Clonal Spread of *Salmonella enterica* Serovar Infantis in Serbia: Acquisition of Mutations in the Topoisomerase Genes *gyrA* and *parC* Leads to Increased Resistance to Fluoroquinolones

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**Impacts**

- *Salmonella* Infantis is the third most common serotype isolated from humans in Serbia, and infection is often associated with the consumption of poultry products. Fluoroquinolones are important drugs for the treatment of human salmonellosis.
- This study showed that *Salmonella* Infantis isolated from humans, poultry and food are clonally related in Serbia and characterized molecular mechanisms of decreased fluoroquinolone susceptibility. Target site mutations and efflux, but not plasmid-mediated genes, seem to be important resistance mechanisms.
- Better management practice and a restricted use of fluoroquinolones in animal production are needed to prevent a further dissemination of *Salmonella* Infantis.

**Keywords:** Foodborne pathogens; microbiology; poultry; public health; *Salmonella* spp; antimicrobial resistance

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**Summary**

Quinolone-resistant *Salmonella* Infantis (*n* = 64) isolated from human stool samples, food and poultry during the years 2006–2011 were analysed for their resistance phenotypes, macrorestriction patterns and molecular mechanisms of decreased susceptibility to fluoroquinolones. Minimum inhibitory concentrations (MICs) of nalidixic acid (NAL) and ciprofloxacin (CIP) were determined by the agar dilution procedure, and the susceptibility to additional antimicrobial agents was determined by the disc diffusion method. To assess the influence of enhanced efflux activity, MICs were determined in the presence and absence of the inhibitor PAβN. The results of pulsed-field gel electrophoresis (PFGE) typing revealed that quinolone-resistant *S*. Infantis in Serbia had similar or indistinguishable PFGE profiles, suggesting a clonal spread. All *S*. Infantis showed combined resistance to NAL and tetracycline, whereas multiple drug resistance to three or more antibiotic classes was rare (2 isolates of human origin). The MICs ranged between 512 and 1024 μg/mL for NAL and 0.125–2 μg/mL for CIP. A single-point mutation in the gene *gyrA* leading to a Ser83→Tyr exchange was detected in all isolates, and a second exchange (Ser80→Arg) in the gene *parC* was only present in eight *S*. Infantis isolates exhibiting slightly higher MICs of CIP (2 μg/mL). The inhibitor PAβN decreased the MIC values of CIP by two dilution steps and of NAL by at minimum 3–6 dilution steps, indicating that enhanced efflux plays an important role in quinolone resistance in these isolates. The plasmid-mediated genes *qnr*, *aac(6’)-Ib-cr* and *qepA* were not detected by PCR assays.
Introduction

Salmonella (S.) enterica serovar Infantis is a common serotype in livestock production, and it is consistently isolated from broiler chickens (Hauser et al., 2012; Sasaki et al., 2012). Besides the occurrence in animals, S. Infantis has been associated with cases of human salmonellosis and is implicated in nosocomial infections in veterinary hospitals (Fonseca et al., 2006; Dunowska et al., 2007) or food poisoning (Kohl and Farley, 2000; Najjar et al., 2012) in several countries. According to the global distribution of reported serotypes, S. Infantis was one of the highest ranked salmonellae (Galanis et al., 2006). During the years 2008 to 2010, in Serbia, it was the third most common serotype isolated from humans after S. Enteritidis and S. Typhimurium (http://thor.dvf.dk/pls/portal/GSS.COUNTRY_DATA_SET_REP.show_parms). Among S. Infantis isolates, multidrug resistance is frequent, and several mobile genetic elements such as plasmids, transposons or integrons harbouring specific resistance genes have been described (Shahada et al., 2006; Nógrády et al., 2007; Gal-Mor et al., 2010). Resistant strains may spread from animals through the food chain, and hence, resistance in S. Infantis represents an important public health concern.

For the treatment of typhoid fever or complicated non-typhoidal Salmonella gastroenteritis cases in humans and animals, fluoroquinolones (FQs) are the drugs of choice (Giraud et al., 2006). The target enzymes for quinolones are the DNA gyrase and topoisomerase IV, both consisting of two subunits encoded by the genes gyrA and gyrB or parC and parE, respectively. Resistance to quinolones emerges typically through alterations in the target enzymes, whereby mutations in salmonellae are found most frequently in the N-terminal part of GyrA, the primary target of quinolone action, at codons 83 (serin) and 87 (aspartic acid). Mutations in the genes parC and parE usually occur in multiple-drug-resistant (MDR) isolates or in cases of clinical resistance to ciprofloxacin (CIP). In addition, the products of plasmid-borne genes, such as Qnr, Aac(6’)-Ib-cr and QepA, may contribute to decreased FQ susceptibility as well. As a major mechanism of resistance, active efflux of FQs was identified, and the overproduction of the efflux pump AcrAB-TolC was considered to be mainly responsible for multiple antibiotic resistance in Salmonella (Giraud et al., 2006; Kehrenberg et al., 2007). Whilst several alterations in the target enzymes have been reported from different Salmonella serovars, mutations in the topoisomerase genes or the impact of efflux pumps was not extensively studied in S. Infantis (Eaves et al., 2004).

In this study, quinolone-resistant S. Infantis isolates from humans, food and poultry from Serbia were investigated for their genetic relationship, and the mechanisms of quinolone resistance were examined.

Material and Methods

Salmonella isolates
Sixty-four nalidixic acid-resistant S. Infantis isolates were included in the study. Forty-four isolates originated from chickens, 19 from human stool samples and a single isolate from a food sample (chicken liver). The human S. Infantis isolates were obtained from patients suffering from salmonellosis and from asymptomatic carriers, too. All cases were epidemiologically unrelated. Poultry isolates originated from different poultry processing plants in Serbia including three farms with a documented prehistory of salmonellae. The serovar of each isolate was confirmed by slide agglutination with Salmonella antisera (Statens Serum Institute, Copenhagen, Denmark) in the Institute of Public Health of Serbia, Reference Laboratory for Salmonella, Shigella, Vibrio cholerae and Yersinia enterocolitica, Belgrade. The isolates were stored at −80°C, and the experiments were conducted after cultivation of the isolates overnight on Mueller-Hinton agar plates.

Resistance phenotype and determination of minimum inhibitory concentrations (MICs)
Antibiotic resistance phenotypes of all isolates were determined by the disc diffusion method on Mueller-Hinton agar (BBL, Franklin Lakes, NJ, USA) according to the guidelines given in the Clinical and Laboratory Standards Institute (CLSI, documents M02-A11 and M100-S22 (CLSI M100-S22, 2012b; CLSI M02-A11, 2012c). For this, the following antibiotics were used: ampicillin (AMP), amoxycillin/clavulanic acid (AMC), chloramphenicol (CAP), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), streptomycin (STR), sulphonamides (SSS), tetracycline (TET), trimethoprim/sulfamethoxazole (1:19) (SXT), trimethoprim (TMP), cefotaxime (CTX) and ceftazidime (CAZ) (Bio-Rad, Marnes-la-Coquette, France), and Escherichia coli ATCC 25922 was included for quality control purposes. MIC values of NAL and CIP (Sigma Aldrich, Milan, Italy) were determined by the agar dilution method, and susceptibility tests were performed and evaluated according to the CLSI guidelines M7-A9 and M100-S22 (CLSI M07-A9, 2012a; CLSI M100-S22, 2012b). In this document, the recommended breakpoint concentration for clinical CIP resistance is ≥4 µg/mL CIP. E. coli ATCC 25922 was used as a control strain for MIC determinations, too. Multidrug resistance was defined as resistance to three or more antibiotic classes.

Efflux pump inhibition
For these experiments, the efflux pump inhibitor Phe-Arg-β-Naphthylamide (PAßN) (Sigma-Aldrich, Munich,
Polymerase chain reaction and sequencing of the topoisomerase genes and qnrS

PCR assays were performed to amplify the quinolone-resistance-determining regions (QRDRs) of the topoisomerase genes and to detect the genes qnrA, qnrB, qnrC, qnrD, qnrS, aac(6’)-Ib-cr and qepA. The gyrA PCR product of all strains was analyzed while for the genes gyrB, parC and parE sequencing was performed for isolates exhibiting MICs of ≥1 μg/mL. In addition, 10 isolates originating from stool (n = 6) and poultry (n = 4) samples and exhibiting a more susceptible CIP phenotype (MIC of 0.25–0.5 μg/mL CIP) were included for comparison reasons. Whole-cell DNA was extracted by the boiling method. For the PCR amplification, previously reported primers and conditions were used (Kehrenberg et al., 2006). The cycling conditions were as follows: initialization 94°C for 15 min, followed by 30 cycles of denaturation 94°C for 1 min, annealing 55°C for gyrA, 58°C for gyrB, 52°C for parC and parE for 1 min, and the elongation was 72°C for 1 min with a final elongation step of 72°C for 10 min. PCR products were purified using the Gene JET PCR purification kit (Fermentas Life Sciences, Vilnius, Lithuania), and subsequently, sequencing was performed on both strands (Macrogen, Amsterdam, the Netherlands or GATC Biotech AG, Konstanz, Germany). Multiple alignments were performed with the DNAMAN version 7 software program (Lynnon Corporation, Quebec, Canada). Recently published primers and protocols for the detection of the genes qnrA, qnrB, qnrC, qnrD, qnrS, aac(6’)-Ib-cr and qepA were used (Kehrenberg et al., 2006, 2007; Yamane et al., 2007; Cavaco et al., 2009; Wang et al., 2009).

Results and Discussion

During the years 2006 to 2011, 44 NALR S. Infantis were isolated from poultry farms located in the central and northern part of Serbia, 19 isolates were collected from human stool samples and one from a food sample. To analyse the epidemiological relationship of all 64 S. Infantis NALR, PFGE was performed, and the resulting DNA restriction patterns are presented in Fig 1. The macrorestriction analysis detected only slight differences in the XbaI patterns, and three major pulse types were identified: Forty-one strains (64%) were nearly indistinguishable from each other, designated as type A1, whilst 19 strains (29.7%) showed an additional band, and this type was assigned as A2. Isolates 8807/9, 9508/9, 8452/9 and 8241/11 (6.3%) did not possess one large band and thus represented the A3 group (Fig 1). According to Tenover et al. (1995), closely related isolates differ in one genetic event and thus in two to three fragment patterns in PFGE profiles. As the restriction profiles of this S. Infantis collection differed in less than three fragments, it was concluded that all Serbian isolates were closely related. Macrorestriction patterns of strains isolated before 2006 are not available from Serbia; therefore, it is not possible to compare changes in the emergence of specific S. Infantis clones. The spread of S. Infantis was a serious threat in poultry industry in Hungary, and they were also prevalent in humans during the recent years (EFSA, 2011). However, registered clones in Hungary (Nögrády et al., 2007) differ in their macrorestriction patterns and resistance phenotypes from S. Infantis from Serbia. In other countries such as Israel, the occurrence of clonally related S. Infantis has been reported, but isolates differ genetically and phenotypically from strains isolated some...
Fig. 1. PFGE profiles of S. Infantis isolates collected during the years 2006–2011. The position of the large fragment (L) that is absent in the A3 group and the additional band (A) that is present in the A2 group is indicated by dotted lines.
years ago (Gal-Mor et al., 2010). In Serbia, epidemiological data imply that food poisoning caused by Salmonella is most often linked to home-made meals resulting in small family epidemics (Petrović et al., 2005).

Susceptibility testing revealed that all NAL-resistant S. Infantis were additionally resistant to TET. Two isolates of human origin (3.1%) were multiple drug resistant, exhibiting the phenotype STR SSS TET NAL, whereas additional resistances were not detected in the other isolates. This finding was different to reports from other countries, where multiple resistance in S. Infantis is very common and most isolates showed a combined resistance pattern (Shahada et al., 2006; Nőgrády et al., 2007; 2 Dionisi et al., 2011; Hauser et al., 2012). The MICs for NAL and CIP ranged between 512 and 1024 μg/mL and 0.125–2 μg/mL, respectively. Clinical CIP resistance was absent in this strain collection. Nevertheless, 16 isolates showed decreased susceptibility to CIP (MIC of 1 or 2 μg/mL) (Table 1).

To investigate the mechanisms of quinolone resistance, mutations in the target genes for quinolones were analysed. A single-point mutation at position Ser83→Tyr was detected in the gene gyrA of all isolates and is normally sufficient to mediate the nalidixic acid-resistant and decreased fluoroquinolone-susceptible phenotype. Double mutants exhibiting exchanges in gyrA and parC at positions Ser83→Tyr (gyrA)/ Ser80→Arg (parC) were found in eight strains and were associated with slightly higher MICs to CIP (2 μg/mL). These isolates originated from poultry (n = 5) or humans (n = 3) exhibited MIC values of 2 μg/mL CIP and belonged to the following PFGE types: poultry isolates 7103/09, and human isolates Mateja/11 and 361/10 belong to the A1 type, poultry isolates 8099/08 and 8505/08 belong to the A2 type whilst poultry isolates 8807/09 and 8452/09 and human isolate 82416/11 was part of the A3 group. The gene parC is a common second target for quinolones in Salmonella, and the Ser80→Arg mutation was previously detected in other Salmonella serotypes from France, China, Spain and Japan. However, in contrast to our findings, all these isolates were clinically resistant to CIP (Velhner and Stojanović, 2012). In CIP-susceptible isolates exhibiting MICs of 0.25–0.5 μg/mL, mutations in the gyrB, parC and parE genes could not be detected.

The use of the efflux pump inhibitor PAβN in association with CIP or NAL resulted in a decreased level of resistance in our S. Infantis. A decrease of 3 to 6 dilutions steps (at minimum) was detected for MICs of CIP, whereas a reduction by two dilution steps was observed for the MIC of NAL (Table 1).

Table 1. Distribution of MIC values of NAL and CIP (in μg/mL)

<table>
<thead>
<tr>
<th>MIC (μg/mL)</th>
<th>NAL</th>
<th>CIP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;1024</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>1024</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>512</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>256</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of S. Infantis isolates with decreased CIP susceptibility (>1 μg/mL): MIC values of NAL and CIP in the presence and absence of an efflux inhibitor, mutations in the topoisomerase genes and results of PCR assays for qnr genes, aac-6′-ib-cr and qepA

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Origin</th>
<th>MIC (μg/mL)</th>
<th>MIC&lt;sub&gt;NAL&lt;/sub&gt; in the presence of 0.25 × MIC&lt;sub&gt;PAβN&lt;/sub&gt; (μg/mL)</th>
<th>MIC&lt;sub&gt;CIP&lt;/sub&gt; in the presence of 0.25 × MIC&lt;sub&gt;PAβN&lt;/sub&gt; (μg/mL)</th>
<th>Mutations in the QRDRs of the genes</th>
<th>PCR to detect the qnr genes, aac-6′-ib-cr and qepA&lt;sup&gt;*&lt;/sup&gt;</th>
<th>PFGE pulstype</th>
</tr>
</thead>
<tbody>
<tr>
<td>8099/08 Poultry</td>
<td>256</td>
<td>&gt;1024 2</td>
<td>128</td>
<td>0.5</td>
<td>Ser83Tyr - Ser80Arg - - A1</td>
<td>CIP, nalidixic acid; PAβN, Phe-Arg-β-naphthylamide; - not detected.</td>
<td>A2</td>
</tr>
<tr>
<td>8505/08 Poultry</td>
<td>256</td>
<td>&gt;1024 2</td>
<td>64</td>
<td>0.5</td>
<td>Ser83Tyr - Ser80Arg - - A2</td>
<td></td>
<td>A2</td>
</tr>
<tr>
<td>8807/09 Poultry</td>
<td>256</td>
<td>&gt;1024 2</td>
<td>64</td>
<td>0.5</td>
<td>Ser83Tyr - Ser80Arg - - A3</td>
<td></td>
<td>A3</td>
</tr>
<tr>
<td>8452/09 Poultry</td>
<td>256</td>
<td>&gt;1024 2</td>
<td>64</td>
<td>0.5</td>
<td>Ser83Tyr - Ser80Arg - - A3</td>
<td></td>
<td>A3</td>
</tr>
<tr>
<td>7103/09 Poultry</td>
<td>256</td>
<td>&gt;1024 2</td>
<td>64</td>
<td>0.25</td>
<td>Ser83Tyr - Ser80Arg - - A1</td>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>208664/08 Stool</td>
<td>256</td>
<td>&gt;1024 1</td>
<td>16</td>
<td>0.25</td>
<td>Ser83Tyr - - - - A1</td>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>287878/08 Stool</td>
<td>256</td>
<td>&gt;1024 1</td>
<td>16</td>
<td>0.125</td>
<td>Ser83Tyr - - - - A1</td>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>5567/09 Stool</td>
<td>256</td>
<td>512</td>
<td>8</td>
<td>0.25</td>
<td>Ser83Tyr - - - - A1</td>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>8-11187/09 Stool</td>
<td>256</td>
<td>&gt;1024 2</td>
<td>64</td>
<td>0.25</td>
<td>Ser83Tyr - - - - A1</td>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>6567/10 Stool</td>
<td>256</td>
<td>&gt;1024 1</td>
<td>16</td>
<td>0.25</td>
<td>Ser83Tyr - - - - A1</td>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>7961/10 Stool</td>
<td>256</td>
<td>512</td>
<td>8</td>
<td>0.25</td>
<td>Ser83Tyr - - - - A1</td>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>361/10 Stool</td>
<td>256</td>
<td>&gt;1024 2</td>
<td>64</td>
<td>0.5</td>
<td>Ser83Tyr - Ser80Arg - - A1</td>
<td>CIP, nalidixic acid; PAβN, Phe-Arg-β-naphthylamide; - not detected.</td>
<td>A1</td>
</tr>
<tr>
<td>7960/10 Stool</td>
<td>256</td>
<td>&gt;1024 1</td>
<td>16</td>
<td>0.25</td>
<td>Ser83Tyr - - - - A1</td>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>Pasteur/10 Food</td>
<td>128</td>
<td>&gt;1024 1</td>
<td>16</td>
<td>0.25</td>
<td>Ser83Tyr - - - - A1</td>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>82416/11 Stool</td>
<td>256</td>
<td>&gt;1024 2</td>
<td>64</td>
<td>0.5</td>
<td>Ser83Tyr - Ser80Arg - - A3</td>
<td>CIP, nalidixic acid; PAβN, Phe-Arg-β-naphthylamide; - not detected.</td>
<td>A3</td>
</tr>
<tr>
<td>Matej/11 Stool</td>
<td>256</td>
<td>&gt;1024 2</td>
<td>64</td>
<td>0.5</td>
<td>Ser83Tyr - Ser80Arg - - A1</td>
<td></td>
<td>A1</td>
</tr>
</tbody>
</table>

CIP, ciprofloxacin; NAL, nalidixic acid; PAβN, Phe-Arg-β-naphthylamide; -, not detected.

*The genes have been monitored in all strains from the collection.
mechanism for decreased FQ susceptibility and was suspected to account for a part of the resistance level. The changes observed in MIC values were even more apparent for NAL than for CIP, and this observation is in good accordance with the results from Escribano et al. (2004) and Kehrenberg et al. (2007), who also noticed a higher reduction in NAL MICs in the presence of PAβN. In S. Infantis strains with and without second mutation in parC, the MIC CIP differed by not more than one dilution step. Therefore, enhanced efflux activity may play a role in these strains. Whether mutations in the local or global regulators as described by Koutsolioutsou et al. (2001) or Kehrenberg et al. (2009) contribute to decreased FQ susceptibility in these isolates remains to be answered (Table 2).

In S. Infantis, the plasmid-borne gene qnrS has been reported some years ago in a single isolate, and the respective isolate exhibited MICs of NAL and CIP of 512 μg/mL and 1 μg/mL, respectively (Kehrenberg et al., 2006). As MICs of our 64 isolates were in the similar range, we performed PCR analysis to detect so far known plasmid-borne quinolone resistance genes. In this strain collection, none of the NALR S. Infantis isolates exhibited positive PCR results for the plasmid-borne genes qnrA, qnrB, qnrC, qnrD, qnrS, aac(6′)-Ib-cr and qepA (Table 2).

These results may be explained by the finding that plasmid-borne FQ resistance genes were most frequently detected in Salmo-nella isolates showing MICs of CIP and NAL between 0.25–1 μg/mL and 8–32 μg/mL (Veldman et al., 2011). In most cases, such a resistance phenotype indicates the involvement of plasmid-borne resistance determinants, and this phenotype was not detected in the Serbian isolates.

In summary, clonally related S. Infantis resistant to NAL and TET have emerged in Serbia in both humans and animals. In eight isolates with MIC CIP of 2 μg/mL, two point mutations in the gyrA and parC genes were detected, and the amino acid exchange Ser80 → Arg in parC has been described. Genes conferring plasmid-mediated resistance were not present, but enhanced efflux was identified as resistance mechanism. Hence, different mechanisms seem to play a role in decreased FQ resistance in this strain collection. The increased MICs to FQs raise concerns about the use of these antibiotics in livestock industry. The finding also underlines the need for a resistance monitoring programme, better farm management practice, education of the consumers about safe food handling and further implementation of the HACCP principle in food production in Serbia.

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