Changes in antimicrobial susceptibility in a population of *Salmonella enterica* serovar Dublin isolated from cattle in Japan from 1976 to 2005

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Objectives: We investigated the antimicrobial susceptibilities and resistance mechanisms of cattle-adapted *Salmonella enterica* serovar Dublin isolated in Japan in the past 30 years. This study is an example of evaluation of the impact of introduction of antimicrobials in veterinary medical practice on the selection of resistance in *S. enterica*.

Methods: The antimicrobial susceptibilities and prevalence of R-plasmids in *Salmonella* Dublin isolated in Japan from 1976 to 2005 were investigated. To evaluate the importance of gyrA mutation and active efflux, we derived the gyrA revertants and acrAB deletion mutants, and then compared with their parental strains the MICs of quinolone antimicrobials such as nalidixic acid, enrofloxacin, ofloxacin and ciprofloxacin.

Results: *Salmonella* Dublin isolates with R-plasmids and resistance to more than three antimicrobials were predominant between 1981 and 1995. From the latter half of the 1990s to the present, *Salmonella* Dublin isolates without R-plasmids became dominant. The introduction of nalidixic acid into the veterinary field in the mid-1980s was followed by the emergence of nalidixic acid-resistant isolates, which are now predominant. We found only a single gyrA mutation (Asp-87 → Tyr) among the nalidixic acid-resistant isolates. Although the reduced susceptibilities to the fluoroquinolones were observed among the nalidixic acid-resistant isolates, none of the isolates was resistant to the fluoroquinolones used in this study. The MIC data for the fluoroquinolones differed up to 4-fold. Results of the susceptibility test using gyrA revertants and acrAB mutants suggest that the isolates with the gyrA mutation were selected by the use of nalidixic acid, and the AcrAB-ToIC system accounts for the decreased fluoroquinolone susceptibilities.

Conclusions: These data suggest that the introduction of nalidixic acid in veterinary medicine seemed to affect the susceptibilities of *Salmonella* Dublin among the cattle population in Japan, whereas the introduction of enrofloxacin has not caused any additional effect. The prudent use of antimicrobials in the veterinary field should be continuously stressed.

Keywords: R-plasmid, quinolone resistance, gyrA mutation, AcrAB-ToIC

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Introduction

The European Union (EU) banned the use of several antibiotic growth promoters including avoparcin, bacitracin, spiramycin, tylosin and virginiamycin by 1999. The Food and Drug Administration of the USA also withdrew approval for administration of enrofloxacin to poultry in 2005. The ban in the EU related to the use of subtherapeutic antimicrobials for growth promotion, whereas the ban in the USA related to therapeutic use of fluoroquinolones in poultry. These bans were based on the ‘precautionary principle’, as the driving forces behind them were consumer and political opinion, and a scientific concern that resistant bacteria selected in animals might be transmitted to humans to the detriment of their health. The propriety of the ban and withdrawing of the approval are however, still controversial.

Casewell et al. reported that the ban on growth-promoting antibiotics has not been beneficial to human health but has been deleterious to animal health. Phillips et al. pointed out that although resistance can be selected in food animals, the chance of transmission to humans seems quite low, and the major cause of drug resistance in humans is inappropriate use of these medications. To understand these interactions better, the severity of the selection pressure in food animals, the risk level for transmitting selective-resistant bacteria from food animals to humans and the effects of transmitted resistant bacteria on human medications must be clarified.

The genus Salmonella consists of two species, Salmonella enterica and Salmonella bongori, and the former is divided into six subspecies: enterica, salamae, arizonae, diarizonae, houtenae and indica. This genus can be differentiated using somatic O and flagellar H antigens, and more than 2500 serovars have been reported based on the Kauffmann–White scheme to date. S. enterica subsp. enterica, to which many problematic serovars belong, is one of the most important food-borne pathogens and is transmissible from food animals to humans through contaminated meats, eggs and milk products. The degree of host adaptation varies among Salmonella serovars and affects the pathogenicity of this bacterium in man and animals alike.

Bovine salmonellosis is an important cause of mortality and morbidity in cattle and poses a threat to both beef and dairy operations. Clinical symptoms of bovine salmonellosis are fever, dullness, loss of appetite, depressed milk yield, diarrhoea, abortion, pneumonia, septicaemia and dry gangrene of the extremities. In acute salmonellosis, no treatment results in a fatality rate of 75%, although this may be reduced to 10% with treatment. Prompt treatment with broad-spectrum antibiotics has been thought to be beneficial, and multiple antimicrobials have been used for the treatment of bovine salmonellosis for a long time.

S. enterica subsp. enterica serovar Dublin is one of the most prevalent Salmonella serovars isolated from cattle in Japan. This serovar is cattle-adapted and is rarely isolated from other animal species. In Japan, there has been no report of milkborne human infections that have been reported in Western Europe and North America. This suggests that the epidemic clones of Salmonella Dublin with antimicrobial resistance traits would have been selected within the cattle reservoir without impact from antimicrobial use in humans.

In this study, we monitored the changes in antimicrobial resistance of Salmonella Dublin isolated in Japan from 1976 to 2005. The mechanisms underlying the resistance were also investigated, especially the resistance to quinolone antimicrobials. This study is an example of evaluation of the impact of introduction of antimicrobials in veterinary medical practice on the selection of resistance in S. enterica. The information would also be beneficial to the field veterinarian for the selection of appropriate antimicrobials for the treatment of bovine salmonellosis caused by Salmonella Dublin.

Materials and methods

Bacterial isolates, plasmids, media and culture conditions

One hundred and sixty-eight Salmonella Dublin isolates were submitted to the National Institute of Animal Health from the prefectural livestock hygiene service centres for diagnostic purposes between 1976 and 2005 and banked in Tryptic Soy (Difco Laboratories, Detroit, MI, USA) broth containing 25% glycerol at −80°C. The isolates were epidemiologically unrelated and were isolated from samples originating in different farms and geographical regions in Japan. The isolates were obtained from 26 of the 47 prefectures in Japan. All isolates were identified as Salmonella spp. based on their colony morphology on selective media, and biochemical testing. Serovar identification was performed using microtitre and slide agglutination methods according to the latest version of the Kauffmann and White scheme.

Salmonella Dublin strains were grown in LB (Difco Laboratories) broth or agar medium at 37°C for 18 h unless stated otherwise. Escherichia coli HB101 was used as a host strain for temperature-sensitive plasmid vectors such as pTH18ks1 and pTH18cs1. The plasmid-containing strains were grown in LS, low-salt LB medium (10 g of Bacto tryptone/5 g of yeast extract/5 g of NaCl, per litre, pH 7.4) containing 15 mg/L kanamycin (pTH18ks1) or 10 mg/L chloramphenicol (pTH18cs1). The gene replacement vectors from pTH18ks1 and pTH18cs1 were introduced to E. coli HB101 or Salmonella Dublin isolates by electroporation using a MicroPulser (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions.

Antimicrobial susceptibility testing

Kirby–Bauer disc diffusion assays were performed using Mueller–Hinton (MH) agar (Difco Laboratories) plates according to the CLSI (formerly NCCLS) standards, using the following antimicrobials: ampicillin (10 μg), cefotaxime (30 μg), ceftaxime (30 μg), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), fosfomycin (50 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), nalidixic acid (30 μg), ofloxacin (5 μg), norfloxacin (10 μg) and ciprofloxacin (5 μg) (BD Diagnostics, Sparks, MD, USA). Reading of inhibition zones was interpreted according to the manufacturer’s instructions.

The MIC assay was performed with an agar dilution method using MH agar (Difco Laboratories), according to the procedure recommended by the CLSI, using the following internal quality control strains: Staphylococcus aureus ATCC 29213, Enterococcus faecalis ATCC 29212, E. coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853. The quinolone antimicrobials listed below were tested: nalidixic acid (Wako Pure Chemical, Osaka, Japan), enrofloxacin (MP Biomedicals, Irvine, CA, USA), ofloxacin (LKT Laboratories, St Paul, MN, USA) and ciprofloxacin (MP Biomedicals). The interpretation was performed according to the CLSI standards except for enrofloxacin. The resistance breakpoint for enrofloxacin was obtained from results of the Japanese
Veterinary Antimicrobial Resistance Monitoring Programme. In this study, the resistance breakpoints for nalidixic acid, enrofloxacin, ofloxacin and ciprofloxacin were ≥32, ≥2, ≥8 and ≥4 mg/L, respectively.

**DNA manipulation**

Restriction enzymes and DNA-modifying enzymes were purchased from Takara Shuzo (Tokyo, Japan) and used according to the manufacturer’s recommendations. Mini-preparations of recombinant plasmids from *E. coli*, isolation of genomic DNAs from *Salmonella* Dublin and transformation of *E. coli* and *Salmonella* Dublin were performed by standard procedures. Oligonucleotides were purchased from Hokkaido System Science (Sapporo, Japan). PCR was performed using an iCycler (Bio-Rad). Takara EX Taq polymerase and a Takara Pyrobest DNA polymerase were used for PCR, according to the manufacturer’s instructions. Electrophoretic analysis of plasmid DNA was performed in 0.8% agarose (Wako Pure Chemical, Osaka, Japan) and TAE buffer (40 mM Tris, 40 mM glacial acetic acid and 1 mM EDTA).

**Plasmid (>10 kb) isolation and characterization**

Plasmid DNAs >10 kb in size were detected by the method of Kado and Liu. To detect the *spvC* gene on the virulence plasmid, Southern hybridization was performed by the method of Rexach et al. Conjugation was done to detect R-plasmids from *Salmonella* Dublin isolates using *E. coli* strain ML1410 (resistant to rifampicin) as a recipient. The strain was grown in TSB with shaking at 37°C for 16 h. Donor isolates were grown in 0.5 mL of TSB using short, small glass tubes static at 37°C for 16 h. One millilitre of the recipient culture was added to the tubes containing the donor culture and incubated at 37°C for 8 h. The mixture was spread on DHL agar plates (Nissui Pharmaceutical, Tokyo, Japan) containing 100 mg/L rifampicin and one of the following antimicrobials: ampicillin (25 mg/L), chloramphenicol (25 mg/L), kanamycin (25 mg/L) or tetracycline (25 mg/L). After incubation at 37°C for 16 h, transconjugants were picked and purified using the same selective plates. Plasmid isolation and antimicrobial susceptibility testing of the transconjugants were done in the same manner to characterize the R-plasmids.

**Sequencing and computer analysis**

Sequencing of cloned DNA fragments and various PCR products was done with fluorescent dye-labelled dideoxynucleotide with an ABI Prism 3100 or 3700 genetic analyser (Applied Biosystems, Foster City, CA, USA). The sequences were assembled with Sequercher Ver. 4 (Hitachi Software Engineering, Yokohama, Japan) and analysed using BLAST and GenBank (National Centre for Biotechnology Information, available on http://www.ncbi.nlm.nih.gov/BLAST/).

**Analysis of topoisomerase gene mutations**

Mutations in topoisomerase genes of 14 *Salmonella* Dublin isolates with and without resistance to nalidixic acid were analysed by the method of Ling et al. Briefly, the quinolone-resistance-determining regions (QRDRs) of genes *gyrA*, *gyrB*, *parC* and *parE* were amplified by PCR using genomic DNA as a template and primers are listed in Table 1. The amplified products were purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) and were then sequenced.

**Gene replacement in Salmonella Dublin**

The primers used for the gene replacement are listed in Table 1. The primer pair n-gyrAF and n-gyrAR was used to amplify a

Table 1. PCR primer pairs used in this study

<table>
<thead>
<tr>
<th>Sequence (5’→3’)</th>
<th>Product size (bp)</th>
<th>Location or description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGTCGAGATGGCCTGAGGC</td>
<td>347</td>
<td>gyrA</td>
<td>25</td>
</tr>
<tr>
<td>TACCCTCATAGTTATCAGCAG</td>
<td>345</td>
<td>gyrB</td>
<td>25</td>
</tr>
<tr>
<td>CAAACCTGGCAGCTGTACAGG</td>
<td>412</td>
<td>parC</td>
<td>25</td>
</tr>
<tr>
<td>ATGACGCGATAGGCGACGCG</td>
<td>272</td>
<td>parE</td>
<td>25</td>
</tr>
<tr>
<td>GACCGAGGATGTCCGGGAGG</td>
<td>677</td>
<td>gyrA</td>
<td>25</td>
</tr>
<tr>
<td>TTTTGGGTAAGATTGGTCG</td>
<td>530</td>
<td>3’ flanking region of <em>acrB</em></td>
<td>present study</td>
</tr>
<tr>
<td>CAGGAATTTGTCATACCTTCCGAGTTGTCG (EcoRI)</td>
<td>540</td>
<td>5’ flanking region of <em>acrR</em></td>
<td>present study</td>
</tr>
<tr>
<td>CCGGGATCCCGGTCTTCGTTTACAGT (BamHI)</td>
<td>960</td>
<td>combine the above two fragments</td>
<td>present study</td>
</tr>
<tr>
<td>TCGGAGGATCCCGGTCTTCGAGGTTGAGC (EcoRI)</td>
<td>463</td>
<td>mutation check (inside)</td>
<td>present study</td>
</tr>
<tr>
<td>ACCTACACGGATGATGTTGG</td>
<td>2800</td>
<td>mutation check (outside)</td>
<td>present study</td>
</tr>
</tbody>
</table>

*Restriction sites are underlined in the primer sequence and the names are identified in parentheses.*

1237
677 bp fragment in gyrA gene from strain 729 by PCR. After digestion by EcoRI and BamHI, the resulting fragment was cloned to temperature-sensitive plasmid vector pTH18ks1 or pTH18cs1 and used as a vector for the gene replacement to construct the gyrA revertants.

The primer pairs ACRP1/ACRP2 and ACRP3/ACRP4 were used to amplify the 3′ flanking region of acrB and the 5′ flanking region of acrR from genomic DNA of Salmonella Dublin strain 729 by PCR, respectively. Twenty nucleotides from the 5′ flanking region of acrR and the 3′ flanking region of acrB were attached to the primers ACRP2 and ACRP3, respectively, to combine both fragments by crossover PCR using the primer pair ACRP5 and ACRP6. After digestion by EcoRI and BamHI, the combined fragment was cloned to the vector pTH18ks1 or pTH18cs1 and used as an acrRAB knockout vector by gene replacement.

Salmonella Dublin strains 729, 974, 1242, 1506, 2226 and 3468 were transformed with a gene replacement vector by electroporation. The cells were spread on LS agar plates containing kanamycin or chloramphenicol and incubated at 28°C for 18 h. The colonies were then streaked on 42°C pre-warmed same agar plates and incubated at 42°C for 18 h. The single crossover strains were purified under the same conditions and then passaged at 28°C several times. The double crossover strains were screened for the loss of vector-mediated resistance and then the mutation was checked by PCR. The primers ACRP7 and ACRP8 were set inside the acrB gene, so that the amplification was negative for the expected deletion mutants. The primers ACRP9 and ACRP10 were set outside the deleted part. A 2.8 kb fragment was amplified by PCR from the deletion mutants. The amplified fragment was sequenced directly to confirm the structure of the deletion sites.

Results

Plasmid typing of Salmonella Dublin

Most Salmonella Dublin isolates used in this study harbour a single or multiple kinds of plasmids, Table 2 shows the results of plasmid typing of Salmonella Dublin isolates defined by the plasmid profiles. Sixty-seven out of the 168 (39.9%) Salmonella Dublin isolates had only the 75 kb virulence plasmid, which was identified by Southern hybridization experiments probed by the spvC gene and classified as plasmid type 1. These isolates were susceptible to all antimicrobial agents used in this study or resistant to any one, two or three of ampicillin, kanamycin, streptomycin and nalidixic acid. Ninety-five out of the 168 (56.5%) Salmonella Dublin isolates harboured a single R-plasmid in addition to the 75 kb virulence plasmid. The results of characterizations of the transconjugants indicated the R-plasmids of types 2–6 and defined the following antimicrobial resistances; type 2, ampicillin, kanamycin, streptomycin and tetracycline; type 3, ampicillin, kanamycin and streptomycin; type 4, chloramphenicol, kanamycin and streptomycin; type 5, ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline; and type 6, ampicillin, chloramphenicol, kanamycin, streptomycin, tetracycline and trimethoprim/sulfamethoxazole.

Remaining isolates did not have any plasmids or contain cryptic plasmids. Type 6, ampicillin, chloramphenicol, kanamycin, streptomycin, tetracycline and trimethoprim/sulfamethoxazole. Remaining isolates did not have any plasmids or contain cryptic plasmids and were termed non-typeable.

Temporal distribution of plasmid types

As shown in Table 3, all seven isolates obtained between 1976 and 1980 were classified as plasmid type 1 and were susceptible to all antimicrobial agents.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7 (100)</td>
<td>2 (25.0)</td>
<td>6 (18.2)</td>
<td>19 (27.9)</td>
<td>25 (75.8)</td>
<td>8 (61.5)</td>
</tr>
<tr>
<td>2</td>
<td>67 (44.0)</td>
<td>52 (78.1)</td>
<td>34 (51.4)</td>
<td>23 (34.3)</td>
<td>20 (29.4)</td>
<td>18 (25.7)</td>
</tr>
<tr>
<td>3</td>
<td>8 (100)</td>
<td>1 (1.5)</td>
<td>2 (2.8)</td>
<td>6 (8.8)</td>
<td>10 (14.3)</td>
<td>1 (1.5)</td>
</tr>
<tr>
<td>4</td>
<td>1 (1.5)</td>
<td>2 (3.0)</td>
<td>3 (4.4)</td>
<td>5 (7.4)</td>
<td>4 (5.8)</td>
<td>1 (1.5)</td>
</tr>
<tr>
<td>5</td>
<td>3 (7.7)</td>
<td>1 (1.5)</td>
<td>1 (1.5)</td>
<td>2 (2.9)</td>
<td>1 (1.5)</td>
<td>1 (1.5)</td>
</tr>
<tr>
<td>6</td>
<td>3 (7.7)</td>
<td>1 (1.5)</td>
<td>1 (1.5)</td>
<td>2 (2.9)</td>
<td>1 (1.5)</td>
<td>1 (1.5)</td>
</tr>
<tr>
<td>Total</td>
<td>7 (100)</td>
<td>8 (100)</td>
<td>12 (17.1)</td>
<td>20 (29.4)</td>
<td>29 (42.9)</td>
<td>13 (18.6)</td>
</tr>
</tbody>
</table>

*Non-typeable.

Table 2. Plasmid types of Salmonella Dublin isolates

Table 3. Temporal distribution of plasmid types of Salmonella Dublin isolates
to all the antimicrobial agents used in this study. Types 2 and 3 isolates were first obtained in 1983 and 1988, respectively. The type 2 isolates were dominant between 1981 and 1995. One of each isolate was sorted to plasmid types 4, 5 and 6 and was isolated in 1991, 1991 and 1992, respectively. The plasmid type 1 isolates became predominant again from 1996 onwards. Antimicrobial resistance profiles of the 33 type 1 isolates obtained between 1996 and 2005 are as follows: 2 isolates were susceptible; 23 isolates were resistant to kanamycin and nalidixic acid; 7 isolates were resistant to kanamycin, streptomycin and nalidixic acid; and 1 isolate was resistant to ampicillin, kanamycin and nalidixic acid.

**Susceptibilities to quinolone antimicrobials of Salmonella Dublin isolates**

All the Salmonella Dublin isolates obtained in and before 1985 were susceptible to nalidixic acid. The MIC distribution became bimodal in the latter half of the 1980s. Since then, nalidixic acid-resistant isolates have been predominant to the present day (Table 4). We also investigated the susceptibilities of Salmonella Dublin isolates to fluoroquinolones. The maximal MIC values of enrofloxacin, ofloxacin and ciprofloxacin for the Salmonella Dublin isolates were 1, 2 and 1 mg/L, respectively (Table 4). Although the reduced susceptibilities to the fluoroquinolones were observed among the nalidixic acid-resistant isolates, none of the isolates was resistant to the fluoroquinolones used in this study.

**Table 4. Temporal changes in susceptibility to quinolone antimicrobials**

<table>
<thead>
<tr>
<th>Time period</th>
<th>No. of isolates</th>
<th>NAL range</th>
<th>NAL MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>EFX range</th>
<th>EFX MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>OFX range</th>
<th>OFX MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>CIP range</th>
<th>CIP MIC&lt;sub&gt;90&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td>1976–80</td>
<td>7</td>
<td>2–4</td>
<td>4</td>
<td>&lt;0.125</td>
<td>4</td>
<td>&lt;0.125</td>
<td>0.5</td>
<td>&lt;0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>1981–85</td>
<td>8</td>
<td>2–4</td>
<td>4</td>
<td>&lt;0.125</td>
<td>0.25–2</td>
<td>1</td>
<td>0.25–1</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>1986–90</td>
<td>39</td>
<td>2 to &gt;512</td>
<td>512</td>
<td>&lt;0.125–1</td>
<td>0.25–2</td>
<td>1</td>
<td>0.25–1</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>1991–95</td>
<td>68</td>
<td>128 to &gt;512</td>
<td>512</td>
<td>&lt;0.125–1</td>
<td>0.25–2</td>
<td>1</td>
<td>0.25–1</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>1996–2000</td>
<td>33</td>
<td>4 to &gt;512</td>
<td>512</td>
<td>&lt;0.125–1</td>
<td>0.25–2</td>
<td>1</td>
<td>0.25–1</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>2001–05</td>
<td>13</td>
<td>256–512</td>
<td>512</td>
<td>0.25–0.5</td>
<td>0.25–0.5</td>
<td>1</td>
<td>0.25–0.5</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

NAL, nalidixic acid; EFX, enrofloxacin; OFX, ofloxacin; CIP, ciprofloxacin.

**Table 5. Susceptibility to quinolone antimicrobials and topoisomerase mutations**

<table>
<thead>
<tr>
<th>Log number</th>
<th>Year of isolation</th>
<th>NAL</th>
<th>EFX</th>
<th>OFX</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>CIP</th>
<th>Topoisomerase mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>974</td>
<td>1985</td>
<td>2</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>ND</td>
<td></td>
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<tr>
<td>489</td>
<td>1981</td>
<td>4</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>729</td>
<td>1983</td>
<td>4</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>ND</td>
<td></td>
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<tr>
<td>2876</td>
<td>1998</td>
<td>4</td>
<td>&lt;0.125</td>
<td>0.125</td>
<td>&lt;0.125</td>
<td>ND</td>
<td></td>
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<tr>
<td>2402</td>
<td>1996</td>
<td>4</td>
<td>&lt;0.125</td>
<td>0.25</td>
<td>&lt;0.125</td>
<td>ND</td>
<td></td>
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<tr>
<td>1886</td>
<td>1992</td>
<td>256</td>
<td>0.5</td>
<td>0.5</td>
<td>0.25</td>
<td>GyrA (Asp-87→Tyr)</td>
<td></td>
</tr>
<tr>
<td>1177</td>
<td>1988</td>
<td>512</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
<td>GyrA (Asp-87→Tyr)</td>
<td></td>
</tr>
<tr>
<td>1242</td>
<td>1989</td>
<td>512</td>
<td>0.5</td>
<td>0.5</td>
<td>0.25</td>
<td>GyrA (Asp-87→Tyr)</td>
<td></td>
</tr>
<tr>
<td>1506</td>
<td>1990</td>
<td>512</td>
<td>0.5</td>
<td>0.5</td>
<td>0.25</td>
<td>GyrA (Asp-87→Tyr)</td>
<td></td>
</tr>
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<td>3486</td>
<td>2004</td>
<td>512</td>
<td>0.5</td>
<td>1</td>
<td>0.25</td>
<td>GyrA (Asp-87→Tyr)</td>
<td></td>
</tr>
<tr>
<td>1563</td>
<td>1990</td>
<td>512</td>
<td>1</td>
<td>1</td>
<td>0.25</td>
<td>GyrA (Asp-87→Tyr)</td>
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<tr>
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<td>1995</td>
<td>512</td>
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<tr>
<td>2226</td>
<td>1994</td>
<td>&gt;512</td>
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<td>2</td>
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<td>GyrA (Asp-87→Tyr)</td>
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</tr>
</tbody>
</table>

The MIC test was performed twice on independent occasions.

NAL, nalidixic acid; EFX, enrofloxacin; OFX, ofloxacin; CIP, ciprofloxacin; ND, not detected.
mutations. The QRDRs of the genes *gyrA*, *gyrB*, *parC* and *parE* were amplified by PCR, and they were sequenced directly. As shown in Table 5, a single point mutation (Asp-87→Tyr) in *gyrA* among the nine nalidixic acid-resistant isolates was observed. No mutations were detected among the five nalidixic acid-susceptible isolates. The nine nalidixic acid-resistant isolates also exhibited higher MIC values of fluoroquinolones than the five susceptible isolates. The MIC values of enrofloxacin, ofloxacin and ciprofloxacin varied up to 4-fold among the nine nalidixic acid-resistant isolates.

### Contribution of the AcrAB-TolC system to the quinolone resistance

The AcrAB-TolC system is one of the most important efflux systems in *E. coli* and *Salmonella*. This system extrudes structurally unrelated chemicals and accounts for both intrinsic and acquired antimicrobial resistance. To investigate the involvement of the AcrAB-TolC system in quinolone resistance of *Salmonella* Dublin, we derived *gyrA* revertants and *acrAB* deletion mutants from six isolates with or without nalidixic acid resistance and compared the MIC values of fluoroquinolones between the mutants and their parental strains. As shown in Table 6, the fold reductions of MIC values for *gyrA* revertants were larger than those of *acrAB* mutants for nalidixic acid, whereas the same or smaller than those of *acrAB* mutants for fluoroquinolones such as enrofloxacin, ofloxacin and ciprofloxacin. The isolates with higher MIC values of fluoroquinolones tend to exhibit larger fold reductions among four nalidixic acid-resistant strains.

### Discussion

*Salmonella* Dublin was first isolated from diseased calves in Oita prefecture, which is located on the southern-most main island in Japan in 1976. Primary isolates were susceptible to all of the antimicrobials used in this study and were isolated from a relatively limited area in the western part of Japan. After acquisition of R-plasmid in the first half of the 1980s, the distribution of *Salmonella* Dublin has drastically increased. We confirmed the isolation of *Salmonella* Dublin in the northern-most main island in Japan in 1988. *Salmonella* Dublin has become one of the most prevalent serovars isolated from diseased cattle in Japan to date.

More than 70% of the *Salmonella* Dublin isolates obtained between 1981 and 1995 exhibited a multidrug-resistant phenotype and harboured an R-plasmid (plasmid types 2–6) in addition to the 75 kb virulence plasmid. Then, the isolates with only the virulence plasmid (plasmid type 1) became predominant from 1996 onwards (Table 3). Resistance to kanamycin and nalidixic acid was predominant among the type 1 isolates. The emergence of the nalidixic acid-resistant isolates was linked with the introduction of a newly developed oral product of nalidixic acid in veterinary medicine. The product was approved by the Ministry of Agriculture, Forestry and Fisheries in 1984 and then widely used for the therapeutic treatment of bovine salmonellosis in Japan. The resistant strains emerged after the introduction of the nalidixic acid product and have now become predominant.
Antimicrobial susceptibility of Salmonella enterica serovar Dublin

It is known that most isolates of S. enterica with nalidixic acid resistance have a single point mutation in gyrA gene. The fact that the accumulation of point mutations within topoisomerase genes, including gyrA, gyrB, parC and parE, led to the emergence of fluoroquinolone-resistant strains motivated us to investigate the susceptibilities of Salmonella Dublin isolates to fluoroquinolones. The maximum MIC values of enrofloxacin, ofloxacin and ciprofloxacin for Salmonella Dublin isolates were 1, 2 and 1 mg/L, respectively. The reduced susceptibilities to the fluoroquinolones were observed among the nalidixic acid-resistant isolates. However, none of the isolates was resistant to the fluoroquinolones used in this study.

Although a single gyrA mutation (Asp-87→Tyr) was observed in the nine examined nalidixic acid-resistant strains, the susceptibilities of the strains to fluoroquinolones including enrofloxacin, ofloxacin and ciprofloxacin differed up to 4-fold (Table 5). This suggests that mechanisms other than the gyrA mutation account partially for the resistance to fluoroquinolones. Decreased accumulation by active efflux was identified as a primary mechanism of resistance to ciprofloxacin in Salmonella Typhimurium. AcrAB, which uses the outer membrane protein TolC as an extruding channel, may be overexpressed and mediate fluoroquinolone resistance. We constructed the acrAB deletion mutants and gyrA revertants using six Salmonella Dublin isolates and compared the MIC values of fluoroquinolones with their parental strains to evaluate the impact of both resistance mechanisms.

gyrA revertants showed a more increased level of susceptibility to nalidixic acid than acrAB mutants, whereas acrAB mutants exhibited more increased levels of susceptibility to fluoroquinolones compared with gyrA revertants (Table 6). Salmonella Dublin isolates with higher MIC values were more likely to exhibit more increased levels of susceptibility to fluoroquinolones by the acrAB mutation. These data suggest that the gyrA mutation was selected for by the veterinary use of nalidixic acid, and the AcrAB-TolC system accounts for the reduced levels of susceptibility to fluoroquinolones. Previous reports have suggested that overexpression of the AcrAB-TolC efflux pump contributes to fluoroquinolone resistance with mutations in gyrA in S. enterica. We have not examined the expression level of the AcrAB-TolC system in this study.

The first fluoroquinolone for veterinary use, enrofloxacin, was approved by the Japanese government in 1991, and these products have been used extensively in veterinary medicine from 1992 onwards. However, no clear impacts on the MICs of fluoroquinolones were detectable in this study (Table 4). Recently, the concept of ‘fitness cost’ has been described for resistant bacteria. Fluoroquinolone resistance in S. enterica is relatively uncommon compared with its frequency in other Enterobacteriaceae, and it has been suggested that the resistance mechanisms in S. enterica may have a prohibitive fitness cost and thus limit the emergence of resistant strains. We also need to consider the successful promotion of the prudent use of fluoroquinolones in veterinary medical practice.2

Taken together, the introduction of nalidixic acid in veterinary medicine seemed to affect the susceptibilities of Salmonella Dublin among the cattle population in Japan, whereas the introduction of enrofloxacin has not caused any additional effect. The prudent use of antimicrobials in the veterinary field should be continuously stressed.

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Transparency declarations

None to declare.

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