Int J Clin Exp Med 2019;12(2):1690-1696
www.ijcem.com /ISSN:1940-5901/IJCEM0079457

Original Article

Detection of integrons in *Escherichia coli* producing plasmid-mediated AmpC β-lactamases

Juanjuan Ding, Zhuochang Chen, Ying Li, Qianqian Zhang, Xiaoliang Li

Department of Respiratory and Critical Care Medicine, Henan Provincial People's Hospital, Zhengzhou, P. R. China

Received October 11, 2017; Accepted October 11, 2018; Epub February 15, 2019; Published February 28, 2019

Abstract: Objective: To investigate the prevalence of antibiotic resistance factors, including the production of plasmid-encoded AmpC (pAmpC) β-lactamases and the presence of integrons among pAmpC positive *E.coli* isolated from clinical specimens. Methods: A total of 252 clinically significant, non-repeat isolates were studied. AmpC disk test was used for phenotypic AmpC-β-lactamase detection. Molecular types of pAmpC were analyzed by using multiplex polymerase chain reaction (PCR) and DNA sequencing. *E.coli* isolates producing pAmpC were screened for the presence of class 1, 2, and 3 integrons by PCR and sequencing. Results: Although 54 isolates (21.4%) were phenotypically positive for AmpC, multiplex PCR detected CMY-2 type of AmpC gene in only 28 isolates (11.1%). In our hospital, the gene type coding pAmpC in the isolates of *Escherichia coli* is CMY-2. Among these 28 isolates, few isolates were resistant to cefepime (21.4%), and all 28 isolates in this study were susceptible to imipenem. Class 1 integrons were detected in 22 of 28 isolates by PCR using primers targeted to conserved regions of class 1, 2, and 3 integrase genes. Three different gene cassette arrays were detected in class 1 integrons, and drug-resistant genes were also present in these integron cassettes, including dfr17-aadA5, dfr17-cmlA, and dfr17-aadA5-aadA4-cmlA1. Integron presence was significantly associated with resistance to certain antibiotics. The resistance ratio to amikacin, cefoperazone/sulbactam, ceftazidime, and cefotaxime and piperacillin/tazobactam was detected in integron-positive *E.coli* strains but not in integron-negative strains ($P < 0.05$). Conclusion: The data suggest that fourth generation cephalosporins and carbapenem should be chosen in clinical empirical medication for plasmid mediated AmpC β-lactamases-producing *E.coli* isolates. The occurrence of CMY-2 pAmpC β-lactamase in *E.coli* is probably increasing rapidly in China. The significant association between class 1 integrons and resistance to amikacin, cefoperazone/sulbactam, ceftazidime, cefotaxime, and piperacillin/tazobactam suggests that class 1 integrons have an important role in resistance to these antibiotics among pAmpC-producing *E.coli*.

Keywords: *Escherichia Coli*, plasmid-mediated ampc β-lactamases (pampc), multiplex polymerase chain reaction (PCR), integrons

Introduction

Escherichia coli is an important etiologic agent for nosocomial- and community-acquired infections in humans [1]. Drug resistance is a serious threat to antimicrobial chemotherapy interventions. The major mechanism of resistance is the production of beta (β)-lactamases including AmpC β-lactamases (AmpC). The genes for AmpC β-lactamases production are chromosomal mediated, however, plasmid-mediated AmpC β-lactamases (pAmpC) have arisen through chromosomal gene transfer to plasmids-and can lead to dissemination of antimicrobial resistance to diverse bacterial populations including *Escherichia coli*, Klebsiella spp., Salmonella spp. and Proteus mirabilis [2]. Typically, pAmpC producing isolates are associated with resistance to multiple antibiotics making the selection of an effective antibiotic difficult [3]. Determination of pAmpC prevalence is important for surveillance and epidemiological studies and for infection control as these genes can spread to other organisms within the hospital setting [2, 4].

Integrons, which are capable of capturing, excising and expressing genes cassettes that encode determinants of antimicrobial-resistance, play important roles in the horizontal dissemination of antibiotic resistance genes in bacteria [5]. Meanwhile, integrons can be pres-

Table 1. Sequences of *ampC*-specific primers, and product size

Name of primer	Sequence (5'-3')	Size of product	Origin of reference
MOXMF	GCTGCTCAAGGAGCACAGGAT	520 bp	[10]
MOXMR	CACATTGACATAGGTGTGGTGC		
CITMF	TGGCCAGAACTGACAGGCAAA	462 bp	[10]
CITMR	TTTCTCCTGAACGTGGCTGGC		
DHAMF	AACTTTCACAGGTGTGCTGGGT	405bp	[10]
DHAMR	CCGTACGCATACTGGCTTTGC		
ACCMF	AACAGCCTCAGCAGCCGGTTA	346 bp	[10]
ACCMR	TTCGCCGCAATCATCCCTAGC		
EBCMF	TCGGTAAAGCCGATGTTGCGG	302 bp	[10]
EBCMR	CTTCCACTGCGGCTGCCAGTT		
FOXMF	AACATGGGGTATCAGGGAGATG	190 bp	[10]
FOXMR	CAAAGCGCGTAACCGGATTGG		

Table 2. Sequence of primers for integrase

Name of primer	Sequence (5'-3')	Size of product	Origin of reference
hep35	TGCGGGTYAARGATBTKGATT	491 bp	[11]
hep36	CAGCACATGCGTRTARTA		

Table 3. RFLP classification of integrase PCR products

PCR product	Enzyme	No. of fragment	Fragment size(s) (bp)
int I 1	Hinf I	1	491
int I 2	Hinf I	2	300,191
int I 3	Hinf I	2	119,372

ent on plasmids or as a part of a transposon and transfer along with them, facilitating the spread of antibiotic resistance genes among bacteria. Class 1 integrons have been examined most extensively and are the most common type of integron found in clinical isolates [6].

Many studies have investigated the presence of integrons in *E.coli* producing extended-spectrum beta-lactamases, and they reported a significant association between antimicrobial resistance and existence of integron [7]. However, there are few literature reports of a correlation between integrons and antibiotic resistance among *E.coli* isolates producing pAmpC. This study was designed to examine the drug resistance pattern and the frequency of the class 1 integrons and pAmpC among *E.coli* isolates in Henan Provincial People's Hospital, China.

Materials and methods

Clinical isolates

Non-duplicate *E.coli* isolates were obtained consecutively from patients during July 2015 and December 2015 from the microbiology laboratory of HeNan Provincial People's Hospital, which is a tertiary care teaching hospital with 4000 beds.

The isolates were identified using automated biochemical system Vitek 2 (bioMerieux, France).

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by agar dilution susceptibility testing method, and interpretation was done according to 2017 Clinical and Laboratory Standards Institute (CLSI) guidelines [8]. Agar dilution susceptibility testing was performed for imipenem, cefepime, ceftazidime, cefotaxime, ceftriaxone, piperacillin/tazobactam, cefoperazone/sulbactam, amikacin, ciprofloxacin and levofloxacin. Agar dilution susceptibility testing methods were performed in accordance with 2017 CLSI guidelines [8]. The Chi-square test was used to calculate the *P* value in terms of resistant and susceptible numbers of integron-positive and -negative isolates.

Phenotypic AmpC activity testing and molecular detection of pAmpC β-lactamase genes

Isolates with a cefoxitin inhibition zone < 18 mm were designated as positive for the AmpC β-lactamase screening test and were further tested by the AmpC disk test as described previously [9]. Isolates with a positive AmpC disk test were selected for multiplex PCR and sequencing analyses. Plasmid DNA was prepared by using Qiagen columns. Multiplex PCR was conducted to detect six families of plasmid-mediated AmpC β-lactamases (FOX, CIT, DHA, EBC, MOX, and ACC), as described in the literature [10]. The primers used for PCR amplification are listed in **Table 1**. The PCR products were purified using the EasyPure™ PCR Purification Kit (Beijing TransGen Biotech Co., Ltd., China). The PCR products were ana-

Table 4. Sequence of primer for Class 1 integron cassette regions

Name of primer	Sequence (5'-3')	Size of product	Origin of reference
hep58	TCATGGCTTGTTATGACTGT	Unknown	[11]
hep59	GTAGGGCTTATTATGCACGC		

Table 5. Agar dilution susceptibility test results against 28 *E.coli* strains producing pAmpC determined by multiplex PCR method

Antimicrobial agents	S	I	R
	No (%)	No (%)	No (%)
Imipenem	28 (100)	0 (0)	0 (0)
Cefepime	22 (78.6)	0 (0)	6 (21.4)
Ceftazidime	5 (17.9)	1 (3.6)	22 (78.6)
Cefotaxime	6 (21.4)	2 (7.1)	20 (71.4)
Ceftrixone	6 (21.4)	1 (3.6)	21 (75.0)
Piperacillin/tazobactam	1 (3.6)	4 (14.3)	23 (82.1)
Cefoperazone/sulbactam	6 (21.4)	0	18 (64.3)
Amikacin	17 (60.7)	0	11 (39.3)
Ciprofloxacin	7 (25)	1 (3.6)	20 (71.4)
Levofloxacin	8 (28.6)	2 (7.1)	18 (64.3)

R = resistant, I = intermediate, S = susceptible.

lyzed and sequenced. Gene sequencing was done via Invitrogen (Beijing, China). The PCR products were sequenced using the Applied Biosystems Automated 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA) and primers used in gene amplification.

Integron detection

Isolates producing pAmpC were tested for characterization of class 1, 2, and 3 integrons (intI) and their resistance-encoding gene cassettes. Plasmids were extracted as described previously. Integrons were detected using PCR with degenerate primers designed to hybridize to conserved regions of integron encoded integrase gene intI1, intI2 and intI3. Primers used are listed in **Table 2**. According to the literature [11], the class of the integron was determined by analyzing integrase PCR products by restriction fragment length polymorphism (RFLP) following digestion using HinfI restriction enzyme (**Table 3**). Restriction analysis revealed that only class 1 integron was detected. Primers described by White et al. were used to amplify the variable region of class 1 integron [12]. Primers used to amplify the class 1 integron cassette region are listed in **Table 4**. PCR amplification parameters have been reported previously [12]. Selected amplicons representing various size classes of integrons were sequenced using the Applied Biosystems Automated 3730XL DNA sequencer. The resulting DNA sequences were analysed by the BLAST program.

Statistical analysis

Data were analyzed using the Chi-squared test (χ^2 test) to determine the significant differences in resistance. Differences were considered significant at $P < 0.05$.

Results

Clinical characteristics of isolates

A total of 252 *E.coli* isolates were recovered from clinical samples in clinical microbiology department during July 2015 and December 2015. Strains were mainly recovered from respiratory secretions (50.8%, n=128), followed by urine (23.8%, n=60), wounds and other clinical samples, and 20.2% belonged to outpatients and 79.8% to hospitalized patients. The cohort included men (56.6%), and the mean age was 55 years.

Susceptibility to antimicrobial agents

Among the 252 clinically isolated *E.coli* strains, 161 (63.9%) isolates were cefoxitin resistant. The proportion of isolates resistant to tested antimicrobial agents included cefepime (21.4%), ceftazidime (78.6%), cefotaxime (71.4%), ceftriaxone (75%), piperacillin/tazobactam (82.1%), cefoperazone/sulbactam (64.3%), amikacin (39.3%), ciprofloxacin (71.4%) and levofloxacin (64.3%). All of isolates in this study were susceptible to imipenem. The results are shown in **Table 5**.

Prevalence and genetic characterization of pAmpC-producing E.coli

Among the 252 clinically isolated *E.coli* strains, 161 (63.9%) isolates were cefoxitin resistant. Of these cefoxitin-resistant isolates, 54 (21.4%) isolates were found to be AmpC producers by AmpC disk test. Among these 54 isolates, 28 (11.1%) isolates were detected producing pAmpC by the multiplex PCR (**Figures 1** and **2**). Sequence analyses of PCR products from

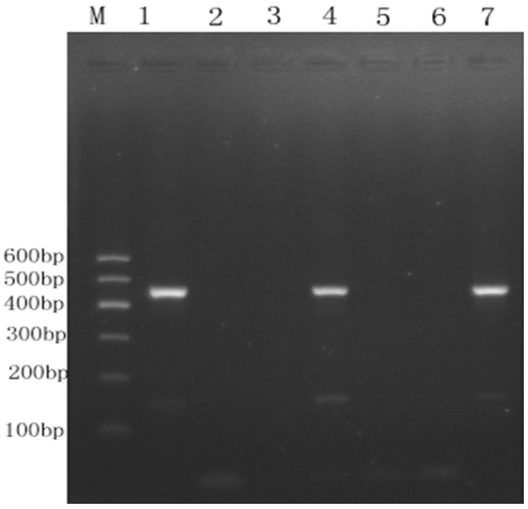

Figure 1. pAmpC Mutiplex PCR amplification of the isolates. M: Marker I; 1, 4, 7 lane: positive results of CMY-2 gene.

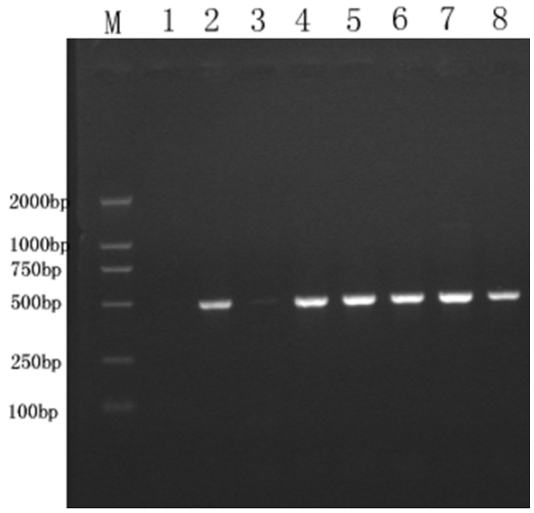

Figure 3. PCR amplification of *intI1*, *intI2*, *intI3* M: DL2000, 1, 2, 3, 4, 5, 6, 7, 8 lane: positive results of *intI1* gene.

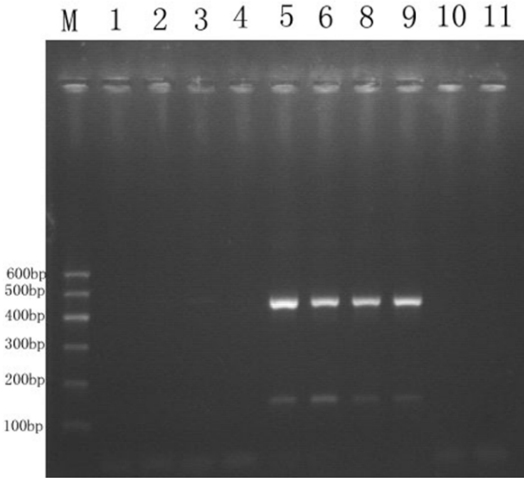

Figure 2. pAmpC Mutiplex PCR amplification of the isolates. M: Marker I. 5, 6, 8, 9 lane: positive results of CMY-2 gene.

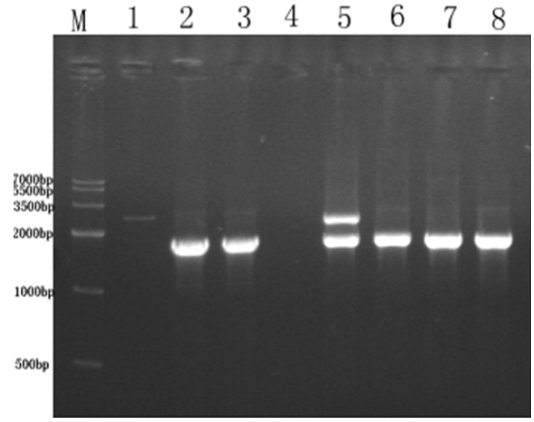

Figure 4. PCR amplification of Class 1 integron cassette region M: Marker IV.

amplification of plasmid AmpC genes showed that the CMY genes were homologues to CMY-2 gene, and CMY-2 was the unique genotype.

Correlation between integron and drug resistance in pAmpC-producing E.coli

Among 28 isolates showing positive pAmpC by multiplex PCR methods, 22 (78.6%) isolates were positive for the presence of *intI1*, while no *intI2* and *intI3* were detected. Among 22 isolates carrying *intI1* (**Figure 3**), 4 isolates harbored integrons without cassettes. *dfr17-aadA5* gene cassette were found in 11 isolates. Six isolates carried *dfr17-cmlA*, and 1 isolates harbored *dfr17-aadA5-aadA4-cmlA1* (**Figure 4**). The resistance ratio to ceftazidime (P=0.023), cefotaxime (P=0.0196), piperacillin/tazobactam (P=0.0203), cefoperazone/sulbactam (P=0.006), amikacin (P=0.0262), was found to be significantly higher in class 1 integron positive *E.coli* strains (**Table 6**).

Discussion

Production of β-lactamases are the main mechanism of resistance to β-lactam antibiotics in bacteria. These enzymes hydrolyze the β-lactam ring, which leads to the inactivation of β-lactam antibiotics [7]. AmpC β-lactamases can hydrolyze penicillins, oxyimino-, 7-α-me-

Table 6. Drug resistance in class 1 integron-positive and integron-negative *E.coli* strains producing pAmpC

Antimicrobial agents	Integron-positve isolate (%R)	Integron-negative isolate (%R)	*P* value
Imipenem	0	0	
Cefepime	5 (22.7)	1 (16.7)	0.7484
Ceftazidime	20 (90.9)	2 (33.3)	0.0023
Cefotaxime	18 (81.8)	2 (33.3)	0.0196
Ceftrixone	17 (77.3)	4 (66.7)	0.5949
Piperacillin/tazobactam	20 (90.9)	3 (50.0)	0.0203
Cefoperazone/sulbactam	17 (77.3)	1 (16.7)	0.006
Amikacin	11 (50.0)	0 (0)	0.0262
Ciprofloxacin	17 (77.3)	3 (50.0)	0.1899
Levofloxacin	15 (68.2)	3 (50.0)	0.4100

thoxy cephalosporins and monobactams. Susceptibility to cefepime or cefpirome is little affected and is unchanged for carbapenems [3]. Plasmid-mediated class C β-lactamases have been discovered frequently in *E.coli* strains worldwide. However, detection of the AmpC enzyme in *E.coli* in the tertiary hospital in the local region was important for clinical management and epidemiological surveillance.

In this study, *E.coli* strains producing pAmpC were resistant to most β-lactam antibiotics, especially for third-generation cephalosporins, but except for carbapenems and fourth-generation cephalosporins (cefepime). Previous studies suggested that cefepime might be effective for the treatment of infections caused by an AmpC-producing organism, since cefepime is a poor inducer of AmpC β-lactamase, rapidly penetrates through the outer cell membrane, and is less hydrolyzed by the enzyme [13].

The USA Committee for Clinical and Laboratory Standards Institute has not recommended use of a standard method for the detection of AmpC [14]. The phenotypic tests have certain drawbacks and cannot differentiate the various families of plasmid-mediated AmpC enzymes and therefore, multiplex PCR has been developed. In the present study, 252 strains of *E.coli* were screened using AmpC disk test, and 54 strains were positive for producing AmpC enzyme. Positive *E.coli* in the primary screening were detected the genotypes using multiplex PCR technique. Twenty-eight (11.1%) strains of *E.coli* amplification were positive for the test. In recent years, the incidence of pAmpC positive *E.coli* has been reported worldwide [15]. CMY-2 is the most common pAmpC in *E.coli* from different geographical areas including Asia, North America, and Europe [16]. In this study, CMY-2 was the unique genotype, indicating our results match these data. The prevalence of pAmpC among clinical isolates differs depending on the countries and institutions. In this study, the detection rate of pAmpC in *E.coli* isolates (11.1%) is lower slightly than that in India (12.3%) [15] and the rate from Xuzhou (12.5%) in China [17], but higher than the prevalence reported from Shanghai in China (1.91%) in a previous research [18]. Therefore, the occurrence of CMY-2 in *E.coli* is probably increasing rapidly in China.

In the present study, Class 1 integrons were the exclusive type integrons among pAmpC-producing *E.coli*, while we couldn't find class 2 and class 3 integrons. Consistent with previous reports, class 1 integrons were the most prevalent compared to the other tested integrons in this study. In accordance with our study, Chang et al. from Taiwan and Li et al. from China detected class 1 integrons in 64% and 66.5% of *E.Coli* isolated from different clinical specimens and blood stream infections, respectively [19, 20]. Three different gene cassette arrays were detected in class 1 integrons, and drug-resistant genes were also present in these integron cassettes, including *dfr17-aadA5*, *dfr17-cmlA*, and *dfr17-aadA5-aadA4-cmlA1*. These gene cassettes have been reported as encoding resistance to aminoglycoside, chloramphenicol and sulfanilamide, respectively [12]. In addition, all resistance gene cassettes in our study were described most commonly elsewhere [21, 22]. In our study, significant differences in the resistance rates between integron-positive and integron-negative groups were observed for certain antibiotics including amikacin, cefoperazone/sulbactam, ceftazidime, cefotaxime and piperacillin/tazobactam, indicating that class 1 integrons have an important role in resistance to these antibiotics among pAmpC-producing *E.coli*.

Conclusion

In antibiotic therapy policy, we recommended carbapenems and cefepime should be used for the treatment of pAmpC producers. The occurrence of CMY-2 pAmpC β-lactamase in *E.coli* is probably increasing rapidly in China. Prevalence of class 1 integron in *E.coli* isolates is a similar trend observed in other published studies, however, co-prevalence with pAmpC and certain drug resistance is a striking feature of our study. The significant association between class 1 integrons and resistance to amikacin, cefoperazone/sulbactam, ceftazidime, cefotaxime, and piperacillin/tazobactam suggests that class 1 integrons have an important role in resistance to these antibiotics among pAmpC-producing *E.coli*. This is a therapeutic concern and requires further investigation taking into account the associated risk factors and study of gene cassettes.

Disclosure of conflict of interest

None.

Address correspondence to: Juanjuan Ding, Department of Respiratory and Critical Care Medicine, Henan Provincial People's Hospital, Room 1103, 11th Floor, 7th Building, No. 5 Weiwu Road, Zhengzhou, Henan Province, P. R. China. Tel: +86-371-65580038; Fax: +86-371-65580098; E-mail: dingjuanhai@163.com

References

[1] Briñas L, Zarazaga M, Sáenz Y, Ruiz-Larrea F and Torres C. β-lactamases in ampicillin resistant Escherichia coli isolates from foods, humans, and healthy animals. Antimicrob Agents Chemother 2002; 46: 156-63.

[2] Shafiq M, Rahman H, Qasim M, Ayub N, Hussain S, Khan J and Naeem M. Prevalence of plasmid-mediated AmpC β-lactamases in Escherichia coli and Klebsiella pneumonia at tertiary care hospital of Islamabad, Pakistan. Eur J Microbiol Immunol (Bp) 2013; 3: 267-71.

[3] Seral C, Gude MJ, Castillo FJ. Emergence of plasmid mediated AmpC β-lactamasas: origin, importance, detection and therapeutical options. Rev Esp Quimioter 2012; 25: 89-99.

[4] Pitout JD. Multiresistant Enterobacteriaceae: new threat of an old problem. Expert Rev Anti Infect Ther 2008; 6: 657-69

[5] Cambray G, Guerout AM, Mazel D. Integrons. Annu Rev Genet 2010; 44: 141-66.

[6] Chen T, Feng Y, Yuan JL, Qi Y, Cao YX and Wu Y. Class 1 integrons contributes to antibiotic resistance among clinical isolates of Escherichia coli producing extended-spectrum beta-lactamases. Indian J Med Microbiol 2013; 31: 385-9.

[7] Khoramrooz SS, Sharifi A, Yazdanpanah M, Malek Hosseini SA, Emaneini M, Gharibpour F, Parhizqari N, Mirzaii M, Zoladi M and Khosravani SA. High frequency of class 1 integrons in escherichia coli isolated from patients with urinary tract infections in Yasuj, Iran. Iran Red Crescent Med J 2016; 18: e26399.

[8] Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 2017.

[9] Black JA, Moland ES, Thpmpson KS. AmpC disk test for detection of plasmid-mediated ampC beta-lactamases in enterobacteriaceae lacking chromosomal ampC beta-lactamases. J Clin Microbiol 2005; 43: 3110-3.

[10] Perez-Perez FJ, Hanson ND. Detection of plasmid-mediated ampC beta-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol 2002; 40: 2153-62.

[11] White PA, McIver CJ, Rawlinson WD. Integrons and gene cassettes in the enterobacteriaceae. Antimicrob Agents Chemother 2001; 45: 2658-61.

[12] White PA, McIver CJ, Deng Y and Rawlinson WD. Characterisation of two new gene cassettes, aadA5 and dfrA17. FEMS Microbiol Lett 2000; 182: 265-9.

[13] Jacoby GA. AmpC beta-lactamases. Clin Microbiol Rev 2009; 22: 161-82.

[14] Clinical and Laboratory Standards Institute: performance standards for antimicrobial susceptibility testing. Sixteenth informational supplement. 2006: M100-S116.

[15] Chakrabory A, Adhikari P, Shenoy S and Saralaya V. Characterization of plasmid mediated AmpC producing Escherichia coli clinical isolates from a tertiary care hospital in South India. Indian J Pathol Microbiol 2014; 57: 255-8.

[16] Guo YF, Zhang WH, Ren SQ, Yang L, Lü DH, Zeng ZL, Liu YH and Jiang HX. IncA/C plasmid-mediated spread of CMY-2 in multidrug-resistant escherichia coli from food animals in China. PLoS One 2014; 9: e96738.

[17] Liu X, Liu Y. Detection of plasmid-mediated AmpC β-lactamase in Escherichia coli. Biomed Rep 2016; 4: 687-90.

[18] Li Y, Li Q, Du Y, Jiang X, Tang J, Wang J, Li G and Jiang Y. Prevalence of plasmid-mediated AmpC β-Lactamases in a Chinese university hospital from 2003 to 2005: first report of CMY-2-Type AmpC β-Lactamase resistance in China. J Clin Microbiol 2008; 46: 1317-21.

[19] Chang CY, Chang LL, Chang YH, Lee TM and Chang SF. Characterisation of drug resistance gene cassettes associated with class 1 inte-

grons in clinical isolates of Escherichia coli from Taiwan, ROC. J Med Microbiol 2000; 49: 1097-102.
[20] Li LM, Wang MY, Yuan XY, Wang HJ, Li Q and Zhu YM. Characterization of integrons among Escherichia coli in a region at high incidence of ESBL-EC. Pak J Med Sci 2014; 30: 177-80.
[21] Su J, Shi L, Yang L, Xiao Z, Li X and Yamasaki S. Analysis of integrons in clinical isolates of Escherichia coli in China during the last six years. FEMS Microbiol Lett 2006; 254: 75-80.
[22] Li J, Zou M, Dou Q, Hu Y, Wang H, Yan Q and Liu WE. Occurrence and characteristics of class I and II integrons in clinical bacterial isolates from patients in south China. J Health Sci 2010; 56: 442-50.