Studies on the Emergence Mechanism of Quinolone Resistance in *Salmonella Enterica* subspecies *Enterica* serovar Choleraesuis

Masaru USUI

The United Graduate School of Veterinary Sciences

Yamaguchi University

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CONTENTS

ABBREVIATIONS

ABPC	ampicillin
ACF	acriflavine
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CFU	colony forming units
CLSI	Clinical and Laboratory Standards Institute
CP	chloramphenicol
CTF	ceftiofur
DMSO	dimethylsulfoxide
ERFX	enrofloxacin
ERY	erythromycin
FAO	Food and Agriculture Organization
GyrA	DNA gyrase A
KM	kanamycin
LB	Luria-Bertani
MH	Mueller-Hinton
MICs	minimum inhibitory concentrations
NAL	nalidixic acid
ND	not determined
O.D.	optical density
OIE	Office International des Epizooties
OMPs	outer membrane proteins

OTC	oxytetracycline
PFGE	pulsed-field gel electrophoresis
QRDR	quinolone resistance-determining region
RND	resistance nodulation cell division
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TMP	trimethoprim
WHO	World Health Organization

PREFACE

Antimicrobial agents are used for prevention, control and treatment of bacterial infections in veterinary medicine (1, 2, 3). However, it is well known that the use of antimicrobials is responsible for the emergence and prevalence of antimicrobial-resistant bacteria (3, 4). The emergence of antimicrobial-resistant bacteria can reduce the efficacy of veterinary antimicrobials, resulting in serious problem in veterinary medicine (5).

In order to discuss related to the use of antimicrobials in food-producing animals, the Expert meetings organized by the international organization has been held since the 1990s. In these meetings, it has been recommended that the monitoring of antimicrobial-resistant bacteria and antimicrobial consumption in food-producing animals is essential. The International Committee of Office International des Epizooties (OIE) amended the OIE international standards (Terrestrial Animal Health Code) on antimicrobial resistance in 2003-2004 (6). In addition, the Expert Workshop on non-human antimicrobial use organized by Food and Agriculture Organization (FAO), OIE and World Health Organization (WHO) were held in Geneva meeting in December 2003 and in Oslo in February 2004, respectively (7, 8). Following the decision of the meeting in Oslo, the lists of Critically Important Antimicrobials in human and veterinary medicine were developed by WHO and OIE, respectively. To debate the both lists of critically important antimicrobials, a Joint

FAO/WHO/OIE Expert Meeting was held again in Rome in November 2007 (9). The meeting recommended that three classes of antimicrobials, fluoroquinolones, 3rd and 4th generation cephalosporins, and macrolides, should be addressed as the highest priority (9). The meeting indicated that foodborne pathogens such as *Salmonella* spp. and *Campylobacter* spp. and the commensal *Escherichia coli* linked to antimicrobial resistance to these groups of antimicrobials should given special consideration for risk analysis (9). And the meeting indicated the importance of identifying the mechanism of antimicrobial resistance in these bacteria.

Quinolones, one of synthetic broad-spectrum antimicrobials, are used for the treatment of several bacterial diseases in humans and non-human animals. The quinolones inhibit the activities of bacterial DNA gyrase or the topoisomerase IV enzyme, thereby inhibiting DNA replication and transcription. Nalidixic acid is considered to be the predecessor of all members of the quinolone family, including the second, third and fourth generations commonly known as fluoroquinolones. The majority of quinolones in clinical use belong to the fluoroquinolones, which have a fluorine atom attached to the central ring system, typically at the 6-position or C-7 position.

In Japan, *Salmonella* is the major cause of bacterial food poisoning next to *Campylobacter* spp., and in 2009 accounted for 22.7% (1,518 patients) of cases which were notified to the National Institute of Infectious Disease,

Food-borne Disease Active Surveillance Network. *Salmonella* spp. infections are zoonotic and can be transferred between humans and nonhuman animals. Although *Salmonella enterica* serovar Choleraesuis is rarely isolated from humans in the United States and the United Kingdom (10, 11), it is a significant serovar for human salmonellosis in several Asian countries (12, 13). In Japan, the number of human cases of infection by *S.* Choleraesuis remains low in spite of increased outbreaks in pigs (14). The symptoms of *Salmonella* infections are gastroenteritis, bacteremia and typhoid fever in humans (15). When severe clinical signs are present, fluoroquinolones are the most commonly used for the treatment of severe salmonellosis in humans (16). However, the emergence of fluoroquinolone-resistant *Salmonella* was observed in several countries, including Japan (16, 17, 18).

Resistance to quinolones in Gram-negative bacteria is generally caused by mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase and topoisomerase IV, decreasing membrane permeability and enhancement of efflux pumps (19, 20, 21). In addition, plasmid-mediated resistance mechanisms, such as the *qnr* and *qep* genes have been reported in Gram-negative bacteria (22, 23). Among these mechanisms, mutations in the QRDR are the major mechanisms responsible for resistance to quinolones in *Salmonella* (24). Porins in *Salmonella* are directly involved in membrane permeability, mediating a passive and nonspecific diffusion of small nutrients and drugs including

quinolones, resulted in contributing to the quinolone resistance (25). Lack of expression of the porin has been reported in some quinolone-resistant *Salmonella* strains (26, 27). Efflux of antimicrobials including quinolones and other substrates toxic to the bacterial cells is also one of the mechanisms of antimicrobial resistance (28). To date, several efflux pumps have been identified in Gram-negative bacteria (29). The main efflux system in Gram-negative bacteria was thought to be AcrAB-TolC system. AcrAB-TolC system is a three-component structure that crosses both inner and the outer membrane. AcrB is the inner membrane component which acts as a proton-motive-force-dependent transporter and belongs to the resistance nodulation cell division (RND) family of transporters (30, 31). AcrA is a periplasmic lipoprotein which has an elongated shape thought to span the periplasmic space thus coordinating the concerted operation of inner and outer membrane components of the three-component structure (32). The functional outer membrane protein TolC is required for the operation of the AcrAB efflux system. AcrAB-TolC excretes several antimicrobials including quinolones and decreases the susceptibility to several antimicrobials (33).

The present study was carried out to clarify the emergence mechanism of quinolone resistance in *S.* Choleraesuis. The intracellular concentration of fluoroquinolones can be associated with the susceptibility of bacteria to fluoroquinolones. Recently, several researchers reported that efflux pumps contribute to the emergence of

fluoroquinolone resistance in *Salmonella* (34). Therefore, we determined the intracellular concentration of enrofloxacin and the frequencies of emergence of quinolones-resistant mutant in *S.* Choleraesuis to clarify the relationship between the intracellular concentration of enrofloxacin and the emergence of quinolone resistance (Chapter 1). Then, we selected mutant strains of *S.* Choleraesuis by passages on enrofloxacin-containing plates and examined efflux systems of the laboratory-derived mutants and their susceptibility to several antimicrobials (Chapter 2).

CHAPTER 1

Intracellular concentrations of enrofloxacin in quinolone-resistant *Salmonella enterica* subspecies *enterica* serovar Choleraesuis

1.1 Introduction

Salmonella enterica subspecies *enterica* serovar Choleraesuis is a causative agent of salmonellosis in pigs and humans (12). The disease causes diarrhea, fever, and septicemia in pigs of all ages as well as elderly and immunocompromised humans (12). Antimicrobials are used to treat the disease in humans and animals when severe clinical signs are present (16). Fluoroquinolones are the most commonly used antimicrobial agents for the treatment of invasive salmonellosis in humans (16). In several countries including Japan, fluoroquinolone-resistant strains of *S.* Choleraesuis have been isolated from pigs and humans (12, 17). As this serovar is host-adapted, contaminated pigs and pig products are considered as infectious sources to humans (12).

Resistance to quinolones in bacteria is generally caused by mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase and topoisomerase IV, decreasing in membrane permeability and enhancement of efflux pumps (24). In addition, plasmid-mediated resistance mechanisms, such as the *qnr* and *qep* genes have been reported in Gram-negative bacteria (23, 24, 35). Mutations in the QRDR of DNA gyrase and topoisomerase IV are the cause of quinolone resistance in *Salmonella* (24). Recently, several investigations have

shown that efflux pumps contribute significantly to the emergence of fluoroquinolone-resistant mutants in Gram-negative bacteria (34). It has also been observed that there is an increase in the number of fluoroquinolone-resistant mutants when the *qnr* gene is present (36). The frequency with which fluoroquinolone resistance emerges can be associated with the susceptibility of bacteria to fluoroquinolone.

In this chapter, we determined the intracellular concentration of enrofloxacin in *S.* Choleraesuis isolates from diseased pigs and clarified the relationship between intracellular enrofloxacin concentration and quinolone resistance.

1.2 Materials and Methods

1.2.1 Bacterial strains

Seventeen isolates of *S.* Choleraesuis from diseased pigs, identified between 2001 and 2005, were used in this study (Table 1); comprising six nalidixic acid-resistant strains and eleven susceptible strains. Biotypes and genotypes of these isolates were determined in a previous study (14).

1.2.2 Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of nalidixic acid and enrofloxacin were determined by the agar dilution method according to the recommendations reported by the Clinical and Laboratory Standards Institute (CLSI) (37). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. The MICs of nalidixic acid and enrofloxacin were interpreted using the CLSI criteria.

1.2.3 DNA sequencing of QRDRs

Mutations in the QRDRs of the *gyrA* gene were detected by direct DNA sequencing. Amplification of the gene and purification of the resulting amplicon was performed as described previously (38). Nucleotide

sequences were determined by using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with a 3130 Genetic Analyzer; (Applied Biosystems, USA).

1.2.4 Accumulation assay

The intracellular concentration of enrofloxacin was assayed using a fluorometric uptake assay (39) with slight modifications. Briefly, a bacterial culture was grown to the logarithmic phase at 37°C in Luria-Bertani (LB) medium (Beckton Dickinson, USA). A 100 μL volume of culture was added to 15 mL LB medium and grown at 37°C to an optical density (O.D.) of 0.8 at 600 nm. Following centrifugation at 10 000 × g for 10 min at 4°C, the pellet was washed twice with 50 mM phosphate buffer (pH 7.0). After the pellet was resuspended and diluted with the same buffer. The 30-fold dilution of suspensions were adjusted to an O.D. at 600 nm of 0.3. The cell suspension (500 μL) was allowed to equilibrate for 10 min at 37°C. Enrofloxacin was then added to the suspension (final concentration 10 mg/L). After incubation for 5 min, 5 μL of dimethylsulfoxide (DMSO) was added to the suspension. To investigate the role of the efflux pump, 5 μL of DMSO containing 10 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma, USA) was added (final concentration, 100 μM) to the cell suspension and incubated for 5 min at room temperature. The cell suspension was diluted in 1 mL ice-cold 50

mM phosphate buffer at pH 7.0 then centrifuged at 10 000 × g for 10 min at 4°C, the pellet was washed with the same buffer and resuspended in 1 mL 0.1 M glycine-HCl at pH 3.0. This suspension was incubated overnight at room temperature, then centrifuged at 10 000 × g for 10 min at 4°C. The concentration of enrofloxacin in the supernatant was estimated using an F-2500 Fluorescence Spectrophotometer (Hitachi, Japan) at the appropriate excitation and emission wavelengths of 279 and 452 nm, respectively. Data represent the mean value of the results from three independent experiments.

1.2.5 Frequencies of emergence of fluoroquinolone-resistant mutants in vitro

Bacteria were grown into the late logarithmic phase at 37°C in Mueller-Hinton (MH) broth (Becton Dickinson), harvested by centrifugation, and resuspended in sodium phosphate buffer (pH 7.2). The total colony forming units (CFU) were measured by serial dilution on MH agar plates. The frequencies of emergence of fluoroquinolone-resistant mutants were calculated on MH agar plates containing 0.5 mg/L enrofloxacin. Data represent the mean value of the results from three independent experiments.

1.2.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Outer membrane proteins (OMPs) were extracted by the method of Schnaitman (40) with slight modifications. Bacteria were grown up to the logarithmic phase at 37°C in LB medium, harvested by centrifugation, washed in 10 mM Tris-HCl (pH 8.0) and resuspended in the same buffer. Crude extracts were sonicated then centrifuged at 10 000 × g for 5 min at 4°C. The supernatant obtained was centrifuged at 13 000 × g for 45 min at 4°C. The pellet was resuspended in suspension buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 2% Triton X-100, pH 8.0), incubated for 30 min at 37°C and then centrifuged at 13 000 × g for 45 min at 4°C. The pellet was resuspended in 100 mM Tris-HCl (pH 8.0), 2% SDS (w/v). Samples were mixed with an equal volume of Laemmli sample buffer (41) and boiled for 10 min at 100°C. SDS-PAGE was performed on 12% polyacrylamide gels. Following electrophoresis, gels were stained with Coomassie blue (Sigma).

1.2.7 Statistical analysis

Student's t-test was used to compare the intracellular concentration of enrofloxacin in *S.* Choleraesuis. And the emergence frequencies of fluoroquinolone-resistant mutants were analyzed by the Tukey's test.

1.3 Results

1.3.1 The MICs of nalidixic acid and enrofloxacin

The MICs of nalidixic acid and enrofloxacin are shown in Table 1. Five quinolone (nalidixic acid)-resistant isolates and one fluoroquinolone (enrofloxacin)-resistant isolate were observed.

1.3.2 Sequencing of QRDRs

Five nalidixic acid-resistant isolates contained single point mutations in the *gyrA* gene (Table 1). The fluoroquinolone-resistant isolate, 13-PLS-6, contained two point mutations in the *gyrA* gene and a mutation in the *parC* gene (17).

1.3.3 Determination of enrofloxacin concentration in bacteria

The intracellular concentration of enrofloxacin ranged from 7.2±1.5–12.7±2.6 ng/10^5 CFU in nalidixic acid-susceptible isolates and from 5.1±0.3–9.1±0.8 ng/10^5 CFU in nalidixic acid-resistant isolates. The intracellular concentration of enrofloxacin was significantly lower in nalidixic acid-resistant isolates compared with susceptible isolates ($P<0.01$; Fig. 1). However, two of the identified susceptible isolates

exhibited low intracellular concentrations of enrofloxacin. The intracellular concentration of enrofloxacin in ZSC-8 and 582, was significantly lower ($P<0.1$) than other susceptible isolates with the exception of 143, ZSC-12 and 1002. The recorded intracellular enrofloxacin concentration for isolates 143, ZSC-12 and 1002 was at an intermediate level. The intracellular concentration of enrofloxacin in biotypes Choleraesuis (group Xa-Ba), Kunzendorf (group Xb-Ba) and Kunzendorf (group Xb-Bb) was 9.1±2.4, 8.1±3.1 and 9.6±2.9 ng/10^5 CFU, respectively. There was no difference in the level of enrofloxacin concentration between two biotypes and among pulsed-field gel electrophoresis (PFGE) genotypes (14).

1.3.4 Frequencies of emergence of fluoroquinolone-resistant mutants in vitro

The emergence frequencies of fluoroquinolone-resistant mutants ranged from 1.2±0.2 × 10^{-9} to 50.2±16.3 × 10^{-9} in nalidixic acid-susceptible isolates (Table 2). Comparison of isolates ZSC-8 and 143 with another susceptible isolate using Tukey's test indicated significantly ($P<0.1$) high frequencies of emergence of resistant mutants.

1.3.5 The effect of CCCP to the enrofloxacin concentration in bacteria

In the presence of CCCP, the intracellular concentration of enrofloxacin in biotypes Choleraesuis (group Xa-Ba), Kunzendorf (group Xb-Ba) and Kunzendorf (group Xb-Bb) was 63.1±13.5, 52.7±10.6 and 57.5±11.5 ng/10^5 CFU, respectively. Intracellular concentration of enrofloxacin increased in the presence of CCCP (Table 2), with concentrations ranging from 43.5±3.5–69.5±2.1 ng/10^5 CFU in nalidixic acid-susceptible isolates and from 41.3±6.9–70.3±24.5 ng/10^5 CFU in resistant isolates. There was no significant difference in the intracellular concentration between susceptible and resistant isolates.

1.3.6 Characterization of outer membrane proteins

SDS-PAGE analysis of the OMPs revealed three bands common to all isolates between approximately 33– 44 kDa. These bands were indicative of the OmpA, OmpC and OmpF proteins.

1.4 Discussion

This chapter has shown that intracellular concentrations of enrofloxacin varied among field isolates of *S.* Choleraesuis. Low level concentrations were observed in quinolone (nalidixic acid)-resistant isolates of *S.* Choleraesuis, although there appeared to be no difference in the level of intracellular concentration between biotypes or genotypes. Conversely, relatively high frequencies of emergence of fluoroquinolone resistance were observed in one of the two susceptible isolates with a low level of intracellular enrofloxacin concentration (isolate ZSC-8). The bacteria with low level of intracellular concentration exhibit reduced quinolone susceptibility in the bacteria, although we could not find the difference in MIC of enrofloxacin. Isolate 582 also exhibited low levels of intracellular enrofloxacin concentration but the frequency of emergence for fluoroquinolone-resistant mutants was not significantly high with the emergence frequencies of fluoroquinolone-resistant mutants approximately four-fold higher than the average of other susceptible isolates. Isolate 143 contained intermediate levels of intracellular enrofloxacin concentration corresponding with significantly high emergence frequencies for fluoroquinolone-resistant mutants. However, the intracellular concentration of enrofloxacin in isolate 143 was relatively lower than other susceptible isolates. *S.* Typhimurium mutants which lack the efflux gene are more susceptible to fluoroquinolone than

the wild type. *S.* Typhimurium mutants that lack the efflux gene are less likely to arise when exposed to ciprofloxacin (34). Jacoby *et al.* showed that emergence frequencies of fluoroquinolone-resistant mutants were elevated in bacteria containing the *qnr* gene (42). From these results arose the hypothesis that a slight reduction of fluoroquinolone susceptibility is a co-factor in the emergence of fluoroquinolone-resistant bacteria.

The low concentration of intracellular fluoroquinolone may be caused by a reduction in the membrane permeability and/or enhancement of efflux systems in bacteria (24). Hydrophilic quinolones, such as ciprofloxacin and enrofloxacin, penetrate bacteria via the porin pathway (25). Porins in the outer membrane of Gram-negative bacteria are formed by trimers of the proteins, OmpA, OmpC and OmpF. The deletion of porin results in decreased membrane permeability of fluoroquinolone in *Salmonella* (43). This chapter showed that the three proteins were found in all isolates. So it is possible that the membrane permeability of all isolates tested was at the same level. It has been reported that active efflux is an important mechanism of fluoroquinolone resistance in *Salmonella* (24). In the present study a protonophore CCCP was used to inhibit efflux of fluoroquinolone. In the presence of CCCP, the level of intracellular enrofloxacin in the resistant isolates increased compared with susceptible isolates. This result indicated that enhancement of efflux pumps may be responsible for low levels of intracellular enrofloxacin

concentration.

This chapter also showed that a decrease in concentration of intracellular enrofloxacin was related to an enhancement of efflux pump. It is likely that the low intracellular concentrations of enrofloxacin might ease the emergence of fluoroquinolone-resistant isolates in *S.* Choleraesuis.

Table 1. Characterization of *S.* Choleraesuis used in this chapter.

Biotype	PFGE profile	Strain No.	NA-MIC(mg/L)	ERFX-MIC(mg/L)	Mutations	
					*gyr*A	*par*C
Choleraesuis	Xa-Ba	ZSC-14-1	4	0.0625	none(wild type)	ND
		ZSC-40	4	0.0625	none(wild type)	ND
		13-PLS-6	256	4	Ser-83-to-Tyr(TCC→TAC) Asp-87-to-Gly(GAC→GGC)[a]	Ser-80-to-Arg(AGC→AGA)[a]
		sal-1372	>512	0.5	Ser-83-to-Tyr(TCC→TAC)	ND
		14-PLS-21	>512	2	Ser-83-to-Phe(TCC→TTC)[a]	none(wild type)[a]
Kunzendorf	Xb-Ba	ZSC-8	8	0.125	none(wild type)	ND
		03-197-1	2	0.0625	none(wild type)	ND
		03-228-1	2	0.0625	none(wild type)	ND
		16-PLS-33	>512	0.25	Asp-87-to-Gly(GAC→GGC)	ND
		16-PLS-45	>512	0.5	Ser-83-to-Tyr(TCC→TAC)	ND
		16-PLS-46	>512	0.5	Ser-83-to-Tyr(TCC→TAC)	ND
	Xb-Bb	143	8	0.0625	none(wild type)	ND
		419	4	0.0625	none(wild type)	ND
		582	8	0.0625	none(wild type)	ND
		916	4	0.125	none(wild type)	ND
		1002	4	0.125	none(wild type)	ND
		ZSC-12	4	0.0625	none(wild type)	ND

a) Data from previous report (14).
PFGE, pulsed-field gel electrophoresis pattern by XbaI and BlnI-digestion; NA, nalidixic acid; ERFX, enrofloxacin; ND, not determined.

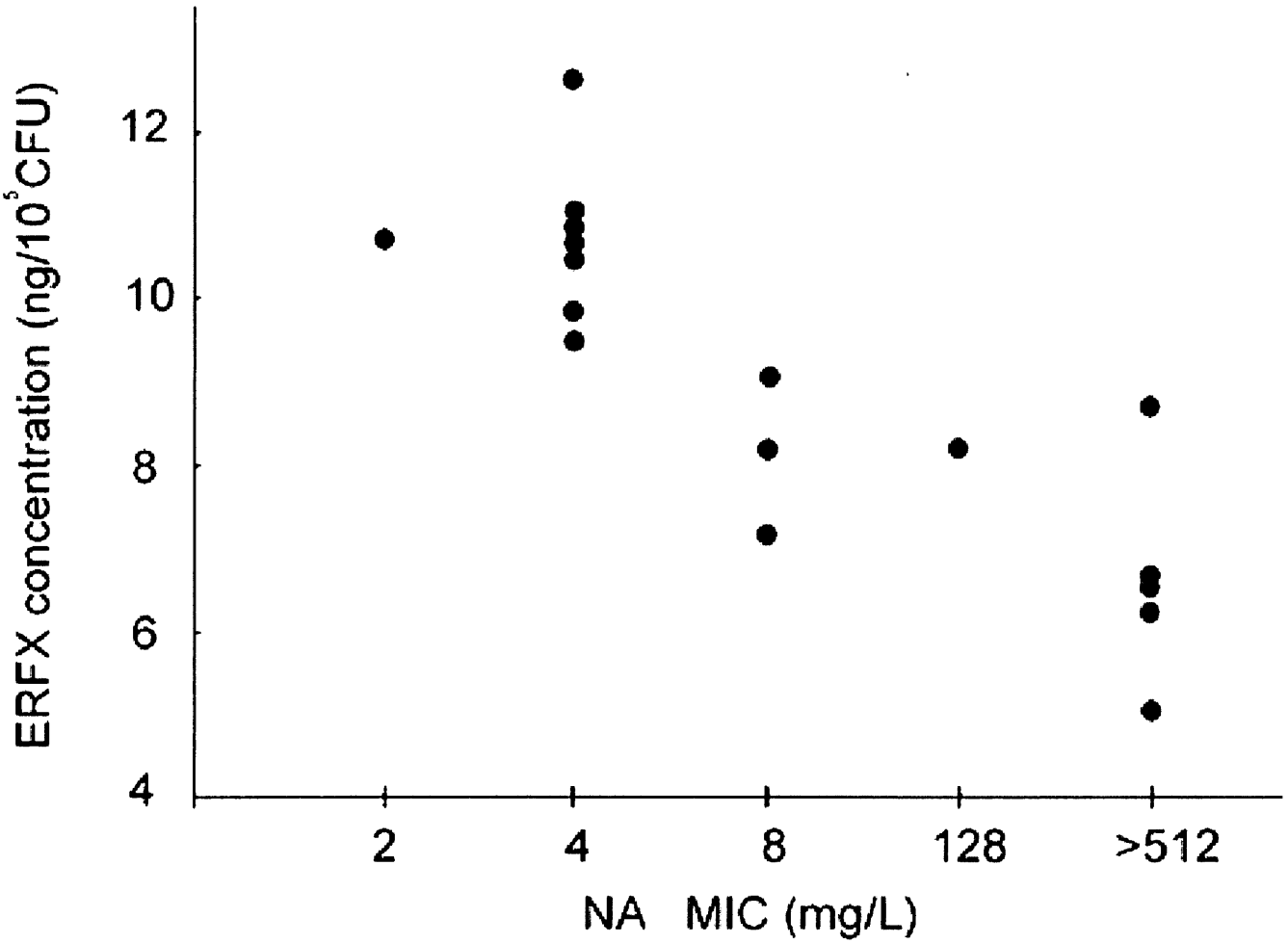

Fig. 1 The relationship between nalidixic acid MIC and enrofloxacin concentration. Nalidixic acid-resistant *S.* Choleraesuis is significantly low intracellular concentration of enrofloxacin. NA, nalidixic acid; ERFX, enrofloxacin

Table 2. Numbers of each isolates and average of enrofloxacin concentration in the absence of CCCP and the presence of CCCP.

Biotype	PFGE profile	Numbers of isolates	Average of ERFX accumulation(ng/10^5CFU)	Average of ERFX accumulation(ng/10^5CFU) in the presence of CCCP
Choleraesuis	Xa-Ba	5	9.1±2.4	63.1±13.5
Kunzendorf	Xb-Ba	6	8.1±3.1	52.7±10.6
	Xb-Bb	6	9.6±2.9	57.5±11.5

PFGE, pulsed-field gel electrophoresis pattern by XbaI and BlnI-digestion ; ERFX, enrofloxacin; CCCP, carbonyl cyanid m-chlorophenylhydrazone.

Table 3. Emergence frequencies of spontaneous resistant isolates and intracellular concentration of enrofloxacin from nalidixic acid-susceptible isolates.

Strain No.	ERFX accumulation(ng/10^5CFU)[b]	Frequencies of emergence of spontaneous resistant mutant($\times10^{-9}$)[c]
ZSC-8	8.1±1.1	50.2±16.3
143	9.1±0.1	37.7±35.6
582	7.2±1.5	10.8±7.9
Average of other isolates[a]	10.8±1.9	2.8±2.6

High frequencies of emergence of the resistant mutants were frequently exhibited low intracellular concentration of enrofloxacin.

a) Average of other isolates were susceptible isolates, except for ZSC-8, 143 and 582.

b) ZSC-8 and 582 indicated significant low level of intracellular concentration of enrofloxacin in susceptible isolate, except for 143, ZSC-12 and 1002 isolates ($P<0.1$).

c) ZSC-8 and 143 indicated significant high frequencies of emergence of resistant mutants ($P<0.1$).

ERFX, enrofloxacin.

CHAPTER 2

Contribution of enhanced efflux to reduced susceptibility of *Salmonella enterica* serovar Choleraesuis to fluoroquinolone and other antimicrobials

2.1 Introduction

The emergence of fluoroquinolone-resistant *Salmonella enterica* serovar Choleraesuis strains is a major public health concern (17). Fluoroquinolone resistance in *Salmonella* is caused by multiple mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase and topoisomerase IV, decreased membrane permeability, increased activities of efflux pumps and plasmid-mediated quinolone resistance (12, 24, 43, 44). Among these causes, mutations in the QRDR are the major mechanisms responsible for resistance to fluoroquinolones in *Salmonella* (12, 17, 21, 24, 45).

Several researchers and Chapter 1 of this thesis have described that enhancement of efflux pumps contributed to the emergence of fluoroquinolone resistance in *Salmonella* (34). The enhancement of efflux pumps as well as decreased membrane permeability has been observed in laboratory-derived mutants of *Salmonella* (43, 44, 46, 47). To date, many kinds of efflux pumps have been identified in Gram-negative bacteria (33).

In this chapter, we selected mutant strains by passage on enrofloxacin-containing plates and examined their susceptibility to several antimicrobials as well as efflux systems in *S.* Choleraesuis.

2.2 Materials and Methods

2.2.1 Bacterial strains

Seven fluoroquinolone-susceptible strains of *S.* Choleraesuis were isolated from diseased pigs between 2001 and 2002 (14), and subjected to selection for quinolone-resistant mutants (Table 4). Each of the seven clinical isolates was plated on Mueller-Hinton (MH) agar plates containing 0.5 mg/L enrofloxacin. Any colonies that grew on these plates were subcultured. Based on minimum inhibitory concentrations (MICs) of nalidixic acid and enrofloxacin (37), mutants with decreased susceptibilities to nalidixic acid (MICs 16 and 32 mg/L) and enrofloxacin (MICs 0.25 and 0.5 mg/L) were designated as R1 strains. Strains resistant to nalidixic acid (MICs 64–1024 mg/L) and enrofloxacin (MICs 0.5 and 1.0 mg/L) were selected and designated as R2 (Table 4). As an example, the mutants derived from strain 03-197-1 were designated as 03-197-1R1 and 03-197-1R2 depending on the MICs of nalidixic acid and enrofloxacin. All the isolates and mutants were stored at -80°C until used.

2.2.2 DNA sequencing of QRDRs

Mutations in the QRDRs of the *gyrA* gene were determined by direct DNA sequencing. Amplification of the gene and purification of the

amplicon was performed as described by Giraud *et al.* (38). Nucleotide sequences were determined using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA) with a 3130 Genetic Analyzer (Applied Biosystems).

2.2.3 Antimicrobial susceptibility testing

The MICs of ampicillin, ceftiofur, chloramphenicol, and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were determined by the micro-broth dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (37). The MICs of oxytetracycline, erythromycin, trimethoprim, acriflavine and kanamycin were determined by the CLSI agar dilution method (37). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

2.2.4 Accumulation assay

The amounts of intracellular enrofloxacin were measured by a fluorometric uptake assay according to the method of Piddock *et al.* (39) as described chapter 1. To investigate the effects of the efflux pump inhibitor on the concentration of intracellular enrofloxacin, the experiment was also carried out in the presence of CCCP (final concentration 100 μM) (48). Data represents the mean value of the

results from three independent experiments.

2.2.5 Expression of 16S rRNA, *acrB, emrA, fsr and mdtK*

To evaluate the genes conferring efflux pumps, quantitative PCR was applied to the detection and quantification of mRNAs. The preparations of cDNA were performed as described by Zheng *et al.* (49) with slight modifications. In brief, extraction of total RNAs from 5 mL of bacterial suspensions were carried out with an ISOGEN (Nippongene, Tokyo, Japan). The cDNA was synthesized from the RNA samples using the Primescript RT reagent kit (TaKaRa, Shiga, Japan). Quantitative PCR was performed with SYBR premix EX taq II (TaKaRa) on a One Step real-time system (Applied Biosystems) according to the manufacturer's instructions. The oligonucleotide primers used for the detection of cDNA specific for 16S rRNA were 5'-CCA GCA GCC GCG GTA AT-3' (forward) and 5'-TTT ACG CCC AGT AAT TCC GAT T-3' (reverse) resulting in a 57 bp amplicon. The *fsr* gene was amplified using the primers 5'-TCC GGC GGA CGT CAT GGT CT-3' (forward) and 5'-CCA GCA GCG GGC CCA ATG AA-3' (reverse), giving a 79 bp amplicon. All primers were designed with Primer3 (http://frodo.wi.mit.edu/primer3/). Primers specific for *acrB* (148 bp) (50), *emrA* (61 bp) (29) and *mdtK* (64 bp) (29) were used according to a previous report. The yield of amplicons from the *acrB, emrA, fsr and mdtK* genes were normalized to those originating from 16S rRNA. Within bacterial cells, the level of 16S rRNA was assumed to be

transcribed at a constant rate throughout the growth conditions in this study. Data represent the mean value of results from three independent total RNA extractions. The correlation coefficient was calculated for the ratio of enrofloxacin accumulation in each parent isolate and the relative expression level of *acrB* mRNA in each parent isolate.

2.2.6 Statistical analysis

Student's *t*-test was used to compare the results between and within experiments.

2.3 Results

2.3.1. Sequencing of QRDRs

Of the seven resistant mutants, six mutants contained nucleotide changes in the *gyrA* gene accompanied by an amino acid substitution in the DNA gyrase A (GyrA) protein. Another mutant (582R2) contained nucleotide changes resulting in two amino acid substitutions (Table 5).

2.3.2 Susceptibilities to several antimicrobials

Ten laboratory-derived mutants (03-197-1R1, 03-197-1R2, 03-228-1R1, 03-228-1R2, 916R1, 916R2, 143R1, 1002R1, 582R1 and ZSC-8R1) exhibited reduced susceptibilities to β-lactams (ampicillin and ceftiofur), oxytetracycline, chloramphenicol, erythromycin, trimethoprim and acriflavine when compared with each parent isolate (Table 4). Three laboratory-derived mutants (143R2, 1002R2 and 582R2) exhibited reduced susceptibilities to ampicillin and/or ceftiofur compared with each parent isolate, whereas their susceptibilities to oxytetracycline, chloramphenicol, erythromycin, trimethoprim and acriflavine were not altered. Susceptibility to CCCP was decreased in laboratory-derived mutants except in the case of two mutants (ZSC-8R1, ZSC-8R2) compared with each parent isolate. The susceptibility to kanamycin of all laboratory-derived mutants was equal to those of each parent isolate.

2.3.3 Determination of the enrofloxacin accumulation

In the accumulation assays, the amounts of intracellular enrofloxacin in the mutants with decreased susceptibilities and those in the resistant mutants ranged from 6.1–8.7 ng/10^5 colony forming units (CFU) and 6.4–7.9 ng/10^5 CFU, respectively, which were significantly ($P < 0.01$) lower than those obtained in the parent isolates (7.3–11.4 ng/10^5 CFU; Table 5). In the presence of CCCP, there was no significant difference in the intracellular enrofloxacin accumulation among parent isolates and mutants.

2.3.4 Expression of efflux pump mRNAs

The expression of *acrB* mRNA was greater in all laboratory-derived mutants, except for the mutant ZSC-8R2, when compared with parent isolates (Table 5). The *acrB* mRNA expression level of the mutant ZSC-8R2 was unchanged from that of the parent isolate. The expression of *emrA* mRNA was greater in laboratory-derived mutants, except for ZSC-8R1 and ZSC-8R2, compared with the parent isolate (Table 5). The mRNA expression of *fsr* and *mdtK* was unchanged in the laboratory-derived mutants (Table 5).

2.4 Discussion

In the present study, nucleotide sequences in the mutants with decreased susceptibilities were not accompanied with any amino acid substitutions in GyrA. It has been previously shown that the amino acid substitutions in GyrA contribute to resistance in quinolones (12, 17, 21, 24, 45).

The laboratory-derived mutants exhibited decreased susceptibilities to not only quinolones but also several other antimicrobials. The decreased susceptibilities to quinolones in laboratory-derived mutants were associated with enhancement of efflux pumps. The enhancement of efflux pumps was commonly observed in the laboratory-derived mutants with and without amino acid substitutions in the GyrA. Giraud *et al.* suggested that enhancement of AcrAB is the primary mechanism before the mutation of QRDRs under fluoroquinolone selective pressure in *S.* Typhimurium (30). Ricci and Piddock demonstrated that antimicrobials, including fluoroquinolone, could select for mutants of *Salmonella* with enhanced efflux mechanisms (51), and Chen *et al.* reported similar results. They suggested that the enhanced efflux mechanisms contributed to the development of resistance to fluoroquinolones and other antimicrobials in laboratory mutants of *S.* Typhimurium. Our results support their finding that enhancement of efflux in mutants selected by culture on fluoroquinolone-containing plates resulted in decreased susceptibility to quinolones and other antimicrobials.

The ratio of enrofloxacin accumulation in each parent isolate was significantly correlated with the relative expression of *acrB* mRNA (correlation coefficient: -0.64, $P < 0.01$; data not shown). These results suggest that *acrB* mRNA expression may contribute to fluoroquinolone resistance in laboratory-derived mutants.

Several investigations have shown that the increased expression of *acrA* and *acrB* mRNAs resulted in decreased susceptibilities to quinolones, β-lactams, oxytetracycline, chloramphenicol, erythromycin, trimethoprim and acriflavine in Gram-negative bacteria, including *Salmonella* (31, 44, 46, 52, 53). There is a possibility that the enhancement of other efflux pumps, like MdfA and MdtABC, makes additional contributions to the abovementioned antimicrobial susceptibilities (33). However, enhancement of AcrAB, MdfA and MdtABC can explain the decreased susceptibility to the abovementioned antimicrobials, but not in the case of CCCP in laboratory-derived mutants.

Nishino and Yamaguchi showed that susceptibility to CCCP was decreased by the enhancement of EmrAB, Fsr (33) and MdtK, also known as YdhE, (54) efflux pumps. The expression of *emrA* mRNA was greater in laboratory-derived mutants. These results suggest that decreased susceptibility to CCCP is caused by increased activities of EmrAB in laboratory-derived mutants. However, expression of EmrAB was not responsible for fluoroquinolone susceptibility in bacteria (33). Further study is required to clarify the mechanism of enhancement of EmrAB in

mutants selected with fluoroquinolone.

Table 4. Susceptibilities of *S.* Choleraesuis parent isolates and laboratory-derived mutants to several antimicrobials.

Strain No. [a]	Strain type	MIC (mg/L) [b]										
		NA	ERFX	ABPC	CTF	OTC	CP	EM	TMP	ACF	KM	CCCP (μM)
03-197-1	Parent susceptible isolate	4	0.0625	>128	1	256	4	64	0.25	512	2	50
03-197-1R1	Reduced susceptible mutant	**16**	**0.25**	>128	**2**	**512**	**16**	**256**	**1**	**>512**	2	**100**
03-197-1R2	Resistant mutant	**64**	**1**	>128	**2**	**512**	**16**	**256**	**1**	**>512**	2	**100**
03-228-1	Parent susceptible isolate	2	0.0625	<1	1	256	4	64	0.25	512	8	50
03-228-1R1	Reduced susceptible mutant	**16**	**0.25**	**4**	**2**	**512**	**8**	**128**	**0.5**	**>512**	8	**100**
03-228-1R2	Resistant mutant	**512**	**0.5**	**4**	**2**	**512**	**8**	**128**	**0.5**	**>512**	8	**100**
916	Parent susceptible isolate	4	0.0625	2	1	2	4	64	0.25	512	2	50
916R1	Reduced susceptible mutant	**16**	**0.5**	**4**	**4**	**4**	**8**	**128**	**0.5**	**>512**	2	**200**
916R2	Resistant mutant	**64**	**0.5**	**8**	**4**	**16**	**32**	**256**	**1**	**>512**	2	**200**
143	Parent susceptible isolate	4	0.0625	<1	1	2	4	64	0.25	512	2	50
143R1	Reduced susceptible mutant	**16**	**0.25**	**4**	**2**	**4**	**8**	**128**	**0.5**	**>512**	2	**100**
143R2	Resistant mutant	**128**	**0.5**	**2**	**2**	2	4	64	0.25	512	2	**100**
1002	Parent susceptible isolate	4	0.0625	2	1	2	4	64	0.25	512	2	50
1002R1	Reduced susceptible mutant	**16**	**0.25**	**4**	**2**	**4**	**16**	**256**	**0.5**	**>512**	2	**200**
1002R2	Resistant mutant	**512**	**1**	2	**2**	2	4	64	0.25	512	2	**100**
582	Parent susceptible isolate	4	0.0625	<1	1	2	4	64	0.25	512	2	50
582R1	Reduced susceptible mutant	**16**	**0.25**	**4**	**2**	**4**	**8**	**128**	**0.5**	**>512**	2	**100**
582R2	Resistant mutant	**512**	**0.5**	**2**	1	2	4	64	0.25	512	2	**100**
ZSC-8	Parent susceptible isolate	8	0.125	<1	1	256	128	64	0.25	>512	>512	100
ZSC-8R1	Reduced susceptible mutant	**32**	**0.25**	**4**	**2**	**512**	128	**128**	**0.5**	>512	>512	100
ZSC-8R2	Resistant mutant	**1024**	**1**	<1	1	256	128	64	0.25	>512	>512	100

MIC, minimum inhibitory concentration; NA, nalidixic acid; ERFX, enrofloxacin; ABPC, ampicillin; CTF, ceftiofur; OTC, oxytetracycline; CP, chloramphenicol; EM, erythromycin; TMP, trimethoprim; ACF, acriflavine; KM, kanamycin; CCCP, carbonyl cyanide m-chlorophenylhydrazone.

a) R1 and R2 are laboratory-derived mutants selected from each susceptible parent isolate. R1 and R2 indicate the reduced-susceptiblility mutants (reduced susceptibility to NA and ERFX) and resistant mutants (high level resistance to NA and ERFX), respectively.

b) Bold type indicates higher MICs compared with the respective parent isolates.

Table 5. Characterization of *S.* Choleraesuis susceptible parent isolates and laboratory-derived mutants used in this study.

Strain No.	ERFX accumulation (ng/10^5 CFU)		Mutations	n-Fold change in efflux pumps gene expression[a)]			
	Without CCCP	With CCCP	GyrA	*acrB*	*emrA*	*fsr*	*mdtK*
03-197-1	9.9	58.7	Wild type	1.0	1.0	1.0	1.0
03-197-1R1	8.5	55.8	Wild type	4.9±1.1	2.1±0.3	1.4±0.5	1.1±0.2
03-197-1R2	7.4	51.0	G81C	5.3±0.8	1.8±0.4	0.9±0.3	1.0±0.3
03-228-1	9.8	56.0	Wild type	1.0	1.0	1.0	1.0
03-228-1R1	8.7	54.2	Wild type	1.6±0.2	1.8±0.2	1.3±0.2	1.3±0.3
03-228-1R2	7.9	55.0	G81C	4.8±0.3	1.6±0.1	1.0±0.3	1.4±0.5
916	10.6	54.5	Wild type	1.0	1.0	1.0	1.0
916R1	8.3	53.8	Wild type	7.0±1.7	1.6±0.3	1.1±0.2	0.8±0.2
916R2	6.4	51.6	L98V	14.5±2.2	1.7±0.2	1.0±0.2	1.4±0.5
143	8.1	49.4	Wild type	1.0	1.0	1.0	1.0
143R1	6.1	48.3	Wild type	3.1±0.5	1.8±0.3	0.9±0.3	0.6±0.3
143R2	7.5	44.4	D82N	3.9±0.8	1.8±0.5	1.3±0.4	1.1±0.2
1002	11.4	55.2	Wild type	1.0	1.0	1.0	1.0
1002R1	7.4	52.9	Wild type	6.7±1.1	2.6±0.5	1.1±0.2	1.1±0.3
1002R2	7.8	55.0	G81C	1.4±0.1	1.8±0.4	1.0±0.1	1.2±0.3
582	7.3	52.3	Wild type	1.0	1.0	1.0	1.0
582R1	6.8	50.9	Wild type	4.3±0.5	1.6±0.1	0.9±0.1	1.0±0.4
582R2	7.0	52.3	D87G, L98V	2.2±0.6	1.4±0.2	1.0±0.1	0.9±0.3
ZSC-8	7.6	48.9	Wild type	1.0	1.0	1.0	1.0
ZSC-8R1	7.2	47.8	Wild type	2.8±0.2	1.1±0.2	0.9±0.2	1.0±0.1
ZSC-8R2	7.6	50.9	D87N	1.0±0.1	0.9±0.2	1.0±0.2	1.0±0.1

ERFX, enrofloxacin; CFU, colony forming units; CCCP, carbonyl cyanide m-chlorophenylhydrazone; GyrA, DNA gyrase A subunit.

a) Relative expression level of *acrB, emrA, fsr* and *mdtK* mRNAs compared to each parent isolate.

CONCLUSION

The purpose of this thesis is to clarify the emergence mechanism of quinolone resistance in *S.* Choleraesuis. We examined the intracellular concentration of enrofloxacin to determine the relationship with the emergence of quinolone resistance in *S.* Choleraesuis. In addition, the efflux systems and several antimicrobial susceptibilities were studied in the laboratory-derived mutants of *S.* Choleraesuis selected by culture on fluoroquinolone-containing plates. The results obtained are as follows:

1, Intracellular concentration of enrofloxacin was determined by fluorimetric uptake assay in *S.* Choleraesuis.

2, Intracellular concentration of enrofloxacin was significantly lower in nalidixic acid-resistant isolates compared with nalidixic acid-susceptible isolates.

3, In the presence of CCCP, the intracellular enrofloxacin concentration increased in all isolates, although there was no significant difference in this intracellular concentration between susceptible and resistant isolates.

4, The frequencies of emergence of fluoroquinolone-resistant mutants was higher in susceptible isolates with a low intracellular concentration of enrofloxacin.

5, Laboratory-derived mutants of *S.* Choleraesuis exhibited decreased susceptibilities to quinolones and several other antimicrobials, although mutations in the quinolone resistance determining region (QRDR) of *gyrA* gene were not always found in the laboratory-derived mutants.

6, Intracellular enrofloxacin concentrations were significantly lower in the laboratory-derived mutants compared with parent isolates.

7, Expressions of *acrB* and *emrA* were greater in laboratory-derived mutants compared with parent strains.

The frequencies of emergence of fluoroquinolone-resistant mutants are different from each strain in *S.* Choleraesuis. Decrease in the intracellular concentration of enrofloxacin is related to the enhancement of efflux pumps and contributes to the emergence of fluoroquinolone resistance. The fluoroquinolone selective pressure is one of the causes of the enhancement of efflux systems, resulted in decreased susceptibilities to several antimicrobials. Therefore, veterinarian should consider the

differences in the frequency of emergence of fluoroquinolone-resistant mutants and the effect of the fluoroquinolone selective pressure to the efflux systems to prevent the emergence of fluoroquinolone-resistant strains in the use of fluoroquinolones. This thesis provides valuable information for preventing the emergence of fluoroquinolone resistance in *Salmonella*.

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SUMMARY IN JAPANESE

和文要旨

動物の各種感染症を治療するために、複数の動物用抗菌剤が使用されている。抗菌剤使用の時間経過に伴い、抗菌剤に対する耐性菌が出現することが知られている。近年、食用動物へ抗菌性物質を使用することにより出現した薬剤耐性菌もしくは耐性遺伝子が食物連鎖を介して人へ伝播し、人の細菌感染症の治療を困難にする可能性があるとして、WHO や OIE などの国際機関において、食用動物への抗菌性物質の使用のリスクについて議論されている。2007 年に開催された FAO/WHO/OIE 合同会議では、医療及び獣医療上、重要な抗菌性物質であるフルオロキノロンに対して耐性を示すサルモネラは、特に注意すべき薬剤耐性菌の一つであることが示された。著者は、ブタから分離されたサルモネラ・コレラスイスを用いて、キノロン耐性の出現機序に着目して研究を行った。

キノロンは菌体内に取り込まれた後、標的酵素（DNAジャイレース、トポイソメラーゼ）の活性を阻害することで抗菌活性を示すため、キノロンの菌体内濃度は、薬剤感受性に影響すると考えられる。そこで、ブタ由来サルモネラ・コレラスイスにおける菌体内エンロフロキサシン濃度とキノロン耐性獲得との関係を明らかにすることを目的に本研究を行った。サルモネラ・コレラスイス野外分

離株17株（キノロン感受性11株と耐性株6株）にエンロフロキサシンを作用させた後、プロトンポンプ阻害剤であるcarbonyl cyanide m-chlorophenylhydrazone（CCCP）存在下及び非存在下で、菌体内エンロフロキサシン濃度をfluorometric uptake assayにより測定した。キノロン耐性株の菌体内エンロフロキサシン濃度は、感受性株よりも有意に低かった（$P<0.01$）。また、CCCP存在下での菌体内エンロフロキサシン濃度について、キノロン耐性株と感受性株の間に有意差は認められなかった。次に、エンロフロキサシンを含む寒天平板培地を用いて、キノロン感受性株の耐性獲得頻度を測定した。感受性株の耐性獲得頻度は、菌体内エンロフロキサシン濃度が低かった2株で50.2×10^{-9}及び10.8×10^{-9}、中程度の1株で37.7×10^{-9}であった。一方、菌体内エンロフロキサシン濃度が高い8株の平均耐性獲得頻度は$2.8\pm2.6\times10^{-9}$であった。このことから、菌体内エンロフロキサシン濃度が低値を示したキノロン感受性株は、耐性獲得頻度が高い事が示された。以上のことから、菌体内エンロフロキサシン濃度は、キノロンに対する耐性獲得と関与し、薬剤排泄ポンプの亢進により低下することが示唆された。

次に、フルオロキノロンによる薬剤排泄ポンプへの影響を調べるために、実験室内においてエンロフロキサシン存在下で作出した耐性株7株（サルモネラ・コレラスイス感受性株由来）の薬剤感受性及び薬剤排泄システムについて親株との比較を行った。作出した耐性株は、キノロンの標的酵素における変異は必ず

しも見られなかったが、キノロン及びその他の薬剤（アンピシリン、セフチオフル、オキシテトラサイクリン、クロラムフェニコール、エリスロマイシン、トリメトプリム、アクリフラビン、CCCP）に対する感受性が親株に比べて低下した。さらに、作出した耐性株の菌体内エンロフロキサシン濃度は、親株よりも低く、グラム陰性菌の多剤排泄ポンプである*acrB* mRNAの発現量が、作出した耐性株で親株よりも増加した。親株に比べて感受性が低下した薬剤は、CCCPを除いてAcrABの基質である。よって、次にCCCPを基質とする排泄ポンプである*emrA* mRNAの発現量を調べたところ、耐性株で親株よりも増加していた。以上のことから、フルオロキノロンによる選択圧は、サルモネラにおいて複数の薬剤排泄ポンプを亢進させる要因の1つとなることが示された。その結果、複数の薬剤に対する感受性の低下に関与することが示唆された。

菌体内エンロフロキサシン濃度の違いにより、耐性獲得頻度が異なったことから、耐性菌出現のリスクは一定ではない。日本において、フルオロキノロンに対して耐性であるサルモネラの報告は、現在のところそれほど多くはない。しかし、菌体内エンロフロキサシン濃度が低下することで耐性菌出現リスクの高い株が広がり、次の段階としてフルオロキノロン耐性サルモネラが広がる可能性がある。フルオロキノロン耐性菌の出現を防ぐため、菌体内エンロフロキサシン濃度やそれに関わる薬剤排泄ポンプをモニタリングすることは、フルオロ

キノロン耐性菌出現のリスクを予測するうえで有用であることが示唆された。

本研究は、フルオロキノロン耐性サルモネラの出現を防ぐために重要な情報になると考えられた。