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Modified combined disc test versus combined disc test in detection of carbapenemase producing *Klebsiella pneumoniae* at a tertiary care hospital, Egypt

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ABSTRACT

Background: Carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp) is considered a distressing healthcare problem owing to limited choices in its therapy. This study aimed to evaluate mCDT for carbapenem's detection in (Kp) in comparison to the traditional CDT and comparing their sensitivity and specificity. **Methods:** Thirty-six carbapenemase producer *Klebsiella pneumoniae* were enrolled in our study, they were previously isolated, conventionally identified and tested for carbapenemase production by screening method in other previous study, they were confirmed and differentiated into classes by combined disc test (CDT) and polymerase Chain reaction (PCR). The PCR positive strains were finally subjected to mCDT. **Results:** Thirty-five of the 36 CR-Kp isolates were positive for CDT and mCDT. mCDT with sensitivity and specificity to be 95.5% and 100%, respectively regarding Class B carbapenemase detection. Whereas Class D carbapenemase sensitivity and specificity of this modification were 77.8% and 100%, respectively. CDT and mCDT were fully agreed when compared with PCR results. **Conclusion:** mCDT is a sensitive and specific phenotypic method with the same accuracy as PCR.

Introduction

K. pneumoniae is capsulated gram-negative rods and lactose-fermenting pathogen which cause many infections including pneumonia, UTIs and bloodstream infections primarily in immunocompromised individuals.[1]

Carbapenems resistant *K. pneumoniae* is mainly mediated by carbapenemases which can be classified as Ambler Class A (encoded by KPCgene), Class B (encoded by NDM, IMP and VIM genes) and Class D (encoded by OXA-48 like gene). Class A and D enzymes have serine based hydrolytic activity (hence called serine

carbapenemase), whereas Class B needs metal e.g. zinc for its activity (thus known as metallo-beta-lactamase). They are obtained through mutations or horizontal gene transfer. [2]

In rare cases, resistance mediated by extended-spectrum β -lactamase (ES β L) and/or AmpC cephalosporinases especially in presence of diminished permeability of the outer membrane. [3]

Carbapenems antibiotics are given as the last choice antibiotics to critically infected patients with resistant bacteria [4]

Carbapenemase is the major element responsible for resistance to almost all commonly

used antibiotics. subsequently, prolonged hospitalization and poor outcomes. As a result, early and proper diagnosis of this bacteria is critical for improved management and better prognosis. [5]

Several phenotypic approaches for rapid detection of carbapenemase production were developed especially if molecular assays were not available [6].

Hence, this study was established to evaluate mCDT for carbapenemase detection in *K. pneumoniae* isolates in comparison to the traditional CDT. Assuming that mCDT is superior to CDT in the detection and differentiation of class A, B, and D carbapenemase producing *Klebsiella pneumoniae*.

Materials and Methods

This cross-sectional study was carried out at the Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University in collaboration with ICU Departments of Al-Ahrar Teaching Hospital from January 2021 to December 2021. The sample size was calculated using (open EPI_7). Assuming that the total number of patients admitted to Intensive care Unit Department in Al-Ahrar Teaching Hospital is 100 patients, and positive predictive value of the modified combined disc test in detection and differentiation of Class A, B and D carbapenemase producing *Klebsiella pneumoniae* is 96.4%, so the sample size is 36 *Klebsiella pneumoniae* isolates.

The study was approved by Zagazig University Institution Review Board (ZU-IRB#5541/10-9-2019). The Code of Ethics of the World Medical Association (Declaration of Helsinki) was followed in this study. Informed consent was obtained from patients or their relatives.

The selected *K. pneumoniae* isolates were previously isolated and conventionally identified. Also detection of antibiotic profile was done by disk diffusion method, as screening method for carbapenemase production, were inoculated on glycerol broth tubes and kept at -18°C.

Confirmation and differentiation of carbapenemase classes were performed using combined disc test (CDT) and polymerase Chain reaction (PCR). The genotypically confirmed positive strains were finally subjected to mCDT.

Detection of classes of carbapenemases by Combined disc test (CDT)

Meropenem (10 µg) disc, Meropenem (10µg) + EDTA (292µg) disc, Meropenem (10µg) +

PBA (400µg) disc, Temocillin (30µg) disc (**Liofilchem, Italy**) were used to perform CDT. [7]

The isolate that showed resistance to meropenem by the screening test was inoculated on Muller Hinton agar plate (MHA) (**Oxoid, UK**). Then one disc of Temocillin (30µg) was added to detect OXA carbapenemase and four discs of Meropenem (10 µg); one disc without any inhibitor, second disc with Phenyl Boronic Acid (PBA) (400 µg), third disc with EDTA (292 µg) and fourth disc with both PBA and EDTA were placed, and the plate was incubated at 37°C for 18-24 hrs. [8]

The diameter of the growth inhibitory zone around the discs was measured and interpreted (**Table 1**).

Genotypic confirmatory test for detection and differentiation of carbapenemase genes

Conventional PCR was done to all phenotypic positive strains. (KPC gene) for detection of class A, (VIM, IMP and NDM-1 genes) for class B and (OXA-48 gene) for class D carbapenemases. DNA was extracted according to the manufacturer's instructions using the i-genomic BYF DNA Extraction Mini Kit (**intron biotechnology, Korea**) and stored at -20°C until amplification was performed by PCR using thermal cycler (**Veriti @96 well thermal cycler, Applied Bio systems, Germany**).

Two multiplex reactions of total volume 20 µL were defined, with number (1) including detection of blaKPC, blaIMP and blaOXA-48 and number (2) including detection of blaNDM and blaVIM.

The reaction mixture for detection of blaKPC, blaIMP and blaOXA-48 genes contained 10 µL of 2X PCR Master mix solution (**DreamTaq Green PCR Master Mix (2X), ThermoFisher Scientific, Germany**), 1 µL of sense 5' primer, 1 µL of antisense 3' primer, 4 µL of template DNA.

The reaction mixture for the detection of blaNDM and blaVIM genes contained 10 µL of 2X PCR Master mix solution (**DreamTaq Green PCR Master Mix (2X), ThermoFisher Scientific, Germany**), 1 µL of sense 5' primer, 1 µL of antisense 3' primer, 6 µL of template DNA.

For the negative control reaction, all components of PCR reaction were added to the tube except for DNA to exclude any source of contamination.

The Primers and PCR conditions are shown in **Table 2** and **Table 3**. Finally, the PCR products

were analyzed by agarose gel electrophoresis on 1.5% agarose (W/Vol.) containing 0.5% mg/mL ethidium bromide (**Sigma, USA**), A 100-1000 bp DNA ladder was used as marker (**Roche, Germany**)

Detection of classes of carbapenemases by modified combined disc test (mCDT)

Genotypically positive strains were subjected to mCDT. One disc of Temocillin (30µg) and 4 discs of Meropenem (10µg); one disc without any inhibitor, second disc with PBA (400 µg), Third disc with EDTA (292 µg) and fourth disc with both PBA plus EDTA were placed on MHA plate. The direct colony suspension of test strain was streaked in a straight line of around 14 mm length from the edge of all five discs in same directions. Four strains were streaked at a time in four directions and plate was incubated at 37°C for 18-24 hrs. [10] The diameter of the growth inhibitory zone near the Meropenem disc with PBA, EDTA, or PBA plus EDTA was compared with that near the plain Meropenem disc and the data were interpreted (**Table 1**).

Klebsiella pneumoniae ATCC 2146 and *Klebsiella pneumoniae* ATCC 1705 were used as positive controls for MβL and serine detection respectively (**Liofilchem, Italy**).

Statistical analysis

Collected data were computerized and statistically analyzed using SPSS program (Statistical package for Social Science) version 21.0. Qualitative data were represented as frequencies and percentages. Chi-Square test (X²) was used to calculate difference between qualitative variables. The test results were considered significant when p-value < 0.05. Quantitative data were represented as

means and standard deviations. Accuracy was represented using the terms sensitivity, specificity, positive predictive value, negative predictive value.

Results

Combined disc test (CDT)

Thirty-five 35 (95.5%) isolates out of the 36 carbapenem resistant isolates were identified as carbapenemase producers by CDT and classes of carbapenemases were differentiated. (**Table 4**)

PCR results for diagnosis of carbapenemase genes

Thirty-two 32 isolates possessed only one gene, while the other 4 harbored combined genes. The most common resistance gene was *blaNDM-1* (22/36) followed by *blaOXA-48* (18/36) while the *blaVIM* gene and *blaKPC* had not been detected in our isolates. (**Table 5**).

mCDTperformance

Thirty-five 35 (95.5%) isolates were identified as carbapenemase producers by mCDT. Class B carbapenemase was detected in 21 *K. pneumoniae* isolates (60%), while class D was detected in 14 isolates (40%). mCDT, when compared with PCR results, showed sensitivity and specificity to be 95.5% and 100%, respectively regarding Class B carbapenemase detection. However, sensitivity and specificity of this modification, to detect Class D carbapenemase were 77.8% and 100%, respectively. CDT also showed total agreement as of mCDT, when compared with PCR. (**Table 6**). When performing the agreement between PCR and mCDT a high kappa value (0.924) with a highly significant p-value (<0.001) indicates strong agreement between the methods. (**Table 7**).

Table 1. Interpretation of CDT and mCDT for the differentiation of class A, B & D carbapenemase producing *K. pneumoniae* [9,10]

Method	Test	Definition of positive test result	Class A	Class B	Class A+B	Class D
CDT	PBA synergy test	Meropenem+ PBA ≥ 5 mm	+	–	–	–
	EDTA synergy test	Meropenem+ EDTA ≥ 5 mm	–	+	–	–
	PBA+EDTA synergy test	Meropenem+ PBA plus EDTA ≥ 5 mm	+	+	+	–
	Temocillin disc _a	Temocillin zone ≤ 10 mm	–	$\pm$	–	+
mCDT	PBA synergy test	Meropenem+ PBA ≥ 2.5 mm	+	–	–	–
	EDTA synergy test	Meropenem+ EDTA ≥ 2.5 mm	–	+	–	–
	PBA+EDTA synergy test	Meropenem+ PBA plus EDTA ≥ 2.5 mm	+	+	+	–
	Temocillin disc _a	Temocillin zone ≤ 3 mm	–	$\pm$	–	+

a: Interpreted only if PBA or EDTA synergy is absent

Table 2. primer sequence of blaNDM-1, blaVIM, blaIMP, blaKPC and blaOXA-48 genes: [11]

Gene target	primers sequence	Product size (bp)
NDM-1	F- GGTTTGGCGATCTGGTTTTC R- CGGAATGGCTCATCACGATC	621
VIM	F- GATGGTGTGTTGGTCGCATA R- CGAATGCGCAGCACCAG	390
IMP	F- GGAATAGAGTGGCTTAAYTCTC R- GGTTTAAAYAAAACAACCACC	232
KPC	Fm- CGTCTAGTTCTGCTGTCTTG Rm- CTTGTCATCCTTGTTAGGCG	798
OXA-48	F- GCGTGGTTAAGGATGAACAC R- CATCAAGTTCAACCCAACCG	438

Table 3. Conditions of PCR cycles: [12]

Cycle	Temperature	Time	Number of cycles
Initial Denaturation	94°C	10 min	1
Denaturation	94°C	30 sec	36
Annealing	52°C	40 sec	
Extension	72°C	50 sec	
Final Extension	72°C	5 min	1

Table 4. Carbapenemase classes in carbapenem resistant *K. pneumoniae* (CR-Kp) isolates as detected by CDT

Carbapenemase class	No. of isolates	%
Class B	21	60
Class D	14	40
Total	35	100

Table 5. PCR results for diagnosis of genes responsible for resistance among carbapenem resistant isolates.

Resistance gene	Positive	
	n=36	%
NDM	18	50
OXA-48	14	38.9
VIM	0	0.0
IMP	0	0.0
KPC	0	0.0
NDM+OXA-48	3	8.3
NDM+IMP+OXA-48	1	2.8

Table 6. Performance parameters of CDT and mCDT in comparison to PCR for the differentiation of Class B and Class D carbapenemase producing *K. pneumonia*.

Carbapenemase class detected by PCR	Phenotypic method	TP (n)	TN (n)	FP (n)	FN (n)	SN (%)	SF (%)	PPV (%)	NPV (%)
Class B (n=22)	CDT	21	14	0	1	95.5	100	100	93.3
Class D (n=18)		14	18	0	4	77.8	100	100	81.8
Class B (n=22)	mCDT	21	14	0	1	95.5	100	100	93.3
Class D (n=18)		14	18	0	4	77.8	100	100	81.8

* TP: True positive, TN: True negative, FP: False positive, FN: False negative, SN: Sensitivity, SF: Specificity, PPV: Positive predictive value, NPV: Negative predictive value

Table 7. Agreement between PCR and mCDT in detection of phenotypic type of carbapenemase producing *Klebsiella pneumoniae*.

		PCR		Total	Kappa test (95%CI)	P.value
		Class B	Class D			
mCDT	Class B	21	0	21	0.924 (0.831-1)	<0.001**
	Class D	1	14	15		
Total		22	14	36		

Figure 1. CDT, used as phenotypic confirmatory test, showing (A); Positive CDT for OXA-48 producing *K. pneumoniae* isolate showing <22mm growth inhibition to Meropenem disc with absence of synergy with the other 3 Meropenem discs with inhibitors and growth near Temocillin disc is less than 10mm. (B); M β L-producing *K. pneumoniae* isolate. Growth inhibition near to Meropenem disc and Meropenem+PBA disc is <22mm, growth inhibition near Meropenem+ EDTA with and without PBA disc is 5mm more than Meropenem disc alone and growth near Temocillin disc is more than 10mm.

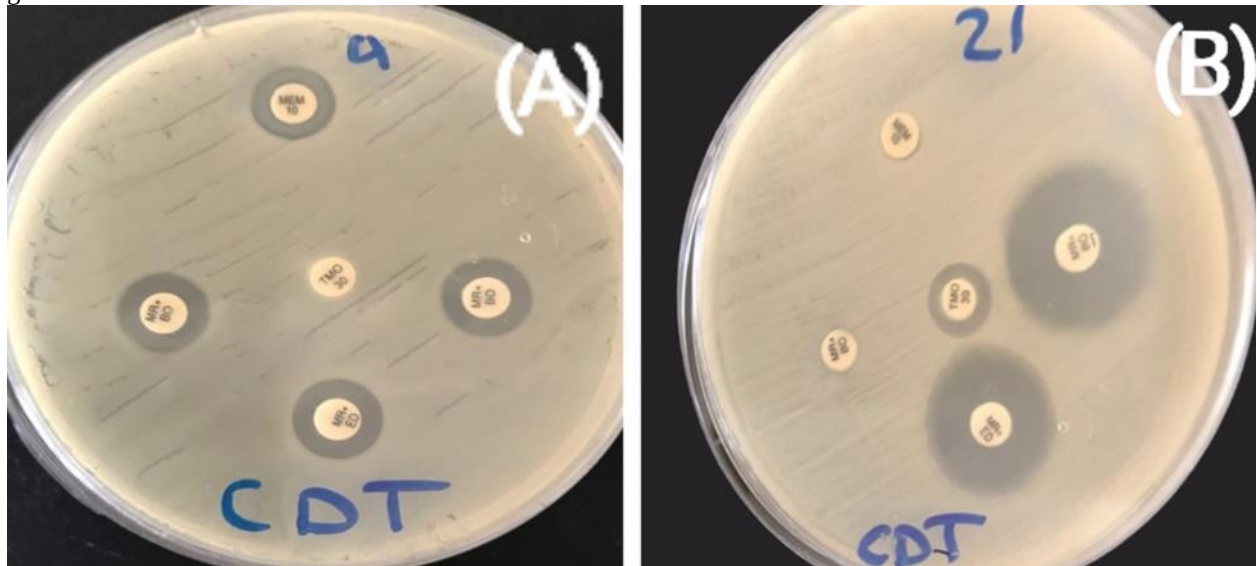
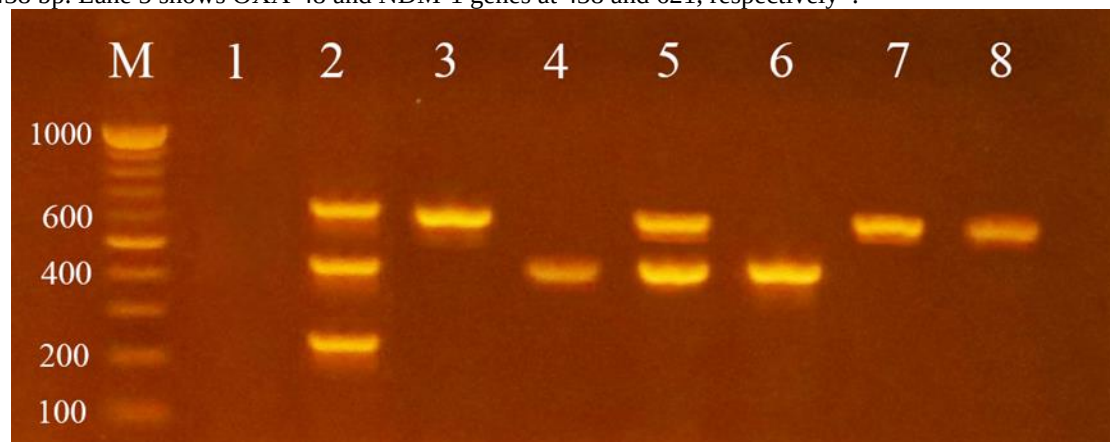
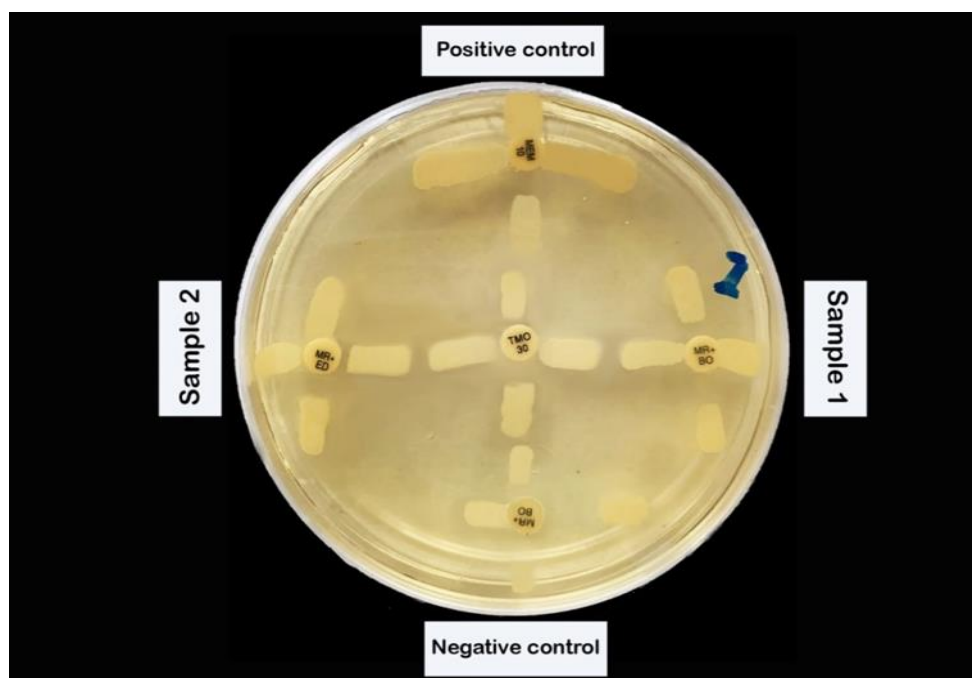


Figure 2. This figure shows UV detection of PCR results of carbapenemase genes. Lane M is a DNA marker with MW from 100 to 1000 bp. Lane 1 shows negative control. Lane 2 shows IMP, OXA-48 and NDM-1 genes at 232, 438 and 621 bp, respectively. Lanes 3,7 and 8 show NDM-1 genes at 621bp. Lanes 4 and 6 show OXA-48 genes at 438 bp. Lane 5 shows OXA-48 and NDM-1 genes at 438 and 621, respectively*.



* (IMP and NDM-1 genes) for class B and (OXA-48 gene) for class D carbapenemases.

Figure 3. mCDT shows positive control (KPC), negative control and two test strains around all five antibiotic discs.



- Sample 1 (MβL (Class B)) Meropenem disc <8mm zone of inhibition and Meropenem+PBA disc, growth inhibition near Meropenem+ EDTA with and without PBA disc is 2.5mm more than Meropenem disc alone and growth near Temocillin disc is more than 3mm.
- Sample 2 (OXA-48 (Class D)) Meropenem disc <8mm growth inhibition with absence of synergy with the other 3 Meropenem discs with inhibitors and growth near Temocillin disc is less than 3mm.

Discussion

The global dissemination of carbapenem-resistant *K pneumoniae* is considered a significant global health problem as they cause serious opportunistic infections and health care associated infections (HAIs) resulting in treatment failure, prolonged hospitalization and high mortality rates. Resistance is mainly attributed to carbapenemases production. Therefore, rapid detection of carbapenemase producers becomes essential for infection control purposes, successful treatment of patients and the preservation of carbapenem efficacy [13].

Regarding carbapenemase class detection by using CDT, as a phenotypic confirmatory test, carbapenemase class B (60%) (21/35) was the most common carbapenemase class of our isolates, followed by class D enzyme (40%) (14/35). The same result was reported by El-Domany and colleges in Kafr Elsheikh city, Egypt that class B carbapenemase enzyme showed the highest percentage (22%) [14]. This finding was matched with other studies that recorded the predominance of *bla*_{NDM-1} in Egypt and other countries. [15,16]

On contrary, Kumar *et al* observed that 82.7% and 2.5% carbapenemase producers belonged to B and D classes respectively. Rachana and his coworkers reported 83.3%, 13% for B and D respectively [10,17]

The prevalence of specific carbapenemase classes vary significantly depending on geographic location, local antimicrobial usage patterns, and infection control practices. [18].

Regarding prevalence of carbapenemase genes, our study reported that *bla*_{NDM-1} and *bla*_{oxa-48} were the most prevalent among carbapenemase encoding genes, and this supports the previous finding that NDM is endemic in northern Africa and OXA-48 is more prevalent in the Mediterranean region of Africa and Europe [19].

The most frequent carbapenemase gene among our isolates was NDM-1 gene with 22 (61.1%) detection rate in our isolates as a single and combined gene. This agreed with Onyeji and colleges who reported *bla*_{NDM-1} as a predominant gene 22 (44%) were positive for the NDM-1 gene among the tested isolates [2]. Also, a study by El-Domany and colleges revealed that the most

prevalent carbapenemase encoding gene was *bla*_{NDM-1} with incidence of 70% [14]

In comparison, another study reported the most frequent carbapenemase gene among CR-Kp isolates was OXA-48 gene (28.57%) [13]. Also, Memish and colleges declared that OXA-48 was the dominant carbapenemase gene among CP-Kp isolates across the kingdom [20].

Our results revealed that *bla*_{OXA-48} was the next prevalent carbapenemase gene detected as a single and combined gene with an incidence of 50% (18/36). In accordance with our results, Domany et al [14] and ElMahallawy *et al* [21] reported comparable detection rate (59% and 52%, respectively) of *bla*_{OXA-48} among their isolates. The *bla*_{OXA-48} gene was first detected in *K. pneumoniae* in Turkey in 2001 and after that, it spread rapidly throughout the Middle East and then all over the world. [22].

According to geographical and epidemiological studies, IMP type was found mainly in Japan, Taiwan and Eastern China, which may account for its lower frequency in our study [15].

On the other hand, our results showed that neither *bla*_{KPC} nor *bla*_{VIM} was detected in our current study. This agreed with ElMahallawy and colleges who weren't reported *bla*_{KPC} and *bla*_{VIM} among the tested isolates [21]. Despite *bla*_{KPC} wasn't detected in our study, other studies reported high prevalence of KPC in Egypt, and this suggested that it is underestimated problem [23]. KPC was first described in USA in the early 2000s, so far disseminated to other countries mainly Greece, China and South America [24].

This variation in prevalence of strains carrying carbapenemase genes may be attributed to differences in the predominance of carbapenem resistant *Enterobacterales* (CRE) between different hospitals and geographic areas [25].

Multiple carbapenemases genes producers tend to be extremely high resistant, and this leads to limited management. Our study showed that 11.1% (4/36) of tested carbapenemase producing isolates carried both *bla*_{OXA-48} and *bla*_{NDM-1}. In contrast, El-Domany and colleges reported a high incidence (48%) of *bla*_{OXA-48} and *bla*_{NDM-1} co-presence among CR-Kp isolates in Kafrelsheikh city, Egypt [14].

An interesting observation in the current results is that one isolate co-harbored a combination of (*bla*_{OXA-48}, *bla*_{NDM-1}, and *bla*_{IMP-1}) genes with an

incidence of 2.8%. A study by Osama and colleges reported the same result that one isolate co-harbored the unusual combination of the 3 carbapenemase genes (*bla*_{OXA-48}, *bla*_{NDM-1}, and *bla*_{IMP-1}) in Cairo, Egypt for the first time [26].

It is considered as an alarming finding confirming that absence of infection control policy and abuse of antimicrobials have contributed to the spread of this unusual combination of genes with the threat of extensively drug resistance (XDR) phenomenon which will limit treatment options, leaving clinicians with only last-resort drugs like colistin, tigecycline, or fosfomycin, which may increase the risk of toxicity and adverse effects.

Also, epidemiological Impact may occur with coexistence of multiple carbapenemase genes in a single isolate increases the potential for horizontal gene transfer to other bacteria, facilitating the spread of resistance genes across species. Moreover, the ordinary Phenotypic detection methods may fail to identify them, and this necessitate advanced molecular methods [27].

When sensitivity and specificity of CDT for detection of class B CP-Kp were compared to PCR results in our study, the finding showed sensitivity of 95.5% and specificity of 100%. This result was in line with a study by Morsi who reported similar sensitivity (94.6 %) but lower specificity (71.4%) for detection of class B CP-Kp. On the other hand, Hojabri and colleges found that sensitivity and specificity of CDT for detection of class B carbapenemase producing *Enterobacterales* were 86.2% and 100%, respectively [28].

This could be due to the false-negative results for the phenotypic test which may be attributed to some isolates producing low levels of carbapenemases, reducing detectability and the presence of hybrid or mixed carbapenemase types. Also, faulty CDT interpretation which needs precise measurement of inhibition zone diameters. [29,30].

By using mCDT for detection of carbapenemase encoding genes, class B CP-Kp isolates showed an incidence rate of 60% (21/35), while class D was 40% (14/35) which is the same obtained by CDT. However, the cost of consumed materials (culture media, antibiotic discs, EDTA, PBA) were four times cheaper in mCDT, because four test strains are streaked on single MHA plate compared to one strain in CDT.

Regarding the evaluation of performance of mCDT for detection and differentiation of

carbapenemase classes, the sensitivity and specificity of class B CP-Kp were 95.5% and 100%, respectively and this result matched with Kumar and his colleges who reported similar results of ours regarding sensitivity and specificity of mCDT for detection of class B CP-Kp (96.4% and 100%, respectively) [10].

Our study demonstrated the co-production of class B and class D carbapenemase in 4 isolates by using PCR. However, it was noticeable that the proposed strategy in mCDT and CDT is unable to detect class D in combination with class B carbapenemases, this finding was agreed by the study done by Van Dijk and colleges [8]. The difficulty to detect the combined carbapenemase classes (class B and class D) by the proposed strategy in mCDT and CDT was explained by Woodford and colleges that temocillin is a narrow-spectrum penicillin active primarily against *Enterobacterales* and resistant to hydrolysis by penicillinases, ES β Ls, and AmpC enzymes. and had some activity in vitro against *Enterobacterales* with KPC-type carbapenemases, while had no activity to OXA-48-like enzymes or the metalloenzymes (including IMP, NDM and VIM types) [31].

The phenotypic detection of carbapenemase classes depends on gene expression levels or enzyme activity which varies among isolates. Some isolates might harbor silent or poorly expressed resistance genes that molecular methods can detect but phenotypic tests cannot. [32].

This obstacle made sensitivity of both mCDT and CDT for overall detection of class D carbapenemase to be 77.8% with specificity 100%. This was agreed with Hojabri and colleges who reported the sensitivity and specificity of CDT for detection of class D carbapenemase producing *Enterobacterales* were 58.5% and 100%, respectively [28].

On the other hand, class D carbapenemase gene was detected as a single gene in 14 isolates in our study by mCDT. This result when compared with PCR result in detection of *bla*_{OXA-48} as a single gene, it showed 100% sensitivity and specificity for both. This agreed with Kumar and colleges who reported the same results [10]. CDT in our study showed the same sensitivity and specificity which was in accordance with results of Van Dijk and colleges [8].

Finally, the agreement between PCR and mCDT in detecting carbapenemase-producing *Klebsiella pneumoniae* phenotypes, shows high

kappa value (0.924) with a highly significant p-value (<0.001) indicates strong agreement. Strong kappa values (>0.8) typically reflect high reliability, ensuring that both methods are effectively interchangeable for clinical purposes.

As shown in this discussion, both CDT and mCDT had the same sensitivity and specificity for each carbapenemase class when compared to results obtained by PCR as a gold standard. However, the cost of consumed materials (culture media, antibiotic discs, EDTA) was four times cheaper in mCDT, as we can streak four strains on single MHA plate compared to one strain in CDT. As a novel phenotypic technique, mCDT has emerged as a practical and reliable tool for detecting carbapenemase production in *Klebsiella pneumoniae*, especially in regions with limited resources.

Conclusion

mCDT is reliable for simultaneous detection and differentiation of carbapenemase-producing *K. pneumoniae* with high sensitivity and specificity.

Limitations of the study

Potential biases may exist due to sample selection and geographic limitations.

Testing mCDT on other *Enterobacteriaceae* species to expand the study's relevance.

Author Contributions

AAA and ASLA contributed to the study conception and design. AAA, ASLA, AMM, AMM contributed to methodology, analysis, and interpretation of data. ASLA wrote the manuscript draft, provided review and editing. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Data availability

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

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