

Full Length Research Paper

Phenotypic and genotypic detection of β -lactams resistance in *Klebsiella* species from Egyptian hospitals revealed carbapenem resistance by OXA and NDM genes

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The β -lactams are frequently used antibiotics and are essential in treatment of serious bacterial infections. The emergence of β -lactamases has been an ongoing serious therapeutic problem. To overcome this problem, scientists utilized several β -lactamases inhibitors such as clavulanic acid and tazobactam that are used in combination with antibiotics. However, pathogenic bacteria have acquired additional mechanisms of resistance such as the acquisition of extended spectrum beta-lactamases (ESBL) and carbapenemases that cannot be inhibited by current inhibitors. Carbapenem-resistant Enterobacteriaceae (CRE) has become an international health threat. In the present work, 100 clinical *Klebsiella* strains were isolated, identified, and their antibiotic profiles were determined by the disc diffusion method. β -lactam resistance was evaluated using phenotypic and genotypic methods. More than 50% of the *Klebsiella* isolates exhibited resistance to tetracyclin, ceftriaxone, (piperacillin/tazobactam), aztreonam, ofloxacin, cefepime and cefoperazone/sulbactam. *Klebsiella* isolates were less resistant to gentamicin (32.4%) and amikacin (14.7%). Few isolates were resistant to meropenem and imipenem (6.86%). Among the 100 clinical isolates, 50% were ESBL producers and 32% were AmpC producers. The 7 imipenem resistant isolates were carbapenemase producers. PCR showed that carbapenem resistance may be due to NDM gene that was present in 43% of the isolates and OXA gene that was found in 28% of the isolates. KPC genes were not detected in any of the isolates. Antibiotic resistance is a worldwide problem that poses a major threat to therapeutic efficacy of available antibiotics, including carbapenem. Determination of causes of resistance is essential for better treatment options.

Key words: *Klebsiella*, beta-lactams, ESBLs, carbapenem-resistant, NDM, OXA, antibiotic resistance

INTRODUCTION

Klebsiella species are important opportunistic pathogens causing community-associated and nosocomial infections

(Brisse et al., 2006). β -lactam antibiotics are widely used in treatment of many bacterial infections. The persistent

exposure of bacterial strains to β -lactams induces production of β -lactamases which are encoded by several genes and selections for mutations (Chaudhary and Aggarwal, 2004). Some of β -lactamases have activity even against 3rd and 4th generation cephalosporins and monobactams, these are called the extended spectrum β -lactamases (ESBLs). ESBLs are a heterogeneous group of plasmid-mediated enzymes (Bush and Jacoby, 2010), which are prevalent in *Klebsiella pneumoniae*. They are frequently isolated from patients with septicemia, pneumonia, or urinary tract infection (Nordmann et al., 2009). It is clearly known that prevalence of ESBL producers in any hospital depends on various factors; antibiotic policies and types of disinfectants used, especially in the Intensive Care Unit (ICU) (Wollheim et al., 2011). More than 300 different ESBL gene variants have been described (Paterson and Bonomo, 2005).

AmpC β -lactamases are a group of enzymes widely found in *K. pneumoniae*. They inactivate cephalosporins such as ceftazidime and cefotaxime. Plasmid-mediated AmpC enzymes have been reported since 1980s (Bradford et al., 1997). Most ESBLs arise as a result of mutations in the genes of TEM, SHV and CTX-M (Bradford, 2001). Carbapenems (for example, imipenem and meropenem) are considered one of the few last resort therapies for serious infections caused by multidrug-resistant (MDR) Gram-negative bacteria. The emergence of novel β -lactamases with direct carbapenem-hydrolyzing activity has contributed to the increased prevalence of carbapenem resistant Enterobacteriaceae (Gupta et al., 2011). Carbapenemases are enzymes that vary in their ability to hydrolyze carbapenems, and they are poorly inhibited by clavulanate (Robledo et al., 2011).

Carbapenemases can be divided into two types based on their mechanism of action. The first (for example, KPC and OXA types) uses a serine residue active site that covalently attacks β -lactam ring. The second (for example, VIM and NDM types) are the Metallo- β -lactamases (MBLs) that use Zn atoms to break the β -lactam ring (Walsh et al., 2005). OXA family encoded carbapenemases are most commonly produced by *Acinetobacter* spp. but have also been reported in *K. pneumoniae* and *E. coli* (Mendes et al., 2009). The most recent MBL, the New Delhi MBL (NDM) showed hydrolytic activity against all β -lactams and has originated in India in 2009 and rapidly spread worldwide (Yong et al., 2009; Li et al., 2014).

In the present study, the antimicrobial susceptibility of *Klebsiella* clinical isolates was evaluated using disc diffusion method, and the causes of increased resistance to β -lactam antibiotics were determined both phenotypically and genotypically using polymerase chain reaction (PCR).

MATERIALS AND METHODS

Bacterial isolation

A total of 100 *Klebsiella* isolates were isolated from clinical specimens obtained from wounds, surgical wounds, urinary catheters, diabetic foot, burns, blood and sputum of patients admitted to Mansoura and Zagazig University Hospitals during the period from April, 2011 to November, 2011. All the isolates were collected under approved ethical standards. The isolates were identified and verified using standard biochemical reactions including: growth on MacConkey agar, citrate utilization, Voges Proskauer, methyl Red, and motility tests (Koneman et al., 1997).

Antibiotic susceptibility tests

The antibiotic resistance pattern of isolates was determined using the disk diffusion method according to the Clinical and Laboratory Standard Institute guidelines (CLSI, 2013). The antibiotic discs were obtained from Oxoid, UK. The tested antibiotics were ofloxacin (OFX, 5 μ g), gentamicin (CN, 10 μ g), aztreonam (ATM, 30 μ g), imipenem (IPM, 10 μ g), sulphamethoxazole/trimethoprim (SXT, 25 μ g), amikacin (AK, 30 μ g), cefepime (FEP, 30 μ g), cefoperazone/sulbactam (SCF, 105 μ g), ceftriaxone (CRO, 30 μ g), meropenem (MEM, 10 μ g), piperacillin/tazobactam (TPZ, 100/10 μ g) and tetracycline (TE, 30 μ g).

Detection of ESBLs producing isolates

Phenotypic detection of ESBL was done by modified double disc synergy test (DDST) according to Jarlier et al. (1998). Briefly, a disc of amoxicillin-clavulanate (20/10 μ g) was placed on the surface of Muller Hinton agar (MHA) plates then discs of cefotaxime (30 μ g) and ceftazidime (30 μ g) were kept 20 mm apart from amoxicillin-clavulanate disc. The plates were incubated aerobically at 37°C overnight. The enhancement of the zone of inhibition of the cephalosporin discs towards amoxicillin-clavulanate disc was taken as evidence of ESBL production.

Determination of AmpC producing isolates

Phenotypic detection of AmpC was done according to Singhal et al. (2005). Briefly, *E. coli* ATCC 25922 was streaked on MHA plate. One colony of *Klebsiella* isolates were inoculated on a sterile moistened 6 mm disc. A cefoxitin disc was placed next to this disc (almost touching). The plates were incubated overnight at 37°C. A flattening of growth or the indentation of the cefoxitin inhibition zone was considered a positive test for the presence of AmpC mediated resistance.

Determination of MBLs producing isolates

Phenotypic detection of Metallo β -lactamases (MBLs) was done by combined disk test according to Yong et al. (2002). Briefly, MHA plates were surface inoculated with overnight broth culture of *Klebsiella* isolates. Two imipenem discs (10 μ g) and two ceftazidime (30 μ g) discs were placed on the plate surface. Five (5) μ l of 0.5 M EDTA solution was added one imipenem and one ceftazidime disc. The inhibition zones of imipenem and ceftazidime and their EDTA-impregnated discs were compared after overnight

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Table 1. Primers used in this study: Beta-lactamases specific primers.

Primer	Sequence (5'-3')	Primer Tm	Amplicon size (bp)	Annealing temp
SHV F	ACTATCGCCAGCAGGATC	58	355	53
SHV R	ATCGTCCCACCATCCACTC	58		
TEM F	GATCTCAACAGCGGTAAG	54	750	50
TEM R	CAGTGAGGCACCTATCTC	56		
CTXM-15 F	GTGATACCACTTCACCTC	54	255	49
CTXM-15 R	AGTAAGTGACCAGAATCAG	54		
NDM-1 F	GCACACTTCCTATCTCGACATGC	57	209	51.5
NDM-1 R	CCATACCGCCCATCTTGTC	56		
OXA-9 F	CGTCGCTCACCATATCTCCC	56	315	51
OXA-9 R	CCTCTCGTGCTTTAGACCCG	56		
KPC F	GTATCGCCGTCTAGTTCTG	51	209	48
KPC R	CCTTGAATGAGCTGCACAGTG	54		

Table 2. Prevalence of *Klebsiella* species isolated from different sources.

Specimen type	NO of specimens	NO of isolates	Percentage %
Urine	83	41	49.4
Sputum	46	21	45.7
Catheters	35	16	45.7
Swabs	25	11	44
Blood	16	9	56.3
Diabetic foot	10	2	20
Total	215	100	46.5

incubation at 37°C. A zone size difference of ≥ 7 mm was taken as indicative of MBLs production.

Determination of carbapenemases producing isolates

Phenotypic detection of carbapenemases was done using modified hodge test [MHT] according to Lee et al. (2001). Briefly, a culture of *E. coli* ATCC 25922 was inoculated onto the surface of MHA plate. A 10 µg imipenem disc was placed at the center of the plate, the *Klebsiella* strains were streaked from edge of the disk to the periphery of the plate. The plate was incubated overnight at 37°C, presence of distorted inhibition zone 'cloverleaf shaped' of *E. coli* was considered as positive result for carbapenemase production by the test strain.

Genotypic detection of resistance genes

Colony PCR was done for detection of resistance genes in *Klebsiella* isolates, including the ESBLs genes (TEM, SHV, CTX-M), the Metallo-β-lactamase gene (NDM-1) and the carbapenemases genes (OXA-9 and KPC-1). The primers were designed and supplied from IDT (Integrated DNA Technologies, Coralville, Iowa, USA). The primers for NDM-1 and OXA-9, KPC were designed according to Geyer and Hanson (2013). All primers

are listed in Table 1. The gDNA was prepared according to Nair and Venkitanarayanan (2006). The PCR mixture was prepared in a final volume of 50 µl and contained: 25 µl of 2x DreamTaq TM Green Master Mix (Thermo Fisher Scientific, <http://www.thermoscientific.com/>), One µl of each primer, 5µl gDNA and nuclease-free water to 50 µl. The cycling conditions were: initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 5 s, annealing for 30 s at temperature (5° below primer Tm) as indicated in Table 1 and extension at 72°C for 1 min and a final extension cycle at 72°C for 5 min.

RESULTS

Prevalence of *Klebsiella* among clinical specimens

In the present study, a total of 100 isolates were identified as *Klebsiella* species using conventional biochemical methods. Those strains were isolated from 215 clinical samples. The highest prevalence of *Klebsiella* isolates (56.3%) was observed in blood specimens, while the lowest prevalence (20%) was observed in diabetic foot specimens (Table 2).

Table 3. Antibiotic susceptibility patterns of *Klebsiella* isolates*

Antibiotic Disk	Klebsiella species (Total Number of isolates =100)					
	Resistant		Intermediate		Sensitive	
	n	%	N	%	N	%
IMP	1	1	6	6	93	93
MEM	1	1	6	6	93	93
AK	15	15	7	7	78	78
CN	33	33	11	11	56	56
FEP	55	55	0	0	45	45
SCF	57	57	5	5	38	38
OFX	57	57	8	8	35	35
TPZ	69	69	8	8	25	25
ATM	75	75	0	0	25	25
CRO	87	87	0	0	13	13
SXT	89	89	1	1	10	10
TE	99	99	0	0	1	1

IMP: imipenem, MEM: meropenem, AK: amikacin, CN: gentamicin, FEP: cefepime, SCF: (cefoperazone/ sulbactam), OFX: ofloxacin, ATM: aztreonam, TPZ: (piperacillin, tazobactam), CRO: ceftriaxone, SXT: (sulphamethoxazole/ trimethoprim), TE: tetracycline. Breakpoints wear according to CLSI (2013).

Determination of antimicrobial susceptibility (AMS) of *Klebsiella* isolates

The AMS pattern of *Klebsiella* isolates were determined by disc diffusion method, the diameters of inhibition zones were recorded and interpreted as resistant, intermediate or sensitive according to CLSI (2013). The results of AMS revealed that *Klebsiella* isolates exhibited high resistance to tetracycline, SXT, ceftriaxone (piperacillin/ tazobactam), aztreonam, ofloxacin, cefoperazone/ sulbactam and cefepime with percentages 97, 87.3, 85.3, 73.5, 67.6, 55.9, 55.9 and 53.9%, respectively. *Klebsiella* isolates exhibited less resistance to gentamicin (32.4%) and amikacin (14.7%). On the other hand the resistance was low to meropenem and imipenem (6.86%) for each (Table 3).

Phenotypic detection of β -lactamase enzymes

Detection of extended spectrum β -lactamase (ESBL) producers

Among 100 *Klebsiella* clinical isolates, 50 isolates were ESBL producers (50%). Enhancement of zone of inhibition between one of ceftazidime, cefotaxime and amoxicillin/ clavulanic disc indicate positive ESBL production (Figure 1A).

Detection of AmpC β -lactamase among *Klebsiella* isolates

AmpC β -lactamase test was carried out on *Klebsiella*. Thirty three isolates (33%) were AmpC producers. The

positive AmpC activity appeared as either flattening or indentation of ceftazidime zone of inhibition (Figure 1B).

Detection of metallo β -lactamase (MBL)

MBLs test were carried out on isolates showing resistance to imipenem and meropenem using combined disk test. Seven *Klebsiella* imipenem resistant isolates (both intermediate (6) and resistant (1) isolates in AMS test), the seven isolates were MBL producers showing a zone size difference of more than 7 mm between imipenem and ceftazidime and their EDTA-impregnated disk (Figure 1C).

Detection of carbapenemases by modified Hodge test

The imipenem and meropenem resistant *Klebsiella* isolates were subjected to modified Hodge test. The seven tested isolates showed a distorted zone of inhibition (clover-leaf shaped zone) due to carbapenemase production (Figure 1D).

Genotypic detection of resistance genes using PCR

Detection of ESBLs genes (TEM, SHV and CTXM-15)

The resistance genes TEM, SHV and CTXM-15 were amplified from gDNA of the 50 ESBL producing *Klebsiella* isolates. For TEM gene, all tested isolates produced a 750 bp band (Figure 2A), for SHV gene, all the ESBL producing isolates gave a 355 bp band (Figure 2B). For CTXM-15 gene, 255 bp band was also detected in all tested ESBL-producing isolates (Figure 2C).

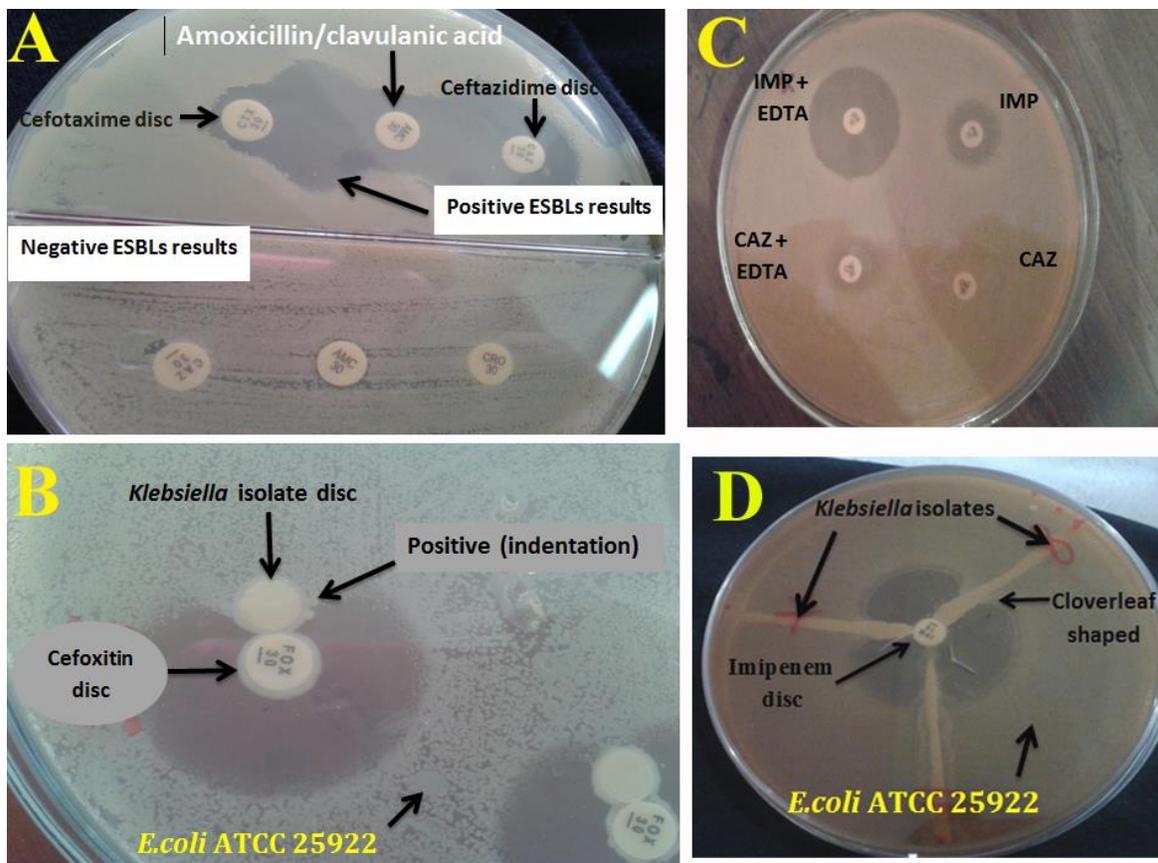


Figure 1. Phenotypic detection of ESBLs, AmpC, MBLs and Carbapenemases. A) Detection of ESBLs by modified double disc synergy test, enhancement of cephalosporin zone of inhibition towards Augmentin disc indicates positive test. B) Detection of AmpC: flattening or indentation of cefoxitin zone of inhibition was considered a positive test. C) Detection of MBLs by combined disk test, 7mm increase in zone of inhibition of EDTA impregnated disc indicates positive result. D) Detection of carbapenemases using Modified Hodge test, distorted inhibition zone 'cloverleaf shaped' was considered as positive result.

Detection of the Metallo- β -lactamase gene (*NDM-1*) and the carbapenemases genes (*OXA-9* and *KPC*)

The resistance gene New Delhi Metallo β -lactamase (*NDM-1*) was amplified from gDNA of the seven imipenem and meropenem resistant *Klebsiella* isolates. It was observed that 3 isolates only harbored *NDM-1* gene of amplicon size 209 bp (Figure 3a). The resistance genes of carbapenemase (*OXA-9* and *KPC*) were amplified from gDNA of the 7 imipenem and meropenem resistant *Klebsiella* isolates. *OXA* gene was observed in two isolates only (amplicon size 300 bp) as shown in Figure 3b, while *KPC* gene was not detected in any of the seven imipenem resistant isolates.

DISCUSSION

The *Klebsiella* species have been shown to be important opportunistic pathogens causing serious infections (Traub et al., 2000). Epidemics caused by MDR *Klebsiella*

species have led to closures of hospital units or even whole hospitals (Casewell and Phillips, 1981).

In the present study *Klebsiella* species were isolated from various clinical specimens. The prevalence of *Klebsiella* in blood, urine, sputum, catheter, swabs and diabetic foot specimens were 56, 49.4, 45.7, 45.7, 44 and 20, respectively. Thosar and Kamble (2014) reported prevalence rate of 45 and 42.9% in sputum and urine, respectively. Our results were higher than those reported by Podschun and Ullmann (1998) and Acheampong et al. (2011). The relative high prevalence of *Klebsiella* may have been due to the increased resistance of these isolates which decreased the possibility of elimination of these isolates, leading to nosocomial outbreaks by these resistant isolates (Brisse et al., 2006).

In the present study, the result of AMS revealed that *Klebsiella* isolates exhibited high resistance to tetracycline (97 %), SXT (88.2%), ceftriaxone (85.3%), TPZ (73.5%), and aztreonam (73.5%). This high resistance rate is more than that reported previously (Acheampong et al., 2011; Midan et al., 2012; El-sharkawy et al., 2013). We

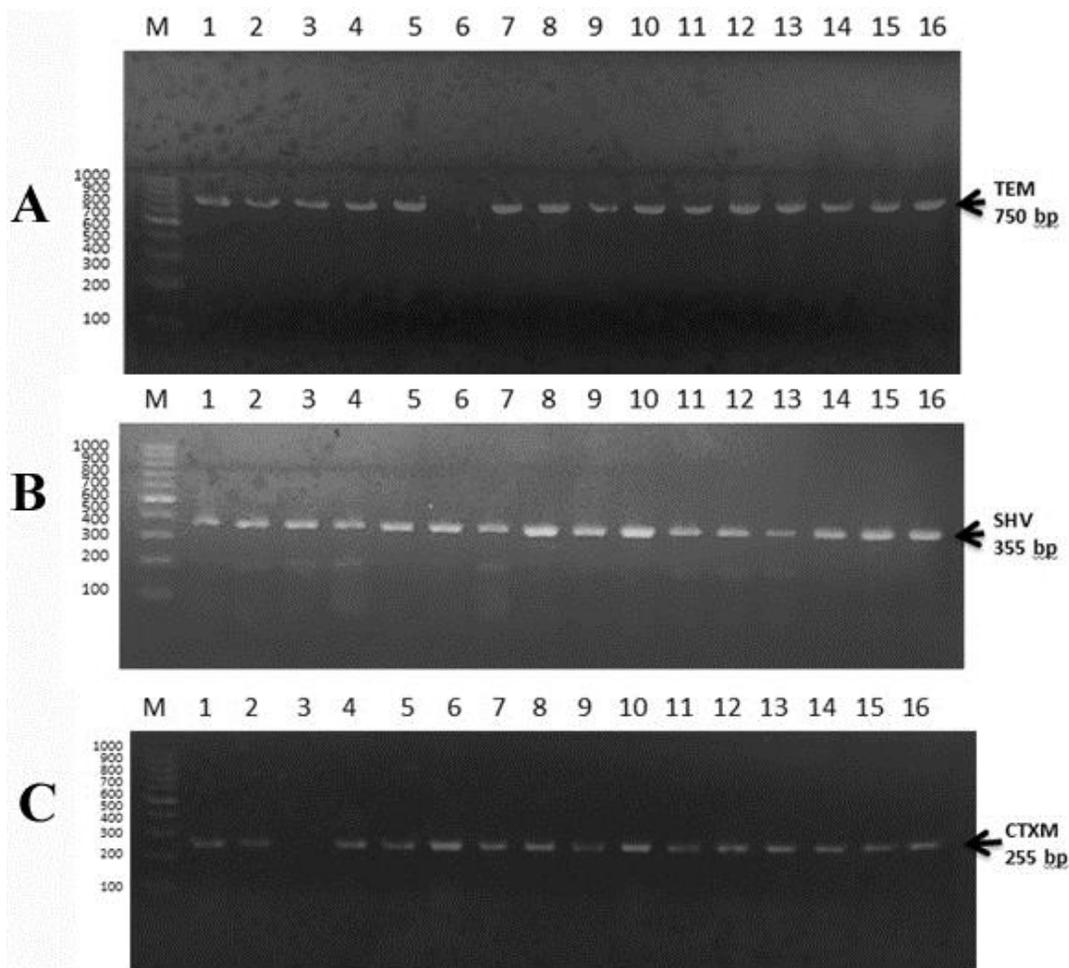


Figure 2. Genotypic detection of ESBLs genes using PCR . A) TEM gene, 750 bp band was detected in all isolates (lane 6 is a negative control), B) SHV, 355 bp band was detected in all isolates and C) CTX-M, 255 bp band was detected in all isolates (lane 3 is a negative control).

concluded that these agents have a limited capacity in treating *Klebsiella* infections. The tested strains exhibited resistance to ofloxacin by 63.7%, while resistance to gentamicin was 43.1%. The data reveals that resistance to quinolones and gentamicin was less than that reported for 3rd generation cephalosporins in this study (ceftriaxone, 87%). The excessive and over use of cephalosporins antibiotics lead to emergence of resistance and therefore shifting to alternative quinolones and aminoglycosides therapy (Sekowska et al., 2002). Less use of quinolone and structural dissimilarities is one of the reasons of comparatively low resistances than cephalosporins. The emergence of resistance to 4th generation cephalosporins (cefepime) was detected in our study by (53%). This susceptibility rate could be attributed to the empirical use of this antibiotic in the treatment of nosocomial infections in Egyptian hospitals.

Resistance to amikacin was 14.7%, which correlated with results obtained by Yasmin (2012). Resistance to imipenem and meropenem were 6.86% but higher than

those reported by El-sharkawy et al. (2013). Yasmin (2012) reported that all *Klebsiella* isolates were sensitive to imipenem and meropenem. According to Rahal et al. (2008), carbapenems are the most effective therapy for ESBL bacterial infections. However, their routine use can select resistant strains leading to the emergence of imipenem-resistant *K. pneumonia* strains.

In the present study, 50% of *Klebsiella* isolates were ESBLs producing. This result was quite similar to that reported by Haque and Salam (2010) and Aladag et al. (2013) who reported 57.9 and 55%, respectively, but was higher than the 40% detected in France (Branger et al., 1998) and 13% in Hong Kong (Ho et al., 2000). High occurrence of ESBLs in *Klebsiella* spp is of great concern since they spread easily, and strains become more efficient at acquiring more resistance plasmids (Chaudhary and Aggarwal, 2004).

In the present study, 32.4% of *Klebsiella* isolates were AmpC producers. This result was higher than that reported by Shivanna and Rao (2014), but lower than that

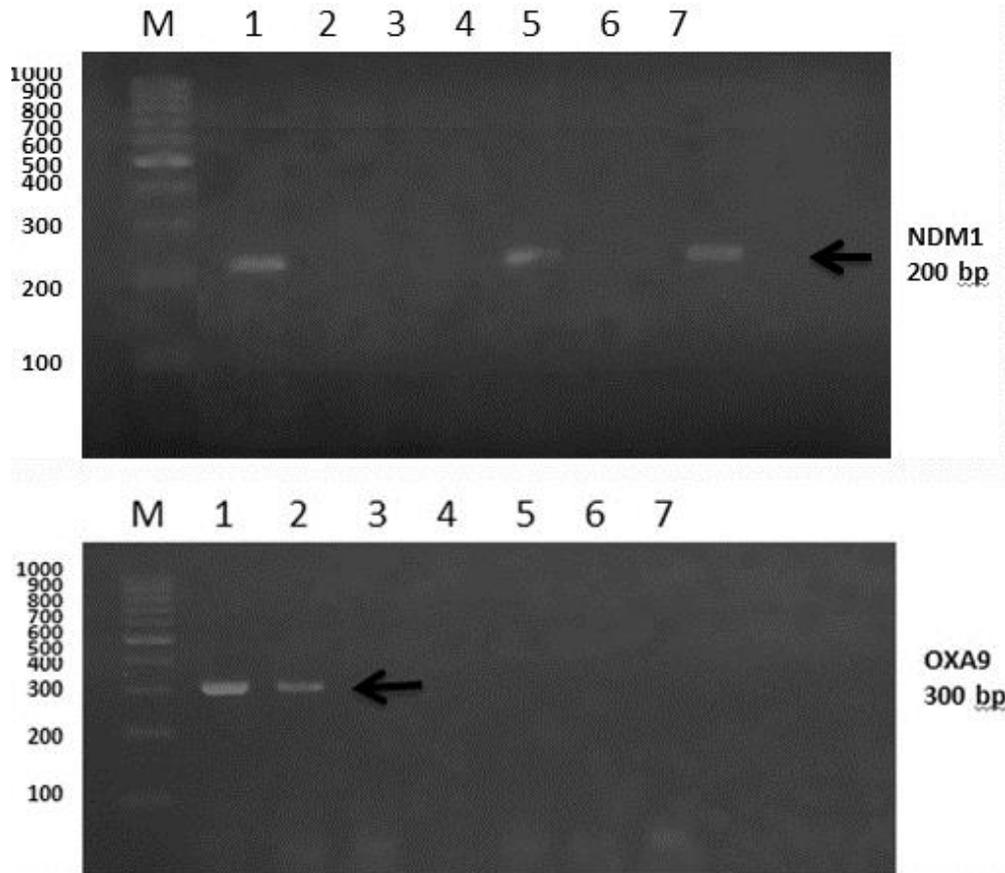


Figure 3. Genotypic detection of the NDM and OXA genes. For NDM, ~200 bp band was detected in 3 isolates only (lanes 1, 5 and 7). OXA gene, a 300 bp band was detected in 2 isolates only.

found by Akujobi et al. (2012) and Fam et al. (2013). The increased presence of plasmid mediated AmpC β -lactamases worldwide is becoming of great concern (Jacoby, 2009). Infections caused by AmpC-positive bacteria cause higher patient morbidity and mortality (Livermore, 2012), demanding new measures of *Klebsiella* infections management. 14.7% of our isolates were both ESBL and AmpC producers, a result that underlines the need for new measures of management of *Klebsiella* infections.

In the current study all *Klebsiella* isolates that exhibit resistance to imipenem and meropenem were MBL and carbapenemase producers by phenotypic detection tests. These results come in complete accordance with the results of Bora et al. (2014). It is reported that carbapenemase-producing *K. pneumoniae* exhibit resistance to important antibiotics, such as aminoglycosides and fluoroquinolones (Livermore et al., 2011). Uncontrolled clinical utility of carbapenems is a serious worldwide threat.

PCR was used in this study to detect the presence of TEM, SHV and CTX-M, these were detected in all the ESBL isolates (100%). Our results come in accordance

with Kiratisin et al. (2008) who detected in ESBL-producing *K. pneumoniae*, CTX-M (99%), SHV (87.4%) and TEM (71.7%); but, were higher than that reported in India, where of the 64 ESBL-*Klebsiella* isolates, 48% had TEM and 20% had SHV (Jain et al., 2008). Another study in USA showed 93% of SHV production and 20% of TEM production among ESBL *Klebsiella* isolates (Bradford et al., 2004).

The incidence of Carbapenem resistant enterobacteriaceae has been increasing worldwide (Bhaskar et al., 2013). The main mechanism of resistance to carbapenem is by enzyme production. The most commonly identified enzymes are the carbapenemases OXA, KPC and the MBL enzyme NDM. They have been identified in different countries (Pfeifer et al., 2012). In the seven imipenem resistance isolates, OXA-9 was detected in 2 isolates (28.5%). Kiratisin et al. (2008) reported OXA-10 in 11.8% of *Klebsiella* isolates in Thailand. However OXA genes were not detected in any of 15 *Klebsiella* isolates from Turkey (Bali et al., 2010).

None of our isolates have the carbapenem resistance gene KPC. Our results differed from those reported in USA by Bradford et al. (2004), who found KPC in 100%

of isolates. Also, Robledo et al. (2011) found KPC in 73% of 457 MDR *Klebsiella* isolates. In this study, the MBL NDM-1 was detected in 42.8% of imipenem resistant isolates. Chaudhary and Payasi (2013) detected NDM in 6.25% (in 24/150) of *Klebsiella* isolates.

We recommend that strict antibiotic policies and measures to limit unnecessary use of cephalosporins and carbapenems should be enforced to minimize the emergence of resistance to these antibiotics. Also rapid routine molecular detection of resistance determinants is essential to optimize therapy, improve outcomes, and limit the spread of such resistance.

Conflict of interest

The authors have not declared any conflict of interest

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