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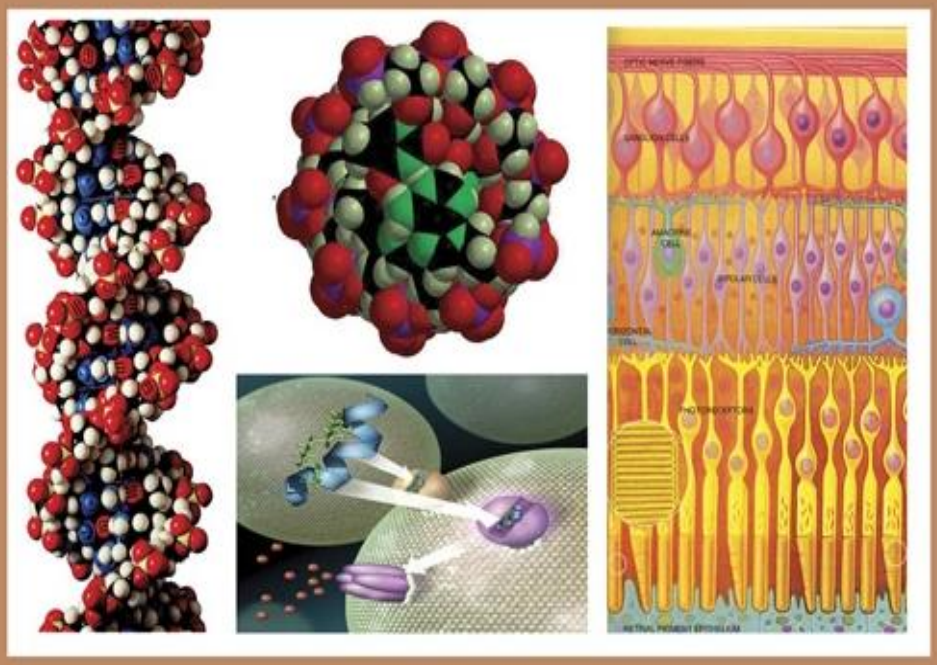

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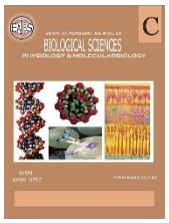

Efficiency of Various Detection Methods for Carbapenem Resistance Gram negative bacteria in King Faisal Medical Complex Hospital, Taif. Saudi Arabia

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ABSTRACT

Carbapenem resistance Gram-Negative bacteria (CR-GNB) impose life-threatening infections with limited treatment options. Rapid detection of CR-GNB-associated infections is usually associated with proper treatment and better disease prognosis. The aim of this study was to evaluate the efficacy of the Phoenix automated system, Modified Hodge Test (MHT) and X´pert Carba-R assay for the detection of CR-GNB. A panel of 167 non-repetitive CR-GNB with reduced susceptibility to carbapenems which was identified by the Kirby-Bauer method was analyzed by means of 1) Phoenix automated system, 2) MHT, and 3) X´pert Carba-R assay. The most accurate identification of resistance determinants was obtained with the Phoenix automated system that diagnosed and confirmed all carbapenem-resistant isolates (n=167/167, 100%). Just 57% of CR-GNB were identified by X´pert Carba-R Assay whereas seventy-nine (n=79/99, 79.8%) of CR *Klebsiella* spp., (n=5/23, 21.7%) of CR *Pseudomonas* spp., (n=10/32, 31.25%) of CR *Acinetobacter baumannii*, and (n=4/13, 30.8%) of CR other bacteria were identified. MHT correctly identified 56/99 (56.6%) strains of CR *Klebsiella* spp., 12/23 (52.2%) strains of CR *Pseudomonas* spp., 18/32 (56.25%) strains of CR *A. baumannii*, and 2/13 (15.4%) strains of other CR bacteria. While according to carbapenemase producers' genes, MHT most successfully identified bla_{NDM}+bla_{OXA} pattern of carbapenem resistance strains (n=25/26, 96.2%), then sensitivity lowered when testing bla_{OXA} positive strain with (n=17/30, 56.7%), and less than half bla_{NDM} positive samples were recognized by MHT with (n=19/42, 45.2%), sensitivity and specificity of MHT to detect carbapenemase producers' bacteria were 69.3% and 60.9%, respectively. Phoenix automated system diagnosed all the carbapenemase producers' bacteria in all genetic patterns as carbapenem resistance isolates with one hundred percent sensitivity but without any specificity to carbapenmase mechanism among other CR mechanisms. In **conclusion, t**o detect and control the spread of CR-GNB with complicated resistance mechanisms, phenotypic automated assays are recommended in the routine diagnostic of clinical laboratories, but genotypic assays are recommended in nosocomial infection control to detect carbapenemase producers.

INTRODUCTION

Mechanisms of resistance in Carbapenem-resistant Gram-Negative bacteria (CR-GNB) could be divided into two categories, adapted from Beatriz and Perez-Gracia. Those producing carbapenemase enzymes and those that do not (Amashah *et al.,* 2022). CR-GNB produce carbapenemases as their primary antimicrobial-resistance mechanism, carbapenemases are b-lactamases that belong to different Amber classes (A, B, and D). Class A carbapenemases are serine b-lactamases, with *Klebsiella pneumoniae* carbapenemases (KPCs) being the most common worldwide. Class B carbapenemases are Metallo-carbapenemases (also known as Metallo-B-Lactamases, MBL), with NDM, IMP, and VIM as common types. Class D is predominantly OXA-48-like serine b-lactamases. Despite the second category of CR mechanism does not produce carbapenemases, they produce other types of β-lactamases such as Type C ampicillinase (Amp C) or Extended spectrum β-lactamases (ESBLs) which are accompanied by alteration in porins or efflux pumps (Abou-assy *et al.,* 2022a; Suay-García and Pérez-Gracia, 2019). Bacteria can limit the entry of carbapenems into the periplasmic space where Penicillin-Binding Proteins (PBPs) exist, this mechanism involves the alterations in the porin-encoding gene or modification of the porin expression gene, leading to either complete loss or defects in the respective (Elshamy and Aboshanab, 2020). For example, the major mechanism of resistance to carbapenems in *P. aeruginosa* isolates is the downregulation of the gene encoding the *orp* D porin (Xu *et al.,* 2020). There is a serious need for rapid and accurate detection of carbapenem resistance (CR) and carbapenemase-producing (CP) isolates, the presence of CR and CP traits can be detected by several phenotypic and genotypic methods in clinical laboratories, these include automated systems or disc diffusion, MICs, modified Hodge test (MHT), selective agar, spectrometric, synergy tests, whole genome sequencing and molecular methods. The baseline test that first predicts CR-GNB is the use of phenotypic methods but the detection of the enzymes is difficult because of the several mechanisms involved and unreliable techniques practiced in some clinical laboratories (Al-Zahrani, 2018; Perez and Van Duin, 2013). X´pert Carba-R assay, a polymerase chain reaction (PCR)-based assay run on the GeneX´pert platform, is designed for the rapid detection and differentiation of 5 carbapenemase genes (*bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{NDM}$, *bla*$_{VIM}$, and *bla*$_{OXA\text{-}48\text{-}like}$) (Amashah e*t al.,* 2022; Tato *et al.,* 2016). The X´pert Carba-R assay operation requires two simple steps that could be conducted within 1 hour, with less than one minute of hands-on time (McMullen et al., 2017; Moore et al., 2017). The result proves X´pert Carba-R analysis is a reliable, accurate, and easy-to-use multiple qualitative analytic tools (Zhou *et al.,* 2019). MHT is a simple phenotypic test for the detection of the presence of carbapenemase enzyme in bacteria, it is relied on the inactivation of a carbapenem by CP strains that enable a carbapenem-sensitive indicator strain to extend growth towards a carbapenem disk, along the streak of inoculum of the tested strain (Datta *et al.,* 2017; Kim *et al.,* 2015). The aim of this study was to evaluate the efficacy of the Phoenix automated system, Modified Hodge Test (MHT) and X´pert Carba-R assay for the detection of CR-GNB.

MATERIALS AND METHODS

1. Carbapenem-Resistance Bacterial Strains:

One hundred and sixty-seven, non-repeat clinical isolates of CR-GNB were included in the study. They were collected in the Microbiology laboratory at KFMC in a previous study (Abou-assy *et al.,* 2022b). These encompassed *Klebsiella* spp., *Acinetobacter baumannii, Pseudomonas* spp., and some other CR-GNBs. All these isolates tested non-susceptible (i.e., intermediate or resistant) to carbapenems using Disk Diffusion Susceptibility Test

Protocol (Yen *et al.,* 2022) which was approved by CLSI as a gold standard clinical antimicrobial susceptibility test (CLSI publication, Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard 9th Edition) (Schumacher et al., 2018). To make sure that the condition of the experiment is set, *Escherichia coli* (ATCC™ 25922) and *Pseudomonas aeruginosa* (ATCC™ 27853) were used as quality control (QC).

2. Carbapenem Resistance Detection by Phoenix™ Automated System:

The bacterial inoculum was prepared from fresh nutrient agar of the pure culture, grown at 35±2°C for 24 hours. The Phoenix panel was inoculated with the prepared ID Broth and the absorbance was adjusted to 0.50–0.60 McFarland (Standard inoculum) by using the Phoenix Spec™ Nephelometer (BD Diagnostic Systems). Then, 25 µl of the prepared ID Broth with one drop from the indicator was added to Phoenix AST broth, placed closure securely on the panel to seal, then panels were loaded into BD Phoenix System 100 (Sparks, MD, USA). After 24 hours of incubation, the sensitivity of all bacterial isolates was obtained through the computer (Jin et al., 2020), QC organisms used were *E. coli* ATCC™ 25922 and *P. aeruginosa* ATCC™ 27853 which were recommended by NCCLS**.**

3. Carbapenem Resistance Detection by Modified Hodge test:

The CR-GNB were tested for carbapenemase production by the Modified Hodge test, as CLSI recommends the MHT to be performed before reporting carbapenem susceptibility results. A 0.5 McFarland dilution of the MHT indicator organism, *E. coli* (ATCC™25922) in 5 ml of saline was suspended and streaked onto a Mueller Hinton agar plate (Watin-Biolif™, KSA, Cat. A150635). A 10 µg imipenem disk (Mast group™, UK, Cat. IMI10C) was placed in the center of the test area. The test organism was streaked in a straight line from the edge of the disk to the edge of the plate with a sterile swab. The plate was incubated overnight at 35 ± 2°C. MHT Positive result showed a clover leaf-like indentation of the *E. coli* (ATCC™25922) growing along the test organism growth streak within the disk diffusion zone. Quality control of the following organisms: MHT Positive *Klebsiella pneumoniae* (ATCC™1705) and MHT Negative *K. pneumoniae* (ATCC™1706) were run with each batch of the test.

4. Detection of Carbapenemase by X´pert Carba-R assay:

X´pert Carba-R (GeneXpert™, Cepheid, USA) was performed on all CR isolates. This assay was performed using the GeneX´pert platform (Cepheid™, USA). The X´pert Carba-R assay is an *in-vitro* real-time PCR assay designed to detect five carbapenemase gene families, including *bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$, and *bla*$_{VIM}$. In more detail, the pure colony of the organism was transferred into the elution reagent tube and vortexed at high speed for 10 seconds. Following the manufacturer's instructions, the contents of the elution reagent tube were transferred using the transfer pipette provided (approximately 1.7 mL) into the specimen chamber of the X´pert Carba-R cartridge, and the run time was 48 minutes. The results were interpreted by the GeneXpert System (Sheth *et al.,* 2022).

RESULTS

Different clinical carbapenem resistance detection methods like real-time PCR (X´pert Carba R-assy), Phoenix automated system and modified Hodge test (MHT) conducted in all 167 strains in this study which was identified as CR-GNB using Disk Diffusion method that's the simplest common method in the medical laboratories. The disk diffusion method is one of the gold standard methods for testing the susceptibility of bacteria. Although the test is reliable, they require extensive manual laboratory work and the results are normally not obtained within the same day, limiting their application. Furthermore, these tests do not offer insights into mechanisms of action. In contrast, the Modified Hodge test is a simple test that can be performed in the

routine lab for the detection of carbapenemases in isolates showing intermediate or sensitive zone diameter on disc diffusion. In another hand, X´pert Carba R-assy is a commercial real-time PCR assay for carbapenemase detection from surveillance cultures, which can result in early implementation measures for infection control.

1. Efficiency of CR Detection Methods Among Various Bacterial Types:

Phoenix automated system diagnosed and confirmed all carbapenem-resistant isolates (167/167, 100%) which were identified by Kirby-Bauer test. By X´pert Carba-R Assay, seventy-nine (79/99, 79.8%) of CR *Klebsiella* spp., (5/23, 21.7%) of CR *Pseudomonas* spp., (10/32, 31.25%) of CR *A. baumannii*, and (4/13, 30.8%) of CR other bacteria were identified, that's mean the most mechanism responsible of carbapenem resistance among *Klebsiella* spp. strains were carbapenemase enzymes that can detect using X´pert Carba R-assay with the most efficiency in CR *Klebsiella* spp. (80%). Whereas, MHT correctly identified 56/99 (56.6%) strains of CR *Klebsiella* spp., 12/23 (52.2%) strains of CR *Pseudomonas* spp., 18/32 (56.25%) strains of CR *A. baumannii*, and 2/13 (15.4%) strains of other CR bacteria. There is a significant association between the biotypes of CR bacteria and CR detection within various studied methods ($P < 0.05$, Chi-Square test) which means that the efficiency of CR detection methods depends on the type of bacteria (Table. 1 & Fig. 1).

Table 1: Phoenix system, X´pert Carba-R Assay and Modified Hodge test efficiency among various CR-GNB biotypes.

Diagnosis method		Diagnosis method			P-value
		Phoenix (%)	Xpert Carba-R Assay (%)	Modified Hodge (%)	
Biotype	*Klebsiella* spp.	99 100	79 79.8	56 56.6	0.012*
	Pseudomonas spp.	23 100	5 21.7	12 52.2	
	Acinetobacter baumannii	32 100	10 31.25	18 56.25	
	Others	13 100	4 30.8	2 15.4	
Total		**167** **100**	**98** **58.7**	**88** **52.7**	

*** Significant association**

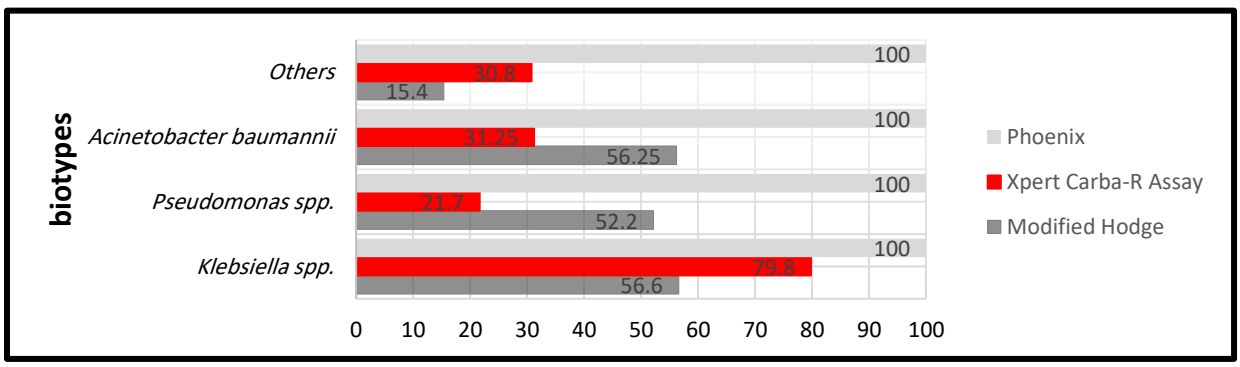

Fig. 1: Diagrams show Phoenix, X´pert Carba-R Assay and Modified Hodge test efficiency percentages among various biotypes.

2. Efficiency of CR Detection Methods Among Various Carbapenemase Molecular Patterns:

According to carbapenemase producers' (CP) genes, MHT most successfully identified *bla*$_{NDM}$+*bla*$_{OXA}$ pattern of CR strains (n=25/26, 96.2%), then sensitivity lowered when testing *bla*$_{OXA}$ positive strain with (n=17/30, 56.7%), and less than half *bla*$_{NDM}$ positive samples were recognized by MHT with (n=19/42, 45.2%), while the *bla*$_{NDM}$ positive sample gave a disturbed edge of the inhibition zone that did not lead to precise identification of the positive sample. Twenty-seven CR strains that didn't have any one of the five most popular carbapenemase genes by Carba-R assay recorded false positive results by MHT (n=27/69, 39.1%) as appeared in (Table. 2, Fig. 2). In our result, sensitivity, and specificity of MHT to detect CP bacteria were 69.3% and 60.9%, respectively. On another flip, Phoenix automated system diagnosed all the CP bacteria in all genetic patterns as CR isolates with one hundred percent sensitivity but without any specificity to carbapenemase mechanism among other CR mechanisms. There is a significant association between carbapenemase gene patterns and the efficiency of detection methods ($P < 0.05$, Chi-Square test) whereas CP bacteria which have *bla*$_{OXA}$+*bla*$_{NDM}$ genes were the best pattern to detect by MHT.

Table 2: Compare Phoenix, X´pert Carba-R Assay and Modified Hodge test efficiency among the most common carbapenemase genes

Diagnosis method		Diagnosis method			P-value
		Phoenix (%)	X´pert Carba-R Assay (%)	Modified Hodge (%)	
CP genes	*bla*$_{NDM}$	42 100	42 100	19 45.2	0.000*
	bla$_{OXA}$	30 100	30 100	17 56.7	
	bla$_{OXA}$ + *bla*$_{NDM}$	26 100	26 100	25 96.2	
	No CP genes	69 100	0 0	27 39.1	
Total		**167** **100**	**98** **58.7**	**88** **52.7**	

*** Significant association; CP: carbapenemase producer**

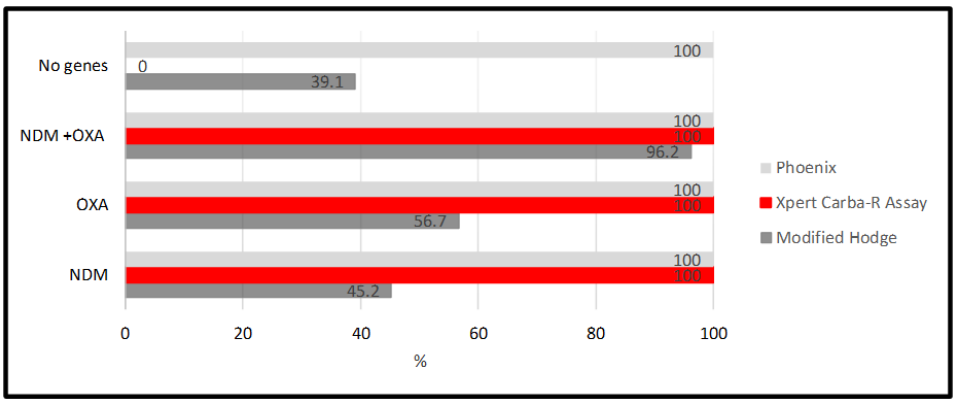

Fig. 2: Diagrams show the Phoenix system, X´pert Carba-R Assay and Modified Hodge test efficiency percentages among the most common carbapenemase genes.

3. Efficiency of CR Detection Methods Among Various Antibiotypes Categories:

Antimicrobial susceptibility test was determined for the panel of 19 antibiotics (ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefalotin, cefoxitin, ceftazidime, ceftriaxone, cefepime, ceftolozane/tazobactam, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, tigecycline, nitrofurantoin, trimethoprim/ sulfamethoxazole, azetronam and colistin) against all 167 CR-GNB clinical isolates using microdilution method by Phoenix system, then antibiotypes categories were determined to three categories including multiple drug resistance (MDR), extensively drug-resistant (XDR) and pan-drug resistant (PDR). MDR is antimicrobial resistance shown by a bacterial species to at least one antimicrobial drug in three or more antimicrobial categories, XDR is the resistance of one bacteria species to all antimicrobial agents except in two or fewer antimicrobial categories but PDR is the non-susceptibility of bacteria to all antimicrobial agents in all antimicrobial categories (Abbott *et al.,* 2013; Pattnaik *et al.,* 2019). Carba R assay successfully identified 85.7% of PDR strains, then sensitivity lowered when testing XDR strain with (n=57/100, 57%), and less than half of MDR samples were recognized (n=17/39, 43.6%). MHT similarly identified MDR, XDR and PDR with 41% (n=16/39), 60% (n=60/100), and 42.9% (n=12/28), respectively. There is no significant association between CR antibiotypes and the CR detection method ($P < 0.05$, Chi-Square test) (Table. 3).

Table 3: Phoenix system, X´pert Carba-R Assay and Modified Hodge test efficiency among various antibiotypes categories**.**

Diagnosis method		**Diagnosis method**			**P-value**
		Phoenix (%)	**X´pert Carba-R Assay (%)**	**Modified Hodge (%)**	
Antibiotype	MDR	39 100	17 43.6	16 41.0	0.231
	XDR	100 100	57 57	60 60	
	PDR	28 100	24 85.7	12 42.9	
Total		**167** **100**	**98** **58.7**	**88** **52.7**	

MDR: multiple drug resistance; XDR: extensively drug-resistant; PDR:pan-drug resistant

DISCUSSION

The massive worldwide spreading of CR-GNB and carbapenemase producers, mainly Enterobacteriaceae, has forced routine analysis to elaborate reliable detection methods. A rapid workflow with high sensitivity and specificity together has become mandatory to describe the treatability of serious CR-GNB and to control and hinder their spread. The epidemiology of CP bacteria has been widely discussed as it has become a major health issue, especially in countries where CP is becoming endemic (Escandón-Vargas *et al.,* 2017; French *et al.,* 2017).

In general, phenotypic tests such as Kirby-Bauer, Phoenix automated system, and MHT failed to characterize isolates harboring multiple carbapenem resistance determinants, which were successfully assessed only by PCR-based analysis like Xpert Carba-R assay (Bartolini et al., 2014). Many antimicrobial sensitivity tests (AST) including Kirby-Bauer considered a gold

standard AST while molecular methods are accepted as the gold standard method for the detection of carbapenemase enzymes especially. However, phenotypic methods have been developed because molecular methods cannot detect new carbapenemase genes, are not suitable for every laboratory, and are costly (Tamma and Simner, 2018; Testing, 2013; CLSI, 2012). The detection of CP by phenotypic methods is quite difficult since it depends on the type of bacteria containing the carbapenemase enzyme, the type of the enzyme, the expression level of the CR gene, and the presence of other resistance mechanisms including permeability reduction or the efflux pump (Abdi *et al.,* 2020; Voulgari *et al.,* 2013). MHT correctly identified 56.6% of CR *Klebsiella* spp.*,* 52.2% of CR *Pseudomonas* spp.*,* 56.25% of CR *A. baumannii,* and 15.4% of other CR organisms in this study.

The MHT is a simple phenotypic test for the detection of carbapenemase enzymes that depends on the ability of the CP strain to decrease the carbapenem concentration and enable the growth of a carbapenem-susceptible *E. coli* strain (Gniadek *et al.,* 2016). The MHT was recommended by the CLSI as a gold standard technique in the past years from 2009-2017 as a confirmatory test for carbapenemases based on its capability to detect bla_{KPC} pattern of CP, and it is therefore used in many clinical microbiology laboratories. It also has good sensitivity in terms of detecting other types of carbapenemases including VIM, IMP and OXA-48 (Lutgring and Limbago, 2016; Nordmann *et al.,* 2012; CLSI, 2012). However, the MHT is characterized by low sensitivity to other carbapenemases such as NDM, MBL, some OXA types, and SME (Gniadek *et al.,* 2016). In our results, MHT most successfully identified bla_{NDM}+bla_{OXA} pattern of CP strains (96.2%) but the sensitivity to detect bla_{OXA} CP strain and bla_{NDM} CP strain decreased to 56.7% and 45.2%, respectively. Furthermore, the MHT suffers from a lack of specificity due to the presence of mechanisms other than carbapenemases and may yield false-positive results when detecting some *Amp*-C-producing isolates combined with porin mutations (Lutgring and Limbago, 2016). Also, the sensitivity of the test is significantly affected by the type of carbapenemase in the isolates studied (Datta *et al.,* 2017; Saito *et al.,* 2015; Terzi *et al.,* 2019). In our result, the sensitivity, and specificity of MHT to detect CP bacteria were 69.3% and 60.9%, similar to previous studies conducted, the sensitivity and specificity of the MHT method varied between 58 - 99% and 38.9 - 100%, respectively (Datta *et al.,* 2017; Saito *et al.,* 2015; Terzi *et al.,* 2019). In a recent study, the overall sensitivity and specificity values for the detection of CP bacteria comparison to the PCR method were (65.62% and 100%) for MHT, (68.65% and 100%) for modified carbapenem inactivation method (mCIM), and (55.22% and 100%) for combined disk test (CDT), respectively (Kamel *et al.,* 2022). Such a finding could be attributed to a failure of MHT to detect 21 CP isolates that carry bla_{KPC} either alone or in combination with bla_{OXA-48} (Workneh *et al.,* 2019; Zhang *et al.,* 2022). Although MHT is applied for screening purposes, it is recommended that it should not be used as a validation test and results should not be given based only on this test.

Rapid detection of CR-GNB infection is of great significance to reduce the mortality rate (Li *et al.,* 2021). Recently, several commercial real time-PCR-based tests for detecting bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48} have been developed and shown to have 100% accuracy (Li *et al.,* 2021). This assay appears to be rapid, robust, useful and reliable for the specific detection of the most dominant carbapenemase genes (McEwan *et al.,* 2013; Nijhuis *et al.,* 2013; Traczewski *et al.,* 2018). These assays include Nucli SENS Easy Q KPC assay, hyplex SuperBug ID, X´pert Carba-R assay and Check Direct CPE (Noster *et al.,* 2021). These commercial assays are useful tools for carbapenemase detection from surveillance cultures, which can result in early

implementation measures for infection control.

X´pert Carba-R assay, as a real-time PCR-based detection instrument, boasts a short turnaround time of less than one hour, simple and fast operation, and a high detection rate (Teo *et al.,* 2018). X´pert Carba-R assay is highly inclusive in terms of the sources of the samples, by which the authors mean that different types of clinical specimens could serve as the samples, including urine, blood, respiratory tract sampling, body fluids, and rectal swabs (McMullen *et al.,* 2017). In another hand, X´pert Carba-R assay has certain limitations, in addition to high costs and specific expertise and equipment required to perform these tests, most of the current PCR-based methods cannot detect new carbapenemase types or new variants of known types (Al-Zahrani, 2018). To mention a case in point, X´pert Carba-R assay could not identify some subtypes of genes, in Findlay et al.'s study, the Carba-R assay cannot identify $bla_{\text{OXA-181}}$, one of the subtypes of the $bla_{\text{OXA-48}}$ family (Findlay *et al.,* 2015), and it is the same case with $bla_{\text{OXA-232}}$ (Tato *et al.,* 2016). Another limitation, the decrease in the positive rate may also be due to the low bacterial load of the samples (Zhou *et al.,* 2019). Moreover, different samples´ and types are always accompanied by varied sensitivities of the tests, which are the problems that await more advanced X´pert Carba-R assay to solve (Terzi *et al.,* 2019).

Conclusion

There are different methods of carbapenem resistance detection, including phenotypic and molecular methods. There is no single detection method that is valid and usable in all laboratories under all conditions. Laboratories should choose a suitable carbapenem resistance and carbapenemase detection method in line with their needs, economic requirements, and infrastructures. Although the detection of the presence of carbapenemase by molecular methods is fast and reliable, low-cost phenotypic tests can be used in laboratories that do not have this possibility. When selecting an ideal detection method for diagnostic use or screening purposes, it is essential to consider the objective of laboratory detection is to find carbapenem resistance traits generally or to find carbapenemase producers' strains for infection control purposes.

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