Molecular Detection of *bla*$_{NDM-1}$ Gene of *Escherichia ~~Coli~~ coli* from Different Clinical Isolates in Khartoum State

Abstract

Background: Increasing reports on NDM-1 producing *E. coli* constitute a serious threat to global health since it is found to be highly resistant to most of the currently available antibiotics including carbapenems.

Objective: This study aim to detect the NDM-1 Gene in *E. coli* isolates recovered from the various clinical samples at Khartoum state in Sudan.

Materials and Methods: A total of 100 *E. coli* isolates were recovered from various clinical samples at Khartoum state. The isolates were identified at the species level using standard biochemical tests. Antibiotic susceptibility tests were performed using the Kirby-Bauer method, the following antibiotics disks were used Cefepime (30µg), Ceftriaxone (30µg), Cefpodoxime (10µg), Meropenem(10µg), Imipenem (10µg) and Amoxicilin(30µg). DNA was extracted using boiling method and they were subjected to the polymerase chain reaction for the detection of bla NDM-1 gene.

Result: The *E. coli* recovered in this study were high resistant to cephalosporins including Cefepime (60%) Ceftriaxone (81%), and found to be complete resistant to the Cefpodoxime (100%). Similar high level of resistance was also observed amongst the *E. coli* isolates to Penicillins including Amoxicilin (82%), Also showed reduced susceptibility to the Carbapenems including meropenem and imipenem at the rate of (94%) and (90%) respectively, in which 9 isolates harbourded NDM-1 gene.

Conclusions: Our finding highlight the incidence of bla NDM-1 gene in *E. coli* isolates (9%). This finding is less than that obtained from previous study. This may be explained by the differences in the time of study, the number and sites of samples collection and the differences in the antibiotics use and consumption.

Keywords: **NDM-1, *Escherichia Coli*, Multidrug resistant (MDR), Clinical Isolates, Khartoum State, Sudan.**

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Introduction:

Escherichia coli is a common inhabitant of the human and animal gut, but can also be found in water, soil and vegetation .it is the leading pathogen causing urinary tract infections, blood stream infections, wounds, otitis media and other complications in human (**Kibret and Abera ,2011**). *Escherichia coli* are gram negative, facultative anaerobic, bacilli belong to the Enterobacteriaceae family. *E. coli* are members of the coliform group, which ferment lactose at 35-37℃ (**Kaper, *et al* .2004**). *E. coli* that infect and cause disease syndrome in the gastrointestinal tract are intestinal pathogenic *E. coli* (IPEC);Those that cause disease syndrome in systems other than gastrointestinal tract are called extra-intestinal *E. coli* (EXPEC) (**Lupindu, 2017**).

In the last few years, the emergence and wide dissemination of *E. coli* strains showing resistance to broad-spectrum of antimicrobial agents has been reported. Multidrug-resistant *Escherichia coli* (MDR *E. coli*) has become a major public health concern in Sudan and many countries, causing failure in treatment with consequent huge health burden (**Ibrahim, *et al.* 2012**).The emergence of Carbapenems resistance among Gram-negative bacteria is a major cause of concern since Carbapenems currently represent the treatment of choice for severe infections caused by multidrug –resistant strains. However, the utility of Carbapenems is under severe threat with the emergence of Carbapenemases including the newly characterized NDM-1(**Fomda, *et al.* 2014**).

Carbapenemase represent the most versatile family of beta lactamase with broad of spectrum unrivaled by other beta-lactam hydrolyzing enzyme, although known as Carbapenemase, many of these enzymes recognize almost all hydrolysable beta lactam and most are resilient against inhibitor by commercially available beta lactamase inhibitors (**Queneen and Bush, 2007**) Carbapenemase is classified according to the ambler categorization system into A, B, and D Carbapenemase; are subdivided into classes of beta lactamase based on the hydrolytic mechanisms in their active sites. serine Carbapenemases are included in class A and D, which referred as being serine dependent because they have serine in the active site, whereas class B Carbapenemase have zinc (zinc dependent) and are referred to as metallo beta lactamas. Among the clinically significant Carbapenemase, the New Delhi metallo beta lactamase (NDM) is currently considered a major concern due to its rapid spread worldwide (**Ismail and Mahmoud, 2018**).

NDM-1 is Carbapenemase beta lactamase enzyme, this enzyme is coded by *bla* $_{\text{NDM-1}}$ or NDM-1gene with molecular mass of approximately 27.5 KD, and it can hydrolyze the beta lactams, particulary carbapenem .They are resistant to clavulanic acid, salbactum and many other commercially available beta lactamase inhibitors (**Charan, *et al.* 2012**). The First Oregon case of NDM -1producing *Escherichia coli* was reported during November 2013 (**Buser, *et al.* 2017**).

NDM-1, cleaves the beta lactam ring, and confers bacterial resistance against most of the beta lactam antibiotics except tigecycline and colistin. Among these two antibiotics, colistin is

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considered toxic, and therefore , its clinical use and dosage need cautious approach .The activity of this enzyme can be inhibited by zinc chelating agents like EDTA(**Kumar, et al. 2018**).

Increasing reports on NDM-1 producing *Escherichia coli* constitute a serious threat to global health since it is found to be highly resistant to most of the currently available antibiotics including carbapenems (**Charan, *et al*. 2012**).

The β-lactam antibiotics constitute one of the oldest classes of antibacterial agents. β -lactam antibiotics are abroad class of antibiotics .The consist of all antibiotic agents that contain a β-lactam ring in their molecular structure (**Dowling, *et al* .2017**).

All β-lactams share the same mode of action: they inhibit the bacterial cell wall synthesis by acting as suicide substrates of the transpeptidase domain of Penicillin Binding Proteins (PBPs). They form a stable covalent adduct with the active site serine residue of PBPs. The PBPs are traditionally partitioned into high molecular weight PBPs (HMW-PBPs), which are further divided in two classes, A and B, and low-molecular weight PBPs (LMW-PBPs), which are also divided in four subclasses based on their tertiary structures (**Zervosen, *et al.*2012**).

Mechanism of action of beta-lactam antibiotics

ß-lactams act as an irreversible inhibitor of the enzyme transpeptidase, an enzyme used by bacteria to make their cell walls (**Dowling, *et al* .2017**).Inhibition of cell wall synthesis is the most common mechanism for antibiotic activity, this prevent the growth of bacteria by inhibiting peptidoglycan synthesis, The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidase knows as penicillin binding proteins (PBPs). PBPs bind to the D-Ala-D-Ala at the end of muropeptides, the peptidoglycan precursors to crosslink the peptidoglycan. B -lactam antibiotics mimic the site and competitively inhibit PBP crosslinking of peptidoglycan.Unlike prokaryotic cells, the eukaryotic cells of humans do not possess peptidoglycan or contain a cell wall. This makes the wall of the bacterial cell an ideal target for antibiotic therapy because the therapy will not target the human cells (**Kaufman, 2011**).

Bacterial resistance to antibiotics can be intrinsic or innate, which is characteristic of a particular bacterium and depends on biology of a microorganism (**Giedraitiene, *et al* .2011**).Where by microorganisms naturally do not possess target sites for the antimicrobials and the antimicrobial does not affect (**Dowling, *et al* .2017**). *E. coli* has innate resistance to vancomycin), and acquired resistance whereby a naturally susceptible microorganism acquires mechanism to not be affected by the antimicrobial (**Dowling, *et al*. 2017**). Acquired resistance occurs from (i) acquisition of exogenous genes by plasmids (conjugation or transformation), transposons (conjugation), integrons and bacteriophages (transduction), (ii) mutation of cellular genes, and (iii) a combination of these mechanisms (**Giedraitiene, *et al* .2011**).

Mechanism of resistance to beta lactam antibiotic

The major mechanism of resistance to β-lactam antimicrobial agents in Gram-negative bacilli is production of β-lactamase hydrolytic enzymes that disrupt the amide bond of the characteristic four-membered β-lactam ring, rendering the antimicrobial ineffective (**Dowling, *et al*. 2017**).

Metallo-B-lactamase (e.g. NDM-1)

Metallobeta_-lactamases (MBLs), a unique group of beta -lactamases both structurally and functionally, are usually produced in combination with a second or third beta –lactamase in clinical isolates. They differ structurally from the other beta-lactamases by their requirement for a zinc ion at the active site. Functionally, they were once distinguished primarily by their ability to hydrolyze carbapenems, but some serine B -lactamases now have also acquired that ability. In contrast to the serine B -lactamases, the MBLs have poor affinity or hydrolytic capability for monobactams and are not inhibited by clavulanic acid or tazobactam. Instead, they are inhibited by metal ion chelators such as EDTA, dipicolinic acid, or 1, 10-*o*-phenanthroline (**Bush and Jacoby, 2010**).

Materials and Methods

Descriptive cross sectional study, conducted in different Khartoum state hospitals and laboratories including: Sharg Al-Neel, Military Medical Hospital, Fedail hospital and Ultra Lab.
Clinical isolates of *E. coli* from different samples (urine, vaginal swab, wound swab and sputum) were collected from different Hospitals and laboratories in Khartoum State.
This study was conducted at period between December 2019 to February 2020. Data in the present study (gender, age, type of specimens from which the clinical isolates were isolated) were obtained from hospitals records.
Ethical approval and informed consent were taken from Al-Neelain University ethical committee and consent was taken from medical director of hospitals included in this study.

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Collection and transport of isolates

Clinical isolates of *E. coli* were collected and transported using- nutrient agar slope media and stored at 4℃.

Identification of clinical isolates

All collected isolates were subjected to purification by sub culturing in MacConkey agar and incubated in incubator aerobically at 37°C for 16-24 h. The purified cultures were examined morphologically for colony characterization on agar media. Characteristic of colonies on MacConkey agar are pink colour indicating lactose ferments. On gram stain ,it appear long gram –negative rods .and they were processed for conventional biochemical testing(Kligler iron agar , Indole test ,Citrate utilization test, Urease test, Motility test, and Methyle red test). *E. coli* are generally motile , Indole positive, Methyle red test positive, Urease negative, citrate utilization test negative and ferment glucose and lactose.

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Antimicrobial susceptibility testing

The susceptibility to antimicrobial agents Cefepime (30µg), Ceftriaxone (30µg), Cefpodoxime (10µg), Meropenem (10µg), Imipenem (10µg) and Amoxicilin (30µg) was determined by Kirby-Bauer discs diffusion method and results will be interpreted as per Clinical and Laboratory Standards Inistitute (CLSI) as guidelines.

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The tested isolates picked up with a sterile wire loop and suspended in sterile normal saline .In a good light, the turbidity of the suspension was prepared equivalent to 0.5 McFarland's standard. A plate of Muller Hinton a gar was inoculated with the suspension using a sterile cotton swab.

The swab evenly over the surface of the medium was streaked. Then discs of antimicrobial were applied by sterile forceps each disc was pressed down to ensure complete contact with the agar surface and they were distributed evenly. The plates were incubated at 37°C for 24h, aerobically then the zone diameter around each antimicrobial disc was manually measured by ruler and recorded.

On the basis of zone diameter, the results were interpreted according to CLSI guidelines.

Molecular detection of NDM-1gene

DNA extraction

Whole- cells DNA were extracted from clinical isolates by boiling methods. Hundred *E. coli* isolates were sub cultured on nutrient agar and the overnight pure bacterial growth were used.

Several bacterial colonies were suspended in 100 µL of sterile distilled water and heated at 100°C for 10 minutes, then centrifuged at 10,000× *g* for 10 minutes. The resulting supernatant contained the bacterial DNA (**Cao, *et al.* 2018**), then transferred to sterile Eppendorf tube and stored at -20°C until used.

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Conventional PCR

The extracted DNA of each isolate was subjected to the polymerase chain reaction (PCR) .detection of blaNDM-1gene with target specific primer set, NDM –Fm (5-GGTTTGGCGATCTGGTTTTC-3, position 133-153): forward primer (FP) and NDM–Rm (5-CGGAAATCACGATC-3, position 734-754): reverse primer (RP) which amplified an internal fragment of 621 bp of the blaNDM-1gene. The amplification of DNA was performed in thermocycler in a final volume of 25 µL containing 12.5 µL of Master Mix (2X), 1 µL of FP (10Mm), 1 µL of RP (10Mm), 8.5 µL of distiller water, and 2 µL of DNA template.The PCR conditions for the amplification of gene were comprised of denaturation at 94°C for10 min,36 cycles of amplification at 94°C for 30 second, 54°C for 40 second, and 72°C for 50 second, and final extension at 72°C for 5 mintues (**Agarwal, *et al*.2018**).

Visualization by Gel electrophoresis

PCR products was separated on 2% agarose gel by electrophoresis, stained with 5 µL ethidium bromide and then visualized under UV gel documentation system. Positive control & negative control were included in PCR reaction, the positive control was obtained from Khartoum University. The negative control containing all components except the DNA template which was replaced by distilled water.

Data analysis

The obtained results were analyzed by the Statistical Package of Social Science (SPSS) soft program version 20, frequency and percent were obtained for frequency tables, cross tables and chi-square statistical analyses were used to determine P value to detect statistical significance.

Results

A number of 100 *E. coli* clinical isolates were collected from different Khartoum state hospitals and laboratories **(**Sharg Al-Neel, Military medical Hospital , Fedail hospital and Ultra Lab),during the period from December 2019 to February 2020 . They were purified and

identified using cultural based technique. After the culture of the samples on MacConkey agar plates, the isolated *E. coli* isolates produced pink-red colonies. On gram stain, it appears long gram –negative rods. The result of the identification studies showed that the *E. coli* isolates were motile, positive for Indole and Methyle red test.

The isolates were obtained from various clinical specimens. Urine specimens represented the majority of specimens, 85(85%) from urine, 10(10 %) wound swabs, 3(3%) sputum and 2(2%) vaginal swabs Figure (.1).

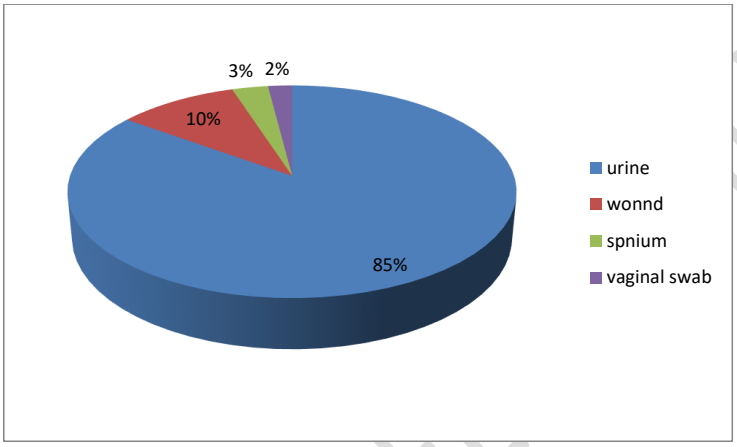

Figure (1): Frequency of *E. coli* isolates among type of specimens

The present study represented that *E. coli* infection was most common among females 71(71%) than males 29(29%) Figure (2).

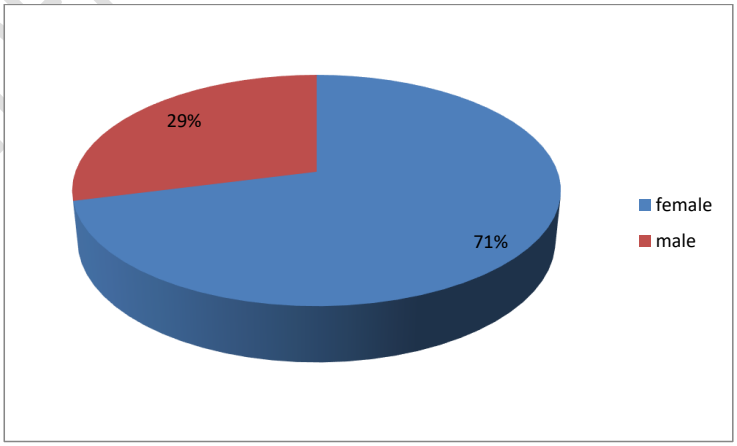

Figure (.2): Gender distribution among study participants

The isolates were collected from patients with age range from 14 to 80 years, the majority of them 80 (80%) were belonging to age group (41-70) years followed by 14(14%) between (10-40) years age group and 6(6%) were belong to age group (71-80) years old Table (1).

Table (1): Frequency of *E. coli* isolates among age groups

Age groups	Frequency
10-40	14
41-70	80
71-80	6

Antimicrobial Susceptibility Testing:

Out of 100 *E. coli* isolates tested by disc diffusion test, the highest percentage of resistance 100% were found in Cefpodoxime, followed by amoxicillin 82%,ceftriaxone 81%,cefepime 60%,imipenem 10%,meropenem 6%.

Polymerase Chain Reaction:

Out of 100 isolates subjected to PCR to detect NDM-1 gene, only 9 (9%) isolates showed a band typical in size (621 bp) to NDM-1 gene Figure (.3) when compared to the standard DNA marker (100 bp), in which 8 isolates (9.4%) was recovered from urine, only one isolates (10%) from wound, 91 isolates (91%) were negative to NDM-1 gene Table (2).

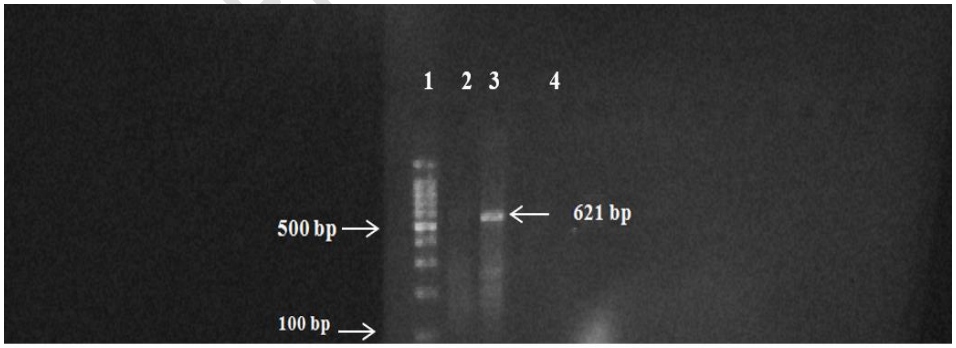

Figure (3): PCR amplification of NDM-1 g e n e on 2% agarose gel electrophoresis, Lane 1 DNA ladder: MW 100bp, La ne 3 showing typical band size of (621 bp) corresponding to the molecular size of NDM-1 gene(NDM-1 Positive), 2, 4(NDM-1Negative).

Table (2): Frequency of NDM-1 gene among different clinical specimens

NDM-1 gene	Sample			Total
	Urine	Wound	Other	
Positive	8(9.4%)	1(10%)	0(0.0%)	9
Negative	77(90.6%)	9(90.0%)	5(100%)	91
Total	85	10	5	100

From females isolates the NDM-1 resistant gene was detected in 5(7%), while in male isolates was detected in 4(13.8%) with insignificant difference (P value =0.667) Table (.3).

Table (3): Frequency of NDM-1 gene among gender

NDM-1 gene	Gender		Total
	Male	Female	
Positive	4(13.8%)	5(7.0%)	9
Negative	25(86.2%)	66(93.0%)	91
Total	29	71	100

The age group of 41 to 70 showed the highest number of patients who were exposed to *E. coli* with a total of 80 (80%) , in which The NDM-1 gene was detected in 6 isolates (7.5) Table (.4).

Table (.4): Frequency of NDM-1 gene among age groups

NDM-1 gene	Age			Total
	10-40	41-70	71-100	
Positive	2(14.3%)	6(7.5%)	1(16.7%)	9
Negative	12(85.7%)	74(92.5%)	5(83.3%)	91
Total	14	80	6	100

81(81%) of isolates were resistant to Ceftriaxone, in which 8 isolates (9.9%) harboured NDM-1 gene (p-value = 0.527).

19(19%) of isolates were sensitive to Ceftriaxone, in which1 isolates (5.3%) harboured NDM-1 gene Table (.5).

Table (5): The association between susceptibility pattern to Ceftriaxone and NDM-1 gene

NDM-1 gene	CTR		Total
	Sensitive	Resist	
Positive	1(5.3%)	8(9.9%)	9
Negative	18(94.7%)	73(90.1%)	91
Total	19	81	100

60(60%) of isolates were resistant to Cefepime, in which 7 isolates (11.7%) harboured NDM-1gene (p-value = 0.254).

40(40%) of isolates were sensitive to Cefepime, in which 2 isolates (5%) harboured NDM-1 gene Table (6).

Table (.6): The association between susceptibility pattern to Cefepime and NDM-1 gene

NDM-1 gene	CFP		Total
	Sensitive	Resist	
Positive	2(5.0%)	7(11.7%)	9
Negative	38(95.0%)	53(88.3%)	91
Total	40	60	100

100(100%) of isolates were resistant to Cefpodoxime, in which 9 isolates (9%) harboured NDM-1gene. (No statistics are computed because CFX is a constant Table (.7).

Table (7) : The association between susceptibility pattern to Cefpodoxime and NDM-1 gene

NDM-1 gene	CFX		Total
	Sensitive	Resist	
Positive	0	9(9.0%)	9
Negative	0	91(91.0%)	91
Total	0	100	100

82(82%) of isolates were resistant to Amoxicillin, in which 7 isolates (8.5%) harboured NDM-1gene (p-value = 0.730).

18 (18%) of isolates were sensitive to Amoxicillin, in which two isolates (11.1%) harboured NDM-1 gene Table (8).

Table (.8) : The association between susceptibility pattern to Amoxicillin and NDM-1 gene

NDM-1 gene	AMC		Total
	Sensitive	Resist	
Positive	2(11.1%)	7(8.5%)	9
Negative	16(88.9%)	75(91.5%)	91
Total	18	82	100

6 (6%) of isolates were resistant to Meropenem, in which 2 isolates (33.3%) harboured NDM-1gene (p-value = 0.033).

94(94%) of isolates were sensitive to Meropenem, in which7isolates (7.4%) harboured NDM-1 gene Table (.9).

Table (9): **The association between susceptibility pattern to Meropenem and NDM-1 gene**

NDM-1	MEM		Total
	Sensitive	Resist	
Positive	7(7.4%)	2(33.3%)	9
Negative	87(92.6%)	4(66.7%)	91
Total	94	6	100

10(10%) of isolates were resistant to Imipenem, in which 3 isolates (30%) harboured NDM-1gene (p-value = 0.014).

90(90%) of isolates were sensitive to Imipenem, in which 6 isolates (6.7%) harboured NDM-1 gene Table (.10).

Table (.10): The association between susceptibility pattern to Imipenem and NDM-1 gene

NDM-1 gene	IPM		Total
	Sensitive	Resist	
Positive	6(6.7%)	3(30.0%)	9
Negative	84(93.3%)	7(70.0%)	91
Total	90	10	100

Discussion

Increasing reports on NDM-1 producing *E. coli* constitute a serious threat to global health since it found to be highly resistant to most of the currently available antibiotics including Carbapenems **(Bora *et al*, 2013)**. This study has been performed to find out the incidence of $bla_{\text{NDM-1}}$ gene in *E. coli* isolates recovered from the various clinical Samples in Khartoum state Sudan.

In the present study the highest number of *E. coli* isolates were from urine 85(85%), which is similar to Agarwal e*t al*. (2018) study to determine the prevalence of NDM-1 gene producing *E. coli* among hospitalized patients in a tertiary care hospital in Northern India, They found 47(77%) *E. coli* isolates from urine (**Agarwal, *et al.* 2018**).

The *E. coli* recovered in this study were high resistant to cephalosporins including Cefepime (60%) Ceftriaxone (81%), and found to be complete resistant to the Cefpodoxime (100%). Similar high level of resistance was also observed amongst the *E. coli* isolates to Penicillins including Amoxicilin (82 %).Also showed reduced susceptibility to the Carbapenems including meropenem and imipenem at the rate of (94%) and (90%) respectively. Also a study done by Elbadawi *et-al*, showed high resistant against Amoxicilin (90%), Ceftriaxone (88.4%), Meropenem(63.1%) and Imipenem (61.6%).(**Elbadawi, *et al* .2019**) were also recovered from outpatients as well as from hospitals, suggesting community-acquired infections as well as inter-hospital dissemination.

Recently, a new type of metallo-β-lactamase, named NDM, was described in K. pneumonia and *E. coli* recovered from a Swedish patient who was hospitalized in New Delhi, India**(Johnson and Woodford, 2013).** The majority of NDM-1 producing bacteria are broadly resistant to various drug classes and also carry a diversity of other resistant mechanisms e.g. to aminoglycosides and fluoroquinolones, which leaves limited treatment options. Since 2011, NDM-1 positive bacteria reported worldwide, Most are Enterobacteriaceae including *E. coli* from patients hospitalized in 2009 and 2010 with an epidemiological link to the Indian subcontinent **(Johnson and Woodford, 2013)**. Among the 100 *E. coli* isolates collected from various clinical samples during a period of December 2019 to February 2020,I detected $bla_{\text{NDM-1}}$ gene in 9 isolates (9%), this study represents the first report of the prevalence of NDM-1 gene among *E. coli* isolates in Khartoum state, Sudan, which was agreed to that reported by Agarwal *et al* in period from January 2014 to August 2014 , were studied the $bla_{\text{NDM-1}}$ gene of *Escherichia coli* in a tertiary care hospital in Northern India , Out of 500 *E. coli* isolates, 36 (7.2%) isolates showed the presence of $bla_{\text{NDM-1}}$ gene.((**Agarwal ,*et al.* 2018**).

There are another study done by Bora *et al*, has been performed to find out the incidence of $bla_{\text{NDM-1}}$ in *E. coli* isolates recovered from the various clinical samples at a tertiary care referral hospital in India, out of 270 *E. coli* isolates, 14 were screened for Carbapenemase production on the basis of their reduced susceptibility to meropenem and eratpenem, All screened isolates were found to be positive for $bla_{\text{NDM-1}}$gene (**Bora, *et al.* 2013**).

Also, in study conducted in India, by Ckakraborty et al, to characterize the NDM-1 producing *E. coli* and their impact on patients clinical outcome: the results showed that: out of 300 isolates ,21 (7%) were MBL producers by phenotypic methods, of this, 17 (81%) were NDM-1positives(**CKaKraborty, *et al.*2015**). The variation in results may be explained by the differences in the time of study, the number and sites of samples collection and the differences in the antibiotics use and consumption.

Conclusion

E. coli recovered in this study were high resistant to cephalosporins including Cefepime (60%) Ceftriaxone (81%), and found to be complete resistant to the Cefpodoxime (100%). Similar high level of resistance was also observed amongst the *E. coli* isolates to Penicillin including Amoxicilin (82 %).

NDM-1 gene detected in 9 isolates, in which 8 isolates was recovered from urine, only one isolates from wound, while 91 isolates were free off NDM-1 gene.

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