FULL PAPER

Bacteriology

Prevalence of plasmid-mediated quinolone resistance in *Escherichia coli* isolated from diseased animals in Taiwan

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ABSTRACT. *Escherichia coli* (*E. coli*) is a zoonotic pathogen that often causes diarrhea, respiratory diseases or septicemia in animals. Fluoroquinolones are antimicrobial agents used to treat pathogenic *E. coli* infections. In this study, 1,221 *E. coli* strains were isolated between March, 2011 and February, 2014. The results of the antimicrobial susceptibility testing showed a high prevalence of quinolone resistance. The antimicrobial resistance rates of these *E. coli* isolates to nalidixic acid (NAL) were 72.0% in swine, 81.9% in chickens, 81.0% in turkeys, 64.0% in ducks and 73.2% in geese. Among these isolates, the positive rate for the plasmid-mediated quinolone resistance (PMQR) determinant was 14.8% (181/1,221); the detection rate for *qnrS1* was the highest (10.2%), followed by *aac(6′)-Ib*-cr (4.5%) and *qnrB2* (0.3%). The quinolone-resistance determining regions (QRDRs) analysis for the PMQR-positive isolates showed that the strains with mutations at codon 83 or 87 in GyrA were resistant to NAL. To the best of our knowledge, this is the first report of occurrence of *qnrB2*, *qnrS1* and *aac(6′)-Ib*-cr genes and high frequency (56.4%; 102/181) of mutation in *gyrA* or *parC* among PMQR-positive *E. coli* strains derived from diseased animals in Taiwan.

KEY WORDS: *Escherichia coli*, plasmid-mediated, quinolones, quinolone resistance, quinolone-resistance determining regions

    Escherichia coli (*E. coli*) is a zoonotic pathogen and an important common bacterium in animals and humans. Pathogenic *E. coli* infection in food-producing animals can cause serious economic losses and can also cause various symptoms of infection in different species. For example, *E. coli* often infects pigs via the gastrointestinal tract and causes diarrhea, septicemia or edema disease [16]. Avian pathogenic *E. coli* (APEC) often infects the respiratory system, such as through the air sacculitis with thickened air sacs, caseous exudates commonly found in the respiratory tract and other systemic infections, including polyserositis and pericarditis in the later stage of infection [10]. Quinolones or fluoroquinolones are antimicrobial agents used to treat infections caused by *E. coli* and other bacteria. In Taiwan, different quinolones are applied to different species. Currently, the most commonly used quinolones in veterinary clinical treatment include nalidixic acid (NAL), oxolinic acid, flumequine (FLU) and enrofloxacin (ENR). Fluoroquinolone-resistance in gram-negative bacteria is a global issue. The resistance mechanisms to quinolones in bacteria might result from mutations in the quinolone-resistance determining regions (QRDRs) of the target proteins and from carriage of the plasmid-mediated quinolone resistance (PMQR) determinants. The QRDRs that have been documented include mutations of DNA gyrase (GyrA and GyrB) and topoisomerase IV (ParC and ParE). These mutations markedly decrease the affinity of the FQ molecules to gyrase [26]. Mutations at codons 83 and 87 in GyrA or codons 80 and 84 in ParC are able to reduce the bacterial susceptibility to quinolones [31]. In addition, a number of PMQR mechanisms have been described, including the following: the *qnr* gene, which protects DNA gyrase (and probably also topoisomerase IV) from binding to the fluoroquinolones; the *aac(6′)-Ib*-cr gene, which confers reduced susceptibility to ciprofloxacin by modifying ciprofloxacin; the *qepA* gene, which encodes an efflux pump belonging to the major facilitator subfamily; and the *oqxAB* gene, which generates multidrug efflux pumps that could enhance resistance to nalidixic acid and ciprofloxacin [14, 17, 28, 35]. The PMQR mechanisms are widely found around the globe in various genera of bacteria and different environments of plasmids [28]. The acquisition of the PMQR genes alone results in a

low level of fluoroquinolone resistance and does not lead to MICs exceeding the breakpoints of these agents. The QRDR mutations develop when resistant bacteria can survive under selective pressure in an environment filled with antimicrobial agents, resulting in high-level resistance to fluoroquinolone [25, 34].

The aims of this study are to investigate the prevalence of resistance to quinolones and to screen for mutations in the QRDRs of the gyrA and parC genes among the PMQR-positive *E. coli* isolates from diseased animals in Taiwan.

MATERIALS AND METHODS

Bacterial isolates

E. coli isolates were recovered from the brains, hearts, livers, lungs, intestines, joints or exudative effusion samples of diseased food-producing animals with diarrhea, respiratory diseases or other diseases, including 650 pigs, 684 chickens, 78 turkeys, 61 ducks and 73 geese in five counties of Taiwan from March 2011 to February 2014 by the Animal Disease Diagnostic Center in the College of Veterinary Medicine, National Chiayi University. Each isolate was from a separate animal, and a total of 1,221 isolates were collected from 363 farms. All samples were seeded on MacConkey agar plates and incubated at 37°C for 24 hr. All the clinical isolates were identified using an API 20E system (bioMérieux, Marcy l'Étoile, France) and conventional laboratory methods, including Gram staining, growth on selective media and analysis of colony morphology [13]. All identified isolates were stored at −80°C in Luria-Bertani broth containing 30% glycerol.

Antimicrobial susceptibility testing

Bacterial susceptibility to antimicrobials was quantitatively tested by broth micro-dilution with cation-adjusted Mueller-Hinton broth (Difco Laboratories, Detroit, MI, U.S.A.) according to the guidelines of the Clinical and Laboratory Standards Institute [8]. The tested antimicrobial agents included nalidixic acid (NA, 0.5–1,024 mg/*l*), flumequine (FLU, 0.5–1,024 mg/*l*), enrofloxacin (ENR, 0.25–512 mg/*l*), ciprofloxacin (CIP, 0.125–256 mg/*l*) and moxifloxacin (MOX, 0.125–256 mg/*l*). The minimum inhibitory concentrations (MICs) were determined after 18 hr of incubation at 35°C. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureas* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as antimicrobial susceptibility testing controls in accordance with the CLSI recommendations. Resistance breakpoints were defined according to the Clinical and Laboratory Standards Institute (CLSI) (M31-A2, 2002, and M-100-S24, 2014) recommendations [7, 9] and British Society for Antimicrobial Chemotherapy (BSAC) guidelines [1]. The breakpoints (resistance levels) for the five antimicrobials tested in this study were defined as follows: nalidixic acid ≥32 mg/*l*, flumequine ≥8 mg/*l*, enrofloxacin ≥2 mg/*l*, ciprofloxacin ≥4 mg/*l* and moxifloxacin ≥1 mg/*l*.

Detection of PMQR determinants

The detection of the PMQR determinants was performed for each isolate of *E. coli*. Plasmid DNA was extracted using Qiagen Mini Kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. Then, the plasmid DNA was tested for the presence of the PMQR genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib*-cr and *qepA*) using PCR amplification [2, 3, 18, 19, 32, 35]. Amplified PCR products were purified with QIAquick PCR purification kit (Qiagen) and sent to the Tri-I Biotech company (Taipei, Taiwan) for DNA sequencing. The results of the sequencing were compared using the BLAST online search engine from GenBank at the National Center for Biotechnology Information Web site (http://blast.ncbi.nlm.nih.gov/).

PCR amplification and DNA sequencing of quinolone resistance-determining regions

The PMQR-positive isolates were analyzed for QRDR mutations. In accordance with the manufacturer's instructions, the InstaGene DNA Purification Matrix kit (Bio-Rad Laboratories Hercules, CA, U.S.A.) was used to extract the total DNA from the *E. coli* isolates. Partial sequences of *gyrA* and *parC* were amplified by PCR using primers and PCR conditions described previously [11, 12]. Purified PCR products were sequenced on both strands, and the results were compared with the sequences of wild-type *E. coli gyrA* (NCBI X06373) and *parC* (P20082). Comparisons were performed using the NCBI BLAST program, the Clustal W Multiple Sequence Alignment Program and the Lasergene sequence analysis software package (DNAstar®, Madison, WI, U.S.A.) [25].

Statistics and analysis

The antimicrobial MIC test results for the strains isolated from each species were statistically analyzed using Microsoft® Excel 2013. The different values of the MIC of the five antimicrobial agents were compared using a *t*-test for the *E. coli* isolates from different species. A *P*-value <0.05 was considered statistically significant.

RESULTS

Isolation and antimicrobial susceptibility testing

Among the 1,221 pathogenic *E. coli* strains tested in this study, 583 were isolated from swine. The remaining samples were isolated from poultry, and they were comprised of 515 samples from chickens, 42 from turkeys, 25 from ducks and 56 from geese. The rates of resistance of all the isolates to NAL, FLU, ENR, CIP and MOX were 76.4, 74.3, 50.6, 42.3 and 51.3%, respectively. The MICs at which 50 and 90% of the isolates were inhibited (MIC_{50} and MIC_{90}), the antimicrobial susceptibility profiles for

Table 1. Resistance percentage of isolates for five pre-selected antimicrobial agents

Isolates (*n*)	MIC range (mg/*l*)				
	NAL	FLU	ENR	CIP	MOX
Total (1,221)					
MIC_{50}	512	16	1	0.5	1
MIC_{90}	>1,024	1,024	128	64	64
R%	76.4	74.3	50.6	42.3	51.3
Swine (583)					
MIC_{50}	512	16	1	0.5	1
MIC_{90}	>1,024	1,024	128	64	32
R%	72.0[a]	71.2[a]	47.5[a]	39.8[a]	46.7[a]
Chicken (515)					
MIC_{50}	>1,024	512	4	2	4
MIC_{90}	>1,024	>1,024	128	64	64
R%	81.9[a,b]	78.8[a,b]	55.2[a,c]	49.5[a,c]	57.3[a–c]
Turkey (42)					
MIC_{50}	>1,024	32	1	0.5	1
MIC_{90}	>1,024	1,024	32	16	16
R%	81.0	69.1	45.2	45.2	50.0
Duck (25)					
MIC_{50}	512	16	0.5	0.5	0.5
MIC_{90}	>1,024	1024	32	16	16
R%	64.0[b]	64.0[b]	44.0	40.0	44.0[b]
Goose (56)					
MIC_{50}	>1,024	16	1	0.5	1
MIC_{90}	>1,024	1024	32	16	16
R%	73.2	73.2	48.2[c]	39.3[c]	48.2[c]

NAL: Nalidixic acid, FLU: Flumequine, ENR: Enrofloxacin, CIP: Ciprofloxacin, MOX: Moxifloxacin. a) The results of MICs between swine and chicken were significant difference calculated by student *t*-test (*P*<0.05). b) The results of MICs between chicken and duck were significant difference calculated by student *t*-test (*P*<0.05). c) The results of MICs between chicken and goose were significant difference calculated by student *t*-test (*P*<0.05).

Table 2. Distribution of PMQR determinants among *E. coli* isolates of different origin

	qnr S1	*aac(6')-Ib*-cr	*qnrB2*
Swine (583)	84	50	0
Chicken (515)	31	5	2
Turkey (42)	0	0	1
Duck (25)	1	0	0
Goose (56)	9	0	0
Total	125	55	3

different animal species and the antimicrobial agents are summarized in Table 1. Isolates from pigs showed significantly lower MICs for all quinolones when compared to the chicken isolates (*P*≤0.05). Among the strains isolated from poultry, the MICs of NAL, FLU and MOX, the MICs of the strains from chickens were significantly higher than the MICs of the strains from ducks (*P*≤0.05). Likewise, isolates from chicken showed significantly higher MICs for ENR, CIP and MOX when compared to the goose isolates (*P*≤0.05) (Table 1).

Prevalence of PMQR genes

Overall, the PMQR determinants were found in 181 *E. coli* isolates (14.8%; 181/1,221). The prevalence of *qnrS1* was the highest (10.2%), followed by *aac(6')-Ib*-cr (4.5%) and *qnrB2* (0.3%). No isolates carrying the *qnrA*, *qnrC*, *qnrD* and *qepA* genes were detected in this study. The prevalence of the PMQR genes in the isolates from different animals was *qnrS1* (14.4%) and *aac(6')-Ib*-cr (8.6%) in swine; in chicken, the rates were *qnrS1* (6.0%), *aac(6')-Ib*-cr (1.0%) and *qnrB2* (0.4%) (Table 2). In 42 *E. coli* isolates from turkeys, only one PMQR gene, *qnrB2* (2.4%), was detected. *qnrS1* was detected in *E. coli* isolates from ducks and geese, with one in 25 (4.0%) for isolates from duck and nine in 56 (16.1%) for isolates from geese. In comparison with the *qnrS1* and the *aac(6')-Ib*-cr genes prevalence in pig isolates, chicken and waterfowl isolates showed low prevalence rates.

Table 3. Co-relationship of genotype and phenotype of 181 PMQR-positive *E. coli* isolates

PMQR	QRDR				n	MIC (mg/*l*)				
	Gyr A-83 Ser	Gyr A-87 Asp	Par C-80 Ser	Par C-84 Glu		NAL	FLU	ENR	CIP	MOX
qnr S1	-	-	-	-	73	4≥1,024	≤0.5–512	≤0.25–32	≤0.125–32	≤0.125–16
	Ala	-	-	-	3	64	16	2	1	4
	Leu	-	-	-	21	256≥1,024	8–1,024	0.5–64	0.25–32	0.5–128
	Leu	-	Arg	-	1	>1,024	512	4	4	4
	Leu	-	Ile	-	1	>1,024	512	32	32	64
	Leu	Asn	Ile	-	24	>1,024	1,024≥1,024	16–128	16–64	16–256
qnr B	-	-	-	-	3	16	4–16	0.5–1	0.25	1–2
aac(6')-Ib-cr	-	-	-	-	3	4–16	≤0.5–2	≤0.25	≤0.125	≤0.125–0.25
	-	Gly	-	-	2	128	4–8	≤0.25	0.25	≤0.125–0.25
	Ala	-	-	-	2	128–256	16	0.5	1	0.5
	Leu	-	Ile	-	1	>1,024	1,024	2	4	1
	Leu	-	Arg	-	3	>1,024	512–1,024	4–16	16	2–8
	Leu	Asn	Ile	-	26	>1,024	512≥1,024	32≥512	32≥256	16–256
	Leu	Tyr	Ile	-	2	>1,024	1,024≥1,024	64–256	64–256	32–256
	Leu	Asn	Ile	Ala	7	>1,024	512≥1,024	64–128	64	32
	Leu	Asn	Ile	Gly	7	>1,024	1,024≥1,024	128≥512	64–256	32–256
aac(6')-Ib-cr, *qnr S*	Leu	Asn	Ile	-	2	>1,024	1,024	16–128	16–128	16–128

NAL: Nalidixic acid, FLU: Flumequine, ENR: Enrofloxacin, CIP: Ciprofloxacin, MOX: Moxifloxacin.

Detection of QRDR in GyrA and ParC from PMQR-positive isolates

Mutations in the QRDRs of GyrA and ParC were analyzed in 181 *E. coli* isolates carrying the PMQR determinant. Alterations in the *gyrA* gene were detected in 102 (56.4%) of the 181 PMQR-positive strains. There were five types of mutations detected in the QRDR of GyrA; 66 (64.7% of the 102 GyrA mutated isolates) isolates had Ser83Leu and Asp87Asn, 27 (26.5%) had Ser83Leu, five (4.9%) had Ser83Ala, two (2.0%) had Ser83Leu, and Asp87Tyr and two (2.0%) had Asp87Gly. Seventy-four (72.5%) isolates out of the 102 strains with *gyrA* mutations were found to harbor additional mutations in the *parC* gene. There were four types of mutations found in ParC: 56 (75.7%) of the 74 mutated isolates had Ser80Ile, seven (9.5%) had Ser80Ile and Glu84Ala, seven (9.5%) had Ser80Ile and Glu84Gly, and four (5.4%) had Ser80Arg. No QRDR mutations were detected in the strains susceptible to NAL, FLU, ENR, CIP and MOX, despite the presence of the *qnrB2*, *qnrS1* or the *aac(6')-Ib*-cr gene. The *E. coli* strains with a single point mutation for GyrA at Ser83Leu/Ala or Asp87Gly showed 100% resistance to NAL (MIC ≥32 mg/*l*). The *E. coli* strains with double point mutations (GyrA Ser83Leu and ParC Ser80Arg/Ile) in the QRDR were highly resistant to NAL and FLU (MIC ≥512 mg/*l*), and the MIC values for the three antimicrobials, ENR, CIP and MOX, were in the range of 1–64 mg/*l*. The MIC values of the *E. coli* strains with three or four point mutations (GyrA Ser83Leu and Asp87Asn/Tyr substitutions and ParC Ser80Ile or Glu84Gly/Ala amino acid substitution) were 16≥512 mg/*l* for ENR, 16≥256 mg/*l* for CIP and 16–256 mg/*l* for MOX. The results suggested that with more point mutations in GyrA and ParC, the MIC values for quinolones became higher (Table 3).

DISCUSSION

This study indicated that the *E. coli* isolated from the diseased animals was highly resistant to the commonly used quinolones NAL (76.4%) and FLU (74.3%). Investigations into the quinolone-resistance of *E. coli* from swine in Taiwan can be backdated to approximately 20 years ago; of the *E. coli* isolated from diseased pigs, 48.3% (29/60) were resistant to ENR [4]. Thereafter, in 2006, of 110 *E. coli* isolated from humans and 61 *E. coli* isolated from diseased swine, 55.5 and 95.1% were resistant to NAL, whereas 38.2 and 85.2% were resistant to CIP, respectively [15]. In this study, 72.0, 47.5 and 39.8%, of the swine isolates were resistant to NAL, ENR and CIP, which indicated that *E. coli* isolated from either healthy or diseased animals have exhibited serious resistance to quinolone antibacterial agents since the 1990s.

Recent studies of APEC in Taiwan show that the resistance to NAL and FLU gradually increased from the 1980s to the 1990s. In the 1980s, *E. coli* isolated from poultry exhibited 100% susceptibility to quinolones at the beginning of FLU use [30]. In the 1990s, after the new generation of fluoroquinolones (ENR, norfloxacin and ofloxacin) was used, the FLU-resistance to APEC increased to approximately 70% [5]. This finding suggests an association between the use of fluoroquinolone agents and the bacterial resistance to these agents. Since May 2005, the government has banned the use of fluoroquinolones in feed additives for food-producing animals. The results of this study showed that even with this ban being enforced, the FLU resistance rate remained above 70%. Therefore, further investigations are needed.

The PMQR determinant is an important public health issue worldwide. This determinant is mainly spread by horizontal transmission [25, 27]. The prevalence of *oqxAB* is the highest among other PMQR genes in China, and this high prevalence is likely due to the frequent use of Olaquindox and Carbadox as medication additives for disease treatment and prevention in swine. Current research in China on *E. coli* PMQR determinants showed that the prevalence of *oqxAB* is the highest, followed by *aac(6')-*

Ib-cr, *qnr* and *qepA* [6, 36, 37]. In South Korea, *E. coli* carrying *qnrB4*, *qnrS1* and *aac(6')-Ib*-cr were isolated from food-producing animals, and *aac(6')-Ib*-cr was also detected in *E. coli* from meat and food products [22, 29]. In 2013, the *aac(6')-Ib*-cr gene was reported in APEC in Japan [21]. Only a few studies regarding the drug resistance in food-producing animals are available in Taiwan. The relevant clinical studies for human medicine suggested that *qnrB2*, *qnrB4*, *qnrS1*, *oqxAB*, *qnrD* and *aac(6')-Ib*-cr were found in clinical cases of bacterial infection [20, 24, 33]. The results of the PMQR determinants detection in this study showed that the prevalence of *qnrS1* was the highest (10.2%), followed by *aac(6')-Ib*-cr (4.5%) and that *qnrB2* was the lowest (0.3%). When compared with the *E. coli* that were previously isolated from healthy animals, the results showed that the detection rates of *qnrS* and *aac(6')-Ib*-cr in diseased animals were higher [23]. The reason is probably that these *E. coli* from diseased animals were under selective pressure in an environment filled with antimicrobial agents used for disease treatment and prevention, and this practice, in turn, causes the spread of the PMQR determinants [25, 34, 37]. In addition, in this study, the majority of *aac(6')-Ib*-cr were detected from swine and chicken isolates, and the detection percentage of *E. coli* from swine (8.6%) was higher than the isolates from chicken (1.0%). The clinical administration of the aminoglycosides is more often seen in swine than in poultry. For example, when diarrhea caused by *E. coli* occurs in new-born piglets, oral gentamicin is often used for treatment, which likely explains the higher positive rate of *aac(6')-Ib*-cr in the *E. coli* isolated from swine.

PMQR determinants confer a low-level of resistance to quinolones and fluoroquinolones and a favorable background in which the selection of additional chromosomally encoded quinolone resistance mechanisms could act during or after treatment with fluoroquinolones [25, 34, 37]. This study showed that the PMQR-positive strains susceptible to NAL, FLU, ENR, CIP and MOX did not have mutations in GyrA and ParC. Point mutations in GyrA (Ser83Leu/Ala or Asp87Gly) confer NAL resistance to the strains with these mutations, and the strains with mutations in GyrA (Ser83Leu) and ParC (Ser80Arg/Ile) showed a high level of resistance to NAL and FLU. The number of mutations in the QRDR of the *gyrA* and/or the *parC* genes was significantly associated with the MICs of quinolones [25]. These results suggest that the quinolone resistance in pathogenic *E. coli* from Taiwan is mainly caused by QRDR mutations. The major mutations in Asia are the substitutions of Ser83Leu and Asp87Asn in GyrA and Ser80Ile in ParC [31], and the results of this study showed a similar pattern of prevalence. In addition, the mutations of Ser83Ala and Asp87Gly/Tyr in GyrA and Glu84Ala/Gly in ParC were also discovered (Table 3).

The pathogenic *E. coli* from diseased animals in Taiwan exhibit high levels of fluoroquinolone resistance. The PMQR could provide *E. coli* with a low level of resistance to quinolones, because this mutation allows the bacteria to survive in an environment with low concentrations of antimicrobials. When animals infected with *E. coli* are treated with antibacterial agents, strains with the QRDR mutations could survive due to selective pressure, leading to higher levels of quinolone resistance. The resistance level found in *E. coli* with multiple mutations in the QRDR was significantly higher than the strains with no QRDR mutations ($P \leq 0.01$). The high prevalence of quinolone-resistant *E. coli* observed in this study suggests the need for improved education and communication on the issue of antimicrobials used in veterinary medicine. Unfortunately, we could not correlate these antimicrobial resistance rates to antibiotic use due to the lack of official data on the use of antibiotics in Taiwan. Therefore, it is important to use antimicrobial agents in a correct and prudent manner. Moreover, in order to control the development and spread of quinolone resistance, comprehensive and stringent regulations and guidelines also need to be established and then enforced.

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