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ORIGINAL ARTICLE

Development of a multiplex PCR system and its application in detection of *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-9}$ and *bla*$_{OXA-1}$ group genes in clinical *Klebsiella pneumoniae* and *Escherichia coli* strains

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Resistance to β-lactam antibiotics through β-lactamase production by *Enterobacteriaceae* continues to burden the health-care sector worldwide. Traditional methods for detection of β-lactamases are time-consuming and labor-intensive and newer methods with varying capabilities continue to be developed. The objective of this study was to develop a multiplex PCR (M-PCR) system for the detection of *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-9}$ and *bla*$_{OXA-1}$ group genes and to apply it in clinical *Klebsiella pneumoniae* and *Escherichia coli* strains. To do this, we used group-specific PCR primers in singleplex reactions followed by optimization into multiplex reactions. Specificity and sensitivity of the M-PCR were then evaluated using 58 reference strains before its application to detect *bla* group genes in 203 clinical *Enterobacteriaceae* strains. PCR amplicons were sequenced to determine the β-lactamase subtypes. The M-PCR system exhibited 100% specificity and sensitivity. In all, 83.7% of *K. pneumoniae* and 89.8% of *E. coli* clinical strains harbored *bla* group genes with 46.9%, 40.1%, 15.0%, 21.1% and 6.1% of *K. pneumoniae* having *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-9}$ and *bla*$_{OXA-1}$ group genes, respectively, whereas 12.2%, 77.6%, 22.4%, 36.7% and 8.2% of *E. coli* had *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-9}$ and *bla*$_{OXA-1}$ group genes, respectively. *Bla*$_{SHV-1}$, *bla*$_{SHV-11}$, *bla*$_{SHV-27}$, *bla*$_{SHV-33}$, *bla*$_{SHV-144}$, *bla*$_{TEM-1}$, *bla*$_{TEM-135}$, *bla*$_{OXA-1}$, *bla*$_{CTX-M-3}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-27}$, *bla*$_{CTX-M-55}$, *bla*$_{CTX-M-65}$ and *bla*$_{CTX-M-104}$ were detected. In conclusion, the M-PCR system was efficient and versatile with an advantage of simultaneously detecting all the targeted *bla* group genes. Hence, it is a potential candidate for developing M-PCR kits for the screening of these genes for clinical or epidemiological purposes.
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INTRODUCTION

With the discovery that microorganisms naturally produce substances that inhibit other microorganisms came the possibility of effectively treating infectious diseases.[1] However, soon after, cases of resistance began to emerge.[2] The β-lactam group of antibiotics is one of the worst affected by antimicrobial resistance through a variety of mechanisms.[3] β-lactamases including broad- and extended-spectrum β-lactamases (ESBLs) have been reported predominantly in *Enterobacteriaceae*, especially *Klebsiella pneumoniae* (*K. pneumoniae*) *and Escherichia coli* (*E. coli*).[4–6] Most extended-spectrum β-lactamases fall into three genotypes: TEM, SHV and CTX-M[7] with hundreds of variants of these gene families having emerged and disseminated widely requiring concerted efforts to effectively control.[6,8] An example is the >130 allelic variants of CTX-M (Lahey Clinic website: http://www.lahey.org/Studies/other.asp#table1).[9] Other β-lactamases including AmpC, carbapenemases and OXA-type enzymes have also been consistently reported to cause antibiotic resistance in *Enterobacteriaceae*.[10,11] OXA-1-like enzymes are sometimes harbored alongside the CTX-M group in *K. pneumoniae* and *E. coli*,[10] whereas the OXA-10 derivatives (OXA-11, -14, -16, -17 and -19) exhibiting ESBL activity, and carbapenem-hydrolyzing class D β-lactamases (CHDLs, e.g., OXA-23, -24/40, -48, -51 and -58), are commonly found in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, respectively.[11–13] Notably, carbapenemases, especially metallo-β-lactamases, present a far greater risk of treatment failure with the currently available drugs in clinical practice. However, by and large,

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the prevalence of these enzymes in clinical *Enterobacteriaceae,* especially *K. pneumoniae* and *E. coli* in China is comparatively lower compared with that of bla_{SHV}-, bla_{TEM}-, $bla_{CTX\text{-}M\text{-}1}$-, $bla_{CTX\text{-}M\text{-}9}$- or $bla_{OXA\text{-}1}$-encoded enzymes.[14] A recent study in Central China has reported that the prevalence of carbapenem resistance in *Enterobacteriaceae* was 1%.[15] With the current overall concern about carbapenemases, it is beneficial to carefully monitor and control any potential spread of carbapenemase-producing bacterial strains in China and other countries in this region.[16]

The phenotypic screening and detection of β-lactamase (*bla*) genes are routinely carried out through tedious culture-based methods, whereas their definitive confirmation in *Enterobacteriaceae* and other bacteria is achieved largely by molecular methods.[17] As the current subtypes and variants are continuously evolving and disseminating both geographically and among the various bacterial species,[7] growing numbers of *Enterobacteriaceae*, especially *K. pneumoniae* and *E. coli* from clinical sources tend to harbor multiple β-lactamases and exhibit multiresistance to the various β-lactams. Consequently, existing efforts geared toward sustained and continuous development of new accurate and rapid molecular methods with the ability to simultaneously detect multiple *bla* genes in single and/or mixed bacterial strains must be sustained.

Different workers have developed multiplex PCR (M-PCR) assays that detect different *bla* genes or combinations of *bla* genes in *Enterobacteriaceae*. For instance, two assays were previously constructed including one that detects the five phylogenetic groups of the $bla_{CTX\text{-}M}$ genotype,[18] and another that targets the bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ genotypes.[19] Recently, a group of workers developed six M-PCR assays that can detect most of the *bla* genes in different combinations.[17] Others have constructed M-PCR assays for detecting carbapenemase[20] and AmpC[21] genes. These assays vary in their achievable capabilities in detecting the different *bla* group genes. Evidently, the currently available M-PCR assays lack the ability to detect bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ and $bla_{OXA\text{-}1}$ group genes simultaneously in a single reaction. As these are the most commonly encountered *bla* genes in this region, we sought to develop an M-PCR system that can detect these five *bla* group genes concurrently in a single reaction. We report a versatile and effective new system and its application in detecting these *bla* group genes in clinical *Enterobacteriaceae* strains from two hospitals in Harbin area of North East China.

MATERIALS AND METHODS

Bacterial strains

K. pneumoniae ($n=45$), *E. coli* ($n=11$) and *Enterobacter cloacae* ($n=2$) were used as reference strains in the optimization and evaluation of the M-PCR system. The distribution of *bla* group genes in these reference strains had been determined previously by at least two singleplex PCR assays with gene-specific primers and DNA sequencing. Another 203 randomly chosen non-repetitive clinical *K. pneumoniae* ($n=147$), *E. coli* ($n=49$), *Enterobacter cloacae* ($n=5$) and *Serratia liquefaciens* ($n=2$) strains previously obtained from patients aged 6 days to 85 years from two hospitals in Harbin area of North East China were used in the application of the developed M-PCR system to detect bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ and $bla_{OXA\text{-}1}$ group genes. These isolates were recovered from urine, stool, blood, sputum, throat swab, pus and aspirate specimens, and were identified using API 20E identification system (BioMérieux, Lyon, France). Identified clinical isolates were stored in 50% glycerol at − 85 °C until used.

Preparation of template DNA

DNA template was prepared according to a previously reported method[22] with a slight modification. A single colony of each strain was subcultured in 2 ml of Luria–Bertani broth (Oxoid, Hampshire, UK) for 16 h at 37 °C with shaking at 200 r.p.m. Cells from 1.5 ml of this culture were harvested by centrifugation at 13 000 *g* for 10 min and the pellet was resuspended in 500 μl of double-distilled water. The cells were then lysed by heating at 99 °C for 15 min, and cellular debris removed by centrifugation at 13 000 *g* for 10 min. The supernatant containing the harvested DNA was collected and stored at − 20 °C until used in the PCR experiments.

Development, optimization and evaluation of the M-PCR system

Group-specific oligonucleotide primers for $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$ group genes were designed using the Primer3 software[23] while the primers specific for bla_{TEM}, bla_{SHV} and $bla_{OXA\text{-}1}$ group genes were obtained from literature (see Table 1). All the primers were manufactured and supplied by Sangon Biotech (Shanghai, China). Initially, the group-specific primers were tested in singleplex PCR reactions under the following reaction conditions: initial denaturation at 94 °C for 10 min; 30 cycles of denaturation at 94 °C for 30 s, annealing

Table 1 Group-specific primers used in this study

Gene targeted	*GenBank accession no.*	*Primer name*	*Sequence (5′–3′)*	*Length (bases)*	*Annealing position*	*Amplicon size (bp)*	*Source*	*Purpose*
Bla_{TEM}	JX268626	M-TEM-F	CATTTCCGTGTCGCCCTTATTC	22	13–34	800	Perez *et al.*[13]	Singleplex and multiplex PCR and sequencing
		M-TEM-R	CGTTCATCCATAGTTGCCTGAC	22	812–791			
Ba_{SHV}	JX268740	M-SHV-F	AGCCGCTTGAGCAAATTAAAC	21	71–91	713	Perez *et al.*[13]	Singleplex and multiplex PCR and sequencing
		M-SHV-R	ATCCCGCAGATAAATCACCAC	21	783–763			
$Bla_{OXA\text{-}1}$	GQ896556	M-OXA-1-F	GGCACCAGATTCAACTTTCAAG	22	201–222	564	Perez *et al.*[13]	Singleplex and multiplex PCR
		M-OXA-1-R	GACCCCAAGTTTCCTGTAAGTG	22	764–743			
$Bla_{CTX\text{-}M\text{-}1}$	X92506	M-CTX-M1-F	TTAGGAAGTGTGCCGCTGTA	20	123–142	655	This study	Singleplex and multiplex PCR
		M-CTX-M1-R	CGGTTTTATCCCCCACAAC	19	777–759			
$Bla_{CTX\text{-}M\text{-}9}$	AJ416345	M-CTXM-9-F	GGTGATGAACGCTTTCCAAT	20	324–340	518	This study	Singleplex and multiplex PCR
		M-CTXM-9-R	TTATCACCYRCAGTCCACGA[a]	20	841–822			
$Bla_{OXA\text{-}1}$	GQ438248	OXA-1-Seq-F	GCCCTTTACCAAACCAATAC	21	433–453	896	This study	Singleplex PCR and sequencing
		OXA-1-Seq-R	ACTTGATTGAAGGGTTGGGC	20	1328–1309			
$Bla_{CTX\text{-}M\text{-}1}$	FJ235692	CTX-M-1-Seq-3F	GACTATTCATGTTGTTGTTATTTC	24	1067–1090	924	Batchelor *et al.*[24]	Singleplex PCR and sequencing
		CTX-M-1-Seq-3R	TTACAAACCGTTGGTGACG	19	1990–1972			
$Bla_{CTX\text{-}M\text{-}9}$	AJ416345	CTX-M-9-Seq-3F	ATGGTGACAAAGAGAGTGCAAC	22	551–568	876	Mena *et al.*[25]	Singleplex PCR and sequencing
		CTX-M-9-Seq-3R	TTACAGCCCTTCGGCGATG	10	1003–987			

Primers were optimized to a common PCR annealing temperature of 59.7 °C allowing a single concurrent run for the M-PCR system.
[a]Y = T or C; R = A or G.

Table 2 Specificity and sensitivity of the M-PCR system for *bla* group gene detection

Genes	*Number of strains tested*	*True (+)* = A	*False (−)* = B	*False (+)* = C	*True (−)* = D	*Specificity (%)*[a]	*Sensitivity (%)*[b]
Bla genes (total)	58	53	0	0	5	100	100
Bla_{SHV}	58	23	0	0	35	100	100
Bla_{TEM}	58	31	0	0	27	100	100
$Bla_{CTX\text{-}M\text{-}1}$	58	22	0	0	36	100	100
$Bla_{CTX\text{-}M\text{-}9}$	58	18	0	0	40	100	100
$Bla_{OXA\text{-}1}$	58	11	0	0	47	100	100

Specificity and sensitivity were calculated based on a previously used formula.[27]
[a]Specificity = $D/C+D\times100$.
[b]Sensitivity = $A/A+B\times100$.

(at 60.1°C, bla_{TEM}; 58 °C, bla_{SHV}; 60.1 °C, $bla_{OXA\text{-}1}$; 57.8 °C, $bla_{CTX\text{-}M\text{-}1}$; 57 °C, $bla_{CTX\text{-}M\text{-}9}$) for 35 s and extension at 72 °C for 1 min; and final extension at 72 °C for 9 min. The amount of each primer and DNA lysate for the 50 µl initial singleplex PCR reaction were 5 nmol and 2 µl, respectively. The primers were then added together in single reactions to develop and optimize the M-PCR system. Reaction conditions and amounts of reactants were adjusted appropriately to achieve optimal amplification of all the five *bla* genes. Annealing temperature gradient PCR was used to determine the optimum annealing temperature. Upon optimization, the PCR conditions used in all the subsequent singleplex and multiplex reactions were: initial denaturation at 94 °C for 10 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 61 °C for 35 s and extension at 72 °C for 1 min; and final extension at 72 °C for 9 min. Primer concentrations of 5 nmol for M-TEM-F and R, M-SHV-F and R, M-OXA-1-F and R and M-CTX-M-1- F and R; and 10 nmol for M-CTX-M-9-F and R were used (Table 1). For each 50 µl reaction, 5 µl DNA lysate and 25 µl 2× Taq Master Mix (Sangon Biotech, Shanghai, China) were added. DNA lysates from each of the 58 reference *Enterobacteriaceae* strains were then used in both singleplex PCR and M-PCR experiments to evaluate and verify the specificity and sensitivity of the newly developed multiplex system as shown in Table 2. Stability and efficiency of the system against varying genomic environments was further tested by mixing DNA lysates from same or different *Enterobacteriaceae* species. PCR products were separated in a 2.5% agarose gel at 80 V for 1 h 30 min and visualized in UV light after staining with ethidium bromide for 3–5 min. A 100 bp DNA ladder (Tiangen Biotech, Beijing, China) was used as a molecular size marker. All PCR experiments were carried out using Bio Rad T100 Thermal cycler (Bio Rad, Singapore city, Singapore).

Application of the M-PCR system in detecting bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ and $bla_{OXA\text{-}1}$ group genes in clinical *Enterobacteriaceae* strains

The newly developed M-PCR system was applied in the multiplex PCR detection of bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ and $bla_{OXA\text{-}1}$ group genes in 203 clinical *Enterobacteriaceae* strains obtained from two hospitals in Harbin area of North East China including 147 *K. pneumoniae*, 49 *E. coli*, 5 *E. cloacae* and *2 S. liquefaciens* clinical strains.

Confirmation of the M-PCR system by direct DNA sequencing of the PCR products

To further ascertain the efficiency of the new M-PCR system, each of the PCR products from the strains that tested positive for the *bla* group genes were subjected to direct bidirectional DNA sequencing using either the M-PCR primers or sequencing primers[24,25] (shown in Table 1). The DNA sequencing data were compared with known *bla* allele sequences (Lahey Clinic website: http://www.lahey.org/Studies/other.asp#table1) by multiple-sequence alignment using the ClustalW2 online program. The gene sequences detected in this study have been deposited with GenBank under accession numbers KP698217–KP698229 and KR338940–KR338951.

Antibiotic sensitivity testing

Each of the 147 *K. pneumoniae*, 49 *E. coli*, 5 *E. cloacae* and 2 *S. liquefaciens* clinical strains was screened for possible resistance to six β-lactam antibiotics by disk diffusion test as recommended by Clinical and Laboratory Standards Institute.[26] Resistance to amoxicillin-clavulanate, AMC (20 µg/10 µg), cefotaxime, CTX (30 µg), ceftazidime, CAZ (30 µg), ceftriaxone, CRO (30 µg), aztreonam, ATM (30 µg) and cefazolin, KZ (30 µg) disks (Oxoid) was tested on Mueller-Hinton agar (Oxoid). Inhibition zone diameters were interpreted according to Clinical and Laboratory Standards Institute recommendations and *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality control strains.[26]

Statistical analysis

Statistical tests including Spearman's rank correlation analysis, χ^2 test and multiple logistic regression analysis were used to evaluate the associations between the determinant *bla* group genes and the various levels of antibiotic resistance phenotypes. A *P*-value of 0.05 was considered statistically significant in this study. All statistical analysis were carried out using IBM SPSS Statistics for Windows, Version 19.0 (IBM, Armonk, New York, NY, USA).

Ethical considerations

This study complied with current ethical stipulations.

RESULTS

Development and optimization of the M-PCR system

Singleplex PCR reactions were initially carried out at different annealing temperatures designated for each primer pair (Table 1) yielding distinct PCR products (bla_{TEM}, 800 bp; bla_{SHV}, 713 bp; $bla_{CTX\text{-}M\text{-}1}$, 655 bp; $bla_{OXA\text{-}1}$, 564 bp; and $bla_{CTX\text{-}M\text{-}9}$, 518 bp) in representative reference *Enterobacteriaceae* strains (Figure 1a). M-PCR at the optimized annealing temperature (61.0 °C) simultaneously amplified all the five *bla* group genes in the reference strains harboring single or multiple *bla* genes (Figure 1b). Distinct PCR products were also obtained after DNA lysates from two or three different reference strains belonging to the same or different species were mixed together before M-PCR amplification of the five *bla* group genes (Figure 1c). These results demonstrate that the M-PCR system can effectively detect all the five *bla* group genes in pure or mixed genomic environments in clinical specimens.

Evaluation of specificity and sensitivity of the multiplex system using reference strains

The newly developed M-PCR system was efficient in detecting the *bla* group genes. Using singleplex PCR to detect the presence of *bla* group genes, 53 of the 58 reference *Enterobacteriaceae* were found to possess at least 1 *bla* group gene. Of them, 26 were found to contain >2 *bla* group genes. The M-PCR amplification of total DNA lysates from the each 58 reference strains was also carried out. There was 100% agreement for all the genes and reference strains tested. Specificity and sensitivity of the M-PCR, calculated according to the following formula: {specificity = $(D/C+D\times100)$; (sensitivity = $A/A+B\times100$), where A is true positive, B is false negative, C is false positive and

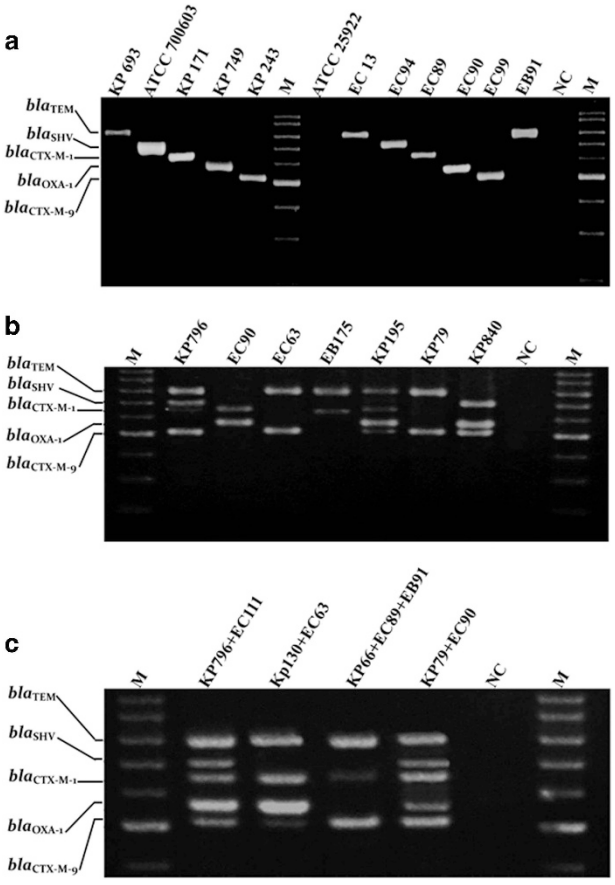

Figure 1 Evaluation of the multiplex PCR (M-PCR) system for the detection of *bla* group genes in the reference *K. pneumoniae* (KP), *E. coli* (EC) and *E. cloacae* (EB) strains with known genotypes. (**a**) Singleplex PCR products in 2.5% agarose gel. Lanes: KP693 (bla_{TEM}); KP ATCC 700603 (bla_{SHV}); KP171 ($bla_{CTX-M-1}$); KP749 (bla_{OXA-1}); KP243 ($bla_{CTX-M-9}$); ATCC 25922 (negative control); EC13 (bla_{TEM}); EC 94 (bla_{SHV}); EC89 ($bla_{CTX-M-1}$); EC90 (bla_{OXA-1}); EC99 ($bla_{CTX-M-9}$); EB91 (bla_{TEM}); NC (negative control-double-distilled water) and M (100 bp molecular marker). ATCC 700603, bla_{SHV} (+) *K. pneumoniae* standard strain and ATCC 25922 *bla* gene (−) *E. coli* standard strain; EB, *Enterobacter cloacae*; EC, *E. coli*; KP, *K. pneumoniae*; M, 100 bp molecular marker. (**b**) M-PCR products in 2.5% agarose gel. Lanes: KP796 ($bla_{TEM/SHV/CTX-M-1/CTX-M-9}$); EC90 ($bla_{SHV/\ CTX-M-1/OXA-1}$); EC63 ($bla_{TEM/CTX-M-9}$); EB175 ($bla_{TEM/CTX-M-1}$); KP195 ($bla_{TEM/CTX-M-1/OXA-1/CTX-M-9}$); KP79 ($bla_{TEM/CTX-M-9}$); KP840 ($bla_{SHV/CTX-M-9}$); NC (negative control-double-distilled water); and M, 100 bp molecular marker. (**c**) Template DNA from *K. pneumoniae, E. coli* and *E. cloacae* strains with different *bla* group genes were mixed, amplified by M-PCR and their PCR products separated in 2.5% agar. Lanes: KP796 ($bla_{TEM/SHV/CTX-M-1/CTX-M-9}$)+EC111 ($bla_{TEM/OXA-1}$); KP130 ($bla_{TEM/OXA-1/CTX-M-1}$)+ EC63 ($bla_{TEM/CTX-M-9}$); KP66 ($bla_{CTX-M-9}$)+ EC89 ($bla_{CTX-M-1}$)+EB91 ($bla_{TEM}$); KP79 ($bla_{TEM/CTX-M-9}$)+EC90 ($bla_{SHV/CTX-M-1/OXA-1}$); NC (negative control-double-distilled water) and M (100 bp molecular marker).

D is true negative},[27] were both determined to be 100% (Table 2) for the targeted *bla* group genes.

Distribution of the bla_{SHV}, bla_{TEM}, $bla_{CTX-M-1}$, $bla_{CTX-M-9}$ and bla_{OXA-1} group genes in clinical *Enterobacteriaceae* strains

Application of the M-PCR system on the clinical *Enterobacteriaceae* strains demonstrated that bla_{SHV} was the most predominant in *K. pneumoniae* (46.9%, 69 strains) followed by bla_{TEM} (40.1%, 59 strains), $bla_{CTX-M-9}$ (21.1%, 31 strains) and $bla_{CTX-M-1}$ (15.0%, 22 strains). Nine strains (6.1%) were found to possess bla_{OXA-1} (Figure 2a). In *E. coli*, bla_{TEM} was the most prevalent (77.6%, 38 strains) followed by $bla_{CTX-M-9}$ (36.7%, 18 strains), $bla_{CTX-M-1}$ (22.4%, 11 strains), bla_{SHV} (12.2%, 6 strains) and bla_{OXA-1} (8.2%, 4 strains) (Figure 2b). Furthermore, we found that 83.7% (123 strains) of the clinical *K. pneumoniae* strains possessed at least 1 *bla* group gene (s) with up to 19 different *bla* genotypes (Table 3). On the other hand, 89.8% (44 strains) of the clinical *E. coli* strains possessed at least 1 *bla* group genes with 11 different genotypes (Table 3). Nearly half of the *K. pneumoniae* (52.4%, 77 strains) carried only a single gene of bla_{SHV} (49 strains), bla_{TEM} (21 strains), $bla_{CTX-M-1}$ (1 strain) or $bla_{CTX-M-9}$ (6 strains) type. Besides, another 46 (31.3%) of *K. pneumoniae* had >2 genes, with 5 (3.4%) and 11 (7.5%) of these carrying four genes ($bla_{TEM/SHV/CTX-M-1/CTX-M-9}$, 4 strains and $bla_{TEM/OXA-1/CTX-M-1/CTX-M-9}$, 1 strain) and three genes ($bla_{TEM/SHV/CTX-M-9}$, 3 strains; $bla_{TEM/CTX-M-1/CTX-M-9}$, 1 strain; $bla_{TEM/SHV/CTX-M-1}$, 4 strains; $bla_{TEM/SHV/OXA-1}$, 1 strain and $bla_{TEM/OXA-1/CTX-M-1}$, 2 strains) each, respectively. Twenty-six (17.7%) of *K. pneumoniae* strains possessed two genes each, whereas 24 *K. pneumoniae* strains did not carry any of the five *bla* group genes. Contrastingly, out of the 49 *E. coli* strains, 18 (36.7%) possessed only one *bla* group gene, whereas 19 (38.8%) and 7 (14.3%) harbored two and three genes, respectively. Five *E. coli* strains (10.2%) did not carry any of the five targeted *bla* group genes (Table 3). Only one of the five *E. cloacae* harbored a *bla* group gene (bla_{TEM}), whereas the two *S. liquefaciens* strains possessed none of the *bla* group genes.

Confirmation of the M-PCR system by direct DNA sequencing of the PCR products

All the results of the M-PCR system coincided with the DNA sequencing results and their distribution in the clinical strains is summarized in Table 4. Briefly, of the 203 studied clinical strains, the CTX-M subtypes were detected in 40.4%, whereas TEM, SHV and OXA-1 subtypes were seen in 47.9%, 38.4% and 6.4%, respectively. Among the *bla* gene-positive clinical *K. pneumoniae* ($n=123$) and *E. coli* strains ($n=44$), CTX-M-9 group subtypes (CTX-M-14, CTX-M-9, CTX-104, CTX-M-65 and CTX-M-27) and CTX-M-1 group subtypes (CTX-M-3, CTX-M-15, CTXM-55 and CTX-M-64) occurrences were 25.2% and 40.9%, and 17.7% and 25% respectively. Four of the five *bla* gene-positive clinical *K. pneumoniae* and *E. coli* strains carrying four genes harbored TEM-1, SHV-1, CTX-M-3 and CTX-M-14, whereas the remaining one strain had TEM-1, OXA-1, CTX-M-55 and CTX-M-14. Among the 18 strains co-harboring three subtypes, six strains had CTX-M-14, five each had CTX-M-15 and CTX-M-3, five had CTX-M-55, whereas one had CTX-M-64. Forty-nine percent ($n=24$) of the 49 strains with two subtypes had CTX-M14. Five SHV subtypes: SHV-1 ($n=53$), SHV-11 ($n=11$), SHV-27 ($n=2$), SHV-33 ($n=2$) and SHV-144 ($n=1$) were detected in clinical *K. pneumoniae*, whereas only one subtype (SHV-1, $n=6$) was seen in clinical *E. coli* strains. All the strains with OXA-1like subtypes (9 *K. pneumoniae* and 4 *E. coli*) harbored the OXA-1 enzyme.

Antibiotic resistance rates of the clinical *Enterobacteriaceae* strains

The clinical *K. pneumoniae* strains harboring bla_{SHV}, bla_{TEM} or $bla_{SHV/OXA-1}$ genotype had lower resistance rates against CTX, CAZ, CRO and ATM (4.1%, 8.2%, 6.1% and 4.1%; 19.1%, 14.3%, 14.3% and 14.3%; or 50%, 0%, 0% and 0%, respectively) compared with those with $bla_{CTX-M-1}$ or $bla_{CTX-M-9}$ genotype alone: 100%, 100%, 100% and 100% or 83.3%, 33.3%, 83.3% and 33.3%, respectively. The resistance rates of *E. coli* strains harboring bla_{TEM} or $bla_{TEM/OXA-1}$ against CTX, CAZ, CRO and ATM were 4%, 6.7%, 6.7% and 0% or 0%, 0%, 0% and 0%. No *E. coli* strain harbored either bla_{SHV} alone or $bla_{SHV/TEM}$.

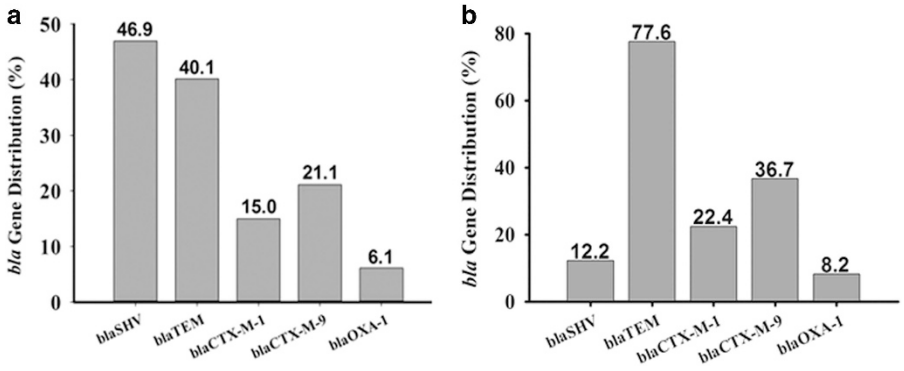

Figure 2 Distribution of the five *bla* group genes in the 147 *K. pneumoniae* (**a**) and 49 *E. coli* (**b**) clinical strains.

Table 3 Distribution of *bla* genotypes among the clinical *K. pneumoniae* and *E. coli* clinical strains and their respective resistance rates to CTX, CAZ, CRO and ATM

	K. pneumoniae						E. coli					
			% Resistance to						% Resistance to			
Genotype	Number of isolates (n = 147)	Percentage	CTX	CAZ	CRO	ATM	Number of isolates (n = 49)	Percentage	CTX	CAZ	CRO	ATM
Bla_{SHV}	49	33.3	4.1	6.1	6.1	4.1	0	0.0	0.0	0.0	0.0	0.0
Bla_{TEM}	**21**	**14.3**	**23.8**	**19.0**	**14.3**	**14.3**	**15**	**30.6**	**40**	**6.7**	**6.7**	**0.0**
$Bla_{CTX\text{-}M\text{-}1}$	**1**	**0.7**	**100**	**100**	**100**	**100**	**1**	**2.0**	**100**	**100**	**100**	**100**
$Bla_{CTX\text{-}M\text{-}9}$	**6**	**4.1**	**83.3**	**33.3**	**83.3**	**33.3**	**2**	**4.1**	**50**	**100**	**50**	**50**
$Bla_{SHV/TEM}$	3	2.0	66.7	66.7	33.3	33.3	0	0.0	0.0	0.0	0.0	0.0
$Bla_{SHV/CTX\text{-}M\text{-}1}$	0	0.0	0.0	0.0	0.0	0.0	1	2.0	100	100	100	100
$Bla_{SHV/CTX\text{-}M\text{-}9}$	3	2.0	100	33.3	100	33.3	0	0.0	0.0	0.0	0.0	0.0
$Bla_{SHV/OXA\text{-}1}$	2	1.4	50	0.0	0.0	0.0	0	0.0	0.0	0.0	0.0	0.0
$Bla_{TEM/CTX\text{-}M\text{-}1}$	**6**	**4.1**	**100**	**100**	**100**	**100**	**3**	**6.1**	**66.7**	**33.3**	**66.7**	**33.3**
$Bla_{TEM/CTX\text{-}M\text{-}9}$	**12**	**8.2**	**100**	**8.3**	**100**	**41.7**	**14**	**28.6**	**78.6**	**21.4**	**78.6**	**50**
$Bla_{TEM/OXA\text{-}1}$	**1**	**0.7**	**100**	**100**	**0.0**	**0.0**	**1**	**2.0**	**0.0**	**0.0**	**0.0**	**0.0**
$Bla_{CTX\text{-}M\text{-}1/OXA\text{-}1}$	2	1.4	100	100	100	100	0	0.0	0.0	0.0	0.0	0.0
$Bla_{CTX\text{-}M\text{-}1/CTX\text{-}M\text{-}9}$	1	0.7	100	100	100	100	0	0.0	0.0	0.0	0.0	0.0
$Bla_{SHV/TEM/CTX\text{-}M\text{-}1}$	**4**	**2.7**	**100**	**50**	**100**	**50**	**3**	**6.1**	**66.7**	**100**	**66.7**	**100**
$Bla_{SHV/TEM/CTX\text{-}M\text{-}9}$	3	2.0	100	66.7	100	66.7	0	0	0.0	0.0	0.0	0.0
$Bla_{SHV/TEM/OXA\text{-}1}$	1	0.7	100	100	100	100	**0**	**0**	**0.0**	**0.0**	**0.0**	**0.0**
$Bla_{SHV/CTX\text{-}M\text{-}1/OXA\text{-}1}$	0	0.0	0.0	0.0	0.0	0.0	2	4.1	50	50	100	100
$Bla_{TEM/CTX\text{-}M\text{-}1/OXA\text{-}1}$	2	1.4	100	0.0	100	50	0	0	0.0	0.0	0.0	0.0
$Bla_{TEM/CTX\text{-}M\text{-}9/OXA\text{-}1}$	0	0	0.0	0.0	0.0	0.0	1	2.0	100	100	100	100
$Bla_{TEM/CTX\text{-}M\text{-}1/CTX\text{-}M\text{-}9}$	**1**	**0.7**	**100**	**100**	**100**	**100**	**1**	**2.0**	**100**	**100**	**100**	**100**
$Bla_{SHV/TEM/CTX\text{-}M\text{-}1/CTX\text{-}M\text{-}9}$	4	2.7	100	75	100	100	0	0	0.0	0.0	0.0	0.0
$Bla_{TEM/CTX\text{-}M\text{-}1/CTX\text{-}M\text{-}9/OXA\text{-}1}$	1	0.7	100	100	100	100	0	0	0.0	0.0	0.0	0.0
Non-$bla_{SHV/TEM/CTX\text{-}M/OXA\text{-}1}$	24	16.3	20.8	12.5	20.8	25	5	12.2	20	0.0	0.0	0.0
Total	147	100.0	—	—	—	—	49	100	—	—	—	—

The *bla* genotypes found in both *K. pneumoniae* and *E. coli* clinical strains are indicated in bold.

One and two clinical *E. coli* strains harbored $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$ genotypes exhibiting uniform resistance rates of 100% and 50%, respectively, to CTX, CAZ, CRO and ATM. The only one *E. cloacae* with bla_{TEM} genotype was sensitive to all the CTX, CAZ and ATM but not CRO, whereas both the *S. liquefaciens* strains were sensitive to the four antibiotics. Clinical strains with bla_{SHV} and bla_{TEM} genotypes alone exhibited relatively low resistance to the four drugs, whereas in combination with $bla_{CTX\text{-}M}$ group genes, their resistance rates against these antibiotics rose markedly. Contrastingly, as shown in Table 3, strains with $bla_{CTX\text{-}M\text{-}1}$ genotypes showed 100% or near-100% resistance rates to the third-generation cephalosporins, regardless of the pattern they took while strains with $bla_{CTX\text{-}M\text{-}9}$ alone exhibited relatively low resistance rate to CAZ, but in combination with $bla_{CTX\text{-}M\text{-}1}$, their resistance to CAZ rose markedly.

Resistance to three or more β-lactam agents was associated with specific gene type and number

As shown in Figure 3, there was a positive correlation between the number of *bla* group genes harbored by the clinical strains and the number of antibiotics to which they exhibited resistance (by

Table 4 The distribution of β-lactamase subtypes among the clinical *K. pneumoniae* and *E. coli* strains

		Distribution in	
Group (number positive)	*Subtype*	K. pneumoniae	E. coli
SHV ($n=78$)	SHV-1	53	6
	SHV-11	11	0
	SHV-27	2	0
	SHV-33	2	0
	SHV-144	1	0
TEM ($n=97$)	TEM-1	58	38
	TEM-135	1	0
CTX-M-1 ($n=33$)	CTX-M-3	12	1
	CTX-M-15	7	2
	CTX-M-55	3	7
	CTX-M-64	0	1
CTX-M-9 ($n=49$)	CTX-M-9	3	0
	CTX-M-14	27	12
	CTX-M-27	0	1
	CTX-M-65	0	5
	CTX-M-104	1	0
OXA-1-like ($n=13$)	OXA-1	9	4

M-PCR products from 123 *K. pneumoniae* and 44 *E. coli* clinical strains were subjected to direct DNA sequencing.

Spearman's rank correlation; $r_s=0.525$, $P<0.05$). Resistance to three or more agents was associated with presence of two *bla* group genes. Specifically, analysis of our data revealed that carriage of bla_{TEM} or $bla_{CTX\text{-}M\text{-}1}$ group genes correlated positively with resistance to three or more antibiotics ($P=0.001$; Table 5). Many strains harboring bla_{TEM} group genes also coharbored $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$ group genes (see Table 3). This positive association between bla_{TEM} and/or $bla_{CTX\text{-}M\text{-}1}$ group gene(s) and the resistance to three or more antibiotics was further confirmed by multiple logistic regression analysis (see Table 6).

DISCUSSION

The dissemination of β-lactamase enzymes, especially ESBLs, among the members of *Enterobacteriaceae* family and other Gram-negative bacteria has been on the rise in the past two to three decades.[28] It creates a public health problem that requires clinical microbiology laboratories to carry out β-lactamase identification and typing. In most clinical settings, especially in the developing countries, the screening and detection of β-lactamases in bacteria has, for the most part, been based on the agar disk tests (such as the disk diffusion tests) and isoelectric focusing. These methods are tedious, time consuming and replete with a myriad of challenges.[17,29] Clinical and reference microbiology laboratories, therefore, must use alternative and/or additional techniques that present rapid, reliable and reproducible test outcomes. Consequently, it is even more beneficial to detect accurately the presence or potential expression of these enzymes in bacteria on large scale. A number of molecular methods including oligotyping, PCR-single-strand conformation polymorphism, PCR-restriction fragment length polymorphism and ligase chain reaction have been used in the identification of ESBL genes.[29] However, most

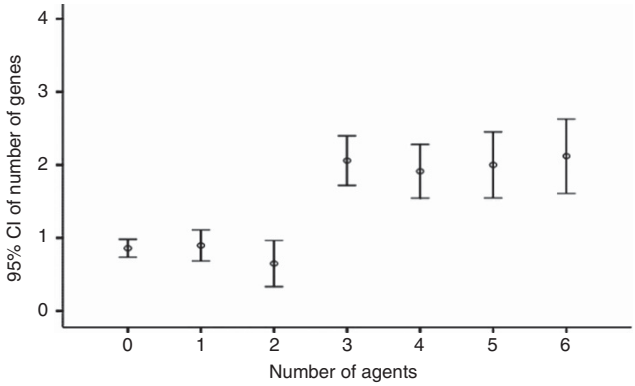

Figure 3 Correlation between the number of *bla* group genes and the number of antibiotics to which the strains are resistant. The X axis represents the resistance phenotype (number of agents), whereas the Y axis represents mean and 95% CI of the number of *bla* genes in each group (by Spearman's rank correlation analysis; $r_s=0.525$; $P<0.05$).

of these techniques have the disadvantage of detecting only a single gene at a time. In contrast, M-PCR has been used to amplify multiple genes simultaneously in a single test.[29] In this way, it has become a more efficient and applicable option to concurrently detect multiple *bla* subtypes in single reactions. In 2005, Woodford *et al.*[18] developed an M-PCR assay that could detect each of the five phylogenetic groups of $bla_{CTX\text{-}M}$ genotype. However, these assays would fail to encompass the detection of the bla_{TEM} and bla_{SHV} genotypes. In 2007, another M-PCR system was described that could detect the bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ genotypes using universal PCR primers for the $bla_{CTX\text{-}M}$ group.[19] That system requires extra singleplex assays to identify the five groups of the $bla_{CTX\text{-}M}$ genotype. Three multiplex assays were later described by Woodford in 2010[30] for the detection of the five CTX-M groups, metallo-β-lactamases and OXA-type carbapenemases. A clearly more comprehensive work to achieve this was carried out by Dallenne *et al.*,[17] in 2010. However, their work relied on two separate assays to screen for the presence bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ and $bla_{OXA\text{-}1}$ genes.

Unlike these and most of the currently available assays, our M-PCR system provides the advantage of detecting the targeted five *bla* group genes (bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$ and $bla_{OXA\text{-}1}$) simultaneously in the clinical strains with high specificity and sensitivity (see Table 2). Thus, it could be a useful tool in China and other countries where $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$ gene clusters, among others, have been reported to be on the rise.[9,31–33] For instance, these enzymes, with varying prevalence rates, have also been reported in many countries in the Asia-Pacific region.[34] Although CTX-M group enzymes emerged later compared with TEM and SHV types, they have spread worldwide and specific subgroups have been localized in different geographic regions.[35,36] Among them, CTX-M-9 group β-lactamases (CTX-M-14 subtype) are the most widespread in China followed by CTX- M-1 group (CTX-M-3).[36–38] The occurrence of these enzymes vary from country to country, with Japan having the lowest *bla* gene prevalence rates among the neighboring countries.[34,36–38] Owing to their very low prevalence rates in China, the scope of application and use this M-PCR system does not include $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8}$ and/or $bla_{CTX\text{-}M\text{-}25}$ group genes.

Our M-PCR data show that among the *K. pneumoniae* clinical strains, bla_{SHV} (46.9%) and bla_{TEM} (40.1%) group genes were the most prevalent in this region followed by group genes $bla_{CTX\text{-}M\text{-}9}$ (21.1%), $bla_{CTX\text{-}M\text{-}1}$ (15.0%) and $bla_{OXA\text{-}1}$ (6.1%). In *E. coli*, bla_{TEM} group (77.6%) was the most prevalent followed by $bla_{CTX\text{-}M\text{-}9}$

Table 5 Relationship between *bla* genes and resistance to three or more antibiotics in the 203 clinical strains (by χ^2 test)

	Resistance rate % (positive/total)														
	bla_{TEM}			bla_{SHV}			bla_{OXA-1}			$bla_{CTX-M-1}$			$bla_{CTX-M-9}$		
Antibiotics	+	–	P-*value*	+	–	P-*value*	+	–	P-*value*	+	–	P-*value*	+	–	P-*value*
Resistance to >3 agents	22.4 (22/98)	6.7 (7/105)	**0.001**	17.3 (13/75)	12.5 (16/128)	0.342	23.1 (3/13)	13.7 (26/190)	0.598	36.4 (12/33)	10.0 (17/170)	<**0.01**	22.4 (11/49)	11.7 (18/154)	0.061

Genotypes found in both K. pneumoniae and *E. coli* strains are indicated in bold.

Table 6 Relationship between bla genes and resistance to three or more antibiotics in the 203 clinical strains (by multiple logistic regression analysis)

	Variables in the logistic regression equation														
	bla_{TEM}			bla_{SHV}			bla_{OXA-1}			$bla_{CTX-M-1}$			$bla_{CTX-M-9}$		
Antibiotics	x^2	*P-value*	*OR (95% CI)*	x^2	*P-value*	*OR (95% CI)*	x^2	*P-value*	*OR (95% CI)*	x^2	*P-value*	*OR (95% CI)*	x^2	*P-value*	*OR (95% CI)*
Resistance to >3 agents	5.681	**0.017**	3.117 (1.224, 7.939)	—	—	—	—	—	—	8.646	**0.003**	3.866 (1.570, 9.520)	—	—	—

Abbreviations: CI, confidence interval; OR, odds ratio.
Genotypes found in both K. pneumoniae and E. coli strains are indicated in bold.

(36.7%), $bla_{CTX\text{-}M\text{-}1}$ (22.4%), bla_{SHV} (12.2%) and $bla_{OXA\text{-}1}$ (8.2%) group genes. Although the prevalence of bla_{SHV} (46.9%) in *K. pneumoniae* in this region appear to be unusually lower compared with previously reported levels such as in Korea (97%) and Brazil (55.8%),[39] they particularly coincide with findings of a previous study elsewhere in China (45.8%).[39] Strains with SHV and TEM genotypes alone exhibited relatively low resistance to the third-generation cephalosporins and monobactam, whereas in combination with $bla_{CTX\text{-}M}$ group genes, their resistance rates against these antibiotics rose markedly. Contrastingly, strains with $bla_{CTX\text{-}M\text{-}1}$ showed high resistance rates to these β-lactams. Strains with $bla_{CTX\text{-}M\text{-}9}$ group gene alone exhibited relatively low resistance rate to CAZ, but in combination with $bla_{CTX\text{-}M\text{-}1}$, resistance to CAZ rose markedly. There could be a possibility that $bla_{CTX\text{-}M\text{-}1}$ group genes in this region may have mediated resistance to both CTX and CAZ strains. On the other hand, the lower resistance rates to third-generation cephalosporins by clinical strains harboring bla_{SHV} or bla_{TEM} alone indicate that most of the SHV or TEM enzymes did not confer extended-spectrum β-lactamase activity. However, there exists some reports that tend to suggest that TEM-1 may also contribute to the ESBL trait in concert with other β-lactamases.[7,40]

Upon DNA sequencing of PCR products from all strains with *bla* group genes, the distribution of the β-lactamase subtypes corresponded with the M-PCR results. CTX-M-14 and CTX-M-3 were the most prevalent among the ESBL-producing clinical *K. pneumoniae* and *E. coli* strains. Besides CTX-M-14, other CTX-M-9 group subtypes included CTX-M-9 and CTX-M-104 in clinical *K. pneumoniae* and CTX-M- 65, and CTX-M-27 in *E. coli*. In the CTX-M-1 group, subtypes other than CTX-M-3 included CTX-M-15, CTX-M-55 in *K. pneumoniae*, and CTX-M-55, CTX-M-15 and CTX-64 in *E. coli*. These results are in agreement with previous reports that have projected CTX-14 to be the most common in different parts of China followed by CTX-M-3.[36–38,41] Our data also indicate that the CTX-M-15 enzyme, which has been reported to have a worldwide distribution, especially in *E. coli* and with a great potential for epidemics,[42] is also available in this region. The four strains of *K. pneumoniae* possessing ESBL-SHV enzymes (SHV-27 and SHV-33) co-harbored CTX-M-14 with most of the rest carrying SHV-11 and SHV-1. The clinical *E. coli* strains did not harbor any ESBL-SHV enzymes. TEM-1 subtype was found in both *K. pneumoniae* (39.5%) and *E. coli* (77.6%) strains. Only one *K. pneumoniae* harbored a TEM-ESBL (TEM-135) subtype, which has also been reported in other parts of China.[43] As reported in other previous works, the OXA-1 subtypes were co-harbored along with other enzymes.[10] Statistical evaluation of our data revealed that bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ group genes were positively associated with resistance to more than three β-lactams. These results consolidate a notion that the M-PCR system could highlight the association of specific *bla* group genes and the resistance to multiple β-lactam agents. That multiresistant strains may harbor large and easily transferrable genetic elements comprising *bla* and other genes is not uncommon.[44] The co-existence of multiple resistance genes in a single strain raises the likelihood of their dissemination to new strains with the diversity of their resistance further complicating their molecular detection and subsequent treatment.

CONCLUSION

In conclusion, this M-PCR system effectively detected the five major *bla* group genes simultaneously in a single PCR reaction in *Enterobacteriaceae* clinical strains. Further confirmation of the M-PCR results by DNA sequencing of PCR products is a proof of its efficiency. By demonstrating high specificity and sensitivity in detecting the targeted group genes both with and without mixing template DNA from different strains, this system can be considered as a potential candidate for the development of M-PCR kits for the identification of these genes in clinical and epidemiology laboratory settings. Most of the *K. pneumoniae* (83.7%) and *E. coli* (89.8%) clinical strains in this study had >1 *bla* genes. Consequently, there is potential problem of resistance to multiple agents resulting from production of specific β-lactamases in this region. Hence, an efficient detection system would help enhance diagnosis, improve treatment decisions, guide antimicrobial stewardship and strengthen infection control programs in this region and elsewhere.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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