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Research article

Detection of AmpC β-lactamases in gram-negative bacteria

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ABSTRACT

AmpC β-lactamase genes are clinically important because they often confer resistance to most β-lactams other than 4th-generation cephalosporins and carbapenems. However, traditional and existing detection methods are expensive, labor-intensive and range-limited. We established an efficient multiplex PCR method to simultaneously identify six families of *ampC* β-lactamase genes, ACC, EBC, CIT, DHA, MOX and FOX, and evaluated the sensitivity and specificity of this assay. The multiplex method could accurately identify ACC, EBC, CIT, DHA, MOX and FOX variants among a total of 175 *ampC* β-lactamase genes. The minimum concentration of genomic DNA that could be detected was 1.0×10^3 copies/μL. We subsequently used this method to analyze 2 *Salmonella* spp. with carrying CMY-2 and DHA-1, and 167 *Enterobacteriaceae* isolates in blinded PCR testing. Positive isolates produced bright bands that corresponded with their genotype. Results were in concordance with those of the traditional method but showed increased sensitivity and accuracy. This indicates that the newly developed multiplex PCR system could be used as a diagnostic tool to accurately distinguish the six families of *ampC* β-lactamase genes with high efficiency, wide range, easy operation and good discrimination.

1. Introduction

Bacteria that produce *ampC* β-lactamase genes are clinically important because they are often resistant to most β-lactams other than 4th-generation cephalosporins and carbapenems (Girlich et al., 2000; Meini et al., 2019). *AmpC* β-lactamase genes exist in most Gram-negative bacteria and represent the main mechanism of resistance of Gram-negative bacteria to broad-spectrum β-lactam antibiotics (Balıkçı et al., 2014; D'Angelo et al., 2016). In recent years, under selection pressure caused by continuous use of cephalosporins and enzyme inhibitors, variation in *ampC* β-lactamase genes has appeared (Philippon et al., 2002; Madec et al., 2017). The increasing variety of *ampC* β-lactamase genes and the expanded spectrum of drug resistance have brought severe challenges to monitoring because of a lack of effective detection methods.

The *ampC* β-lactamase genes were first named after the discovery of ampicillin-resistant *Escherichia coli* (*E. coli*) and can be harbored either chromosomally or on plasmids (Päivärinta et al., 2020). Since Bauernfeind first reported CMY-1 in 1989, many plasmid-mediated *ampC* β-lactamase genes have been reported all over the world (Bauernfeind et al., 1989), with more than 200 types discovered so far. In 1990, Papanicolaou found that resistance of some *Klebsiella pneumoniae* (*K. pneumoniae*) to cefoxitin and cefotetan could be transferred to *E. coli* through plasmid conjugation, and the sequence of the gene responsible (MIR-1) was 90% similar to that of the chromosomal *ampC* β-lactamase gene, which first confirmed the existence of plasmid-mediated *ampC* β-lactamase genes at the molecular biological level (Papanicolaou et al., 1990). The homology suggested that the plasmid-mediated *ampC* β-lactamase gene was derived from chromosomes, but the mechanism was not clear; it may be related to gene transfer elements such as transposons or integrons (Wachino et al., 2006). Different from the chromosomal *ampC* β-lactamase gene, plasmid-mediated *ampC* β-lactamase genes were continuously expressed at a high level and can be transferred to other strains through transformation and conjugation, resulting in widespread drug resistance (Toleman et al., 2006; Jacoby, 2009; Tamma et al., 2019). The commonly reported *ampC* genotypes can be divided into six groups based on sequence: CIT, EBC, DHA, ACC, FOX and MOX (Barlow and Hall, 2003; Hossain et al., 2004). The CIT group of Citrobacter origin includes the BIL-1, CFE-1, LAT type and partial CMY type, mainly in *E. coli*, *K. pneumoniae* and *Proteus mirabilis* (*P. mirabilis*) (Zorgani et al., 2017). The sequence similarity of each genotype ranges from 94% to 95%. The EBC group of *Enterobacter cloacae* (*E. cloacae*) origin includes the MIR type and ACT type, with sequence similarity of more than 90% (Bauernfeind et al., 1998). The DHA group of *Morganella morganii* (*M. morganii*) origin includes seven DHA hypotypes with sequence

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similarity of 99% (Fortineau et al., 2001). The ACC group of *Hafnia alvei* (*H. alvei*) origin includes such as ACC-1, ACC-3 and ACC-4 (Jacoby, 2009). The FOX group of *Aeromonas* origin includes nine FOX hypotypes with sequence similarity of 95% (Jacoby, 2009). The MOX group includes the MOX type and partial CMY type. Above all, *amp*C β-lactamase genes show centralized evolution; however, point mutations may lead to the continuous emergence of new genes (Huang et al., 2011; Kohlmann et al., 2018).

Traditional PCR with specific primers is widely used to determine whether bacteria carry the *amp*C β-lactamase genes. Pérez-Pérez and Hanson designed six groups of specific primers according to the sequence similarity of *amp*C genes, only 29 plasmid-mediated *amp*C β-lactamase genes were considered for primer design in the multiple PCR (Pérez-Pérez and Hanson, 2002). This method has been adopted in many studies, and is widely used as a gold standard for detection of plasmid-mediated *amp*C β-lactamase genes (Geyer and Hanson, 2014). However, non-specific amplification occurs with this method, which interferes with the interpretation of positive bands. Typically, six pairs of single primers are used for repeated tests, resulting in increased workload. In addition, other methods such as the cefoxitin E-test (Ding et al., 2008), cefoxitin three-dimensional test and DNA chip have also been applied in experimental research, but are often accompanied by problems such as false positive, cumbersome operation and considerable time consumption (Coudron et al., 2000; Lee et al., 2002; Zhu et al., 2007).

Currently, there are not many methods for detecting *amp*C β-lactamase genes, and the several established methods have their own advantages and disadvantages. It is generally difficult to accurately detect *amp*C β-lactamase genes through phenotypic screening carried out in clinical laboratories, and detection at the gene level is more reliable. The main purpose of this study was to establish a simple, reliable and exercisable method for many laboratories with high levels of accuracy for detecting more *amp*C β-lactamase genes both from chromosomes and plasmids.

2. Materials and methods

2.1. Bacterial strains

169 strains were isolated from pig or chicken origin during routine monitoring in previous researches. Among them two strains of *Salmonella* with *amp*C β-lactamase genes were previously characterized and sequencing for the expression of specific plasmid-mediated CMY-2 and DHA-1 *amp*C genes variants, 167strains were classified as unknown for *ampC* type and included 37 strains of *Salmonella* Typhimurium (S. Typhimurium), 113 strains of *E. coli*, 11 strains of *K. pneumoniae*, 2 strains of *E. cloacae*, 1 strain of *K. pseudomonas*, 1 strain of *Citrobacter freundii* (*C. freundii*), 1 strain of *M. morganii* and 1 strain of *P. mirabilis*.

2.2. Designing PCR primers using bioinformatics analysis

To develop a sequence- and PCR-based genotyping method for identifying differences in *amp*C β-lactamase genes, all reported gene sequences were downloaded from GenBank. MEGA X software (https://www.megasoftware.net/) was applied for comparing the sequences and constructing the dendrogram, and 175 *amp*C β-lactamase genes were divided into six groups: ACC, EBC, CIT, DHA, MOX and FOX according to their sequence similarity and origin. The sequences of each cluster were aligned using Clustal W (version 6.5, Oxford Molecular Ltd), the number of aligned sequences to display was set to a maximum value of 20 000, and other parameters were set to default values. Six pairs of primers for the targets were designed using Primer Premier 5 (Premier, Palo Alto, CA, USA). Target homology of the designed primers was compared using the basic local alignment search tool (BLAST) algorithm to ensure primer specificity.

2.3. Template DNA preparation

Standard sequences of six groups of *amp*C β-lactamase genes, ACC, EBC, CIT, DHA, MOX and FOX, were selected from GenBank to synthesize genes (GenBank accession no. AJ133121, M37839, HM565135, JX495964, D13304 and X77455). Each product was cloned into the plasmid pUC57. After mixing with *E. coli* competent cells, recombinant bacteria were cultured on LB media. Colonies containing cloned inserts were verified by sequencing (Sangon Biotech, Shanghai, China). Purified cultures were suspended in 500 μL PBS and washed twice. DNA from viable bacteria was extracted using a Rapid Bacterial Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China) following the manufacturer's guidelines. The concentration of DNA was assessed using a DNA concentration detector (Nano Drop One ND-2000, Thermo, American).

2.4. Multiplex PCR procedure and optimization

Polymerase chain reactions were performed initially in a volume of 25 μL, the primers used for PCR amplification are listed in Table 1. Each reaction consisted of 12.5 μL of Extaq premix (Takara Biotechnology Co., Dalian, China), 1 μL of mix template DNA, 0.5 μL of ACC/EBC/CIT/DHA/MOX/FOX forward and reverse primers (10 μmol/L), and 5.5 μL ddH_2O. The reaction was performed as follows: 95 °C for 1 min, 30 cycles of 95 °C for 30 s and 65 °C for 1 min, followed by a final extension at 72 °C for 5 min. Gel Red stain (Biotium, Fremont, CA, USA) was added to 2 μL of PCR product and run on a 1.5% agarose gel for 60 min at 100 V. Agarose gels were visualized under UV light using a G: BOX imaging system (Syngene, Cambridge, UK).

Annealing temperatures and primer concentrations were optimized. Annealing temperature was set to 59 °C, 61 °C, 63 °C, 65 °C, 67 °C and 69 °C. Optimization of primer concentration was conducted by adding primers in different amounts.

2.5. Specificity of the multiplex PCR assay

The specificity of the multiplex PCR method was determined by using mixed template DNA in the simplex PCR and single template DNA in the multiplex PCR. Multiplex PCR specificity was proved if the simplex PCR could detect the target gene but no other genes. For example, in the AAC simplex PCR (only AAC primer involved), mixed DNA was used as template. And in the multiplex PCR (six primer pairs involved), ACC, EBC, CIT, DHA, MOX and FOX template DNA were used as template, respectively.

2.6. Sensitivity of the multiplex PCR assay

Template DNA of six groups of *amp*C β-lactamase genes (ACC, EBC, FOX, DHA, MOX and CIT) were continuously diluted 10-fold from 1.0×10^{14} copies/μL to 1.0×10^{1} copies/μL, respectively. The mixed template DNA consisted of DNA from all six groups. The simplex PCR assay used 1 μL of template DNA to determine the minimum DNA that could be detected by the PCR method. Similarly, 1 μL of mixed template DNA was used in the multiplex PCR assay to determine the minimum amount of DNA that could be detected by the PCR method.

To validate the accuracy and compare the advantages and disadvantages, all isolates (positives, negatives and unknowns) tested in the multiplex PCR assay were compared to data obtained using the gold standard of the Pérez-Pérez and Hanson genotyping approach (Pérez-Pérez and Hanson, 2002).

2.7. Repeatability and stability of the multiplex PCR

To evaluate the repeatability and stability of the multiplex PCR, all diluted DNA samples were used separately as template in three

Table 1. Multiplex PCR primers used for amplification.

Flocks	GenBank accession no.	Targets	No. of targets	Primer sequence (5' to 3')	Expected amplicon size (bp)
CIT	HM565135	CMY-2, 4 to 7, 13 to 17, 21 to 32, 34, 35, 37, 39, 42, 43, 46, 49, 55 to 60, 62 to 64, 69, 71, 77, 80. 86, 94 to 96, 102, 106 to 108, 111, 119, 121, 124 to 127, 129, 130, 132 to 134, 138 to 140, 142 to 148, 153 to 156	77	TCAGCCTGCTGCACTTAGCCAC GTGGATCCCGTTTTATGCACCC	646
ACC	AJ133121	ACC-1 to 7, 1a, 1a2, 1a4, 1b, 1c and 1d	13	GAATATGGAGATTTTGGGTAACGAAGC CGTTAGTTGATCCGGTCTTATTAATCC	326
FOX	X77455	FOX-1 to 10, 12 to 15 and 7a	15	CCAACATGGGGTATCAGGGAGAT CCAGTCGAGCCCGTCTTGTTATAG	247
MOX	D13304	MOX-1 to 4, 6, 8, 9, 12 and 13, CMY-1, 8 to 11, 19 and 8b	16	GATTACTCGGTAGGCGGGATGAC GGTCGAGCCGGTCTTGTTGAAG	174
DHA	JX495964	DHA-1, 3 to 7, 9, 10, 12 to 25	22	TGCGTCTGTATGCAAACAGCAGT TGCGCCCGTTTTATGCACCC	512
EBC	M37839	MIR-1 to 22, ACT-1 to 4, 8, 14, 15, 17, 18, 24	32	GCTGGACCATACCTGGATTAACG CGTCGAGCCTGTTTTATGGACCC	409

independent multiplex PCR assays. Consistency of results provided an estimate for multiplex PCR assay repeatability and stability.

2.8. Application of the multiplex PCR method to clinical samples

The multiplex PCR method was used to detect *Salmonella* genomic DNA containing naturally occurring CMY-2 and DHA-1 *amp*C β-lactamase gene variants, and 167 strains of unknown genotype. Bacterial strains were cultured by standard methods, and genomic DNA was extracted by the boiling method for use as template. The results obtained from the assays were compared with the results of the traditional genotyping.

3. Results

3.1. In silico analysis and primer design

Six family-specific gene clusters—CIT, ACC, FOX, MOX, DHA and EBC—were identified and grouped based on percentage sequence similarities (Figure 1). *amp*C β-lactamase gene sequences were downloaded from the GenBank database. The sequences of each cluster were aligned using Clustal W (version 6.5, Oxford Molecular Ltd), and the aligned sequences were used as a reference for primer design using Primer 5. To avoid cross-hybridization, the newly designed primers were compared with all members of the different clusters and evaluated for length. The theoretical formation of primer dimers was found to be insignificant. The 12 primers designed for multiplex PCR are listed in Table 1.

3.2. PCR optimization

Six family-specific gene clusters were successfully identified using an established multiplex PCR system as shown in Figure 2. The PCR used six sets of *amp*C specific primers resulting in amplicons ranging from 174 bp to 646 bp and that could be easily distinguished by gel electrophoresis.

The annealing temperature and primer concentration were optimized. Conventional agarose gel electrophoresis (1.5%, w/v) (Figure 3) revealed that the multiplex PCR assay was successful at annealing temperatures of both 63 °C and 65 °C. Bright and clear bands were amplified at 63 °C, so we chose this as the optimal annealing temperature. The concentration of primers was 10 μmol/L, and the brightest bands were generated by addition of different volumes of each primer (Figure 4). Therefore, in the multiplex PCR assay we added 0.3 μL of CIT, ACC, FOX and MOX primers and 0.15 μL of DHA and EBC primers.

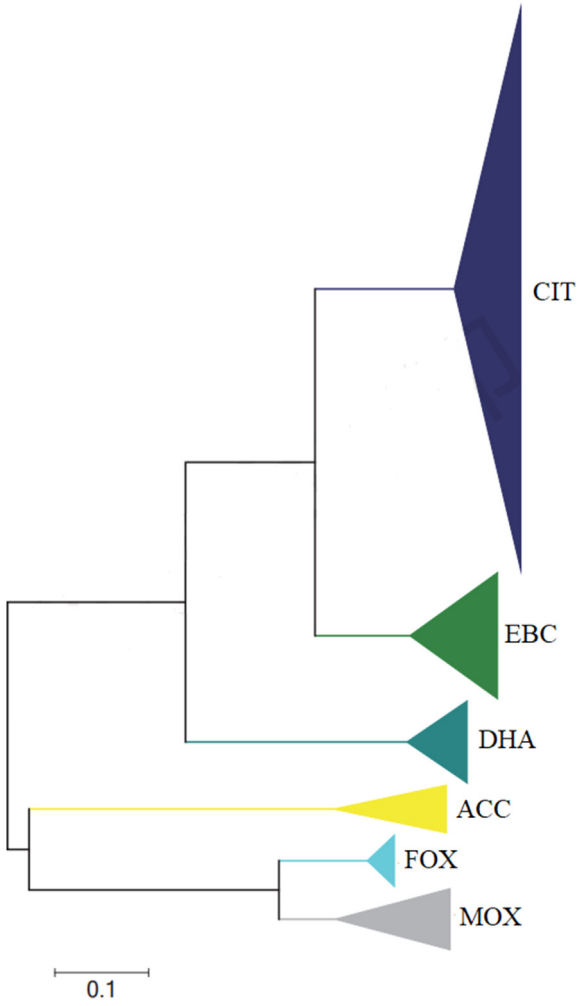

Figure 1. Dendrogram constructed using 175 *ampC* β-lactamase gene sequences downloaded from the GenBank database. The ruler length represents a 0.1% nucleotide sequence difference. Targets (175 *ampC* β-lactamase genes) belonging to the six families are listed in Table 1.

3.3. Repeatability and stability of the multiplex PCR assay

All diluted DNA template was amplified successfully in three independent multiplex PCR assays. Results from independent tests and two

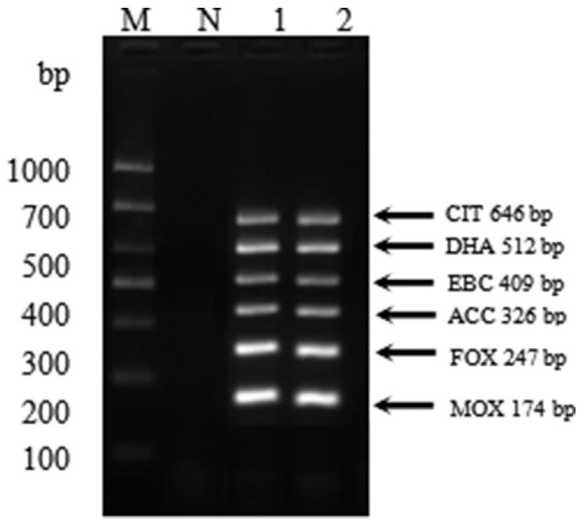

Figure 2. Amplified products of the multiplex PCR method for identification of six *ampC* β-lactamase genes. M: DL 1000 DNA Marker; N: negative control; lanes 1–2 show successful amplification of ACC, FOX, MOX, CIT, DHA and EBC.

strains of *Salmonella* carrying CMY-2 and DHA gene variants proved the repeatability and stability of the assay.

3.4. Specificity and sensitivity of the multiplex PCR assay

We determined the specificity of the multiplex PCR method by adding mixed template DNA to the simplex PCR and single template DNA to the multiplex PCR. The six specific primers amplified their targets, but no amplification product was observed with any other template DNA.

To evaluate the sensitivity of the multiplex PCR method, we consecutively diluted template DNA of ACC, EBC, CIT, DHA, MOX and FOX. In simplex PCR assays, ACC, EBC, FOX and DHA fragments could be amplified at a lowest template concentration of 1.0×10^3 copies/μL, while MOX and CIT fragments could be amplified at a lowest concentration of 1.0×10^2 copies/μL. In the multiplex PCR assay, the minimum template concentration for the six targeted fragments was 1.0×10^3 copies/μL (Figure 5). Using the Pérez-Pérez and Haonson genotyping approach (Pérez-Pérez and Hanson, 2002) (Figure 6), the minimum concentration of detection was 1.0×10^4 copies/μL; the amplicons ranged from 190 bp to 520 bp but were harder to recognize than the method established in this study.

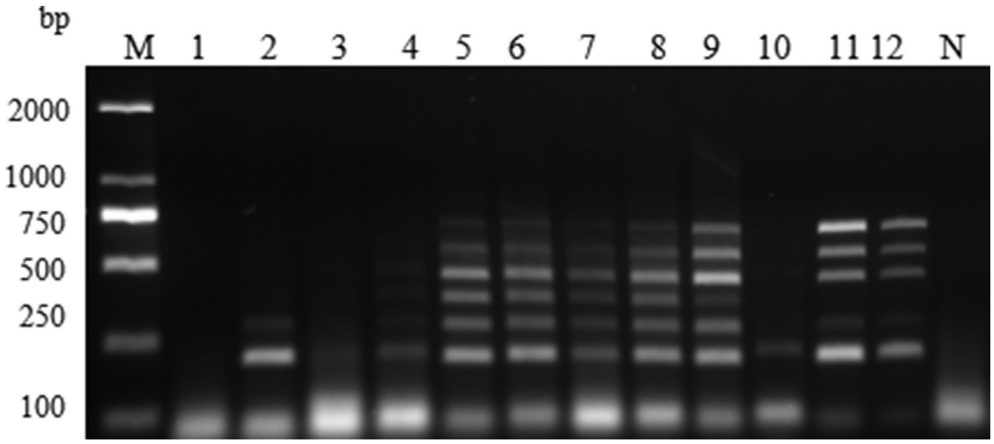

Figure 3. Amplified products of the multiplex PCR method during optimization of annealing temperature. M: DL 2000 DNA Marker; N: negative control; lanes 1–12 represent amplification at different annealing temperatures. 1 and 2: 59 °C, 3 and 4: 61 °C, 5 and 6: 63 °C, 7 and 8: 65 °C, 9 and 10: 67 °C, 11 and 12: 69 °C.

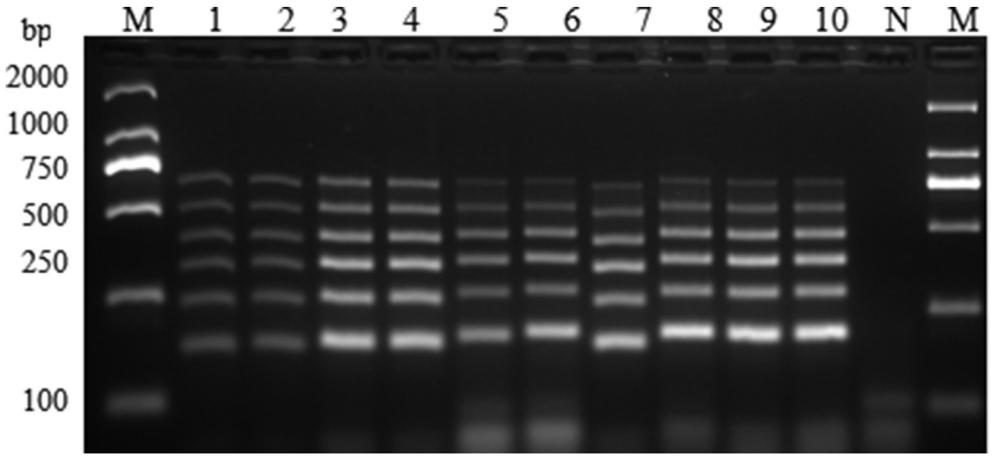

Figure 4. Amplified products of the multiplex PCR method during optimization of primer concentration. M: DL 2000 DNA Marker; N: negative control; lanes 1–10 represent different primer concentration, primer concentration was conducted by adding primers in different amounts and the concentrations of all primers were 10 μmol/L; lanes 1 and 2: 0.2 μL of CIT, 0.2 μL of ACC, 0.2 μL of FOX, 0.2 μL of MOX, 0.1 μL of DHA, 0.1 μL of EBC; lanes 3 and 4: 0.3 μL of CIT, 0.3 μL of ACC, 0.3 μL of FOX, 0.3 μL of MOX, 0.15 μL of DHA, 0.15 μL of EBC; lanes 5 and 6: 0.4 μL of CIT, 0.4 μL of ACC, 0.4 μL of FOX, 0.4 μL of MOX, 0.2 μL of DHA, 0.2 μL of EBC; lanes 7 and 8: 0.5 μL of CIT, 0.5 μL of ACC, 0.5 μL of FOX, 0.5 μL of MOX, 0.25 μL of DHA, 0.25 μL of EBC; lanes 9 and 10: 0.6 μL of CIT, 0.6 μL of ACC, 0.6 μL of FOX, 0.6 μL of MOX, 0.3 μL of DHA, 0.3 μL of EBC.-

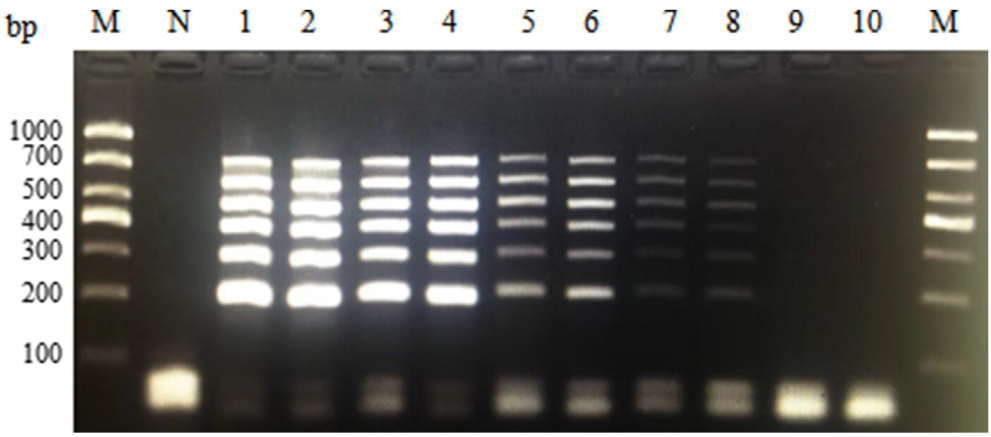

Figure 5. Amplified products of the multiplex PCR method showing the sensitivity of primer concentration. M: DL 2000 DNA Marker; N: negative control; lanes 1–10 represent different concentrations of template DNA. 1 and 2: 1.0×10^6 copies/μL, 3 and 4: 1.0×10^5 copies/μL, 5 and 6: 1.0×10^4 copies/μL, 7 and 8: 1.0×10^3 copies/μL, 9 and 10: 1.0×10^2 copies/μL.

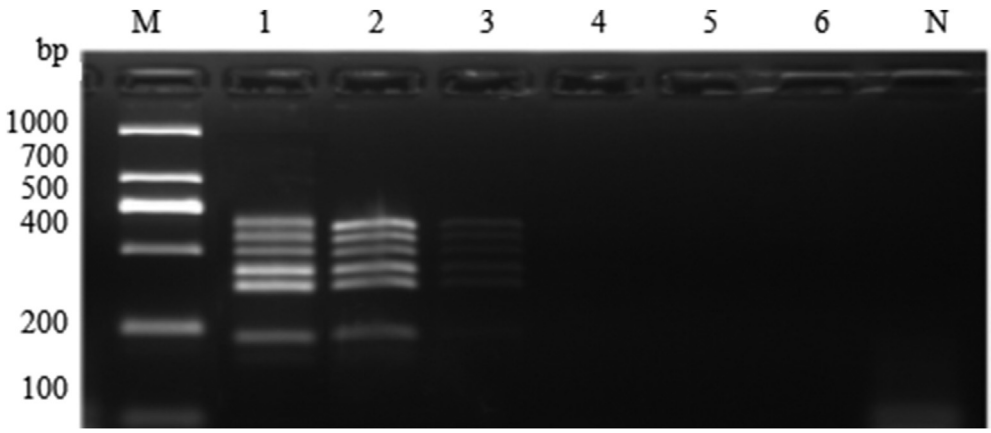

Figure 6. Amplified products using the Pérez-Pérez and Hanson multiplex PCR method. M: DL 1000 DNA Marker; N: negative control; lanes 1–6 revealed represent different concentrations of template DNA. 1: 1.0×10^6 copies/μL, 2: 1.0×10^5 copies/μL, 3: 1.0×10^4 copies/μL, 4: 1.0×10^3 copies/μL, 5: 1.0×10^2 copies/μL, 6: 1.0×10^1 copies/μL.

Table 2. Detection results of 169 strains and sequencing analysis of PCR-positive isolates.

Strain	No.	No. of *ampC*-positive strain						Genotype (strains numbers)
		CIT	DHA	EBC	ACC	FOX	MOX	
ampC-positive strains	2	1	1	-	-	-	-	CMY-2 (1) and DHA-1 (1)
S. typhimurium	37	6	-	-	-	-	-	CMY-2 (6)
E. coli	113	23	3	1	-	-	-	CMY-2 (12), CMY-42[a] (4), CMY-49 (Barlow and Hall, 2003; Bauernfeind et al., 1998[a] (2), CMY-55[a] (5), ACT-2[a] (1), and DHA-1 (3)
K. pneumoniae	11	-	2	-	-	-	-	DHA-1 (2)
E. cloacae	2	-	-	1	-	-	-	MIR-2[a] (1)
K. Pseudomonas	1	-	-	-	-	-	-	-
C. freundii	1	-	-	-	-	-	-	-
M. morganii	1	-	-	-	-	-	-	-
P. mirabilis	1	-	-	-	-	-	-	-

[a] Strains expressing β-lactamase genes were detected in multiplex PCR assay presented in this study but cannot be produced in traditional method.

3.5. Application of the multiplex PCR method

To determine the effectiveness of the newly developed multiplex PCR method, we examined two *Salmonella* strains of known CMY-2-positive and DHA-1-positive, and 167 strains of unknown genotypes. No gel electrophoresis analysis was associated with the *ampC*-negative isolates, because no PCR product was generated. As shown in Table 2 and Figure 7, Of the 169 isolates, Two *Salmonella* samples harboring *ampC* produced the specific 646 bp target band of CIT and the 512 bp target band of DHA, confirming the presence of these gene variants. By comparing the summary data to gold standard method, PCR results were confirmed by the traditional genotyping and showed concordance

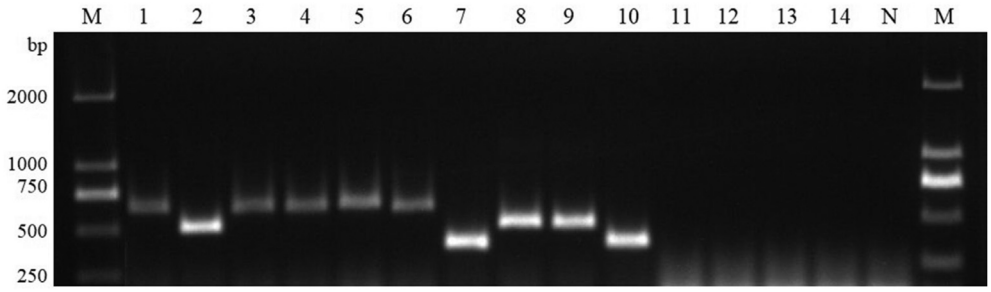

Figure 7. Analysis of clinical isolates. Multiplex PCR products were separated in a 1.5% agarose gel. M: DL 1000 DNA Marker; N: negative control; lanes 1–14 revealed represent different *ampC*-positive strains and *ampC*-negative isolates: lane 1, *Salmonella* expressing CMY-2, lane 2, *Salmonella* expressing DHA-1; lane 3 to 6, *E.coli* expressing CMY-2, -42, -49 and -55, lane 7, *E.coli* expressing ACT-2, lane 8, *E.coli* expressing DHA-1; lane 9, *K.pneumoniae* expressing DHA-1; lane 10, *E. cloacae* expressing MIR-2; lane 11 to 14, negative results of *K. pseudomonas*, *C. freundii*, *M.morganii*, *P.mirabilis*.

between the two methods, however, five strains were generated products of CMY-42, CMY-48, CMY-65, ACT-2 and MIR-2 in this multiplex PCR assay but cannot be detected in the Pérez-Pérez and Hanson genotyping approach. No strains expressing more than one *ampC* β-lactamase and no strains were available for testing ACC, FOX and MOX because of limited strains. Therefore, the *ampC* multiplex PCR assay presented here has the capability of detecting *ampC*-producing isolates.

Above all, the optimal conditions for multiplex PCR detection of *ampC* β-lactamase genes are shown in Table 3. The reaction was performed as follows: 95 °C for 1 min; 30 cycles of 95 °C for 30 s and 63 °C for 1 min, and a final extension at 72 °C for 5 min. This PCR assay was also very rapid, taking less than 2 h to complete.

4. Discussion

Not all genotypes can typically be detected in traditional screening methods for different drug-resistant phenotypes, so there are limitations in the detection scope (Gupta et al., 2014; Pérez-Pérez and Hanson, 2002; Geyer and Hanson, 2014). In addition, when a single strain contains multiple mechanisms of drug resistance, phenotypic results can be complex and difficult to determine (Sepp et al., 2019). It is difficult for many laboratories to detect *amp*C β-lactamase genes accurately and timely with a simple and exercisable method. Plasmid-mediated *amp*C β-lactamase genes are derived from the chromosome and lead to the continuous emergence of new genotypes through point mutation (Mammeri et al., 2010; Coertze and Bezuidenhout, 2018). plasmid-mediated *amp*C β-lactamase genes can mobilize in various organisms to confer a resistance pattern of overproduction of chromosomal *amp*C β-lactamase, resulting in multidrug resistance in Gram-negative bacteria (Dorado-García et al., 2018; Mirelis et al., 2006). Therefore, we designed primers specific for six families of *amp*C β-lactamase genes in the conserved region, allowing detection of the six groups to be completed in the same reaction, providing obvious advantages in specificity, accuracy, detection range and detection limit compared with previous reported methods.

For multiple detection methods, sensitivity and specificity are important indexes reflecting the performance of the detection system (Xiong et al., 2018). six pairs of primers theoretically using BLAST online analysis software were analyzed. In the actual reaction, the specificity of primers was investigated in six single systems and one multiplex PCR system, respectively. The six pairs of group-specific primers for *amp*C β-lactamase genes had good compatibility in the same reaction, and there was no cross-reaction between groups. In addition, *amp*C variants were detected in 167 bacteria strains by multiplex PCR, no strains were expressed more than one *amp*C β-lactamase. It is possible that organisms may not be able to express two or more *amp*C β-lactamase, there is a limit to the amount of *ampC* genes that a bacterial cell can accommodate. However, if multiplex *ampC* genes can be expressed in one single organism, then the multiplex PCR assay described in this report can be used to differentiate them.

Through extensive analysis of *amp*C β-lactamase gene sequences, optimization of the reaction system, system verification and investigation of clinical strains, the multiplex PCR system established in this study has many advantages over other detection methods.

- High sensitivity. The detection sensitivity of *amp*C β-lactamase genes was 1.0×10^3 copies/μL.
- Good specificity. Group-specific primers led to no cross-reaction between groups.
- Comprehensive detection. With the help of the GenBank nucleic acids data-base, the detection capability of this system covers 175 genotypes of plasmid-mediated and chromosomally mediated *amp*C that have been reported so far.
- High accuracy. The effectiveness of primers was verified using positive templates representing six families of *amp*C. Meanwhile, the accuracy of the system was also verified by the detection of two *Salmonella* strains carrying CMY-2 and DHA gene and 167 additional isolates of unknown genotypes, the results were consistent with sequencing results.
- Feasibility. PCR and electrophoresis required a total time of less than 2 h. No special instruments or reagents were needed, it could be promoted to use in many laboratories. The Gel Red imaging system allowed us to easily determine the *amp*C β-lactamase gene variants in a strain.

In summary, we established a simple and efficient multiplex PCR method targeting six families of *amp*C β-lactamase genes, ACC, EBC, CIT, DHA, MOX and FOX, and confirmed the specificity, sensitivity, repeatability and stability of the multiplex PCR method. This efficient multiplex

Table 3. Conditions for the multiplex PCR method for detection of *ampC* β-lactamase genes.

Reagent	Reagent concentration	Adding volume in 25 μL system/μL
2×Ex taq premix	-	12.5
Forward and reverse primers of CIT	10 μmol/μL	0.3
Forward and reverse primers of DHA		0.3
Forward and reverse primers of EBC		0.3
Forward and reverse primers of ACC		0.3
Forward and reverse primers of FOX		0.15
Forward and reverse primers of MOX		0.15
Template DNA	-	1
ddH_2O	-	8.5

Note 1: The amount of each reagent in the reaction system can be adjusted according to the specific situation or the total volume of different reactions.
Note 2: Two parallel reactions should be set for each reaction system.

PCR assay could be used as an efficient diagnostic tool for simultaneous identification of 175 *ampC* β-lactamase gene variants. Our method provides increased accuracy and detection range that will lead to improvements in gene surveillance and infection control.

Declarations

Author contributions

Qian Zhou and Mengjun Tang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Qian Zhou Writed the manuscrpit.

Xiaoyan Zhang: Performed the experiments.

Junxian Lu; Xiujun Tang: Contributed reagents, materials, analysis tools or data.

Yushi Gao: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest

Additional information

No additional information is available for this paper.

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