

ORIGINAL ARTICLE

# Phenotypic and Genotypic Detection of *klebsiella Pneumoniae* Carbapenemase and New Delhi Metallo- $\beta$ -Lactamase of *Enterobacteriaceae* in Benha University Hospitals

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## ABSTRACT

### Key words:

CPE,  
*bla* KPC gene,  
*bla* NDM gene,  
PCR

**Background:** The prevalence of carbapenem-resistant *Enterobacteriaceae* is on the rise worldwide, posing a major public health threat as well as a serious concern for infection control management. The accurate identification and reporting of carbapenemase producing *Enterobacteriaceae* (CPE) will aid infection control practitioners in preventing the spread of these multidrug-resistant isolates. **Objectives:** This study aimed to detect the prevalence of CPE in Benha university hospitals and to confirm the presence of *k. Pneumoniae* carbapenemase (KPC) and New Delhi Metallo- $\beta$ -Lactamase (NDM) in *Enterobacteriaceae*. **Methodology:** This study was conducted on 100 *Enterobacteriaceae* strains collected from patients admitted to Benha University Hospitals. The isolated *Enterobacteriaceae* strains were screened for CPE by chromID CARBA agar with species identification by using API 20 E test strips as a biochemical identification system. KPC and NDM carbapenemases detection was confirmed phenotypically by Mast Disc ID carbapenemase detection set (MDI) and genotypically by multiplex PCR. **Results:** Out of 100 *Enterobacteriaceae* isolates, 40 strains (40 %) are carbapenemase producers and 27 strains of them (67.5 %) are carrying genes responsible for carbapenem resistant. Twenty six strains (65%) are carrying KPC gene and only one *k. Pneumoniae* strain (2.5%) is carrying NDM-1 gene for M $\beta$ L production. MDI detected 60% of KPC and 25% of M $\beta$ L producers with high sensitivity (92.6 %) in comparison to PCR. Intensive Care Unit patients harbored most of carbapenem-resistant isolates with the highest percentage of carbapenemases genes in *k. Pneumoniae*. **Conclusion:** Emergence of CPE pathogens in our setting create an important challenge for clinicians and hospital epidemiologists with the possibility of outbreak eruption by these difficult-to-treat pathogens, in the future.

## INTRODUCTION

Carbapenems are potent and broad-spectrum  $\beta$ -lactam antibiotics traditionally reserved for the treatment of the most serious infections. Carbapenem resistant *Enterobacteriaceae* (CRE) can be defined as *Enterobacteriaceae* that are resistant to one or all of the following carbapenems: ertapenem, meropenem, imipenem or doripenem; and resistant to all of the following third-generation cephalosporins: ceftriaxone, cefotaxime, and ceftazidime.<sup>1</sup>

The US Centers for Disease Control and Prevention identified carbapenem-resistant *Enterobacteriaceae* as 1 of the 3 most urgent MDR threats. Carbapenem resistance has been associated with different mechanisms including carbapenemase production,

overexpression of chromosomal AmpC or ESBL production combined to porin mutations. *Enterobacteriaceae* harbouring carbapenemases belong to one of three major classes according to the Ambler classification system: class A carbapenemases, class B metallo- $\beta$ -lactamases (MBL), and class D  $\beta$ -lactamases (OXA).<sup>2</sup>

Two carbapenemase subclasses are especially problematic: *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo- $\beta$ -lactamase-1 (NDM-1).<sup>3</sup> The horizontal transmission of carbapenemase genes mediated by mobile genetic elements carrying additional resistance elements, which confer resistance to various groups of antibiotics, results in multidrug resistance, including bacteria resistant to all available antibiotics.<sup>4</sup>

The most clinically important CPE is KPC-producing *K. pneumoniae*, which are no longer limited to *K. pneumoniae*. They have also been found in many other *Enterobacteriaceae* including *E. coli*, *Enterobacter* species, and *Citrobacter freundii* and has

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become endemic in many countries around the world.<sup>5</sup> In Egypt, KPC-producing *Enterobacteriaceae* isolates have been also reported.<sup>6</sup>

Among the newly emerged carbapenemases, New Delhi metallo- $\beta$ -lactamase-1 (NDM-1)-producing strains, NDM-1-positive *Enterobacteriaceae* is now the focus of worldwide attention. Most patients from whom NDM-1 is isolated have an epidemiologic link to the Indian subcontinent, but NDM-1 has also recently become endemic to the Balkans and Middle East suggesting that those areas might constitute a secondary reservoir of NDM-1-producing strains.<sup>7</sup>

Unfortunately, the prevalence of CPE has increased during the past 10 years, seriously compromising the therapeutic choice. This increasing prevalence of CPE poses a challenge in the treatment of healthcare-associated infections.<sup>8</sup>

CPE cause infections with mortality rates of 40% to 50%.<sup>9</sup> Therefore, emphasis should be placed on the early detection of carbapenemase enzymes, the prevention of the spread of carbapenemase-producing bacteria and the development of new drugs resistant to carbapenemase hydrolysis.<sup>10</sup>

Detection of carbapenemase activity in *Enterobacteriaceae* has been identified as a challenge for both KPC enzymes and MBLs. Isolation of carbapenemase producing bacteria that is not resistant to carbapenems by current carbapenem antimicrobial susceptibility testing methods is a frequent problem.<sup>11</sup>

Series of non-molecular-based tests have been proposed for the detection and identification of carbapenemases including a screening step followed by a phenotypic and genotypic confirmation step by using either simplex or multiplex PCR assays in order to deal with the problems of phenotypic detection methods and reduce the detection time.<sup>12</sup>

Surveillance and infection control are of paramount importance to limit dissemination at the local, national and international levels.<sup>13</sup> Preventing transmission is presently the starting point to win this war.<sup>14</sup>

## METHODOLOGY

This work was carried out in Medical Microbiology & Immunology department, Faculty of Medicine, Benha University in the period from March 2015 to April 2016.

### 1. Subjects and samples:

The study was conducted on 100 strains of *Enterobacteriaceae* isolated from 157 random clinical samples including: (22) bronchial aspirate, (27) sputum, (14) pus, (32) urine, (5) stool. The clinical samples were collected from 157 hospitalized patients admitted to Benha University hospital and outpatient clinics. They were 86 males and 71 females and their ages ranged from 11 years to 80 years.

This study was approved by Benha University ethical committee and consent was obtained from all patients under study.

### a. Isolation and identification of *Enterobacteriaceae*

Clinical samples were cultured on MacConkey's agar plates while urine samples were cultured on CLED agar plates then incubated at 37°C for 24h. The growing organisms were identified as *Enterobacteriaceae* by the standard laboratory technique including: Gram staining, colony morphology, sugar utilization tests and oxidase reaction.

Isolates identified as Gram negative bacilli, glucose fermenters and oxidase negative were considered *Enterobacteriaceae*.

### b. Screening for Carbapenemase Producing *Enterobacteriaceae*

*Enterobacteriaceae* isolates were screened for carbapenemase production by culturing on chromID CARBA agar (CARB) (bioMérieux, France). ChromID CARBA agar contains a mixture of antibiotics which enable the selective growth of CPE particularly KPC and NDM-1 and chromogenic substrates which enable the identification of the most frequently isolated CPE. As following 24h of incubation, carbapenemase producing colonies appear with different colors according to their specific enzymatic properties: *E. coli* appear as pink to burgundy colonies and KESC (*Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter*) as bluish to green colonies.

### c. Differentiation of bacterial species

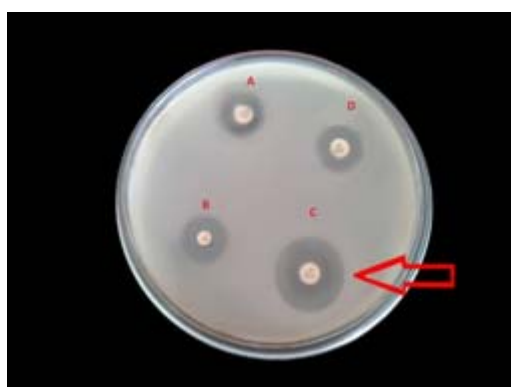
API 20E test strips (BioMérieux, France) was used as a biochemical identification system for identification of the isolated genera to the species level.

### 2. Phenotypic detection of carbapenemases producing *Enterobacteriaceae*

Phenotypic confirmatory step was employed by Mast Discs ID (MDI) carbapenemase (*Enterobacteriaceae*) detection set. (D70C) (Mast Diagnostics). Mast D70C is a four disc detection set comprising Meropenem (10  $\mu$ g) (A), Meropenem with M $\beta$ L inhibitor (B), Meropenem with KPC inhibitor (C) and AmpC inhibitor discs (D). MDI detects M $\beta$ L positive strains, KPC positive strains, and also can differentiate KPC positive isolates from isolates expressing AmpC coupled with porin loss (impermeability). The interpretation of the test according to the manufacture instructions: 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  $\geq 5$  mm from disk A, the organism is recorded as demonstrating M $\beta$ L activity. If disk C shows a zone difference of  $\geq 4$  mm from disk A, the organism is recorded as demonstrating KPC activity. If disk C and disk D both show a zone difference of  $\geq 5$  mm from disk A, the organism is recorded as demonstrating AmpC activity coupled with porin loss (impermeability). (Figures 1, 2).



**Fig. 1:** A plate of Muller Hinton agar showing Mast Disc ID test indicating MβL producing isolate



**Fig. 2:** A plate of Muller Hinton agar showing Mast Disc ID test indicating KPC producing isolate

### 3. Multiplex PCR for detection of carbapenemases genes:

Multiplex PCR testing of all CPE isolates for the presence of *bla* KPC and *bla* NDM carbapenemases genes. Two primer pairs (Biosearch technologies, USA), specific for each gene of carbapenemases were used.

#### DNA extraction:

Total DNAs of the different bacterial isolates were extracted by the DNA extraction kit (Thermo Scientific GeneJET Genomic DNA Purification Kit #K0721) according to manufacturer instructions. The extracted DNA was then stored at -20°C until further processing.

#### DNA amplification:

Amplification was done using My Taq Red PCR Master Mix (2x) (Thermo Scientific, EU Lithuania). The PCR mix contained 25ul of PCR master Mix, 1ul of each forward primer, 1ul of each reverse primer, 5ul of the template DNA and the amount completed with nuclease free water to reach a final volume of 50ul. Thermal cycler (Biometra, Germany) was used for amplification according to the following program: initial denaturation at 95 °C for 1mins, 35 cycles of denaturation at 95 °C for 15 sec , annealing at 50 °C for 15 sec followed by extension at 72 °C for 10 sec.

#### DNA detection by agarose gel electrophoresis:

Ten μl of each amplified DNA & 100 bp ladder (molecular weight marker) were separated on 1.5 % agarose gel containing 2μl of ethidium bromide. The bands were visualized using UV trans- illuminator (312 nm), photographed and analyzed.<sup>19</sup>

The *bla* KPC and *bla* NDM genes were determined by the site of amplified product in comparisons with known ladder bands as *bla* KPC gives a band at 785 bp<sup>[23]</sup> and *bla* NDM gives a band at 621 bp<sup>[31]</sup>

## RESULTS

Out of 100 strains of *Enterobacteriaceae* obtained during the study period, 40 isolates (40 %) are carbapenemase producers as detected by using chromogenic medium ChromID CARBA agar.

API 20E test strips was used as a biochemical identification system for identification of the species of the resistant isolated strains; *K. pneumoniae* is the most common bacterial isolate accounted for 62.5% followed by *E.coli* (27.5%) then *Enterobacter sakazakii* (7.5%). *Citrobacter farmeri* is the least common species isolated (2.5%).

Multiplex PCR was applied for carbapenemases genes detection and revealed that out of 40 strains, 27 strains (67.5 %) are carrying carbapenemases genes. 26 strains (65%) are carrying KPC gene and 1 strain (2.5%) is carrying NDM gene for MβL production. (Table 2), (Figure 3).

Mast Disc ID Carbapenemase Detection Set (D70C) was used for all 40 carbapenemase producers isolated for phenotypic confirmation, KPC (60%) and MβL (25%) producers are detected with (92.6%) sensitivity and (30.8%) specificity in comparison to PCR as a gold standard method. (Table 1)

This study shows that the urine samples represent the most common site for CPE production (35 %) followed by bronchial aspirate, sputum samples and pus are (30 %, 25 % and 10 % respectively). The major percentage of the study population infected with CPE are from intensive care units (ICU) (30 %) followed by urology department (22.5 %).

**Table 1: Sensitivity and specificity of Mast Disc ID (MDI) carbapenemase detection set versus Multiplex PCR.**

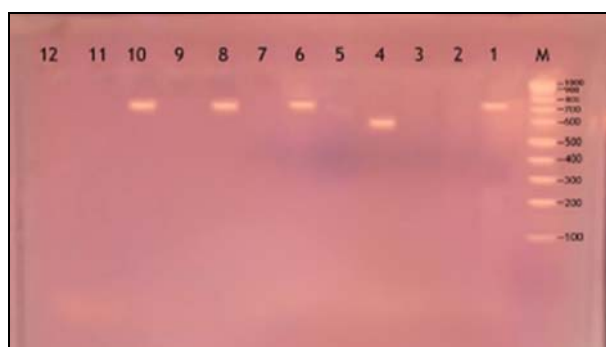
		Multiplex PCR		Total
		CPE (n= 40)		
		-ve PCR (n = 13)	+ ve PCR (n = 27)	
MDI	Negative	4	2	6
	Positive	9	25	34
Total		13	27	40

Sensitivity =92.6 %  
PPV =73.5 %

Specificity = 30.8%  
NPV = 66.7%

**Table 2: Results of multiplex PCR for detection of *bla* KPC and *bla* NDM genes among 40 CPE isolates**

Genotype	CPE (n=40)	
	No.	%
<i>bla</i> KPC	26	65.0 %
<i>bla</i> NDM	1	2.5 %
<b>Total PCR- positive isolates</b>	<b>27</b>	<b>67.5%</b>



**Fig. 3: Agarose gel electrophoresis for the multiplex PCR amplified products of *bla*KPC and *bla*NDM genes of carbapenemases producing *Enterobacteriaceae***  
**Lane M:** DNA molecular size marker (1000 bp); **lanes 1, 6, 8, 10:** represent positive *bla*KPC (785 bp) **lane 4:** represents positive *bla*NDM-1 (621 bp) and **lanes 2, 3, 5, 7, 9, 10, 11 and 12:** represent negative samples.

## DISCUSSION

Carbapenemases represent the strict threat for human health worldwide and a medical challenge, implicating an extremely high cost for hospitals.<sup>15</sup> The most clinically important carbapenemase is *K. pneumoniae* carbapenemase (KPC) which has become endemic in many countries around the world.<sup>5</sup> NDM is one of the most recently described carbapenemases not only nosocomial enterobacterial pathogens, but also community acquired *Enterobacteriaceae*.<sup>16</sup>

In the present study, out of 100 *Enterobacteriaceae* strains, 40 isolates (40%) was carbapenemases producers detected by using chromID CARBA agar. This result is similar to another study conducted by *El-Sweify et al.*<sup>17</sup> in Suez Canal University Hospitals, Ismailiya, Egypt who found that 44.3% of *Enterobacteriaceae* isolates are carbapenem-resistant. However, lower prevalence rates were reported by another Egyptian study, in Sohag University Hospital, Egypt, *Fattouh et al.*<sup>18</sup> who found that the rate of carbapenem resistant isolates was 27.4% detected by screening methods.

A higher prevalence of carbapenem resistance was reported in a study conducted by *Yusuf et al.*<sup>20</sup> in a teaching hospital in Nigeria where out of 135 *Enterobacteriaceae* isolates, 91% are non-susceptible to carbapenem. *Nordmann and Poire*<sup>21</sup> expected that

specific enzyme responsible for carbapenem resistance will emerge in a given geographical area where many favourable conditions exist, such as a high-density population, poor hygiene, and high selective pressure linked to overuse and misuse of antibiotics and that could be an explanation for differences in carbapenem resistance rates among different countries and geographical regions.

The present study applied the commercial multiplex PCR technique for the detection of *bla* KPC, *bla* NDM genes among 40 strains of carbapenemases producing *Enterobacteriaceae*. Out of them 26 strains (65%) are carrying KPC gene and only one strain (2.5%) is carrying NDM gene. (Table 2). These results are in accordance with another study in Egypt conducted by *Metwally et al.*<sup>22</sup> who confirmed the presence of *bla* KPC genes in (70.0%) of carbapenem non susceptible *K. pneumoniae* isolates.

Also, *Pournaras et al.*<sup>23</sup> who reported identification of CPE isolates in 51.3% of samples. They were subsequently confirmed by phenotypic and molecular assays. KPC positive strains are (61.8%). In Italy, *Lombardi et al.*<sup>24</sup> performed a study to detect isolation rate of *bla*KPC in Cardiac Surgery Division of their hospital. (90.8%) of isolates were positive with the primer specific for *bla*KPC gene.

In the present study, *bla* NDM gene is detected only in one isolate of *K. pneumoniae*. (table2) This results in accordance with that stated by *Abdelaziz et al.*<sup>25</sup> who reported the first identification of NDM-1-producing *K. pneumoniae* in Egypt. Two *K. pneumoniae* isolates recovered from two hospitalized patients in the same intensive care unit of a cancer hospital in Cairo, Egypt. PCR was used to detect  $\beta$ -lactamase genes. One of the two isolate of *K. pneumoniae* carried the *bla*NDM-1 gene. These added further evidence to the spread of NDM-1-producing Gram-negative organisms in North Africa and Egypt.

The spread of NDM-1 in the Middle East may be explained by a close relationship with the Indian subcontinent which is the main reservoir of NDM (population exchange and trade).

In contrast to our results, high prevalence rate of *bla*NDM-1 gene was reported by *Gamal et al.*<sup>26</sup> in a tertiary care hospital in Egypt and found that a total of 157 *Klebsiella spp* isolates are recovered. 13 *K. pneumoniae* are found to be carbapenem resistant with 8 of these isolates (61.5%) are carbapenem-resistant NDM-1-producing *K. pneumoniae* isolates.

Concomitant detection of *bla* KPC and *bla* NDM genes in CPE isolate is reported by *Giani et al.*<sup>27</sup> who found that KPC-type enzymes are by far the most common (89.5%) of carbapenemase producers while NDM gene is not detected. On the other hand, *Shibl et al.*<sup>28</sup> in Riyadh, Saudi Arabia, found that carbapenemase gene *bla* NDM-1 is detected in 20% of *K. pneumoniae* isolates, none of the isolates harbored *bla* KPC.

This controversy in the incidence of strains carrying carbapenemase genes may be due to variations in the prevalence of CPE between different hospitals and geographic areas.

Another reasons for these discrepancies in findings since the profiles of study organisms were largely different. Some studies included both colonized patients and those with clinically-relevant infections; sites and species of infecting organisms also varied.

The high prevalence of carbapenem resistance in the current study could be explained by the fact that the majority of our samples were collected from the ICU unit. Patients within the ICU usually are subjected to invasive procedures, treatment with antibiotic combinations, and are sharing life with other patients with multi-drug resistant pathogens.

Mast Disc ID (MDI) carbapenemase detection set was used for phenotypic confirmation of carbapenemase production in *Enterobacteriaceae*. Out of 40 CPE, 85% of carbapenemase are detected. The percentage of KPC carbapenemase (60%) is more than MBL (25%). In the present study, out of 27 PCR-positive isolates, MDI detected 25 isolates of CPE (92.6%). Considering PCR as the gold standard, MDI has 92.6 % sensitivity and 30.8 % specificity. (Table 1).

In comparison to Doyle *et al.* [29] who reported a study that evaluated different phenotypic confirmatory tests for the detection of CPE. MDI detected 98% of KPC producing isolates, 100% of NDM producers. MDI has 78% sensitivity and 93% Specificity in relation to PCR results.

In the present study, Mast Disc ID carbapenemase detection set has an excellent sensitivity (92.6 %), indicating the usefulness of doing this phenotypic testing. This will provide a cost-effective and rapid approach for the detection of carbapenemase in *Enterobacteriaceae*. Specificity of MDI decreased in this work (30.8%) because only NDM gene of MBL could be detected with the presence of other carbapenemase genes not screened for in this study which is the main limitation of our study. This could be the reason in bacterial strains which are carbapenem resistant but negative by PCR.

## CONCLUSIONS

This study shows a significant rate of carbapenemase producing *Enterobacteriaceae* in our setting as reported in similar studies from other parts of Egypt that provides a clearer picture of the current CPE dissemination in the hospital setup of the country and thus further emphasizes the need for strict implementation of infection control practices within the community including active antimicrobial stewardship programs, this should include restriction of carbapenem use.

We recommend that hospitals routinely screen *Enterobacteriaceae* strains for both KPC and NDM

genes, even in regions where they are not yet endemic. So, this study should be repeated in other hospitals (especially the public hospitals) to assess the level of the problem.

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