High Prevalence of Plasmid-Mediated Quinolone Resistance Determinants *qnr*, *aac(6′)-Ib-cr*, and *qepA* among Ceftiofur-Resistant *Enterobacteriaceae* Isolates from Companion and Food-Producing Animals[†]

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Three kinds of plasmid-mediated quinolone resistance (PMQR) determinants have been discovered and have been shown to be widely distributed among clinical isolates: *qnr* genes, *aac(6′)-Ib-cr*, and *qepA*. Few data on the prevalence of these determinants in strains from animals are available. The presence of PMQR genes in isolates from animals was determined by PCR amplification and DNA sequencing. The production of extended-spectrum β -lactamases (ESBLs) and AmpC β -lactamases in the strains was detected, and their genotypes were determined. The genetic environment of PMQR determinants in selected plasmids was analyzed. All samples of ceftiofur-resistant (MICs ≥ 8 μ g/ml) isolates of the family *Enterobacteriaceae* were selected from 36 companion animals and 65 food-producing animals in Guangdong Province, China, between November 2003 and April 2007, including 89 *Escherichia coli* isolates, 9 *Klebsiella pneumoniae* isolates, and isolates of three other genera. A total of 68.3% (69/101) of the isolates produced ESBLs and/or AmpC β -lactamases, mainly those of the CTX-M and CMY types. Of the 101 strains, PMQR determinants were present in 35 (34.7%) isolates, with *qnr*, *aac(6′)-Ib-cr*, and *qepA* detected alone or in combination in 8 (7.9%), 19 (18.8%), and 16 (15.8%) strains, respectively. The *qnr* genes detected included one *qnrB4* gene, four *qnrB6* genes, and three *qnrS1* genes. Five strains were positive for both *aac(6′)-Ib-cr* and *qepA*, while one strain was positive for *qnrS1*, *aac(6′)-Ib-cr*, and *qepA*. *qnrB6* was flanked by two copies of *ISCR1* with an intervening *dfr* gene downstream and *sul1* and *qacEΔ1* genes upstream. In another plasmid, *aac(6′)-Ib-cr* followed *int11* and *arr-3* was downstream. PMQR determinants are highly prevalent in ceftiofur-resistant *Enterobacteriaceae* strains isolated from animals in China. This is the first report of the occurrence of PMQR determinants among isolates from companion animals.

Since 1998, three kinds of plasmid-mediated quinolone resistance (PMQR) determinants have been described: Qnr (QnrA, QnrB, QnrS) (8, 13, 21), AAC(6′)-Ib-cr (32), and QepA (40). Qnr proteins belong to the pentapeptide-repeat family and protect DNA gyrase and topoisomerase IV from quinolone inhibition (30). AAC(6′)-Ib-cr is a variant of AAC(6′)-Ib and is responsible for reduced susceptibility to ciprofloxacin or norfloxacin by N-acetylation of a piperazinyl amine (32). QepA is a quinolone efflux pump protein and shows a considerable similarity to the MFS types of efflux pumps belonging to the 14-transmembrane segment family of environmental actinomycetes (27, 40).

qnr and *aac(6′)-Ib-cr* determinants are widely distributed in human clinical isolates of the family *Enterobacteriaceae* around

the world (24, 28, 30, 37, 38), while only a few surveys on the prevalence of the newer *qepA* gene are available (18, 39). The transferability of resistant bacteria or mobile resistance determinants between animals and humans via the food chain or by direct contact has been indicated in several studies (6, 20, 22). Quinolones have also been widely used to treat livestock and for pet animal therapy in some countries. However, few reports on the occurrence of PMQR determinants among bacteria from companion animals and food-producing animals have been published, and the total strain number is limited (1, 2, 15, 42).

Quinolones and β -lactams are among the most commonly used antimicrobials in both human and veterinary clinical medicine. The association of *qnr* and *aac(6′)-Ib-cr* determinants with extended-spectrum β -lactamases (ESBLs) and AmpC β -lactamases has been documented (14, 23, 25, 31). A variety of β -lactamases, including extended-spectrum CTX-M and plasmid-encoded AmpC-type β -lactamases, have been identified in bacteria derived from food-producing and companion animals. Such strains may provide a reservoir for β -lactamase-producing bacteria in humans (3, 17, 19, 41). In a previous study, we showed that PMQR determinants were present among 16S rRNA methylase RmtB-producing *Escherichia coli* strains isolated from pigs and demonstrated a strong linkage between *qepA* and *rmtB*, with *qepA* being present in 28 (58.%)

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TABLE 1. Characteristics of the *qnrB*-, *qnrS*-, *qepA*-, and *aac(6')-Ib-cr*-positive *Enterobacteriaceae* strains

Strain	Animal source ^a	Origin	Date of isolation	Ciprofloxacin MIC (μg/ml)	PMQR determinant	ESBL or AmpC β-lactamase
<i>K. pneumoniae</i> C1	PH	Cat feces	4 Sept. 2006	8	<i>aac(6')-Ib-cr</i>	ND ^b
<i>K. pneumoniae</i> C7	PH	Cat sneeze	5 Sept. 2006	8	<i>aac(6')-Ib-cr</i>	ND
<i>K. pneumoniae</i> D10	PH	Dog feces	8 Sept. 2006	32	<i>aac(6')-Ib-cr</i> , <i>qnrB4</i>	CTX-M-1G, ^c DHA-1
<i>K. pneumoniae</i> D20	PH	Dog vomitus	15 Oct. 2006	32	<i>aac(6')-Ib-cr</i>	CTX-M-9G, DHA-1
<i>K. pneumoniae</i> D24	PH	Dog vomitus	18 Oct. 2006	2	<i>qnrB6</i>	CTX-M-9G
<i>K. pneumoniae</i> D34	PH	Dog sneeze	22 Nov. 2006	32	<i>aac(6')-Ib-cr</i> , <i>qepA</i>	CTX-M-9G
<i>K. pneumoniae</i> D61'	PH	Dog feces	12 Mar. 2007	16	<i>aac(6')-Ib-cr</i>	CTX-M-9G
<i>E. cloacae</i> D32	PH	Dog feces	20 Nov. 2006	32	<i>qepA</i>	CTX-M-9G
<i>E. cloacae</i> D73	PH	Dog feces	7 Apr. 2007	16	<i>aac(6')-Ib-cr</i>	CTX-M-9G
<i>C. freundii</i> D26	PH	Dog feces	2 Nov. 2006	4	<i>qnrB6</i>	CTX-M-9G
<i>E. coli</i> D3	PH	Dog feces	4 Sept. 2006	16	<i>aac(6')-Ib-cr</i> , <i>qepA</i>	CTX-M-1G, CTX-M-9G
<i>E. coli</i> D23	PH	Dog feces	17 Oct. 2006	32	<i>aac(6')-Ib-cr</i> , <i>qepA</i>	CTX-M-9G
<i>E. coli</i> D40	PH	Dog feces	4 Dec. 2006	8	<i>qepA</i>	CTX-M-1G, CTX-M-9G
<i>E. coli</i> D47	PH	Dog feces	7 Dec. 2006	32	<i>aac(6')-Ib-cr</i> , <i>qepA</i>	CTX-M-9G
<i>E. coli</i> D55	PH	Dog feces	9 Dec. 2006	32	<i>qepA</i>	CTX-M-9G
<i>E. coli</i> D57	PH	Dog feces	9 Mar. 2007	4	<i>qepA</i>	CTX-M-9G
<i>E. coli</i> D60	PH	Dog feces	10 Mar. 2007	0.125	<i>qepA</i>	CTX-M-1G, CTX-M-9G
<i>E. coli</i> D61	PH	Dog feces	11 Mar. 2007	16	<i>aac(6')-Ib-cr</i>	CTX-M-9G
<i>E. coli</i> D71	PH	Dog feces	5 Apr. 2007	64	<i>qepA</i>	CTX-M-1G, CTX-M-9G
<i>E. coli</i> D76	PH	Dog sneeze	9 Apr. 2007	16	<i>qepA</i>	ND
<i>E. coli</i> D77	PH	Dog feces	4 Apr. 2007	4	<i>aac(6')-Ib-cr</i>	CTX-M-9G
<i>E. coli</i> D81'	PH	Dog feces	2 May 2007	16	<i>aac(6')-Ib-cr</i>	CTX-M-9G, CMY-2
<i>E. coli</i> D86	PH	Dog feces	7 May 2007	0.06	<i>aac(6')-Ib-cr</i>	CTX-M-9G
<i>E. coli</i> D87	PH	Dog feces	8 May 2007	16	<i>aac(6')-Ib-cr</i>	CTX-M-9G
<i>E. coli</i> Du6	F1	Duck feces	21 Apr. 2007	2	<i>qnrB6</i>	CTX-M-9G
<i>E. coli</i> Du27	F1	Duck feces	21 Apr. 2007	32	<i>qepA</i>	CTX-M-1G
<i>E. coli</i> Du19'	F2	Duck liver	21 Apr. 2007	64	<i>qnrB6</i>	CTX-M-9G
<i>E. coli</i> Du89	F8	Duck feces	12 Sept. 2004	0.5	<i>qnrS1</i>	ND
<i>E. coli</i> P1	F3	Pig liver	15 Apr. 2007	32	<i>qepA</i>	ND
<i>E. coli</i> P17'	F4	Pig liver	15 Apr. 2007	32	<i>qepA</i>	CTX-M-1G
<i>E. coli</i> P498	F6	Pig feces	27 Apr. 2005	64	<i>qnrS1</i>	ND
<i>E. coli</i> Chi197	F5	Chicken feces	20 Jan. 2005	64	<i>aac(6')-Ib-cr</i>	CTX-M-9G
<i>E. coli</i> PA09	F7	Partridge feces	23 May 2005	64	<i>aac(6')-Ib-cr</i>	ND
<i>E. coli</i> PA11	F7	Partridge feces	23 May 2005	>128	<i>qnrS1</i> , <i>qepA</i> , <i>aac(6')-Ib-cr</i>	ND
<i>E. coli</i> PA14	F7	Partridge liver	23 May 2005	>128	<i>aac(6')-Ib-cr</i> , <i>qepA</i>	ND

^a PH, pet hospital; F1 to F8, animal farms 1 to 8, respectively.

^b ND, not detected.

^c G, group.

of 48 *mtB*-carrying strains (18). The aim of the present study was to investigate the prevalence of PMQR determinants among ceftiofur-resistant *Enterobacteriaceae* isolates from companion and food-producing animals and to identify whether these strains produce ESBLs and/or plasmid-mediated AmpC β-lactamases. The genetic environment of *qnr* and *aac(6')-Ib-cr* in selected plasmids was also characterized.

MATERIALS AND METHODS

Bacterial isolates. *Enterobacteriaceae* isolates were collected from 20 animal farms and a veterinary hospital; each isolate was from a separate animal, and all 101 unique ceftiofur-resistant (MICs ≥ 8 μg/ml) strains were selected. Thirty-six strains, including 25 *E. coli* strains, 8 *Klebsiella pneumoniae* strains, 2 *Enterobacter cloacae* strains, and 1 *Citrobacter freundii* strain, were isolated from feces, vomitus, or sneeze samples from diseased pet animals (31 dogs and 5 cats) at the South China Agricultural University Veterinary Hospital in Guangzhou, China, between September 2006 and May 2007. Sixty-five strains (64 *E. coli* strains and 1 *K. pneumoniae* strain) were collected from feces or liver samples of diseased food-producing animals (29 ducks, 18 pigs, 11 chickens, 6 partridges, and 1 goose) in Guangdong Province of China from November 2003 to April 2007. The bacterial strains were identified by classical biochemical methods and by using the Microlog (release 4.2) program (Biolog). Additional strains used were *E. coli* V517 and *E. coli* J53 containing plasmid R1, *Plac*, or R27, which were used as standards for determination of plasmid sizes (36). *E. coli* J53Azi^r, which is resistant to azide, was used as the recipient strain in conjugation experiments

(12). *E. coli* UAB1 (21), *E. coli* J53 containing plasmid PMG298 (13), a *K. pneumoniae* strain containing *qnrS1* (10), *E. coli* 12 (36), and *E. coli* GZ3 (18) were used as positive control strains for *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr*, and *qepA*, respectively, in PCR screening experiments. Sequenced strains that were positive for ESBL and AmpC β-lactamase genes and that were obtained in our previous studies (10, 19) were used as positive controls in PCR screening experiments. *E. coli* ATCC 25922 was used as a quality control strain in antimicrobial susceptibility testing experiments.

Antimicrobial susceptibility testing and β-lactamase determination. The MICs of nalidixic acid, ciprofloxacin, levofloxacin, and other antimicrobials were determined by agar dilution, in accordance with the guidelines of the CLSI (4, 5). The Etest (Biodisk AB, Solna, Sweden) was used to detect minimal changes in ciprofloxacin and levofloxacin susceptibility. The production of ESBLs by the strains was determined by the phenotypic confirmatory test (5), and AmpC expression was evaluated by the three-dimensional test with cefoxitin disks (16). The genotypes of the ESBL- and AmpC-positive strains were determined by PCR and sequencing.

Detection of PMQR determinants and β-lactamase-encoding genes. The isolates were investigated for the presence of *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib*, *bla_{TEM}*, *bla_{SHV}*, and *bla_{CTX-M}* genes by PCR amplification with the primer sets described previously (3, 19, 24, 31, 37). Two sets of primers for *qnrB* and *qnrS* were used to ensure the amplification of all subtypes (11). AmpC β-lactamases were amplified with the primers described by Perez-Perez and Hanson (26). The primers used for *qepA* were 5'-CGGCGGCGTGTGCTGCTGAGTTCTT and 5'-CCGA CAGGCCACGACGAGGATGC. Both strands of the purified PCR products were sequenced. The DNA sequences obtained and the deduced amino acid sequences were compared with those available in the GenBank database to

identify the subtypes of the *qnr* determinants and the β -lactamase genes. All positive PCR products of *aac(6')-Ib* were further analyzed by digestion with FokI (TaKaRa Biotechnology, Dalian, China) and/or direct sequencing to identify *aac(6')-Ib-cr*, which lacks the FokI restriction site present in the wild-type gene.

ERIC-PCR analysis. Enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) was performed with all PMQR determinant-positive isolates. The PCR amplifications were performed in 25- μ l volumes containing 2.5 mM MgCl₂, 0.5 U of ExTaq polymerase, 0.25 mM each deoxynucleoside triphosphate, 2.5 μ l of 10 $\times$ amplification buffer, 10 ng of crude template DNA, and 25 pmol each of two opposing primers, primers ERIC1R (5'-ATGTAAGCTCCTGGGGATTCA-3') and ERIC2 (5'-AAGTAAGTACTGGGGTGAGCG-3') (TaKaRa Bio, Dalian, China) (35). A negative control consisting of the same reaction mixture without a DNA template was included in each PCR. The samples were amplified as follows: 95°C for 5 min to denature the template; four-low stringency cycles of 94°C for 1 min, 26°C for 1 min, and 72°C for 2 min; 40 cycles of 94°C for 30 s, 40°C for 30 s, and 72°C for 1 min; and finally, 72°C for 10 min.

Conjugation experiments and plasmid analysis. Conjugation experiments were performed with the *qnr*-positive strains by the liquid mating-out assay, as described previously (36). Transconjugants were selected on tryptic soy agar plates containing sodium azide (100 μ g/ml) and sulfamethoxazole (300 μ g/ml) or ampicillin (100 μ g/ml). Plasmid DNA extraction was performed by a rapid alkaline lysis procedure (34). Plasmid DNAs from the *qnr*-positive isolates and transconjugants or reference strains were subjected to electrophoresis in 0.7% agarose gels. Plasmid sizes were estimated as described previously (36).

Structure analysis of the *qnrB*- or *aac(6')-Ib-cr*-containing plasmids. To determine the genetic environment of *aac(6')-Ib-cr* or the *qnr* genes, plasmid DNA was digested with EcoRI or HindIII, ligated to pUC18 (which is resistant to ampicillin; TaKaRa Bio, Otsu, Japan), and introduced into *E. coli* DH5 α . Transformants were selected on tryptic soy agar plates containing ampicillin at 50 μ g/ml and ciprofloxacin at 0.06 μ g/ml. The sequencing of both DNA strands was carried out by primer walking. The DNA sequences and the deduced amino acid sequences were analyzed and compared by use of the NCBI BLAST program.

Nucleotide sequence accession numbers. The nucleotide sequences in plasmid pHND2 containing *qnrB6* and pHND1 harboring *aac(6')-Ib-cr* have been submitted to GenBank and assigned accession numbers EU543271 and EU543272, respectively.

RESULTS

Prevalence of *qnr*, *qepA*, and *aac(6')-Ib-cr* genes. Among the total of 101 animal isolates, PMQR determinants were present in 35 (34.7%), with *qnr*, *aac(6')-Ib-cr*, and *qepA* being detected alone or in combination in 8 (7.9%), 19 (18.8%), and 16 (15.8%) strains, respectively. The *qnr* genes included one *qnrB4* gene, four *qnrB6* genes, and three *qnrS1* genes; *qnrA* was not detected. Five strains were positive for *aac(6')-Ib-cr*, in addition to *qepA*, while one strain harbored both *qnrB4* and *aac(6')-Ib-cr*. *qnrS1*, *aac(6')-Ib-cr*, and *qepA* coexisted in a strain of *E. coli* isolated from partridge feces. Detailed information on these PMQR determinant-positive isolates is given in Table 1.

Among the 36 isolates from companion animals, 24 (66.7%) strains carried at least one PMQR determinant; 3 (8.3%), 15 (41.7%), and 11 (30.6%) strains were positive for *qnr* genes, *aac(6')-Ib-cr*, and *qepA*, respectively. Of the 65 isolates from food-producing animals, 11 (16.9%) contained one or more PMQR determinants; 5 (7.7%), 4 (6.2%), and 5 (7.7%) strains were positive for *qnr* genes, *aac(6')-Ib-cr*, and *qepA*, respectively. The prevalence of PMQR determinants was significantly higher in companion animal strains (66.7%) than in food-producing strains (16.9%) (by the Pearson chi-square test, $\chi^2 = 25.32$; $P < 0.001$). The difference contributed to the higher prevalence of *aac(6')-Ib-cr* and *qepA* in pet animals than in food-producing animals.

ERIC-PCR analysis indicated that most PMQR determinant-positive isolates were not clonally related. However, two

TABLE 2. Characteristics of donor strains and *E. coli* J53 transconjugants

Strain ^a	Plasmid (size [kb])	PMQR determinant	ESBL or AmpC β - lactamase	MIC (μ g/ml) ^b														
				NAL	CIP	LEV	AMP	CTF	CTX	CAZ	CEO	GEN	AMK	CHL	SMZ	TMP		
<i>E. coli</i> J53	NA ^c	NA	NA	4	0.008	0.016	2	0.25	0.06	0.125	0.06	0.125	0.06	0.125	0.5	8	16	0.25
<i>K. pneumoniae</i> D10	MP ^d	<i>qnrB4 aac(6')-Ib-cr</i>	CTX-M-1G, DHA-1	>128	32	16	>128	128	128	128	128	64	>128	>128	>128	>128	>256	>256
Tc-D10	pHND1 (>180)	<i>qnrB4 aac(6')-Ib-cr</i>	DHA-1	16	0.125	0.25	>128	0.5	0.125	0.25	0.125	0.25	0.06	>128	>128	>128	>256	>256
<i>K. pneumoniae</i> D24	MP	<i>qnrB6</i>	CTX-M-9G	64	2	1	>128	>128	64	64	2	64	128	2	>128	>256	>256	>256
Tc-D24	pHND2 (75)	<i>qnrB6</i>	CTX-M-9G	16	0.5	0.25	>128	64	32	1	8	0.5	>128	>256	>256	>256	>256	>256
<i>C. freundii</i> D26	MP	<i>qnrB6</i>	CTX-M-9G	128	4	2	>128	128	64	64	1	64	>128	>128	64	>256	>256	>256
Tc-D26	pHND3 (145)	<i>qnrB6</i>	ND ^e	4	0.06	0.06	>128	0.25	0.06	0.125	0.06	16	1	8	4	>256	>256	>256
<i>E. coli</i> Du19 ^f	MP	<i>qnrB6</i>	CTX-M-9G	>128	16	16	>128	128	128	16	32	>128	>128	>128	>128	>128	128	0.5
Tc-Du19 ^f	pHNDU1 (70)	<i>qnrB6</i>	ND	64	0.5	2	64	0.25	1	0.5	0.25	>128	>128	>128	>128	>128	128	0.5
<i>E. coli</i> PA11	MP	<i>qnrS1</i> , <i>qepA</i> , <i>aac(6')-Ib-cr</i>	ND	>128	>128	128	>256	>128	16	32	64	>256	>256	>256	>256	>256	>256	>256
Tc-PA11	pHNPAl (85)	<i>qnrS1</i>	ND	32	0.5	1	>256	0.5	0.5	1	0.25	0.5	0.25	16	>256	>256	>256	>256

^a Tc-D10, Tc-D24, Tc-Du19^f, and Tc-PA11, transconjugants of *K. pneumoniae* D10, *K. pneumoniae* D24, *C. freundii* D26, *E. coli* Du19^f, and *E. coli* PA11, respectively.

^b CIP, ciprofloxacin; LEV, levofloxacin; NAL, nalidixic acid; AMP, ampicillin; CTF, ceftiofur; CTX, cefotaxime; CAZ, ceftazidime; CEO, cequinome; GEN, gentamicin; AMK, amikacin; CHL, chloramphenicol; SMZ, sulfamethoxazole; TMP, trimethoprim. Ciprofloxacin and levofloxacin MICs were determined by Etest.

^c NA, not applicable.

^d MP, multiple plasmids.

^e ND, not detected.

^a Tc-D10, Tc-D24, Tc-D26, Tc-Du19^f, and Tc-PA11, transconjugants of *K. pneumoniae* D10, *K. pneumoniae* D24, *C. freundii* D26, *E. coli* Du19^f, and *E. coli* PA11, respectively.

^b CIP, ciprofloxacin; LEV, levofloxacin; NAL, nalidixic acid; AMP, ampicillin; CTF, ceftriaxone; CAZ, ceftazidime; CEO, cefquinome; GEN, gentamicin; AMK, amikacin; CHL, chloramphenicol; SMZ, sulfamethoxazole; TMP, trimethoprim. Ciprofloxacin and levofloxacin MICs were determined by Etest.

^c NA, not applicable.

^d MP, multiple plasmids.

^e ND, not detected.

FIG. 1. Comparison of regions flanking *qnrB6* in pHND2 and in three other plasmids carrying *qnrB2*, *qnrB6*, and *qnrA1*, respectively. (A) *Salmonella enterica* plasmid (GenBank accession no. AM234698); (B) pMG252 (GenBank accession no. DQ831140); (C) *K. pneumoniae* plasmid (GenBank accession no. EF667294); (D) pHND2 (GenBank accession no. EU543271).

K. pneumoniae isolates from cats (isolates C1 and C7) had indistinguishable ERIC patterns. Both carried *aac(6')-Ib-cr* but contained different β -lactamase genes: *bla*_{TEM-1} in *K. pneumoniae* C1 but *bla*_{SHV-1} in *K. pneumoniae* C7. Three isolates from food producers, two *E. coli* strains from partridges (strains PA11 and PA14) and one *E. coli* strain from a chicken (strain Chi197), also showed indistinguishable ERIC-PCR patterns, although they carried different PMQR determinants.

Identification of ESBLs and plasmid-mediated AmpC β -lactamases. ESBLs and/or AmpC β -lactamases were detected in 68.3% (69/101) of all strains and in 74.3% (26/35) and 65.2% (43/66) of the PMQR determinant-positive and -negative strains, respectively ($\chi^2 = 0.88$; $P = 0.348$). Among the ESBLs detected in ESBL-positive strains, 53 belonged to the CTX-M-9 group, 13 belonged to the CTX-M-1 group, and 1 was an SHV type. Among the AmpC-positive strains, eight had the CMY-2 β -lactamase and two had the DHA-1 β -lactamase. Eleven strains had two ESBLs or both ESBL and AmpC enzymes. The ESBLs or AmpC β -lactamases present in 35 strains positive for PMQR determinants are listed in Table 1.

PMQR determinants were detected in 26 (37.7%) of 69 isolates positive for ESBLs and/or AmpC β -lactamases and in 9 (28.1%) of 32 ESBL- and/or AmpC-negative isolates ($P = 0.348$).

Conjugation experiments and plasmid analysis. Quinolone resistance could be transferred by conjugation from four *qnrB*-positive strains and one *qnrS1*-positive strain. These transconjugants contained single plasmids of the same size as those in their respective donors. *aac(6')-Ib-cr* and *bla*_{DHA-1} were cotransferred with *qnrB4* carried by plasmid pHND1 from donor strain *K. pneumoniae* D10, which was isolated from dog feces. *bla*_{CTX-M-9G} was cotransferred with *qnrB6* carried by plasmid pHND2 from *K. pneumoniae* D24, but *bla*_{CTX-M-9G} was not detected on the transfer of *qnrB6*-carrying plasmid pHND3 from *C. freundii* D26 or *qnrB6*-carrying plasmid pHNDU1 from *E. coli* Du19'. A donor strain of *E. coli* PA11 isolated from a partridge carried three PMQR determinants (*qnrS1*, *aac(6')-Ib-cr*, and *qepA*), but a transconjugant carrying only *qnrS1* was obtained, indicating that *qnrS1* was located on a plasmid separate from plasmids carrying the other two determinants. The sizes of the plasmids harboring the *qnr* genes were between 70 kb and >180 kb. The ciprofloxacin MICs of four *qnrB* transconjugants and one *qnrS1* transconjugant

ranged from 0.06 to 0.5 μ g/ml, an increase of 8- to 64-fold over that of the recipient strain (Table 2).

Genetic structures adjacent to *qnrB6* and *aac(6')-Ib-cr* genes. DNA from *qnrB6*-carrying plasmids pHND2, pHND3, and pHNDU1 was digested with EcoRI and cloned into pUC18 by selection for ampicillin and ciprofloxacin resistance. Recombinant plasmids from all three donors contained an identical 5.5-kb sequence in which *qnrB6* was flanked by two copies of *ISCR1* and an intervening *dfr* gene downstream and *sul1* and *qacEΔ1* upstream (Fig. 1). An EcoRI-digested fragment of about 10.0 kb from plasmid pHND1 carrying *aac(6')-Ib-cr* was also cloned and sequenced. *aac(6')-Ib-cr* was located in a class 1 integron cassette, following *int11*, with *arr-3*, *sul1*, and *qacEΔ1* being located downstream (Fig. 2).

DISCUSSION

PMQR determinants were highly prevalent (34.7%) in the set of ceftiofur-resistant *Enterobacteriaceae* isolates from companion and food-producing animals from China evaluated in this study. In these strains, *qepA* and *aac(6')-Ib-cr* were more common than *qnr* determinants. Prior to our study, only a few investigations evaluated animal strains for PMQR determinants. The *qnrS* gene was identified in a strain of *Salmonella enterica* serovar Infantis isolated from a chicken in Germany (15) and in three strains of *Salmonella enterica* serovar Virchow obtained from chickens in Turkey (2). *qnrB*, *qnrS*, and *aac(6')-Ib-cr* were found in 10 strains of gram-negative bacteria isolates from zoo animals in Japan (1) and recently in *E. coli* isolates of food-producing animals from China (18, 42). Interestingly, as shown in this study, *qnrA* has not been detected in any reported animal isolates. *qepA*, a newly identified PMQR determinant, often seems to be carried along with the *rmtB* gene, which encodes a 16S rRNA methyltransferase (18, 27, 40). A recent study showed that the prevalence of *qepA* was low (0.3%) in *E. coli* clinical isolates collected from 140 Japanese hospitals between 2002 and 2006 (39). We previously showed that *qepA* is common in *rmtB*-positive *E. coli* isolates from pigs (18), and the present study also revealed a very high prevalence of *qepA* (15.8%) in *Enterobacteriaceae* isolates from food-producing and companion animals. To the best of our knowledge, *qepA* has been identified only in *E. coli* strains from Japan (39,

FIG. 2. Comparison of regions flanking *aac(6')-Ib-cr* in pHND1 and in other three plasmids. (A) pHSH2 (GenBank accession no. AY259086); (B) *K. pneumoniae* plasmid (GenBank accession no. EF636461); (C) pAS1 (GenBank accession no. DQ310703); (D) pHND1 (GenBank accession no. EU543272).

40), Belgium (27), and China (18). Our study extended the set of strains carrying *qepA* to *K. pneumoniae* and *E. cloacae* (Table 1).

This study found that the prevalence of PMQR determinants was significantly higher in isolates from companion animals (66.7%) than isolates from food-producing animals (16.9%). 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 (9). Many fewer data on the public health impact of antimicrobial usage in companion animals are available. In Denmark, a comparatively small number of dogs and cats consumed approximately the same amount of fluoroquinolones and cephalosporins as was consumed annually by the much larger population of food-producing animals (9). Thus, bacteria in pets are more likely to be subjected to rather high levels of antibiotic selection pressure, which may in turn explain the high rate of detection of these PMQR determinants. Since cats and dogs are in close contact with humans, the transfer of bacteria between animals and humans is also possible. The prudent use of antibiotics in companion animals is desirable.

The levels of production of ESBLs and/or AmpC β -lactamases were high (68.3%) in the ceftiofur-resistant *Enterobacteriaceae* strains evaluated in this study, with the main genotypes identified being the *bla*_{CTX-M-9} group, the *bla*_{CTX-M-1} group, and *bla*_{CMY-2}. Among the five transferable plasmids obtained during the conjugation experiment in this study, two plasmids harbored a *qnr* gene and an ESBL or AmpC gene simultaneously: *qnrB4* and *bla*_{DHA-1} in pHND1 and *qnrB6* and *bla*_{CTX-M-9} in pHND2. The linkage of the DHA-1 β -lactamase and *QnrB4* has been described in several recent reports (23, 25, 38). Our previous study found that *bla*_{DHA-1} is located downstream of *qnrB4* and is separated by *psp* operons and *orf1* (10).

qnrB6 was flanked by two copies of *ISCR1*, with *sul1* and *qacEΔ1* located upstream. This organization is similar to that seen in plasmid pMG252 carrying *qnrA1* (30), in a *Salmonella enterica* serovar Keurmassar plasmid (GenBank accession no. AM234698) carrying *qnrB2* (7), and in a *K. pneumoniae* plasmid carrying *qnrB6* (GenBank accession no. EF667294); but there are three differences among these four plasmids. The first is that *orf5* and *orf6* replaced one copy of *ISCR1* upstream of *qnrB6* in the *K. pneumoniae* plasmid. The second is that *sul1* and *qacEΔ1* are located upstream of *qnrB6* or *qnrB2* in plasmid pHND2, the *K. pneumoniae* plasmid and the *S. enterica* serovar

Keurmassar plasmid but they are downstream of *qnrA1* in pMG252. The third is that the orientation of *qnrB6* and *qnrB2* is reversed with respect to the location of *ISCR1* in pHND2, the *K. pneumoniae* plasmid, and the *S. enterica* serovar Keurmassar plasmid; but *qnrA1* has the same orientation as *ISCR1* in pMG252. Just as in In37 (36), in a *qnrB6*-bearing *K. pneumoniae* plasmid (GenBank accession no. EF636461) (29) and *Vibrio cholerae* plasmid pAS1 (GenBank accession no. DQ310703) (33), *aac(6')-Ib-cr* was located in the variable region of a class 1 integron and was always found in the first position of the gene cassettes directly following *intI1*.

In summary, we have found a high prevalence of PMQR determinants in ceftiofur-resistant isolates from companion and food-producing animals in China. *qepA* and *aac(6')-Ib-cr* were more prevalent in companion animal isolates than in food-producing animal isolates. The presence of these genes in bacteria isolated from pets and food-producing animals is an important fact that constitutes a public health concern. The prudent use of antimicrobial agents is thus necessary in veterinary medicine as well as in human medicine to minimize the spread of these resistance genes.

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