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Co-prevalance of PMQR and 16S rRNA methylase genes in clinical *Escherichia coli* isolates with high diversity of CTX-M from diseased farmed pigeons

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ABSTRACT

In the present study, we determined the molecular epidemiology of extended-spectrum β-lactamases (ESBLs) in *Escherichia coli* isolated from diseased farmed pigeons in China. A total of 71 *E. coli* isolates were collected from three pigeon farms from 2011 to 2012 and screened for the presence of the ESBL genes. The ESBLs producers were further tested for the presence of PMQR-encoding genes as well as the 16S rRNA methylase gene using PCR and DNA sequence analysis. Co-transfer of plasmids encoding for ESBLs, PMQR determinants and/or 16S rRNA methylase gene was performed by conjugation into *E. coli*. The genetic relatedness and plasmid replicon type were determined. A total of 41 ESBLs producers were identified. Only CTX-M type ESBLs were detected, with the most common CTX-M types being CTX-M-65 ($n = 17$), CTX-M-27 ($n = 11$), CTX-M-55 ($n = 10$). Thirty-eight CTX-M-positive isolates were found to harbor at least one PMQR gene, with *aac*(6′)-*Ib-cr* ($n = 32$) and *oqxAB* ($n = 21$) being the most prevalent. The *rmtB* was the only prevalent 16S rRNA methylase gene detected in 24 (58.1%) CTX-M-positive isolates. Although most of the CTX-M producers had distinct pulsotypes, clonal transmission in the same farm was observed. $bla_{CTX\text{-}M}$ genes were carried by IncF alone or in combination with IncK plasmids with three different sizes, including 76.8 Kb ($n = 20$), 194 Kb ($n = 5$), 104.5 Kb ($n = 2$). PFGE profiles of CTX-M-positive *E. coli* isolates indicated potential horizontal spread of these multidrug resistant strains along with those CTX-M encoding genes. Our findings highlight the importance of pigeons as a reservoir of multiple antimicrobial resistance genes.

1. Introduction

Development of antimicrobial resistance by microbial pathogens and commensals represents a major threat to animal and public health (Clarke, 2006). The emergence of antimicrobial resistance in food-producing animals is of major public health significance arising from the risk of these bacteria entering the food chain (Van den Bogaard and Stobberingh, 2000). Growing evidence indicates that intensive agricultural and veterinary usage of antimicrobial compounds contributes to the emergence and dissemination of antimicrobial resistance in bacteria derived from food-producing animals (Molbak, 2004).

β-lactams, fluoroquinolones and aminoglycosides continue to play important roles in the treatment of serious infections caused by Gram-negative pathogens. However, *Enterobacteriaceae* with multidrug resistance phenotypes of these antibiotics have been increasing worldwide (Canton and Ruiz-Garbajosa, 2011). The most common cause of bacterial resistance to β-lactams is the production of β-lactamase (especially extended-spectrum β-lactamases, ESBLs). A dramatic increase in the number of class A and class D β-lactamases has been described since the 1980s (Bush and Jacoby, 2010). ESBLs of class A mainly include enzymes of TEM, SHV, CTX-M, VEB, and GES, which represent public health concerns because of their ability to hydrolyze expanded-spectrum cephalosporins (such as cefotaxime, ceftriaxone) (Pitout and Laupland, 2008). Studies over the last ten years have revealed that the CTX-M enzymes have nearly displaced other ESBL enzymes in *Enterobacteriaceae* (Bush, 2010; Canton et al., 2012). One reason for this displacement is the extraordinary dissemination of the

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corresponding bla_{CTX-M} genes in highly mobilizable genetic platforms, including plasmids and transposons (Canton et al., 2012; Woodford et al., 2011). Another reason for this increase is the co-resistance phenomenon in CTX-M-producing bacteria, particularly to fluoroquinolones and aminoglycosides, which might facilitate co-selection processes from which multidrug resistant organisms arise (Canton and Ruiz-Garbajosa, 2011; D'Andrea et al., 2013). The presence of different resistance genes undoubtedly gives advantages to the bacteria under antibiotic selective force and increases the opportunity for persistence of the bacteria and resistance genes (Canton et al., 2003).

In China, high antimicrobial resistance rates of β-lactams, fluoroquinolones and aminoglycosides in *Enterobacteriaceae* have been reported frequently in a variety of food-producing animals, which are considered to be reservoirs of commensals carrying various resistant determinants (Jiang et al., 2012; Jiang et al., 2011; Liu et al., 2013; Ma et al., 2012). Pigeons are normally considered as food-producing animals in China, some countries of the Western Europe and Southeast Asia, in which case the same restrictions on antimicrobial usages are applied as previously discussed for other food-producing animals (Maron et al., 2013). For these reasons the pigeon industry has also been considered a potential reservoir of resistance-gene-producing Gram-negative bacteria that may be acquired by humans through handling or consumption of contaminated products. However, the carriage of these bacteria by pigeons in China has not been well characterized.

This study was designed to determine the extent to which antimicrobial resistant *E. coli* strains occur in pigeons in China in order to determine whether this animal species might serve as a reservoir or vehicle for the dissemination of antimicrobial resistant strains into food-chains. We performed a pilot study to gain insight into the occurrence of antimicrobial resistance in *E. coli* isolated from pigeons. Our studies reported co-prevalence of CTX-M-type extended-spectrum β-lactamases (ESBLs) genes with plasmid-mediated quinolone resistance (PMQR) genes and those encoding 16S rRNA methylase that may display reduced susceptibility to multi-antimicrobial agents in *E. coli* isolates of pigeon origin.

2. Materials and methods

2.1. Bacterial isolates

A total of 97 samples of intestinal tract ($n = 46$), viscera ($n = 31$), blood ($n = 16$) and other sites ($n = 4$) were collected from 97 pigeons aged between 20 and 25-days from three different pigeon farms (named Farm 1–3: 34 samples from Farm 1, 32 from Farm 2, 31 from Farm 3) in Guangdong Province in China during the period of February 2011 and December 2011. Many of the pigeons appeared very sick with poor appetite, going to die or already dead at the time received at the Institute of Animal Health. Samples were collected on-site when the birds in the herds presented symptoms before being transported to the Institute of Animal Health. Diagnosis of New Castle disease was made by PCR. The sampling was carried out in a sterile room using sterile cotton swabs. All samples were dispatched within 12 h to the Veterinary Research Institute, Guangdong Academy of Agricultural Science, where they were seeded onto MacConkey agar and incubated at 37 °C for 18 h. One colony with typical *E. coli* morphology was selected from each sample and was identified with API20E systems (BioMerieux, Beijing, China). All isolates were stored at −80 °C in Luria–Bertani broth containing 30% glycerol.

2.2. Antimicrobial susceptibility

The minimal inhibitory concentrations (MICs) of 16 antibiotics, including ampicillin (AMP), cefotaxime (CTX), cefoxitin (CXT), ceftiofur (CTF), ceftazidime (CAZ), ceftriaxone (CTR), nalidixic acid (NAL), ciprofloxacin (CIP), kanamycin (KAN), gentamycin (GEN), amikacin (AMK), tetracycline (TET), chloramphenicol (CHL), florfenicol (FFC), olaquindox (OLA), were determined by a agar dilution method according to the standards described by Clinical and Laboratory Standards Institution (M100-S25 and VET01-A4/VET01-S2).

Isolates were classified as either susceptible or resistant according to the interpretative criteria recommended by the CLSI (M100-S25) (ampicillin, cefotaxime, ceftiofur, ceftriaxone, cefoxitin, ceftazidime, nalidixic acid, ciprofloxacin, kanamycin, amikacin), veterinary CLSI (gentamycin, enrofloxacin, tetracycline, chloramphenicol, florfenicol) (VET01-S2) and DANMAP 98 (olaquindox ≥ 64 μg/mL) (DANMAP, 1999). *E. coli* ATCC strain 25922 was used as quality control in the antimicrobial susceptibility testing.

2.3. Detection of ESBL genes

E. coli isolates that displayed resistance to cefotaxime or ceftiofur were screened for ESBL-genes using PCR as described previously for bla_{CTX-M} genotype groups 1, 2, 8, 9 and 25, bla_{TEM}, bla_{SHV}, bla_{OXA} (Jiang et al., 2012). The obtained DNA amplicons were submitted to BGI Life Tech Co., Ltd. (Beijing, China) for sequencing and sequences were compared with those included in the GenBank database by using the BLAST algorithm (www.ncbi.nlm.nih.gov) and at www.lahey.org/Studies/ in order to identify the specific β-lactamase genes. ESBL confirmatory testing was not included in this study.

2.4. Chromosomal mutation, PMQR genes and 16srRNA genes in CTX-M-positive isolates

All bla_{CTX-M}-positive isolates were screened for PMQR genes [*aac(6′)-Ib-cr, qnrA, B, C, D, S, qepA* and *oqxAB* and mutations in the quinolone resistance-determining regions (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* using previously described primers and protocols (Shaheen et al., 2013).

The presence of 16S rRNA methylase genes (*armA*, *rmtA*, *rmtB*, *rmtC* and *rmtD*, *npmA*) were also analyzed by PCR in bla_{CTX-M}-positive isolates, using specific primers and conditions described previously (Doi and Arakawa, 2007).

2.5. Strain typing

Genetic relatedness of all isolates containing CTX-M-encoding genes was analyzed by pulsed-field gel electrophoresis (PFGE) of XbaI-digested chromosomal DNA (CHEF Mapper®, Bio-Rad Laboratories, Hercules, CA) as described previously(Jiang et al., 2014). Resulting PFGE patterns were interpreted according to the method of Tenover et al. (1995).

2.6. Conjugation assay and plasmid analyses

E. coli isolates positive to bla_{CTX-M} were selected to perform conjugation experiments by the broth-mating method using *E. coli* C600 as the recipient. Transconjugants were selected on MacConkey agar plates containing streptomycin (1000 μg/ml) and cefotaxime (2 μg/ml) as described previously (Jiang et al., 2014). Gene analyses of the bla_{CTX-M} positive transconjugants were confirmed by PCR as described above. Plasmid replicon typing of all transconjugants was done by a PCR based method using 18 pairs of primers described previously (Carattoli et al., 2005). PFGE with S1 nuclease (Takara Biotechnology, Dalian, China) digestion of whole genomic DNA was performed for all transconjugants, as described previously (Barton et al., 1995). Primers used to design