NOTE *Bacteriology*

Resistance phenotypes and genotypes among multiple-antimicrobial-resistant *Salmonella enterica* subspecies *enterica* serovar Choleraesuis strains isolated between 2008 and 2012 from slaughter pigs in Okinawa Prefecture, Japan

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(Received 22 December 2014/Accepted 20 January 2015/Published online in J-STAGE 21 February 2015)

ABSTRACT. A total of 349 *Salmonella enterica* subspecies *enterica* serovar Choleraesuis (*S.* Choleraesuis) strains, which were isolated between 2008 and 2012 from 349 pigs at two slaughterhouses in Okinawa Prefecture, Japan, were investigated for antimicrobial susceptibility and the presence of antimicrobial resistance genes. All isolates were resistant to at least four antimicrobial agents. The antimicrobial agents for which isolates showed a high incidence of resistance were as follows: ampicillin (100%) and streptomycin (100%), followed by gentamicin (99.7%), oxytetracycline (99.7%), sulfamethoxazole/trimethoprim (99.4%), nalidixic acid (40.1%) and oxolinic acid (40.1%). All isolates were sensitive to cefuroxime, ceftiofur, colistin, fosfomycin, enrofloxacin, orbifloxacin and danofloxacin. The predominant resistance phenotypes and genotypes were: resistance to ampicillin, streptomycin, gentamicin, oxytetracycline and sulfamethoxazole/trimethoprim (58.5%, 204/349) and *bla*TEM-*strA-strB-aadA1-aadA2-aacC2-tet (B)-sul1-sul2-dhfrXII-dhfrXIII* (36.1%, 126/349). The quinolone resistance-determining regions (QRDRs) of *gyrA, gyrB, parC* and *parE* of the quinolone-resistant isolates (n=12) showed amino acid substitutions of Ser-83→Phe or Asp-87→Tyr in GyrA and Ser-107→Ala in ParC. To our knowledge, this is the first report on the molecular characterization of antimicrobial resistance among *S.* Choleraesuis strains in Japan.
KEY WORDS: antimicrobial resistance, pig, resistance genes, *Salmonella* Choleraesuis, zoonosis

doi: 10.1292/jvms.14-0683; *J. Vet. Med. Sci.* 77(6): 705–710, 2015

The emergence of antimicrobial-resistant *Salmonella enterica* has become a major public health hazard worldwide. In Southeast Asia, pigs may be an important reservoir of *Salmonella* for humans, and there have been many reports on isolating antimicrobial-resistant *S. enterica* from pork, pork products and the slaughter environment [2–5, 8, 9, 28, 29]. *Salmonella enterica* subspecies *enterica* serovar Choleraesuis (*S.* Choleraesuis) is a highly swine-adapted organism that causes several different disease syndromes [6, 15]. This pathogen is a zoonotic agent that is transmitted to humans via contaminated food (e.g., raw meat and liver), usually causing septicemic infections that require antimicrobial treatment [6, 7]. *S.* Choleraesuis infection is designated as a notifiable infectious disease in Japan. Thus, diseased pigs containing the organism should be condemned at postmortem inspection. In recent years, multidrug-resistant (MDR) *S.* Choleraesuis has become a public concern in Japan's neighbors, that is, Taiwan and other countries [5, 19, 20, 25–28].

In Okinawa Prefecture, *S.* Choleraesuis was not detected at slaughterhouses until May 2008. Since then, *S.* Choleraesuis isolates have been regularly recovered from slaughter pigs (85–163 cases per year between 2008 and 2012). In general, pigs can remain subclinical carriers of Salmonella, and these pigs are found in meat inspections, contributing to economic losses of swine producers. Therefore, antimicrobial therapy is essential in the treatment of *S.* Choleraesuis infection [6, 15]. Consequently, collection of detailed information on the *in vitro* activities of antimicrobial agents is an important strategy for both meat producers and clinicians.

The objectives of this study were 1) to investigate the antimicrobial resistance phenotypes and genotypes of *S.* Choleraesuis isolates from slaughter pigs and 2) to provide trace-back information on this foodborne zoonosis.

Salmonella Choleraesuis isolates: A total of 349 strains of *Salmonella* Choleraesuis were isolated from organs, livers, spleens, mesenteric lymph nodes and tracheal lymph nodes, of 349 slaughter pigs from 25 farms in 8 municipalities at slaughterhouses in Okinawa Prefecture, Japan, between May 2008 and January 2012 (Table 2). The isolates were identified biochemically (API® 20 E, bioMérieux, Marcy-l'Étoile, France) and were serotyped by slide and tube agglutination tests with commercially available antisera (Denka Seiken Co., Ltd., Tokyo, Japan).

Antimicrobial susceptibility test: Antimicrobial susceptibility testing was carried out using the disk agar diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [10]. Mueller-Hinton agar (Nippon Becton Dickinson, Fukushima, Japan) batches were used as the culture medium. The following antimicrobial agents were used: ampicillin (ABPC, 10 μg), streptomycin (SM, 10 μg), kanamycin (KM, 30 μg), gentamicin (GM,

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Table 1. PCR primers used for antimicrobial resistance genes and sequencing of QRDR

Antimicrobial family	Resistance gene	Forward PCR primer sequence (5′-3′)	Reverse PCR primer sequence (5′-3′)	Reference
Beta-lactams	*bla*TEM	GAGTATTCAACATTTTCGT	ACCAATGCTTAATCAGTGA	[22]
	*bla*SHV	TCGCCTGTGTATTATCTCCC	CGCAGATAAATCACCACAATG	[22]
	*bla*OXA	TCAACTTTCAAGATCGCA	GTGTGTTTAGAATGGTGA	[1]
	*bla*PSE	GCAAGTAGGGCAGGCAATCA	GAGCTAGATAGATGCTCACAA	[8]
	*bla*CMY	GACAGCCTCTTTCTCCACA	TGGAACGAAGGCTACGTA	[29]
Aminoglycosides	*strA*	TGGCAGGAGGAACAGGAGG	AGGTCGATCAGACCCGTGC	[9]
	strB	GCGGACACCTTTTCCAGCCT	TCCGCCATCTGTGCAATGCG	[9]
	aadA1	TATCAGAGGTAGTTGGCGTCAT	GTTCCATAGCGTTAAGGTTTCATT	[24]
	aadA2	TGTTGGTTACTGTGGCCGTA	GATCTCGCCTTTCACAAAGC	[24]
	aadB	GAGCGAAATCTGCCGCTCTGG	CTGTTACAACGGACTGGCCGC	[21]
	aphA1	ATGGGCTCGCGATAATGTC	CTCACCGAGGCAGTTCCAT	[22]
	aphA2	GAACAAGATGGATTGCACGC	GCTCTTCAGCAATATCACGG	[22]
	aac (3)-IV	GTGTGCTGCTGGTCCACAGC	AGTTGACCCAGGGCTGTCGC	[22]
	aacC2	CGGAAGGCAATAACGGAG	TCGAACAGGTAGCACTGAG	[22]
Tetracycline	*tet (A)*	GTGAAACCCAACATACCCC	GAAGGCAAGCAGGATGTAG	[22]
	tet (B)	CCTTATCATGCCAGTCTTGC	ACTGCCGTTTTTTCGCC	[22]
	tet (C)	ACTTGGAGCCACTATCGAC	CTACAATCCATGCCAACCC	[22]
	tet (D)	TGGGCAGATGGTCAGATAAG	CAGCACACCCTGTAGTTTTC	[22]
	tet (E)	TTAATGGCAACAGCCAGC	TCCATACCCATCCATTCCAC	[22]
	tet (G)	CCGGTCTTATGGGTGCTCTA	CCAGAAGAACGAAGCCAGTC	[24]
Phenicols	*floR*	CGCCGTCATTCCTCACCTTC	GATCACGGGCCACGCTGTGTC	[22]
	cmlA	CCGCCACGGTGTTGTTGTTATC	CACCTTGCCTGCCCATCATTAG	[18]
	catA1	AGTTGCTCAATGTACCTATAACC	TTGTAATTCATTAAGCATTCTGCC	[22]
Sulfonamides/ trimethoprim	*sulI*	TTCGGCATTCTGAATCTCAC	ATGATCTAACCCTCGGTCTC	[22]
	sulII	CGGCATCGTCAACATAACC	GTGTGCGGATGAAGTCAG	[22]
	dhfrI	AAGAATGGAGTTATCGGGAATG	GGGTAAAAACTGGCCTAAAATTG	[22]
	dhfrV	CTGCAAAAGCGAAAAACGG	AGCAATAGTTAATGTTTGAGCTAAAG	[22]
	dhfrVII	GGTAATGGCCCTGATATCCC	TGTAGATTTGACCGCCACC	[22]
	dhfrIX	TCTAAACATGATTGTCGCTGTC	TTGTTTTCAGTAATGGTCGGG	[22]
	dhfrX	ACCAGAGCATTCGGTAATCA	TTGGATCACCTACCCATAGA	[4]
	dhfrXII	AAATTCCGGGTGAGCAGAAG	CCCGTTGACGGAATGGTTAG	[4]
	dhfrXIII	CAGGTGAGCAGAAGATTTTT	CCTCAAAGGTTTGATGTACC	[22]
QRDR	*gyrA*	GCTGAAGAGCTCCTATCTGG	GGTCGGCATGACGTCCGG	[9]
	gyrB	GCGCGCTCGATTTAGCCG	TGATAGCGCAGCTTGTCCG	[9]
	parC	GTACGTGATCATGGATCGTG	TTCCTGCATGGTGCCGTCG	[9]
	parE	GCGATCGCGAATATCAGGCG	CAGTTGTTCCAGTACGCCC	[9]

10 μg), cefuroxime (CXM, 30 μg), ceftiofur (CTF, 30 μg) oxytetracycline (OTC, 30 μg), colistin (CL, 10 μg), chloramphenicol (CP, 30 μg), sulfamethoxazole/trimethoprim (ST, 23.75/1.25 μg), fosfomycin (FOM, 50 μg), nalidixic acid (NA, 30 μg), oxolinic acid (OA, 10 μg), enrofloxacin (ERFX, 5 μg), orbifloxacin (OBFX, 10 μg) and danofloxacin (DNFX, 5 μg). Antibiotic discs used in the test were purchased from Nippon Becton Dickinson, except for oxolinic acid, enrofloxacin, orbifloxacin and danofloxacin, which were kindly donated by Eiken Chemical Co., Ltd. (Tokyo, Japan), Bayer Yakuhin, Ltd. (Tokyo, Japan), Merial Japan Ltd. (Tokyo, Japan) and Pfizer Japan Inc. (Tokyo, Japan), respectively. Reading of inhibition zones was interpreted according to the manufacturer's instructions. Quality control strains were routinely used: *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

Detection of antimicrobial resistance genes: The choice of resistance genes to be studied was based on their relative importance, as observed in resistant *Salmonella enterica* isolates. For PCR amplification of antimicrobial resistance genes, 32 different oligonucleotide primer sets were used in the study (Table 1). We tested genotypes for resistance to ampicillin (*bla*TEM, *bla*SHV, *bla*PSE, *bla*OXA and *bla*CMY), streptomycin (*strA*, *strB*, *aadA1* and *aadA2*), kanamycin (*aadB*, *aphA1* and *aphA2*), gentamicin [*aadB*, *aac (3) IV*, *aacC2* and *aphA2*], oxytetracycline [*tet (A)*, *tet (B)*, *tet (C)*, *tet (D), tet (E)* and *tet (G)*], chloramphenicol (*floR*, *cmlA* and *catA1*), and sulfamethoxazole/trimethoprim [*sul1*, *sul2* (sulfonamide resistance), *dhfrI*, *dhfrV, dhfrVII*, *dhfrIX*, *dhfrX*, *dhfrXII* and *dhfrXIII* (trimethoprim resistance)]. All primers were commercially synthesized (Hokkaido System Science Co., Ltd., Sapporo, Japan). To prepare DNA templates, bacterial cells were suspended in distilled water and boiled for 10 min, and the cells were pelleted by centrifugation for 1 min. Amplifications were carried out with 5 μl of the supernatants. PCR was performed in a final volume of 25

Table 2. Trends in antimicrobial resistance genes in *Salmonella* Choleraesuis isolates (n=349)

Resistance phenotype (No. of isolates)	Resistance genes	No. (%) of resistant isolates by isolation period					
		2008 (n=128)	2009 (n=26)	2010 (n=93)	2011 (n=86)	2012 (n=16)	Total (n=349)
Ampicillin	*bla*TEM	128 (100)	26 (100)	93 (100)	86 (100)	16 (100)	349 (100)
(349)	*bla*SHV, *bla*PSE, *bla*OXA, *bla*CMY	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Streptomycin	*strA*	126 (98.4)	26 (100)	93 (100)	86 (100)	16 (100)	347 (99.4)
(349)	*strB*	126 (98.4)	26 (100)	93 (100)	86 (100)	16 (100)	347 (99.4)
	aadA1	71 (55.5)	16 (61.5)	78 (83.9)	37 (43.0)	7 (43.8)	209 (59.9)
	aadA2	127 (99.2)	26 (100)	93 (100)	86 (100)	16 (100)	348 (99.7)
Kanamycin	*aphA1*	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)
(1)	*aadB, aphA2*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gentamicin	*aphA1, aphA2, aac (3)-IV*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
(348)	*aacC2*	127 (99.2)	25 (100)	93 (100)	86 (100)	16 (100)	347 (99.4)
Tetracycline	*tet (A)*	0 (0)	1 (3.8)	0 (0)	0 (0)	0 (0)	1 (0.3)
(348)	*tet (B)*	127 (100)	25 (96.2)	93 (100)	85 (98.8)	16 (100)	346 (99.4)
	tet (C), tet (D), tet (E), tet (G)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Phenicols	*catA1*	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)
(1)	*floR, cmlA*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	sulI	124 (98.4)	26 (100)	90 (96.8)	85 (98.8)	16 (100)	341 (98.3)
	sulII	126 (100)	26 (100)	93 (100)	86 (100)	16 (100)	347 (100)
Sulfonamides/trimethoprim	*dhfrI, dhfrV, dhfrVII, dhfrIX, dhfrX*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
(347)	*dhfrXII*	126 (100)	26 (100)	93 (100)	86 (100)	16 (100)	347 (100)
	dhfrXIII	126 (100)	26 (100)	93 (100)	86 (100)	16 (100)	347 (100)

µl using GoTaq® Green Master Mix, 2× (Promega, Madison, WI, U.S.A.), according to the manufacturer's instructions.

Sequencing of gyrA, gyrB, parC and parE genes: The nalidixic and oxolinic acid-resistant isolates (defined as isolates derived from 12 farms) were examined for mutations of the quinolone-resistance determining regions (QRDRs) of *gyrA*, *gyrB*, *parC* and *parE* by PCR [9]. All PCR products were purified using a QIAquick® PCR Purification Kit (QIAGEN, Tokyo, Japan) and submitted to Macrogen Japan Corp. (Tokyo, Japan) for sequencing. The resulting DNA sequence data were compared to data in the GenBank database using the BLAST algorithm available at the National Center for Biotechnology Information Web site (www.ncbi.nlm.nih.gov).

Salmonella Choleraesuis isolates: None of the isolates showed H_2S production, which was determined by Api 20 E systems, and therefore, they were classified into biotype Choleraesuis (H_2S^-).

Antimicrobial susceptibility test: The isolates showed high levels of resistance to ampicillin (100% of the isolates were resistant) and streptomycin (100%), followed by gentamicin (99.7%), oxytetracycline (99.7%), sulfamethoxazole/trimethoprim (99.4%), nalidixic acid (40.1%) and oxolinic acid (40.1%). In contrast, a single isolate was resistant to kanamycin and chloramphenicol. None of the isolates exhibited resistance to cefuroxime, ceftiofur, colistin, fosfomycin, enrofloxacin, orbifloxacin or danofloxacin.

All isolates could be grouped into five phenotypes (Table 3). The most frequent multiple resistance phenotype was ABPC-SM-GM-OTC-ST (58.5%) followed by ABPC-SM-GM-OTC-ST-NA/OA (40.4%). All isolates were resistant to four or more antimicrobials.

Detection of antimicrobial resistance genes: Antimicrobial-resistant genotypes are shown in Table 3.

Beta-lactams. All 349 beta-lactam-resistant isolates possessed *bla*TEM genes. None of the isolates tested were positive for *bla*SHV, *bla*PSE, *bla*OXA and *bla*CMY.

Aminoglycosides. Of the nine aminoglycoside resistance genes for which tests were conducted, 99.4% of streptomycin-resistant isolates possessed *strA*, *strB* and *aadA2*, while 59.9% possessed *strA*, *strB* and *aadA1*. One kanamycin-resistant isolate possessed only the *aphA1* gene. One gentamicin-resistance gene, *aacC2,* was found among the gentamicin-resistant isolates, and one isolate was negative for the genes tested.

Tetracycline. Of the six tetracycline-resistance genes targeted, *tet (A)* and *tet (B)* were detected. The *tet (B)* gene was found exclusively in 99.4% of all tetracycline-resistant isolates, except for one isolate possessing *tet (A)*, and only one tetracycline-resistant isolate possessed none of the *tet* genes tested.

Phenicols. One chloramphenicol-resistant isolate possessed only the *catA1* gene.

Sulfamethoxazole/trimethoprim. Among the sulfamethoxazole/trimethoprim-resistant isolates, almost all (98.3 to 100%) of the resistant isolates possessed *sul1* and/or *sul2* genes, and both the *dhfrXIIand dhfrXIII* genes were detected in all sulfamethoxazole/trimethoprim-resistant isolates.

Sequencing of gyrA, gyrB, parC and parE genes: Twelve isolates harbored mutations that encoded an amino acid substitution within the QRDRs of *gyrA* and *parC.* Nine isolates possessed a change at Ser-83 to Phe (TCC→TTC), and three

Table 3. Antimicrobial resistance phenotypes and genotypes of *Salmonella* Choleraesuis from slaughter pigs

Resistance phenotype	No. of isolates (%)	Resistance genotype
ABPC·SM·KM·OTC·CP·ST	1 (0.3)	*bla*TEM, *strA, strB, aadA1, aadA2, aphA1, tetA, catA1, sul1, sul2, dhfrXII, dhfrXIII*
ABPC·SM·GM·OTC·ST·NA/OA	74 (21.2)	*bla*TEM, *strA, strB, aadA1, aadA2, aacC2, tetB, sul1, sul2, dhfrXII, dhfrXIII*
	62 (17.8)	*bla*TEM, *strA, strB, aadA2, aacC2, tetB, sul1, sul2, dhfrXII, dhfrXIII,*
	3 (0.9)	*bla*TEM, *strA, strB, aadA1, aadA2, aacC2, tetB, sul2, dhfrXII, dhfrXIII*
	1 (0.3)	*bla*TEM, *strA, strB, aadA1, aadA2, aacC2, sul1, sul2, dhfrXII, dhfrXIII*
	1 (0.3)	*bla*TEM, *strA, strB, aadA2, aacC2, tetB, sul2, dhfrXII, dhfrXIII*
ABPC·SM·GM·OTC·ST	126 (36.1)	*bla*TEM, *strA, strB, aadA1, aadA2, aacC2, tetB, sul1, sul2, dhfrXII, dhfrXIII*
	73 (20.9)	*bla*TEM, *strA, strB, aadA2, aacC2, tetB, sul1, sul2, dhfrXII, dhfrXIII*
	1 (0.3)	*bla*TEM, *strA, strB, aadA2, aacC2, tetB, sul2, dhfrXII, dhfrXIII*
	2 (0.6)	*bla*TEM, *strA, strB, aadA1, aadA2, aacC2, tetB, sul2, dhfrXII, dhfrXIII*
	1 (0.3)	*bla*TEM, *strA, strB, aadA2, aacC2, tetB, sul1, sul2, dhfrXII, dhfrXIII*
	1 (0.3)	*bla*TEM, *aadA2, tetB, sul1, dhfrXII, dhfrXIII*
ABPC·SM·GM·OTC	1 (0.3)	*bla*TEM, *strA, strB, aadA1, aacC2, tetB, sul1, sul2*
	1 (0.3)	*bla*TEM, *aadA2, aacC2, tetB*
ABPC·SM·GM·ST	1 (0.3)	*bla*TEM, *strA, strB, aadA1, aadA2, aacC2, sul2, dhfrXII, dhfrXIII*
Total	349 (100)	

ABPC, ampicillin; SM, streptomycin; KM, kanamycin; GM, gentamicin; OTC, oxytetracycline; CP, chloramphenicol; ST, sulfamethoxazole/trimethoprim; NA, nalidixic acid; OA, oxolinic acid.

isolates possessed a change at Asp-87 to Tyr (GAC→TAC) in GyrA, respectively; 12 isolates possessed Thr-57-Ser (ACC→AGC) substitutions in ParC. No mutations were observed in the QRDRs of *gyrB* and *parE*.

In this study, all the isolates exhibited multiple resistances to four or more of the antimicrobials tested. Almost all (98.9%) displayed very similar antimicrobial resistance phenotypes, such as ABPC-SM-GM-OTC-ST (58.5%) and ABPC-SM-GM-OTC-ST-NA/OA (40.4%). In the Japanese veterinary fields, especially the swine industry, ampicillin, streptomycin and tetracycline antibiotics are the most commonly used antimicrobials, and aminoglycosides are, in particular, recommended for treatment of swine salmonellosis. Thus, resistance to streptomycin and gentamicin is a serious concern. Furthermore, conventional antimicrobials, such as ampicillin, sulfamethoxazole/trimethoprim, fosfomycin, extended-spectrum cephalosporins and fluoroquinolones, have been recommended for invasive salmonellosis of humans in Japan. Hence, the high prevalence of resistance to ampicillin and sulfamethoxazole/trimethoprim may cause serious problems with respect to public health as a result of zoonotic infections.

A single enrofloxacin-resistant *S*. Choleraesuis isolate was recovered from a diseased pig in the Japanese Veterinary Antimicrobial Resistance Monitoring Program [3, 13, 14]. Therefore, fluoroquinolone-resistant *S.* Choleraesuis is not widespread in Japan. Our current data are consistent with these findings.

In this study, *aacC2*, which has rarely been detected among *Salmonella* [4, 21], was exclusively detected from gentamicin-resistant isolates. Furthermore, trimethoprim-resistant isolates exclusively possessed both the *dhfrXIIand dhfrXIII* genes, and almost all isolates displayed very similar genotype-phenotype correlations in antimicrobial resistance. Thus, all isolates showed strong similarities at both phenotypic and genotypic levels, suggesting that the isolates were of clonal origin. The hypothesis may be supported by the finding of indistinguishable pulsed-field gel electrophoresis (PFGE) patterns for twenty *S*. Choleraesuis strains, isolated between May 2008 and November 2008 at a slaughterhouse in Okinawa Prefecture, (Goto N, personal communication, 2009). However, further expandable studies involving PFGE analysis and other typing methods are necessary to more precisely determine whether or not the multiple-antimicrobial-resistant *S.* Choleraesuis isolates are derived from the same origin and are widely distributed.

Interestingly, the prevalence of nalidixic and oxolinic acid-resistant isolates has significantly increased, from 0% in 2008 to 87.5% in 2012 (2009, 15.4%; 2010, 58.1%; 2011, 79.1%). Nalidixic acid has not been approved for therapeutic use in pigs in Japan, whereas oxolinic acid was first approved for food-producing animals including cattle, swine and poultry in 1975. Oxolinic acid has been particularly used for decades to treat colibacillosis and salmonellosis in piglets with diarrhea. In general, inappropriate or intensive use of antimicrobials in farming practices can potentially lead to the emergence of antimicrobial resistance among bacteria. However, oxolinic acid had rarely been used on the farms investigated. The reason for the rapid dissemination of *S*. Choleraesuis resistant to an old quinolone is unknown.

As in other bacteria, the mechanisms of resistance to quinolone in *Salmonella* have been attributed to point mutations in the QRDRs of the *gyrA* gene coding for the A subunit of DNA gyrase and *parC* coding for the ParC subunit of topoisomerase IV [11]. Amino acid changes in GyrA at Ser-83 (to Phe, Tyr or Ala) or at Asp-87 (to Gly, Asn or Tyr) are the most frequently observed changes in quinolone-resistant strains [5, 12, 13, 16, 17, 23, 27]. Recently, several researchers [9, 12, 13, 27] reported on the ParC mutations (Thr57-Ser, Thr66-Ile and Ser80-Arg). The present study

revealed that similar amino acid substitutions were detected in the nalidixic and oxolinic acid-resistant isolates. Ling *et al.* [20] reported that mutations in *gyrA* conferred low-level fluoroquinolone resistance, while addition of another *gyrA* mutation together with *parC* and/or a *parE* mutation increased the resistance to a high level. Furthermore, in Japan, an *S.* Choleraesuis isolate with a single mutation in *gyrA* showed minimum inhibitory concentrations of 512 μg/m*l* for nalidixic acid and 2 μg/m*l* for enrofloxacin, and those of *S.* Choleraesuis with double mutations in *gyrA* were 512 μg/m*l* and 4 μg/m*l*, respectively [13]. Our results were consistent with these findings.

In conclusion, fluoroquinolones were very active against *S.* Choleraesuis. Thus, these agents are recommended for treatment and control of this foodborne pathogen. However, in Okinawa, use of fluoroquinolones for therapy and prophylaxis has gradually increased on swine farms, and thus, excessive use of fluoroquinolones may cause the emergence of resistance to these drugs in the future. The widespread nature of MDR *S.* Choleraesuis carrying multiple resistance genes is of serious concern, and it should be carefully monitored.

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