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ORIGINAL ARTICLE

Evaluation of Chromogenic Medium chromID OXA-48 for Detection of OXA-48 Producing *Enterobacteriaceae*

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ABSTRACT

Background: *In the last years there has been an increased incidence of carbapenemase-producing Enterobacteriaceae which has become a major public health concern. One of the major concerns for controlling the spread of OXA-48- producers is the absence of reliable phenotypical tests that might contribute to their easy recognition.* ***Objectives:*** *This study aimed to determine the presence of OXA-48 carbapenemases among a group of carbapenem resistant Enterobacteriaceae (CRE) isolates and to assess the performance of chromID OXA-48 medium for rapid detection of OXA-48 producing Enterobacteriaceae in comparison with blaOXA-48 gene amplification by PCR.* ***Methodology:*** *Different clinical samples were obtained from 100 patients. They were outpatient clinic attendants and inpatients admitted to the Intensive Care Unit and different wards of Bolak EL-Dakror Hospital over the period from July 2015 to July 2016. Samples were cultured and Enterobacteriaceae isolates were identified. The antimicrobial susceptibility patterns were determined by disc diffusion test and Minimum Inhibitory Concentration of carbapenem resistant isolates was confirmed by E test. Carbapenem-resistant isolates were selected, cultured on chromID OXA-48 (bioMérieux) and subjected to real time PCR for detection of blaOXA-48 gene.* ***Results:*** *Ninety five Enterobacteriaceae isolates were cultured from the different samples. Screening for carbapenem resistance by disc diffusion and E test revealed 28 of the isolates (29.5%) were CRE. All CRE isolates were Klebsiella pneumoniae. Among CRE, 8 isolates (28.6%) were positive for blaOXA-48 gene. On chrom ID OXA-48 medium, 7 isolates gave visible growth of blue colonies. The sensitivity and specificity of chromID OXA-48 medium was 75%, and 95% respectively. C****onclusion:*** *OXA-48 carbapenemases pose a rising threat in our country. Early detection of OXA-48-producing Enterobacteriaceae isolates is important to start early administration of appropriate antimicrobial therapy as well as the enforcement of infection control measures. The chromID OXA-48 medium can be used for presumptive identification of OXA-48 carbapenemase producers. The medium is easy to interpret, inexpensive, and has acceptable specificity.*

INTRODUCTION

Carbapenemase production is the most common resistance mechanism against carbapenems among the members of the *Enterobacteriaceae* family and its production is often associated with resistance to other β-lactams. The genes encoding carbapenemases are often located in genetic elements together with genes encoding other resistant determinants such as those for aminoglycosides, fluoroquinolones, tetracyclines and sulphonamides[1]. In the last years there has been an increased incidence of carbapenemase-producing *Enterobacteriaceae (CPE)* which has become a major public health concern[2].

Based on amino acid homologies, carbapenemases are included in classes A, B, C and D of the Ambler classification. Class A, C and D β-lactamases share a serine residue in the active site, whereas class B enzymes (also referred to as metallo-β-lactamases, MBL) require the presence of zinc for their activity[3].

Class D β-lactamases were referred to as oxacillinases (OXAs) because they commonly hydrolyze isoxazolylpenicillins (oxacillin, cloxacillin, and dicloxacillin) much faster than benzylpenicillin. OXA-48 is the most efficient class D carbapenemase for imipenem and is one of the most prevalent class D carbapenemases[4]. The OXA-48 was first identified in *K. pneumoniae* in Turkey in 2003[5]. Since 2003, the endemic spread of these bacteria has been reported in countries such as Turkey, Morocco, Libya, Egypt, Tunisia, and India[6].

One of the major concerns for controlling the spread of OXA-48- producers is the absence of reliable phenotypical tests that might contribute to their easy recognition. This is due in part to relatively low carbapenem MICs, a lack of suitable inhibitor

compounds for use in confirmatory tests, and the very low expression of carbapenemase activity which cannot be detected readily by recently developed biochemical methods[7]. PCR is the gold standard for the detection of OXA-48, but is costly and usually available only in larger laboratories or academic institutions[8].

Chromogenic medium enables rapid identification of carbapenem resistant pathogens either from clinical samples or from isolates. chrom ID OXA-48 is the only available chromogenic medium for selective identification of OXA-48. The medium contains antibiotic for the inhibition of other microorganism and biochemical markers to differentiate species or groups of species using either chromogenic substrates or fermentable carbohydrates with a pH indicator. The sensitivity of chromogenic medium can be increased by incubating the plates for complete 24 hours[9].

While there have been several studies on carbapenem-resistant *Enterobacteriaceae (CRE)* from various countries, Egyptian research in this area is limited. This study was conducted to determine the presence of OXA-48 carbapenemases among a group of *CRE* isolates and to assess the performance of chromID OXA-48 medium for rapid detection of OXA-48-producing *Enterobacteriaceae* in comparison with *blaOXA-48* gene amplification by PCR.

METHODOLOGY

This is an observational cross sectional study that was conducted during the period from July 2015 to July 2016. Outpatient clinic attendants and inpatients admitted to the Intensive Care Unit and different wards of Bolak EL-Dakror Hospital were enrolled.

Sample Collection, Cultivation and Identification of isolates:

Midstream urine, sputa, endotracheal tube aspirates, pus and blood samples were collected from 100 patients. All samples were collected under complete aseptic conditions and transported immediately to the Microbiology Laboratory for processing.

All clinical samples were plated directly in parallel onto MacConkey's agar and blood agar. The cultured plates were then incubated aerobically at 37°C to be inspected for growth after 18- 24 hours. Blood samples were inserted in Trypticase soy broth bottles and subculture was done on both media after giving a positive signal. Identification of the isolates was done based on colony morphology, microscopic examination of Gram stained films and conventional biochemical reaction of the isolated organisms [10].

Antimicrobial susceptibility testing and detection of carbapenem resistance

Isolates biochemically identified as *Enterobacteriaceae* were subjected to antimicrobial susceptibility testing by the Bauer-Kirby disc diffusion technique using the following discs (Oxoid, United Kingdom): Ceftriaxone (30µg), Cefotaxime (30µg), Cefepime (30µg), Ceftazidime (30µg), Ampicillin/ sulbactam (20µg), trimethoprim/ sulphamethoxazole (25µg), Ciprofloxacin (5µg), Amikacin (30µg), Aztreonam (30µg), Colistin (10µg), Imipenem (10µg), and Meropenem (10µg). The results were interpreted in accordance with the guidelines established by the Clinical and Laboratory Standards Institute[11]. For both Imipenem and Meropenem (≥23mm indicated sensitivity, ≤19 mm indicated resistance and 20-22 mm indicated intermediate sensitivity).Carbapenem resistance was defined by being non-susceptible to at least 1 of the 2 carbapenems tested.

Isolates with intermediate sensitivity or resistance to carbapenem discs were subjected to E test (BioMérieux, France) to detect Minimum Inhibitory Concentration (MIC) of these *Enterobacteriaceae* isolates to Imipenem and Meropenem. For both Imipenem and Meropenem (≤1µg/ml indicated sensitivity, ≥4µg/ml mm indicated resistance and 2µg/ml indicated intermediate sensitivity).

Culture on chromID OXA-48 medium

The chromogenic chromID OXA-48 medium (bioMérieux, Marcy l'Etoile, France) was obtained from the manufacturer as a prepared plate medium. The medium consists of a rich nutritive base combining different peptones. It contains: a mixture of antibiotics allowing growth selection of OXA-48 *CRE* and three chromogenic substrates which enable the identification of most frequently isolated *CRE*. *CRE* isolates were streaked onto the plates that were incubated aerobically overnight at 37°C before being read.

E. coli isolates should produce pink to burgundy coloration of beta-glucuronidase and/or beta-galactosidase producing colonies; while *Klebsiella* should show green and/or blue coloration of beta-glucosidase producing colonies.

Amplification of *blaOXA-48* gene by real time PCR

CRE isolates were further analyzed for the presence of *blaOXA-48* gene using real time PCR. DNA was extracted from glycerol broth bacterial suspension using QIAamp DNA mini kit (cat. no. 51304, Qiagen, Germany), through the protocol for cultured cells. The purified DNA was stored at -20 °C until used.

Amplification and detection of *blaOXA-48* gene was performed using Microbial DNA qPCR Assay kit (cat. no. 330025, Qiagen, Germany) following the manufacturer's protocol, and Applied Biosystem StepOne instrument. Reaction mixes were prepared for the specific Microbial DNA qPCR Assay with microbial DNA positive control, negative template control and the sample. The PCR reaction was performed in a final volume of 25 µL with 5 µL of genomic DNA sample,1 µL of microbial DNA qPCR Assay, 12.5 µL of microbial qPCR mastermix, and the volume was completed to 25µL by using 6.5 µL of microbial DNA-free water. For negative template control, microbial

DNA-free water was added instead of genomic DNA. The real-time thermal cycle PCR cycler was programmed as: initial PCR activation step at 95°C for 10min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 2 min. Calculation of the threshold cycle for each well was done using the cycler's software.

Statistical analysis:

Collected data were computerized and statistically analyzed using SPSS program version 18.0.Data were statistically described in terms of frequencies (number of cases) and percentages. The performance of the chromID OXA-48 agar was assessed using real time PCR for *blaOXA-48* gene as the gold standard. Sensitivity = a ×100/a+c, Specificity = d ×100/b+d, where a= true positives, b=false Positives, c=false negatives, d=true negatives[12].

RESULTS

The current study was conducted on 100 different clinical specimens that yielded 95 *Enterobacteriaceae* isolates. The isolates were obtained from 57 outpatients and 38 inpatients (25 from NICU, 11 from ICU, 1 from orthopedics, and 1 from surgical wards). The isolates were: 53 *K. pneumoniae*, 37 *E.coli*, 3 *Citrobacter spp.*, and 2 *Proteus spp.* Screening for carbapenem resistance by disc diffusion and E test revealed 28 of the isolates (29.5%) were *CRE*. All *CRE* isolates were *K. pneumoniae.* The distribution of *CRE* according to the department and site of infection is shown in (table 1).

Table 1: The distribution of *CRE* according to the department and site of infection.

		***CRE*(N0.=28)**		
		***blaOXA-48* positive No.(%)**	***blaOXA-48* negative No.(%)**	***Total* No.(%)**
Department	Outpatient	0(0%)	3(10.7%)	3(10.7%)
	NICU	7(25%)	16(57.1%)	23(82.1%)
	ICU	1(3.6%)	1(3.6%)	2(7.1%)
	Orthopedic ward	0(0%)	0(0%)	0(0%)
	Surgical ward	0(0%)	0(0%)	0(0%)
Site of infection	Respiratory tract infection	7(25%)	10(35.7%)	17(60.7%)
	Bacteremia	1(3.6%)	7(25%)	8(28.6%)
	Urinary tract infection	0(0%)	3(10.7%)	3(10.7%)
	Wound infection	0(0%)	0(0%)	0(0%)
Total		8(28.6%)	20(71.4%)	28(100%)

Among *CRE*, 8 isolates (28.6%) were positive for *blaOXA-48* gene by real time PCR (fig 1). Among *blaOXA-48* positive *K. pneumoniae,* 7 isolates (87.5%) were grown from NICU patients, and 1 isolate (12.5%) was grown from an ICU patient. Seven out of 8 (87.5%) *blaOXA-48* positive *K. pneumoniae* were grown from respiratory tract infections, and 1(12.5%) from bacteremia case.

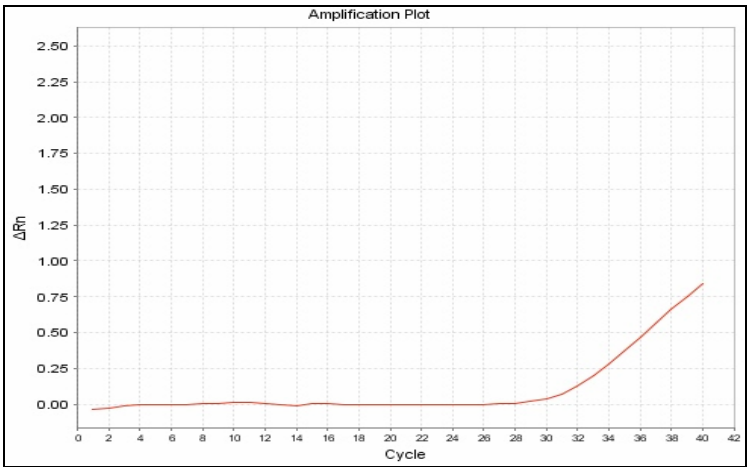

Fig 1: Amplification curve of *blaOXA-48* gene in microbial DNA positive sample by microbial DNA qPCR assay.

The susceptibility pattern of the 8 *OXA-48* producing isolates to antimicrobial agents is shown in (fig 2). They were found to be 100% resistant to Imipenem, Meropenem, Amikacin, Ampicillin/ Sulbactam, Sulphamethoxazole/ Trimethoprime, Cefepime, Ceftriaxone, Cefotaxime, Ceftazidime. High resistance rates were also observed to Ciprofloxacin (87.5%), Aztreonam (87.5%), and Colistin (75%). The MIC of Imipenem ranged from 12-32 µg/ml, while the MIC of Meropenem was 32 µg/ml.

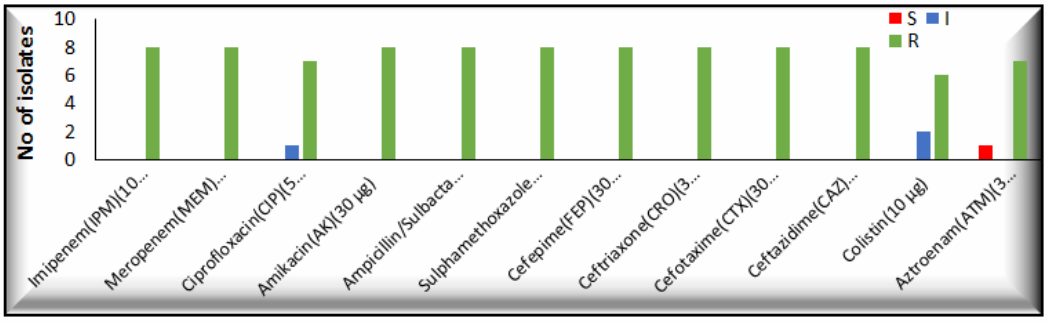

Fig 2: Results of antimicrobial susceptibility testing of 8 OXA-48 producing isolates to different antibiotics.

Assessment of the performance of chromID OXA-48 medium for detection of OXA-48 carbapenemases is presented in (table 2). On chromID OXA-48 medium, 7 carbapenem resistant isolates gave visible growth of blue colonies (fig. 3). Six isolates were positive for *blaOXA-48* gene (true positive), 1 isolate was negative for the gene (false positive). Two isolates gave visible growth of colorless colonies, and were positive for the gene (false negative). The remaining 19 isolates didn't grow on the medium and were negative for the gene (true negative) by PCR. Using real time PCR as the gold standard, the sensitivity and specificity of chromID OXA-48 medium was 75%, and 95% respectively.

Table 2: Results of ChromID OXA-48 medium and real time PCR assay for *blaOXA-48* gene on carbapenem resistant *Klebsiella pneumoniae* isolates (n = 28).

Test		PCR			Characteristics	
		Positive	**Negative**	**Total**	**Sensitivity %**	**Specificity %**
Growth on chromID OXA-48 medium	**Positive**	6	1	7		
	Negative	2	19	21	75	95
	Total	8	20	28		

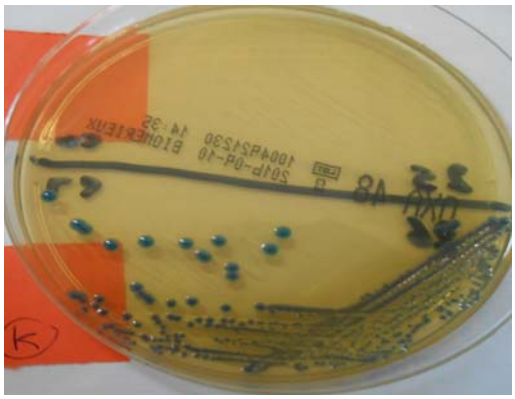

Fig. 3: Blue colonies of OXA-48- producing *K. pneumoniae* on chrom ID OXA-48 medium.

DISCUSSION

Recent epidemiological data have highlighted that the prevalence and incidence of OXA-48 variants are rapidly increasing and that OXA-48 is currently becoming the predominant carbapenemase type in *Enterobacteriaceae* in many countries in Europe, as well as in other areas such as North Africa, Turkey and the Middle East. All currently used phenotypic methods are suboptimal and there are no single tests apart from molecular methods that allow the direct positive confirmation of this carbapenemase[13].

In this study, screening for carbapenem resistance by disc diffusion and E test revealed 29.5% of the isolates were *CRE*. All *CRE* isolates were *K. pneumoniae.* Mechanisms described for carbapenem resistance include, production of different classes of

carbapenemase, hyperproduction of AmpC β-lactamase with an outer membrane porin mutation, and production of ESBL with a porin mutation or drug efflux. Production of carbapenemases is the most commonly reported mechanism of carbapenem resistance in *K. pneumoniae* [14].

Screening for *blaOXA-48* gene by real time PCR was detected in 28.6% of carbapenem resistant isolates. Similar results were reported by *Poirel et al.*[15], in a specialized cancer hospital in Cairo, Egypt. They found 3 OXA-48 producers (30%) out of 10 *CRE* isolates recovered from cancer patients. *Morsi et al.*[16], in a study conducted in Zagazig university hospitals, found 42 *K. pneumoniae* isolates (42%) were carbapenem-resistant. The *blaOXA-48* gene was detected in 28.57% of carbapenem resistant isolates. They attributed the high frequency of carbapenem resistant *K. pneumoniae* in their study to the excessive empirical use of carbapenems in their hospital, and the improper application of the infection control measures. In a study by *Amer et al.*[17], in Tanta university hospitals, *CRE* was detected in 47 (62.7%) isolates. The *blaOXA-48* gene was detected in 11(23.4%) isolates. *CRE* were more frequently found in *Enterobacter cloacae, K. pneumoniae and Klebsiella spp.* isolates. This may be attributed to their ability to transfer multidrug resistance plasmids. *Assem et al.*[18], in a study conducted in a surgical ICU at Cairo University Hospital, found 21.8% of isolated Gram negative bacilli were resistant to carbapenems. PCR analysis revealed 48% of the isolates carried *blaOXA-48* gene, which was higher than our study. *Moemen and Massalat* [19], in their study at different ICUs of Mansoura university hospitals, found the prevalence of carbapenem resistance was 33.3% among *K. pneumoniae* isolates; 10.9% were *blaOXA-48* positive. They attributed that high trend to the frequent use of carbapenems as an empiric therapy in ICUs at their institution, as well as lack of implementation of antimicrobial stewardship program. Other studies showed varying prevalence rate, in United Arab Emirates OXA-48-like was found in 11 isolates (32.4%) out of 34 *CRE* while in Kuwait one OXA-48 isolate was detected incidentally in 2011[20].

In the present study, all *blaOXA-48* positive *K. pneumoniae* were isolated from NICU and ICU. Our findings are in accordance with other researchers who found most of their isolates detected from ICUs concluding that ICU stay was a risk factor for carbapenem-resistant *K. pneumoniae* acquisition[16,21].

We found respiratory tract infection was the most frequent infection (87.5%) from which OXA-48 were isolated. This agreed with the study by *Amer et al.*[17], where cases of septicaemia and ventilator associated pneumonia with previous history of carbapenem intake, especially in neonatal ICU had higher risk for *CRE*. This may be explained that patients suffering from severe underlying disease are routinely and extensively given broad-spectrum anti-infective agents as prophylaxis or targeted therapy. Broad-spectrum antibiotic therapy exerts a selective pressure towards resistant organisms and affects the normal body flora. Furthermore, intensive care medicine, including use of medical devices, allows pathogens to colonize or infect patients. As in many low-income countries, the current infection control practices face many obstacles both in terms of financial support and healthcare workers' compliance, with no specific infection control measures in place regarding *CRE*.

Also, *other studies*[18,19] found that respiratory infections yielded the highest number of carbapenem-resistant Gram negative bacilli. They explained that cross-infection with multi-resistant clones or long-term exposure of respiratory tract microbiota to antibiotics, caused accumulation of resistance determinants. These resistant organisms may later cause respiratory tract infection.

Treatment of infections caused by OXA-producing *K. pneumoniae* is very difficult since most isolates are resistant to all β-lactams and fluoroqinolones[22]. The antibiotic susceptibility profile of our isolates is in keeping with the reported multidrug-resistant phenotype associated with isolates harboring OXA48[23,24], however their isolates remained susceptible to colistin. Our finding of colistin resistant OXA-48-producers is of major concern.

In our study, the growth of the 8 OXA-48 positive isolates on chromID OXA-48 medium was as follows; 6 isolates gave visible growth of blue colonies, while 2 isolates gave visible growth of colorless colonies. One isolate gave visible growth of blue colonies, but was OXA-48 negative by real time PCR. The sensitivity and specificity of the medium was 75%, and 95% respectively. These results are in agreement with previous studies where the chromID OXA-48 has been tested; however, this study provided new information using a different strain collection from another region. In France, a study was done by *Dévigne et al.*[25] using internal collection strains and clinical specimens (stool and rectal swabs) to evaluate the performance of chromID OXA-48. The sensitivity of the medium was excellent despite the low inoculum used, and the specificity was 98.7%. In Turkey, *Zarakolu et al.*[26] screened rectal swabs collected from 302 hospitalized patients for carriage of *CPE*, comparing between chromID OXA-48 agar and chromID CARBA. The sensitivity and specificity of detection of *CPE* was 75.8% and 99.3% for chromID OXA-48 agar, 57.6% and 98.9% for chromID CARBA. In Belgium, a study was done by *Saegeman et al.*[27] to evaluate the intestinal carriage of *CPE* in a geriatric/ chronic care population. They compared the performance of chromID OXA-48, chromID CARBA agars and MacConkey agar after enrichment broth followed by a molecular assay. The specificity of the different methods was at least 97%. A false-positive result (product of low specificity) would result in further follow-up testing; however, a false-

negative result (product of low sensitivity) may result in the spread of the *CPE*, potentially adding very significant costs for the hospital to care for infected patients[28].

On the other hand, *Girlich et al.*[29], reported a higher sensitivity for chromID OXA-48 than our study. They showed that chromID OXA-48 and SUPERCARBA media have the highest sensitivity for detection of OXA-48 producing *Enterobacteriaceae* (91% and 93%) comparatively to chromID CARBA (21%).The chromID OXA-48 has the highest specificity, with 100%, as compared to 53% and 68% for the SUPERCARBA and chromID CARBA media for detecting those OXA-48 producers. In our study, two OXA-48 producing *K. pneumoniae* isolates showed colorless colonies (probably due to lack of the β-glucosidase enzyme in these strains). This may explain the decrease in sensitivity achieved by this medium when the chromogenic criterion was taken into account, however, if we considered total growth (including colorless colonies), we would get 100% sensitivity.

One major advantage of this chromogenic medium is that, it allows identification of OXA-48 carbapenemases in 24 hours directly from bacterial culture, thus easier to implement in the routine workflow of a clinical microbiology laboratory compared to carbapenemase inhibitor-based disc tests, which are much more complex. Another advantage is cost effectiveness; it costs 22.5LE per plate. Use of chromogenic medium should contribute to more effective control of the spread of organisms possessing these resistance mechanisms in outbreak settings. The potential benefits would possibly also include better patient management and a reduction in the escalation of antibiotic resistance through better infection control.

CONCLUSION

OXA-48 carbapenemases pose a rising threat in our country. Early detection of OXA-48-producing *Enterobacteriaceae* isolates is important to start early administration of appropriate antimicrobial therapy as well as the enforcement of infection control measures. The chromID OXA-48 medium can be used for presumptive identification of OXA-48 carbapenemase producers. The medium is easy to interpret, inexpensive, and has acceptable specificity.

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