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RESEARCH

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Carbapenemase producing Enterobacterales clinical isolates from a tertiary care hospital in Egypt

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Abstract

Background Carbapenem resistant Enterobacterales (CRE) is on the rise globally, triggering a significant health threat and a substantial concern for infection control management. We aimed to detect and characterize carbapenemases producing Enterobacterales (CPE) clinical isolates over a period of nearly one-year duration in Theodor Bilharz Research Institute, a tertiary care hospital in Egypt through molecular and phenotypic methods using carbapenemase detection combination inhibitor disk set (Enterobacterales) MASTDISCS ID (MDI) (MAST, UK), with the addition of temocillin disk.

Results CRE represented 6.5% of Enterobacterales. Healthcare-associated infections were frequently high representing 87% of the CRE isolated from hospitalized patients. Most of the CRE isolates were *Klebsiella pneumonia* (68%) followed by *Escherichia coli* (22%), *Enterobacter cloacae* (4%), *Serratia marcescens* (4%) and *Citrobacter freundii* (2%). Phenotypic detection revealed metallo-β lactamases in 84% of isolates, followed by oxacillinase-48 {(OXA-48) 6%} and *Klebsiella pneumoniae* carbapenemase in 2% of the isolates. The most prevalent gene detected by conventional PCR was bla_{NDM} (84%) followed by bla_{OXA-48} (6%) and bla_{KPC} (2%). Excellent agreement was found between PCR and MDI for detection of carbapenemase production.

Conclusions NDM carbapenemase is prevalent in our hospital. Carbapenemase detection combination inhibitor disk set (Enterobacterales) MASTDISCS ID is a useful tool for rapid and precise confirmation of the detection of CPE.

Keywords CRE, MASTDISCS ID, MBLs, NDM, OXA-48, KPC, Infection control

1 Background

Carbapenem-resistant Enterobacterales (CRE) is a worldwide urgent public health concern, causing serious outbreaks [1]. Resistance to carbapenems, the drugs of choice used for treatment of multi-drug resistant (MDR) bacterial strains, renders CRE as one of the biggest health challenges as it results in further limitation in therapeutic options and i increased likelihood of morbidity and mortality [2, 3].

Resistance to carbapenems among Enterobacterales isolates is predominantly attributed to carbapenemases; a diverse group of enzymes that possess carbapenem hydrolyzing activity with varying levels of carbapenem resistance [4]. Carbapenemases may confer resistance to nearly all β-lactams, and are readily transferable as they are mostly encoded by highly transferable plasmids [5]. Carbapenemases belong to one of three classes of

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β-lactamases, class A as *Klebsiella pneumoniae* carbapenemases (KPCs), class B metallo β-lactamases (MβLs) including NDM, IMP, and VIM, and class D with oxacillinases (OXAs) carbapenemases such as OXA-23 and OXA-48 [6]. Recognizing the mechanism of carbapenem resistance is crucial in choosing the most effective antimicrobial treatment and the appropriate infection control method. Although molecular identification of carbapenemase genes is the gold standard [7], rapid diagnostic phenotypic tests are extremely valuable and economic to be used routinely in reducing length and/or cost of hospitalization especially in the intensive care units and could detect the un-expressed genes [8].

This work aimed to detect and characterize carbapenemases producing Enterobacterales (CPE) clinical isolates over a period of nearly one-year duration in Theodor Bilharz Research Institute (TBRI), a tertiary care hospital in Egypt through molecular and phenotypic methods.

2 Methodology

2.1 Sample collection, bacterial identification and antimicrobial susceptibility testing

Fifty (50) CRE isolates out of a total of 775 Enterobacterales isolates were obtained from various clinical samples received and handled in the Microbiology Laboratory of TBRI, during the study period from June 2018 to July 2019.

2.1.1 Identification of bacterial isolates to the genus and species level

Bacterial isolates were identified preliminary by conventional methods and API 20E (BioMérieux, France), and then identification and antimicrobial susceptibility testing (AST) were confirmed using VITEK2 Compact System (BioMérieux, France).

Following the Epidemiological cut-off breakpoints (ECOFFs) recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) on detection of resistance mechanisms in CRE using Kirby Bauer disk diffusion method; Enterobacterales isolates that showed zone diameter < 25 mm for meropenem (MEM) (10 µg) and/or ertapenem (ERT) (10 µg) and/or < 23 mm for imipenem (IPM) (10 µg) were considered carbapenem resistant and used for subsequent investigations [9].

The CRE isolates were stored at − 70 °C as recommended until further phenotypic and genotypic carbapenemase production confirmatory tests were done [10].

All isolates included in the study were archived, and codes were used instead of patient names. The protocol of the study was approved by TBRI Research Ethics Committee (TBRI-REC) under Federal Wide Assurance (FWA00010609) and the work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for Experiments in Humans and its later amendments (GCP guidelines) or comparable ethical standards.

2.2 Phenotypic detection of carbapenemase activity

2.2.1 Combination disk test

Fifty CRE isolates; detected by Kirby Bauer and VITEK2 Compact system; were subjected to carbapenemase detection combination inhibitor disk set (Enterobacterales) (MDI) *(MAST, UK)*. The test was performed according to manufacturer's instructions. Four disks were included: disk A, containing meropenem (10 µg); disk B, consisting of meropenem (10 µg) and an MBL inhibitor; disk C, consisting of meropenem (10 µg) with a KPC inhibitor; and disk D, containing meropenem (10 µg) with an Amp C inhibitor. The interpretation of the test was as follows; the zone of inhibition of disk A is compared to the zones of inhibition of each of disks B, C, and D. If disk B shows a zone difference of ≥ 5 mm from disk A, the organism is considered as MBL producer. If disk C shows a zone difference of ≥ 4 mm from disk A, the organism is considered having KPC activity. If disk C and disk D both show a zone difference of ≥ 5 mm from disk A, the organism is considered as Amp C producer coupled with porin loss mechanism [9].

2.2.2 Detection of OXA-48

OXA-48 carbapenemase resistance was detected using Temocillin disk (30 µg). The temocillin zone diameter was only interpreted if no significant zone difference was observed with disks B, C or D. Isolates in which temocillin zone diameter ≤ 10 mm were considered OXA-48 carbapenemase positive [9, 11].

2.3 Genotypic detection of carbapenemase-encoded genes using conventional polymerase chain reaction (PCR) assay

Bacterial DNA was extracted from fresh overnight cultures by the boiling method; five colonies were added to 200 µl of sterile distilled water and boiled for 5 min, followed by centrifugation for 2 min at 8000 rpm. Supernatants were stored in duplicate copies at − 20 °C for further use [12]. PCR was performed for the following carbapenemase-encoding genes (bla_{KPC}, bla_{NDM}, bla_{VIM} bla_{IMP} and bla_{OXA-48} genes). Primers used in PCR assay are listed in (Table 1).

The PCR assay was performed as previously described by [13]. Briefly 2µL of extracted DNA was added to PCR reaction mixture (final volume of 50 µL) consisting of: 25 µl Maxima hot start green *(ThermoScientific, USA)*, 22 µl nuclease free water *(ThermoScientific, USA)*, 0.5 µl forward primer, 0.5 µl reverse primer. PCR amplifications were carried out in a T-personal PCR Thermal Cycler

Table 1 Primer sequences and sizes of expected amplicons of each PCR assay

Target gene	Primers (5′–3′)	Size of amplicon (bp)	Annealing temp	References
bla_{VIM}	F: GATGGTGTTTGGTCGCATA R: CGAATGCGCAGCACCAG	390	52 °C	[14]
bla_{IMP}	F: GGAATAGAGTGGCTTAAYTCTC R: GGTTTAAYAAAACAACCACC	232	52 °C	[15]
bla_{NDM}	F: GGTTTGGCGATCTGGTTTTC R: CGGAATGGCTCATCACGATC	621	52 °C	[14]
bla_{OXA-48}	F: GCGTGGTTAAGGATGAACAC R: CATCAAGTTCAACCCAACCG	438	52 °C	[15]
bla_{KPC}	F: CGTCTAGTTCTGCTGTGTTG R: CTTGTCATCCTTGTTAGGCG	798	52 °C	[15]

(Biometra, Uk) with the following PCR protocol; initial activation at 94 °C for 10 min, denaturation: 94 °C for 30 s., annealing at 52 °C for 50 s., extension at 72 °C for 50 s. Denaturation, annealing and extension steps were repeated for 30 successive cycles that were finally followed by a maintenance temperature of 4 °C.

Each PCR end-product was analyzed using a 1.5% agarose *(Promega, USA)* gel containing 0.5 µg/µl Ethidium Bromide *(Promega, USA)*. The gel was electrophoresed in 1×TBE buffer *(Sigma, USA)* at 120 V for 60 min in an electrophoreses system *(Cleaver Scientific, UK).* A 100 bp ladder *(Promega, USA)* was used as a molecular size marker. DNA bands were visualized with a gel documentation system *(Bio-Rad, USA).*

3 Results

Fifty (50) CRE isolates were identified out of 775 Enterobacterales isolates recovered from different clinical samples such as urine (n = 433), sputum (n = 96), wound swab (n = 108), blood (n = 97), ascitic fluid (n = 41). CRE isolates represented 6.5% of the isolated Enterobacterales. Most of the CRE isolates were obtained from the inpatients representing (46/50; 92%) while the outpatients represented (4/50; 8%).

Health-care associated CRE infections were 40/46 representing 87%, while community acquired infections were 6/46 representing 13% of infections caused by CRE isolates during the study period. The CRE healthcare-associated infections were mostly recovered from the ICU and the Urology Departments (12/46 each) each representing 26% from the 46 CRE isolates that were recovered from different hospital departments.

Most of CRE isolates were *K. pneumoniae* (34/50; 68%) followed by *E. coli* (11/50; 22%), *E. cloacae* (2/50; 4%), *S. marcescens* (2/50; 4%) and *C. freundii* (1/50; 2%).

MID set with the addition of a temocillin disk confirmed carbapenemase production in 92% (46/50) of the 50 tested CRE isolates. MBL represented the main class (42/50; 84%) (Fig. 1a) followed by OXA-48 (3/50; 6%) (Fig. 1b) and KPC (1/50; 2%) (Fig. 1c). Two isolates (2/50; 4%) had an Amp C activity (Fig. 1d) and two isolates (2/50; 4%) revealed a non-conclusive result (Fig. 1e).

By applying conventional PCR to detect bla_{KPC}, bla_{NDM}, bla_{OXA-48}, bla_{VIM} and bla_{IMP} genes in the isolates, 46 isolates harbored carbapenemase gene; bla_{NDM} gene showed the highest percentage (92%) (Fig. 2) followed by bla_{OXA-48} (6%) (Fig. 3) and bla_{KPC} (2%). Isolates harboring more than one carbapenem resistant gene, as well as bla_{VIM} and bla_{IMP} weren't detected in any of the tested isolates.

Phenotypic combination carbapenemase detection disk test MASTDISCS ID (MDI) set with the addition of a temocillin disk showed 100% sensitivity and specificity with all tested genes in comparison to PCR assay as the gold standard method.

3.1 Statistical methods

Results were expressed as number (%). Comparison between categorical data was done using Chi Square test. Agreement between different methods of detection was done using kappa coefficient test. Diagnostic indices (sensitivity, specificity, positive, negative and efficacy) was performed according to Statistical Package for Social Sciences (SPSS) version 21. P values < 0.05 were considered significant and < 0.01 was considered highly significant.

4 Discussion

CRE has been disseminated and recognized globally causing serious hospital and community acquired infections, with significant limitation in treatment options. Carbapenemase production is the most common cause of carbapenem resistance [16] and therapy differs according to the type of β-Lactamases produced [17]. Rapid identification by simple methods is therefore crucial and allows for timely approaches in prevention and applying prevention control measures. Therefore, we aimed to detect and

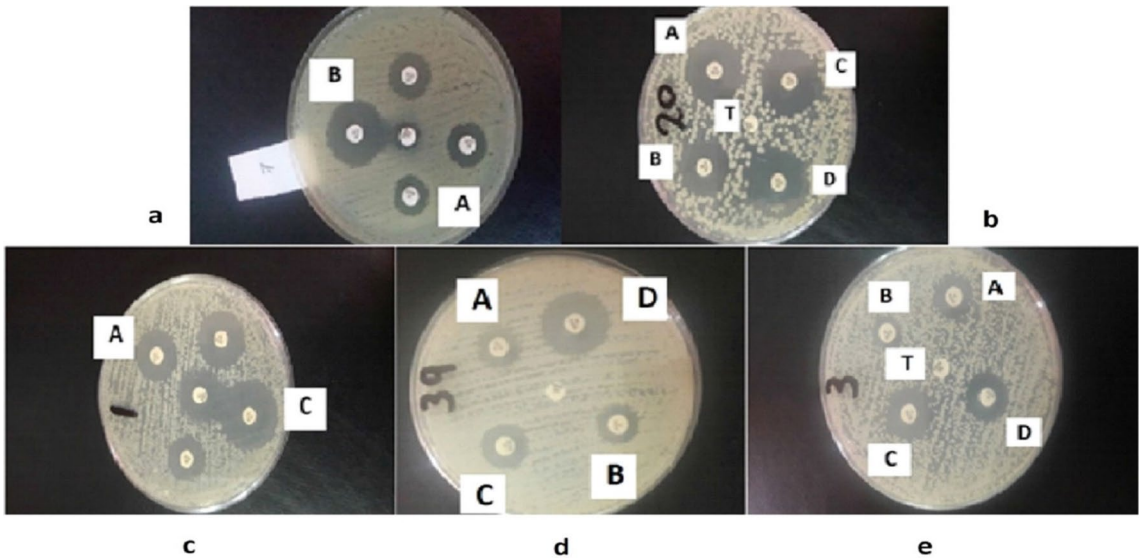

Fig. 1 **a** Confirmation of carbapenemase production through expression of MBL activity detected by the combination test: A carbapenem resistant isolate showing > 5 mm difference in zone diameter between disk A (meropenem 10 µg) and disk B (meropenem 10 µg + MBL inhibitor) indicating MBL activity. **b** Confirmation of carbapenemase production through expression of OXA-48 activity detected by the combination test: A carbapenem resistant isolate showing resistance to T (temocillin30 µg) disk &no significant zone difference was observed between disk A (meropenem 10µ) and disks B (meropenem 10 µg + MBL inhibitor), C (meropenem 10 µg + KPC inhibitor) or D (meropenem 10 µg + AmpC inhibitor) indicating OXA-48 activity. **c** Confirmation of carbapenemase production through expression of KPC activity detected by the combination test: A carbapenem resistant isolate showing > 4 mm difference in zone diameter between disk A (meropenem 10µg) and disk C (meropenem 10 µg + KPC inhibitor) indicating KPC activity. **d** Amp C activity detected by the combination test: A carbapenem resistant isolate in which both C (meropenem 10 µg + KPC inhibitor) and D (meropenem 10 µg + AmpC inhibitor) disks show significant zone differences (≥ 4 mm and ≥ 5 mm respectively) compared to disk A (meropenem 10 µg) while the zone difference between disks B (meropenem 10 µg + MBL inhibitor) and A (meropenem 10 µg) is less than 4 mm (B-A < 4 mm) indicating Amp-C activity. **e** Non-conclusive result by the combination test: A carbapenem resistant isolate in which a non-conclusive result was detected. Disk A: meropenem 10 µg, disk B: meropenem 10 µg + MBL inhibitor, disk C: meropenem 10 µg + KPC inhibitor, disk D: meropenem 10 µg + AmpC inhibitor, disk T: temocillin disk 30 µg.

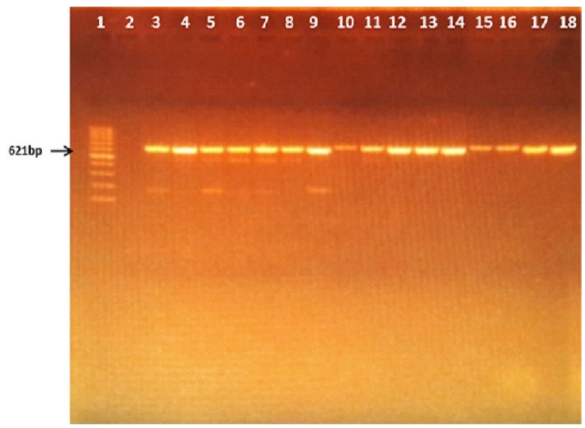

Fig. 2 Agarose gel electrophosresis of PCR products of Enterobacterales bla_{NDM} positive gene (621 bp). Lane 1: Molecular weight marker (ladder 100 bp). Lane 2: Negative control. Lane 3: Positive control. Lane (4–18): Positive DNA samples. The arrow points to bla_{NDM} PCR amplification product

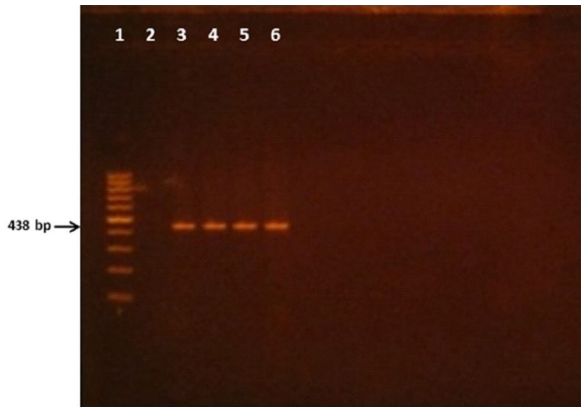

Fig. 3 Agarose gel electrophosresis of PCR products of Enterobacterales bla_{OXA-48} positive gene (438 bp). Lane 1: Molecular weight marker (ladder 100 bp). Lane 2: Negative control. Lane 3: Positive control. Lane (4, 5, 6): Positive DNA samples. The arrow points to bla_{OXA-48} PCR amplification product

characterize CPE clinical isolates through molecular and phenotypic methods.

It has been established that CRE transmission is much more common in healthcare care facilities than in community [3, 18]. Therefore, it is obvious that most of the CRE isolates in the current study (87%) were obtained mainly from inpatients rather than from outpatients (13%). CRE isolates were chiefly recovered from ICU and Urology Departments being 26% in each, as patients are more prone to intense therapy regimens and invasive procedures. This is consistent with findings from Menoufia University Hospitals, Egypt, in which highest rate of CRE (32.5%) was obtained from hospital ICU [19]. Similarly, Kotb and colleagues [3] reported that CRE formed 54.1% of hospital acquired infections (HAI) in ICU cases. This drives more attention to antimicrobial and infection control policies implemented in ICUs.

The prevalence rate of carbapenem resistance among Enterobacterales in our hospital located in Giza Governorate was 6.5% compared to 19.9% in Sohag in the South [20]. Higher rates (40%) were detected in Benha [21] and Tanta cities (62.7%) [22] located to the north of Cairo. In Ismailia Governorate in the North-East of Cairo CRE rate was 34.1% [23]. Such variation in CRE rates could be attributed to differences in geographic distribution, specimen type, the causative pathogen, location prior to admission, length of stay in hospital and ICU stay, and most importantly to antibiotic policies used in different health care settings. Broad spectrum antibiotics aid in the selection for antimicrobial resistance by selective pressures, leading to higher prevalence of CRE. On the other hand, strict implementation of infection control measures and better selection of antibiotic policies may lead to lower CRE rates [24].

Comparable results were detected from Kuwait hospitals as 8% of isolates were CRE detected from different clinical samples [25]. In a study done by *Zowawi* et al. [26] Enterobacterales isolates collected from six participating institutes across the Gulf Corporate Council countries, (Saudi Arabia, United Arab Emirates, Kuwait, Qatar, Oman and Bahrain) showed that CRE prevalence was 15%. In North African countries, 11.4% CRE isolates were recovered from Tunisia and Libya [27].

Both *K. pneumoniae* and *E. coli* were the most prevalent CRE in the studied isolates (68%, 22% respectively) and comparable results in Egypt (51.4 K. *pneumoniae* and 28.6% *E. coli*) were found by [20]. *El-Kholy* and colleagues [28] revealed that the former pathogens were the mostly detected among their multi drug resistant (MDR) isolates.

The EUCAST recommended the combination disk test to identify and differentiate carbapenemases in Enterobacterales using zone size comparison of combined disks, incorporating specific enzyme inhibitors [9]. MDI set with an added temocillin disk showed 100% sensitivity and revealed that MBL was the dominating carbapenemase (84%), followed by OXA-48 (6%). Previous Egyptian study by Shaker et al. [29] using the same confirmatory detection set, showed that MBLs rate (82%) were comparable to ours (84%) although OXA-48 was the predominant enzyme (96%). A study previously done in Alexandria Main University Hospital (AMUH) revealed that 70% of their CRE isolates tested by carbapenemase production phenotypic tests were metallo- β Lactamase producing CRE of which NDM formed 67.5% [30].

The studied carbapenem resistance genes were detected by conventional PCR assay in 92% of the studied isolates. Interestingly, *bla* $_{NDM}$ producers prevailed among the isolates (84%) followed by bla_{OXA-48} (6%) and bla_{KPC} (2%), bla_{VIM} and bla_{IMP} carbapenemase genes couldn't be determined in any of the studied strains. In Egypt *bla* $_{NDM}$ was first detected in 2013 [31], but outbreaks of NDM were uncommon. Few recent studies reported that NDM is starting to predominate carbapenemase genes. Shawky et al. [32] reported that 75% of the isolates included in their study in Al Kasr AL-Ainy hospital, Egypt showed that the highest prevalence of carbapenemase encoded genes were bla_{NDM} followed by bla_{OXA-48} (59%). In addition, *Gamal* and colleagues [33] confirmed the emerging threat of NDM variants dissemination in carbapenem resistant *K. pneumoniae* (CRKP) in 94% of the studied isolates. In addition, *bla* $_{NDM}$ was the most recognized carbapenemase genotype (68.88%) followed by bla_{OXA-48} (32.59%) in cancer institutes in Egypt [34]. On the contrary, Shaker et al. [29] revealed that bla_{OXA-48} gene showed the highest percentage of prevalence (96%) followed by *bla* $_{VIM}$, bla_{NDM}, bla_{KPC}, and bla_{IMP} in which they represented 94%, 54%, 46% and 40%, respectively, El-Kholy et al. [28] also showed that bla_{OXA-48} was the dominating (40.6%) carbapenem resistance gene, followed by bla_{NDM1} (23.7%) and $bla_{OXA-232}$ (4.5%). NDM carbapenemase is considered a global health threat due to its ongoing worldwide dissemination and being linked to hospital recent outbreaks [35]. A recent review by El-Kholy and colleagues in Egypt reported that NDM and oxacillin carbapenemase 48 (OXA-48) genes were the most prevalent in Enterobacterales reaching 26.04–68.88% and 30–58.62%, respectively [36].

In Kuwait [25] reported that *bla* $_{NDM}$ was the most common carbapenem resistant gene detected (34%) in Enterobacterales strains, collected from four governmental hospitals in Kuwait. Also, Perovic et al. [37] stated that *bla* $_{NDM}$ was the most common detected carbapenemase producing gene in Enterobacterales

strains (59%) collected from National Health Laboratory Service in South Africa, followed by $bla_{\text{OXA-48}}$ (29%) then bla_{KPC} (1%).

Dominance of the bla_{NDM} in the current study and other studies from the Middle-East may be attributed to the existence of favorable conditions, such as dense populations, neglected hygienic practices, high selective pressure caused by the irrational antibiotic use and increased migration especially from the Indian continent [38]. In addition, strains carrying NDM gene are frequently MDR, as the plasmid which carry the NDM gene also carries resistance genes against aminoglycosides, macrolides and sulphamethoxazole, which leaves very few therapeutic options [39].

Rapid diagnostics in routine Laboratoy work are promptly required to detect MDR bacteria including carbapenemases with reliable sensitivity and specificity. Using MDI inhibitor set (Enteobacterales) with the addition of a temocillin disk in this study, showed 100% sensitivity and specificity with all tested genes in comparison to PCR assay as the gold standard method. Comparable results were obtained by Morsi [40] at Zagazig University Hospitals as the test showed 100% sensitivity and 88.9% specificity for $bla_{\text{OXA-48}}$, bla_{NDM} bla_{IMP}, bla_{VIM} and bla_{KPC}.

The predominant carbapenemase in our study was bla_{NDM} unlike that previously detected by *Shaker and colleagues* [29] as $bla_{\text{OXA-48}}$ was the main carbapenemase. In addition, the sensitivity and specificity of the test for whole carbapenemases were 82.1% and 70% respectively. The predominance of the weak OXA-48 in the previous study, may have lowered the specificity for the temocillin disk as well as the specificity of combined use of temocillin disk (50%) as it was often missed in detection. This difference in carbapenemase types has major clinical outcome that completely alters therapeutic options to patients, as with OXA-48 we still may use carbapnemes in high concentration and over prolonged infusion period or we may use the novel expensive antimicrobial available now in Egypt; ceftazidime/avibactam which on the contrary will not be effective against NDM-producing isolates and mandates the usage of colistin combination therapy with all its known side effects. Recent studies stated that NDM is the predominant carbapenemase in contrast to previous older studies indicating continuous and expected change of the type of prevalent carbapenemase reported over the time within each country and region [41, 42]. Previously, OXA-48 was the predominant carbapenemase in Egypt until few years ago where metallo beta- lactamases took the upper hand, as in our work.

5 Conclusion

As the use of carbapenems has significantly increased over time in our region, it is associated with increased incidence of CRE. The prevalent NDM carbapenemase in our hospital, with its high tendency to disseminate, represents a threatening health issue. The carbapenemase detection combination inhibitor disk set (Enterobacterales) MASTDISCS ID (MID) is a useful tool for rapid and precise confirmation of the diagnosis of CPE. Effective antimicrobial stewardship strategy and infection control measures are urgently required to slow down the dissemination of these CRE isolates.

Abbreviations

CRE	Carbapenem resistant Enterobacterales
MDR	Multi-drug resistance
KPC	*Klebsiella pneumoniae* Carbapenemase
MβLs	Metallo β-lactamases
OXAs	Oxacillinases
OXA-23	Oxacillinase-23
OXA-48	Oxacillinase-48
CPE	Carbapenemases producing Enterobacterales
TBRI	Theodor Bilharz Research Institute
AST	Antimicrobial susceptibility testing
ECOFFs	Epidemiological cut-off breakpoints
EUCAST	European Committee on Antimicrobial Susceptibility Testing
MEM	Meropenem
ERT	Ertapenem
IPM	Imipenem
TBRI-REC	Theodor Bilharz Research Institute Research Ethics Committee
MDI set	Mast discs combination detection inhibitor set
PCR	Polymerase chain reaction
ICU	Intensive Care Unit
K. pneumonia	*Klebsiella pneumonia*
E. coli	*Escherichia coli*
E. cloacae	*Enterobacter cloacae*
S. marcescens	*Serratia marcescens*
C. freundii	*Citrobacter freundii*
MASTDISCS ID	Mast discs detection inhibitor
CRKP	Carbapenem resistant *Klebsiella pneumoniae*

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Author contributions

EI is the PI of TBRI internal project 99D; from which this study aroused. EI and DM designed the study, supervised all the work, and contributed in the analysis of the results. SD and GD Contributed in the research work, supervised of technical work, and analyzed the data. SD is a major contributor in writing and revising the manuscript. AG performed laboratory work, interpreted the results, and performed statistical analyses. EE supervised the molecular work and contributed in data analysis. AAS and SM supervised all the work and contributed in data analysis. All authors critically revised the manuscript, and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

All specimens included in the study were archived, and codes were used instead of patient names. The protocol of the study was approved by TBRI Research Ethics Committee (TBRI-REC) under Federal Wide Assurance (FWA00010609) and the work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for Experiments in Humans and its later amendments (GCP guidelines) or comparable ethical standards.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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