Prevalence of tetracycline resistance genes and identification of tet(M) in clinical isolates of Escherichia coli from sick ducks in China

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Tetracycline resistance is one of the most frequently encountered resistance properties in bacteria of animal origin. The aim of the present study was to investigate the prevalence and diversity of tetracycline resistance (tet) genes among Escherichia coli clinical isolates from diseased ducks in China and to report the identification and sequencing of the tet(M) gene. The susceptibility of 85 Escherichia coli strains to tetracyclines was determined by broth microdilution, and the presence of tet genes was investigated by multiplex PCR. All of the 85 isolates were fully resistant to both oxytetracycline and tetracycline, and 76.5% were resistant to doxycycline. Seventy-seven of the isolates (90.6%) encoded multiple tet genes, with 17.6, 38.8 and 34.1% encoding two, three and four tet genes, respectively, and only 7.1% encoded a single tet(A) gene. The MICs of oxytetracycline and tetracycline for all isolates ranged from 16 to ≥128 μg ml⁻¹ with a MIC₉₀ of >128 μg ml⁻¹, regardless of the type or number of tet genes encoded. Isolates containing tet(M) commonly had more than one tet gene per strain. The doxycycline resistance rate in the tet(M)-positive isolates was significantly higher than in the tet(M)-negative isolates (P<0.05). A full-length tet(M) gene, including the promoter region, was obtained by PCR in seven of the 41 tet(M)-positive isolates and was sequenced and cloned. The cloned tet(M) gene conferred resistance to tetracyclines in the recombinant Escherichia coli host strain. These results revealed that, in these isolates, the prevalence of multiple tet genes was strikingly high and that tet(M) played a role in doxycycline resistance.

INTRODUCTION

As a result of the widespread use of tetracyclines for the treatment of various infections in humans, animals and plants, there has been a dramatic increase in the number of tetracycline-resistant bacteria (Chopra & Roberts, 2001). Tetracycline resistance in bacteria is mediated mainly by two mechanisms: drug efflux and ribosomal protection. Both resistance mechanisms are associated with mobile elements. Enzymatic inactivation can also confer resistance to tetracyclines. So far, 30 genes encoding efflux pumps, 12 genes encoding ribosomal protection proteins and three genes encoding an inactivating enzyme have been identified in bacteria (Thaker et al., 2010; Warburton et al., 2013).

Several studies have examined the prevalence and/or distribution of a large number of tetracycline resistance (tet) genes among Escherichia coli isolates, including non-clinical isolates from diverse human and animal species (Blake et al., 2003; Bryan et al., 2004; Karami et al., 2006), and clinical isolates from patients and diseased pigs, cattle and broiler chickens (Sengeløv et al., 2003; Tuckman et al., 2007). In contrast, data on the distribution of tet genes in Escherichia coli isolates from ducks are still relatively scarce.

The tet(M) gene, which imparts resistance to tetracyclines by encoding a ribosomal protection mechanism, is widely distributed among both Gram-negative and Gram-positive bacteria and has been found in 59 genera (Roberts, 2005; de Vries et al., 2009). However, only a limited number of studies have examined tet(M) in Escherichia coli strains from humans and animals (Schwaiger et al., 2010; Tuckman et al., 2007; Zhang et al., 2010) since the first report of the presence of tet(M) in Escherichia coli strains of human origin (Jones et al., 1992). The complete tet(M) sequence, including flanking regions, was not identified in clinical Escherichia coli isolates from humans until 2006.

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Abbreviations: ERIC, enterobacterial repetitive consensus; RAPD, random amplified polymorphic DNA.
(Jones et al., 2006). To date, neither a partial nor a complete sequence of the tet(M) gene has been reported in Escherichia coli isolates from ducks.

Doxycycline is a synthetic broad-spectrum antibiotic derived from oxytetracycline and is more active than tetracycline against many bacterial species. In veterinary medicine, it is used to treat infections in several animal species, such as ehrlichiosis or respiratory tract diseases in dogs, pneumonia in cattle and pigs, and colibacillosis and psittacosis in poultry. However, a high prevalence and widespread distribution of doxycycline-resistant Escherichia coli isolates in pigs and poultry in China and in meat and meat products in Korea have been found (Jiang et al., 2011; Koo & Woo, 2011). In this study, we investigated the prevalence of tet genes that encode tetracycline and possible doxycycline resistance, and report what we believe to be the first identification of full-length tet(M) genes among 85 Escherichia coli clinical strains isolated from sick ducks in China.

METHODS

Bacterial strains. Eighty-five non-duplicate Escherichia coli isolates were collected between February 2009 and September 2011 from liver samples of dead ducks at Henan Agricultural University Animal Hospital, Zhengzhou, PR China. All samples, which originated from 27 duck farms in five provinces in China, were isolated aseptically from liver swabs and seeded immediately in Mueller–Hinton agar. Each isolate was collected from a different duck. The isolates were identified as Escherichia coli using the VITEK-32 system (bioMérieux) and showed resistance to tetracycline according to the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (CLSI, 2009). Azide-resistant Escherichia coli DH5α was the host for cloning experiments.

Antimicrobial susceptibility testing. The susceptibilities of isolates to oxytetracycline, tetracycline and doxycycline were determined using broth microdilution according to CLSI guidelines (CLSI, 2009). Escherichia coli ATCC 25922 was used as a quality control in MIC determinations and Escherichia coli O73 (CVC1547) was used as a tetracycline-sensitive control. MIC determinations were repeated at least twice to ensure reproducibility.

Multiplex PCR detection of tet genes. All isolates were examined for the presence of the tet(A), tet(B), tet(C), tet(D), tet(E), tet(K), tet(L), tet(M) and tet(O) genes by multiplex PCR using previously described primers and conditions (Ng et al., 2001; Xu et al., 2011). Bacterial whole-cell lysates were used as a DNA template. The amplification products were separated by electrophoresis on a 1.0% agarose gel with ethidium bromide staining. Each PCR cycle contained corresponding positive controls and one negative control (PCR mix without template DNA). Escherichia coli strains DY16, A21 (identified by our laboratory), DJ14 and DJ23, harbouring tet(B), tet(A) + tet(C), tet(D) and tet(E) genes, respectively, and Enterococcus faecalis strains CQ11, CQ15, CQ27 and CQ34 (provided by Henan Province Key Laboratory of Animal Food Safety, Zhengzhou, PR China), harbouring tet(K), tet(L), tet(M) and tet(O) genes, respectively, were used as positive controls.

The representative positive amplicons for tet(M) were purified using a TIANgel Midi Purification kit (Tiangen), ligated into a pGEM-T Easy vector and then sequenced on both strands. The nucleotide and deduced amino acid sequences were analysed using DNASTAR (DNASTAR) and BLAST (http://www.ncbi.nlm.nih.gov).

PCR amplification of full-length tet(M), cloning and sequencing. The genetic environment of tet(M) was characterized by PCR assays using specific primers and sequencing. PCR mapping analysis was performed on genomic DNA from all the tet(M)-positive isolates. The forward primer UM-F based on GenBank accession no. U09422 for Tn916 from Enterococcus faecalis [located 600 bp upstream of tet(M)] or on GenBank accession no. DQ534550 from an Escherichia coli isolate [located 1400 bp upstream of tet(M)] together with a reverse primer internal to tet(M) (UM-R) was used to amplify the tet(M) upstream region. Primers CM-F and CM-R were used to amplify the 3’ end of the tet(M) gene. Primers DM1-F [internal to the tet(M) gene] and DM1-R [based on IS51 sequences 300 bp downstream of the Escherichia coli tet(M) gene] or DM2-F [internal to the tet(M) gene] and DM2-R [based on Tn916 sequences 200 bp downstream of the Enterococcus faecalis tet(M) gene] were used for the identification of the tet(M) downstream region. All positive results were confirmed by direct sequencing of both strands of amplification products. All primers used in this study are shown in Table 1.

Enterobacterial repetitive consensus (ERIC) PCR. The clonal relationship of the randomly selected isolates with tet(M) and other efflux gene combinations was assessed by studying ERIC-PCR genomic DNA profiles, generated using primer ERIC2 (Versalovic et al., 1991). Amplified PCR products were separated using 1.5% agarose gels and were visualized by UV transillumination. DNA fingerprints were compared by visual inspection. Random amplified polymorphic DNA (RAPD) patterns were regarded as different if there were different bands on visual inspection (Rasschaert et al., 2005; Johnson & O’Bryan, 2000; Li et al., 2008).

Statistical analysis. Statistical analysis was performed with SPSS version 11.5 (SPSS). Data were compared using a χ2 test or Fisher’s exact test. Correlation analysis was further carried out using Pearson correlation calculations when a significant difference was observed. A P value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Antimicrobial susceptibility analysis

All 85 Escherichia coli isolates were fully resistant to both oxytetracycline and tetracycline (MIC ≥16 μg ml⁻¹) and 65 (76.5%) were resistant to doxycycline. The 50 and 90%
MIC (MIC_{50}/MIC_{90}) values of oxytetracycline, tetracycline and doxycycline for the *Escherichia coli* isolates were 0/0, 128/128, 128/128 and 16/64 mg l^{-1}, respectively. The distribution of oxytetracycline, tetracycline and doxycycline MICs for the isolates are presented in Table 2. It was noteworthy that the resistance rate of the isolates to tetracyclines was strikingly high, which may in part be due to the frequent, heavy and long-term use of this antibiotic group for the control of bacterial infections in animals in China (Wei-hong *et al.*, 2006; Zhang *et al.*, 2010).

### Prevalence of tet genes

Of the 85 *Escherichia coli* isolates tested, 83 (97.6 %) carried at least one of the tet genes examined, with 83 (97.6 %), 67 (78.8 %), 60 (70.6 %) and 41 (48.2 %) isolates carrying tet(A), tet(B), tet(C) and tet(M), respectively, whereas two of the isolates (2.4 %) did not contain any tet genes (Table 3). None of the tested strains contained tet(D), tet(E), tet(K), tet(L) or tet(O). The most prevalent tet gene was tet(A). Although the tet(A) gene has been reported to be predominant in *Escherichia coli* isolates from animals in many countries (Lanz *et al.*, 2003; Maynard *et al.*, 2004; Roberts, 2005; Sengeløv *et al.*, 2003; Schwaiger *et al.*, 2010; Zhang *et al.*, 2010), it was still remarkable that, in this study, 97.7 % of the *Escherichia coli* isolates carried tet(A). We have therefore reported the highest prevalence, to the best of our knowledge, of tet(A) among tetracycline-resistant *Escherichia coli* and demonstrated that the tet(A) gene is highly endemic in China. Consistent with previous studies reporting that tet(B) is common in *Escherichia coli* isolates of animal origin (Schwaiger *et al.*, 2010; Bryan *et al.*, 2004; Zhang *et al.*, 2010), tet(B) was found to be very widespread in duck sources in this study. The tet(B) gene has also been found to be predominant in *Escherichia coli* isolates from humans (Tuckman *et al.*, 2007; Schwaiger *et al.*, 2010; Bryan *et al.*, 2004), as well as in *Escherichia coli* isolates from catfish in the USA (Nawaz *et al.*, 2009). The tet(C) gene, often detected in human strains, was also found to be common in duck strains. The tet(M) gene, a

### Table 2. Distribution of tetracycline, oxytetracycline and doxycycline MICs among the 85 *Escherichia coli* isolates

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. of samples with MIC (µg ml^{-1}) of:</th>
<th>MIC_{50}</th>
<th>MIC_{90}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>2</td>
<td>3</td>
<td>15</td>
</tr>
</tbody>
</table>
The frequency of *Escherichia coli* isolates from ducks with more than one *tet* gene was higher than in isolates from humans or other animals (Bryan et al., 2004; Lanz et al., 2003; Sengelov et al., 2003). In most previous studies, <40% of the *Escherichia coli* isolates carried multiple *tet* genes. One study of *Escherichia coli* O157:H7 isolates in the mid-western USA found that four (33%) of 12 human tetracycline-resistant isolates, with known *tet* genes, carried two different *tet* genes (Wilkerson et al., 2004). Bryan et al. (2004) found that >30% of *Escherichia coli* isolated from pigs, turkeys and horses in the USA carried two different *tet* genes and 4.5% of the pig isolates carried three different *tet* genes, whilst none of the *Escherichia coli* from deer, ducks, rats, cows or goats carried more than a single *tet* gene. Approximately 20% of the chicken *Escherichia coli* carried multiple *tet* genes, whilst >10% of the sheep and human isolates carried multiple *tet* genes. Schwaiger et al. (2010) reported that 10% of porcine *Escherichia coli* isolates and 17% of human isolates in Germany carried multiple different *tet* genes. Tuckman et al. (2007) reported that 33% of *Escherichia coli* clinical isolates from patients enrolled worldwide encoded more than one *tet* gene, with 2.5% of isolates encoding three or four *tet* genes. Therefore, these surveys indicate not only geographical variations but also variations in strain origins in the prevalence of multiple *tet* genes. In China, Zhang et al. (2010) reported that 27.8% of commensal *Escherichia coli* from clinically healthy chickens and pigs carried multiple *tet* genes. This study suggests that the frequency of multiple *tet* genes in *Escherichia coli* isolates from animals in China is increasing.

The MICs of oxytetracycline and tetracycline for all isolates ranged from 16 to \( \geq 128 \) \( \mu \text{g ml}^{-1} \) with a MIC\(_{90}\) of \( \geq 128 \) \( \mu \text{g ml}^{-1} \), regardless of the type or number of *tet* genes encoded or the presence of the *tet(M)* gene (Tables 2 and 3). Resistance rates to doxycycline ranged from 66.7 to 86.2% among the isolates with various numbers of *tet* genes and from 50 to 79.1% among isolates with a *tet(A)*, *tet(B)* or *tet(C)* gene and those without *tet(A)*, *tet(B)* or *tet(C)*, respectively, but the differences were not significant \((P>0.05)\). Isolates containing *tet(M)* commonly had more than one *tet* gene per strain. The *tet(M)* gene coexisted with *tet(A)*, *tet(A) + tet(B)* or *tet(A) + tet(B) + tet(C)*

### Table 3. Prevalence and distribution of *tet* genes in the *Escherichia coli* isolates showing various susceptibility patterns to tetracyclines

<table>
<thead>
<tr>
<th><em>tet</em> Gene pattern</th>
<th>No. strains</th>
<th>Antibiotic</th>
<th>MIC (( \mu \text{g ml}^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND 2 OXY</td>
<td></td>
<td>DOXY</td>
<td>32–128 NA NA</td>
</tr>
<tr>
<td>AB 5 OXY</td>
<td></td>
<td>TC</td>
<td>32–128 NA NA</td>
</tr>
<tr>
<td>AM 2 OXY</td>
<td></td>
<td>TC</td>
<td>128–128 &gt;128 &gt;128</td>
</tr>
<tr>
<td>ABC 23 OXY</td>
<td></td>
<td>TC</td>
<td>128–128 &gt;128 &gt;128</td>
</tr>
<tr>
<td>ABCM 29 OXY</td>
<td></td>
<td>TC</td>
<td>128–128 &gt;128 &gt;128</td>
</tr>
<tr>
<td>+ M 41 OXY</td>
<td></td>
<td>TC</td>
<td>128–128 &gt;128 &gt;128</td>
</tr>
<tr>
<td>− M 44 OXY</td>
<td></td>
<td>DOXY</td>
<td>128–128 &gt;128 &gt;128</td>
</tr>
</tbody>
</table>

rarer-occurring efflux gene in *Escherichia coli*, was found in 48.2% of the duck *Escherichia coli* isolates, and this detection rate was surprisingly high. Jones et al. (1992) detected *tet(M)* in three of 567 tetracycline-resistant *Escherichia coli*, which was the first report of this gene in strains of human origin. Bryan et al. (2004) were the first to report the presence of *tet(M)* in *Escherichia coli* isolated from pigs and chickens (<5%) but not from humans and other animals, whilst Schwaiger et al. (2010) found that 3% of porcine and 7% of human *Escherichia coli* isolates tested carried *tet(M)*.

The results of our studies also showed that 90.6% (77/85) of the strains encoded two or more *tet* genes (up to four genes). Among the 85 tetracycline-resistant isolates, 17.6% (15/85), 38.8% (33/85) and 34.1% (29/85) were found to encode two, three and four *tet* genes, respectively, and only 7.1% (6/85) encoded a single *tet(A)* gene. The distribution of multiple *tet* genes was as follows: the combination of *tet(A) + tet(B)* was found in five isolates, *tet(A) + tet(C)* in eight isolates, *tet(A) + tet(M)* in two isolates, *tet(A) + tet(B) + tet(C)* in 23 isolates, *tet(A) + tet(B) + tet(M)* in ten isolates and *tet(A) + tet(B) + tet(C) + tet(M)* in 29 isolates. None of the isolates were found to carry *tet(B), tet(C) or tet(M)* alone. To our knowledge, this is the first report not only documenting the presence of *tet(M)* in *Escherichia coli* clinical isolates from ducks but also describing *Escherichia coli* clinical isolates with *tet(A), tet(B), tet(C)* and *tet(M)* combinations.
genes. The doxycycline resistance rate in the tet(M)-positive isolates was significantly higher than in the tet(M)-negative isolates (36/41, 87.8 % versus 29/44, 65.9 %; \( P < 0.05 \)). Among the strains with doxycycline MICs of \( \leq 4,8,16,32 \) and \( \geq 64 \mu g ml^{-1} \), tet(M) was found in none (0/5), five (5/15, 33.3 %), 15 (15/35, 42.9 %), 13 (13/20, 65 %) and eight (8/10, 80 %) isolates, respectively. The calculated correlation between the presence of a tet(M) gene and the level of doxycycline resistance (MIC) was significant (\( P < 0.05 \)). These results suggested that the tet(M) gene may contribute to doxycycline resistance in the tested isolates.

Most tetracycline-specific efflux pumps confer resistance to tetracycline only. In contrast, tet(B) confers a wider spectrum of resistance to tetracyclines. The tet(M) gene imparts resistance to tetracycline, doxycycline and minocycline (Chopra & Roberts, 2001). There were 14 isolates that, although PCR positive for tet(B) and/or tet(M), showed intermediate resistance for doxycycline (MIC 8 \( \mu g ml^{-1} \)) and resistance to tetracycline and oxytetracycline (MIC \( \geq 64 \mu g ml^{-1} \)). These isolates encoded one or more additional tet genes: tet(A) or tet(A) + tet(C). Therefore, it is possible that the tet(B), tet(M) or both tet(B) and tet(M) genes are either poorly expressed or not expressed, or that tetracycline resistance in these strains is mediated by tet(A) or tet(A) + tet(C).

In two strains, no tet gene was detectable, despite expressed tetracycline or both tetracycline and doxycycline resistance. This phenomenon, also reported by other authors (Tuckman et al., 2007; Koo & Woo, 2011; Schwaiger et al., 2010), could be explained by point mutations in primer-binding sites, which would prevent synthesis of the relevant amplicons. Additionally, these resistance phenotypes may be due to the existence of an unidentified tet gene in these two isolates.

**Identification and cloning of Escherichia coli tet(M)**

The tet(M) gene has been found in *Escherichia coli* associated with different genetic elements such as Tn916, IS26 and ISV8 insertion sequences (Jones et al., 2006). Interestingly, the PCR product obtained in seven (17.1 %) of the 41 tet(M)-positive isolates with UM-F and UM-R was \( \sim 1.3 \) kb instead of 2.1 kb, suggesting that the region upstream from the *Escherichia coli* tet(M)-coding sequence diverged from the published *Escherichia coli* sequence using these primers. GenBank accession numbers of the sequences are indicated on the right.
The Escherichia coli tet isolates from an animal origin including duck.
tet resident promoter, from the seven full-length respectively. To our knowledge, this is the first report of the strains) were positive isolates and was expressed in Staphylococcus rostri Tn (GenBank accession no. AP012053) and Streptococcus gallolyticus faecalis Tn916 (GenBank accession no. DQ534550), Lactococcus lactis Tn (GenBank accession no. DQ060148), Klebsiella pneumoniae Tn6009 (GenBank accession no. EU239355), Enterococcus faecalis Tn916 (GenBank accession no. U09422), Streptococcus gallolyticus (GenBank accession no. AP012053) and Staphylococcus rostri Tn916 (GenBank accession no. FN550102), differing by 15, 15, 20, 16, 15 and 13 residues, respectively. To our knowledge, this is the first report of the identification of a full-length tet(M) gene in Escherichia coli isolates from an animal origin including duck.

The Escherichia coli tet(M) gene was cloned, along with its resident promoter, from the seven full-length tet(M)-positive isolates and was expressed in Escherichia coli. The MICs of oxytetracycline, tetracycline and doxycycline for all the transformants (recombinant Escherichia coli host strains) were >128, >128 and 64–128 μg ml⁻¹, respectively (data not shown), but were more than 128-fold higher than those observed for the host strain (1, 1 and 0.25 μg ml⁻¹, respectively), suggesting that the cloned tet(M) gene was responsible for the decreased susceptibility to tetracyclines in the recombinant Escherichia coli host strain.

The tet(M) gene was associated with Tn916/Tn1545-like conjugative transposons in Gram-positive streptococci and enterococci, with Tn5801-like conjugative transposons in Staphylococcus aureus Mu50 (de Vries et al., 2009) and with Tn5397-like transposons in Clostridium difficile from human and in Enterococcus faecium from broilers (Agersø et al., 2006). However, Tn916-likexis, Tn5397-like tndx and Tn5801-likeint genes were not found in the seven full-length tet(M)-positive isolates. These results indicated that only limited Tn916-derived sequences remained associated with the tet(M) gene in these seven strains and that most Tn916 sequences had been lost. We propose that the Escherichia coli tet(M) gene originated in a Tn916 host and that there may have been one or more intermediate hosts between the original host and the clinical Escherichia coli strains. No PCR product for the upstream or downstream regions of tet(M) was obtained in the remaining 34 of the 41 tet(M)-positive strains, which suggested that another unknown genetic environment of tet(M) was present in these isolates and should be further analysed.

**Strain typing**

A total of 14 RAPD patterns were observed among the 20 representative Escherichia coli strains with tet(A) + tet(B) + tet(C) + tet(M) (Fig. 2), comprising five main banding patterns, designated clusters I–V, and nine RAPD unique patterns. Three, two, two and two isolates belonged to clusters I, II, III, IV and V, respectively, and were found to be almost equally distributed among the tested isolates. The ERIC-PCR results in this study indicated that both clonal and horizontal spread of the tet genes had occurred in Escherichia coli isolates and suggest the possibility of further spread of these genes in the future.

**Conclusions**

In summary, we have reported the first extensive study of the prevalence and distribution of tetracycline efflux genes and ribosomal protection genes in clinical Escherichia coli isolates from sick ducks in China. Our data support the recent report (Zhang et al., 2010) of tet(M) in commensal Escherichia coli isolates from healthy chickens and pigs, and extend the finding that the ribosomal protection mechanism of tetracycline resistance, mediated by tet(M), has migrated into animal clinical isolates of Escherichia coli. These surveys clearly indicated increasing trends not only
in the prevalence of tet genes but also in the number of tet genes present in China. This is the first time that the full-length tet(M) gene has been identified and sequenced among *Escherichia coli* clinical isolates from sick ducks in China. A more comprehensive study of the tet(M) gene in *Escherichia coli* isolates from ducks is now needed.

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