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RESEARCH ARTICLE

Detection of Plasmid-Mediated Quinolone Resistance Genes in Swine Clinical Isolates of *Escherichia coli*

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ABSTRACT

The drug resistance was investigated in swine clinical isolates of *Escherichia coli* in East China, and the prevalence of plasmid-mediated quinolone resistance (PMQR) genes was detected by PCR and sequencing. Among 53 clinical *E. coli* isolates, 47 (88.7%) isolates exhibited resistance to one or more antimicrobial agents, with the greatest resistance in norfloxacin (69.8%) and ciprofloxacin (67.9%) and the lowest resistance in amikacin (17.0%). In total, *aac(6')-Ib*, *qnr* and *qepA* were detected in 10 (18.9%), 10 (18.9%) and 1 (1.9%) of 53 *E. coli* isolates. The 10 *qnr*-positive isolates include 5 for *qnrB* and 5 for *qnrS*, whereas no *qnrA* and *qnrD* were detected. Noteworthy, 90% (9/10) *aac(6')-Ib*-positve isolates were the *cr* variant allele. The fact that, none resistance gene was detected in some isolates with severe drug resistance, suggests that there may be other potential genes or mechanisms mediating it.

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INTRODUCTION

Escherichia coli is the most common pathogen, which can cause infectious diseases such as enteritis or septicemia in both humans and animals (Croxen *et al.*, 2013), and the antimicrobial resistance of *E. coli* is on the rise in recent decades, which complicated the treatment of serious infections and even may lead to death (Jim, 2016). According to the statistics, there're over 0.7 million people dying from the infection of drug-resistant bacteria all over the world (Jim, 2016). *E. coli* is one of the bacteria most often resistant to drug and by 2050, over 3 million people will die from infection of *E. coli* if measures are not taken to reduce it (Jim, 2016). The mechanisms of plasmid-mediated quinolone resistance (PMQR) include 1) *qnr* proteins (*qnrA*, *qnrS*, *qnrB*, *qnrC*, *qnrD*), which belong to the large pentapeptide repeat family 2) the *aac(6')-Ib-cr*, which is a bifunctional aminoglycoside acetyltransferase variant that can acetylate fluroquinolones such as ciprofloxacin and norfloxacin, and 3) the efflux pump, *qepA* and *oqxAB*, which has been recently discovered to reduce susceptibility to hydrophilic fluoroquinolones (Hooper and Jacoby, 2015; Jacoby *et al.*, 2014). The plasmid-mediated mechanisms only provide low-level resistance to quinolones, but it is reported that PMQR genes can facilitate the mutant selection of high-level resistance to quinolone (Jacoby *et al.*, 2014).

The prevalence of PMQR genes has been widely reported in the world in human's clinical infectious bacteria, like *Escherichia coli* and *Klebsiella* spp. (Karah *et al.*, 2010; Wang *et al.*, 2004). However, few articles have reported the PMQR genes in veterinary clinical *E. coli* isolates. Because of the great use of aminoglycoside and fluoroquinolone in treating swine infectious diseases, the emergence and spread of resistant strains is becoming a serious problem. The objectives of this study were: 1) to investigate the relatedness between the drug resistance and prevalence of PMQR genes; 2) to investigate the aberration rate of *aac(6')-Ib* and whether it is associated with the resistance to fluoroquinolones.

MATERIALS AND METHODS

Bacteria strains: The *Escherichia coli* used in this study was isolated from diseased or dead swines from East China (Shanghai, Jiangsu Province, Anhui Province and Shandong Province) between March 2015 to April 2017.

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The clinical samples of liver, duodenum, kidney, spleen, lung and heart were seeded in blood agar and the single colony was then seeded in Mac-Conkey agar for purification. For further identification, the colonies turning pink were smeared for Gram staining and observed under the oil-immersion microscope and all *E. coli*-like colonies were identified by conventional biochemical analyses.

Detection of PMQR genes and sequencing: The presence of PMQR genes including *qnrA*, *qnrS*, *qnrB*, *qnrD*, *aac(6')-Ib* and *qepA* was investigated by PCR amplification and gel electrophoresis. All primers were shown in (Table 1). The DNA templates were prepared by boiling lysis. The PCR conditions for *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib* were as follows: initial step of 94℃ for 5 min, 30 cycles consisting of 94℃ for 1 min, 48℃ for 45 sec for *qnrA* as well as 52℃ for 45 sec for *qnrB*, *qnrS* and *aac(6')-Ib*, and 72℃ for 1 min, and a final extension step of 72℃ for 8 min. The detection of *qnrD* and *qepA* was conducted as previously described (Cavaco *et al.*, 2009; Yamane *et al.*, 2008). All positives were further confirmed by sequencing and compared with GenBank sequences using BLAST algorithms (www.ncbi.nlm.nih.gov).

Susceptibility testing: According to the judgment standard of the Clinical and Laboratory Standards Institutes (CLSI, 2009), the susceptibility testing was determined by Kirby-Bauer Disk Diffusion. The types of antimicrobial agents and the breakpoints were shown in (Table 2).

Table 1: Primers used in this study.

Gene	Primer Sequence (5' -3')	Size (bp)	Reference
aac(6')-Ib	TTGCGATGCTCTATGAGTGGCTA CTCGAATGCCTGGCGTGTTT	482	Park *et al.*, 2006
qnrA	TCAGCAAGAGGATTTCTCA GGCAGCACTATTACTCCCA	627	Wang *et al.*, 2004
qnrB	GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC	469	Gay *et al.*, 2006
qnrS	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	417	Gay *et al.*, 2006
qnrD	CGAGATCAATTTACGGGGAATA AACAAGCTGAAGCGCCTG	533	Cavaco *et al.*, 2009
qepA	GCAGGTCCAGCAGCGGGTAG CTTCCTGCCCGAGTATCGTG	199	Yamane *et al.*, 2008

Table 2: Judgment standard of drug resistance.

Drug class	Antimicrobial drug[a]	Drug content/μg	Breakpoints		
			Resistant	Intermediate	Susceptible
Fluoroquinolones	CIP	5	≤15	16-20	≥21
	NOR	10	≤12	13-16	≥17
	LVX	5	≤13	14-16	≥17
Aminoglycosides	GEN	10	≤12	13-14	≥15
	TOB	10	≤12	13-14	≥15
	AMK	30	≤14	15-16	≥17
β-lactams	MEZ	75	≤17	18-20	≥21
	CRO	30	≤13	14-20	≥21

[a] CIP, ciprofloxacin; NOR, norfloxacin; LVX, levofloxacin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; MEZ, mezlocillin; CRO, ceftriaxone.

RESULTS

Isolation and identification of *E. coli*: Totally, 53 *E. coli* strains were isolated from diseased organs or tissues (22 from duodenum, 9 from liver, 3 from lung, 7 from kidney, 6 from spleen and 6 from heart or hydropericardium) of sick and dead pigs in East China, which were sent to Animal Hospital of Yangzhou University from March 2015 to April 2017. All 53 strains grew well with smooth, moist and round colonies and turned pink on Mac-Conkey agar.

The strains were red and dispersedly distributed under the microscopy, which were Gram-negative bacteria. As for the conventional biochemical analyses, all strains can ferment glucose, lactose, maltose, mannose and sucrose. The IMViC tests showed that the strains were positive to indole test and methyl red test and were negative to VP test and citrate test. All these outcomes correspond to the biochemical characteristics of *E. coli*.

Prevalence of PMQR genes: Among the detected PMQR genes, there were 10 (18.9%, 10/53) strains being positive for *qnr* genes, with 5 for *qnrB* and 5 for *qnrS* respectively. And only 1 strain carried the efflux pump gene *qepA*. In addition, no *qnrA* and *qnrD* was detected.

As for aminoglycoside acetyltransferase, 10 (18.9%, 10/53) strains were detected carrying the *aac(6')-Ib*. 1 strain carried both *aac(6')-Ib-cr* and *qnrB*. The 10 *aac(6')-Ib*-positive strains were used to conduct phylogenetic analysis. Noteworthy, nine strains were phylogenetically related to previously published strain of *Escherichia coli* WJ1 (GenBank N0: KY924928) with the aberrant gene of *aac(6')-Ib-cr*, which can also acetylate the fluoroquinolones, and only one strain were related to previously published strain of *Enterobacter cloacae* CY01 (GenBank No: NC025003) with the normal gene of *aac(6')-Ib* (Fig. 1). As shown in Fig. 2, all the *–cr* variant has two substitutions: Trp128Arg and Asp205Tyr, compared with the other *aac(6')-Ib* and the standard CY01.

All the sequenced genes have been submitted to the GenBank (*aac(6')-Ib*: No.MF999208-217; *qnrB*: No. MF 999218-219; *qnrS*: No.MF999210-211; *qepA*:MF999222).

Antimicrobial drug resistance: 88.7% (47/53) strains showed resistance to one or more antimicrobial agents, among which there are 10 strains resistant to three kinds of antimicrobials. As shown in (Table 3), the most serious resistance is norfloxacin and ciprofloxacin, with the resistance rate up to 69.8% and 67.9%. The resistance rate of amikacin is the lowest, which is only 17.0%.

Genotype-phenotype correlation: The genotype-phenotype correlation is discussed by the agreement rate, which represents the proportion of gene-carrying isolates among the drug-resistant isolates. The genotype-phenotype agreement rate of PMQR genes to aminoglycosides or fluoroquinolones has been shown in (Table 4). The gene *aac(6')-Ib* has the highest rate to gentamicin and the lowest rate to amikacin. With regard to fluoroquinolones, the *-cr* variant has the highest rate to ciprofloxacin and norfloxacin, which is 25.0% and 24.3% respectively. The agreement rates of *qnrB*, *qnrS* and *qepA* are all less than 10%.

As to 9-*cr* variant-positive isolates, all of them are resistant to ciprofloxacin and norfloxacin, with the proportion reaching to 100% (Table 5). What's more, in 19 PMQR-positive strains, 11 (57.9%) strains are also resistant to β-lactams at the same time.

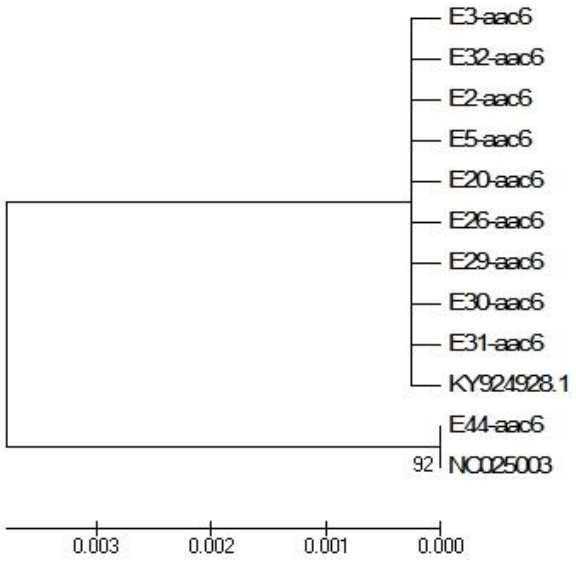

Fig. 1: Phylogenetic analysis of *aac(6')-Ib*-positive strains based on Neighbor-joining method with 1000 bootstrap replicas using Mega 4.0. DNA sequences of *Escherichia coli* WJ1 (GenBank No: KY924928) and *Enterobacter cloacae* CY01 (GenBank No: NC025003) were downloaded from the NCBI Webpage (www. ncbi.nlm.nih.gov). Numbers at the node are bootstrap values and the bar represents nucleotide changes.

Table 3: Antimicrobial resistance of *E. coli.*

Antimicrobial drug	Resistant		Intermediate		susceptible	
	No. of isolates	Ratio	No. of isolates	Ratio	No. of isolates	Ratio
CIP	36	67.9	4	7.5	13	24.5
NOR	37	69.8	3	5.7	13	24.5
LVX	32	60.4	7	13.2	14	26.4
GEN	25	47.2	8	15.1	20	37.7
TOB	18	34.0	5	9.4	30	56.6
AMK	9	17.0	1	1.9	43	81.1
MEZ	30	56.6	9	17.0	14	26.4
CRO	11	20.8	10	18.9	32	60.4

DISCUSSION

In recent years, the drug resistance is always on the rise in animals or food, which has killed many lives of animals and also threatens the human health. From 2008 to 2015, the antimicrobial resistance was severe with high prevalence of MDR *E. coli* in swine and chicken in China (Zhang *et al.*, 2017). In meat products, Jiang *et al* have found over 90% resistance and demonstrated the prevalence of resistant genes, which may transfer to humans via the food chain (Jiang *et al.*, 2014). In this study, the 53 isolates of *E. coli* were all isolated from East China from March 2015 to April 2017, hence, the detection results of resistance genes can reflect the current situation in swine and help us to take effective measures.

According to the drug sensitivity results, the drug resistance of *E. coli* in swine in this area is very serious, with the drug resistance rate up to 88.7% and MDR (multidrug resistance) rate up to 18.9%. And proportion of the potential MDR strains may be much higher than this data if we add more drug categories into the test. Generally, the resistance to fluoroquinolones is much more severe than the resistance to aminoglycosides and β-lactams, which may relate to the clinical medication. Corresponding to Jiang *et al.*'s research results of *E. coli* (Jiang *et al.*, 2014), amikacin has the lowest rate of 17.0%, and this suggests that amikacin can be used as the first choice to treat for serious infection of *E. coli* in the future.

The prevalence of PMQR genes in *E. coli* has been reported in many hospitals or laboratories all over the world (Strahilevitz *et al.*, 2007; Karah *et al.*, 2010), but only few studies investigated the *E. coli* isolated from swine.

According to the phylogenetic analysis (Fig 1), the *aac(6')-Ib* has a high proportion of aberrance, which is consistent to many other studies (Park *et al.*, 2006; Karah *et al.*, 2010; Jiang *et al.*, 2014). The amino acid substitutions (Trp128Arg and Asp205Tyr) are analogous to Trp102Arg and Asp179Tyr (Jacoby *et al.*, 2014), and this 26 amino acid larger *aac(6')-Ib-cr* has also been reported (de Toro *et al.*, 2013). The recent studies showed that the *aac(6')-Ib-cr* can mediate the resistance to ciprofloxacin and norfloxacin (Jacoby *et al.*, 2014). Although the agreement rates of these two agents are only 25.0% and 24.3%, the resistance rate reaches 100% in the 9 *-cr* variant-positive strains, which suggests the high concordance in the function to the two agents.

As an aminoglycoside acetyltransferase, *aac(6')-Ib* mainly modifies gentamicin and amikacin (Jacoby *et al.*, 2014), which does not agree with the observed low prevalence of amikacin resistance. Similar phenomenon has been reported in many other articles (Lindemann *et al.*, 2012; Haldorsen *et al.*, 2014), which suggests that the current breakpoint of amikacin cannot reflect the prevalence of aminoglycoside acetyltransferase precisely. Study also shows that there are differences in *aac(6')-I*-caused resistance affected by host bacterium(Haldorsen *et al.*, 2014). In addition, it has been suggested that the acitivity towatds amikacin can be influenced by the mutations in *aac(6')-Ib* (Shmara *et al.*, 2001).

In many studies, only 1 or 2 *E. coli* isolates from human beings have been detected to carry *qnrA*, and most of them were resistant to ciprofloxacin or producing extended-spectrum β-lactamases (ESBLs) (Wang *et al.*, 2004; Karah *et al.*, 2010). Further, there's study showing that the detection rate of *qnrA* was 0% in animals in Guangzhou, China (Yue *et al.*, 2008). Our study also hasn't found *qnrA*, so the prevalence of *qnrA* was generally low in both human and veterinary clinical isolates. *QnrD* was first defined by Cavaco (Cavaco *et al.*, 2009) in 2009 but the prevalence trend of *qnrD* in *E. coli* was not clear so far. Compared with *qnrA* and *qnrD*, *qnrB* and *qnrS* is somewhat more common, which corresponds to studies in recent years (Yue *et al.*, 2008; Karah *et al.*, 2010). The detection of *qnr* genes has been increasing in studies of the past decade, but it is still less than 10% in unselected clinical samples. What's more, in comparison with *E. cloacae* and *K. pneumonia*, the prevalence of *qnr* in *E. coli* is the least (Kim *et al.*, 2013; Jacoby *et al.*, 2014).

In this study, only 1 (1.9%) of 53 strains was *qepA*-positive. Although *qepA* gene has been detected in Japan and Korea many times (Yamane *et al.*, 2008; Kim *et al.*, 2009), the prevalence of it was less than 2%. The *qepA* was first found in multidrug resistant *E. coli*, carrying several other drug resistant genes (Cantón, 2009). The only 1 *qepA*-positive strain in this study also carries *aac(6')-Ib*, which explains the cause of multidrug resistance of this strain.

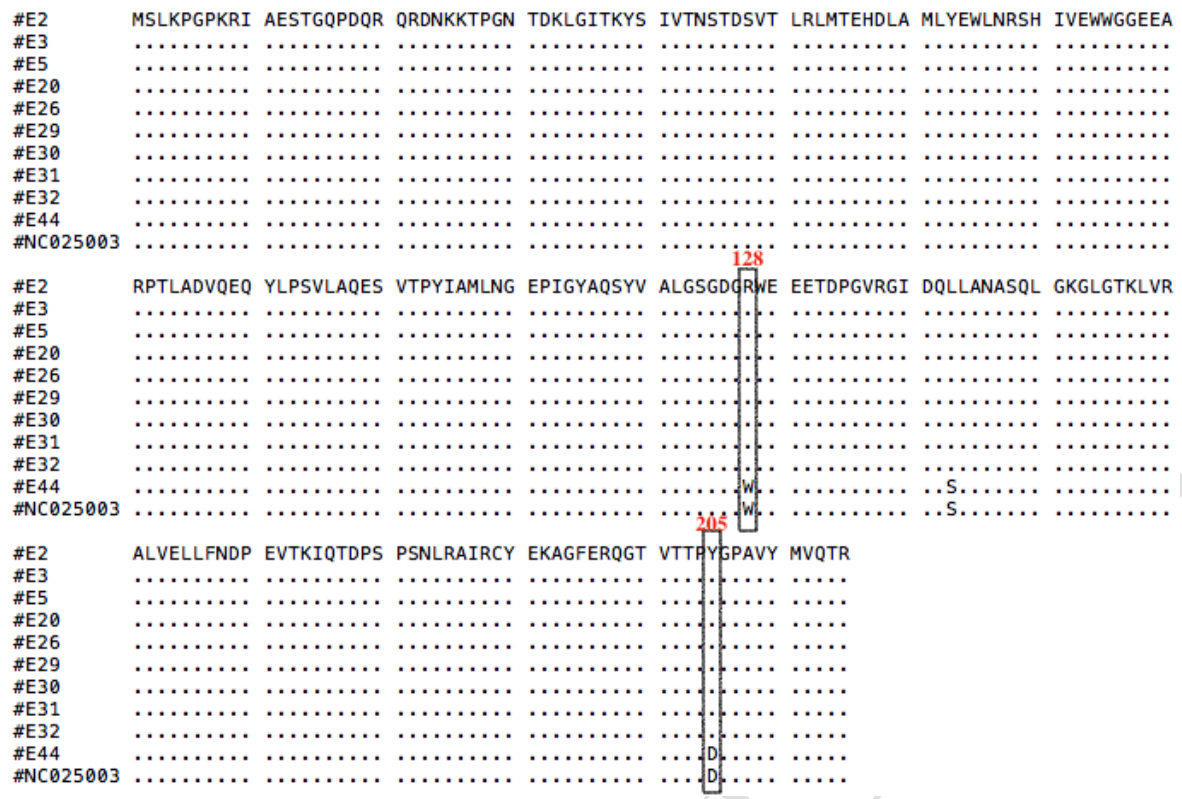

Fig. 2: The DNA sequences of 10 detected *aac(6')-Ib*-positive strains and *Enterobacter cloacae* CY01 have been translated and aligned by ClustalW using MEGA 4.0. The amino acid of location 128 and 205 has been substituted.

Table 4: Genotype-phenotype agreement rate[a].

Antimicrobial drug	*aac(6')-Ib*	Antimicrobial drug	*aac(6')-Ib-cr*	*qnrB*	*qnrS*	*qepA*
GEN	28.0(7/25)	CIP	25.0(9/36)	8.3(3/36)	5.6(2/36)	2.8(1/36)
TOB	44.4(8/18)	NOR	24.3(9/27)	5.4(2/37)	5.4(2/37)	2.7(1/37)
AMK	22.2(2/9)	LVX	15.6(5/32)	6.3(2/32)	6.3(2/32)	3.1(1/32)

[a]agreement rate=No. of isolates carrying genes/No. of resistant isolates×100%.

Table 5: Distribution of PMQR genes among *E. coli* clinical isolates.

Sample ID	*Aac(6')-Ib*	*qnr*	*qepA*	Fluoroquinolones phenotype	Aminoglycosides phenotype	β-lactams phenotype
E2	+, *-cr*[a]	—	—	CIP, NOR, LVX	TOB	
E3	+, *-cr*	—	—	CIP, NOR, LVX	GEN, TOB	
E5	+, *-cr*	*qnrB*	—	CIP, NOR	GEN, TOB	
E9	—	*qnrB*	—			MEZ
E12	—	*qnrS*	—	CIP, NOR, LVX	GEN	MEZ
E15	—	*qnrB*	—	CIP, NOR, LVX		MEZ, CRO
E16	—	*qnrS*	—			MEZ
E17	—	*qnrS*	—	CIP, NOR, LVX	GEN	MEZ
E20	+, *-cr*	—	—	CIP, NOR	TOB	
E26	+, *-cr*	—	—	CIP, NOR, LVX	GEN, TOB	MEZ
E29	+, *-cr*	—	—	CIP, NOR, LVX	GEN	MEZ
E30	+, *-cr*	—	—	CIP, NOR, LVX	GEN, TOB, AK	MEZ
E31	+, *-cr*	—	—	CIP, NOR	TOB	
E32	+, *-cr*	—	—	CIP, NOR	GEN	MEZ
E37	—	*qnrS*	—			
E43	—	*qnrS*	—		TOB	MEZ
E44	+	—	+	CIP, NOR, LVX	GEN, TOB, AMK	MEZ, CRO
E45	—	*qnrB*	—	CIP, NOR, LVX		
E47	—	*qnrB*	—	CIP		

[a]-cr, cr variant.

In the 19 PMQR-positive strains, 57.9% (11/19) strains were also resistant to β-lactams, such as Mezlocillin and Ceftriaxone, suggesting that PMQR genes may be associated with the resistane to β-lactams (Table 5). There're studies proving that the prevalence of *aac(6')-Ib-cr* and *qnr* among ESBL-producing strains was significantly higher than non-ESBL-producing strains (Karah *et al.*, 2010; Briales *et al.*, 2012; Haldorsen *et al.*, 2014).

The prevalence of PMQR genes in this study is generally low, while the drug sensitivity results showed that the resistance to fluoroquinolones was the severest. This difference may be influenced by some other drug resistant mechanisms, like the mutations in DNA gyrase

and topoisomerase IV. These plasmid-mediated genes can only confer low-level resistance, but it is proved that PMQR genes can facilitate bacterium to acquire other resistant genes, which can lead to high-level resistance (Karah *et al.*, 2010; Jacoby *et al.*, 2014). Hence, more focus and research should be given to it in the future.

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Authors contribution: Study design: WL and YW; Experimentation: WL, YY and JL; Article writing: WL, YY and YW; Data analysis: WL and YW.

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