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Original article

EVIDENCE OF *gyrA* MUTATIONS IN NALIDIXIC ACID-RESISTANT *SALMONELLA ENTERICA* SEROTYPE ENTERITIDIS

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Summary

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The goal of our research was to detect mutations on *gyrA* gene in a small collection of *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) isolated from stool specimens, food and poultry in quinolone resistant strains. Out of 60 randomly selected isolates, resistance to nalidixic acid was exhibited by 15%. Five isolates were from poultry, three isolates were from stools and one was found in a pastry collected from a biscuit factory. Polymerase chain reaction and sequencing revealed different mutations in *gyrA* gene. The following amino acid exchanges were found: Asp87→Asn (in three isolates from stool and four isolates from poultry), Ser83→Phe mutation was found in one isolate from poultry and Asp87→Gly substitution was detected in one isolate from food. Multiple resistance in *S.* Enteritidis from a day-old chicken was attributed to ampicillin, cephalothin, nalidixic acid and tetracycline. In two strains from human stools the following resistotypes were noted: ampicillin, tetracycline, trimethoprim-sulfamethoxazole and ampicillin, tetracycline, trimethoprim-sulfamethoxazole and neomycin. This was the first research on distribution of *gyrA* mutations in *S.* Enteritidis in Serbia.

Key words: food, *gyrA*, poultry, resistance, *Salmonella*, stool

INTRODUCTION

Salmonella spp. is one of the most important food borne pathogens worldwide. The clinical symptoms in poultry are quite seldom, but through the food, they could infect humans. People infected with salmonella could experience mild symptoms of a disease or just opposite they may require treatment with antibiotics and sometimes have to be hospitalized. Of the highest concern is infection of children, immunocompromised patients and elderly people which in most circumstances need therapy. *Salmonella* infection in humans occurs most frequently from home made

meals in Serbia (Petrović *et al.*, 2005). The food is contaminated from table eggs or meat and meat product. Cross contamination of food borne pathogens in the kitchen has been described and most frequently present the source of food poisoning in some European countries (Gorman et al 2002). The worldwide dissemination of multiple resistant *Salmonella* strains is also very important (Velhner *et al.*, 2013).

Bacteria can develop various mechanisms of resistance during their life time. Resistance to quinolones is chromosomally and/or plasmid encoded and often is driven by therapy of people and food producing animals. The target sites for quinolones are topisomerase genes. They encode enzymes Gyrase A and topoisomerase IV. In Gram negative bacteria Gyrase A is a primary target while in Gram positive bacteria the target for quinolones is topoisomerase IV. Both enzymes are important for replication of bacteria and their inactivation is detrimental to the cell. Gyrase A and topoisomerase IV are encoded from two genes called *gyrA*/*gyrB* and *parC* /*parE* respectively. Mutations induced by quinolones are located in the part of a gene called quinolone resistance determining region – QRDR. It is known that a single point mutation in the *gyrA* gene induces resistance to quinolones in *Salmonella* (Velhner *et al.*, 2010; Velhner & Stojanović, 2012). Easy access to medications, in some countries, is the reason for the development of increased resistance to fluoroquinolone. They are attributed to multiple mutations in topoisomerase genes (Cui *et al.*, 2008).

We prepared a collection of *Salmonella enetrica* serovar Enteritidis (*S*. Enteritidis) isolates from humans, food and poultry, to carry on research about their resistance to several classes of antimicrobials and to monitor mutations on QRDR from the *gyrA* gene.

MATERIALS AND METHODS

Isolates

Collection of 60 randomly selected *S*. Enteritidis were included in the study. Poultry isolates (30) originate from different farms located in northern part of Serbia. Isolates from stool were from patients with enterocolitis (15) and additional 15 isolates were from marketed poultry products and eggs.

Antimicrobial resistance and MIC determination

Antimicrobial resistance monitoring was done according to the recommendation of the Clinical and Laboratory Standard Institute (CLSI, 2006a). In agar disc diffusion method the following disc from BioRad (Marnes-la-Coquette, France) were used in the study: amikacin (AMK, 30 μg), ampicillin (AMP, 10 μg), amoxicillin-clavulanic acid (AXC, 20-10 μg), chloramphenicol (CAP, 30 μg), ceftazidime (CAZ, 30 μg), cefquinone (CEQ, 10 μg), cephalothin (CFT, 30 μg), ciprofloxacin (CIP, 5 μg), colistin (COL, 10 μg), ceftriaxone (CRO, 30 μg), doxycycline (DOX, 30 μg), enrofloxacin (ENR, 5 μg), fluorphenicol (FFC, 30 μg), gentamicin (GMC, 10 μg), nalidixic acid (NAL, 30 μg), neomycin (NEO, 30 μg), trimethoprim-sulfamethoxazole (SXT, 1.25 μg +23.75 μg), tetracycline (TET, 30 μg).

Nine isolates from the whole collection were resistant to NAL and sensitive to CIP. The distribution of minimal inhibitory concentration (MIC) to NAL and CIP was determined for these nine isolates and for 30 strains selected randomly (15 from poultry and 15 from food and stool). The

MIC test was done by agar dilution procedure (CLSI, 2006b) with the following breakpoints: for NAL susceptible ≤16 μg/mL, resistant ≥32 μg/mL and for CIP susceptible ≥1 μg/mL and resistant ≥4 μg/mL.

Polymerase chain reaction (PCR) and sequencing of the QRDR from the gyrA gene

The DNA was extracted from NAL resistant isolates after boiling bacteria. Primers and PCR protocol were described by Giraud *et al.*, (1999). The forward primer sequence is: STGYRA1 5'-TGT CCG AGA TGG CCT GAA GC-3` and the reverse primer sequence is STGYRA12 5`-CGT TGA TGA CTT CCG TCA G-3`. The PCR products were purified with QIA quick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced in Macrogen (Seoul, Republic of Korea). Alignment was done using Clustal W program. The nucleotide sequences from the fragment of 451 bp were compared to *Salmonella* Typhimurium NCTC 74, accession number X78977 from the EMBL GenBank database (Griggs *et al.*, 1996).

RESULTS

Multiple resistances to several classes of antimicrobials were found in three isolates (one from a day-old chicken and two from stools). *S.* Enteritidis from a day-old chicken was resistant to AMP, CFT, NAL and TET, while two strains from human stool were resistant to: AMP, TET, SXT and AMP, TET, SXT and NEO. Single resistance to AMP was found in one poultry isolate and one isolate from stool. Resistance to NAL was found in five poultry strains, one isolate found in a pastry from a biscuit factory and in three isolates from stools.

From nine isolates resistant to NAL, seven had a MIC for NAL of 256 μg/mL. One isolate had MIC to NAL of 128 and

Table 1. Obtained MIC to nalidixic acid (NAL) and ciprofloxacin (CIP) in 30 randomly selected and 9 NAL-resistant isolates

MIC NAL, μg/mL	MIC CIP, μg/mL	Number of isolates
NAL-sensitive CIP-sensitive		
2	0.016	1
4	0.016	7
4	0.032	20
4	0.064	1
8	0.032	1
NAL-resistant CIP-sensitive		
128	0.256	1
256	0.256	5
256	0.512	2
512	0.512	1

Table 2. Mutations at the quinolone resistance determining region (QRDR) of *gyrA* gene in nalidixic acid-resistant isolates

Isolate number	Resistance pattern	Mutation at QRDR of the *gyrA* gene
P: 9568, 501, 74	NAL	Asp87→Asn
F: 1898	NAL	Asp87→Gly
P: 7938	NAL	Ser83→Phe
P: 75	AMP, CFT, NAL, TET	Asp87→Asn
S: 33641	NAL	Asp87→Asn
S: 317526	NAL	Asp87→Asn
S: 228020	NAL	Asp87→Asn

P: poultry, F: food: S: stool; NAL: nalidixic acid; AMP: ampicillin; CFT: cephalothin; TET: tetracycline.

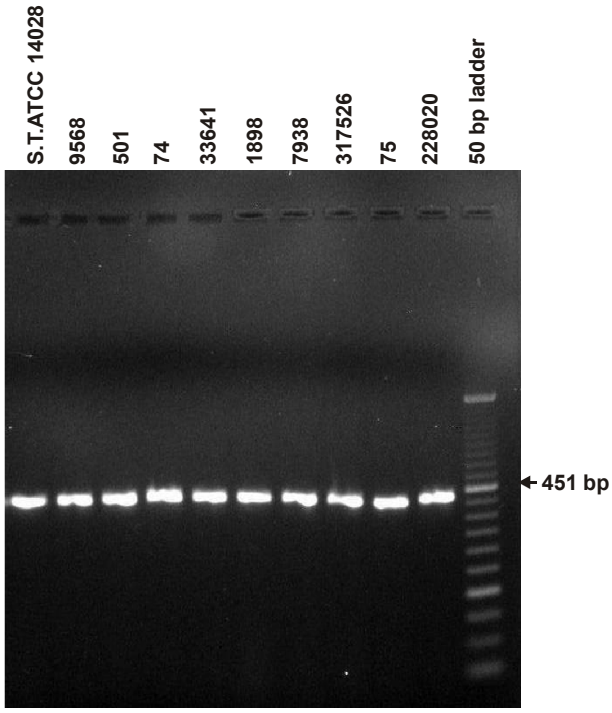

Fig. 1. PCR product of the *gyrA* gene in *Salmonella* Enteritidis, resistant to nalidixic acid.

one isolate had MIC of 512 µg/mL. MIC to CIP was 0.256 (6 strains) or 0.512 µg/mL (3 strains). Distribution of the MICs for all tested isolates is presented in Table 1. Isolates susceptible to quinolones had MIC to NAL of 2–8 µg/mL.

In isolates resistant to NAL, mutation on a *gyrA* gene inside the QRDR, were detected. Mutations were found on codons 83 and 87. Double mutant were not found. In four isolates from poultry and three from stool the transition Asp87→Asn was

detected. In one isolate from food the mutations occurs at Asp87→Gly while in one *S*. Enteritidis from poultry Ser→83Phe amino acid exchange was found. The results are presented in Table 2 and Fig. 1.

DISCUSSION

In this research multiple antibiotic resistances was found in only three isolates. The quinolone resistance was represented in 15% of *S*. Enteritidis mostly from poultry but also in stool specimens and from one food. Obtained results do not reflect the actual situation about the resistance to quinolone since in clinical material (Kozoderović *et al.*, 2012) and also in poultry (unpublished data) *S*. Enteritidis in most circumstances reflects susceptible phenotype. *S*. Enteritidis is the most frequently reported from humans (http://thor.dfvf.dk/pls/portal/GSS.COUNTRY_DATA_SET_REP.show) and in food producing animals in Serbia therefore, it is very important to perform resistotyping in human and veterinary laboratories. In NAL-resistant CIP-sensitive isolates the MIC was 0.256 and 0.512 μg/mL which is two to four folds higher than in NAL-sensitive CIP-sensitive strains. This is important for clinicians who prescribe fluoroquinolone therapy in humans. For treatment of enteric diseases in poultry the drug of choice is enrofloxacin, a synthetic chemotherapeutic, usually prescribed if birds are infected with *Salmonella* and/or *Escherichia coli*. However, it is known that antibiotic treatment of poultry against *Salmonella* is not adequate since after the therapy these bacteria colonize the intestinal tract of chickens repeatable. The shedding could be induced after poultry is exposed to stress or also if chickens are infected from contaminated litter. The next important obstacle in therapy with enrofloxacin is resistance development in *Campylobacter* spp. A single point mutation on the *gyrA* gene in these bacteria is sufficient to induce high level resistance to fluoroquinolone. Because of that Baytril was withdrawn from the therapy of poultry and cattle in the USA and nowadays is prescribed exclusively to treat pets and small animals in North America. Good management practice, vaccination and other therapeutic options are the most important principles in animal farming, to sustain the good health of animals, and prevent infection of people through food (Velhner *et al.*, 2013).

This work is a part of recently published paper by Kozoderović *et al.* (2011) in which we have shown, for the first time, distribution of *gyrA* mutations in *S*. Enteritidis from stool, food and poultry. We found that even in a low number of isolates (60) three different point mutations on *gyrA* gene were found. This is in good agreement with the work of Eaves *et al.* (2004) who concluded that different mutations in QRDR from the *gyrA* gene could be found in the same serovars. It was proposed by Giraud *et al.* (2006) that Ser83→Phe substitution is most frequently selected after treatment with enrofloxacin and this was different from our observation. Namely, the Ser83→Phe transition was found in this research in one poultry isolate while Asp87→Asn exchange dominated in *S*. Enteritidis from chickens. Mutations on other topoisomerase genes in most cases develop if *gyrA* is already mutated and if strains are resistant to CIP. There are as many as three to four mutations found in such highly resistant strains (Ling *et al.* 2003). Easy accesses to the medication are the main reason for the prevalence of highly resistant mutants in some countries (Cui *et al.*, 2008).

The efficacy of antibiotic treatment of poultry infected with *Salmonella* is doubtful therefore it should be avoided. We do not have National monitoring program for *Salmonella* in agriculture. The HACCP principle is however implemented on many farms but such preventive measures did not minimize actual problems related to *Salmonella*. Strict management in primary production is needed to reduce the shedding and intestinal colonisation and to prevent contamination of food and environment. Fewer outbreaks with *Salmonella* are recorded in humans probably due to the fact that in fast food restaurants, day care centers, nursing homes and other settings, better hygiene measures are undertaken. Homemade meals still present significant risk for human infection and because of that education about proper food handling is necessary.

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