A multidrug-resistance region containing bla<sub>CTX-M-65</sub>, fosA3 and rmtB on conjugative IncFII plasmids in Escherichia coli ST117 isolates from chicken

The bla<sub>CTX-M</sub> gene has become the most common gene encoding extended-spectrum β-lactamase (ESBL) in multidrug-resistant Gram-negative bacteria worldwide. To date, 147 subtypes of the CTX-M enzyme have been reported (http://www.lahey.org/Studies/other.asp#table1). Since 2007, bla<sub>CTX-M-65</sub> has become one of the predominant bla<sub>CTX-M</sub> genes in ESBL-producing bacterial isolates from animals in China (Yuan et al., 2009; Deng et al., 2011). The bla<sub>CTX-M</sub> genes often coexist with genes encoding 16S rRNA methylases such as armA and rmtB, or with fosA3 that confers resistance to fosfomycin (Hou et al., 2012; Lee et al., 2012; Ho et al., 2013). In China, rmtB is the most prevalent 16S rRNA methylase gene among the Enterobacteriaceae.

Plasmids in which the four resistance genes bla<sub>TEM-1</sub>, bla<sub>CTX-M-65</sub>, fosA3 and rmtB coexist were recently found in Escherichia coli isolates from ducks and dogs in China (Sun et al., 2012; He et al., 2013). Mobile elements mobilize resistance genes. The IS<sub>Ecp1</sub>-bla<sub>CTX-M</sub>-IS<sub>903</sub>-iro<sup>N</sup> structure is a typical transposition unit in the bla<sub>CTX-M-9</sub> cluster, to which bla<sub>CTX-M-65</sub> belongs (He et al., 2013). The rmtB gene is commonly carried by the Tn3 transposon (Doi et al., 2004) and fosA3 has been found in the IS26-formed composite transposon (Lee et al., 2012). Since chicken was considered as a reservoir for extraintestinal pathogenic E. coli in humans (Bergeron et al., 2012), the detection of bla<sub>TEM-1</sub>, bla<sub>CTX-M-65</sub>, rmtB and fosA3 in an isolate of animal origin may represent an emerging threat to public health. In our present study, we characterized a multidrug-resistance region (MRR) of an F33-A::B-plasmid carrying bla<sub>TEM-1</sub>, bla<sub>CTX-M-65</sub>, rmtB and fosA3 that was isolated from an E. coli avian strain of the sequence type 117 (ST117).

In a survey on antimicrobial-resistant bacterial strains in China in 2009, an E. coli strain, EC011, was isolated from chicken in Changchun Province, North-east China. The species identification was performed using the VITEK 32 automated identification system (bioMérieux). Susceptibility tests to ampicillin, ceftazidime, cefotaxime, cefoxitin, amikacin, gentamicin, ciprofloxacin, levofloxacin, doxycycline and florfenicol were performed using the broth microdilution method, and were interpreted according to the recommendations by the Clinical and Laboratory Standards Institute (CLSI, 2010). The MIC of fosfomycin was determined using the agar dilution method on Mueller–Hinton agar containing 25 µg glucose 6-phosphate ml<sup>−1</sup> as described previously (Hou et al., 2012). E. coli strain ATCC 25922 was used as the control. The EC011 strain was resistant to ampicillin (MIC >256 µg ml<sup>−1</sup>), cefotaxime (MIC=64 µg ml<sup>−1</sup>), ciprofloxacin (MIC=16 µg ml<sup>−1</sup>), amikacin (MIC >256 µg ml<sup>−1</sup>), gentamicin (MIC >256 µg ml<sup>−1</sup>), doxycycline (MIC=64 µg ml<sup>−1</sup>), florfenicol (MIC=256 µg ml<sup>−1</sup>) and fosfomycin (MIC >256 µg ml<sup>−1</sup>). The presence of genes encoding ESBLs, 16S rRNA methylases and the plasmid-encoded fosfomycin resistance determinants were detected using PCR as described previously (Hou et al., 2012; Pan et al., 2013). Amplicons were sequenced using 3730xl DNA Analyser (Applied Biosystems). The EC011 strain had the bla<sub>TEM-1</sub>, bla<sub>CTX-M-65</sub>, rmtB and fosA3 genes. This isolate belonged to the phylogenetic group D as determined using multiplex PCR (Clermont et al., 2000), which has been associated with extraintestinal virulence. Multilocus sequence typing was performed according to the protocols recommended at http://mlst.ucc.ie/mlst/dbs/Ecoli and EC011 was assigned to ST117.

The conjugation experiments were performed using the rifampicin-resistant E. coli strain, C600, as the recipient. Transconjugants were selected on MacConkey agar supplemented with 4 µg cefotaxime ml<sup>−1</sup> and 450 µg rifampicin ml<sup>−1</sup>, and E. coli DH5α was transformed by electroporation using the plasmid DNA isolated from the transconjugants. The transformants were selected on Luria–Bertani agar supplemented with 4 µg cefotaxime ml<sup>−1</sup>. The MIC results revealed that both the transconjugants and the transformants were resistant to ampicillin, cefotaxime, amikacin, gentamicin and fosfomycin, which is consistent with the antimicrobial resistance profile of EC011.

Plasmid DNA was extracted from the transformants using the Plasmid Midi Kit (Qiagen), and the size of the undigested plasmid was estimated by comparison with E. coli V517 DNA using agarose gel electrophoresis. Gel electrophoresis revealed that the transformants contained only a single plasmid designated pEC011, which was approximately 70 kb in size. The PCR-based replicon typing performed as described previously (Carattoli et al., 2005) revealed that pEC011 had only an IncFII replicon. Further typing analysis using a previously described method (Villa et al., 2010) revealed that pEC011 was an F33-A::B– plasmid.

PCR mapping was performed to investigate the genetic context of the MRR on pEC011 with primers listed in Table 1. The nucleotide sequence of the pEC011 MRR was deposited in GenBank (accession no. KF537631). The pEC011 MRR was in a complex structure and was bounded by IS1294 at both ends as seen in pHN7A8 and pXZ (GenBank accession nos JN232517 and JF972996, respectively; Fig. 1). The MRR consisted of the IS26-αIS<sub>Ecp1</sub>-bla<sub>CTX-M-65</sub>-IS<sub>903D</sub>-iro<sup>N</sup>-IS26, IS26-fosA3-orf1A-IS1294 and AtnpR-bla<sub>TEM-1</sub>-rmtB-IS26 modules that might have been adjoined by three IS26 elements in the same direction and one IS1294 element (Fig. 1).
Table 1. Primers used for the PCR amplification of the MRR DNA sequences

<table>
<thead>
<tr>
<th>PCR target</th>
<th>Primer name</th>
<th>Nucleotide sequence (5’→3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upstream flanking region of pemK</td>
<td>CA1</td>
<td>ATGTCGCASACHGAAATGC*</td>
<td>3559</td>
<td>Osborn et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>pemK-R</td>
<td>TATGCCAACACCATCAACGA</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>peml-F</td>
<td>CAATTACTGCTGAGCTGTCAC</td>
<td>3191</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>blac_{CTX-M-65}$\text{.}^*{}$-R1</td>
<td>ACCTTACTCGTAGTCACAT</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>Upstream flanking region of rmtB</td>
<td>blac_{CTX-M-65}$\text{.}^*{}$-F</td>
<td>ATGGTGCAAGAGAGTGCA</td>
<td>9193</td>
<td>Pan et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>rmtB-R</td>
<td>TTCCAGCGCCGCTAAACT</td>
<td></td>
<td>Pan et al. (2013)</td>
</tr>
<tr>
<td>Downstream flanking region of rmtB</td>
<td>rmtB-F</td>
<td>ATCAAGTGCCTACCTCC</td>
<td>2019</td>
<td>Pan et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>IS1-R</td>
<td>TATGTCAGATAATGCGCAT</td>
<td></td>
<td>This study</td>
</tr>
</tbody>
</table>

*S= G or C; H = A or C or T.

A similar structure was recently found in the pH7A8 (F33:A-B-) and pXZ (F2:A-B-) plasmids in *E. coli* isolated from dogs (He *et al.*, 2013) and ducks (Sun *et al.*, 2012), respectively, in China. The MRR of the pH7A8 and pXZ plasmids were almost identical (Fig. 1), suggesting that a common MRR has been mobilized into different plasmids. The IS26-foxA3-orf1-orf2-orf3

**Fig. 1.** Structural features of plasmid pEC011 MRR compared to those of IncFII-type plasmids EC096TF (GenBank accession number JQ343850), pHK23a (GenBank accession number JQ432559), pXZ (GenBank accession number JF927996) and pH7A8 (GenBank accession number JN232517). Arrows indicate open reading frames and the arrowheads indicate the direction of transcription. The similar regions are indicated by grey shading, except for the segments of IS26-$\Delta$Ecp1-blac_{CTX-M-65}$\text{.}^*{}$-orf477-IS26 in pHK23a and IS26-$\Delta$Ecp1-blac_{CTX-M-65}$\text{.}^*{}$-iroN-IS26 in pXZ.
element in the MRR in our study exhibited greater variability than the IS26-foSA3-orf1-orf2A-IS26-IS1294 element. As a member of the IS91 family, the IS1294 element is an atypical insertion sequence that lacks terminal inverted repeats, transposes using rolling-circle replication, and does not duplicate the target nucleotide sequence (Tavakoli et al., 2000). The insertion-site (CAAG) footprint left by IS1294 reflects the deletion of orf1A-orf2A-IS26. Moreover, the clustering of these components of the IS26-AISEcp1-blaCTX-M-65-IS903D-iroN-IS26, IS26-AISEcp1-blaCTX-M-3-orf477-IS26 and IS26-foSA3-orf1-orf2A-orf3-IS26 modules were likely to be the result of homologous recombination between two copies of IS26 that generated the different MRR (Fig. 1) (Lee et al., 2012; Sun et al., 2012; He et al., 2013; Ho et al., 2013).

E. coli ST117 is not only an avian pathogenic E. coli but it can also cause clinical infection such as septicaemia and urinary tract infections in humans (Bergeron et al., 2012; Mora et al., 2012). Furthermore, chicken was considered as a reservoir of extraintestinal pathogenic E. coli in humans (Bergeron et al., 2012). In the present study, the strain EC011 from chicken carrying pEC011 (F33:A-:B-) belonged to ST117 and phylogenetic group D. As ST117 is a lineage associated with avian diseases and clinical infections, the introduction of a plasmid such as pEC011 carrying four resistant genes is significant and may represent a challenge for treatment and public health. It is worth noting that the strain HN7A8 from dog carrying pHN7A8 (F33:A-:B-) belonged to phylogenetic group B1, suggesting that the horizontal transmission of a multidrug-resistance plasmid may have occurred between commensal E. coli and pathogenic E. coli. Therefore, this conjugative plasmid from avian E. coli belonging to ST117, carrying four resistant genes conferring resistance to different antimicrobial agents, may be worrying.

In conclusion, we characterized a MRR on a conjugative plasmid that contained the blaTEM-1, blaCTX-M-65, foSA3 and rmtB antimicrobial resistance genes, which was isolated from an ST117 E. coli of avian strain. The mobile genetic elements IS26, IS1294 and Tn3 may have contributed to the evolution of the MRR through recombination. However, it is unclear whether the structure of the MRR was generated through classical transposition, one-ended transposition, homologous recombination or IS26-mediated co-integration. Further investigations of the mechanism responsible for generating this MRR are needed. The spread of IncFII plasmids with MRRs harbouiring blaTEM-1, blaCTX-M-65, rmtB and foSA3 among E. coli strains of animal origin may represent an emerging threat to public health through contact with or the consumption of infected animals.

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