Characterization of plasmid-mediated quinolone resistance by the qnrS gene in Escherichia coli isolated from healthy chickens and pigs

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ABSTRACT: The prevalence of qnr and qepA genes in 660 Escherichia coli isolates was investigated in healthy animals from 30 pig farms and 30 chicken farms in Taiwan from January 2005 to February 2006 by the polymerase chain reaction. The qnrS gene, but not qnrA, qnrB, and qepA were detected in 12/360 pig isolates (3.33%) and in 6/300 chicken isolates (2%). Southern blot hybridization analysis indicated that qnrS was located on plasmids ranging in size from 50–165 kb. Eleven of the 18 qnrS positive isolates which showed a high ciprofloxacin resistance phenotype (minimum inhibitory concentration ≥ 8 mg/l) also had amino acid sequence variations in chromosomal quinolone resistance-determining regions of gyrA and parC. Only two qnrS-positive isolates carried the aac(6')-Ib-cr variant that mediates FQ acetylation. For the high percentage resistance of cephalosporins, the blaCTX-M gene was also examined in qnrS-positive isolates. The blaCTX-M gene was detected in fifteen isolates (15/18, 83.3%) of which 12 isolates were blaCTX-M-1 and three isolates were blaCTX-M-15. This study demonstrated a close linkage between the qnrS gene and blaCTX-M-1, suggesting CTX-M and Qnr-based mechanisms might be co-emerging in E. coli strains isolated from healthy chickens and pigs under selective pressure of quinolone and cephalosporin administration.

Keywords: Escherichia coli; qnr gene; blaCTX-M; pig; chicken

Multiple mechanisms are involved in resistance to fluoroquinolones (FQ) in Enterobacteriaceae. Besides mutations in chromosomal genes encoding for DNA gyrase and topoisomerase IV (Ferrero et al., 1994; Kumagai et al., 1996), plasmid mediated quinolone resistance (PMQR) has also been reported, including a Qnr-mediated inhibition of quinolone binding to DNA (Tran and Jacoby, 2002; Poirel et al., 2005b; Tran et al., 2005), a QepA encoded efflux pump (Yamane et al., 2007), and the aac(6')-Ib-cr mediated FQ acetylation (Park et al., 2006; Robicsek et al., 2006a).

The PMQR determinants are widely distributed in clinical Enterobacteriaceae isolates around the world and are usually associated with mobile elements, including integrons, insertion sequences, and transposons (Martinez-Martinez et al., 1998; Mammeri et al., 2005; Nordmann and Poirel, 2005; Robicsek et al., 2006a,b; Lascols et al., 2007; Wu et al., 2007; Yamane et al., 2008). The genetic linkage of low-level quinolone resistance with multidrug resistance (β-lactams, aminoglycosides, and sulfonamides) promotes co-selection of quinolone resistance.

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upon exposure to other antimicrobials (Lavigne et al., 2006; Robicsek et al., 2006b; Wu et al., 2007). Despite the existence of many clinical studies (Jacoby et al., 2003; Kehrenberg et al., 2006; Wu et al., 2008; Yue et al., 2008), the role of plasmid mediated quinolone resistance, especially in healthy animals, is still uncharacterized. The aim of this study was to investigate the prevalence of the PMQR in *Escherichia coli* isolated from healthy chickens and pigs and to characterize its association with extended-spectrum β-lactamases (ESBLs) or other β-lactamases.

**MATERIAL AND METHODS**

**Sampling and questionnaire**

During the period from January 2005 to February 2006, a total of 660 samples comprising 360 rectal swabs from 360 healthy pigs (120 nursery pigs, 120 grower-finisher pigs and 120 sows) in 30 pig farms, and 300 cloacal swabs from 300 healthy broilers in 30 poultry farms were collected in Taiwan. Based on the age and stage of production, individual animals that did not receive antimicrobial treatment at least one month prior to sampling were randomly selected from different pens of each farm for sampling (i.e., the animals might have received different antimicrobial treatments thirty days previously). The rectal samples from pigs were collected at three different production stages: nursery pigs (*n* = 4), grower-finisher pigs (*n* = 4), and sows (*n* = 4) in each farm. Ten cloacal swabs taken from broilers were collected evenly from each pen of broiler production farm and chickens were less than one week old prior to slaughter (36–42 days). In addition, each farmer was asked to fill in a questionnaire about any recent mass medication of their pigs or chickens with antimicrobials, as well as any recent stay in hospital or personal use of antimicrobials by the farmer or family members during the preceding one month before sampling.

**Bacterial isolation and identification**

Swabs were inoculated onto MacConkey agar (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) and incubated for 24 h at 37°C. Primary plates were carefully inspected for colonies of *E. coli*, which were plated onto sheep blood agar plates, which were incubated at 37°C for 24 h. Suspected colonies were identified as *E. coli* using standard techniques, including indol, methyl red-Voges Proskauer (MR-VP), citrate biochemical tests (Ewing, 1986) and the API-20E system (BioMérieux, Marcy l’Etoile, France).

**Screening for qnrA, qnrB, qnrS, qepA, and aac(6’)-Ib-cr genes**

PCR analyses for *qepA* and the three *qnr* genes were performed for all of the isolates. PCR amplification of the *aac(6’)-Ib* gene was performed only in the *qnr*- or *qepA*-positive strains. The total DNA of *E. coli* isolates was extracted using the InstaGene DNA Purification Matrix kit (Bio-Rad; CA, USA) according to the manufacturer’s instructions. The primer sets used for detection of *qnrA*, *qnrB*, *qnrS* (Cattoir et al., 2007b), *qepA* (Yamane et al., 2008) and *aac(6’)-Ib* (Jiang et al., 2008) were described previously. Amplified PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and sent to the Tri-I Biotech company (Taipei, Taiwan) for DNA sequencing. The results of sequencing were compared using the BLAST online search engine from GenBank at the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/blast) (Altschul et al., 1997).

**Antimicrobial susceptibility testing**

The susceptibility to antimicrobial agents was tested using the broth dilution method in accordance with the standards of the Clinical and Laboratory Standards Institute (CLSI), 2007 (Anonymous, 2007). Resistance to 11 antimicrobial drugs was tested: nalidixic acid, chloramphenicol, florfenicol, ampicillin, ceftazidime, cefotaxime, gentamicin, kanamycin (Sigma Aldrich, St Louis, MO, USA), enrofloxacin, ciprofloxacin (Fluka Chemie, Buchs, Switzerland), and ceftiofur (Excenel RTU, Pfizer Animal Health, Karlsruhe, Germany). *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 were used as control strains.

**Plasmid extraction and Southern blot hybridization**

Plasmid DNA was extracted using the Qiagen Midi Kit (Qiagen, Valencia, CA, USA) according
to the manufacturer’s instructions. Plasmid DNA was analyzed on 0.8% agarose gels accompanied by BAC-Tracker supercoiled DNA ladder (Epiconcentre Biotechnologies, Madison, WI, USA), and the approximate size of plasmids was determined. Southern blot hybridization analysis was performed according to Sambrook et al. (1989). DNA from the agarose gel was transferred onto a Hybond N+ nylon membrane (Nycomed Amersham plc, Buckinghamshire, UK) with a vacuum blotting system. Digoxigenin (DIG)-labeled DNA probes were prepared by PCR amplification with primers for qnrS and labeled with a PCR DIG-labeling kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the instructions provided by the manufacturer. The membrane with blotted DNA was subjected to pre-hybridization followed by hybridization with a labeled probe. After hybridization and following the recommendations of the manufacturer, the DIG-High Prime DNA labeling and detection system (Digoxigenin Labeling and Detection Kit; Roche Diagnostics, Mannheim, Germany) was used for signal detection.

**PCR amplification and DNA sequencing of quinolone resistance-determining regions and β-lactamase genes**

Total DNA of *E. coli* isolates was extracted as described above. Partial sequences of *gyrA*, *gyrB*, *parC*, *parE*, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>CTX-M</sub> were amplified by PCR using primers and PCR conditions described previously (Everett et al., 1996; Wu et al., 2007). Purified PCR products were sequenced on both strands and results were compared with the sequences of wild-type *E. coli* *gyrA* (NCBI X06373), *gyrB* (P06982), *parC* (P20082), and *parE* (P20083). Comparisons were performed using NCBI BLAST program, Clustal W Multiple Sequence Alignment Program and Lasergene sequence analysis software package (DNA Star software version 4.0, Madison, WI, USA; Thompson et al., 1994).

**RESULTS**

According to the questionnaire results and the farm medical records, many of the same antibiotics had been used by veterinarians to treat as well as prevent diseases, i.e., the use of oxytetracycline in feed in pig farms and enrofloxacin in water in poultry farms. Among the pig and chicken farms investigated, enrofloxacin had been administered to nursery pigs in 15 (50%) pig farms and 20 (66.7%) poultry farms, primarily to treat respiratory diseases on at least one occasion one year prior to sampling. The remaining antibiotics used in pig farms for disease therapy included amoxicillin (60%), florfenicol (56.7%), tetracycline (56.7%), and ceftiofur (30%). For chicken farms, those administered were ampicillin (66.7%), florfenicol (60%), tetracycline (56.7%), and sulfadiazine (26.7%). According to the questionnaire results, the farmers and their family members didn’t take any antibiotics or stay in hospital in the month prior to sampling.

PCR screening using gene-specific primers failed to detect *qnrA*, *qnrB*, and *qepA* genes in any *E. coli* isolates. In comparison, the frequency of *qnrS* positive *E. coli* strains in pigs was 12/360 (3.33%), including 7/120 (5.83%) in nursery pigs, 3/120 (2.5%) in grower-finisher pigs and 2/120 (1.67%) in sows (Table 1). In comparison, the frequency of *qnrS*-positive *E. coli* isolates from chicken was 6/300 (2%; Table 1).

DNA sequencing of PCR products using primers specific for *aac(6)Ib* were performed. Seventeen of the eighteen *qnrS*-positive isolates were also positive for *aac(6)Ib*, among which only two strains (Farm-4-14 and C-2-111) shared 100% identity with the nucleotide sequence of the *aac(6)Ib*-cr variant (Table 1).

Minimum inhibitory concentration (MIC) data, together with the resistance breakpoints defined by the CLSI, of the eighteen *qnrS*-positive isolates against the 11 antimicrobial agents tested are listed in Table 1. All *qnrS*-positive strains were resistant to nalidixic acid, chloramphenicol, florfenicol, and ampicillin (Table 1). For these strains, a high resistance ratio was also observed against enrofloxacin (94.4%, 17/18), kanamycin (88.9%, 16/18), cefotaxime (83.3%, 15/18), ciprofloxacin (77.8%, 14/18), ceftiofur (77.8%, 14/18), and gentamicin (55.6%, 10/18). The lowest level of resistance was found against ceftazidime (11.1%, 2/18).

To characterize the percentage of *qnrS* genes localized in plasmids, plasmids from the eighteen *qnrS*-positive strains were prepared. The size of these plasmids ranged from 50 and 165 kb (Figure 1). Southern blot hybridization using a *qnrS*-specific probe showed that *qnrS* was detected in 11/18 (61.1%) of plasmids (Figure 1). No hybridization to plasmids was found for the remaining
<table>
<thead>
<tr>
<th>Strains</th>
<th>Sources</th>
<th>QRDR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MIC (mg/l)&lt;sup&gt;bc&lt;/sup&gt;</th>
<th>ESBLs</th>
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<tr>
<td></td>
<td></td>
<td>gyrA–83 Ser</td>
<td>gyrA–87 Asp parC–80 Ser</td>
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<td>Asn (AAC)</td>
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</tbody>
</table>

No mutations were identified in gyrB and parE sequences. In the column "strains", the number after the first dash represented the sampled farm. Ex: Farm-1-... represented the isolate came from pig farm 1.

<sup>a</sup>wt refer to wild type; amino acids: Ser = serine, Asp = aspartic acid, Leu = leucine, Tyr = tyrosine, Ile = isoleucine, Asn = asparagines

<sup>b</sup>NA = nalidixic acid, EN = enrofloxacin, CIP = ciprofloxacin, CHL = chloramphenicol, FLO = florfenicol, KM = kanamycin, GA = gentamicin, AMP = ampicillin, CFO = ceftiofur, CT = cefotaxime, TZ = ceftazidime,

<sup>c</sup>MIC breakpoints were recommended by the CLSI: enrofloxacin ≥ 2 mg/l; ciprofloxacin ≥ 4 mg/l; florfenicol, ceftiofur ≥ 8 mg/l; gentamicin ≥ 16 mg/l; nalidixic acid, chloramphenicol, ampicillin, cefotaxime, ceftazidime ≥ 32 mg/l; kanamycin ≥ 64 mg/l

<sup>d</sup>strain harbouring qnrS gene in addition to aac (6')-Ib-c

<sup>e</sup>strain harbouring qnrS gene in addition to bla<sub>SHV-1</sub>
seven strains indicating that *qnrS* was either located on the chromosomal DNA or that it could not be purified by this method.

After PCR, the DNA sequences of quinolone resistance-determining regions (QRDR), such as *gyrA*, *gyrB*, *parC*, and *parE* were analyzed in *qnr*-positive *E. coli* isolates. Point mutations were observed in *gyrA* and *parC*, whereas no *gyrB* and *parE* mutations were detected (Table 1). Substitutions at codons 83 and/or 87 in the *gyrA* gene were detected in 83.3% (15/18) of the strains. Among the 15 strains with *gyrA* mutations, eight strains had an additional mutation at codon 80 in the *parC* gene. There were no strains with a *parC* QRDR mutation alone. The MICs of enrofloxacin for *qnrS*-positive strains with the mutations in the QRDR of *gyrA* with or without *parC* ranged from 4 to 256 mg/l.

Using PCR and DNA sequencing, the *bla*<sub>CTX-M</sub> gene was detected from 15/18 *qnrS*-positive isolates, most being *bla*<sub>CTX-M-1-like</sub> (CTX-M-1 in 12 isolates and CTX-M-15 in three isolates). Moreover, TEM-1-type β-lactamase and SHV-1-type β-lactamase

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Figure 1. Plasmid DNAs of *E. coli* strains isolated in this study (A) and results of Southern blot hybridization of plasmid DNAs with the *qnrS*-specific probe (B)

Lanes: 1 = C-2-111; 2 = C-1-794; 3 = C-1-822; 4 = C-3-2367; 5 = C-3-2369; 6 = C-5-2389; 7 = Farm2-50; 8 = Farm5-26; 9 = Farm5-28; 10 = Farm6-121; 11 = Farm5-30; 12 = Farm5-32; 13 = Farm1-7; 14 = Farm3-121; 15 = Farm4-14; 16 = Farm5-25; 17 = Farm 6-91; 18 = Farm6-104; M = BAC-Tracker supercoiled DNA ladder used as a negative control and a reference for estimation of plasmid size

*southern-hybridization positive strain; ← = qnrS-positive plasmid
were also detected in all and two qnrS positive isolates (Table 1).

DISCUSSION

According to the questionnaire results and the farm medical records, use of animal growth promoters was frequent, especially for nursery pigs, grower pigs, and chickens. Based on the “Handbook of Animal Drug Management”, administration of growth promoters in animal feed, including tetracycline (less than 50 ppm), nalidixic acid (less than 20 ppm), tylosin (less than 20 ppm), bacitracin (less than 55 ppm), or colistin (less than 20 ppm), is most common for growth promotion in Taiwan (BAPHIQ, 2008). The questionnaire also indicated that the isolation of qnrS-positive isolates from pigs and chickens was due to the administration of enrofloxacin, ceftiofur, or florfenicol for the treatment of respiratory diseases. These drugs were also used for treatment of other diseases, such as diarrhea (four farms) or arthritis (two farms). However, all treatments were stopped at least 30 days before sampling. Antimicrobial drugs used in food animals are often administered in a way that increases drug resistance, e.g., by sub-therapeutic dosage and repeated mass treatments, long-term administration as antimicrobial growth promoter or addition to food and water for prophylaxis purposes (Van den Bogaard and Stobberingh, 1999; Rajic et al., 2006). Jørgensen et al. (2007) reported that the use of ceftiofur reduced susceptibility to cefotaxime due to acquisition of CTX-M-1 β-lactamase. In this study, among the 18 qnrS-positive isolates, 15 isolates carried CTX-M-1 or CTX-M-15. Thus, our data support those from the previous report that selection pressures from the use of fluoroquinolones and ceftiofur would promote an increase in the prevalence of qnr and bla_CTX-M genes (Robicsek et al., 2006b).

Several reports had shown a low prevalence (0–0.6%) of the plasmid-mediated quinolone resistance gene in food-producing animals (Cavaco et al., 2007; Cavaco et al., 2008; Lapierre et al., 2008; Cerquetti et al., 2009). Ma et al. (2009) also showed that the prevalence of PMQR determinants was significantly higher in isolates from companion animals than in isolates from food-producing animals. This finding may be related to the extensive use of broad-spectrum agents, including antimicrobial preparations also used in human medicine, in small-animal veterinary practice (Heuer et al., 2005). Based on previous reports and our sample sources, our results also demonstrated that a low incidence of the plasmid-mediated quinolone resistance gene is present in healthy pigs and chickens.

In the present study, most qnrS-positive strains isolated from nursery pigs and chickens showed a high-level resistance to enrofloxacin and/or ciprofloxacin. This might be associated with the fact that (1) a total of five qnrS-positive strains were found on Farm 5 (three strains from nursery pigs and two from sows; Table 1) that had been administering enrofloxacin and ceftiofur for treatment of respiratory disease to nursery pigs and without a history of quinolone and cephalosporine usage in the sows; (2) although the water medication of chickens with enrofloxacin was banned in Taiwan in November 2005, the qnr-positive chicken farms still used enrofloxacin in water six months prior to sampling; and (3) approximately 60% of the production units administered enrofloxacin to nursery pigs, primarily to treat respiratory disease. Thus, our data are in broad agreement with those from previous reports which show that PMQR determinants confer lower level resistance to quinolones and fluoroquinolones and may provide a favourable background in which the selection of additional chromosomally-encoded quinolone resistance mechanisms can act during or after treatment with fluoroquinolones (Robicsek et al., 2006b; Cesaro et al., 2008).

Cesaro et al. (2008) and Lascols et al. (2007) have shown that ciprofloxacin resistance (≥ 4 mg/l) is closely related to the number of topoisomerase mutations in gyrA and parC, and further to the additional harbouring of qnr gene in Enterobacter cloacae and E. coli strains. Similar results were found in this study that most qnrS-positive strains (11/18; 61.1%) with higher MICs against enrofloxacin (≥ 16 mg/l) and ciprofloxacin (≥ 8 mg/l) were down to mutations in the QRDR of gyrA and parC genes. Among the 18 qnrS-positive strains, a single mutation (Ser83Leu) in gyrA was identified in seven strains which showed high nalidixic acid resistance (MIC 128 to > 1 024 mg/l) and low to high ciprofloxacin resistance (MIC 1–16 mg/l; Table 1). Three isolates, two from nursery pigs (Farm-5-26 and Farm-5-28) and one from chicken (C-4-2369) which possessed mutations in gyrA (Ser83Ile) and parC (Ser80Ile) showed high level resistance to ciprofloxacin (MIC ≥ 16 mg/l) and enrofloxacin (MIC = 64 mg/l). In addition, double mutations in gyrA at codon 83 (Ser83Leu) and 87 (Asp87Asn,
in the present study we showed that currently there is no significant relationship between the two strains (Farm-4-14 and C-2-111), or 64 mg/l (Farm-3-121). One possibility might be the existence of an additional resistance gene, a potential efflux mechanism (e.g., AcrAB-TolC) or other unidentified factors contributing to the resistance phenotype (Mazzariol et al., 2000; Hopkins et al., 2005; Kehrenberg et al., 2007). The mechanism of resistance in these isolates remains a topic for further study.

The aac(6')-Ib gene encodes a common aminoglycoside acetyltransferase responsible for resistance to aminoglycoside antibiotics such as kanamycin, amikacin and tobramycin (Tolmasy et al., 1986; Vakulenko and Mobashery, 2003). The aac(6')-Ib-cr is a variant of the aac(6')-Ib gene, in which twelve base pairs at the 5' end are different, and which harbours mutations at codons 102 (Trp102Arg) and 179 (Asp179Tyr). Consequently, the variant enzyme acetylates ciprofloxacin and norfloxacin, conferring slightly higher MICs (a 2- to 4-fold increase; Robicsek et al., 2006a). The co-transmission of qnr with aac(6')-Ib-cr genes which speeds up the formation of multidrug resistance in Enterobacteriaceae has been previously reported in China (Yang et al., 2008). However, in the present study we showed that currently there is no significant relationship between aac(6')-Ib-cr prevalence and the presence of the qnrS gene in Taiwan. However, why the two strains (Farm-4-14 from a pig farm and C-2-111 from a chicken farm) harboured the aac(6')-Ib-cr remains to be determined. According to the results of questionnaire and the aac(6')-Ib-cr-positive farm’s medical records, a possible explanation might be a steady increase in clinical use of quinolone and kanamycin pressure for this specific variant.

Southern blot hybridization using a qnrS-specific probe showed that qnrS was detected in 11 of 18 isolates. No hybridization to plasmids was found for the remaining seven strains. Even after repeated tests on the seven strains, no hybridization to plasmids was found. It is important to consider that the qnr gene has been reported to be located in a mobile resistance determinant or insertion element that might jump to the chromosome (Mammeri et al., 2005; Nordmann and Poirel, 2005; Gay et al., 2006; Jacoby et al., 2006). Several studies did have identified the plasmid-mediated qnr gene in the chromosomes of E. coli, Citrobacter werkmanii, Shewanella algae and the Vibrionaceae family (Poirel et al., 2005a; Cattoir et al., 2007; Cavaco et al., 2007; Kehrenberg et al., 2008). Thus, the presence of chromosome-encoded Qnr-like determinants should be possible in these seven strains.

Our study indicated that a high prevalence of multidrug resistance percentage occurs among qnrS-positive strains isolated from healthy pigs or broilers. Besides this, the presence of plasmid-mediated quinolone resistance among E. coli isolates recovered from food producing animals raises a potential public health concern about the spread of genetic resistance elements from food animal products to humans by direct contact or through the food chain. Further investigation might be required to assess the risk of zoonotic transmission via the food chain and by contact with animals. Based on the results of this study, it is suggested that the large-scale administration of quinolones and/or cephalosporines to food-production animals might select for cephalosporin-resistant (blaCTX-M) and plasmid-mediated quinolone resistant E. coli strains in animals. This has to be taken into consideration when using antimicrobial agents for prophylaxis or growth-promoting purposes in animals.

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