In our study co-resistance of NDM with OXA–48, VIM and IMP were reported in few strains. Co-resistance with ESBL (CTXM, SHV and TEM) was detected in high prevalence 87/107 (81.3%) of NDM positive isolates. Most of the strains carried NDM with one ESBL gene in (43.5%), NDM with two ESBL genes in (39.2%) and NDM with three ESBL genes in (17.3%). That with harmony with various studies reported co-resistance among clinical strains [41,42]. These co-production genes among some isolates as observed in this study are indicative of the existence of multi-drug resistant bacteria pathogens. That may be responsible for treatment failure and outbreaks of infections caused by resistant organisms. Resulting in more hospitalization and higher treatment costs as well as disease complications [43].

Conclusion

The prevalence of carbapenemase producing bacilli has been increasing in our setting which worries microbiologists as well as clinicians to prescribing carbapenems. NDM was found to be the most prevalent carbapenemase genes among clinical isolates and belong to Indian lineage. That we need for implementation of drastic infection control measures and regular surveillance to prevent further spread of these resistant organisms among the hospital isolates.In addition,screening for carbapenemase production should be performed in any Gram-negative isolates with any slight decrease in susceptibility to carbapenems.

Declarations

Acknowledgments

We would like to thank the medical and technical staff of Medical Microbiology Department in Soba University Hospital, University of Khartoum for their help in strain and data collection.

Funding

This research received partial research fund from the Ministry of Higher Education and Scientific Research, Sudan (grant number SUD/MOH/MMAH/08/2016).

Authors' contributions

HE, MA and KE designed the study. HE carried out the microbiological and molecular analysis. HE and HA analysed the data. HE and MA wrote the first draft, KE, EM, and HA were contributors in revising the manuscript. All authors read and approved the final manuscript

Ethics approval

Formal permission was obtained from the managers of Soba University Hospital and the Institutional Research Ethics Committee of the Institute of Endemic Diseases, University of Khartoum, approved this study under reference number IEND_REC 12/2017. Patient consent was waived by the Research Ethics Committee.

Data availability

Data is available upon request from the first author.

Conflict of interests

All the authors declare on conflicts of interests.

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Tables

Due to technical limitations, Tables 1 - 5 are only available for download from the Supplementary Files section.

Figures

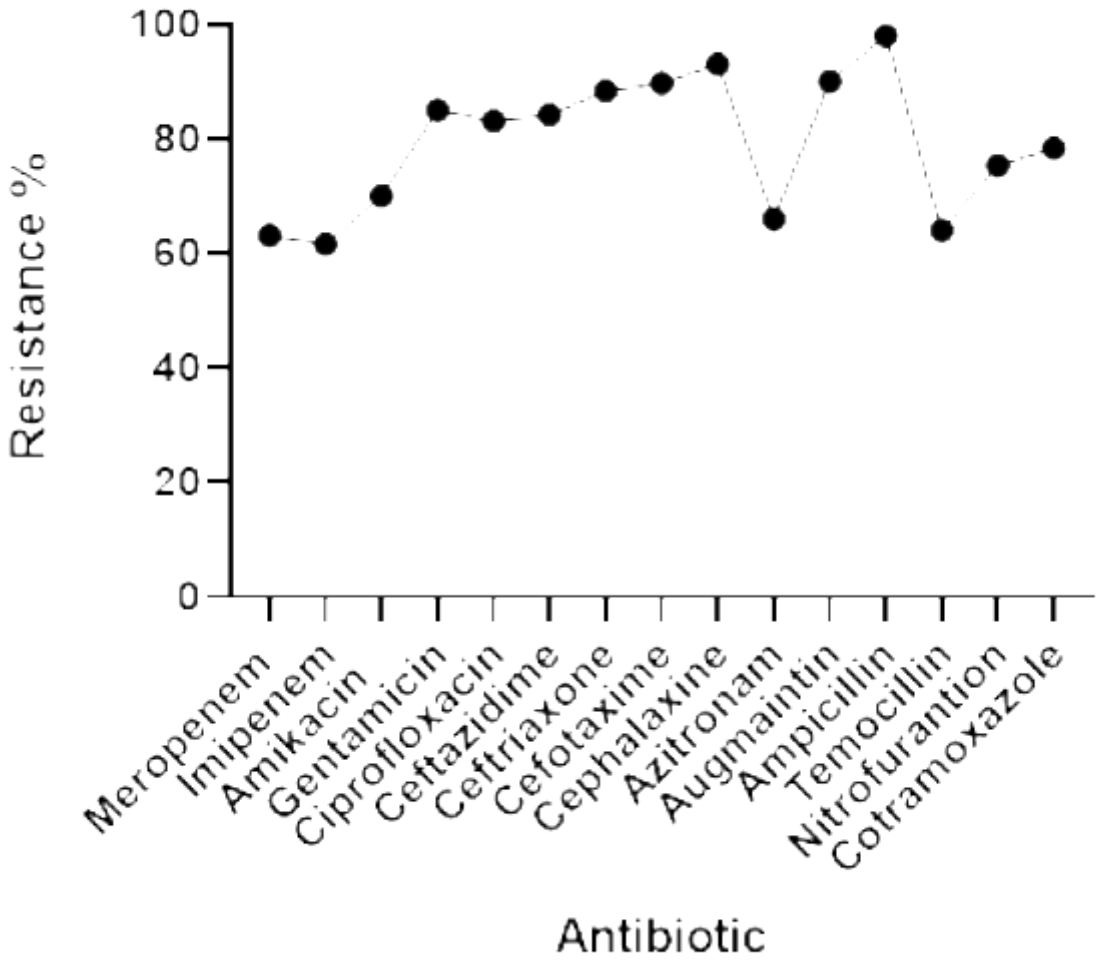

Figure 1

Antimicrobial Resistance pattern among different Gram-negative bacilli isolated from patients treated at Khartoum state hospitals.

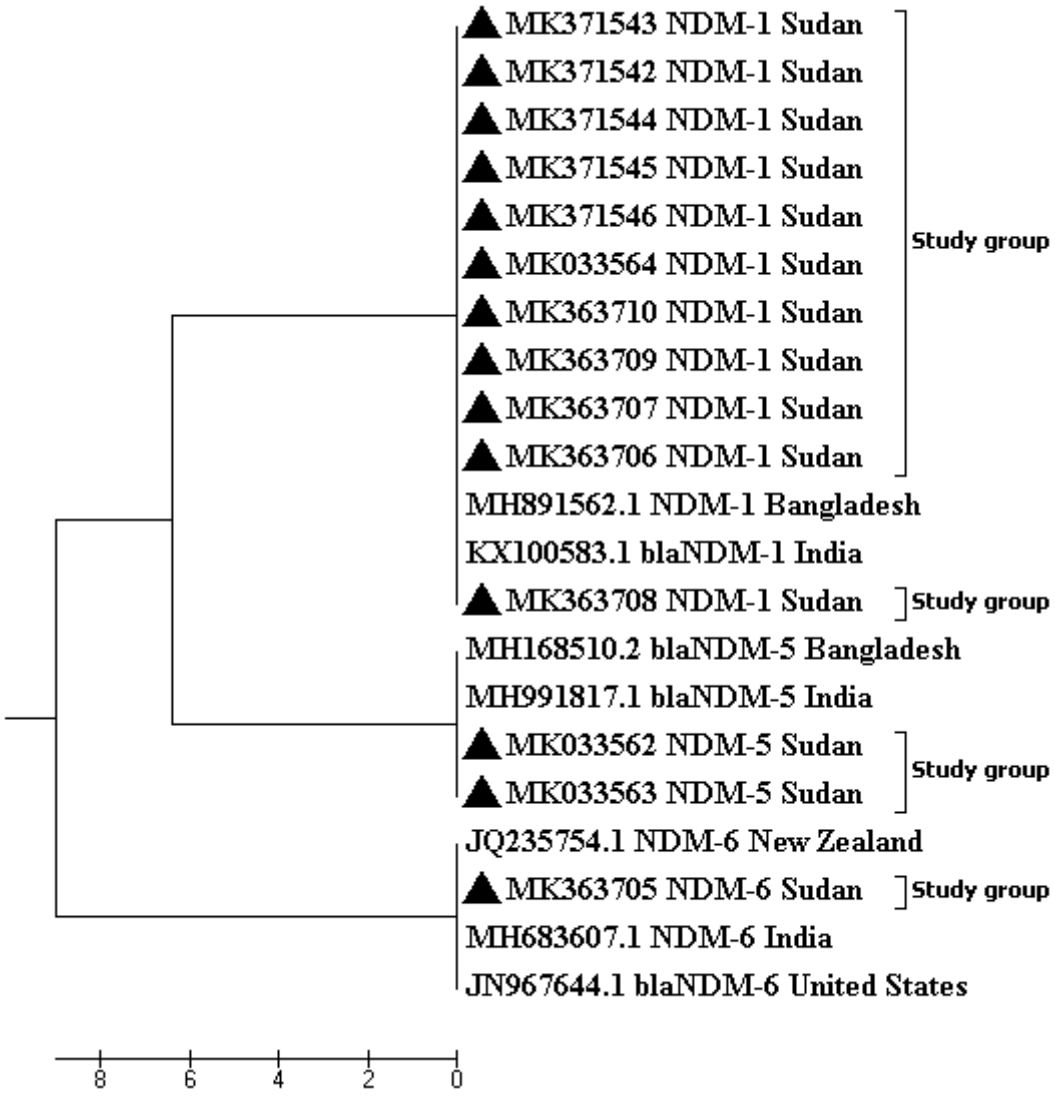

Figure 2

Phylogenetic tree of the 14 NDM isolated from different Gram-negative bacilli: Phylogenetic tree of the 14 NDM genes, Sequences were analysed using MEGA7, the neighbor-joining method, and bootstrap analysis (1,000 replicates) based on the ClustalW algorithm. The scale bar indicates 0.1 nucleotide substitutions per site. Reference sequences shown as: accession number, gene subtype, country. Sequences isolated in this study are designated by grey triangle.

Supplementary Files

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