FULL PAPER *Bacteriology*

Dynamics of Quinolone Resistance in Fecal *Escherichia coli* of Finishing Pigs after Ciprofloxacin Administration

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ABSTRACT. *Escherichia coli* resistance to quinolones has now become a serious issue in large-scale pig farms of China. It is necessary to study the dynamics of quinolone resistance in fecal *Escherichia coli* of pigs after antimicrobial administration. Here, we present the hypothesis that the emergence of resistance in pigs requires drug accumulation for 7 days or more. To test this hypothesis, 26 pigs (90 days old, about 30 kg) not fed any antimicrobial after weaning were selected and divided into 2 equal groups: the experimental (EP) group and control (CP) group. Pigs in the EP group were orally treated daily with 5 mg ciprofloxacin/kg of body weight for 30 days, and pigs in the CP group were fed a normal diet. Fresh feces were collected at 16 time points from day 0 to day 61. At each time point, ten *E. coli* clones were tested for susceptibility to quinolones and mutations of *gyrA* and *parC*. The results showed that the minimal inhibitory concentration (MIC) for ciprofloxacin increased 16-fold compared with the initial MIC (0.5 μg/m*l*) after ciprofloxacin administration for 3 days and decreased 256-fold compared with the initial MIC (0.5 μg/m*l*) after ciprofloxacin withdrawal for 26 days. *GyrA* (S83L, D87N/ D87Y) and *parC* (S80I) substitutions were observed in all quinolone-resistant *E. coli* (QREC) clones with an MIC ≥8 μg/m*l*. This study provides scientific theoretical guidance for the rational use of antimicrobials and the control of bacterial resistance.

KEY WORDS: ciprofloxacin, dynamics, *Escherichia coli*, quinolone resistance, swine

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Escherichia coli is a common porcine enteric bacterium. There exist *E. coli* strains that cause neonatal and postweaning diarrhea and edema disease [10, 16]. These 2 diseases are responsible for considerable economic losses worldwide due to mortality, morbidity, decreased growth rate and the cost of medication [3, 10]. To reduce the economic losses, different kinds of antimicrobials have been used for many years in pig farms all over the world [33]. However, the use of antimicrobials leads to selection of resistant bacteria, such as antimicrobial-resistant *E. coli* [32]. In China, quinolones have been widely used in medical science and veterinary clinic for many years. For this reason, *E. coli* resistance to quinolones has now become a serious issue in China [6, 18, 33]. Recent research shows that quinolone-resistant *E. coli* (QREC) from farm animals in China is more prevalent than in other countries [17].

As a natural reservoir of bacterial resistance, the fecal microbiota of animals plays an important role in the spread of bacterial resistance, because it contains great numbers and various kinds of bacteria [2]. There are mainly 4 reasons for the appearance of resistance in the fecal microbiota: 1. *de novo* selection of resistance mutants from the initial microbiota, 2. enrichment of QREC in the initial gut microbiota, 3. horizontal transfer of plasmid-mediated quinolone resistance genes and 4. exogenously acquired resistant bacteria [8, 11].

E. coli is a prevalent intestinal bacterium in animals and humans, and the level of antimicrobial resistance in intestinal *E. coli* can be used as a valid indicator for study about selection pressure in bacteria exposed to antimicrobials [5]. Selective pressure exerted by the use of antimicrobials appears to induce the *E. coli* resistance [32]. How QREC is induced has been well reported by *in vitro* studies for many years [9, 14]. But, conclusions from these studies do not well reflect the reality *in vivo*, as the environment *in vivo* is complex. Recently, the dynamics and diversity of *E. coli* populations in swine intestines under large-scale farming conditions have been reported [20]. Also, how antimicrobial use influences gut bacterial resistance in pigs has been explained by using metagenomic sequencing and quantitative PCR-based approaches [19, 34]. However, the ciprofloxacin resistance of *E. coli* from feces of pigs on large-scale pig farms and its dynamic changing pattern have not been clearly revealed. The time required for development of resistance is also unclear. In the pig industry in China, prophylactic antimicrobial therapy is usually used for 7 days to prevent disease. Based on this reality, the aim of our study was to investigate the effect of in-feed ciprofloxacin on the dynamics of resistance to quinolones in fecal *E. coli* of pigs

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Table 1. Primers used in this study

Target gene	Primers	Sequence (5′-3′)	Size (bp)	Annealing temperature (°C)	Accession no.
qnrB	*qnrB* -F	CGACCTGAGCGGCACTGAAT	515	57.5	DQ351241
	qnrB -R	TGAGCAACGATGCCTGGTAG			
qnrS	*qnrS* -F	CATACATATCGGCACCACAAC	637	56	EF683584
	qnrS -R	CAGGATAAACAACAATACCCAGT			
aac (6') Ib-cr	aac -F	TTGCGATGCTCTATGAGTGGCTA	482	55	EF465463
	aac -R	CTCGAATGCCTGGCGTGTTT			
oqxAB	*oqxAB* -F	GATCAGTCAGTGGGATAGTTT	627	55	HQ674771
	oqxAB -R	TACTCGGCGTTAACTGATTA			

on a large-scale pig farm and to test the hypothesis that the emergence of resistance in pigs requires drug accumulation for 7 days or more.

MATERIALS AND METHODS

Study design: The study was performed from July 2011 to September 2011 at a commercial pig farm located in Jitian Village, Shuangliu County, Chengdu, Sichuan Province, China. The pig farm was far from the city and has normal breeding management. There had been no mass outbreaks of disease and less antimicrobial use at the farm in the past year. A total of 26 finishing pigs were selected and divided into the following 2 groups: the experimental (EP) group and the control (CP) group. Each group of pigs was placed in different pens, and the 2 groups were managed by a single stockman.

The detailed characteristics of the pigs were as follows: 1. Landrace, Duroc and Yorkshire crossbred pigs (mixed males and females), 2. same ages (90 days old) with an average weight of 30 kg, 3. normal body temperature and normal fecal consistency and 4. no antimicrobial treatment in the past month.

Antimicrobial administration and sampling: Pigs were fed a control diet for 2 weeks before sampling. Pigs in the EP group were orally fed ciprofloxacin (150 mg per pig) once per day at noon for 30 days. We performed the feeding procedure in 2 steps: 1) Half the fodder (by weight) was taken out, and an antimicrobial solution was sprayed onto it by mixing; the mixture was then added into the corresponding feeder. 2) After the pigs ate all the mixture, the other half of the fodder without the antimicrobial was added in so that all the antimicrobials were eaten by pigs. No antimicrobials were used for the pigs in the CP group. The feeder would clean the floor of each pen after feeding the pigs. Fresh feces (>5 g) were picked from 5 different locations in each pen and mixed immediately. Fecal samples were collected at noon on each sampling day within 1 hr post feeding. They were collected on days 0, 1, 2, 4, 6, 11, 16, 21, 26, 31, 36, 41, 46, 51, 56 and 61. The fecal samples were placed into an ice box and transported to laboratory within 2 hr.

Counting of drug-resistant E. coli and total E. coli in fecal samples: One gram of each fecal sample was suspended in 10 m*l* PBS buffer (0.05 M), and the supernatant was collected after the sample was mixed fully. The supernatant was serially diluted 10-fold by using PBS buffer (0.05 M), and then 0.1 m*l* of each dilution, including the original dilution, was spread onto 2 types of culture medium: EMB agar (Becton, Dickinson and Co., Sparks, MD, U.S.A.) containing no ciprofloxacin and ciprofloxacin-plus EMB (CIP+EMB) agar containing 4 μg/m*l* ciprofloxacin. After 24 hr of incubation at 37°C, the numbers of QREC and total *E. coli* in feces were calculated according to the countable isolates on agar plates, and 10 *E. coli* clones were randomly picked from EMB agar for further analysis.

Bacterial isolates and susceptibility testing: All the *E. coli* clones were identified by classical biochemical methods and confirmed with an API 20E system (bioMérieux, France). The susceptibility to quinolones of the *E. coli* clones was tested by determining the minimal inhibitory concentration (MIC, 0.0078125–256 μg/m*l*). The agar dilution method described by the Clinical and Laboratory Standards Institute (CLSI) was used to test the MIC of the antimicrobials, ciprofloxacin, nalidixic acid, norfloxacin and levofloxacin [4]. *E. coli* clones were considered resistant or susceptible according to a standard introduced by the CLSI [4]. The reference *E. coli* strain ATCC 25922 was used as an internal control in this study.

Statistical analysis for the MICs of E. coli clones in the EP and CP groups: After the MICs of *E. coli* clones from both the EP and CP groups were determined, we used the *t*-test in SPSS (Statistical Product and Service Solutions) 13.0 software to analyze the salient differences in the MICs of *E. coli* clones between different points of time in the EP group and between the EP and CP groups. The *E. coli* clones were from the fecal samples collected on day 0, 1, 4, 6, 11, 31, 41, 51 and 61.

DNA sequence analysis and genotypic comparison of E. coli clones: By using the boiling method, DNA of *E. coli* clones was extracted and reserved at −80°C until use. The quinolone resistance-determining region (QRDR) genes (*gyrA*, *parC*) were found to have point mutations with primers described previously [13, 23]. Four plasmid-mediated quinolone resistance (PMQR) genes (*qnrB*, *qnrS*, *aac (6')-Ib-cr*, and *oqxAB*) were detected by PCR with the primers shown in Table 1. All the reactions were carried out in a final volume of 25 μl containing 2.5 μl of 10×Ex Taq Buffer, 2 μl of $MgCl_2$ (25 mM), 2 μl of dNTP (2.5 mM),

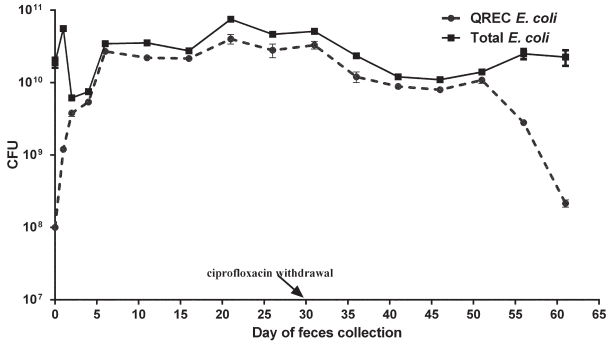

Fig. 1. The number of QREC and total *E. coli* strains in fecal samples collected from experimentally treated pigs at 16 time points.

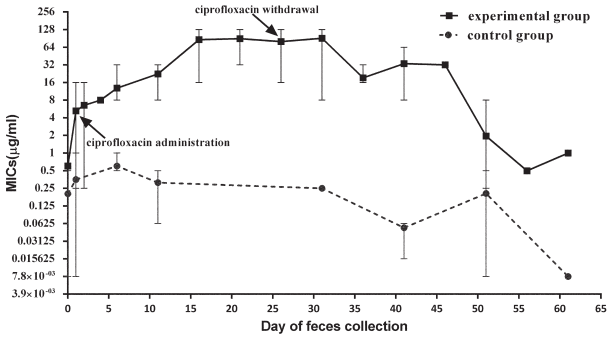

Fig. 2. The average MIC of *E. coli* clones (n=10) from fecal samples at 16 time points in the experimental group of pigs and at 8 time points in the control group of pigs. Bars indicate the standard deviation between MICs.

0.25 μl of TaKaRa Ex Taq (5 U/μl), 0.5 μl of each primer (25 M) and 1 μl of DNA samples. Homologous analysis of *E. coli* clones was performed through enterobacterial repetitive intergenic consensus (ERIC) PCR amplification as described previously [7]. PCR amplicons were analyzed on 1.5% (wt/vol) agarose gels. The Quantity One 4.6.2 Software was used to analyze genetic relationships among different isolates. Similarity between fingerprints was calculated with the Dice coefficient. Cluster analysis was performed using the unweighted pair-group method with average linkages.

Monitoring of bacterial growth: The growth rates of *E. coli* clones from 3 stages (before ciprofloxacin administration, after ciprofloxacin administration and after ciprofloxacin withdrawal) were determined by the method described previously [12]. For each of the 3 stages, 5 *E. coli* clones were selected to measure the optical density at 600 nm (OD_{600}) in Luria-Bertani (LB) medium. All the clones were initially cultured in LB medium at 37°C for 16 hr, and then, the bacterial suspension was diluted to a determined density (10^5 CFU); finally, 0.1 m*l* of the diluted culture was transferred into 3 m*l* LB medium for measuring the OD_{600}. The optical density was detected every 20 or 30 min.

RESULTS

The number of QREC and total E. coli isolates: Fecal samples contained a certain amount (10^8 CFU per gram of feces) of QREC isolates before ciprofloxacin administration, and the amount of total *E. coli* isolates was 10^{10} CFU per gram of feces. After ciprofloxacin administration, the number of QREC isolates increased from 10^8 CFU to 10^9 CFU, and on day 6, the amount was stable (10^{10} CFU). The number of total *E. coli* isolates decreased obviously from 10^{10} CFU to 10^9 CFU after ciprofloxacin administration, but returned to a stable level on day 6 (10^{10} CFU). After withdrawal, the numbers of QREC isolates and total *E. coli* isolates gradually decreased, and on day 56, they finally decreased to the initial level seen before ciprofloxacin administration (Fig. 1).

Antimicrobial resistance testing and statistical analysis: A total of 240 *E. coli* clones (160 clones from the EP group and 80 clones from the CP group) were examined with the API 20E system. The results concerning the dynamics of the MICs for the 240 *E. coli* clones are shown in Fig. 2. In the EP group, after 3 days of ciprofloxacin administration, the MIC for ciprofloxacin increased from 0.5 to 8 μg/m*l* (16-fold); after 26 days of ciprofloxacin withdrawal, the MIC decreased from 128 to 0.5 μg/m*l* in the EP group. The MICs for the 80 *E. coli* clones in the CP group maintained a stable value (<1 μg/m*l*). According to the statistical analysis, the MICs for the *E. coli* clones showed no significant difference between the EP and CP groups, in which the fecal samples were collected on days 0, 51 and 61 (P>0.05), but there were significant differences between the 2 groups in which the fecal samples were collected on days 1, 6, 11, 31 and 41 (P<0.05). In the EP group, after 3 days of ciprofloxacin administration, the MICs for the *E. coli* clones showed significant differences compared with the *E. coli* clones from before ciprofloxacin administration (day 0).

Detection of PMQR genes and mutations in QRDR genes: The PMQR genes (*qnrB*, *qnrS*, *aac (6')-Ib-cr* and *oqxAB*) were not detected in both the EP and CP groups. Only the position 83 mutation of *gyrA* was detected in some of the *E. coli* clones in the CP group during the whole study. In the EP group, only the position 83 mutation of *gyrA* was detected before ciprofloxacin administration. After ciprofloxacin administration, the position 83 and 87 mutations of *gyrA* and the position 80 mutation of *parC* were detected in *E. coli* clones with an MIC ≥8 μg/m*l*. After 26 days of ciprofloxacin withdrawal, only the position 83 mutation of *gyrA* was detected (Table 2).

Homology of E. coli clones: Cluster analysis of ERIC-PCR profiles of the *E. coli* clones from the 3 stages (before ciprofloxacin administration, after ciprofloxacin administration and after ciprofloxacin withdrawal) revealed a low level of similarity among the strains (61–79%).

Growth rates of E. coli clones: The *E. coli* clones we used to measure growth rates were quinolones susceptible *E. coli* (QSEC) from feces before ciprofloxacin administration (day 0) and after ciprofloxacin withdrawal (day 61); and the *E. coli* clones after ciprofloxacin administration (day 26) were QREC clones. The QREC clones showed lower growth rates

Table 2. Characteristics of *E. coli* isolates isolated from the fecal samples of pigs fed ciprofloxacin from days 1 to 30

CIP use	Day of feces collection	Max MIC (μg/m*l*)				*gyrA*		*parC*
		CIP	NAL	NOR	LVX	mut 83 (n)	mut 87 (n)	mut 80 (n)
Before ciprofloxacin administration	0	1	64	1	1	S83L (4)	...	...
After CIP administration	1	16	512	16	16	S83L (6)	D87N (4)	S80I (4)
	2	16	512	16	16	S83L (6)	D87N (4)	S80I (6)
	4	16	512	16	32	S83L (10)	D87N (10)	S80I (10)
	6	32	>1024	32	32	S83L (10)	D87N (10)	S80I (10)
	11	32	>1024	32	32	S83L (10)	D87N (4)	S80I (6)
	16	128	>1024	32	32	S83L (10)	D87N (4) D87Y (6)	S80I (10)
	21	128	>1024	32	32	S83L (10)	D87N (10)	S80I (10)
	26	128	>1024	32	32	S83L (10)	D87N (10)	S80I (10)
After CIP withdrawal	31	128	>1024	32	32	S83L (10)	D87N (10)	S80I (10)
	36	32	>1024	32	32	S83L (10)	D87N (2) D87Y (8)	S80I (10)
	41	64	>1024	16	16	S83L (10)	D87N (10)	S80I (10)
	46	32	512	16	32	S83L (10)	D87N (10)	S80I (10)
	51	8	512	16	16	S83L (10)	D87N (4)	S80I (4)
	56	0.5	64	2	2	S83L (10)	...	...
	61	1	64	2	2	S83L (10)	...	...

CIP: Ciprofloxacin, NAL: Nalidixic acid, NOR: Norfloxacin, LVX: Levofloxacin, MIC: Minimal inhibitory concentration, n: number of *E. coli* with a point mutation.

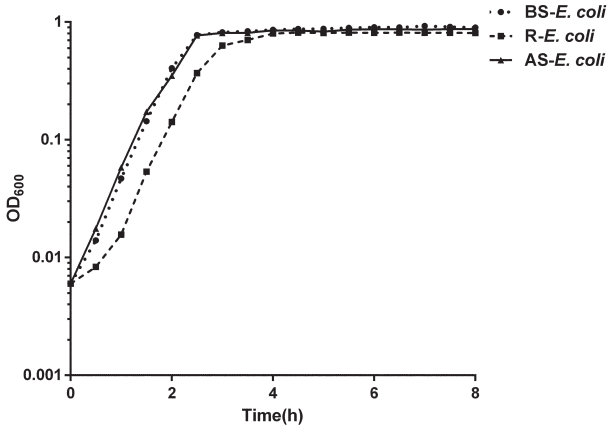

Fig. 3. Growth profiles of *E. coli* clones (n=5) from before ciprofloxacin administration (BS-*E. coli*, QSEC), after ciprofloxacin administration (R-*E. coli*, QREC) and after ciprofloxacin administration (AS-*E. coli*, QSEC).

than QSEC clones (Fig. 3).

DISCUSSION

This study was specifically designed to analyze the time-effect relationship between ciprofloxacin administration and quinolones resistance in fecal *E. coli* of finishing pigs and to test the hypothesis that the emergence of resistance in pigs requires drug accumulation for 7 days or more. A certain amount of QREC isolates was present in the intestine of pigs before ciprofloxacin administration, suggesting that resistant isolates could colonize the swine intestine, which is consistent with previous research [27]. The use of antimicrobials can influence the number of intestinal microbiota in swine immediately [22], but the influence becomes smaller as time goes on, as the swine intestinal ecosystem was previously shown to remain stable with or without antimicrobial pressure [30]. The dynamic changes in the number of QREC and total *E. coli* isolates suggest this. We found that a low-level dose (5 mg/kg) of ciprofloxacin alone could alter the number of QREC isolates, but not the total number of *E. coli* isolates from feces of pigs. A recent study also showed that chlortetracycline alone at a low-level dose (50 g/ton) had little effect on the microbial population of swine feces [24]. But, in another study that investigated the intestinal microbiota after feeding a performance-enhancing mixture of 3 antimicrobials (chlortetracycline 100 g/ton, sulfamethazine 100 g/ton and penicillin 50 g/ton) to pigs, an increase was found in the *E. coli* population [20].

The correlation between emergence of ciprofloxacin-resistant bacteria and ciprofloxacin treatment in humans or swine has been well characterized in recent years [11, 22]. However, how medication time influences the emergence of resistance has not been revealed. According to the dynamics of the MIC, we found that just 3 days were needed to develop quinolone resistance to fecal *E. coli* of pigs, which proves that our hypothesis that the emergence of resistance in pigs requires drug accumulation for 7 days or more is invalid. Removing antimicrobial pressure can reduce the number of resistant bacteria, but the seeds of resistance could colonize in the animal farm environment for a long time [31]. The dynamics of the MIC after ciprofloxacin withdrawal suggest this view.

In China, quinolones have been used as therapy or feed additives in animals for many years, which has resulted in high quinolone resistance in bacteria [6, 18, 33]. The mechanism of quinolone resistance is mainly mediated by the mutation of *gyrA* and *parC* genes [15]. In this study, when the max

MIC of QSEC to CIP was 1 μg/ml or less, only the *gyrA* mutation was detected; but, when the max MIC was 16 μg/ml or more, mutations of *gyrA* and *parC* were both detected. These results suggest that mutations of the *gyrA* gene could not mediate high-level quinolone resistance, but are necessary for high-level resistance, which is in agreement with previous reports [25, 29]. Low-level quinolone resistance can also be mediated by PMQR genes [28]. However, we detected none of the described PMQR genes, suggesting that the emergency of QREC has little relation to the PMQR genes in this study.

There was no similarity between *E. coli* clones that were separated from feces before ciprofloxacin administration, during ciprofloxacin administration and after ciprofloxacin withdrawal, illustrating that *de novo* selection of resistance mutants from the initial microbiota was not the cause of emergence of QREC. Moreover, no plasmid-mediated resistance genes were detected in QSEC and QREC clones. Thus, the emergence of QREC may be caused by 2 possibilities: (1) enrichment of QREC in the initial gut microbiota and (2) exogenously acquired resistant bacteria. In both the EP and CP groups, we found a certain number of QREC isolates (10^8 CFU) in feces from pigs before ciprofloxacin administration. Therefore, we are inclined to believe that the more likely cause of QREC emergence was the enrichment of QREC in the initial gut microbiota.

It has been reported that some mutations can influence bacterial fitness [21]. In this study, we found that the QREC had a selective advantage from day 31 to day 56, suggesting that the antimicrobial pressure did not disappear immediately after ciprofloxacin withdrawal, which is in agreement with a previous report [22]. QREC with mutations in *gyrA* and *parC* may have a fitness cost without antimicrobial pressure [1, 26]. However, our findings indicate that without antimicrobial pressure, QREC lost its selective advantage after 26 days of ciprofloxacin treatment.

The potential limitations of our study should be addressed. First, fecal samples were picked from just 5 different locations in the pens, but there were 13 pigs in each pen. This may have led to potential bias in some results of our study. Second, QREC isolates, which were present in feces at a low concentration before ciprofloxacin administration, may have been missed when we selected only 10 clones for further research. Finally, we did not determine the concentration of ciprofloxacin in feces. This may have led to a lack of information about the relationship between *E. coli* resistance and antimicrobial residues.

The emergence of QREC in fecal microbiota is a complex phenomenon. It has been shown that exogenous acquisition of resistant isolates is the cause for the emergence of QREC from an individual's feces [8]. However, our results suggest that the enrichment of QREC in the initial gut microbiota is the more probable cause for the emergence of fecal QREC after feeding ciprofloxacin to pigs.

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