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ORIGINAL ARTICLE

Detection of extended-spectrum and plasmid-mediated AmpC β-lactamases in nosocomial *Klebsiella* isolates

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ABSTRACT

Objective: The coexistence of ESBLs and pAmpCs enzymes in the same *Klebsiella* strain may result in false-negative tests for the detection of ESBLs as pAmpCs resist inhibition by clavulanic acid so masking ESBL presence. This study was to highlight the detection rates of ESBLs and pAmpCs by using phenotypic method; MAST 4-disc test and multiplex polymerase chain reaction (PCR) method. In addition, it aimed to evaluate the sensitivity of the phenotypic method in detection of these enzymes.

Methods: *Klebsiella* isolates were collected from clinical samples in different wards in Zagazig University Hospitals. The antibiogram of these bacteria was determined by disc diffusion method. The presence of ESBLs and pAmpCs within the isolates was determined using the phenotypic MAST 4-disc test followed by a multiplex PCR method.

Results: In total, 38 *Klebsiella pneumoniae* strains were evaluated. Among these isolates, 65.8% were ESBL producers, 2.6% were pAmpC producers, and 31.6% were neither ESBL nor pAmpC producers. The most frequent genotype of ESBL was *CMY* (84%); followed by *CMY* (44%) before pAmpC producers were of *CMY* genotype. The distribution of different ESBL genotypes was *CMY, CMY* and *CTX-M* II genotype (28%) and followed by *CMY* and *CTX-M* IV genotype (24%). Using multiplex PCR as a reference method, MAST 4-disc test was of 92% sensitivity and 86.7% specificity.

Conclusion: A rising alarm of ESBL producing strains among *K. pneumoniae* isolates. The exact detection of ESBLs in isolates that produce both enzymes is important for both treatment and epidemiology. *J Microbiol Infect Dis 2013; 3(1): 24-30*

Key words: ESBL, pAmpC, B-Lactasmases, Nosocomial, *Klebsiella*

Nozokomiyal *Klebsiella* suşlarında genişlemiş spektrumlu ve plazmid aracılı AmpC β-lactamazların saptanması

ÖZET

Amaç: Aynı *Klebsiella* suşunda heriki enzimin bir arada bulunması GSBL için yanlış-negatif test sonucuna neden olabilir; pAmpC'lerin klavulanik asit tarafından inhibisyona direnç göstermesi sonucu GSBL maskelenebilir. Bu çalışma fenotipik yöntem olan MAST 4-disk testi ile ve multipleks polimeraz zincir reaksiyonu (PCR) yöntemi ile GSBL ve pAmpC oranlarının saptanmasını amaçladı. Buna ek olarak bu enzimlerin tespitinde fenotipik yöntemin duyarlılığının değerlendirmesi amaçlandı.

Yöntemler: *Klebsiella* izolatları Zagazig Üniversitesi Hastaneleri'nin farklı koğuşlarından elde edilen klinik örneklerden toplandı. Bu bakterilerin antibiyogramı disk difüzyon yöntemi ile belirlendi. İzolatlarda GSBL ve pAmpC varlığı fenotipik MAST 4-disk testi ve akabinde multiplex PCR yöntemi kullanılarak tespit edildi.

Bulgular: Toplam olarak 38 *Klebsiella pneumoniae* suşu değerlendirildi. Bu izolatların % 65,8'i GSBL üretiyordu, % 2,6'sı pAmpC üretiyordu ve % 31,6'sı ne GSBL ne de pAmpC üretiyordu. En sık GSBL genotipi *CMY* (% 84) idi; bunu *CMY* (%44) izledi, PAmpC üretenler *CMY* genotipindeydi. Farklı GSBL genotiplerinin dağılımı *CMY* (% 84), *CMY* (% 44), *CTX-M* II genotipi (28%), *CMY* ve *CTX-M* IV genotipi (% 24) şeklinde idi. Referans yöntem olarak multipleks PCR alındığında MAST 4-disk testinin duyarlılığı % 92 ve özgüllüğü % 86,7 idi.

Sonuç: *K. pneumoniae* arasında GSBL üreten suşların yükselen alarmı. Her iki enzimi üreten izolatlarda GSBL'lerin kesin tespiti, hem tedavi hem de epidemiyolojide önemlidir.

Anahtar kelimeler: GSBL, pAmpC, B-Laktamazlar, Hastane, *Klebsiella*

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INTRODUCTION

Klebsiellae are opportunistic pathogens that are incriminated in many nosocomial infections including pneumonia, bacteremia, thrombophlebitis, urinary tract infection, cholecystitis, diarrhea, upper respiratory tract infection, wound infection, osteomyelitis and meningitis.[1]

There is an increase in the prevalence of resistance to extended-spectrum cephalosporins in *Klebsiella* species. Such resistance is often mediated by the production of Extended Spectrum B-Lactamases (ESBLs) or Ambler Class C β-lactamases (AmpC).[2] Genes coding for AmpC enzyme may be chromosomally-encoded or plasmid-mediated. *Klebsiellae* strains possess only the plasmid-mediated form of the enzyme.[3]

ESBLs are capable of conferring resistance to the penicillin, cephalosporins and monobactams (but not cephamycins; cefoxitin and cefotetan or carbapenems) by hydrolysis of these antibiotics. They are inhibited by β-lactamase inhibitor combinations such as clavulanic acid, sulbactam and tazobactam.[4] On the other hand, AmpC β-lactamases are usually resistant to penicillins, cephalosporins including the cephamycins and monobactams. They are resistant to β-lactamase inhibitor combinations but are usually sensitive to carbapenems.[5]

Notably, the coexistence of both ESBLs and pAmpC β-lactamases in the same strain may result in false-negative tests for the detection of ESBLs. This may be explained by the ability of pAmpCs to pAmpCs to resist inhibition by clavulanic acid and hence the presence of an ESBL can be masked by activity of a pAmpC.[6]

Therefore, there is a need, for an alternative method that can detect ESBLs in *Klebsiella* isolates with a high sensitivity even though the isolates simultaneously harbor pAmpCs β-lactamases.[7]

Although a susceptibility screening test using a cephamycin (usually cefoxitin) disc is available, it is not one hundred percent accurate as a reduced outer membrane permeability may also cause insusceptibility.[3] Fortunately, boronic acid, an AmpC β-lactamases inhibitor, can be used as an alternative screening test for detection of AmpC producing bacteria. Its advantages over other β-lactam inhibitors are the following: it is unaffected by porin channel mutations, not induce AmpC expression and not hydrolysed by mutant β-lactamases.[8]

Despite of those highly sensitive phenotypic methods for detection of ESBLs and pAmpCs, the genotypic detection ones remain the gold standard because they are less time-consuming, less complicated and more accurate. Moreover, a multiplex polymerase chain reaction (PCR) method has been used for the detection and classification of these enzymes.[9]

This study was designed to highlight the detection rates of ESBLs & pAmpCs β-lactamases among nosocomial *Klebsiella* isolates. In addition, to evaluate the sensitivity of a MAST 4-disc test as a phenotypic method for detection of ESBLs and pAmpCs β-lactamases using multiplex PCR as a gold standard.

METHODS

Patients

This study was carried out at the Microbiology and Immunology Department, Faculty of Medicine, Zagazig University in the period from June 2010 to November 2011. The patients were included from different wards in Zagazig University Hospitals. They had acquired different types of nosocomial infections.

Sample collection

Blood, urine, sputum and wound swabs samples were collected from each patient according to the site of infection under complete aseptic conditions and before the start of antimicrobial therapy when possible. All clinical specimens were subjected to the following: direct microscopic examination of smears stained with Gram's stain. Blood samples were inoculated into blood culture bottles (Egyptian Diagnostic Media, Egypt) then incubated at 37°C for 7-14 days. Subcultures were done every 48 hours on blood agar and MacConkey's agar (Oxoid, UK) plates. Other samples were cultured on nutrient agar (Oxoid, UK) blood agar and MacConkey's agar.

Identification of *Klebsiella* isolates was performed by the following methods: a) Microscopic examination of Gram-stained films, b) Colonial morphology, c) Conventional biochemical reactions; oxidase reagent (Tetramethyl p-phenylene diamine dihydrochloride) (Oxoid, UK) for oxidase test, triple sugar iron medium (Merck, UK), peptone water (Oxoid, UK) for indole test, p- dimethyl- aminobenzaldehyde (Sigma, USA) for indole test, Simmon's citrate agar medium for citrate test and semi-solid agar to detect the motility and d) Biochemical reactions utilizing API 20E (Bio-Mérieux, France). All *Klebsiella* isolates were subjected to antimicrobial susceptibility testing by disc diffusion method for determination of the susceptibility pattern to β-lactams to includ-

ing ampicillin, ampicillin-sulbactam, amoxacillin-clavulanic acid, piperacillin-tazobactam, cefuroxime, cefoxitin, ceftazidime, ceftriaxone, cefepime, aztreonam, imipenem, meropenem, gentamycin, amikacin, ciprofloxacin, levofloxacin and non B-lactams including (Oxoid, UK).

All *Klebsiella* isolates were examined for the presence of extended-spectrum β-lactamases (ESBLs) and plasmid mediated AmpC β-lactamases (pAmpCs) using MAST 4-disc test (MAST Group Ltd., MAST House, Derby Road, Bottle, Merseyside, L20 1EA) and Multiplex PCR. Multiplex PCR was performed for all isolates for detecting the presence or absence of genes coding for ESBLs and pAmpCs using PCR-GOLD Master-Mix Beads (Bioron, The ENZYME Company, Germany). DNA extraction was done using QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The extracts were then kept at -20°C until they were used in PCR reactions. Fourteen PCR primers were designed to be used in two multiplex PCR reactions, one targeting 3 different regions (Table 1) and the other targeting four different regions (Table 2). For each multiplex PCR reaction, the following materials were added to each tube containing a PCR bead: 4 µl (40 pmol) of each forward primer and 4 µl (40 pmol) of each reverse primer, 10 µl of template DNA, 3 µl of MgCl of 25 mM and sterile deionized distilled water to a total volume of 50 µl mixed well by automatic pipette. The reaction was performed in a thermal cycler (Biometra, Goettingen, Germany) using the amplification program consisting of: initial denaturation step at 94°C for 5 min., followed by 30 cycles of denaturation at 94°C for 1 min., anneling at 61°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 5 min.[9] Each run of PCR amplification included negative control with no template DNA to avoid false positive results caused by possible contamination. Amplicon detection was done using agarose gel electrophoresis.[10]

Table 1. Primers for set I multiplex PCR

Gene	Forward primer	Reverse primer	Amplicon size (bp)
CTX-M IV	(5'-GACAAAGAGAGT GCAACGGATG- 3')	(5'-TCAGTGCGAT CCAGACGAAA-3')	501
CMY	(5'-AGTGCTGCCATA ACCATGAGTG- 3')	(5'CTGACTCCCCGT CGTGTAGATA- 3')	431
CMY	(5'-GATGAACGC TTTCCCATGATG-3')	(5'-CGCTGTTATC GCTCATGGTAA-3')	214

Table 2. Primers for set II multiplex PCR

Gene	Forward primer	Reverse primer	Amplicon size (bp)
CMY II	(5'-AGCGATCCGG TCACGAAATA- 3')	(5'-CCCGTTTTATGC ACCCATGA- 3')	695
CTX-M I	(5'TCCAGAATAAGG AATCCCATGG- 3')	(5'-TGCTTTACC CAGCGTCAGAT- 3')	621
CTX-M II	(5'-ACCGCCGATA ATTCGCAGAT- 3')	(5'-GATATCGTTGGTGGT GCCATAA- 3')	588
CMY	(5'-GTGGTGGA CAGCACCATTAAA- 3')	(5'-CCTGCGGTATA GGTAGCCAGAT- 3')	314

Statistical analysis

Data were checked, entered and analyzed by using SPSS version 17. All Data were expressed as number and percentage for the categorical variables. Chi-square or Fisher-exact test was used when appropriate. Kappa coefficient and validity assessment were done. $P<0.05$ was considered statistically significant.

Ethical considerations

The study was reviewed and approved by the review boards of the Research Ethics Committee, Faculty of Medicine, Zagazig University. Informed consent was obtained from all participants after explanation of the procedure and the purpose of the study.

RESULTS

Thirty-eight *K. pneumoniae* isolates was isolated. *K. pneumoniae* isolates. They were mostly sensitive to imipenem (97.4%) and meropenem (94.7%) followed by amikacin (71.1%) and levofloxacin (71.1%) (Figure 1).

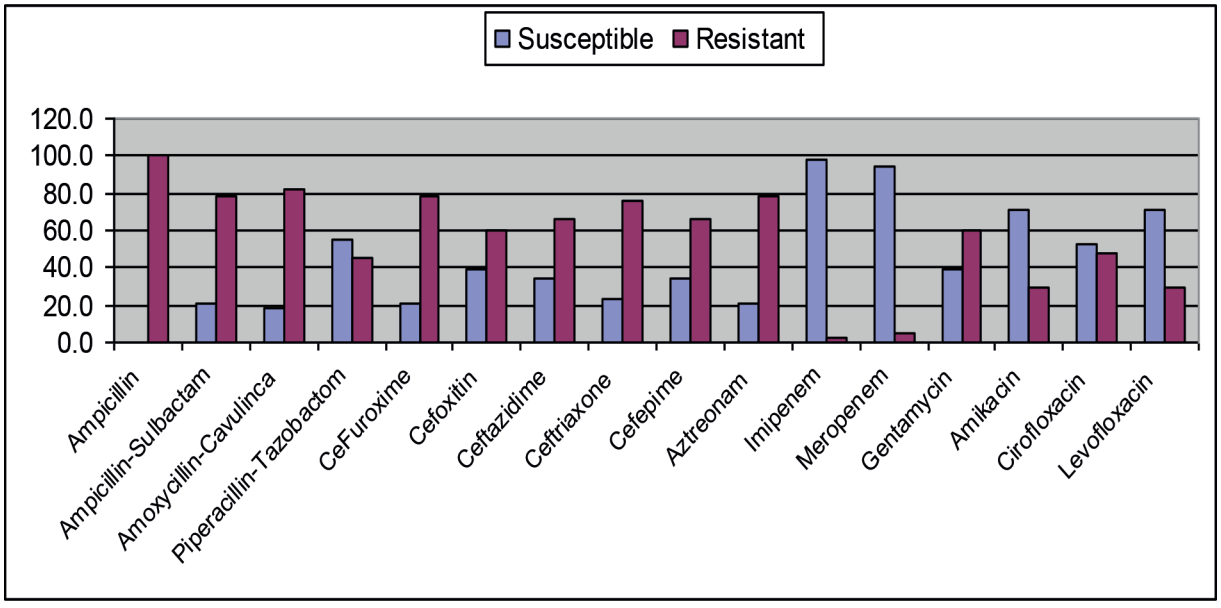

Figure 1. Antimicrobial susceptibility patterns of the 38 *K. pneumoniae* isolates.

Using MAST 4-DISC test the frequency of ESBL-, pAmpC, ESBL and pAmpC-, and neither ESBL nor pAmpC- producing *K. pneumonia* isolates by was 23 (60.5%), 1 (2.6%), 1 (2.6%) and 13 (34.2%); respec-tively (Table 3 and figures 2, 3 and 4). Also, it can be observed that by using multiplex PCR method the frequency of ESBL, pAmpC, ESBL and pAmpC-, and neither ESBL nor pAmpC- producing *K. pneumonia* isolates. The most frequent genotpe was *CMY, CMY* and *CTX-M II* genotype (28%) and followed by *CMY* and *CTX-M IV* genotype (24%). 25 (65.8%), 1 (2.6%), 0 (0.0%) and 12 (31.6%); respectively. In addition, it can be observed that when comparing MAST 4-DISC test with multiplex PCR, it has achieved 92.0% sensitivity and 86.7% specificity with 90.0% accuracy. There was no statistically significant difference between both methods (P>0.05.

The most frequent genotype of ESBL was *CMY* (84%), followed by *CMY* (44%), *CTX-M II* (32%), *CTX-M IV* (28%), and the lowest was *CTX-M I* (4%). Table 4 showed the distribution of different ESBL genotypes among ESBL producing *K. pneumonia* isolates. All of pAmpC-producers were of *CMY* type.

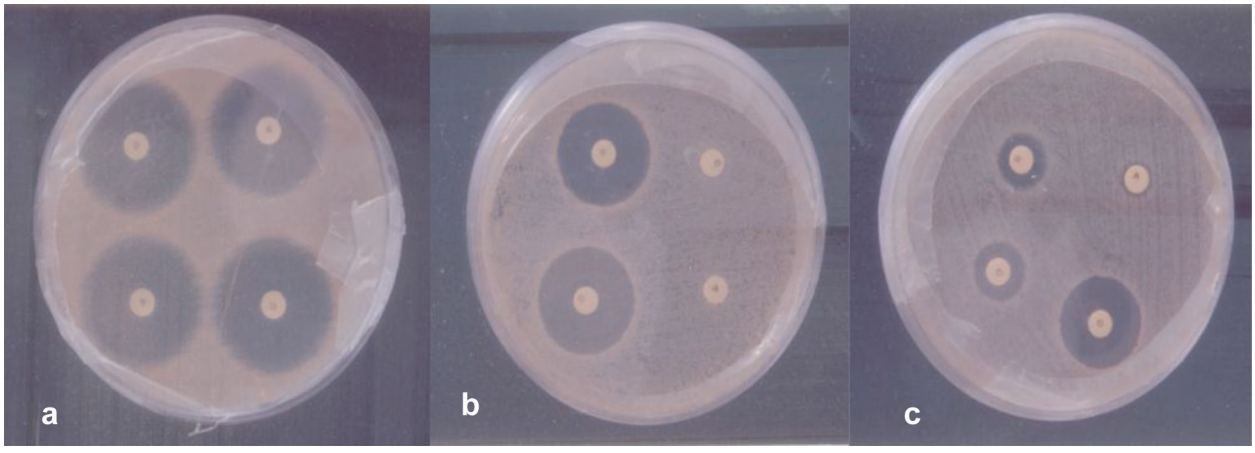

Figure 2. Testing *K. pneumoniae* isolates using MAST 4-disc method.
a: Neither ESBL nor pAmpC activity.
b: ESBL activity
c: pAmpC activity

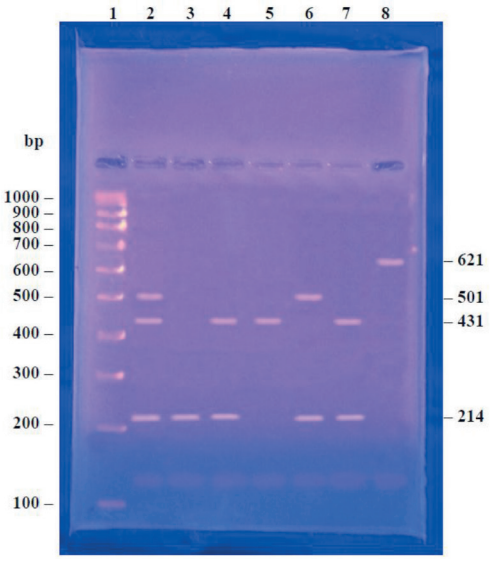

Figure 3. A sample of agarose gel electrophoresis
Lane 1: MW DNA marker (100-1000 bp)
Lane 2: *CMY*, *CMY* and *CTX-M* IV in the same isolate
Lane 3: An isolate with *SHV* gene only
Lane 4: *CMY* and *CMY* in the same isolate
Lane 5: An isolate with *CMY* gene only
Lane 6: *CMY* and *CTX-M IV* in the same isolate
Lane 7 & 8: An isolate with *CMY* and *CMY* genes in one set and *CTX-M I* in another set

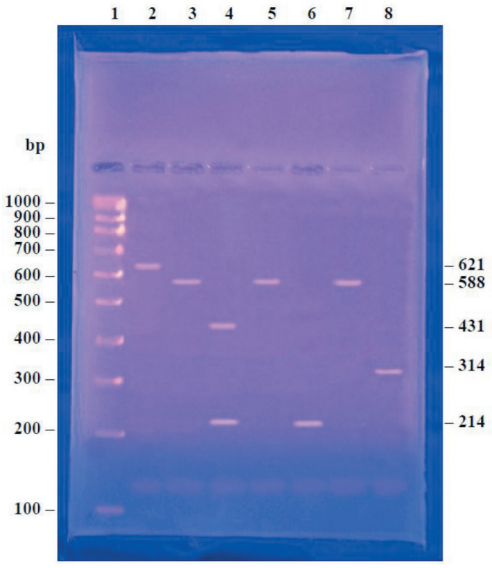

Figure 4. A sample of agarose gel electrophoresis.
Lane 1: MW DNA marker (100-200-300----1000)
Lane 2: An isolate with *CTX-M I* gene only
Lane 3: An isolate with *CTX-M II* gene only
For the same isolate, lane 4 shows *CMY* and *CMY* genes and lane 5 shows *CTX-M II* gene.
For the same isolate, lane 6 shows *CMY* gene and lane 7 shows *CTX-M II* gene.
Lane 8: An isolate with *CMY* gene only

Table 3. Comparison between MAST 4-disc test as a phenotypic method and multiplex PCR

Isolates	MAST 4-disc test No (%)	Multiplex PCR method No (%)	p
ESBL producers	23 (60.5)	25 (65.8)	> 0.05
pAmpC producers	1 (2.6)	1 (2.6)	> 0.05
ESBL& pAmpC producers	1 (2.6)	0 (0.0)	> 0.05
Neither ESBL nor pAmpC producers	13 (34.2)	12 (31.6)	> 0.05

Table 4. Distribution of different ESBL genotypes among ESBL producing *K. pneumonia* strains as yielded by multiplex PCR method

ESBL Producers	*CMY* alone	*CMY* alone	*CMY* & *CMY*	*CTX-M I* alone	*CMY* & *CTX.M IV*	*CTX.M II*	*CMY*, *CMY*& *CTX-M II*	*CMY*, *CMY* & *CTX.M IV*
n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
25 (100)	5 (20)	1 (4)	3 (12)	1 (4)	6(24)	1 (4)	7 (28)	1 (4)

DISCUSSION

Hospital-acquired *K. pneumoniae* infections have increased due to outbreaks of strains producing ESBLs with prevalence ranges from 10% to 40% in several parts of the world.[11] In addition, infections with these strains harboring an acquired pAmpC β-lactamase are associated with a higher mortality and a high risk of therapeutic failure of 3rd generation cephalosporins.[12]

There were low susceptibility rates in our study and they could be attributed to the empirical usage of these antibiotics in treatment of nosocomial infections in our hospitals.[13]

Although definition of ESBLs as they are capable of conferring resistance to penicillins, first-, second-, third-, and fourth- generation cephalosporins, and monobactams (but not cephamycins; cefoxitin and cefotetan or carbapenems) by hydrolysis of these antibiotics. They are inhibited by β-lactamase inhibitor combinations such as clavulinic acid, sulbactam and tazobactam. This interferes with what we actually found in our work as not all ESBLs confer show sensitivity to cefoxitin as one of cephamycins and amoxacillin-clavulanic acid and ampicillin-sulbactam as β-lactamase inhibitor combinations which may be due to other mechanisms of resistance.[14]

So, in this work a phenotypic method for ESBLs and pAmpCs detection; D68C MASTDISCS™ using clavulanic acid as ESBL inhibitor and boronic acid as pAmpC inhibitor. Then, 2 sets of multiplex PCR were used to detect the presence of genes coding for ESBLs and pAmpCs.

The incidence of ESBL-producing *K. pneumoniae* is varying from country to another.[15] Also, it is clearly understandable that the prevalence of ESBL producers in any hospital depends upon various factors, like antibiotic policy, the carriage rate among hospital personnel, and the type of disinfection used especially in the ICU.[16]

In this work, the low incidence of AmpC β-lactamases among *K. pneumonia* isolates in comparison with high ESBL prevalence can be explained by that pAmpCs are less common than ESBLs, although they have been found all over the world.

Despite being just a phenotypic method, D68C MASTDISCS™ has proved its efficacy in detection of ESBL and/or pAmpC producing isolates. Our results are in agreement with the results yielded by Ellem and colleagues who found that the ability of MAST 4-disc test to detect AmpC was of 100% sensitivity and 67% specificity, while its ability to detect ESBL producers was of 92% sensitivity and 89% specificity. The relatively low specificity can be explained by its inability to differentiate AmpC and MBL activity highlighting the continuing need for genetic information.[17]

In our work, the most dominant genotype of ESBL producing *K. pneumoniae* was *CMY*, *CMY* and *CTX-M II* (28%), followed by *CMY* and *CTX-M IV* (24%). In Egypt, the study conducted by Al-Agamy et al about phenotypic detection revealed a high rate of ESBLs (60.9%), where all ESBL producers were polymerase chain reaction-positive for *blaTEM* and *blaCTX-M* genes.[18] Also, Yoo and coworkers documented that 54% of their ESBL producing *K. pneumoniae* simultaneously harbored *CMY* and *CTX-M* 14).[19] On the other hand in Taiwan, Chia and associates reported that their *K. pneumoniae* were more likely to harbor *CTX-M* type ESBLs.[20]

The used multiplex PCR assay enabled us to detect different types of *CTX-M* genes; *CTX-M I*, *CTX-M II*, and *CTX-M IV*. Of those, *CTX-M II* was the most dominant (32%), followed by *CTX-M IV* being pre-sent in 28% of ESBL producing isolates, while *CTX-M I* came last with 4% incidence. The study per-formed in Taiwan by Chia and colleagues showed that 51.6% of their *K. pneumoniae* isolates were *CTX-M* producers. Moreover in their study, types 3, 15 from *CTX-M I* group and types 9,13 and 14 from *CTX-M IV* group were detected. Although *K. pneumoniae* with *CTX-M* enzymes are an emerging problem in Slo-venian hospitals, an epidemiological study of nosocomial *K. pneumoniae* isolates showed that those with *CTX-M* represented a minority (34%) of ESBL producing isolates.[20]

In this study, the detected type of AmpC was *CMY* and no isolates were proved to harbor the *CMY* enzyme. This comes in agreement with the study conducted by Yoo and coworkers who reported that in their *K. pneumoniae*, only *CMY-1* was detected at the same time when their E. coli isolates harbored *CMY* and *CMY* types of pAmpCs.[19] In another study performed by Park and coworkers they have documented that the most prevalent pAmpC genotype has been reported as *CMY-1* in *K. pneumoniae*, where 95% of their *K. pneumoniae* isolates harbored *CMY-1* gene and the remaining harbored the *CMY-1* gene.[21]

Conclusions

A rising alarm of ESBL producing strains among *K. pneumoniae* isolates was detected. The exact detection of ESBLs in isolates that produce both ESBLs and pAmpCs is important for both treatment and epidemiology. Detection of ESBLs and pAmpCs production among *Klebsiella* is essential, especially in critical sites as ICUs. Application of a multiplex PCR technique serves as a simple and rapid molecular method to study the genes encoding various ESBL and pAmpCs with extension of its use to discriminate other resistance determinants.

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