Microbes and Infectious Diseases 2020; 1 (1): 24-33

Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Detection of carbapenemase enzymes and genes among carbapenem-resistant *Enterobacteriaceae* isolates in Suez Canal University Hospitals in Ismailia, Egypt

Atef S. Raheel*, Hasnaa A. Mohamed, Waheed F. Hessam, Said H. Abbadi[1], Abeer E. El Sayed

Department of Microbiology and Immunology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.

ARTICLE INFO

Article history:
Received 7 March 2020
Received in revised form 19 April 2020
Accepted 20 April 2020

Keywords:
CRE
Carbapenems
PCR
mCIM
eCIM

ABSTRACT

Background: Carbapenem antibiotics are important therapeutic agents in the health care setting, they are frequently used as an empiric therapy for life-threatening infections as well as infections with multi-drug-resistant gram-negative bacilli· Carbapenemase-producing Carbapenem-Resistant *Enterobacteriaceae* (CRE) are a significant public health challenge worldwide. The detection of carbapenemases productions in CRE strains is performed by phenotypic and genotypic methods. The phenotypic methods target carbapenemases production but provide no guidance regarding the specific carbapenemases types, while the genotypic diagnosis has the benefit of determining the exact mechanism conferring carbapenems resistance**. Aim:** Improvement of the antibiotic policy and infection control strategies in Suez Canal University Hospitals in Ismailia; through adequate detection of carbapenem resistance in the hospitals. **Methods:** All the CRE isolates were tested by the phenotypic methods (mCIM & eCIM) test to detect carbapenemases production, and screened by the conventional PCR for the presence of five carbapenemase genes, namely *bla*KPC, *bla*IMI, *bla*VIM, *bla*NDM, *bla*OXA-48. **Results:** The study showed that (53/155) 34.1% of the *Enterobacteriaceae* isolates were carbapenems resistant. Carbapenemases activity was detected in (36/53) 67.9% of the examined CRE isolates using mCIM test (20/36)37.8 % showed Metallo-carbapenemases and (16/36) 30.2% showed Serine- carbapenemases by eCIM test. 60.4% (32/53) were sensitive to colistin. While by PCR, all the isolates (100%) harbor one or more carbapenemases genes. (51/53) 96.2% were proved to harbor blaOXA-48 gene, (47/53) 88.7% were proved to harbor blaNDM gene, (28/53) 52.8%, were proved to harbor blaVIM,gene, the percentage of blaIMI, blaKPC isolation was (17/53) 32.1%, (4/53)7.5% respectively. **Conclusion:** High frequencies of carbapenemase genes among CRE isolates.

Introduction

Infections by Carbapenem-Resistant *Enterobacteriaceae* (CRE) are a significant public health challenge worldwide. The prognosis of infections by CRE is particularly poor in high risk immunocompromised populations as intensive care unit, solid organ transplant, hematological malignancies and stem cell transplant patients [1].

Carbapenem antibiotics are important therapeutic agents in the health care setting. Since these drugs have a broad-spectrum activity against both Gram-positive, Gram-negative bacteria, and anaerobes [2]. The epidemiological situation for CRE had worsened in the last years, due to the international transfer of patients coming from

DOI: 10.21608/MID.2020.25702.1007
* *Corresponding author:* Atef S. Raheel
E-mail address: atefraheel@yahoo.com

endemic areas [3].

There are several mechanisms for carbapenem resistance in *Enterobacteriaceae* which are: enzymatic degradation of carbapenem antibiotics via the production of carbapenemases, reduced accessibility of carbapenems to the periplasmic space via mutations in outer membrane porins and increased carbapenem export via augmented expression of efflux pump components .The most important carbapenemase are: K. pneumoniae carbapenemase (KPC), Verona integron metallo-β lactamases types (VIM), oxacillinase-48 (OXA-48), Imipenem hydrolysing β-lactamase carbapenemase (IMI) and New Delhi metallo-βlactamase (NDM) [4].

The detection of carbapenemases production in CRE strains is performed by phenotypic and genotypic methods .The presence of carbapenemases is suspected when *Enterobacteriaceae* isolates are resistant to one or all of the following carbapenems: ertapenem, meropenem, imipenem or doripenem; and resistant to third-generation cephalosporins: ceftriaxone, cefotaxime, or ceftazidime [5].

Phenotypic confirmation can be performed by the modified Hodge test (MHT), the Carba NP test and its variants, and the modified carbapenem inactivation method (mCIM); all target carbapenemase production but provide no guidance regarding the specific carbapenemase type [6].

The genotypic diagnosis includes: PCR, microarrays, and whole-genome sequencing (WGS). These methods have the benefit of determining the exact mechanism conferring carbapenems resistance, which can be especially helpful during outbreak investigations and while performing epidemiological research [6].

Carbapenem resistant plasmids have the potential to promote dissemination of carbapenem resistance to new populations of *enterobacteriaceae*, raising the spectrum of untreatable community-associated infections [7]. Therefore, this study was conducted to assess the prevalence and genotypes of carbapenemase genes among carbapenem resistant *Enterobacteriaceae* isolated from patients at Suez Canal University Hospitals (SCUH), Egypt .

Aim of the work

Improvement of the antibiotic policy and infection control strategies in Suez Canal University Hospitals in Ismailia, Egypt; through adequate detection of carbapenem resistance in the hospitals.

Materials and Methods

This cross-sectional descriptive study was conducted in Suez Canal University Hospitals in Ismailia starting from November 2016 till December 2018. It included 53 carbapenem resistant *Enterobacteriaceae* isolates obtained from 320 patients admitted in SCUHs,Egypt. Isolates obtained from 320 patients admitted in the Urology, Internal medicine, Surgical wards, Intensive Care Unit (ICU) and Neonatal ICU (NICU) in SCUHs. Patients were 32 males and 21 females, from all age groups. A detailed history was taken with reference to name, age, occupation, history of prior antibiotic therapy, time of hospital admission . Consent was taken from each patient to use their data in the current research work. The study was conducted in accordance with the Declaration of Helsinki).

320 samples collected under aseptic conditions and put in Amies transport media and processed in the laboratory of Microbiology Department, Faculty of Medicine, Suez Canal University for the isolation of *Enterobacteriaceae* strains. Culture was done on blood agar (OXOID) and MacConkey's type I agar (OXOID) plates and incubated overnight at 35°C–37°C. Tube indole test, citrate test, methyl red vogus-Voges Proskauer (MRVP) test, motility indole ornithine (MIO) test, lysine iron agar (LIA) test, triple sugar iron (TSI) test, urease test and carbohydrate fermentation tests was used for identification of the family *Enterobacteriaceae* [8].

Antimicrobial susceptibility testing and phenotypic screening Done for 155 *enterobacteriaceace* (CRE) isolates were performed by Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Oxoid, UK) incubated at 35ºC ± 2˚C for 16-18 hours . [(5)]. Meropenem (10 µg), Ceftazidime 30µg, Ciprofloxacin 5µg, Levofloxacin 5µg Gentamicin 10µg, Amikacin 30µg, Cefepime 30µg, Amoxicillin-clavulanate 20/10µg, Ceftazidime 30µg, Trimethoprim-sulfamethoxazole 1.25/23.75µg, Cefoxitin 30µg, Tetracyclin 30µg and Nitrofurantoin 30µg only for CRE urinary pathogens.

For Colistin susceptibility, the minimum inhibitory concentration method (MIC) was done as the following for (CRE) isolates:Broth Micro Dilution (BMD) was done to determine the MICs of

the CRE isolates to Colistin [9]. Plain un-treated 96-well, polystyrene microplates were used for broth microdilution experiments. A serial two-fold dilution of colistin sulfate (Sigma Aldrich) was prepared (0.25 to 128 mg/liter). Each isolate was examined for growth by visual inspection following overnight incubation of the plate at 37°C for 20 hours. Enterobacterial isolates with colistin MICs of ≤ 2 mg/liter were categorized as wild type (susceptible), and those with MICs of ≥ 4 mg/liter were categorized as non-wild type (resistant) [9].

Phenotypic confirmatory method (mCIM) performed for isolates that show positive screening test, together with eCIM (EDTA-modified carbapenem inactivation method) at the same time to differentiate metallo-β-lactamases from serine carbapenemases in *Enterobacteriaceae* strains . A two labelled tubes of 2-mL TSB (Trypticase soy broth) were prepared in parallel for the mCIM and eCIM tests for each isolate.A 20 µL of the 0.5 M EDTA to the 2-mL TSB tube for eCIM test was added to obtain a final concentration of 5 mM EDTA. A suspension was made by suspending a 1-µl inoculation loop of culture, taken from over-night blood agar plate in each tube.

In eCIM, a susceptibility-testing disk containing 10 µg meropenem (Oxoid) was immersed in the suspension and incubated for a minimum of two hours at 35°C. After incubation, the disk was removed from the suspension using an inoculation loop, placed on a Mueller-Hinton agar plate inoculated with a susceptible *E. coli* indicator strain (ATCC 29522) and (kindly gifted from *NAMRU-3)*subsequently incubated at 35°C. For mCIM zone diameter of 6–15 mm or presence of pinpoint colonies within a 16–18 mm zone was considered as carbapenemase producer isolate,For eCIM ; a ≥ 5-mm increase in zone diameter for eCIM versus zone diameter for mCIM [5].

Plasmid DNA was extracted from the test isolates by using QIAprep Mini-Prep Kit (QIAGEN, Germany). All the CRE isolates were screened by conventional PCR for the presence of the five carbapenemases genes, namely *bla*IMI [10], *bla*KPC [11], *bla*NDM [12], *bla*OXA [13], *bla*VIM [14], 48 genes with a set of primers as described in **table (1).**

The reaction mixture was prepared in a total volume of 25µl including: 5µl of template DNA, 12.5 µl of 2X ABT Red master mix (Applied Biotechnology Co. Ltd, Egypt), and (2 µl) picomoles of both forward and reverse primers then the volume completed with sterile distilled water up to 25µl. Reaction mixtures without a DNA template served as negative controls. Amplification was carried out in a thermal cycler (Peltier Thermal cycler, MJ Research, U.S.A) with the following thermal cycling conditions for each gene:

For IMI gene:

Five minutes at 94°C as the initial denaturation step and 25 cycles of amplification consisting of: 30 Sec of denaturation at 94°C, 30 sec of primer annealing at 50 C ,1 minute of extension at 72°C and 7 minutes at 72°C for the final extension [10].

For KPC gene:

Ten minutes at 94°C as the initial denaturation step and 35 cycles of amplification consisting of : 1 minute of denaturation at 94°C, 1 minute of primer annealing at 52 C ,1 minute of extension at 72°C and 10 minutes at 72°C for the final extension [11].

For NDM gene:

Five minutes at 95°C as the initial denaturation step and 30 cycles of amplification consisting of: 30 sec. of denaturation at 95°C, 30 sec. of primer annealing at 55C ,30 sec of extension at 72°C and 3 minutes at 72°C for the final extension [12].

For OXA-48 gene:

Five minutes at 95°C as the initial denaturation step and 35 cycles of amplification consisting of: 1 minute of denaturation at 95°C, 1 minute of primer annealing at 56 C and single final extension for 5 minutes at 72°C [13].

For VIM gene:

Five minutes at 95°C as the initial denaturation step and 30 cycles of amplification consisting of: 30 sec of denaturation at 94°C, 30 sec of primer annealing at 55 C and single final extension for 30 sec. at 72°C [14].

Amplicons obtained from PCR reactions were analyzed by gel electrophoresis in 1.5 % agarose gel in 1X Tris-Borate-EDTA (TBE) buffer containing 0.1 µl/mL ethidium bromide at 120 volts for 45 minutes and finally visualized with ultraviolet light [15]. Amplicon size (bp) of the tested gene was identified and compared to a100 bp molecular size standard DNA ladder (Cleaver scientific).

Statistical analysis

Collected data were entered into a database file. All

statistical analyses were performed using Statistical Package for Social Science program (SPSS version 22 for windows).

Table 1. Primers for the detection of carbapenemases genes in *Enteroacteriaceae*

Target	Sequence (5′ → 3')	Annealing temp.	Amplicon size (bp)	Ref.
IMI	**F**:5′TGCGGTCGATTGGAGATAAA -3' **R**:5′CGATTCTTGAAGCTTCTGCG -3'	50°C	399	[10]
KPC	**F**:5′TCGCTAAACTCGAACAGG -3' **R**:5′TTACTGCCCGTTGACGCCCAATCC -3'	52 °C	785	[11]
NDM	**F**:5′CACTTCCTATCTCGACATGC -3' **R**:5′GGGCCGTATGAGTGATTG-3'	55 °C	608	[12)]
OXA-48	**F**:5′AACGGGCGAACCAAGCATTTT -3' **R**:5′TGAGCACTTCTTTTGTGATGGCT -3'	56° °C	597	[13]
VIM	**F**:5′AGTGGTGAGTATCCGACAG -3' **R**:5′ATGAAAGTGCGTGGAGAC -3'	55°°C	261	[14]

Results

Among 320 clinical specimens, *Enterobacteriaceae* were isolated at a rate of 48.4% (155/320). Carbapenems resistant *Enterobacteriaceae* were 34.1% (53/155)of isolates**.** Study population showed that 85% used carbapenems 1 month before specimen collection, while 15% have no history of carbapenems use**.**

The source of 79.3 % of infection by CRE strains was Health care associated, while 20.7% was Community acquired before patient admission. The highest rate of CRE isolation was from patients admitted in the ICU having Ventilator associated pneumonia (39.7%) as illustrated in **table (2).**

Table (3) shows that the largest numbers of the *Enterobacteriaceae* isolates were *K.pneumonia* (70/155) 45.1%, followed by *E. coli* (40/155) 25.8% and the smallest numbers was for *Hafnia* (5/155) 3.2%.

Among the examined CRE isolates, the antibiotics susceptibility was as following **:** All the CRE isolates were resistant to, Amoxicillin-clavulanate, Trimethoprim-Sulfamethoxazole, Tetracyclin and Ceftazidime,(52/53) 98.4% of the isolates were resistant to cefoxitin, (48/53) 90.5% were resistant to Cefepime 66% were resistant to Amikacin, (26/53) 50.9% were resistant to Gentamicin, (43/53) 84.9%% were resistant to Levofloxacin and (42/53) 81.1% were resistant to Ciprofloxacin.

Regarding the susceptibility to Colistin by the MIC for isolates *E. coli* and *K. pneumonia* (n=46), 60.8% (n=32) of the CRE isolates (*E. coli* and *K. pneumoniae*) were sensitive.

Table (4) shows that Carbapenemases activity was detected in 67.9% of the examined CRE isolates using mCIM test; 37.2 % showed Metallo-carbapenemases and 30.2% showed Serine-carbapenemases by eCIM test.

By PCR, all the isolates (100%) harbor one or more carbapenemases genes (90.6% of the isolates carry 2 or more genes. The largest number of isolates (45.3%) carried 3 genes mainly of blaVIM, blaOXA-48 and blaNDM types (32.1%).while26.4% of the isolates carry 2 genes mainly of blaOXA-48 , blaNDM type (24.5%), 15.1% carried 4 genes and only 3.8%. of the isolates carried 5 genes) .The most commonly present gene among CRE isolates was *bla*OXA-48 (51/53) 96.2 % ,followed by *bla*NDM (47/53)88.7%, *bla*VIM (28/53) 52.8%, *bla* IMI (17/53) 32.1% and least was the *bla*KPC (4/53) 7.5%, as shown in **table (5).**

The genotypic method PCR showed that all the isolates (53/53)100% harbor carbapenemases genes while only (36/53) 67.9 detected by phenotypic methods as shown in table (6).

Figures 1-3 illustrate the results of detection of **IMI, KPC, NDM, OXA-48 a**nd **VIM** genes,

respectively.

Table 2. Frequency distribution CRE isolates regarding the type of infection (N = 53).

Variable		Frequency	Percentage
Type of infection	Ventilator associated pneumonia (VAP)	21	39.7%
	Catheter associated urinary tract infections (CAUTI)	9	16.9%
	Sepsis & Lab Confirmed Blood Stream Infections(LCBSI)	8	15.1%
	Pneumonia	8	15.1%
	Surgical sites infections (SSI)	7	13.2%

Table 3. frequency distribution of the studied *Enterobacteriaceae* specimens according to their species (N = 155).

Variable		**Frequency**	**Percentage**
***Enterobacteriaceae* species**	*K.pneumonia*	70	45.1%
	K. Oxytoca	10	6.4%
	E. coli	40	25.8%
	E. cloacae	8	5.1%
	P. mirabilis	10	6.4%
	S. marcescens	12	7.7%
	Hafnia	5	3.2%

Table 4. Frequency distribution of the studied CRE isolates according to the presence of carbapenemases activity and its type according to mCIM and eCIM phenotypic tests (N = 53)

Variable			**No.**	**%**	**Total**	
					No.	**%**
mCIM & eCIM test	Positive	Metallo-carbapenemases	20	37.8%	36	67.9%
		Serine-carbapenemases	16	30.2%		
	Negative		17	32.1%	17	32.1%

Table 5. Frequency distribution of carbapenemases genes among the CRE isolates (N = 53)

Variable		**No.**	**%**
carbapenemases genes	*bla*OXA-48	51	96.2%
	*bla*NDM	47	88.7%
	*bla*VIM	28	52.8%
	*bla*IMI	17	32.1%
	*bla*KPC	4	7.5%

Table (6): Comparison between phenotypic and genotypic methods for detection of carbapenemases .

Variable	Positive	Negative	Total	%
mCIM & eCIM test	**36**	17	53	67.9%
PCR	**53**	**0**	53	100

Figure 1. Agarose gel electrophoresis of IMI gene amplicons (399 bp).

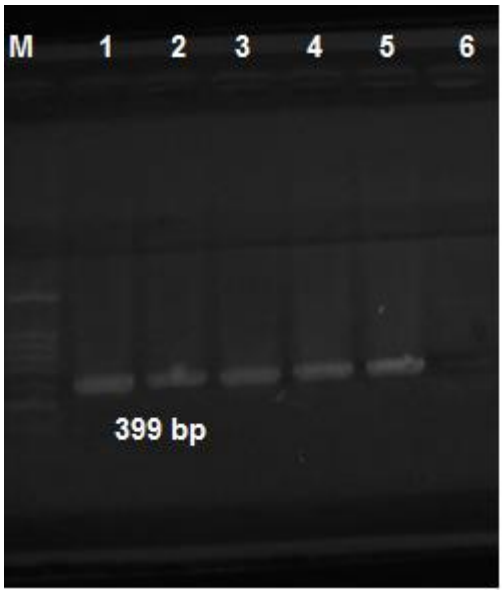


Figure 2. Agarose gel electrophoresis of KPC gene amplicons (785 bp) Rt., NDM gene amplicons (608 bp) Lt.

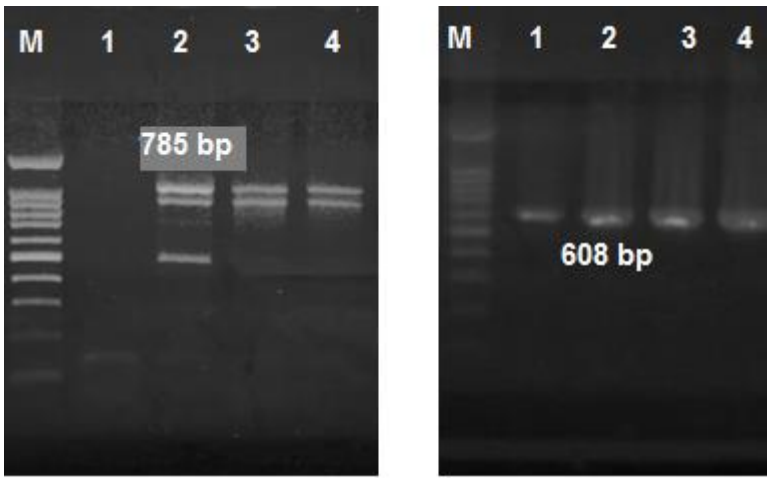
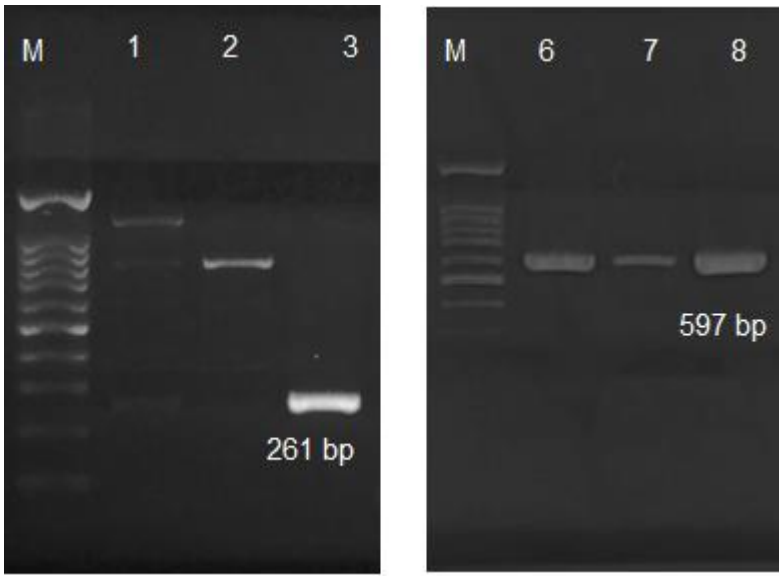


Figure 3. Agarose gel electrophoresis of VIM gene amplicons (261 bp) Rt., OXA-48 gene amplicons (597 bp) Lt.



Discussion

Carbapenem-Resistant *Enterobacteriaceae* (CRE) infections are a significant public health challenge worldwide and associated with high morbidity and mortality [10]. The prevalence of CRE varies widely between different species and different geographical regions and despite several attempts to control the spread of these infections [3].

In this study; a total of 155 *Enterobacteriaceae* isolates, collected from the specimens of 320 patients. *Enterobacteriaceae* were isolated at a rate of 48.4 %. Higher results (51.6%) reported by **Marei *et al*** [16]. A study in Aseer Central Hospital and Abha General Hospital, Kingdom of Saudi Arabia, the isolation rate of *Enterobacteriaceae* was 53.2% [17].

This study showed that 34.1% of the *Enterobacteriaceae* isolated from SCUHs in Ismailia were carbapenem resistant. **El-Sweify *et al*** [18] reported carbapenem resistance about 44.3% among *K. pneumoniae* isolates in SCUHs. The difference in the prevalence may be due to the proper application of infection control measures. Another studies in Egypt revealed higher results as **Amer *et al*** reported a high CRE prevalence rate (62.7%) in a study conducted in Tanta University Hospitals in Egypt [19].

One of the main predisposing factors for the dissemination of CRE is the carbapenems abuse .The present study and another in Tanta University Hospitals show that 85%, 76.6% respectively of cases harboring CRE were previously exposed to carbapenem intake one month before specimen's collection [19]. In a study conducted in 14 provinces in China, 32.2% of the studied patients had a previous history of carbapenems intake [20].

Regarding Colistin; 60.8% of the CRE isolates (*E. coli* and *k. pneumonia)* were sensitive. Colistin is considered as one of the last-resort agents for the treatment of multidrug-resistant Gram-negative bacteria. All CRE specimens collected from the patients in a tertiary cancer hospital showed 100% sensitivity to colistin [21]. In a study conducted on Henan General Hospital resistance rate of CRE isolates to Colistin was 0.025% [22]. The increase in the rate of resistance to Colistin in our hospitals; resulted from the rise in colistin consumption and plasmids in the CRE often carry additional resistance elements.

Results revealed that by using the phenotypic methods (mCIM test), carbapenemases activity was detected in 67.9% of the examined CRE isolates. The eCIM testShowed that 37.8% of carbapenemases positive CRE isolates showed Metallo-carbapenemases and 30.2% showed Serine-

carbapenemases. **Kuchibiro *et al*** [23] stated that carbapenem inactivation method is better than the other phenotypic tests (modified Hodge test, Carba NP test) in its performance, with both sensitivity and specificity of 100% for detection of carbapenemases positive isolates. While in a study conducted by **Song *et al*** the mCIM showed positive results for all the carbapenemase producing CRE isolates, except one of the OXA-48-like *Enterobacteriaceae* isolate and three of the GES-5 (class A carbapenemases) producing *Klebsiella pneumonia*. The sensitivity and specificity of CIM in CRE was (93% sensitivity and 100% specificity) [24].

Conventional PCR showed that all the isolates were positive for one or more carbapenemases genes. Also results of this study showed that, the most commonly present gene among CRE isolates was *bla*OXA-48(96.2%), followed by *bla*NDM (88.7%), *bla*VIM (52.8%), *bla*IMI (32.1%) and least was the *bla*KPC (7.5%). A recent report from Saudi Arabia also reported that *OXA*-48 and NDM1 were dominant among *Enterobacteriaceae*, specifically *E. coli* and *K. pneumonia*, and *bla*KPC was not detected [25]. Similar results were also found by **ElMahallawy *et al*** [21]. The most common resistance genes identified among the CRE isolates there were *bla*OXA-48 (61.8%) followed by *bla*NDM-1 (45.4%). The *bla*IMP-1 gene was detected in only one *K. pneumoniae* isolate. No KPC- or VIM-type b-lactamases were detected. Carbapenemase association of OXA-48 like and NDM type was detected in (27%) isolates. Another study revealed that Carbapenemase genes were detected in 50.8% of CRE isolates. The most prevalent carbapenemase gene was *bla*OXA-48 (49.2%) followed by *bla*NDM-1 (47.6%), *bla*VIM was found in 26% of the CRE isolates [26].

Conclusion

Carbapenem resistant *Enterobacteriaceae* is an emerging threatening problem. Plasmid mediated carbapenemases genes are present in high frequencies though isolates.

Recommendations

Carbapenems use should be prescribed strictly on bases of susceptibility results, Infection control guidelines should be revised based on local bacterial culture data.

Funding

Non declared

Competing interests

Non declared

Ethical approval

Waived by the institutional review board

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