A strategy design based on antibiotic-resistance and plasmid replicons genes of clinical Escherichia coli strains

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ABSTRACT
Since antimicrobial resistance, especially β-lactam resistance genes were common in clinical Escherichia coli strains, this study designed and developed multiplex amplification platform for rapid and accurate detection of such resistance genes in 542 clinical E. coli isolates. The obtained specimens were subjected to bacteriological examination, antimicrobial susceptibility testing, and detection of β-lactamase genes and plasmid replicons. The major virulence genes were detected by 7 groups of multiplex PCR and eight groups of multiplex PCR were designed to detect 8 different plasmid replicons including parA-parB, iteron, repA, and RNAI. It was found that most MDR isolates were co-resistant to penicillins (AMP) and fluoroquinolones (LVX, CIP) and distribution of LVX and CIP resistance was significantly higher among female than male gender. RNAI (AY234375) showed the highest detection rate, followed by the iteron (J01724) and repA (M26308), indicating the relatively higher carriage rate of corresponding plasmids. BlaCTXM, acquired the highest carriage rate, followed by group 2 BlaCTX-M and BlaQAP, indicating their prevalence among clinical E. coli. Among the β-lactamase genes, BlaCTXM acquired the highest carriage rate, followed by group 2 BlaCTX-M and BlaQAP, indicating their prevalence among clinical E. coli. The RNAI (AY234375) showed the highest detection rate, followed by the iteron (J01724) and repA (M26308), indicating the relatively higher carriage rate of the corresponding plasmids by clinical E. coli isolates. It is shown that the developed multiplex amplification methodology is applicable to AMR detection, and such identification of plasmid replicons and β-lactamase genes may aid in the understanding of clinical E. coli isolate epidemiology.
1. Introduction

In recent decades, antimicrobial resistance (AMR) in microorganisms has been considered to be a leading concern for human health, which has posed a significant obstacle for therapeutic treatment on various clinical infections. In 2016, the United Nations High-Level meeting had declared that progress toward achieving several of the Sustainable Development Goals (SDGs) is threatened by AMR [1]. In 2020, the World Health Organization (WHO) had announced the launch of 2 new AMR indicators in the monitoring framework of the SDGs linked to the health target 3, which monitor proportion of bloodstream infections (BSIs) due to 2 typical Super-bugs, Escherichia coli and Staphylococcus aureus [2]. Consequently, in this study, a strategy design based on multiplex amplification and further application for rapid detection on antimicrobial resistance and β-lactam resistance genes had been performed on a large scale of clinical Escherichia coli strains [3].

Bacterial factors are reported to be associated with E. coli pathogenesis and progression [4]. Moreover, pathogenic E. coli strains show great diversity in gene content, virulence factors, genomic Islands, and pathogenicity Islands [5]. Pathogenic Escherichia coli are capable of causing various diseases in humans, including several types of diarrhea, urinary tract infections, sepsis, and meningitis [6,7]. Due to the widely application of antibiotics, the emergence of E. coli isolates with multiple antibiotic-resistant phenotypes, involving co-resistance to four or more unrelated families of antibiotics, has been considered a serious health concern [8,9]. A large number of genes are responsible for antibiotic resistance. As the first identified antibiotic, β-lactams have become widely applied. Introduction of β-lactams in clinical settings was quickly followed by the emergence of numerous β-lactamases [10]. Identification of β-lactamase genes is important to understand resistance epidemiology and identify resistant strains [11]. β-lactamase genes have been reported to frequently transferred by plasmids [11–13]. The spread of extended-spectrum β-lactamases (ESBLs) is an emerging global public health problem. Until the late 1990s, TEM and SHV enzymes were the most common extended-spectrum β-lactamases (ESBLs). In the late 1990s, the prevalence of both TEM and SHV decreased, whereas that of CTX-M increased, especially associated with Escherichia coli species. Within a few years, CTX-M ESBL-producing E. coli had spread across the world, involved in both nosocomial outbreaks and community-acquired infections [14].

Plasmids are capable of increasing bacterial genetic diversity, acquiring and losing genes, and can be horizontally exchanged among bacterial populations by conjugation or mobilization [15]. Plasmids contain genes essential for initiation and control of replication and accessory genes [16,17]. Replicons contribute to the replication of plasmid and represent the acquisition of plasmids.

This study collected and analyzed the 10-year antimicrobial resistance data of clinical E. coli isolates and aimed in identifying the distribution of β-lactamase genes and plasmid replicons, which may aid in the understanding of its epidemiology and the development of control strategies.

2. Material and methods

2.1 Bacterial strain

During 2008 to 2018, a total of 542 E. coli isolates were isolated from the First Affiliated Hospital of Guangzhou Medical University (FAHGMU). The large proportion of patients in FAHGMU are from Guangdong and other adjacent provinces, which can represent the epidemiology of E. coli in Southern China. Both inpatients and outpatients were included in this study. For the criteria of bacterial selection, strains were averagely distributed in different months, with an average of 4 to 5 strains isolated in 1 month. Also, strains were selected according to the location of medical settings and wards, as well as their antimicrobial resistance profile. The isolates datas included demographic information such as date, age, gender of patients, department of isolation and infection types.
2.2 Bacterial Isolation and Identification

Bacterial identification to the species level on all tested strains had been performed by standard procedures: colony morphology, Gram staining, the API commercial kit and the Vitek 2 automated system [18]. Isolation of *E. coli* was performed using standard bacteriological methods. The clinical samples were incubated in Violet Red Bile Agar (VRBA, Huankai Microbial, China) for 24 h at 37°C and those pink colored presumptive *E. coli* were sub-cultured in Eosin Methylene Blue (EMB, Huankai Microbial, China) agar to get a pure colony. Colonies with metallic green sheen were characterized microscopically using Gram stain and other different biochemical tests such as indole production, methyl-red test, Voges-Proskauer test, citrate utilization (IMViC) test, oxidase test, sugar fermentation, triple sugar iron [19]. Furthermore, the identified isolates were confirmed by using a species-specific set of primers targeting 16S rRNA gene of *E. coli* (27FYM: 5’-AGAGTTTGATYMTGGCTCAG-3’; 1492 R: 5’-GGTTACCTTGTTACGACTT-3’) [20].

2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by disk diffusion according to the Clinical and Laboratory Standard Institute (CLSI), and minimum inhibitory concentrations (MICs) determined through Vitek 2 automated system (Vitek AMS; bioMerieux Vitek Systems Inc., Hazelwood, MO) [17–19]. The 13 tested antimicrobial agents classified into 7 antibiotic groups: penicillins (AMP: Ampicillins), β-lactam (TZP: piperacillin-tazobactam; AMC: Amoxicillin-clavulanate), cephalos (CAZ: cefazidime; FEP: cefepime), monobactams (ATM: aztreonam), carbapenems (IPM: imipenem; MEM: meropenem), aminoglycosides (GEN: gentamicin; TOB: tobramycin; AMK: amikacin) and fluoroquinolones (LVX: levofloxacin; CIP: ciprofloxacin). In addition, the susceptibility analysis was interpreted based on the criteria on *E. coli* of CLSI (2019).

Multidrug-resistant (MDR) was defined as acquired non-susceptibility to at least one agent in more than three antimicrobial categories, and extensively drug-resistant (XDR) was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories [21]. Pandrug-resistant (PDR) was defined as non-susceptibility to in all antimicrobial categories listed.

2.4 Detection of β-lactamase genes

The major β-lactamase genes were detected by seven groups of multiplex PCR [22,23]. Three sets of primers were used in group 1 for the amplification of *bla*TEM (*bla*TEM-1 and *bla*TEM-2), *bla*SHV-1, and *bla*OXA (*bla*OXA-1, *bla*OXA-4 and *bla*OXA-30) genes, respectively. Group 2 and group 3 mainly amplified *bla*CTX-M, including group 1 *bla*CTX-M (*bla*CTX-M-1, *bla*CTX-M-3 and *bla*CTX-M-15), group 2 *bla*CTX-M (*bla*CTX-M-2) and group 9 *bla*CTX-M (*bla*CTX-M-9 and *bla*CTX-M-14), and group 8/25 *bla*CTX-M (*bla*CTX-M-8, *bla*CTX-M-25, *bla*CTX-M-26 and *bla*CTX-M-39 to *bla*CTX-M-41). Five sets of primers were used in group 4 to detect *bla*AAC (*bla*AAC-1 and *bla*AAC-2), *bla*FOX (*bla*FOX-1 to *bla*FOX-5), *bla*MOX (*bla*MOX-1, *bla*MOX-2, *bla*CMY-1, *bla*CMY-8 to *bla*CMY-11 and *bla*CMY-19), *bla*DHA (*bla*DHA-1 and *bla*DHA-2), *bla*CT (*bla*CT-1 to *bla*CT-7, *bla*CMY-2 to *bla*CMY-7, *bla*CMY-12 to *bla*CMY-18 and *bla*CMY-21 to *bla*CMY-23) and *bla*EBC (*bla*EBC-1 and *bla*EBC-2). *Blav*erb (*bla*ereb-1, *bla*ereb-2, *bla*ereb-3) and *bla*GES (*bla*GES-1 to *bla*GES-9 and *bla*GES-11) genes were detected by group 5. *Blav*esg (*bla*esg-1 to *bla*esg-9 and *bla*esg-11) and *bla*OXA-18 were amplified by group 6, while *bla*IMP, *bla*VIM (*bla*VIM-1 and *bla*VIM-2) and *bla*KPC (*bla*KPC-1 to *bla*KPC-5) were detected by group 7. Annealing temperature at 60°C was used for group 1–5, while 57°C and 55°C were selected for group 6 and 7, respectively. One min was chosen for extension time since all the PCR products were shorter than 1 kb. All PCR products were run on 2% agarose gel. To further avoid false-positive results from multiplex PCR, all positive samples were further subjected to separate PCR confirmation using single pairs of primers from the corresponding multiplex PCR. For samples yielding positive results from single PCR reaction, Sanger sequencing was further performed to ensure accuracy. All experiments were performed in triplicate.
2.5 Detection of plasmid replicons

Eight groups of multiplex PCR were designed to detect 8 different plasmid replicons [22,23]. Group 1 targeted on \textit{para-parB} (AF250878), \textit{iteron} (BX664015), and RNAI (M20413). The target genes in group 2 were \textit{ori} (Y00768), \textit{repABC} (U27345), and \textit{repA} (NC_003292). One iteron (J01724) and two \textit{repA} genes (M26308 and U12441) were selected to be detected in group 3. Similarly, iteron (M20134), \textit{repA} (K02380), and \textit{repA2} (AH003523) were detected in group 4. Three \textit{repA} genes (X73674, K00053, and AE006471) were amplified in group 5. In group 6–8, three different RNAI (AY234375, M93063, and M28718) were detected, respectively. For the multiplex PCR groups except for group 6, annealing temperature at 60°C and extension for 1 min were used. In group 6, 52°C was selected for annealing temperature. All PCR products were run on 2% agarose gel.

2.6 Statistical analysis

In this study, the susceptibility of \textit{E. coli} was defined as resistant (resistant and intermediate resistant) and susceptible in this study. The antimicrobial susceptibility test results were collected and managed in WHONET (version 5.6). Chi-square test or Fisher’ s exact test were used to analyze the difference of proportions, if appropriate. A value of \(p < 0.05\) was defined as statistically significant.

3. Results

The obtained \textit{E. coli} isolates were subjected to bacteriological examination, antimicrobial susceptibility testing, detection of \(\beta\)-lactamase genes and plasmid replicons. The identification of plasmid replicons and \(\beta\)-lactamase genes, especially MDR, XDR, and PDR strains, may aid in the understanding of clinical \textit{E. coli} isolates epidemiology.

3.1 Phenotypic characteristics of the recovered isolates

The colonies of the recovered \textit{E. coli} isolates showed bright pink colonies on VRBA agar plates and showed a distinctive metallic green sheen on EMB agar. All \textit{E. coli} isolates were lactose fermenting colonies, methyl red, pidole-positive, catalase, oxidase-negative, urea hydrolysis, criritate utilization, Voges-Proskauer, and did produce \(\text{H}_2\text{~S}\) in biochemical tests. Moreover, all isolates PCR amplicons were compared in the GenBank database using the BLAST program and the result indicated that these strains belonged to \textit{Escherichia coli}.

3.2 Characteristics of antimicrobial resistance of \textit{E. coli}

\textit{E. coli} isolates collected from 7 different specimen sources during 10-year period were compared antimicrobial resistance in this study (Table 1). Females were the most affected group of patients (286 samples, 58.4%) as compared to males (181 samples, 37.0%). Maximum number of \textit{E. coli} were isolated in the age group of 41–70 (271 samples, 55.3%). Urine was the most predominant specimen source of \textit{E. coli} (46.9%), followed by blood (24.7%) and sputum (12.2%). Other remaining sources included secretion, pleural fluid, pus and wound, only making up a small proportion.

The resistance rates of penicillins (AMP) and fluoroquinolones (LVX, CIP) remained high during the study period (vary between 58.8% and 91.2%, 79.7% and 90.2%, 54.9% and 81.3% over the study period, respectively; Figure 1). Monobactams (ATM) and aminoglycosides (GEN, TOB) kept fluctant (between 31.9% and 74.5%). Meanwhile, increased rates in \(\beta\)-lactam use for clinical treatment accompanied these increasing resistance rates, the resistance rate of \(\beta\)-lactam (TZP, AMC, 3.5% to 40.7%) and cephems (CAZ, FEP, 16.7% to 40.3%) increased during 10-year period.

3.3 MDR, XDR and PDR profiles in \textit{E. coli}

Approximately 92.9% of isolates were resistant to at least one antimicrobial agent (Table 1). Among them, 76.9% 27.9%, 1.4% were MDR, XDR, and PDR, respectively (Table 2). The results showed that many MDR isolates were co-resistant to penicillins (AMP) and fluoroquinolones (LVX, CIP) and distribution of LVX
and CIP resistance was significantly higher among female than male gender. These results were not exactly consistent with previous research [24,25], its reasons may be due to the geographical difference of E. coli.

Strains Most of XDR strains were resistant to penicillins, cephems, monobactams, and fluoroquinolones (Figure 2). Only 7 strains were identified as PDR strains, isolated in 2008, 2009, 2012, 2013, 2016, and 2017 (Table 2, Figure 3).

3.4 Distribution of β-lactamase genes

According to characteristics of antimicrobial resistance to β-lactam (TZP, AMC), the resistance rate increased during the study period (from 3.5% to 40.8%). Among all β-lactamase genes, blaOXA-1 acquired the highest carriage rate (195/542, 35.98%), followed by group 2 blaCTX-M (90/542, 16.61%), blaSHV-1 (78/542, 14.39%) and group 9 blaCTX-M (39/542, 7.20%), indicating their prevalence among clinical E. coli in South China (Table 3). The carriage rate of blaOXA-1 was high (40.82–62.50%) from 2008 to 2012, 2015, and 2018, but decreased (4.08–5.56%) in 2013–2014 and 2016–2017. The carriage rate of group 2 blaCTX-M and blaSHV-1 was fluctuant.

Among the 378 MDR strains, blaOXA-1 acquired the highest carriage rate (137/378, 36.24%), followed by group 2 blaCTX-M (71/378, 18.78%), blaSHV-1 (65/378, 17.20%), group 9 blaCTX-M (32/378, 8.47%), and blactX (27/378, 7.14%) (Figure 4). Among the 137 XDR strains, blaOXA-1 acquired the highest carriage rate (49/137, 35.77%), followed by blaSHV-1 (29/137, 21.17%), group 2 blaCTX-M (23/137, 16.79%), and group 9 blaCTX-M (19/137, 13.87%) (Figure 4). Among the 7 PDR strains, 2 strains carried blaOXA-1, 1 strain carried group 2 blaCTX-M (23/137, 18.78%), and another one carried group 9 blaCTX-M, respectively.

3.5 Distribution of plasmid replicons

Eight groups of plasmid replicons were detected among the 542 E. coli isolates (Table 2). The RNAI (AY234375) in group 6 showed the highest detection rate (285/542, 52.58%), followed by the iteron (J01724, 233/542, 42.99%) and repA (M26308, 135/542, 24.91%) in group 3, indicating the relatively higher carriage rate of corresponding plasmids by clinical E. coli isolates. In group 1, RNAI (M20413) showed relatively higher detection rate (61/542, 11.25%), followed by parA-parB (AF250878, 31/542, 5.72%) and iteron (BX664015, 6/542, 1.11%). The identification rate of RNAI (M20413) was relatively stable among strains isolated in different years, except for in 2013 (none detected). In group 2, the identification rates of ory (Y00768), repABC (U27345), and repA (NC_003292) were 2.40%, 0.92%, and 4.43%, respectively. The ory (Y00768) was only detected in 2012 and 2018, while the repABC (U27345) was only detected in 2009, 2011, and 2018. In group 3, except for iteron (J01724) and repA (M26308), repA (U12441) was carried by 135 (8.49%) E. coli strains isolated in 2012 and 2014–2016. The detection rate of the three genes in group 4 was relatively low, but the lowest identification rate was acquired by group 5. One of the repA genes (K00053) in group 5 was not detected. Only 1 strain isolated in 2010 carried the RNAI from group 7 (M93063).

Among those MDR strains, the identification rates of RNAI (AY234375)/repA (AE006471), iteron (J01724), repA (M26308) were 55.03% (208/378), 44.71% (169/378), and 30.16% (114/378), respectively (Figure 5). Approximately 51.10% (70/137) and 47.45% (65/137) XDR strains carried RNAI (AY234375)/repA (AE006471) or iteron (J01724).

4. Discussion

In this study, females were more susceptible to E. coli infection, which is consistent with global trends that higher prevalence of urinary tract infections in female patients than in males [26–28]. Our result showed that urine was the main source of E. coli. Urinary tract infections (UTIs) caused by uropathogenic Escherichia coli (UPEC) are one of the most common outpatient bacterial infections [29]. The emergence of high rates of antibiotic resistance and multidrug-resistant MDR-phenotype from urinary tract infections related bacteria becomes a public health concern worldwide [30]. Moreover, the high resistance rates of penicillins (AMP) and fluoroquinolones
## Table 1. Characteristics of specimen and drug resistance profiles of E. coli.

| Specimen Source | Total | MDR (≥1) | XDR (≥3) | PDR (≥4) |
|-----------------|-------|----------|----------|----------|
| Sputum          |       |          |          |          |
| Female          | 16(33.3)/16(33.3) | 3(6.3)/0(0.0) | 0(0.0)/0(0.0) | 0(0.0)/0(0.0) |
| Male            | 33(67.3)/33(67.3) | 6(11.8)/6(11.8) | 0(0.0)/0(0.0) | 0(0.0)/0(0.0) |
| Blood           | 10(42.6)/10(42.6) | 2(8.3)/2(8.3) | 0(0.0)/0(0.0) | 0(0.0)/0(0.0) |
| Urine           | 36(76.6)/36(76.6) | 6(13.3)/6(13.3) | 0(0.0)/0(0.0) | 0(0.0)/0(0.0) |
| Urinary tract   | 37(81.3)/37(81.3) | 7(14.6)/7(14.6) | 0(0.0)/0(0.0) | 0(0.0)/0(0.0) |
| Ampicillin      | 38(79.2)/38(79.2) | 7(14.6)/7(14.6) | 0(0.0)/0(0.0) | 0(0.0)/0(0.0) |
| Ticarcillin      | 37(79.2)/37(79.2) | 7(14.6)/7(14.6) | 0(0.0)/0(0.0) | 0(0.0)/0(0.0) |
| Cefepime        | 36(76.6)/36(76.6) | 6(13.3)/6(13.3) | 0(0.0)/0(0.0) | 0(0.0)/0(0.0) |
| Tobramycin      | 35(74.5)/35(74.5) | 7(14.6)/7(14.6) | 0(0.0)/0(0.0) | 0(0.0)/0(0.0) |
| Gentamicin      | 36(76.6)/36(76.6) | 6(13.3)/6(13.3) | 0(0.0)/0(0.0) | 0(0.0)/0(0.0) |

**Notes:**
- **AMP:** Ampicillin; **T2P:** Piperacillin-tazobactam; **AMC:** Amoxicillin-clavulanate; **CAZ:** Cefazidime; **FEP:** Cefepime; **ATM:** Aztreonam; **IPM:** Imipenem; **MEM:** Mecillinam; **TOB:** Tobramycin; **AMK:** Amikacin; **LVX:** Levofloxacin; **CIP:** Ciprofloxacin.
- **MDR:** non-susceptibility to ≥1 agent in ≥3 antimicrobial categories; **XDR:** non-susceptibility to ≥1 agent in all but ≤2 antimicrobial categories; **PDR:** non-susceptibility to all antimicrobial categories listed.
- *N (%)*, b NT: Not Tested.
(LVX, CIP) during this period was in agreement with previous study [27,31].

Multidrug resistance has been increased all over the world that is considered a public health threat gave a warning to the potential and proper use of antibiotics [32,33]. Several recent investigations reported the emergence of multidrug-resistant bacterial pathogens from different origins including humans, birds, cattle, and fish that increase the need for routine application of the antimicrobial susceptibility testing to detect the antibiotic of choice as well as the screening of the emerging MDR strains [34–39]. The rate of MDR was higher than in other study from China, which reported only 47.9% of MDR strains [24]. It is known that susceptibility patterns may vary in different geographical regions and can be change over time. It is worth noting that MDR E. coli usually implies a significant increase in resistance and pathogenic potential and indicates complicated treatment and bad prognosis of infections [40]. Interestingly, resistance rates to antimicrobial drugs were higher in isolates from females than in those from male patients. Thus, it may be more difficult to eradicate female infections and susceptibility analysis of isolates to antibiotics prior to treatment choice is recommended. For example, German national guides as a first choice for the treatment of uncomplicated cystitis [30].

Over the last decade, blaCTX-M has become increasingly common worldwide, to the point that their prevalence easily surpassing those of blaSHV and blaTEM ESBL genes [14]. Based on CTX-M amino acid sequences, these enzymes have been classified into five major groups, groups 1, 2, 8, 9 and 25. Previous study revealed that the incidence of these blaCTX-M genotypes varies geographically. Our results showed that group 2 blaCTX-M may be the most widely disseminated genotype in southern China, followed by group 9 and group 1.

Strains carrying blaCRT were mainly isolated in 2010 and 2011. Six each group 1 blaCTX-M and blaAAC were detected, with group 1 blaCTX-M identified in strains isolated in 2008 and 2009, and blaAAC carried by the strains isolated from 2010 to 2012. Two strains isolated in 2008 and 2011, respectively, carried blaFOX. Only one each strain isolated in 2011, 2018, and 2010 was identified to carry blaTEM, blaEBC, and blaKPC, respectively. In contrast, no group 8/25 blaCTX-M, blaAAC, blaFOX, blaDHA, blagES, blaper, blaveb, blaOXA-48, blaIMP, and blaVIM genes were identified.

Among the 94 isolates that were resistant to β-Lactam, surprisingly 64 did not have any of the 21

![Figure 1. Phenotypic Multidrug Resistance of MDR Strains.](image-url)
Table 2. Characteristics of antimicrobial susceptibility testing profiles of *E. coli*.

| Year (Total) | 2008(48) | 2009(47) | 2010(49) | 2011(47) | 2012(57) | 2013(56) | 2014(48) | 2015(33) | 2016(74) | 2017(51) |
|--------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| **MDR**      | 36(75.0) | 34(76.6) | 39(79.6) | 39(80.9) | 30(77.7) | 24(66.7) | 40(83.3) | 25(75.8) | 56(75.7) | 33(64.5) |
| **XDR**      | 9(18.8)  | 13(27.7) | 10(20.4) | 13(27.7) | 17(29.8) | 9(23.6)  | 19(39.6) | 11(33.3) | 24(32.4) | 12(23.5) |
| **PDR**      | 1(2.0)   | 2(2.1)   | 0(0.0)   | 0(0.0)   | 1(1.1)   | 1(2.8)   | 0(0.0)   | 0(0.0)   | 22.7      | 12(2.0)  |

**Antimicrobial resistance of MDR/XDR/PDR**

| Antibiotics | Penicillins | β-Lactam | Cephalosporins | Carbapenems | Ćephem | Fluoroquinolones | Fluoroquinolones |
|-------------|-------------|-----------|----------------|-------------|--------|----------------|----------------|
| **AMP**     | 34(74.4)/9(100/100/0) | 35(97.2)/2)/1292.3/1(100) | 3897.4/10(100/0/0) | 3794.8/13(0/0/0) | 30(100)/17(100/1/100) | 24(100)/19(100/0/0) | 24(100)/11(100/0/0) | 55(98.2)/2(100/0/0) | 28(84.8)/11(99.7/1/100) |
| **AMC**     | NT         | NT       | NT             | NT          | NT     | NT             | NT             | NT          | NT         |
| **TZP**     | 1/2/6/0/0 (0/100/100/0) | 2/5/5/1/1/1/100 | 2/1/1/100/0/0 | 1/2/6/0/0/0/0 | 3/1/1/1/1/100 | 1/2/6/0/0/0/0 | 24/0/1/0/1/100 | 1/2/6/0/0/0/0 | 9/16/1/5/20/2/100 |
| **FEP**     | 10/27/8/7/7/6/7/100 | 20/55/6/13/100/100 | 1/1/2/1/100/0 | 0/0/0/0/1/100 | 0/0/0/0/1/100 | 0/0/0/0/1/100 | 0/0/0/0/1/100 | 0/0/0/0/1/100 | 26/4/4/15/62/5/2/100 |
| **CAZ**     | 11/10/6/6/6/6/6/100 | 8/22/5/5/5/5/0/0 | 5/1/2/1/100/0 | 1/5/5/1/100/0 | 1/5/5/1/100/0 | 1/5/5/1/100/0 | 1/5/5/1/100/0 | 1/5/5/1/100/0 | 11/16/1/20/1/200 |
| **IPM**     | NT         | NT       | NT             | NT          | NT     | NT             | NT             | NT          | NT         |
| **MEM**     | NT         | NT       | NT             | NT          | NT     | NT             | NT             | NT          | NT         |
| **AMK**     | NT         | NT       | NT             | NT          | NT     | NT             | NT             | NT          | NT         |
| **TOB**     | NT         | NT       | NT             | NT          | NT     | NT             | NT             | NT          | NT         |
| **LVX**     | NT         | NT       | NT             | NT          | NT     | NT             | NT             | NT          | NT         |
| **CIP**     | NT         | NT       | NT             | NT          | NT     | NT             | NT             | NT          | NT         |

**AMP:** Ampicillin; **TZP:** Piperacillin-tazobactam; **AMC:** Amoxicillin-clavulanate; **CAZ:** Cefazidime; **FEP:** Cefepime; **ATM:** Aztreonam; **IPM:** Imipenem; **MEM:** Meropenem; **GEN:** Gentamicin; **TOB:** Tobramycin; **AMK:** Amikacin; **LVX:** Levofloxacin; **CIP:** Ciprofloxacin.

MDR: non-susceptibility to ≥1 agent in ≥3 antimicrobial categories; XDR: non-susceptibility to ≥1 agent in all but 2 antimicrobial categories; PDR: non-susceptibility to in all antimicrobial categories listed.

*N (%)*, **NT:** No Tested
β-lactam resistance genes. These results suggested that there may be no relation between the presence of \( \text{bla}_{\text{TEM}} \), \( \text{bla}_{\text{SHV}} \), \( \text{bla}_{\text{OXA-1-like}} \), \( \text{bla}_{\text{CTX-M}} \), \( \text{bla}_{\text{ACC}} \), \( \text{bla}_{\text{MOX}} \), \( \text{bla}_{\text{CIT}} \), \( \text{bla}_{\text{EBC}} \) and \( \text{bla}_{\text{KPC}} \) with β-lactam production and other types of β-lactam resistant genes may be responsible for the phenotype. More
Table 3. Distribution of β-lactamase genes and plasmid replicons of *E. coli*.

| Genes | Primer sequence (5’-3’) | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 | 2015 | 2016 | 2017 | 2018 | Total |
|-------|------------------------|------|------|------|------|------|------|------|------|------|------|------|-------|
| TEM   | F: CATTTCCTGCTGGCCCTATTTC | 1(0.0) | 0(0.0) | 0(0.0) | 1(2.1) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 1(2.1) | 0(0.0) | 0(0.0) | 1(2.1) |
|       | R: CCTGCATCGATTGCCCCTGAC | 2(4.2) | 0(0.0) | 5(10.2) | 36(6.3) | 1(1.8) | 8(2.2) | 3(16.5) | 0(0.0) | 20(27.8) | 25(49.0) | 1(2.0) | 78(14.4) |
| SHV   | F: AGGCGCTTCAGAAATAAAC | 30(62.3) | 28(57.4) | 20(40.8) | 23(47.9) | 32(56.1) | 25(6.5) | 2(4.1) | 16(44.4) | 5(6.9) | 47(8.8) | 34(69.4) | 195(36.0) |
|       | R: ATCCGCGAGATAATACACG | 1(2.1) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 1(2.1) |
| OXA-1 | F: GCGGCAAGCACTACCTGCAAG | 19(39.6) | 20(42.6) | 4(8.2) | 5(10.4) | 19(33.3) | 0(0.0) | 21(6) | 11(15.3) | 5(9.8) | 5(10.2) | 90(16.6) |
|       | R: CGATCGTGCCTGGTGCCTCAGT | 2(4.2) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 6(1.1) |
| CTX-1 | F: TGCAAGATGGTGCTGCCTGAC | 4(8.3) | 3(6.4) | 4(8.2) | 5(10.4) | 12(21.1) | 0(0.0) | 4(8.2) | 0(0.0) | 1(2.4) | 0(0.0) | 6(12.2) | 39(7.2) |
|       | R: TATCTCTGGCGCCTCAGAC | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) |
| CTX-2 | F: CGTACGACGAATCGTGTGTT | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) |
|       | R: CCATTGCGGCGGACCTGGA | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) |
| CTX-9 | F: TCAAGCTGATGGCTGCCTGAC | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) |
|       | R: TATGCTGCTGGCCTCAGAC | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) |

(Continued)
Table 3. (Continued).

| repA  | F: TTGGCCCTTGTGCTAACCAC | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) |
|-------|--------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|       | R: CGTGGATTACACTTAGCTTTG | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 2(3.5) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 2(0.4) |
| repA  | F: CTGCGTAAAGTGTAGGC    | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) |
|       | R: CTCTGCCGCAAGCTTCAAGC | 40(83.3) | 40(85.1) | 0(0.0) | 0(0.0) | 43(75.4) | 0(0.0) | 22(44.9) | 28(77.8) | 48(66.7) | 36(70.6) | 28(57.1) | 285(52.6) |
| RNA   | F: TGATGGTTGAAAGGATTTGG | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 10(2.2) |
|       | R: TGATGGTTGAAAGGATTTGG | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 10(2.2) |
| RNA   | F: CGTGCGGAAGCCAGAAAC   | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 0(0.0) | 10(2.2) |
|       | R: GAGTGGTTGAAAGGATTTGG | 12(2.1) | 0(0.0) | 0(0.0) | 0(0.0) | 1(1.8) | 0(0.0) | 6(12.2) | 21(5.6) | 1(1.4) | 12(2.4) | 12(2.4) | 13(2.4) |

a Annealing position within the corresponding open reading frame (from the base A of start codon ATG). b Y = T or C; R = A or G; S = G or C; D = A or G or T. c This primer pair was previously described.

Figure 4. Detection Rate of β-lactamase genes and MDR/XDR/PDR.

Figure 5. Detection Rate of Plasmid Replicons and MDR/XDR/PDR.
importantly, the expression of β-lactam genes depends upon the environmental conditions such as the presence of antibiotics and genes presence shown by PCR does not necessarily indicate its expression.

Plasmids are extra-chromosomal circular fragments of DNA that replicate autonomously in E. coli. Plasmids appear to increase bacterial genetic diversity, containing genes essential for initiation and control of replication and accessory genes that may be useful to their bacterial host such as antimicrobial resistance or virulence genes [41]. Since the origin of replication is a constant and conserved part of a plasmid, replicon typing focused on this portion of the plasmid is a more sensitive and specific method for identifying phylogenetically related plasmids than restriction-based analysis of the entire plasmid. The classification of plasmid is usually based on PCR-based replicon typing (PBRT) method which relied on the phenomenon of incompatibility that closely related plasmids cannot coexist stably in the same cell [42].

Plasmids are significantly correlated with bacterial pathogenicity. IncF1 (pEM4) and IncI (pEM6) are found to determine virulence and colicine I production and resistance to tetracycline [43]. RNAI, a small countertranscript RNA which can inhibit translation of repA mRNA, expressed the incompatibility of IncI plasmids in the PBRT scheme. IncI plasmids generate both a thick pilus (tra genes) for DNA transfer and a thin pilus (pil genes) that appears to stabilize the mating apparatus in liquid media but not on solid surfaces, which is reported to result in different conjugation efficiencies and biofilm and/or adherence properties [44,45]. ESBL and plasmid-mediated (p) AmpC genes have been described on IncI plasmids in E. coli. bla\textsubscript{CTX-M-1} is the most often identified gene on IncI plasmid, followed by bla\textsubscript{CMY-2} and bla\textsubscript{CTX-M-15} [46]. RepA, broadly distributed throughout the low G + C Gram-positive bacteria, is the target site of IncA/C, IncN, IncF and IncT plasmid. In the PBRT (PCR-based replicon typing) scheme. Incompatibility group IncA/C plasmids are large, low copy plasmids that associated with enteric pathogens of humans and animals [47,48] IncA/C plasmids are associated with MDR and can encode ESBLs (bla\textsubscript{TEM}, bla\textsubscript{SHV}, but rarely bla\textsubscript{CTX-M}), AmpC (bla\textsubscript{CMY}, bla\textsubscript{DHA}), carbapenemases (bla\textsubscript{OXA}, bla\textsubscript{NDM}, bla\textsubscript{IMP}) and enzymes modifying groups of antibiotics: sulfonamides (sul1, sul2), aminoglycosides (aphA1, aadA, aadB, strA, strB, aacC), tetracyclines, chloramphenicol (floR, catA1) and trimethoprim (dfrA) [49,50]. IncN plasmids carry a great variety of resistance determinants against ESBL, sulfonamides, quinolones, aminoglycosides, tetracyclines and streptomycin [51]. The most frequently described resistance genes on IncF plasmids are ESBL genes (genes encoding carbapenemases, aminoglycoside-modifying enzymes and plasmid-mediated quinolone resistance (PMQR) genes). IncF plasmids also drive the spread of bla\textsubscript{NDM} and the rmtB gene (mostly reported in China) [52]. IncT plasmids usually carry kanamycin- (RstI) or sulfonamide resistance genes [53]. ParA-parB is the target site for typing IncHI plasmids in the PBRT scheme, which has been reported to contain large virulence plasmid pWR100 [54]. IncH plasmids are reported to be associated with multidrug resistance because, besides ESBL genes, they often carry genes encoding for resistance to sulfonamides, aminoglycosides, tetracyclines and streptomycin [55]. IncP is a group of broad-host-range, low-copy-number plasmids, the copy number of which is controlled by iterons [56]. A recurring theme in the duplication of prokaryotic replications is the recognition of the replication origin by cis-encoded initiators that bind to repeated nucleotide sequences called iterons. They were reported to carry genes conferring resistance to ESBL, sulfonamides, aminoglycosides and tetracyclines [57,58]. Ori\textsubscript{Y} is the target site of IncX, a group of narrow-host-range plasmids encoding primarily AMR determinants against ESBL and quinolones. These plasmids encode primarily AMR determinants against ESBL and quinolones [59,60]. Its six known subtypes (X1-X6) were relevant to tetracycline and trimethoprim resistance determinants. IncX plasmids were able to form cointegrants with
Salmonella serotype-specific plasmid-carrying virulence genes which resulted in a broadening of the host range of the new plasmid [61].

5. Conclusions

This study had designed and developed multiplex amplification platform for rapid and accurate detection of such resistance genes in 542 clinical E. coli isolates. It was found that most MDR isolates were co-resistant to penicillins (AMP) and fluoroquinolones (LVX, CIP) and distribution of LVX and CIP resistance was significantly higher among female than male gender. Among the β-lactamase genes, blaOXA acquired the highest carriage rate, followed by group 2 blaCTX-M and blaSHV-1, indicating their prevalence among clinical E. coli in South China. The RNAI (AY234375) showed the highest detection rate, followed by the iteron (J01724) and repA (M26308), indicating the relatively higher carriage rate of corresponding plasmids by clinical E. coli isolates. A high percentage of the isolated E. coli strains were multidrug resistant (MDR) to penicillins: ampicillin, aminoglycosides, and fluoroquinolones; and are harboring the blaOXA-1, group 2 blaCTX-M, and blaSHV-1 genes, as well as RNAI(AY234375)/repA (AE006471), iterons (J01724) genes. It is shown that such identification of plasmid replicons and β-lactamase genes may aid in the understanding of clinical E. coli isolates epidemiology. A strategy design based on multiplex amplification and further application for rapid detection on antimicrobial resistance and β-lactam resistance genes in clinical Escherichia coli strains.

Data availability

All data generated or analyzed during this study are included in this article.

The authors confirm that the data supporting the findings of this study are available within the article

Disclosure statement

No potential conflict of interest was reported by the author(s).

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