

ORIGINAL ARTICLE

Carpabenem Resistant *Klebsiella* Species in Neonatal ICU in Beni-Suef University Hospital: Molecular Characterization and In-vitro Efficacy of Synergistic Antibiotic Combinations

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ABSTRACT

Key words:
CRK, *Klebsiella* spp.,
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Background: Carapenem resistant *Klebsiella* species [CRK] is increasing worldwide that may necessitate the use of combination therapy of antibiotics. **Objectives:** our aim was to characterize the main mechanisms of resistance carried by CRK isolates and to investigate the in-vitro efficacy of antibiotic combinations. **Methodology:** One hundred *Klebsiella* isolates were used for detection of KPC by modified Hodge test and boronic acid combination disc. PCR was done for detection of (OXA-48, KPC, OMP-35 and OMP-36) genes. The efficacy of antimicrobials combination against these isolates was also tested. **Results:** By boronic acid combination disc 62% were positive, by modified Hodge test 12% were positive. By PCR; 18% of isolates were positive for OMP-35, 16% for KPC, 14% OXA-48 and 6% for OMP36 genes. Antibiotic combination results showed that 64 and 26 isolates were sensitive to gentamicin- ciprofloxacin, meropenem-ciprofloxacin combinations respectively. **Conclusion:** We concluded that OXA-48, KPC, OMP-35 and OMP-36 genes were circulating in our hospital. Gentamycin and ciprofloxacin Combination may be effective.

INTRODUCTION

Klebsiella species have multiple resistance mechanisms against Carbapenems and other β -lactams. The common mechanisms of resistance are either lack of drug penetration (i.e., outer membrane protein (OMP) mutations and efflux pumps), hyper production of an AmpC-type β -lactamase, and/or Carbapenem-hydrolyzing β -lactamases¹.

Carbapenem-resistant *Klebsiella* species (CRK) are gaining increasing importance in healthcare settings, especially among critically ill patients². They are associated with high mortality rates due to inappropriate or inadequate antimicrobial therapy³.

These bacteria are also frequently resistant to all antibiotics except colistin, some aminoglycosides and variably tigecycline, posing a serious challenge for treatment⁴.

These "last-line antibiotics" are prone to the rapid emergence of resistance when used as monotherapy, are often more toxic, or have significant pharmacokinetic limitations for treating CRK infections in the urine, bloodstream, or lung⁵.

Combination therapy has become the standard of care for many physicians. It is used in critically ill patients due to widespread emergence of multidrug resistance organisms (MDR)⁶.

Combination regimen including a carbapenem may be associated with increased survival rates. The use of a carbapenem for treatment of carbapenem-resistant Gram-negative infections is suggested based on *in-vitro* synergistic activity and clinical experience⁷.

Our study aimed to characterize the main mechanisms of resistance carried by CRK isolates from neonatal ICU in Beni-Suef University hospital by phenotypic and genotypic methods and to investigate the *in-vitro* efficacy of synergistic antibiotic combinations against these isolates.

METHODOLOGY

Bacterial isolates:

This study was conducted on clinical isolates of **100 CRK** isolated from samples sent from Neonatal ICU to the Microbiology Unit of Clinical Pathology department; Faculty of Medicine; Beni-Suef University; in a one year period from May 2015 to May 2016.

The study was approved by Ethics Committee, Faculty of Medicine, Beni-Suef University.

Susceptibility testing:

This was done by Kirby Bauer method on Mueller-Hinton agar plates (Oxoid cop. England)⁸.

Phenotypic screening of isolates for carbapenemases using:

Boronic acid combination disc:

Phenotypic detection of KPC possessing *Klebsiella* isolates was tested with boronic acid combination disk tests (Liofilchem s.r.l. Italy) according to Girgis et al.⁹.

Modified Hodge test for KPC: was done according to Rania et al.¹⁰.

Conventional polymerase chain reaction:

This was done for detection of OXA-48, KPC, OMP 35 and OMP 36 genes in *Klebsiella* species.

DNA extraction:

DNA templates were prepared by boiling a bacterial suspension for 10 min (Heat block technique)¹¹.

PCR amplification and cycling conditions:

PCR amplification for KPC¹², blaOXA-48¹³, OMP-35¹⁴ and OMP-36¹⁴ were performed using the sets of primers listed in table 1.

Table 1: Primers used for PCR reactions:

Gene	Forward primer	Reverse primer	Reference
KPC	GATACCACGTTCCGTCTGG	GCAGGTTCCGGTTTTGTCTC	12
OXA-48	TTGGTGGCATCGATTATCGG	GAGCACTTCTTTTGTGATGGC	13
OMP 35	CAGACACCAAACCTCTCATCAAGGG	AGAATTGGTAAACGATACCCACG	14
OMP 36	CAGCACAATGAATATAGCCGAC	GCTGTTGTCGTCACGAGGTTG	14

PCR amplification was performed in a total volume of 25 µl containing:

- 1 µl of each primer: OXA-48, KPC, OMP-35, OMP-36.
- 12.5 µl Dream Taq Green PCR Master Mix(2X),

- 250 ng of genomic DNA,
- The volume was completed to 25 µl with nuclease-free water¹⁵.

PCR cycling conditions for each gene are shown in table 2.

Table 2: PCR cycling conditions for each gene:

	OXA-48	KPC	OMP-35	OMP-36
Initial denaturation	95°C for 30 s	95°C for 30 s	95°C for 30 s	95°C for 30 s
Annealing	55°C for 90 s	51°C for 60 s	51°C for 60 s	55°C for 90 s
Extension	72°C for 60 s	72°C for 60 s	72°C for 60 s	72°C for 60 s
Final extension	72°C for 5 min	72°C for 5 min	72°C for 5 min	72°C for 5 min

Repeated for 35 cycles¹⁵.

Gel electrophoresis and visualization of PCR products under UV light was done.

The amplified gene product is expected at molecular size of 743 bp for OXA-48, 246 bp for KPC, 1183 bp for OMP-35 and 598 bp for OMP-36.

Synergy antibiotic combinations:

In-vitro interactions of the following antibiotic combinations were studied using broth micro-dilution method¹⁶⁻¹⁸.

Data analysis:

It was done using Statistical Package of Social Science Software program (SPSS), Version 22.0 (IBM Corp., New York, USA).

RESULTS

Our study was conducted in Clinical Pathology Department of Beni-Suef University Hospital on all Carbapenem resistant *Klebsiella* isolates collected at Microbiology Unit from clinical specimens obtained

from patients who were admitted to neonatal ICU in the period from June 2015 to June 2016.

During the study period, 228 clinical samples were culture-positive for *Enterobacteriaceae*. From these samples, *Klebsiella spp.* were detected (120/228; 52.6%). Based on susceptibility testing using disc diffusion 83.3% of these *Klebsiella pneumoniae* isolates (100/120) were carbapenem resistant (CRK), which were included in our study figure 1.

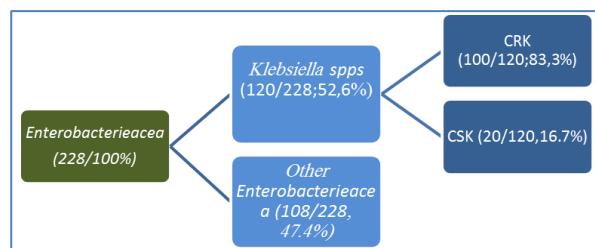


Fig. 1: Distribution of CRK among clinical samples in NICU

CRK: carbapenem resistant klebsiella
CSK: carbapenem sensitive klebsiella

Demographic data of the studied cases:

The 100 patients from which CRK were isolated; 62% were males and 38% were females. Out of them 80% were neonates (1-40 days) and 20% were infants (41 days- 2 years) table 3.

Table 3: The demographic data of the studied cases:

Age	Sex		Total
	Male	Female	
Neonates	52	28	80
Infants	10	10	20
Total	62	38	100

The CRK isolates were obtained from different specimens. The most common specimens were sputum 62% followed by urine 22% and blood 16% figure 2.

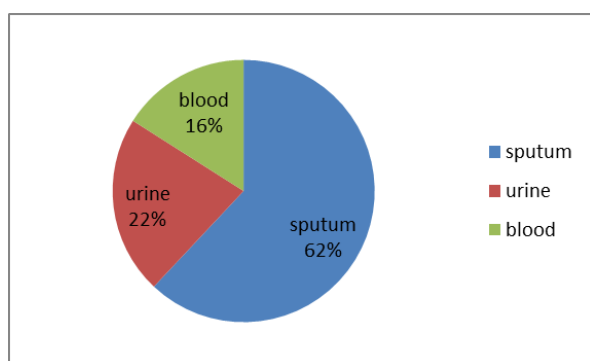


Fig. 2: Distribution of CRK isolates among different specimens

All patients had history of recent receiving antimicrobial therapy including Carbapenems (58%), quinolones (15%), β-lactams (7%) and other antibiotics (20%).

Antibiotic susceptibility testing:

All *Klebsiella* isolates were resistant to beta-lactam antibiotics including aztreonam, and carbapenems Imipenem (IPM), meropenem (MEM) and to ertapenem (ETP).

The resistance rate to amikacin, gentamicin and ciprofloxacin were 83%, 86% and 95% respectively table 4.

Phenotypic screening of isolates for carbapenemases:

Modified Hodge test (MHT) was positive in 12 (12%) isolates while boronic acid combination disc test (BACDT) was positive in 62 isolates (62%) table 4.

Table 4 antibiotics resistance and phenotypic tests results:

	Percentage	Total
β-lactams	100%	100 (100%)
Aztreonam	100%	
Aminoglycoside	AK	83%
	CN	86%
Quinolone	CIP	95%
Modified Hodge test (MHT) positive	12%	
boronic acid combination disc test (BACDT) positive	62%	

AK: Amikacin CN: Getamycin CIP :Ciprofloxacin

Polymerase chain reaction (PCR): 18% of isolates were OMP-35 gene positive, while 16%, 14% and 6% of isolates were positive to KPC, OXA-48, and OMP36 genes respectively.

The MHT (Modified Hodge Test) detected 3 (3%) of blaKPC positive isolates. Considering PCR as the gold standard [19], the sensitivity of modified Hodge test in detecting blaKPC was 18.8 and specificity was 89.3%; while the sensitivity of boronic acid combination disc test (BACDT) was 23.7% and specificity was 88.7% table 5.

Table 5 sensitivities and specificities of MHT and BACDT:

Item	Sensitivity	Specificity
MHT	18.8%	89.3%
BACDT	23.7%	88.7%

Synergy studies of antibiotics on CRK isolates:

Among the 100 CRK isolates 64, 26, and 16 isolates were sensitive to gentamicin-ciprofloxacin, meropenem-ciprofloxacin and amikacin-imipenem combinations respectively while only 4% were sensitive to tazobactam/ piperacillin- imipenem combination table 6.

Table 6 Synergy studies of antibiotics on CRK isolates:

	GM+CIP						MEM+CIP						AK+IPM			TZP+IPM
	C in ug/ml	64/64	32/32	16/16	8/8	4/4	2/2	16/64	8/32	4/16	2/8	1/4	0.5/2	128/8	32/2	8/0.5
N	15	15	15	11	4	4	7	6	4	4	3	2	6	5	5	4

C= concentration, N= number of sensitive isolates

GM: gentamicin, CIP: ciprofloxacin, MEM: meropenem, AK: amikacin, TZP: piperacillin-tazobactam, IPM: imipenem.

DISCUSSION

In the present study, the rate of isolated *Klebsiella* spp. among *Enterobacteriaceae* isolates was (52.6%) throughout 12 months period from June 2015 to June 2016.

A similar figure was reported in a study done in Iran²⁰ in which 46.4% of isolates were *Klebsiella* spp. Lower figures of 32.5% were reported in a study done in USA²¹.

Among *Klebsiella* isolates, the incidence of CRK was 83.3%, which is much higher than several studies; where the prevalence rates of carbapenem resistance in *K. pneumoniae* isolates were higher than 30% in some institutions in the eastern United States, in association with nosocomial outbreaks²². Surveillance cultures of hospitals in the New York City area had reported rates of carbapenem resistance of *K. pneumoniae* isolates ranging up to 24%²³, 5.2% was reported in study done in a tertiary hospital in Lagos²⁴ and 2.9 in a study that was done in USA²¹.

The high incidence of CRK in our study may be due to antibiotic misuse and lack of applications of infection control measures effectively.

The *klebsiella* isolates showed co-resistance rates of 83% and 95% to Amikacin and Ciprofloxacin respectively which were higher than that detected Moini et al.²⁰ study in Iran the reported resistance rates were 48.1% and 46.3% respectively. In the study of Shatalov²⁵ in Equatorial Guinea 62.5% resistance rate was reported for Levofloxacin and 25.9% for amikacin. Mitchell et al.²⁶ had reported a figure of 27% for both Levofloxacin and Amikacin resistance.

Taking PCR as a gold standard; the sensitivity of the MHT in our study was 18.8% and the specificity was 89.3% while Doyle et al.²⁷ had found that the sensitivity and specificity for MHT was 61% and 93%, respectively. Netikul and Kiratisin²⁸ showed that MHT has 100% sensitivity and 76.3% specificity. In a previous Egyptian study the sensitivity of modified Hodge test in detecting blaKPC was 80%, the specificity was 86.27%²⁹. Other authors had reported false positive results for MHT^{30, 31} which probably results from low-level carbapenem hydrolysis by ESBLs, particularly those of the CTX-M type³² and were also observed in isolates in which carbapenem resistance is due to ESBL production coupled with porin loss^{30, 33}.

MHT is time-consuming, sometimes is difficult to interpret and delays the availability of results by at least 24 hours; therefore, its result should be carefully interpreted.

Boronic acid combination disc test (BACDT) was positive in 62% of isolates with 23.7% sensitivity and 88.7% specificity. Other studies; Taskaris et al.³⁴ and

Girgis et al.⁹ showed higher sensitivities 77.7%, and 100% respectively.

In the present study 14% CRK isolates were OXA-48 positive. Similarly, Oduyebo et al.³⁵ had found (12.4%) OXA-48 producers among carbapenemase producing *Enterobacteriaceae* isolates in a tertiary hospital in Lagos.

However; Al Tamimi et al.³⁶ revealed high prevalence of OXA-48 type carbapenemase-producing in (38.33%) of isolates and Zaman et al.³⁷ had reported that the OXA-48 gene was also found in all multi-drug carbapenem-resistant *Klebsiella pneumoniae* isolates causing an outbreak in a tertiary care hospital in Riyadh, Saudi Arabia.

On the other hand in a Tanzanian study OXA-48 was detected in 4.8% of all multidrug-resistant *Enterobacteriaceae* isolates tested³⁸, and in a study that was done in Morocco, 2.2% of carbapenemase producing *Enterobacteriaceae* isolates were OXA-48 producers³⁹.

We reported that the prevalence rate of KPC gene among CRK was (16%) by PCR.

A previous Egyptian study that was done in 2010-2011 in Cairo had reported a CPK rate of 20.8%²⁹.

Other mechanisms of carbapenem resistance may explain the isolates that were negative for KPC and OXA-48 such as combination of an ESBL or combined AmpC- enzyme with porin loss.

We found that (6%) CRK isolates were OMP-36 positive. Zaman et al.³⁷ stated that the expression of Omp-36 has been shown to be a major factor in conferring resistance against carbapenems in 17.4% of *K. pneumoniae* isolates causing an outbreak in a tertiary care hospital in Riyadh, Saudi Arabia.

There were 64, 26, and 16 *klebsiella* isolates sensitive to gentamicin-ciprofloxacin, meropenem-ciprofloxacin and amikacin- imipenem combinations respectively while only 4% were sensitive to other combinations (IPM+TZP, IPM+MEM and IPM +OMP) and colistin alone.

Al-Hasan et al.⁴⁰ had shown that combination therapy of β -lactam and fluoroquinolone was associated with lower 28-days mortality compared to β -lactam monotherapy (4.2% versus 8.8%)

Another study of Rafailidis and Falagas¹⁸ found that the combination of a carbapenem with colistin or high-dose tigecycline or aminoglycoside or even triple carbapenem-containing combinations seems to have an advantage over monotherapy.

CONCLUSIONS

To the best of our knowledge; our study is the first study to characterize the molecular epidemiology of CRK isolates and to test for synergetic antibiotic combinations in Beni Suf University hospital in Egypt.

OXA-48, KPC, OMP-35 and OMP-36 were found in different percentages among *Klebsiella* isolates in neonatal ICU of Beni-Suef university hospital which is an alarming finding due to the limited therapeutic options and the poor outcome of patients infected with these isolates.

In vitro data suggest that combination therapy of gentamycin and ciprofloxacin may be effective in treatment of CRK at different concentrations even if the bacteria are resistant to the individual drugs.

RECOMMENDATIONS

Screening for blaKPC production by carbapenem disc diffusion according to CLSI breakpoints is the first step that could be taken to address this problem.

Because resistance to carbapenems and other broad-spectrum beta-lactams is increasing, leaving to limited effectiveness of the remaining antibiotics. It is urgent to explore the potential use of antibiotic combinations to enhance the antibacterial effects of the available drugs. Clinical data are needed to support the choice and the efficacy of different combinations.

Strict adherence to infection control measures and avoid miss use of antibiotics are key factors to stop the circle of antimicrobial resistance.

Ethics approval and consent to participate:

The study was approved from Clinical Pathology Department, Faculty of Medicine, Beni-Suef University. A signed informed consent was obtained from patients. The Ethical Committee signed on the work approval.

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Conflict of interest:

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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