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Detection of OXA-48-Carbapenemase-Producing *Enterobacteriaceae* using ChromID OXA-48 in Critical Care Patients in Egypt

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Carbapenems are a class of beta-lactam antibiotics with broad spectrum of activity. They are often considered as a last resort in treatment of infections caused by multidrug resistant organisms. Carbapenemase-producing *Enterobacteriaceae* (CPE) have been reported worldwide. Class D OXA-48 carbapenemases is rapidly disseminating in *Enterobacteriaceae* leading to high mortality from resistant and invasive CPE infections. In the present study, we attempted to isolate OXA-48 carbapenemase-producing *Enterobacteriaceae* from different clinical specimens obtained from hospitalized patients at different ICU of kasr Kasr Al-Ainy hospital, Cairo University. Initial screening for carbapenemase-producing *Enterobacteriaceae* was done using ertapenem disc diffusion method and direct inoculation of the specimens into ChromID OXA-48. The phenotypic Modified Hodge Test (MHT) was used for confirmation of carbapenemse production among screened carbapenem resistant isolates.Out of 112 collected samples, 94 *Enterobacteriaceae* were isolated. Fifty five isolates (58.5%) were ertapenem disc resistant and 50 isolates (53%) showed positive growth on ChromID OXA-48. Fifty two (94.5%) out of 55 suspected carbapenemase-producing isolates by disc diffusion method and the 50 isolates (100%) grown on ChromID OXA-48 were MHT positive. Our study underlines the need to detect OXA-48 CPE as early as possible to minimize its spread in ICU and apply appropriate infection control measures.

Keywords: *Enterobacteriaceae*, class D OXA-48 carbapenemase, ChromID OXA-48, Modified Hodge Test.

ICU patients are important potential reservoirs of CPE because of the severity of illness and the antibiotic selection pressure. The most important challenges for ICU practitioners are to identify admissions with the highest risk of communicable disease and to rapidly implement preventive measures in order to avoid nosocomial transmission (Memish *et al*., 2015).

Carbapenems are often used as last-line agents when patients with infections become gravely ill or are suspected of harboring resistant bacteria. The rapid emergence and dissemination of *Enterobacteriaceae* that are resistant to carbapenems poses a considerable threat to clinical patient care and public health. Carbapenemase-producing strains are characterized by their resistance to virtually all beta-lactam antibiotics, including the cephalosporins and carbapenems,

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as well as to flouroquinolones, aminoglycosides and co-trimoxazole. Invasive infections with these strains are associated with high rates of morbidity and mortality (Gaibani *et al*., 2011; Lee *et al*., 2016).

Carbapenemase-producing *Enterobacteriaceae* (CPE) have been increasingly identified worldwide. Class A (KPC), Class B (VIM and IMP) and Class D (OXA-48) carbapenemase producing isolates have been involved in nosocomial outbreaks (Djahmi *et al*., 2014). This global spread of carbapenemase-producing *Enterobactericeae* has made the development of a simple test a desirable goal (Kim *et al*., 2015). A disc diffusion test using ertapenem (10µg) is the most sensitive screening test to identify CPE (CLSI, 2016).

Chromogenic media allow the presumptive identification of bacteria within 24 hours of incubation on the basis of clonal morphology and distinctive color patterns. These media have been shown to improve the culture based methodology by decreasing the turnaround time and by increasing the differentiation and detection of the target microbes of interest (Akter *et al*., 2014). Studies have highlighted the potential difficulty in isolating CPE, as such isolates often have low carbapenem MIC and maybe inhibited by some selective media that contain carbapenems (Yamamoto *et al*., 2017).

A chromogenic media (ChromID OXA-48) has been shown to identify class D CPE (Girlish *et al*., 2013). ChromID OXA-48 is the only selective media for identification of class D OXA-48 with very accurate sensitivity and specificity (Zarakolu *et al*., 2015).

Aim of the work

The purpose of our study was to detect the frequency of carbapenemase-producing *Enterobacteriaceae* by ertapenem disc diffusion test and the frequency of OXA-48 carbapenemases using ChromID OXA-48 and confirm both methods by Modified Hodge test.

Patients and Methods

The present study was conducted on 105 patients admitted at general surgery, neonatal, obstetric and chest intensive care units (ICU) of Kasr El-Ainy hospitals, Cairo University during the period from February 2016 through August 2016. The study included 60 males and 45 females their ages range from few days to 87years. A total of 112 different samples were obtained including 38 urine samples, 27 wound swabs, 25 sputum samples, 15 endotracheal tube aspirates and 7 blood samples. The study was carried out in the Department of Microbiology and Immunology, Faculty of Medicine, Cairo University. This study has been approved by the institutional research ethics committee and have been performed in accordance with the ethical standards.

Sample collection

Different clinical samples were collected from ICU hospitalized patients after taking their informed consent, under complete aseptic conditions using sterile containers, swabs, suction catheters, syringes as follows:

Wound swabs

Twenty seven wound swab samples were collected from surgical site infections using sterile swabs.

Sputum samples

Twenty five morning sputum samples were collected using screw capped universal containers.

Endotracheal tube aspirates

Fifteen endotracheal aspirates were obtained from patients by mean of sterile suction catheters. A portion of the catheter containing significant amount of the aspirate was aseptically cut into a sterile container.

Urine samples

Thirty eight catheter specimens of urine (CSU) were collected after clamping the catheter for 10 minutes, then the wall of the catheter was disinfected above the level of clamping and CSU were aspirated with syringe through the disinfected part.

Blood samples

Seven blood samples were collected using a sterile syringe; 5 ml of blood was withdrawn after disinfection of the skin.

All specimens were labeled with the date, patient's name, patient's number, time of collection, specimen type, and then transported immediately to the microbiology department.

Cultivation of the samples

Different clinical samples other than the blood were cultured on MacConkey's agar plates number 3 (CM 0115) (Oxoid Ltd., Basingstoke, Hampshire, England) and the chromogenic media Chrom ID OXA-48 agar

plates (BioMérieux, France). The plates were incubated aerobically at 37 °C for 24-48 hours.

Chrom ID OXA-48 agar is a selective chromogenic medium for the screening of OXA-48 type carbapenemase–producing *Enterobacteriaceae* (CPE). It consists of a rich nutritive base combining different peptones that contains a mixture of antibiotics which enable selective growth of CPE in addition to three chromogenic substrates which enable the identification of the most frequently isolated CPE.

Blood samples, 5 ml each, were inoculated into blood culture bottles and mixed with the medium (Oxoid Ltd., Basingstoke, Hampshire, England), then incubated aerobically at 35 °C . The medium is designed to create pressure in the sealed bottle when the organisms are growing. The detection of positive pressure is by means of a growth indicator device which is connected to the bottle after the blood sample is added. A positive result is indicated when the blood/broth mixture rises above the green locking sleeve of the growth indicator device. Subcultures were done on MacConkey's and chrom agar. The blood culture bottles were considered negative after 14 days if no growth of the subculture was detected.

Identification of the isolates

Identification of the isolates was done according to the conventional microbiological standard tests (Gram stain, glucose fermentation test and oxidase test). Isolates on MacConkey's agar identified as Gram negative bacilli, glucose fermenters and oxidase negative were considered *Enterobactericeae* (Brooks *et al*, 2007). Identification of different isolates was done using biochemical reactions (TSI, urease test, citrate test, MIO, LIA and oxidase tests) the interpretation in discussed in Table (1). Colonies on chromogenic media were subjected to biochemical reactions for further confirmation.

Carbapenemase Screening Tests

Isolates were screened for carbapenemase production using ertapenem (ETP) disc (Oxoid, UK) and the chromogenic medium ChromID OXA-48.The results of ertapenem disc were interpreted according to the standard guidelines (CLSI., 2016).

Disc diffusion method (CLSI, 2016)

- The diameters of the inhibition zones were measured in millimeters and compared to the reference table (Table 1) to differentiate the isolates into susceptible (S), intermediate (I) or resistant (R).
- Carbapenemase –producing isolates of *Enterobacteriaceae* usually test intermediate or resistant to one or more carbapenems using the interpretive criteria according to (CLSI, 2016).

Chromogenic media (ChromID OXA-48)

On the chromogenic media ChromID OXA-48, carbapenemase producing *E.coli* produce pink to burgundy color, while carbapenemase producing *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* isolates produce bluish-green to bluish-grey color.

Phenotypic confirmatory test for carbapenemase production

Isolates that were intermediate or resistant to ertapenem or yeilded positive growth on ChromID OXA-48 media were subjected to the confirmatory Modified Hodge Test (CLSI, 2016).

- A 0.5 McFarland turbidity standard suspension of *E.coli* ATCC 25922 was diluted 1:10 in sterile physiological saline solution (NaCl 0.9%).
- A Muller-Hinton agar plate was inoculated with this dilution as for disc diffusion test.
- An ertapenem disc (Oxoid, UK) was placed on the plate.
- An inoculation loop or swab was used to pick up three to five colonies of test organism grown overnight on blood agar and were streaked from the edge of the ertapenrm disc. The streak was 20-25 mm long. Three to four isolates were examined in the same plate.
- The plate was incubated overnight at 35 ± 2 °C in an aerobic atmosphere.
- Following incubation for 16-20 hours, the intersection of the edge of the inhibition zone and the streak of the test isolate was examined.

Reading and Interpretation

- Indentation of the inhibition zone(s) indicated that the test organism hydrolyzes carbapenems.
- The test result is non –determinable if the growth of *E.coli* ATCC 25922 was inhibited by the test isolate (inhibition zone parallel to the streak of the test isolate)

- The test is considered negative for carbapenemase production if there was no enhanced growth.

Statistical analysis

Data were statistically described in terms of mean ± standard deviation (± SD), and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Chi-square (χ^2) test. Exact test was used instead when the expected frequency is less than 5. *P values* less than 0.05 was considered statistically significant. All statistical calculations were done using computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 22 for Microsoft Windows.

RESULTS

The current study was conducted on 105 patients of different intensive care units of Kasr Al-Ainy hospitals, Cairo University during the period from February 2016 through August 2016. A total of 134 isolates were recovered. The distribution of isolates among collected samples was as follows: 40 isolates (30%) were isolated from urine samples, 35 isolates (26%) from wound swabs, 30 isolates (22.4%) from sputum samples, 22 isolates (16.4%) from endotracheal tube aspirates and 7 isolates (5.2%) from blood samples. The isolates were identified using the standard biochemical reactions (TSI, urease test, citrate test, MIO, LIA and oxidase tests) as 94 (70.1%) *Enterobacteriaceae* species,

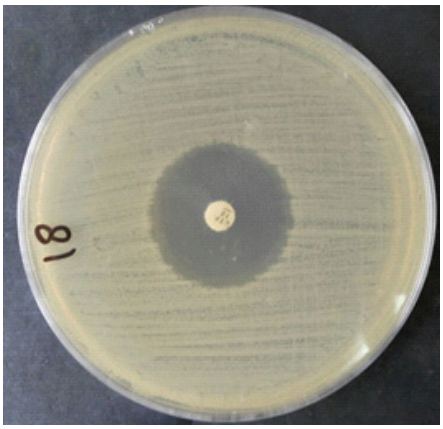


Fig. 1a. Ertapenem sensitive isolate (zone diameter >22mm)

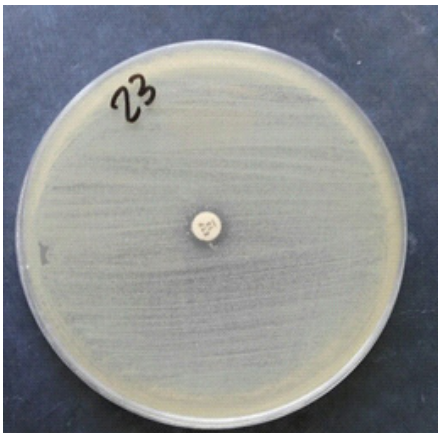
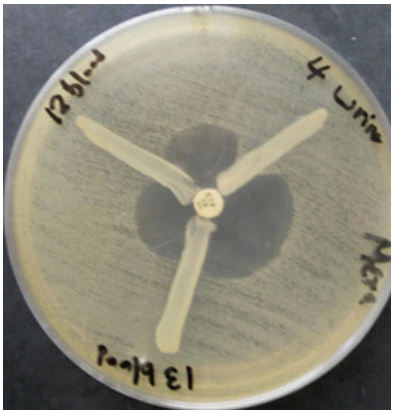


Fig. 1b. Ertapenem resistant isolate (zone diameter <18mm)



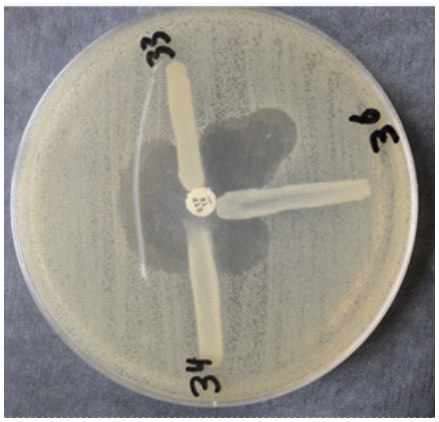


Fig. (2a, 2b). Modified Hodge Test
Isolates number 4, 12, 33 and 36 showed positive results. Isolates number 13 and 34 were negative

25 (18.7%) *Acinetobacter spp*. and 15 (11.2%) *Pseudomonas spp*.. The *Enterobactericeae* isolates were further identified to 35 (37.23) *E. coli*, 57 (60.64) *Klebsiella spp*. and 2 (2.13) *Proteus spp*. as shown in Table 2.

I- Carbapenemase screening tests

Disc diffusion method

Out of 94 *Enterobacteriaceae* isolates, 55 isolates (58.5 %) were resistant to ertapenem disc (49 *Klebsiella* and 6 *E.coli* isolates), while 39 isolates (41.5%) were sensitive (8 *Klebsiella.*, 29 *E.coli* and 2 *Proteus* isolates) fig. 1.

Chromogenic media (ChromID OXA-48)

Out of 94 *Enterobacteriaceae* isolated from 112 clinical samples which were directly inoculated into ChromID OXA-48, 50 *Enterobactericeae* isolates were observed. Forty two *Klebsiella* isolates were observed as green colonies, while 8 *E.coli* isolates showed pink colonies on the chromogenic media. All the 50 isolates grown on ChromID OXA-48 were resistant to ertapenem disc. Number and percentage of *Enterobacteriaceae* grown on Chrom ID OXA-48 agar are illustrated in table 3.

Comparison between ChromID OXA-48 and ertapenem disc diffusion results

Out of 55 ertapenem disc resistant isolates, 50 (91%) *Enterobacteriaceae* isolates grew on the ChromID OXA-48 agar Table 4.

Modified Hodge Test as phenotypic confirmatory test

Out of the 55 suspected carbapenemase-producing *Enterobacteriaceae* isolates (resistant to ertapenem), 52 isolates (94.5%) were found to be MHT positive as evidenced by the indentation of the inhibition zones of ertapenem discs, while 3 isolates (5.5%) were found to be MHT negative as there was no indentation of the inhibition zones Table 5 & fig. 2.

Testing the 50 isolates by ChromID OXA-48 using MHT showed statistically significant agreement between both results (p value = 0.001), the 50 positive isolates by ChromID OXA-48 gave also positive results with MHT Table 6.

The results of the screening tests (ertapenem disc diffusion and ChromID OXA-

Table 1. Inhibition zone diameters interpretive standards for *Enterobacteriaceae* (CLSI, 2016)

Antimicrobial agent	Disc content (µg)	Zone diameter(mm)		
		S	I	R
Ertapenem	10	≥ 22	19-21	≤18

Ertapenem non susceptibility is the most sensitive indicator of carbapenemase production (CLSI, 2016).

Table 2. Distribution of Gram-negative bacilli isolates recovered from different clinical samples

Type and number of specimens	*E.coli*	*Klebsiella Spp.*	*Proteus Spp.*	*Acinetobacter Spp.*	*Pseudomonas Spp.*	Total number of isolates
Urine (38)	25	11	0	3	1	40
Wound swab(27)	7	15	0	8	5	35
Sputum (25)	1	13	0	11	5	30
Endotracheal tube aspirates(15)	2	11	2	3	4	22
Blood (7)	0	7	0	0	0	7
Total (112)	35	57	2	25	15	134

Table 3. Number and percentage of *Enterobacteriaceae* isolated on Chrom ID OXA-48 agar

Growth on chrom agar	No & percentage
E. coli	8 (8.5%)
Klebsiella spp.	42 (44.6%)
Total number of *Enterobacteriaceae*	94 (100%)

Table 4. Comparison between ChromID OXA-48 and ertapenem disc diffusion results for 94 *Enterobacteriaceae* isolates

Ertapenem disc resistant isolates	Isolates grown on ChromID OXA-48
55 (58.5%)	50 (53.2%)

48) and the confirmatory MHT for each sample are illusterated in Tables 7 & 8.

The total prevalence of CPE among 94 *Enterobactericeae* isolates was 55% (52/94). The frequency of CPE spread among 112 samples collected from ICUs was 46.5% (52/112).

DISCUSSION

Carbapenemase producing Gram-negative pathogens are of great concern for physicians. The challenging aspects are the treatment options and infection control measures. Monitoring of the respective carbapenem resistance mechanism is necessary to prevent outbreaks. Carbapenemase production is the main mechanism of carbapenem resistance in *Enterobacteriaceae*. The most prevalent carbapenemases in *Enterobacteriaceae* are KPC, VIM, NDM and OXA-48 (Shahcheraghi *et al*., 2017).

The rapid emergence of oxacillinases (OXA-48 and OXA-48 like enzymes) is alarming. The frequency of OXA-48 is more than the classical carbapenemases (KPC, NDM, IMP, and VIM) across the world (Bakthavatchalam *et al*., 2016). Class D OXA-48 is commonly identified in *E. coli* and *Klebsiella pneumoniae*. The transferrable plasmid of OXA-48 is associated with rapid spread and inter-species dissemination (Temkin *et al*., 2014).

Identification of carbapenem resistance among *Enterobacteriaceae* is of primary importance since carbapenemase producers are resistant not only to most (if not all) β-lactams but also to other main classes of antibiotics including quinolones, aminoglycosides and trimethoprim–sulfamethoxazole (Hara *et al*., 2013).

Reliable detection of carbapenemases is necessary to implement contact precautions to prevent hospital outbreaks (Cuzon *et al*., 2010 and Melake *et al*., 2016). The CLSI guidelines for the phenotypic detection of carbapenemase-producing *Enterobacteriaceae* is based on an initial screening

Table 5. Confirmation of disc diffusion test results by Modified Hodge Test

MHT	No & percentage
Positive	52 (94.5%)
Negative	3 (5.5%)
Total ertapenem disc resistant isolates	55 (100%)

Table 6. Confirmation of ChromID OXA-48 results by Modified Hodge Test

MHT	No & percentage
Positive	50
Negative	-
Total number of isolates grown on ChromID OXA-48	50 (100%)

*(*p value* < 0 .001 is considered statistically significant)

Table 7. Screening tests results for each sample

Type of samples / number of *Enterobacteriaceae* Isolates	Growth on ChromID OXA-48		ETP disc diffusion	
	Positive	Negative	Resistant	Sensitive
Urine samples (36)	8	28	10	26
Wound swabs (22)	13	9	16	6
Endotracheal tube aspirates (15)	10	5	11	4
Sputum samples (14)	14	0	12	2
Blood samples (7)	5	2	6	1
Total (94)	50	44	55	39

Table 8. Modified Hodge Test results as a confirmatory phenotypic test

	MHT	
	Positive	Negative
ETP resistant isolates (55)	52 (94.5%)	3 (5.5%)
ChromID OXA-48 positive growth isolates (50)	50 (100%)	-

test for carbapenem resistance by the disc diffusion method or MIC determination, followed by the MHT for confirmation (CLSI., 2016)

In the present study, we attempted to determine the presence of class D OXA-48 carbapenemases among 112 clinical samples by disc diffusion method and the chromogenic media (ChromID OXA-48), to confirm both methods using Modified Hodge Test and to estimate the frequency of CPE in critical care patients of Kasr Al-Ainy hospital.

In the current study, out of 112 clinical samples, 94 *Enterobacteriaceae* were isolated on MacConkey's agar plates and identified by the conventional biochemical reactions. The antimicrobial susceptibility test was done for the 94 *Enterobacteriaceae* isolates using ertapenem discs as an initial screening method. Fifty five isolates (58.5%) showed resistance to ETP disc, while 39 isolates (41.5%) were sensitive. The high prevalence of carbapenem non-susceptibility in the current study could be explained by the fact that our samples were collected from ICU. Patients admitted to ICU undergo invasive procedures, receive combined antibiotic therapy and are exposed to patients with multi-drug resistant pathogens. (Cisneros *et al*., 2005; Parveen *et al*., 2010 and Morrill *et al*., 2015).

However, lower rates were reported by study done by Mcgettigan *et al*., (2009) who stated that 3% of *Enterobacteriaceae* isolates were ETP resistant. Another study performed at Suez Canal university hospital by (Metwally *et al*., 2013) stated that 44.4% of *Enterobacteriaceae* isolates were ertapenem resistant.

On the other hand, a higher prevalence of ETP resistance was reported in a study conducted by Birgy *et al*, (2012) in a teaching hospital in Paris, where the disc diffusion method revealed that 91% of *Enterobacteriaceae* isolates were non susceptible to ETP. Those variations in the prevalence of carpabenemase-producing *Enterobacteriaceae* could be explained by different sample size as well as different geographical distribution.

In the present study, 55 isolates (49.1%) fulfilled the CLSI criterion for performing carbapenemase detection by the MHT (they were non- susceptible to ETP). These isolates were tested for the presence of carbapenemases by the MHT using substrates recommended by the CLSI (2016). Out of 55 isolates, 52 (94.5%) were carbapenem resistant.

This result was in line with previous study done by Deshpande *et al*., (2010) showed that 22 (91.6%) out of 24 carbapenem resistant *Enterobacteriaceae* were MHT positive. Birgy *et al*., (2012) stated that 95% (21/22) of tested isolates produced positive results with MHT. Mosca *et al*., (2013) showed that 32 isolates out of 38 (84%) gave positive results by MHT.

In disagreement with the current study, Lari *et al*., (2013) reported that 19 out of 35 (54%) gave positive results with MHT. The later result showed partial agreement with studies done by Castanheira *et al*., (2011) and Ambretti *et al*., (2013) who reported that 26/39 (66.7%) and 71/108 (65.7%) *Enterobacteriaceae* isolates were positive by MHT, respectively. Meanwhile, lower rates than that of the current study was reported by Kiratisin and Henprasert, (2010) who found that out of 35 isolates suspected of carbapenemase production, only 6 (17%) isolates yielded positive results with MHT. Saharman and Lestari, (2013) also stated that 8 out of 29 CRE (27.59%) were MHT positive. Another study done in Bayero University, Kano, Nigeria by Yusuf *et al*., (2012) found that out of 135 *Enterobacteriaceae*, 19 isolates (14%) gave positive results by MHT. Moreover, Balan *et al*., (2012) tested 200 *Enterobacteriaceae* for carbapenemases production by MHT and reported that 45 isolates (22.5%) found to be CPE.

In the current study, results of Chrom ID OXA-48 were different from disc diffusion results as 50 *Enterobactericaea* isolates (53%) were observed as growth of colored colonies. All these fifty isolates were MHT positive (100% agreement). *Klebsiella pneumoniae* was the most dominant species and was isolated from 42 (44.6%) patients.

A study in Christian Medical College, India done by Bakthavatchalam *et al*., (2016) reported that out of 117 CPE screened isolates, 106 isolates (90.5%) were positive by Chrom ID OXA-48 media. Chrom ID OXA-48 is the only available chromogenic medium for selective identification of OXA-48. This result showed agreement with study conducted by Girlich *et al*., (2013) who showed 54 (94.5%) out of 57 class D OXA-48 isolates were recovered on ChromID OXA-48 medium.

A lower rate was reported by Zarakolu *et al*., (2015) at a university hospital in Turkey who showed that 25 out of 33 CPE (75.8%) were found to be colonized by class D OXA-48 CPE. The failure to isolate CPE on ChromID OXA-48 was probably attributable to either relatively low amount of CPE in the sample or over growth by other bacteria. Another explanation of this finding maybe due to presence of other classes of carbapenemases.

In our study the most prevalent CPE isolates were *Klebsiella* (44.6%) and *E.coli* (8.5%). Similarly, Ling *et al*., (2015) found that CPE were commonly seen in *Klebsiella pneumoniae* (42.2 %), *E.coli* (24.3 %) in 268 isolates. Another study done by Gijón *et al*., (2012) showed that the most common carbapenemase-producing *Enterobacteriaceae* identified from fecal samples were *Klebsiella pneumoniae* (72.7%) and *E.coli* (9.1%).

CONCLUSION

Carbapenem resistance is spreading rapidly due to antibiotic selective pressure causing serious outcomes. OXA-48 carbapenemases are emerging as an important mechanism of resistance in *Enterobacteriaceae*.

The rapid dissemination of OXA-48 CPE among ICU patients leads to severe infection and emergence of multidrug-resistant isolates with high mortality rate. Early detection of outbreak using rapid ChromID OXA-48 and handling infection control properly in ICU are essential to minimize spread of such strains.

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