**INTRODUCTION**

Aminoglycosides comprise one of the key classes of antimicrobial agents in the treatment of infections caused by Gram-negative bacteria. However, exogenously acquired 16S rRNA methylase genes, including armA, rmtA–rmtH and nmpA, which are responsible for a very high level of resistance to various aminoglycosides, are widely distributed among Enterobacteriaceae, posing a serious threat to the clinical use of these important antimicrobials (van Hoek et al., 2011; O’Hara et al., 2013). The armA and rmtB genes are the predominant 16S rRNA methylase genes in Enterobacteriaceae in North America, with rmtD in Latin America, and armA in Europe (Fritsche et al., 2008). The positive rate for the rmtB gene was found to be much lower among Klebsiella pneumoniae and Proteus mirabilis in humans in Greece (Galani et al., 2012). In China, the rmtB gene is highly prevalent among Enterobacteriaceae strains from humans (Wu et al., 2009; Yu et al., 2010; Yang et al., 2011), and is also the most widespread among Enterobacteriaceae strains from pigs, chickens and companion animals (Chen et al., 2007; Du et al., 2009; Deng et al., 2011; Sun et al., 2012). The rmtB gene is located between the Tn2 transposon and insertion elements such as IS26, ISCR1 and ISCR3 (Doi et al., 2008; Du et al., 2009). Recently, the rmtB gene has often been found to coexist with blac_{TEM,1} and fosA3 (Hou et al., 2012; Lee et al., 2012; Sun et al., 2012; Pan et al., 2014), which could pose a threat to animal husbandry and public health. The dissemination of plasmids carrying rmtB has frequently been observed to be associated with the IncF and IncA/C incompatibility groups (Kang et al., 2008; Yu et al., 2010; Hou et al., 2012), whereas IncI1 plasmids harbouring rmtB are scarce. The aim of this study was to characterize the IncI1 plasmid carrying rmtB from avian Escherichia coli strains.

**METHODS**

**Bacterial strains.** In September 2012, eight non-duplicate E. coli strains were isolated from the livers of sick chickens on a traditional farm in Henan province, China. Each isolate was collected from a single chicken. All isolates were identified using a VITEK 32 automated identification system (bioMérieux) and designated EC001–EC008. The rifampicin-resistant E. coli strain C600 was used as the recipient in conjugation experiments and E. coli DH5α was used for the general cloning experiment and transformation experiments. E. coli ATCC 25922 and K. pneumoniae ATCC 700603 were used as the quality-control strains.
Antimicrobial susceptibility testing and confirmation of extended-spectrum β-lactamases (ESBLs). Antibiotic susceptibility was determined as the MIC using the broth microdilution method, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013). The following antimicrobial agents were used: ampicillin, cefotaxime, gentamicin, kanamycin, amikacin, enrofloxacin, ciprofloxacin, doxycycline and florfenicol (China Institute of Veterinary Drugs Control). Production of ESBLs was confirmed with a double-disc synergy test, according to the CLSI recommendations (CLSI, 2013).

Detection of 16S rRNA methylase genes. In order to investigate the presence of genes encoding 16S rRNA methylase (armA, rmtA–rmtH and npmA), PCR was performed for those strains displaying high-level resistance to amikacin, and the corresponding primers were used as described previously (O’Hara et al., 2013; Pan et al., 2013). The positive amplicons were sequenced on both strands directly using an ABI Prism3730 DNA analyser (Applied Biosystems), and the nucleotide sequences were compared using a BLAST procedure (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Epidemiological typing. Multiplex PCR was performed to determine whether these strains were commensal (phylogroups A and B1) or associated with phylogenic groups exhibiting extra-intestinal virulence (phylogroups B2 and D), as described previously (Clermont et al., 2000). The epidemiological relationships among the eight strains were analysed using enterobacterial repetitive intergeneric consensus sequence (ERIC) PCR, as described previously (Versalovic et al., 1991). Seven housekeeping genes (adk, fumC, gyrB, icd, mdh, purA and recA) were amplified and sequenced as described previously (Wirth et al., 2006). The sequences were analysed further by multilocus sequence typing (MLST) according to the protocols recommended at http://mlst.warwick.ac.uk/mlst/dbs/Ecoli.

Conjugation experiments, transformation experiments and PCR-based replicon typing. The conjugation experiments were performed as described previously (Pan et al., 2013). Briefly, the eight strains were inoculated separately into Luria–Bertani broth and incubated at 37°C for 12 h. Subsequently, the strains were mixed at a ratio of 1:10 (by volume) and incubated at 37°C. After 4 h incubation, 0.1 ml mixed culture was plated onto MacConkey agar (Beijing Land Bridge Technology Co.) supplemented with 450 μg rifampicin ml⁻¹ and 20 μg amikacin ml⁻¹. The transformation experiments were carried out using E. coli DH5α as the recipient strain when conjugation failed. The transformants were selected on Luria–Bertani agar plates supplemented with 20 μg amikacin ml⁻¹. Transconjugants and transformants were selected and tested for antimicrobial susceptibility by the broth microdilution method. The presence of the rmtB gene in transconjugants and transformants was verified by PCR. The plasmid incompatibility groups of these trans-conjugants and transformants were typed by a PCR-based method as described previously (Carattoli et al., 2005). All IncI1-positive plasmids were selected for further investigation. Five genes (repl, ard, trbA, sogS and pilL) from the IncI1 plasmid were amplified and sequenced as described previously (García-Fernández et al., 2008). They were analysed by plasmid MLST according to the protocols recommended at http://pubmlst.org/plasmid/.

Determination of the flanking regions of the rmtB gene. To elucidate the genetic environment of the rmtB gene, plasmid DNA

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Phylogroup</th>
<th>MLST</th>
<th>pMLST (IncI1)</th>
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<tr>
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AM, ampicillin; AMK, amikacin; CIP, ciprofloxacin; CTX, cefotaxime; DOX, doxycycline; ENR, enrofloxacin; FFC, florfenicol; GEN, gentamicin; KAN, kanamycin.
was extracted from transconjugants TEC001–TEC008 using a Qiagen Plasmid Mini kit (Qiagen), and was used to clone and amplify the flanking regions of the \textit{rmtB} gene, designated pEC001–pEC008. A \textit{Bam}HI-digested fragment containing the \textit{rmtB} gene derived from pEC008 was cloned into the pBluescript II SK(2) (Stratagene) and then sequenced by PCR walking. According to the partial sequence of pEC008, the flanking regions of the \textit{rmtB} gene were amplified for other plasmids using two pairs of primers (\textit{IScfr1}-F: 5'-TGGGGTG-GCGGGAGATGA-3', and \textit{rmtB}-R: 5'-TTCCACGCCCCCTAAAACCT-3'; and \textit{rmtB}-F: 5'-ATCAACGATGCCCTACCC-3', and \textit{qacE}Δ1-F: 5'-TCGCAACATCCGCATTAAAA-3') as described previously (Eckert et al., 2006; Pan et al., 2013).

RESULTS AND DISCUSSION

Antibiotic susceptibility, genotype analysis and genetic relatedness

All strains exhibited high-level resistance to ampicillin, gentamicin, kanamycin, amikacin, enrofloxacin and ciprofloxacin. Two strains were also resistant to doxycycline and florfenicol; however, they were all susceptible to cefotaxime (Table 1). Screening of the 16S rRNA methylase genes showed that all strains harboured the \textit{rmtB} gene. The results of confirmatory testing of the ESBLs showed that the eight strains were non-ESBL producers. ERIC-PCR was successfully carried out for these eight strains, which were grouped into two clonal patterns. Six strains belonged to one ERIC pattern, while the other two strains showed a different pattern (Fig. S1, available in the online Supplementary Material). Phylogenetic grouping and MLST demonstrated that two of these strains belonged to phylogroup D, sequence type (ST) D-ST117 and the other strains belonged to A-ST156 (Table 1). This was consistent with the results from the ERIC-PCR. D-ST117 is reported to be a lineage usually associated with avian infection and clinical disease (Bergeron et al., 2012), and the presence of \textit{rmtB}-bearing \textit{E. coli} ST117 will accelerate its dissemination.

Transfer of aminoglycoside resistance and plasmid replicon typing

The high-level resistance to amikacin in five strains was found to be transferable. The other three strains were successfully transformed by electroporation. The MIC results revealed that the transconjugants and transformants displayed a higher level of resistance to kanamycin, amikacin and gentamicin compared with the recipient, but were more susceptible to cefotaxime, enrofloxacin and

\begin{figure}
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\includegraphics[width=\textwidth]{fig1}
\caption{Major structural features of plasmid pEC008 compared with those of IncL/M-type plasmids pCTX-M3 (GenBank accession no. AF550415), pNDM-HK (GenBank accession no. HQ451074) and pE11573 (GenBank accession no. JX101693), and the IncFII-type plasmid pGUE-NDM (GenBank accession no. JQ364967). Similar regions are indicated by grey shading.}
\end{figure}
ciprofloxacin compared with the WT strains (Table 1). The plasmid replicon typing results revealed that all plasmids in the transconjugants and transformants belonged to the incompatibility group IncI1, which was further assigned to ST136 (Table 1). This indicated that both horizontal gene transfer and clonal spread were responsible for the dissemination of the rmtB gene in the E. coli strains in this study. The prevalence of plasmids carrying rmtB has frequently been observed to be associated with the incompatibility group F (IncF) in humans and other animals in China (Yu et al., 2010; Hou et al., 2012), and with IncA/C in humans and IncF in companion animals in Korea (Kang et al., 2008; So et al., 2012). However, all rmtB-carrying plasmids in the present study belonged to IncI1. To the best of our knowledge, this is the first identification of an rmtB-carrying IncI1 ST136 plasmid.

Genetic environment of the rmtB gene

A BamHI-digested fragment containing the rmtB gene derived from pEC008 was successfully cloned into pBlueScript II SK(−) and sequenced. The DNA sequence analysis showed that there was a class 1 integron cassette array (intI1-dfrA12-orfF-aadA2-qacEΔ1-sul1) and an insertion sequence ISCR1 on the downstream side of the rmtB gene in plasmid pEC008. Upstream of the rmtB gene, the structure blatem-1-IScrf1-aacC2-IS26 was found, which has also been reported in the IncL/M plasmid pCTX-M3, pNDM-HK and pE11573, isolated from Citrobacter freundii, E. coli and Enterobacter cloacae, respectively (Golebiowski et al., 2007; Ho et al., 2011; Partridge et al., 2012) (Fig. 1). According to the partial sequence of pEC008 that we determined, amplicons of the same length were successfully produced using the two pairs of primers (IScrf1-F/rmtB-R and rmtB-F/qacEΔ1-F), indicating that the genetic environment of the rmtB gene from pEC001–pEC008 was the same. In this study, the rmtB gene was located between the Tn2 transposon and an ISCR1 element. Usually located downstream of a class 1 integron cassette array, ISCR1 is responsible for the mobilization of many classes of antibiotic resistance genes, including catAII, dfrA, armA, blacMY, blachBLA1, blactXM, blacbEB3, bladbPER1 and qnr genes located in complex class 1 integrons (Toleman et al., 2006). In our previous study, a 4.6 kb EcoRI-digested fragment (GenBank accession no. FJ556900) was obtained, which only covered the partial genetic environment of the rmtB gene (Du et al., 2009). In the present study, we obtained an 18.7 kb BamHI-digested fragment carrying the IScrf1 element that was located immediately upstream of blatem-1-rmtB, rather than a 2.9 kb BamHI-digested tmpr-Blam-1-rmtB-IS26 fragment, as reported previously (Doi et al., 2008). Although the number of strains studied was small, the results suggest that the IncI1 ST136 plasmid may be one of the more important plasmids carrying the rmtB gene. Therefore, attention should be paid to the IncI1 ST136 conjugative plasmid carrying the rmtB gene, the class 1 integron cassette array (intI1-dfrA12-orfF-aadA2-qacEΔ1-sul1) and aacC2.

In conclusion, this study identified a new rearrangement of a multidrug-resistant region carrying rmtB, a class 1 integron cassette array (intI1-dfrA12-orfF-aadA2-qacEΔ1-sul1) and aacC2 on a conjugative IncI1 ST136 plasmid in an avian E. coli isolate. Both the Tn2 and ISCR1 element may participate in mobilization of the rmtB gene. Co-location of the rmtB gene with the class 1 integron cassette array and aacC2 on the conjugative plasmid may facilitate its maintenance and dissemination, reflecting a sophisticated response to antibiotic pressure.

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