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A novel multidrug resistance plasmid isolated from an *Escherichia coli* strain resistant to aminoglycosides

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Objectives: Previous studies have reported several different plasmids that confer multidrug resistance (MDR) including resistance to aminoglycosides. In this study, we investigated the aminoglycoside resistance patterns for 224 *Escherichia coli* isolates from diseased chickens and ducks in China, characterized a novel MDR plasmid, and collected prevalence data on similar resistance plasmids.

Methods: Antibiotic susceptibilities were determined using disc diffusion and the microdilution method. The plasmid pXZ was analysed by restriction fragment length polymorphism (RFLP) with EcoRI and SalI, and sequenced. The prevalence of similar resistance plasmids was assessed by multiplex PCR and by RFLP analysis.

Results: Among the 224 *E. coli* isolates, 189 (84.4%) were resistant to streptomycin, 125 (55.8%) were resistant to kanamycin, 116 (51.8%) were resistant to gentamicin, 106 (47.3%) were resistant to neomycin and 98 (43.8%) were resistant to amikacin. Among the 224 *E. coli* isolates, 17 contained a plasmid with the MDR-encoding region of pXZ, which showed high-level resistance to aminoglycosides (MICs of gentamicin and amikacin $\geq$512 mg/L). The plasmid pXZ was digested into five fragments by EcoRI and six fragments by SalI. The plasmid pXZ was a circular DNA molecule of 76 635 bp with a 51.65% guanine+cytosine content and included four resistance genes (*rmtB*, *fosA3*, $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-24}}$).

Conclusions: A novel MDR plasmid, pXZ, harbouring four resistance genes (*rmtB*, *fosA3*, $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M}}$) was identified. To our knowledge, this is the first report of an aminoglycoside resistance plasmid harbouring the *fosA3* gene.

Keywords: avian *Escherichia coli*, aminoglycoside resistance, $bla_{\text{CTX-M}}$ gene, *fosA3* gene

Introduction

Aminoglycosides are broad-spectrum antibacterials to which some bacteria have acquired resistance. The primary mechanism of resistance to aminoglycosides is an enzymatic modification of the drug by different classes of enzymes. To date, six plasmid-encoded 16S rRNA methylases, including ArmA, RmtA, RmtB, RmtC, RmtD and NpmA, have been identified in Gram-negative bacilli isolated from different countries.[1] In China, the *rmtB* gene is the most prevalent 16S rRNA methylase gene among Enterobacteriaceae isolates.[1–3]

Bacterial resistance plasmids constitute an important part of the so-called horizontal gene pool that can be acquired by various bacterial strains by means of horizontal gene transfer.[4] Different plasmids that produce extended-spectrum β-lactamases (ESBLs)[5] and 16S rRNA methylases[6,7] have been found in *Escherichia coli*. Data regarding multidrug resistance (MDR) plasmids that can confer resistance to the majority of β-lactams and aminoglycosides are lacking for China. The objective of the present study is to characterize an MDR plasmid from an *E. coli* isolate, which mediates resistance to β-lactams, fosfomycin and aminoglycosides, and to detect the prevalence of similar resistance plasmids in 224 *E. coli* isolates by designing a multiplex PCR assay.

Materials and methods

Strains and growth conditions

E. coli ATCC 25922 was originally purchased from ATCC, maintained in our laboratory and used as a control in this study. During March 2003 to October 2010, a total of 224 *E. coli* isolates were isolated from the

livers of sick chickens and ducks in different geographic locations in China. All isolates were identified using standard biochemical tests and commercial typing antiserum according to the manufacturers' instructions.

All *E. coli* strains were grown in Luria–Bertani (LB) medium at 37°C; selective LB plates were made by adding amikacin (1024 mg/L) to normal LB plates.

Antibiotic susceptibility testing assay

Several assays for testing antibiotic susceptibility were conducted. According to the criteria recommended by the CLSI, the disc diffusion method was employed to test the antimicrobial susceptibility of the 224 *E. coli* isolates to different antibiotics, including kanamycin, streptomycin and neomycin. The MICs of amikacin and gentamicin for the *E. coli* isolates were determined by the microdilution method according to CLSI guidelines.

Identification and sequencing of aminoglycoside resistance plasmid

One of the 224 *E. coli* strains, isolated from the liver of a sick duck in Jiangsu Province, China in May 2008 and displaying a high degree of resistance to many aminoglycosides, was designated XZDC and chosen for the plasmid isolation. XZDC was cultured in LB medium containing amikacin (1024 mg/L), with shaking at 37°C. Plasmid preparation was undertaken using the Plasmid Miniprep Kit (Omega Ltd). The isolated plasmid, designated pXZ, was transferred to *E. coli* DH5α cells three times consecutively and then was analysed by restriction fragment length polymorphism (RFLP) with EcoRI and SalI. pXZ was sequenced using an ABI PRISM® 3730xl DNA Sequencer. DNA database searches were performed with the Basic Local Alignment Tool (BLASTn) and sequences were compared using DNAStar analysis programs (DNAStar Inc.).

Transfer of antibiotic resistance

A conjugation experiment was carried out to determine whether the antibiotic resistance of pXZ could be transferred. Transconjugants were selected on LB agar plates containing 1024 mg/L amikacin and analysed by RFLP as above when *E. coli* DH5α was used as the recipient. The antibiotic susceptibilities of DH5α strains with or without pXZ to a panel of antimicrobial drugs (shown in Table 1) were determined.

Determination of the occurrence of the MDR-encoding region of pXZ in E. coli clinical isolates

In order to investigate the occurrence of the MDR-encoding region of pXZ in the 224 *E. coli* isolates, three pairs of primers were designed according to the sequence of pXZ: *fosA3*-F, 5′-GCTGCAGGGATTGAATCATCT-3′; *fosA3*-R, 5′-AAGACCATCCCCTTGTAGG-3′; $bla_{\text{CTX-M-24}}$-F, 5′-GGTGACAAAG AGAGTGCAACG-3′; $bla_{\text{CTX-M-24}}$-R, 5′-ACAGTCCACGACGTCGGT-3′; $bla_{\text{TEM-1}}$-*rmt*B-F, 5′-GTGTCGCCCTTATTCCCTT-3′; and $bla_{\text{TEM-1}}$-*rmt*B-R, 5′-TTAT CCATTCTTTTTTATCAAGTATAT-3′. A multiplex PCR method was established and was performed in a final volume of 25 μL containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 2 mM dNTPs, 1 U of r*Taq*, 1 nM *FosA3*-F, *FosA3*-R, $bla_{\text{CTX-M-24}}$-F and $bla_{\text{CTX-M-24}}$-R, 2 nM $bla_{\text{TEM-1}}$-*rmt*B-F and $bla_{\text{TEM-1}}$-*rmt*B-R, and 1 μL of plasmid DNA (0.05–50 ng). The cycling conditions were: 94°C for 5 min, 32 cycles at 94°C for 50 s, 50°C for 50 s, 72°C for 2 min and then 72°C for 10 min.

All the isolates positive by the multiplex PCR were cultured in LB medium containing amikacin (1024 mg/L) at 37°C. Then, the plasmids were extracted, transferred and analysed by RFLP as for pXZ. The $bla_{\text{CTX-M}}$ genes in the plasmids were amplified with the primers $bla_{\text{CTX-M}}$-F (5′-ATGGTGACAAAGAGAGTGCAA-3′) and $bla_{\text{CTX-M}}$-R (5′-TTACAGCCCTTCG GCGATGATTC-3′). All the DNA sequences of the PCR products were determined and analysed using the same method and tools as described above.

Results

Among the 224 *E. coli* isolates, 189 (84.4%) were resistant to streptomycin, 125 (55.8%) were resistant to kanamycin, 116

Table 1. Antimicrobial resistance of DH5α strains with or without the pXZ plasmid

Antibiotic	Content of drug-sensitive slip (μg/slip)	Diameter of inhibitory bacterium zone (mm)	
		DH5α without pXZ	DH5α with pXZ
Amikacin	30	31	6
Gentamicin	10	30	6
Kanamycin	30	29	6
Streptomycin	10	25	20
Neomycin	30	26	23
Spectinomycin	100	24	22
Cefotaxime	30	23	6
Ceftriaxone	30	21	6
Cefalotin	30	19	6
Cefalexin	30	17	6
Cefradine	30	16	6
Cefoperazone	75	21	10
Piperacillin	100	22	6
Ampicillin	10	16	6
Amoxicillin	10 IU	13	6
Carbenicillin	100	28	10
Benzylpenicillin	10 IU	6	6
Fosfomycin	50	28	6
Ciprofloxacin	5	23	16
Pefloxacin	10	28	23
Lomefloxacin	10	22	21
Norfloxacin	10	19	19
Levofloxacin	5	6	6
Ofloxacin	5	6	6
Clarithromycin	15	20	17
Erythromycin	15	12	11
Azithromycin	15	26	26
Roxithromycin	15	6	6
Polymyxin B	300 IU	13	13
Bacitracin	0.04 IU	6	6
Vancomycin	30	6	6
Clindamycin	2	6	6
Chloramphenicol	30	20	20
Rifampicin	5	10	10
Tetracycline	30	16	16
Nitrofurantoin	300	16	15
Furazolidone	300	20	20
Sulfamethoxazole	300	17	15
Sulfamethoxazole/ trimethoprim	23.75/12.25	6	6

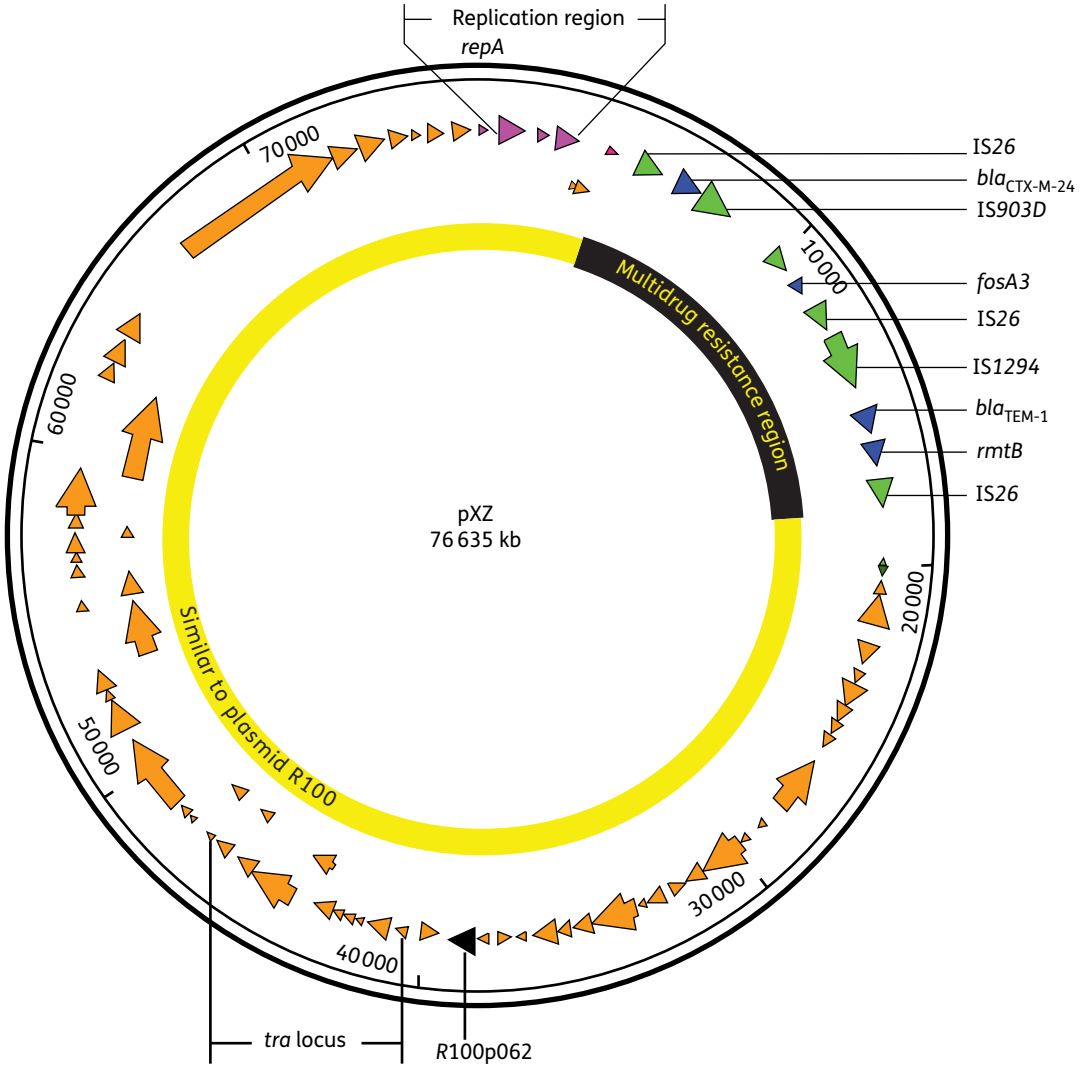

Figure 1. Circular map of plasmid pXZ. Open reading frames are colour coded as follows: pink, associated with replication; green, transposition-related elements; orange, function protein and integron region; blue, associated with antimicrobial resistance; black, hypothetical protein. The arrows show the direction of transcription. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

(51.8%) were resistant to gentamicin, 106 (47.3%) were resistant to neomycin and 98 (43.8%) were resistant to amikacin. Forty-six isolates displayed a very high level of resistance to gentamicin (MIC ≥512 mg/L), 32 isolates displayed a very high level of resistance to amikacin (MIC ≥512 mg/L), and 26 isolates displayed a very high level of resistance to both gentamicin (MIC ≥512 mg/L) and amikacin (MIC ≥512 mg/L).

pXZ was extracted from *E. coli* XZDC and analysed by restriction enzyme digestion and sequencing. The result of the RFLP showed that pXZ was digested into five fragments by EcoRI and six fragments by SalI (data not shown). pXZ was a circular DNA molecule of 76635 bp with a 51.65% guanine+cytosine content, and contained two genetically and physically distinguishable components: a 62 kb region essentially homologous to the non-R-determinant region of plasmid R100 except for several point mutations; and a 15 kb resistance (R-determinant) component (Figure 1). Four resistance genes were found in the resistance component: the *rmtB* gene (pos. 16341…17097), encoding the 16S rRNA methylase RmtB that mediates resistance to aminoglycosides (the numbering of pXZ commences at the first nucleotide of the ATG start codon of *repA*, a gene predicted to encode a replication protein); the *fosA3* gene (pos. 10938…11354) encoding the FosA3 enzyme that mediates fosfomycin resistance; and two β-lactamase genes, bla_{TEM-1} (pos. 15311…16171) and $bla_{CTX-M-24}$ (pos. 6219…7194), encoding ESBLs that mediate resistance to β-lactams. The annotated sequence of pXZ has been submitted to GenBank and assigned accession number JF927996.

To determine whether the antibiotic resistance of *E. coli* isolates carrying plasmid pXZ could be transferred to an antibiotic-susceptible strain, conjugation experiments were carried out in LB medium using *E. coli* DH5α as the recipient. The results showed that the MICs of amikacin and gentamicin for the DH5α strain carrying pXZ were >1024 and 512 mg/L,

respectively, while for DH5α without pXZ they were only 2 and 0.5 mg/L, respectively. Compared with the DH5α strain without pXZ, the DH5α strain with pXZ significantly was resistant to aminoglycosides, β-lactam antibiotics and fosfomycin, while the DH5α strains with and without pXZ displayed similar susceptibility to the other drugs (Table 1). The results suggested that plasmid pXZ conferred resistance to multiple antibiotics in *E. coli*.

Using the optimized multiplex PCR and RFLP, 17 of the 224 *E. coli* isolates were found to be positive for plasmid with the MDR-encoding region of pXZ. Among the 17 isolates, 16 were positive for the $bla_{CTX\text{-}M\text{-}24}$ gene and 1 for the $bla_{CTX\text{-}M\text{-}14}$ gene (GenBank accession numbers JQ003799–JQ003815). All the 26 *rmtB*-positive *E. coli* isolates confirmed by the multiplex PCR showed a very high level of resistance to aminoglycosides (gentamicin and amikacin MICs ≥512 mg/L).

Discussion

In the present study, we sequenced the multiplex resistance plasmid pXZ and detected the prevalence of similar resistance plasmids. Through sequence analysis, it was found that pXZ contained four resistance genes (*rmtB*, *fosA3*, $bla_{TEM\text{-}1}$ and $bla_{CTX\text{-}M\text{-}24}$). In an earlier study, a high prevalence of the plasmid-mediated *rmtB* gene was found among clinical *E. coli* isolates (36/680) from a Chinese teaching hospital.[3] In this paper, we found that the *rmtB* gene has a higher prevalence in avian *E. coli* isolates (26/224) in China and that RmtB conferred high-level resistance to aminoglycosides in avian *E. coli*. A recent study showed that $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}24}$ and $bla_{CTX\text{-}M\text{-}15}$ were co-transferred with *rmtB* on plasmid to recipients.[3] Similar to previous studies,[3,6,8] besides the *rmtB* gene, the $bla_{TEM\text{-}1}$ and $bla_{CTX\text{-}M\text{-}24}$ genes were found to be located on plasmid pXZ. On the quinolone resistance plasmid pKP96, it has been found that the fosfomycin resistance gene *fosA* co-associated with the $bla_{CTX\text{-}M\text{-}24}$ gene.[9] In this study, the *fosA3* gene was first found harboured on the aminoglycoside resistance plasmid. Cottell *et al*.[10] discovered the plasmid pCT that carried a single resistance gene, $bla_{CTX\text{-}M\text{-}14}$, and detected pCT-like plasmids with a series of PCRs that amplified the characteristic region of pCT10. Plasmids with the MDR-encoding region of pXZ were detected in 17 *E. coli* isolates by multiplex PCR and RFLP, which indicated that this type of plasmid harbouring four resistance genes (*rmtB*, *fosA3*, $bla_{TEM\text{-}1}$ and $bla_{CTX\text{-}M}$) has a high prevalence (17/224) among avian *E. coli* isolates in China.

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Transparency declarations

None to declare.

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