

Detection of Multi-Drug Resistant *Klebsiella Pneumoniae* in Al-Zahraa University Hospital

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ABSTRACT

Background : the rate of multidrug resistant (MDR) *klebsiella pneumonia* is increasing worldwide, its detection by available phenotypic methods represents a challenge. However, every lab should estimate its frequency for infection control measures and antibiotic stewardship program. **Objective:** The aim of the study was to estimate the frequency of MDR *klebsiella pneumoniae* in Al-Zahraa University Hospital, Cairo, Egypt, and to determine their different phenotypic methods. **Methods:** frequency of MDR *K. pneumoniae* isolates from different clinical samples provided to the microbiology laboratory, Al-Zahraa University Hospital, Cairo, Egypt during the period from May 2016 to January 2017 was detected by different phenotypic methods including ESBL detection (ESBL combined disk test, the double disk synergy test and the ESBL NDP test), AmpC production, carbapenemase production, the temocillin disk diffusion test, the Carba NP test, the Blue carba test and the genotypic detection of OXA-48 gene). **Results:** out of 2058 samples provided to Al-Zahraa University Microbiology Lab, 143 isolates were *K. pneumoniae* (6.94 %), 120 out of these 143 (83.9 %) were MDR. Their incidence rate (0.1) from the total and (0.8) and from the *K. pneumoniae* isolates respectively. 98.3% of these MDR were ESBL producers, 45.8% were AmpC B lactamase, 39.16% were carbapenem resistant while quinolone and aminoglycoside resistance were 63.6% and 68.3% respectively. **Conclusion:** continuous laboratory surveillance for different types of resistance in all *K. pneumoniae* isolates is recommended. ESBL NDP test and blue carba test could be used as routine tests in the microbiology lab for rapid detection of ESBL and carbapenem isolates.

Keywords: MDR *klebsiella pneumoniae*, ESBL, Carbapenem resistance, AmpC B lactamase.

INTRODUCTION

Klebsiella pneumoniae is a member of the *Enterobacteriaceae* family, a natural population of the nasal pharynx and the digestive tract of healthy humans and animals. It is a common pathogen associated with the opportunity, accounting for about one-third of all negative infections in grams. It is involved in a wide range of infections acquired from hospitals and society ⁽¹⁾.

The European Center for Disease Control (ECDC) and the Centers for Disease Control and Prevention MDR know that at least one factor is not obtained in three or more antimicrobial groups ⁽²⁾.

Many risk factors can contribute to multi-drug resistant *K. pneumoniae*. Including: age, previous hospitalization, in particular ICU admission, long hospital stay, and use of associated gaseous devices and associated diseases ⁽³⁾. Antibiotic resistance in *K. pneumoniae* occurs by inhibiting the enzyme with antibiotics, modifying the target or decreasing the concentration of antibiotics by reducing its permeability and increasing flow activity. These are encoded either substantially or by acquired resistance genes, too, as a result of environmental stresses through the formation of biofilm ⁽⁴⁾.

The prevalence and incidence rate of MDR *klebsiella pneumoniae* is different according to geographical area and even within the same country according to strict adherence to infection control strategies and strict antibiotic policy.

Detection of multidrug resistant *K. pneumoniae* becomes challenging as these strains co-carry diverse and

numerous multiple resistance determinants with very limited treatment options ⁽⁵⁾.

The aim of this study was to detect frequency of MDR *K. pneumoniae* in Al-Zahraa University Hospital, Cairo, Egypt and to assess risk factors for acquiring infections with these MDR *K. pneumoniae*.

MATERIALS AND METHODS:

This study included a total of 143 *K. pneumoniae* isolates recovered from all inpatient different clinical specimens provided to the Microbiology Laboratory, Al-Zahraa University Hospital, for culture and antimicrobial susceptibility testing. For patients from whom multiple isolates were collected, only the initial isolate was included. **Approval of the ethical committee was obtained.**

Isolates were identified as *K. pneumoniae* by the standard microbiological methods. These isolates were subjected to:

- A. **Antibiotic susceptibility tests:** by modified Kirby bauer using antibiotic discs (Ceftazidime (30 µg), Aztreonam (30 µg), Cefotaxime (30 ug), Cefepime (30 ug), Cefoxitin (30 ug), Cefuroxime (30 ug) , Gentamycin (10 ug), Amikacin (10 ug), Amoxicillin/clavulanic acid (30/10 ug), Meropenem (10µg), Trimethoprim/Sulfamethoxazole ((1.25/23.75 ug), Ciprofloxacin (5 ug), Pipracillin (100 ug), (Oxoid, UK). according to the recommendation of the *Clinical and Laboratory Standard Institute (CLSI)* ⁽⁶⁾.

klebsiella pneumoniae isolate was identified as multidrug resistant when it was resistance to ≥ 1 antibiotic in ≥ 3 different antibiotic groups ⁽²⁾.

B. Phenotypic confirmatory tests for ESBL production: for isolates conferring resistance to cephalosporins and or monobactam.

1. **ESBL Combined disk test:** by using cefepime, cefepime/clavulanic acid (Mast diagnostics D 36 C), cefotaxime, cefotaxime/clavulanic acid (Biorad) ⁽⁶⁾.
 2. **Double-Disc Synergy Test (DDST):** cefotaxime, ceftazidime, cefepime and aztreonam were used and applied next to a disc with clavulanic acid with a distance of 20 mm centre-to-centre. The inhibitory effect appears as distortion or increased of cephalosporin inhibition zone in the direction of the disc containing clavulanic acid ⁽⁷⁾.
 3. **The ESBL NDP (Nordmann-Dortet-Poirel) test:** Bacterial isolates were added to 100 μ l of Bacterial Protein Extraction Reagent (B-PERII; Thermo Scientific), 10 μ l of 0.8 mg/ L tazobactam solution (Sigma-Aldrich) was added to tube C; 100 μ l revelation solution [phenol red (0.5 %, w/v); Merck KGaA] was added to tube A and 100 μ l revelation solution supplemented with cefotaxime 6 mg/ ml (Claforan; Sanofi-Aventis) to tubes B and C. After 15 min incubation at 37 C, the colour of solution in tubes was observed. Positive results is indicated by changing the colour from red to yellow in tube B while tube A and C remain red in colour ⁽⁸⁾.
- C. Confirmatory test of AmpC detection:** it was done to all isolates with cefoxitin resistance by disk diffusion test ≤ 14 mm.
1. **AmpC disk test:** by applying a filter paper disk containing the tested organism, beside the cefoxitin disk in a plate with a lawn susceptible indicator strain (*E. coli* ATCC 25922) resulting in indentation or flattening of the zone of inhibition of the susceptible indicator *E. coli* strain around cefoxitin by AmpC producing bacteria ⁽⁹⁾.
 2. **Three-dimensional extract test:** using a crude enzyme extract of the tested organism, was prepared by repeated thawing and freezing of the bacterial pellet and applied to a slit done in the Muller Hinton media, with the use of a lawn culture of *E. coli* ATCC 25922 as indicator. Positive test is presented in the form of indentation towards the cefoxitin disc seen at the junction of the slit along the line of inhibition⁽¹⁰⁾.

D. Confirmatory tests for carbapenemase production:

1. **Combined inhibitory disk test for carbapenemase:** by using the MASTCDS (Mast Group, Merseyside, UK: D70 C) which contain 4 discs. Disc A contains meropenem with no inhibitor, disc B contains MBLs inhibitor, C contains KPC inhibitor and D contains AmpC inhibitors. Differences in zone sizes between A and B (≥ 5 mm) and A and C (≥ 4 mm) was read as MBL and KPC-type positive respectively. Where zone size differences of ≥ 4 mm and ≥ 5 mm were observed in both C and D respectively, compared to A, then test isolate was considered porin deficient AmpC producers ⁽¹¹⁾.
2. **The temocillin disk diffusion method:** Temocillin disk test was done on with carbapenemase negative or equivocal results by the MASTCDS kit. Decreased zone diameters for temocillin (< 11 mm) was used as a cutoff for temocillin resistance and indication of presence of OXA carbapenemase genes ⁽¹²⁾.
3. **The Carba NP test:** It is a biochemical test for detection of carbapenemase, This test is based on invitro hydrolysis of the β -lactam ring of imipenem by carbapenemases, resulting in acid product that can be detected by the change of the colour of the PH indicator (phenol red) from red to yellow or orange ⁽³⁾.
4. **The Blue Carba test:** It is based on in-vitro hydrolysis of the β -lactam ring of imipenem by carbapenemases, resulting in acid product that can be detected by the change of the colour of the PH indicator (bromothymol blue) from blue to yellow⁽¹³⁾.
5. **Genotypic detection of OXA-48 gene:**
6. It was done on the basis of real time PCR in 14 isolates that show discrepant results by combined inhibitory tests for carbapenemase detection and temocillin disk test.

RESULTS

Out of 2058 patient samples, 143 *K. pneumoniae* (6.94 %) were isolated from these specimens and 120 MDR *K. pneumoniae* were detected representing (5.83%) from total samples and (83.9%) from *K. pneumoniae* isolates.

Their incidence rate was (0.1) from the total and (0.8) from the *klebsiella pneumoniae* isolates respectively.

The distribution of MDR *klebsiella pneumoniae* isolates according to sample type and ward of isolation was illustrated in fig (1) and table (2).

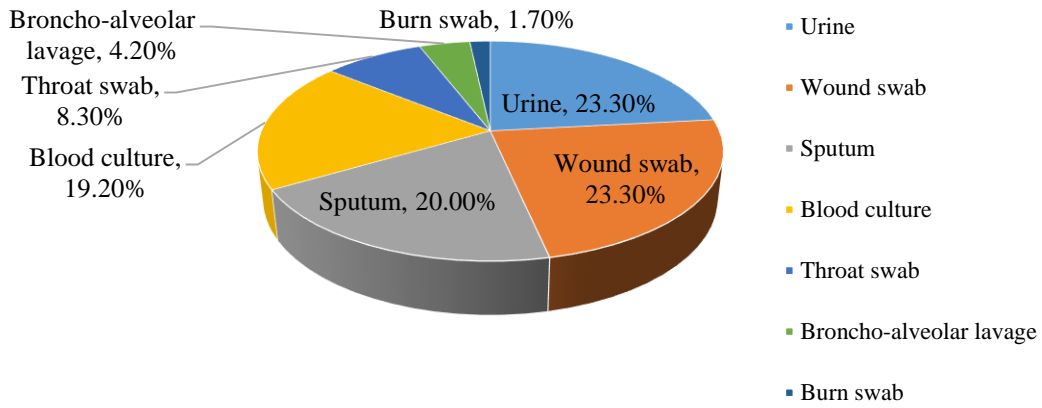


Fig (1): Distribution of MDR *klebsiella pneumoniae* isolates according to sample type

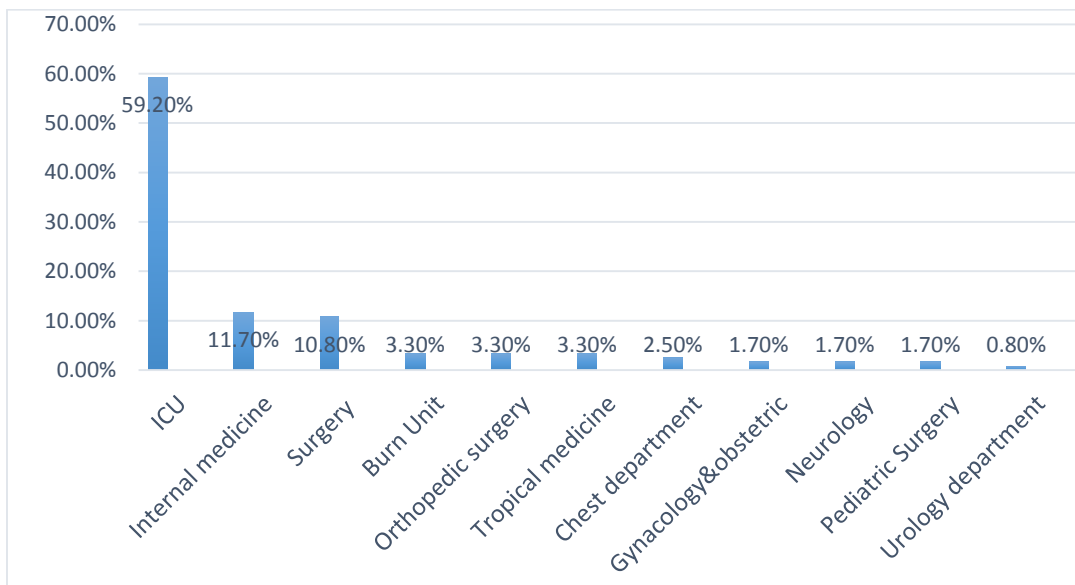


Fig (2): Distribution of MDR *K. pneumoniae* isolates according to the ward of isolation.

The demographic data of patients with MDR *K. pneumoniae*, (48.3%) were males and (51.7%) were females, (40.0%) patients gave history of antibiotic intake during the last month, (57.5%) patients gave history of previous hospital admission during the last year. (82.5%) infected patients had associated Comorbidities with the cardiovascular diseases were the predominant one (43.3%) followed by diabetes mellitus (23.3%) and neurological diseases (22.5%).

The distribution of different type of antimicrobial resistance was shown in fig (3).

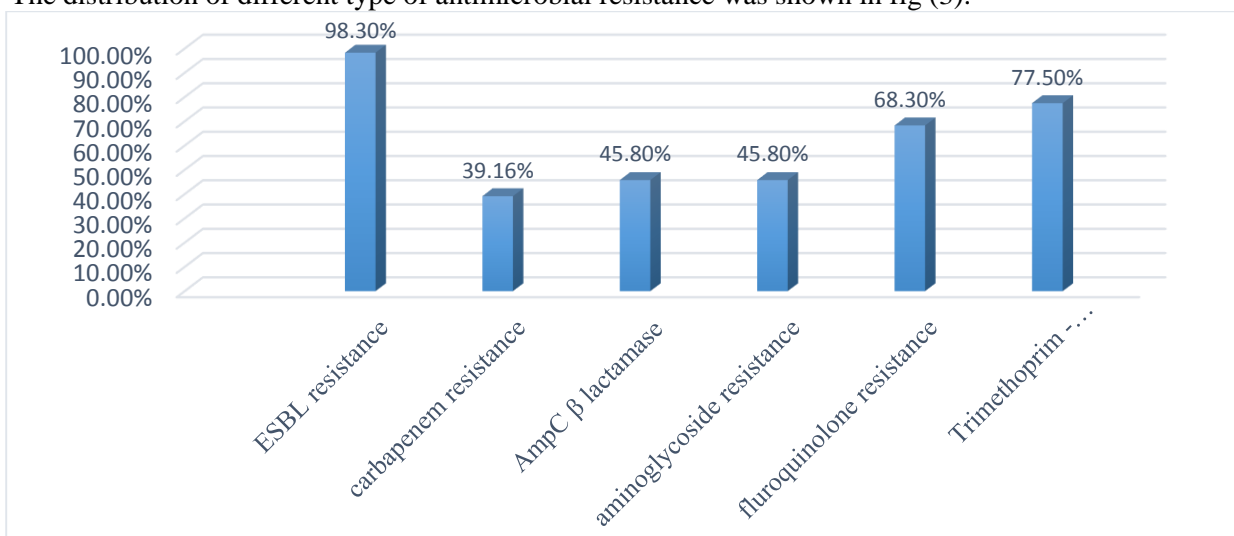


Fig (3): The antimicrobial resistance type of studied MDR *klebsiella pneumoniae* by disc diffusion.

ESBL detection: The results and performance of different confirmatory tests for ESBL production was illustrated in table (1and 2) and fig (4):

Table (1): Results of the confirmatory ESBL tests:

Method	Positive		Negative		Total		
	NO.	%	NO.	%	NO	%	
Combined disk test	97	80.8%	21	17.5%	118	98.3%	
Double disk test	93	77.5%	25	20.8%	118	98.3%	
ESBL NDP test	89	74.2%	29	24.1%	118	98.3%	
Total	120					100%	

Table (2): The performance of the Double disk synergy test and ESBL NDP test in ESBL detection:

		Combined disk test				Total		P value
		Negative		Positive		No	%	
		No	%	No	%			
Double disk synergy test	N	21	100%	4	4.1%	25	21.2%	0.000*
	P	0	0.0%	93	95.9%	93	78.8%	
ESBL NDP test	N	19	90.5%	10	10.3%	29	24.6%	0.000*
	P	2	9.5%	87	89.7%	89	75.4%	
Total		21	100%	97	100%	118	100%	

*P value = highly significance < 0.001 .

The sensitivity of ESBL NDP test in comparison to CDT was 89.7 %, specificity 90.5%, Positive predictive value (PPV) 97.8%, Negative predictive value (NPP) 65.5% and efficacy was 89.8%.

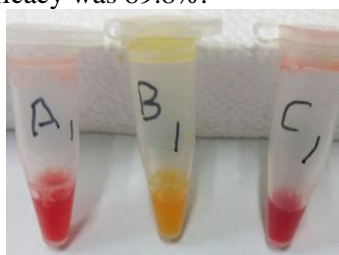


Fig (4): Positive results of ESBL NDP test; positive ESBL NDP test; tube A (without antibiotic), red in color, tube b (containing cefotaxime)gave yellow color (hydrolysis of cefotaxime), tube c (cefotaxime +tazobactam) no color changes (no hydrolysis of cefotaxim in presence of tazobactam).

AmpC detection:

Results of confirmatory tests for AmpC detection were illustrated in table (3)

Table (3): Results of the modified three dentionl test and AmpC disk test in the MDR klebsiella pneumoniae isolates.

Confirmatory tests for AmpC production	AmpC positive		AmpC negative		Total	
	No	(%)	No	(%)	No	(%)
Modified three-dimensiona l test	38	-31.70%	17	-14.10%	55	-0.458
AmpC disk test	33	-27.50%	22	-18.30%		
Total	120					100.00 %

Carbapenemase detection:

Table (4): Distribution of carbapenemase resistance among the MDR *K. pneumoniae* isolates:

Carbapenemase detection kits (D70 C and temocillin)	NO	%	Total	%
KPC	4	3.33%	47	39.16%
MBL	29	24.16%		
OXA- 48	14	11.67%		
Total	120		100	

We used real time PCR for confirmation of OXA-48 production in 14 isolates showing positive temocillin disc and the 14 isolates were positive for OXA-48 gene.

The Carba NP test and blue carba test were used as a confirmatory test for carbapenemase production and results are shown in table (5) and fig (5and6).

Table (5): The results of combined disk test, carba NP and blue carba test for carbapenemase detection in the carbapenem resistant studied isolates:

	Positive		Negative		Total	
	No	%	No	%	No	%
Carba NP	46	38.33%	1	0.83%	47	39.16%
Blue carba	47	39.16%	0	0.0%	47	39.16%
Total	120					100.0%

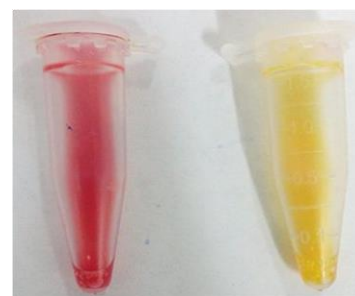


Fig (5): Positive carba NP test: tube A; negative control tube solution without imipenem. Tube B: solution with

imipenem. Change the color of the solution from red to yellow (hydrolysis of imipenem).

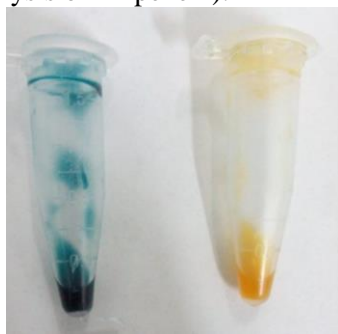


Fig (6): Positive blue carba test: Tube (A): control tube solution without imipenem (blue) . Tube (B): solution with imipenem with positive results, change the color of the solution to yellow (hydrolysis of imipenem).

DISCUSSION

In our study, out of 2058 clinical specimens, 143 isolates were *K. pneumoniae* (6.94 %), 120 of these isolates were MDR representing 83.9 % of the *K. pneumoniae* isolates and 5.83% of the total clinical isolates.

Similar to our results, in a study conducted in Iraq, 32 out of 464 (6.89%) were *K. pneumoniae* and MDR represented 5.81% and 84.37 of the total isolates and *K. pneumoniae* respectively. **Tohamy *et al.***⁽¹⁴⁾ reported 4.5% MDR among the studied samples.

Similarly, **Ferreira *et al.***⁽¹⁵⁾ reported that 21 out of 25(84%) isolates of *K. pneumoniae* isolates were MDR. While, in Egypt **Kamel *et al.***⁽¹⁶⁾ reported that MDR *K. pneumoniae* represented (95.77%) of the *Klebsiella* isolates.

While **El- Kady and Gouda**⁽¹⁷⁾ at El-Mansoura University Hospital reported MDR *K. pneumoniae* represented (49.2%) of the *K. pneumoniae* isolates. The difference in MDR *klebsiella* prevalence in different studies may be due to the difference in clinical state of patients, the sample included in the study, the antibiotic use and the implementation of the infection control measures. The isolation of MDR *K. pneumoniae* was highest from ICU (59.2%), followed by the internal medicine (11.7%), (66.6%) of these patients had invasive devices, the urinary catheter was the most one used (35.8%). We found that 47.5 % of patients had previous hospital stay during the last year and 40 % of patients had previous antibiotic exposure in the last month. Similarly **Zheng *et al.***⁽¹⁸⁾ found that; ICU stay, use of invasive intervention, medical history of previous hospitalization, exposure to antibiotics 90 days before infection, and antibiotic exposure during hospital stay are risk factors for acquiring MDR carbapenem resistant *K. pneumoniae*.

Most of ESBL Producing isolates are MDR. In our work, ESBL producers represented 98.3 % of MDR *K. pneumoniae* isolates. **El-Kady and Gouda**⁽¹⁷⁾ verified ESBL production in 71.2% of their MDR *Klebsiella* studied isolates.

The use of the ESBL combined disc test as a reference method in our work was based on the reported high sensitivity and specificity of the test and was recommended by the EUCAST 2017 for the detection of ESBL-producing isolates^(7,12).

In the double disk test, the use of cefepime along with the 3rd generation cephalosporins and monobactam, demonstrates the synergy which arises from the inhibition of ESBL by clavulanate in the presence of the AmpC enzyme enhancing the test sensitivity⁽⁹⁾. So, we used cefepime in the double disk synergy.

We found that, the double disk test had 95.5 % sensitivity, 100.0% specificity, (PPV) 100.0%, (NPP) 84.0% and efficacy 96.6% when compared with the combined disk test. In contrast to our results **Sarojamma and Ramakrishna**⁽²⁰⁾ in an Indian study reported sensitivity of double disk test 88% compared with the CDT. This lower sensitivity may be attributed to discrepancy between geographical areas with different genotypic profile, the lower number of tested ESBL producers and the use of cefotaxime and ceftazidime only, without cefepime and aztreonam used in our study.

We found that the ESBL NDP test was able to detect ESBL-producing isolates in 20 minutes with 89.7 % sensitivity and 97.8% positive predicative value.

In Banha University, **Saeed *et al.***⁽²¹⁾ reported a sensitivity of ESBL NDP of 90.5% from urine sample with 100 % specificity and PPV and 95 % NPV, while in blood culture had 100% sensitivity, specificity, PPV and NPV for ESBL NDP test. The ESBL NDP was effective in detecting ESBL producers carrying all genes detected in their study except two negative cases that showed coexistence of multiple genes.

While, **Poriel *et al.***⁽²²⁾ reported 95% sensitivity of the ESBL NDP test and explained that the inclusion of internal control allows better interpretation especially for weak positive and allows the detection of false positive due to no specific reaction.

In contrary to our results, **Affolabi *et al.***⁽²³⁾ found complete agreement between DDST and the ESBL NDP test, both had 100% sensitivity, specificity, PPV and NPP, but the ESBL NDP test gave results within 37 min in their study, so they recommended it as a rapid and reliable test.

The discrepancy among these results may be attributed to the coexistence of several resistance genes⁽⁸⁾, and the involvement of other types of cephalosporinases causing hydrolysis of cefotaxime not inhibited by clavulanic acid or tazobactam in ESBL producing isolates (AmpC producers and MBL carbapenemase producers) resulting in yellow/orange colour in b and c tubes (leading to a false-negative result)⁽⁸⁾.

Detection of isolates producing AmpC beta-lactamases are often associated with potentially false susceptibility to B- lactams phenotypically. Thus, their accurate, authentic and valid detection are important from epidemiological, clinical, laboratory, and infection control methods⁽²⁴⁾.

Out of 120 MDR isolates, 55 isolates (45.8%) were cefoxitin resistant, among them only 38 isolates (31.7%) were confirmed as AmpC B lactamase producers

by the modified three dimensional test and only (27.5%) were confirmed by AmpC disk test. These two methods detect AmpC B-lactamase as a mechanism of cefoxitin resistance, not other mechanisms as porin loss.

Liu and Liu ⁽²⁵⁾ reported that AmpC production in *K. pneumoniae* was (47.77%). While **Saffar et al.** ⁽²⁶⁾ reported that (15.6%) isolates were AmpC producers

The modified three dimensional test using enzyme extract has better sensitivity over AmpC disk test using bacterial colonies, but, the test is laborious, technically demanding requiring careful cutting of slit and well, time consuming, and needs experience ⁽²⁷⁾. All of the AmpC producing isolates in our study were ESBL producers 100%. **Sheemar et al.** ⁽²⁸⁾ found (25%) of *K. pneumoniae* ESBL producers, were co-producer of AmpC beta-lactamase using phenotypic methods.

In our work we evaluated the production of carbapenemases among the studied MDR *K. pneumoniae* isolates by Carba NP test and Blue carba test in comparison to the combined inhibitory disc test for carbapenemase production and the temocillin disc test.

The EUCAST ⁽¹²⁾ recommendation reported the use of carbapenem combined inhibitory test and temocillin disk diffusion in an algorithmic manner to detect and differentiate between KPC, MBL, OXA-48 phenotypically, differentiating them ESBL or AmpC production with porin loss as a mechanism of carbapenem resistance. The isolates with discrepant results for temocillin disk test and combined disk test were further evaluated for carbapenemase OXA-48 gene by real time PCR that revealed positive results in all discrepant isolates. So, temocillin disk test has 100% sensitivity in phenotypic detection of OXA-48 type resistance.

Although the carbapenemase combined inhibitory disk test is simple, cheaper and relatively efficient in detecting and differentiating between carbapenemases, it requires at least 18 h incubation to obtain results. This longer turnaround time is the main drawback of this inhibitor test. As with all phenotypic tests, the potential exists for false negative results in the event of unexpressed or minimally expressed resistance genes ⁽²⁹⁾.

Despite high sensitivity of Temocillin disk test, it was reported to have poor specificity, as it can give false positive results in MBL producing isolates. So, it cannot be used as initial method but it can be used as apart of algorithm in isolates that yield a negative result by the combined inhibitory test for carbapenemase production ⁽²⁹⁾. The biochemical tests hold much promise over other phenotypic methods, due to their relatively lower cost, faster turnaround time, simplicity, and minimum skill required in conducting them. They are also able to differentiate between carbapenemase producers and noncarbapenemase-producing CREs, ESBLs and/or AmpCs hyperproducers with membrane impermeability ⁽²⁹⁾.

The advantage of the Blue carba over the carba NP test is the increased protocol simplicity and the significantly reduced cost per reaction due to the direct use of colonies instead of bacterial extracts by extraction

buffer (B-PER II) with the use of bromothymol blue as the indicator, since it includes the optimal pH for most β -lactamases (pH = 6.8), and the validation of the test for the detection of OXA-type carbapenemases ⁽³⁰⁾. However, their inability to differentiate between carbapenemase types, especially for isolates expressing multiple carbapenemases, and to detect isolates with low carbapenemase hydrolysis activity, makes them less effective than molecular tests ⁽³⁰⁾. So, they can be used routinely as initial screen for carbapenemase production for CRE isolates with the need for molecular methods in negative results. In our work, the blue carba NP test gave 100.0 % sensitivity, accuracy and PPV. Similar to our results **Pires et al.** ⁽³⁰⁾ and **Kamel et al.** ⁽¹⁶⁾ found that the blue carba test was able to rapidly detect all variants of carbapenemases gene with 100.0% sensitivity and PPV and accuracy.

While, **Pasteran et al.** ⁽³¹⁾ and **García-Fernández et al.** ⁽¹³⁾ found the blue carba sensitivity was 97.0% and 98.0% respectively. These lower results can be attributed to the involvement of OXA-48 enzymes in hypermuroid phenotype included in the first study, decreasing test sensitivity, also in the second study, involvement of enterobacteracea, pseudomonas and acinetobacter with OXA-163 producers, in addition to the use of a modified protocol using 5 uL loopful bacteria resulted in lower sensitivity.

In our work, the Carba NP test had a 97.7% sensitivity and 97.9% for accuracy and PPV. **García-Fernández et al.** ⁽¹³⁾ reported Carba NP test had 100% sensitivity. However, they gave these results after modification of the carba NP test by the use of 200 ul lysis buffer, while before this modification the sensitivity was 96.23%, near to our results. In contrary to our results **Pancotto et al.** ⁽³¹⁾ found that the sensitivity of the Carba NP test was 62.7%. It detected all metallo-enzymes tested and all KPC isolates while, the reduced sensitivity of Carba NP was mostly observed in OXA-370 isolates in the second study. Molecular detection of MDR-coding genes is interesting but remains costly, requires expertise, and does not detect all genes encoding enzymes exhibiting MDR activity (ESBL encoding genes, AmpC B lactamase genes, carbapenemase genes, aminoglycoside resistant genes and can not detect resistance due to new undiscovered genes.

In conclusion, high frequency of MDR *Klebsiella pneumoniae* was found in our hospital mainly in ICU patients, in patients with inserted devices and associated comorbidities. It is of value to estimate the frequency of these MDR isolates by available phenotypic methods available in most routine laboratories.

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