Prevalence of quinolone resistance and mutations in the topoisomerase genes in Salmonella enterica serotype Enteritidis isolates from Serbia

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A B S T R A C T

The prevalence of quinolone resistance was studied in Salmonella enterica serotype Enteritidis isolates collected during 2005–2010 in Southern Bačka County, Serbia. A total of 878 clinical isolates were examined, among which 19 (2.2%) nalidixic acid (NAL)-resistant S. Enteritidis were detected by selection on agar plates containing 64 mg/L NAL. Antimicrobial susceptibility of the isolates was tested by the agar dilution method. According to Clinical and Laboratory Standards Institute (CLSI) breakpoints, ciprofloxacin (CIP) resistance was not present in the strains. Multiple drug resistance was rare, and resistance to NAL was most often present as a single resistance property. All but one NAL-resistant S. Enteritidis showed reduced susceptibility to CIP [i.e. minimum inhibitory concentration (MIC) ≥ 0.125 mg/L]. This isolate of human origin had a CIP MIC of 0.064 mg/L and DNA sequencing revealed that it contained an Asp87Gly gyrA mutation. Most of the remaining isolates had MICs for NAL and CIP of 256 mg/L and 0.256 mg/L, respectively. Mutations in the Asp87 codon resulted in substitutions to Asn in most of the isolates, but Asp87Gly and Ser83Phex exchanges were also detected. No mutations were present in the gyrB, parC or parE genes. Although CIP resistance was absent, reduced susceptibility characterised by mutations in gyrA was apparent among S. Enteritidis isolates from Serbia.

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1. Introduction

Salmonellae are one of the most important food-borne pathogens. Owing to a high prevalence in livestock, they tend to spread through the food chain, exposing humans to the risk of salmonellosis. Clinical resistance to fluoroquinolones is rare, but reduced susceptibility in Salmonella is an important concern since these antimicrobials are used to treat humans and animals against severe Salmonella infections. The mechanisms of quinolone resistance in Enterobacteriaceae are well documented. The target enzymes for quinolones are DNA gyrase and topoisomerase IV, which are necessary for bacterial replication. Genes encoding the DNA gyrase subunits are designated gyrA and gyrB, whilst parC and parE encode topoisomerase IV. Reduced susceptibility to fluoroquinolones is mainly based on mutations in the quinolone resistance-determining region (QRDR) of the gyrA gene, which is the primary target of quinolone action. The QRDR of gyrA comprises a region from amino acid 67 to 106 [1], and the most frequently detected mutations are at codons 83 and 87 [2]. Single mutations normally cause resistance to nalidixic acid (NAL) and decreased susceptibility to fluoroquinolones such as ciprofloxacin (CIP). In addition, other mechanisms such as overexpression of multidrug efflux pumps or plasmid-mediated resistance genes like ζgrq, aac(6’)-Ib-cr and qepA may also contribute to decreased susceptibility [3,4].

In a previous investigation of a limited number of isolates, resistance to fluoroquinolones was not found, but 16.7% of Salmonella enterica serotype Enteritidis from humans were resistant to NAL [5]. The aim of the present study was to investigate the occurrence of quinolone resistance in a larger collection of S. Enteritidis isolates from human samples and to elucidate the mechanisms behind it. Epidemiological aspects are briefly discussed.

2. Materials and methods

A total of 878 S. Enteritidis isolates of human origin were selected from a strain collection of the Centre for Microbiology, Institute of Public Health of Vojvodina (Novi Sad, Serbia). All isolates
were collected during the years 2005–2010 from Southern Bačka County (Serbia). Isolates originated from hospitalised patients, outpatients and Salmonella carriers discovered during epidemiological surveillance studies. Only one isolate per patient was included in the study.

2.1. Screening for nalidixic acid-resistant isolates of Salmonella Enteritidis

Isolates were screened for resistance to NAL by striking 3 μL of overnight broth cultures on Mueller-Hinton agar plates containing 64 mg/L NAL. Following 24 h of incubation, surviving colonies were picked and tested for their susceptibility to a panel of selected antibiotics.

2.2. Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) for ampicillin, cefalothin, ceftriaxone, tetracycline, trimethoprim/sulphamethoxazole (SXT), NAL and CIP were determined by the agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [6]. Current CLSI breakpoints were used for classifying the isolates as susceptible, intermediate or resistant [7]. Escherichia coli ATCC 25922 was used for quality control purposes.

2.3. PCR and sequencing

Overnight broth cultures were centrifuged and the pellets were re-suspended in 200 μL of bidistilled water and subsequently boiled for 5 min. PCR reactions were performed in a volume of 50 μL using the Hot Start Kit from Qiagen (Hilden, Germany). To detect resistance-mediating mutations in the QRDRs of the target genes gyrA, gyrB, parC and parE, the regions were amplified and sequenced using previously described primer sets [3]. The cycling conditions were as follows: initialisation at 94 °C for 15 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for gyrA, 58 °C for gyrB and 52 °C for parC and parE for 1 min, and elongation at 72 °C for 1 min; and final elongation for 10 min at 72 °C. Purification of the PCR products and sequencing on both strands were performed at Macrogen (Amsterdam, The Netherlands).

3. Results and discussion

Salmonella Enteritidis is the most frequently detected serotype among humans in Southern Bačka County, although a trend of decrease in the total number of salmonellosis cases during recent years was observed [8]. In this study, 19 S. Enteritidis isolates resistant to NAL were detected over a period of 6 years. None of these isolates appeared to be clinically resistant to CIP. The isolates were either resistant to NAL alone or in combination with resistance to ampicillin and either cefalothin or SXT (Table 1). Multiple drug resistance to three antimicrobial agents was detected in only two

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**Table 1**

<table>
<thead>
<tr>
<th>Resistance</th>
<th>Total no. of isolates</th>
<th>Year of isolation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2005</td>
<td>2006</td>
</tr>
<tr>
<td>NAL</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>AMP/CT/NAL</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>AMP/SXT/NAL</td>
<td>1</td>
<td>–</td>
</tr>
</tbody>
</table>

NAL, nalidixic acid; AMP, ampicillin; CT, cefalothin; SXT, trimethoprim/sulphamethoxazole.
isolates from 2006. The highest number of NAL-resistant S. Enteritidis was found in 2005, when six isolates were detected. In 2008 there was a conspicuous absence of NAL-resistant S. Enteritidis, although the reason for this finding remains unclear. Nevertheless, the overall frequency of resistance to nalidixic acid amounted to 2.2% and thus appears to be relatively low in Serbia compared with other countries in East and West Europe. On the other hand, none of the isolates tested appeared to be clinically resistant to CIP and this finding is in good agreement with reports from most European countries using CLSI breakpoints [9].

All of the NAL-resistant isolates contained mutations in the QRDR region of the gyrA gene. Mutations in the other topoisomerase genes (gyrB, parC and parE) were not detected. MICs of the 19 NAL-resistant S. Enteritidis ranged from 128 mg/L to 1024 mg/L for NAL and from 0.064 mg/L to 0.512 mg/L for CIP. The most common combination of MIC values for NAL and CIP was 256 mg/L and 0.256 mg/L, respectively. Nearly all isolates showed reduced susceptibility to CIP (MIC > 0.125 mg/L) with the exception of one isolate that yielded a CIP MIC value of 0.064 mg/L [7]. However, this isolate possessed an Asp87Gly substitution in the gyrA gene (Table 2). Similar findings have been reported previously. Hakanen et al. detected NAL-resistant Salmonella isolates with single mutations in gyrA (Asp87Asn, Asp87Tyr or Asp87Gly) that were also inhibited by 0.064 mg/L CIP [10]. In CIP-susceptible Salmonella enterica serotype Typhimurium isolates from humans and pigs in Taiwan (CIP MICs of 0.03–0.25 mg/L), single nucleotide exchanges in gyrA were identified that led to amino acid exchange at positions Ser83Phe or Ser83Tyr [11]. Furthermore, Cavaco and Aarestrup [12] detected single mutations in the gyrA gene of S. Enteritidis isolates of animal origin that also showed CIP MICs of 0.064 mg/L. In the isolates from Southern Bačka County, the most frequent mutation was found in codon 87 and resulted in an amino acid substitution Asp87Asn. This exchange was detected in 47.4% (9/19) of the NAL-resistant isolates. Asp87Gly (7/19; 36.8%) and Ser83Phe (3/19; 15.8%) exchanges were also detected. None of the S. Enteritidis isolated during the 6-year time period in this county contained a double gyrA mutation and this was in agreement with the MIC values for CIP (Table 2). Nevertheless, there was no correlation between different gyrA mutations and MICs, meaning that each mutation type displayed various MICs for NAL and CIP.

Resistance to quinolones is explained by a loss of interaction of gyrase and quinolones caused by a substitution of the small polar amino acid at 83 (Ser) with a large hydrophobic amino acid (Phe), or substitution of the negatively charged Asp at position 87. In this study, mutations in the QRDR of gyrA were found either in codon 83 or 87, as reported from other countries [13]. The fact that different mutation types appeared during the 6-year period in different samples suggests that there was no clonal spread of NAL-resistant S. Enteritidis.

The absence of clinical resistance and the low prevalence of reduced susceptibility to CIP among S. Enteritidis of human origin are most likely attributed to the fact that layer flocks on Serbian farms are not intensively treated with antibiotics. Since in Southern Bačka County the most frequent sources of infection are homemade meals with raw eggs, continuous education of the public sector is very important to reduce the risk of food poisoning in the future.

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Ethical approval: Not required; isolates used in the research were from an existing collection of isolates.

References