*Acta Microbiologica et Immunologica Hungarica, 63 (1), pp. 69–81 (2016)*
*DOI: 10.1556/030.63.2016.1.5*

# COMPARISON OF MOLECULAR AND PHENOTYPIC METHODS FOR THE DETECTION AND CHARACTERIZATION OF CARBAPENEM RESISTANT ENTEROBACTERIACEAE

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(Received: 8 July 2015; accepted: 15 January 2016)

In recent years, there has been a rapid dissemination of carbapenem resistant *Enterobacteriaceae* (CRE). This study aimed to compare phenotypic and molecular methods for detection and characterization of CRE isolates at a large tertiary care hospital in Saudi Arabia. This study was carried out between January 2011 and November 2013 at the King Khalid University Hospital (KKUH) in Saudi Arabia. Determination of presence of extended-spectrum beta-lactamases (ESBL) and carbapenem resistance was in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. Phenotypic classification was done by the MASTDISCS™ ID inhibitor combination disk method. Genotypic characterization of ESBL and carbapenemase genes was performed by the Check-MDR CT102. Diversilab rep-PCR was used for the determination of clonal relationship. Of the 883 ESBL-positive *Enterobacteriaceae* detected during the study period, 14 (1.6%) isolates were carbapenem resistant. Both the molecular genotypic characterization and phenotypic testing were in agreement in the detection of all 8 metalo-beta-lactamases (MBL) producing isolates. Of these 8 MBL-producers, 5 were positive for $bla_{NDM}$ gene and 3 were positive for $bla_{VIM}$ gene. Molecular method identified additional $bla_{OXA}$ gene isolates while MASTDISCS™ ID detected one AmpC producer isolate. Both methods agreed in identifying 2 carbapenem resistant isolates which were negative for carbapenemase genes. Diversilab rep-PCR analysis of the 9 *Klebsiella pneumoniae*

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isolates revealed polyclonal distribution into eight clusters. MASTDISCS™ ID is a reliable simple cheap phenotypic method for detection of majority of carbapenemase genes with the exception of the *bla*$_{OXA}$ gene. We recommend to use such method in the clinical laboratory.

**Keywords:** *Enterobacteriaceae,* carbapenem resistance, Modified Hodge Test, *bla*$_{NDM}$ gene, *bla*$_{VIM}$ gene, *bla*$_{OXA}$ gene, molecular characterization, Check-MDR, DNA microarray, MASTDISCS™ ID

## Introduction

The rapid dissemination of extended-spectrum beta-lactamase (ESBL)-producing bacteria has led to the increased utilization of carbapenems as antimicrobials of last resort for the treatment of infections caused by these multidrug resistant (MDR) microorganisms [1]. Thus in recent years, carbapenem-resistant *Enterobacteriaceae* (CRE) harbouring resistance genes for carbapenemases have emerged as major causes of nosocomial infections globally [2–3]. Among isolates reported to the National Healthcare Safety Network (NHSN) in 2009 and 2010, carbapenem resistance was reported in up to 77.2% of *Escherichia coli* and *Klebsiella pneumoniae* [4]. Clinically significant carbapenmases in *Enterobacteriaceae,* are the Ambler molecular Class A (KPC), Class B (VIM, IMP, NDM) and Class D (OXA-48) types [5]. Infections caused by CRE harbouring these resistance genes have limited treatment options, spread easily in healthcare settings and are associated with high mortality [2, 6–7].

To date, there is a paucity of data on the prevalence of CRE isolates in the Kingdom of Saudi Arabia. Two recently published studies indicate that CRE isolates positive for *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ are circulating in hospitals in Riyadh, Saudi Arabia [8–9]. This study aimed to determine the prevalence of carbapenem resistance as well as evaluate molecular and phenotypic methods for the detection and characterization of CRE isolates at a large tertiary care hospital in Saudi Arabia.

## Materials and Methods

### *Clinical specimens, bacterial isolates and their identification*

This study was carried out between January 2011 and September 2013 at the King Khalid University Hospital, Riyadh Saudi Arabia. All ESBL-positive isolates identified from routine investigation in the bacteriology laboratory dur-

ing the study period were included in the study. The isolates were obtained from specimens submitted for routine bacteriology investigations. These specimens were blood, wound swabs, endotracheal secretions, sputum, body fluids and urine. Isolates were stored at –80 °C for further analysis. Identification of organism and antimicrobial susceptibility testing were carried out by the MicroScan Walkaway 96 plus System (Siemen Healthcare Diagnostic Inc.). ESBL screening was carried out by both double disk and E-test according to the Clinical and Laboratory Standards Institute (CLSI), 2013 recommended guidelines [10]. Only the first representative isolate per patient was included and all other repeat isolates were excluded. Carbapenem resistant but carbapenemase-negative isolates were used as negative controls.

*Antimicrobial susceptibility testing*

Antibiotic susceptibility using the Kirby-Bauer method was carried out on Mueller-Hinton agar with commercially available disks from Oxoid in accordance with CLSI guidelines [10]. Antibiotics tested were amikacin (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg) and sulfamethoxazole (23.75/1.25 μg). The minimum inhibitory concentration for imipenem, meropenem, colistin and tigecycline was determined by E-test (bioMerieux, Marcy l'Etoile, France) following the manufacturer's instructions and results were interpreted as per CLSI guidelines [10]. *Escherichia coli* ATCC 25922 was used for the quality control. All the carbapenem resistant isolates were confirmed by using modified Hodge Test following CLSI guidelines [10].

*Phenotypic confirmation of carbapenemase production*

The MASTDISCS™ ID inhibitor combination disk method (Mast Diagnostics) for the phenotypic confirmation of carbapenemases production was used according to the manufacturer's instructions. This methodology utilizes four disks: disk A, containing a carbapenem (meropenem, 10 μg); disk B, consisting of meropenem (10 μg) and an MBL inhibitor; disk C, consisting of meropenem (10 μg) with a KPC inhibitor; and disk D, containing meropenem (10 μg) with an AmpC inhibitor. Interpretation of the test requires comparison of the zone of inhibition of disk A with the zones of inhibition of each of disks B, C, and D. If disk B shows a zone difference of ≥5 mm from disk A this is indicative of MBL activity. Zone difference of ≥4 mm for disk C compared to disk A,

indicates KPC activity. If disk C and disk D show a zone difference of ≥4 and ≥5 mm, respectively, from disk A, this is indicative of AmpC activity coupled with porin loss (impermeability) [11].

*Genotypic testing for ESBL and carbapenemases genes*

This was carried out using the Check-MDR CT102 DNA microarray (CheckPoints BV, Wageningen, Netherlands) at the Antimicrobial Resistance Research Laboratory, Alfaisal University, Riyadh Saudi Arabia. At the time of the genotypic testing, the research laboratory was blinded to the findings of the phenotypic assays. The Check-MDR CT102 combines ligation-mediated amplification with detection of amplified products on a microarray to detect carbapenemase genes ($bla_{\text{OXA-48}}$, $bla_{\text{NDM}}$, $bla_{\text{IMP}}$, $bla_{\text{VIM}}$ and $bla_{\text{KPC}}$), $bla_{\text{CTX-M}}$ groups ($bla_{\text{CTX-M}}$ groups 1, 2 and 9, or combined 8/25), and common ESBL-associated single nucleotide polymorphisms in $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$ variants [12]. Whole-cell DNAs were extracted from overnight bacterial cultures using the Mo Bio UltraClean™ Microbial DNA Isolation Kit. Microarray assays were performed according to the manufacturer's instructions with provided tubes and reagents as previously described [12–13].

*Determination of clonal relationship*

This was carried out using the DiversiLab repetitive extragenic palindromic PCR (rep-PCR) system (bioMérieux). This was carried out only for *K. pneumoniae* which constituted the bulk of our isolates. Briefly, genomic DNA extraction and preparation was carried out using the Mo Bio UltraClean™ Microbial DNA Isolation Kit. As previously described, the rep-PCR amplification was performed using the DiversiLab Klebsiella fingerprinting kit and detection done using the microfluidic LabChip kits on Agilent 2100 bioanalyzer according to the manufacturer's instructions (bioMérieux) [14]. The amplified rep-PCR DNA fragment patterns were analyzed with the DiversiLab analysis software using the modified Kullback–Leibler statistical method.

## Results

A 883 ESBL-positive *Enterobacteriaceae* were detected during this period. A total of 14 isolates suspected to have carbapenemase enzymes were identi-

**Table Ia.** Patient demographics, laboratory and clinical details (n = 13)

| **Gender** | **No. (%)** |
|---|---|
| Male | 10 (76.9) |
| Female | 3 (23.1) |
| **Age (Years)** | |
| Range (Mean) | 1–93 (49) |
| **Source** | |
| Wound | 5 (38.5) |
| Sterile Body Fluid | 3 (23.1) |
| Urine | 2 (15.4) |
| Blood | 1 (7.7) |
| Respiratory | 1 (7.7) |
| Unknown | 1 (7.7) |
| **Isolates** | |
| *E. coli* | 2 (15.4) |
| *K. pneumoniae** | 8 (61.5) |
| *K. oxytoca* | 1 (7.7) |
| *E. cloacae* | 2 (15.4) |
| **Risk factors** | |
| Abdominal surgery | 7 (53.8) |
| Catheterized | 6 (46.2) |
| **Clinical presentation** | |
| Surgical diseases | 6 (46.2) |
| Acute infections | 3 (23.1) |
| Chronic medical diseases | 4 (30.8) |
| **Wards** | |
| Internal medicine | 5 (38.5) |
| Surgical | 4 (30.8) |
| Oncology | 2 (15.4) |
| ICUs | 2 (15.4) |
| **Outcome** | |
| Survived | 12 (92.3) |
| Died | 1 (7.7) |

*: From Patient #6 two different strains isolated.
ICUs: Intensive care units

**Table Ib.** The antibiogram pattern of isolates

| | Isolates | Imipenem | Meropenem | Colistin | Tigecycline | AMK | GENT | SXT | CIP |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *E. coli* | 2 | 1.5 | 0.25 | 0.38 | I | R | R | R |
| 2 | *K. pneumoniae* | 4 | 3 | 0.38 | 1 | S | S | R | I |
| 3 | *K. pneumoniae* | 2 | 3 | 0.25 | 1.5 | S | S | R | I |
| 4 | *K. oxytoca* | 32 | 16 | 0.19 | 0.75 | R | R | R | S |
| 5 | *K. pneumoniae* | 32 | 8 | 0.25 | 1.5 | S | R | R | S |
| 6a* | *K. pneumoniae* | >32 | >32 | 0.38 | 2 | R | R | R | R |
| 6b* | *K. pneumoniae* | >32 | >32 | 0.125 | 0.25 | R | R | R | R |
| 7 | *K. pneumoniae* | >32 | >32 | 0.5 | 8 | R | R | R | R |
| 9 | *K. pneumoniae* | 4 | 8 | 0.38 | 1.5 | S | R | R | R |
| 10 | *K. pneumoniae* | >32 | >32 | 0.25 | 2 | R | R | R | R |
| 11 | *E. cloacae* complex | 32 | 12 | 0.125 | 1 | S | R | R | I |
| 12 | *E. coli* | 2 | 1.5 | 0.25 | 0.38 | I | R | R | R |
| 13 | *E. cloacae* | 32 | 32 | 16 | 0.75 | S | S | R | R |
| 14 | *K. pneumoniae* | 12 | R | 0.4 | 0.5 | R | R | R | R |

AMK: Amikacin; GENT: Gentamicin; SXT: Trimethoprim-sulfamethoxazole; CIP: Ciprofloxacin
*Two isolates obtained from the same patient
S: Sensitive; I: Intermediate resistant; R: Resistant

fied. Two isolates (6a and 6b) were obtained from the same patient. Tables Ia and Ib show the clinical characteristics of the patients from whom these isolates were obtained and the antibiogram of the isolates. The findings of the genotypic testing using the Check-MDR CT102 DNA microarray for the 14 isolates are shown in Table II. Twelve isolates were positive for ESBL genes of which there were 10$bla_{\text{CTX-M}}$, 6$bla_{\text{TEM}}$ and 9$bla_{\text{SHV}}$. Eight isolates were positive for more than one ESBL gene (Table II). Of the 10$bla_{\text{CTX-M}}$ positive isolates, 7 haboured only $bla_{\text{CTX-M1}}$, 2 had only $bla_{\text{CTX-M9}}$, while one isolate harboured both $bla_{\text{CTX-M1}}$ and $bla_{\text{CTX-M9}}$. Ten isolates were positive for carbapenemase genes of which 5 were $bla_{\text{NDM-1}}$, 3$bla_{\text{OXA-48}}$ and 3$bla_{\text{VIM-1}}$. One isolate was positive for both $bla_{\text{OXA-48}}$ and $bla_{\text{VIM-1}}$. Nine isolates were positive for both ESBL and carbapenemase genes (Table II). All isolates were negative for $bla_{\text{KPC}}$, $bla_{\text{IMP}}$ and $bla_{\text{VIM-2.}}$ Comparison of genotypic and phenotypic testing for carbapenemase producers revealed that $bla_{\text{VIM-1}}$ positive isolates were detected as NDM-1 on phenotypic testing (Table III). All isolates which were negative for carbapenemase genes were also negative for NDM-1 by phenotypic testing. Isolates positive for only

**Table II.** Distribution of ESBL and carbapenemase genes

| Isolate # | Microorganism | ESBL genes | | | | Carbapenemase genes | | | Interpretation |
|---|---|---|---|---|---|---|---|---|---|
| | | TEM | SHV | CTX-M1 | CTX-M9 | NDM-1 | OXA-48 | VIM I | |
| 1 | *E. coli* | – | – | + | – | – | – | – | ESBL |
| 2 | *K. pneumoniae* | – | – | + | – | + | – | – | ESBL CARBA |
| 3 | *K. pneumoniae* | + | + | + | – | + | – | – | ESBL CARBA |
| 4 | *K. oxytoca* | – | – | – | – | – | + | + | CARBA |
| 5 | *K. pneumoniae* | – | + | – | – | – | – | + | ESBL CARBA |
| 6a* | *K. pneumoniae* | + | + | + | – | + | – | – | ESBL CARBA |
| 6b* | *K. pneumoniae* | + | + | – | + | – | + | – | ESBL CARBA |
| 7 | *K. pneumoniae* | + | + | + | – | + | – | – | ESBL CARBA |
| 9 | *K. pneumoniae* | – | + | + | – | + | – | – | ESBL CARBA |
| 10 | *K. pneumoniae* | – | + | + | + | – | + | – | ESBL CARBA |
| 11 | *E. clocae* complex | + | + | – | – | – | – | + | ESBL CARBA |
| 12 | *E. coli* | – | – | + | – | – | – | – | ESBL |
| 13 | *E. cloacae* | – | – | – | – | – | – | – | Negative |
| 14 | *K. pneumoniae* | + | + | | + | | + | | ESBL CARBA |

*All isolates were negative for KPC, IMP and VIM_II; *Two isolates obtained from the same patient. +: positive; –: negative

**Table III.** Comparison of genotypic and phenotypic testing for carbapenemase and ESBL

| Isolate # | Genotypic testing | Phenotypic testing | | | Hodge's Test |
|---|---|---|---|---|---|
| | Carbarpenemase genes | KPC | NDM | AmpC | |
| 1 | – | – | – | – | Negative |
| 2 | NDM-1 | – | + | – | Negative |
| 3 | NDM-1 | – | + | – | Negative |
| 4 | OXA-48; VIM-1 | – | + | – | Positive |
| 5 | VIM-1 | – | + | – | Positive |
| 6a | NDM-1 | – | + | – | Negative |
| 6b | OXA 48 | – | – | – | Positive |
| 7 | NDM-1 | – | + | – | Negative |
| 9 | NDM-1 | – | + | – | Negative |
| 10 | OXA 48 | – | – | – | Positive |
| 11 | VIM1 | – | + | – | Positive |
| 12 | – | – | – | – | Negative |
| 13 | – | – | – | + | Negative |
| 14 | OXA 48 | – | – | – | Positive |

+: positive; –: negative

**Table IV.** Correlation between phenotypic and genotypic methods for detection of carpeneamases

| | | NEG | MBL | | OXA-48 | Total |
|---|---|---|---|---|---|---|
| | | | NDM | VIM | | |
| Interpretation | AmpC | 1 | 0 | 0 | 0 | 1 |
| | MBL | 0 | 5 | 3 | 1 | 8 |
| | NEG | 2 | 0 | 0 | 3 | 5 |
| Total | | 3 | 5 | 3 | 4 | 14 |

*bla*$_{\text{OXA-48}}$ were reported as negative on phenotypic testing (Table III). Both phenotypic and genotypic methods were in agreement in the identification of 2 carbapenem resistant isolates which were negative for carbapenemase genes (Table III). The correlation between phenotypic and genotypic tests is show in Table IV. Two *K. pneumoniae* isolates (6a and 6b) were from a single patient. These two isolates showed different antibiogram patterns (Table Ib) and genotypic characteristics (Table II). Diversilab rep-PCR analysis of the 9 *K. pneumo-*

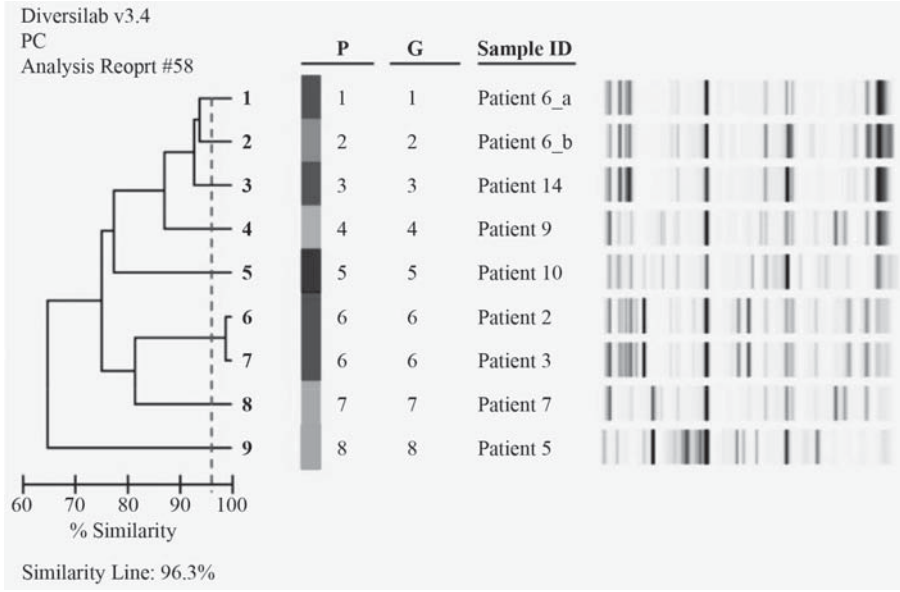


**Figure I.** Diversilab rep-PCR analysis of *K. pneumoniae* isolates.

*niae* isolates revealed distribution into eight clusters supportive of a polyclonal origin of these isolates in our setting (Fig. 1). The two *K. pneumoniae* isolates (6a and 6b) obtained from the same patient were clustered into two different groups (Groups 1 and 2) on the basis of rep-PCR patterns (Fig. 2).

## Discussion

We have described the molecular epidemiology of carbapenemase resistance genes and comparison of detection methods of such mechanism in *Enterobacteriaceae*. Although the prevalence of CRE (1.6%) in our study is lower compared to other reported data [15–16], the risk factors and CRE clinical infections seen in our patients are in keeping with reported literature [17]. The most prevalent mechanism of carbapenem resistance among *Enterobacteriaceae* is the acquisition of carbapenemases with KPC-, NDM-, and OXA-48-type enzymes being the commonest [18]. In our study the predominant mechanism of carbapenem resistance was due to NDM enzyme with prevalence similar to data from India where 31%–55% of *Enterobacteriaceae* isolates were reported to be NDM producers [19–20]. However, it is higher than the 5%–18.5% in Indian and Pakistani hospitals reported by Dortet et al. [21]. This disparity is

attributable to geographical variation, isolates number studied and detection method. Within our region, sporadic reports of NDM producers have been reported in hospitals in neighbouring countries of the Arabian Gulf region and its international transfer from patients previously hospitalized in the Middle East have been reported [22–23].

The OXA-48 carbapenemases which were first described in *K. pneumoniae* epidemic isolates from Turkey have now superseded VIMs and IMPs as the most common MBLs [24]. Genes for OXA-48 and related enzymes are present widely in North Africa, the Middle East and the Indian subcontinent reflecting the high level of occurrence we have observed [25]. For three isolates (two *E. coli* and one *E. cloacae*) no carbapenemase activity was identified suggesting a non-carbapenemase related resistance mechanism. The two *E. coli* isolates genetically demonstrated $bla_{\text{CTX-M1}}$ gene with probable hyperproduction along with porin loss being responsible for apparent carbapenem resistance. However, it was quite interesting to witness *E. cloacae* carbapenem resistance in our study which apparently lacked any of the tested ESBLs and carbapenemase genes and we speculate that the resistance might be via porins loss or efflux mechanism. None of the isolates was found to produce the KPC, or IMP type beta lactamase, although these enzymes have been previously documented in Saudi Arabia and Kuwait [26–27]. Our Diversilab rep-PCR analysis of the 9 *K. pneumoniae* isolates revealed a polyclonal origin of these isolates. Two *K. pneumoniae* clones from the same patient (one harboured $bla_{\text{NDM-1}}$ gene and the other $bla_{\text{OXA-48}}$ gene) were resistant to amikacin, gentamicin, ciprofloxacin and cotrimoxazole but susceptible to colistin and tigecycline. This phenomenon is rare and could be attributed to plasmid transfer.

Our evaluation of the phenotypic confirmation test (MHT) and commercial system (MASTDISCS™ ID inhibitor combination disks) for the detection of carbapenemase production in *Enterobacteriaceae* showed that in keeping with previous report, the MDI disks detected all MBL positive isolates with 100% agreement with molecular genotyping data [11]. However, MDI disks are not designed to detect class D enzymes like OXA-48 and one isolate (#4) which coexpressed VIM and OXA-48 was identified as MBL. Although the MHT assay reliably detects KPC and OXA-48 producers it performs poorly in the detection of MBL producers and this is reflected in our findings as none of the NDM-1 was detected by MHT [28]. Therefore for the optimal detection of CRE we recommend the use of molecular based methods. However, in resource-limited settings, a combination of the two phenotypic tests we have evaluated is a good alternative. The MASTDISCS™ will identify KPC and NDM producers, while the mod-

ified Hodge test will ensure that OXA-48 producers are detected. This will provide a cost-effective and rapid approach for the detection of carbapenemases in *Enterobacteriaceae*.

## Acknowledgement

We would like to thank all the technical staff at King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia, for their work in identifying the isolates as per standard guidelines and for their help in data collection and analysis.

## Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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