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Quinolone resistance among *Salmonella enterica* and *Escherichia coli* in Lithuania

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Lithuanian isolates of *Escherichia coli* and *Salmonella enterica* from animals and humans were examined for resistance to quinolones, fluoroquinolones and for resistance-associated mutations. 9% of *S. enterica* from animals and 4% of isolates from clinical samples of humans were resistant to nalidixic acid and susceptible to fluoroquinolones. DNA analysis of nalidixic acid-resistant *S. enterica* strains from animals revealed a single mutation at codon 83 (Ser→Phe) in *gyrA* gene, whereas resistant clinical strains contained a single *gyrA* mutation at codon 87 (Asp→Tyr). 10% of human isolates of *E. coli* were resistant to nalidixic acid and ciprofloxacin. 22% of *E. coli* isolates from calves were resistant to nalidixic acid. 40% and 20% of *E. coli* isolates from pigs were resistant to nalidixic acid and to fluoroquinolones, respectively. *E. coli* isolates of animal and human origin analyzed for nalidixic acid resistance-associated mutations carried single mutations at codon 83 (Ser→Leu) or at codon 87 (Asp→Tyr) in *gyrA* gene. Fluoroquinolone-resistant *E. coli* isolates from calves and humans carried multiple mutations within *gyrA* (83Ser→Leu, 87Asp→Gly or Asn) and *parC* (80Ser→Ile or Arg, 84Glu→Val or Lys) genes.

Key words: *Escherichia coli, Salmonella enterica*, quinolone resistance, *gyrA*, *parC*

INTRODUCTION

Quinolones (nalidixic acid) and fluoroquinolones (norfloxacin, ciprofloxacin, and enrofloxacin) are the classes of synthetic antimicrobial agents with an excellent activity against *Escherichia coli* and other gram-negative bacteria used in human and veterinary medicine. The mechanisms of resistance to quinolones in *S. enterica* and *E. coli* include target gene mutations, active efflux, and a decreased outer membrane permeability [1, 2]. The major resistance mechanism is developed by occurrence of the mutations in bacterial genes coding for target enzymes – DNA gyrase of *E. coli* and *S. enterica,* and topoisomerase IV of *E. coli* [2]. DNA gyrase catalyzes the negative supercoiling of DNA required for chromosome replication and transcription and is composed of two subunits, GyrA and GyrB, the products of the *gyrA* and *gyrB* genes, respectively [3]. Topoisomerase IV, which is essential for chromosome partitioning, is also composed of two subunits, ParC and ParE, encoded by genes *parC* and *parE*, respectively [4]. Quinolones inhibit these enzymes by stabilizing the complex between DNA and the enzyme and thus block the progression of DNA replication. Chromosomal point mutations preventing binding of quinolones to the active site of the enzyme are clustered in *gyrA* and *parC* gene regions termed the quinolone resistance-determining region, QRDR [5, 6].

The aim of this study was to examine the frequency of resistance to quinolones and fluoroquinolones of *Salmonella enterica* and *Escherichia coli* isolates of human and animal origin in Lithuania and to determine mutations in the QRDR regions of *gyrA* and *parC* in resistant strains.

MATERIALS AND METHODS

Bacterial strains. Lithuanian isolates of *S. enterica* (animal isolates n = 63, human isolates n = 73) and *E. coli*

(animal isolates n = 18 from calves and n = 5 from pigs, human isolates n = 140) were obtained in 2004 to 2005. Clinical isolates from humans were recovered mostly from patients with urinary tract infections (*E. coli*) and from stools of patients with diarrhea (*S. enterica*). Veterinary isolates were mostly obtained from pathological material of pigs and cattle (*E. coli)* and chicken slush water or carcass (*S. enterica*). For isolation of *Salmonella,* the pre-enrichment medium Buffered Peptone Water (Oxoid, Great Britain) and enrichment medium Rappaport_Vassiliadis Media (BBL, Great Britain) were used. XLD Media (Oxoid) and SS Agar (Oxoid) were used for further isolation. Identification of *Salmonella* was done using the Microbact identification system (Oxoid). Results were interpreted using Microbact 2000 (Oxoid). Isolation of *E. coli* was done using MacConkey Agar (Oxoid). Identification was done like for *Salmonella*.

Antibiotic resistance testing. Susceptibility to nalidixic acid, norfloxacin and ciprofloxacin was determined by the disc-diffusion method [7] using Mueller Hinton Agar (Oxoid). The concentrations of antimicrobials were 30 μg of nalidixic acid, 10 μg of norfloxacin and 5 μg of ciprofloxacin (Oxoid). Tests were carried out and interpretation of results was performed according to the CLSI (formerly NCCLS) guidelines [7].

Amplification of *gyrA* and *parC* QRDR DNA. QRDR regions of *gyrA* and *parC* genes were amplified by PCR from the DNA of quinolone-resistant isolates. The DNA template for PCR (2 μl) was prepared as follows: a single colony was picked and resuspended in 200 μl of distilled water, the suspension was boiled for 5 min and the supernatant was collected after centrifugation for 2 min. A 343-bp region covering the QRDR of *gyrA* was amplified with primers 5'-AAATCTGCCCGTGTCGTTGGT-3'and 5'-GCCATACCTACTGCGATACC-3'. The 964-bp QRDR fragment from *parC* was amplified with primers 5'-GTGGTAGCGAAGAGGTGGTT-3' and 5'- GACCGTGCG TTGCCGTTTAT-3'. Amplifications were carried out in 25-μl volumes containing 0.4 μM reverse and forward primers, 2.5 mM $MgCl_2$, 0.2 mM of dNTP, 0.3 U *Taq* DNA polymerase (AB Fermentas). PCR was initiated by denaturation at 94 °C for 2 min, followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final cycle of 72 °C for 7 min. The amplification products were visualized by 2% agarose gel electrophoresis and etidium bromide staining to assess the sizes of the gene fragments.

DNA sequencing. PCR products were purified by using Perfect Prep Gel Cleanup Kit (Eppendorf) prior to DNA sequencing. The purified PCR products were sequenced at the Institute of Biotechnology Sequencing Center (Lithuania). The sequenced *gyrA* DNA was compared to native *gyrA* DNA (AE000312), and *parC* DNA was compared to native *parC* DNA (AE000384) by using ClustalW [8].

RESULTS AND DISCUSSION

In the 1980s, first and second generation quinolones with limited activity, such as nalidixic acid and pipemidic acid, were replaced by fluoroquinolones which are highly effective against gram-negative bacteria and some gram-positive pathogenic species [9]. Today they are widely used as antimicrobials for the treatment of a broad range of infections in humans worldwide [9]. Fluoroquinolones are extensively used for therapy of *E. coli* urinary tract infections in most countries of Western Europe and North America [10]. They are also drugs of choice for treatment of gastrointestinal infections caused by zoonotic bacteria, such as salmonellosis [11]. With their broad spectrum of activity, quinolones and fluoroquinolones are widely used in livestock for treatment of animal diseases [12]. However, the frequency of resistance to fluoroquinolones among *E. coli* and *Salmonella* isolates are rising year by year [13–15]. The use of quinolones and fluoroquinolones in veterinary medicine represents a particular concern because of the possibility of the emergence of highly resistant veterinary *E. coli, Salmonella, Campylobacter* strains which could be later transferred to humans via food chain and lead to the failure of clinical treatment.

Quinolones and particularly fluoroquinolones are widely used in Lithuania for animal treatment. Enrofloxacin, which is chemically closely related to norfloxacin, is one of the most often used antimicrobials in the treatment of poultry and pigs. Enrofloxacin was used by oral administration as a prophylactic measure for prevention of animal diseases. Such an extensive usage of fluoroquinolones in veterinary bears a potential risk of emerging highly resistant zoonotic agents. However, there are no data on the consumption of antimicrobials in veterinary medicine in Lithuania, but the registration of fluoroquinolones is rising, including new substances such as marbofloxacin or danofloxacin. Ciprofloxacin and norfloxacin are also used for treatment of companion animals and thus also have a potential risk for future effectivity.

Systematic studies on the use of fluoroquinolones for the treatment of human infections and microbial resistance levels in Lithuania are lacking [16]. In the study conducted 10 years ago on the antibiotic resistance of *Salmonella* and *Shigella* in Kaunas region, no quinolone (nalidixic acid) and fluoroquinolone (ciprofloxacin) resistant *Shigella* were observed. All tested *Salmonella* were also susceptible to ciprofloxacin [17]. A more recent similar study on the use of fluoroquinolones (norfloxacin) in the Vilnius University Hospital (Vilnius region) revealed lower use than in Huddinge University Hospital (Sweden), but the resistance level of clinical *E. coli* isolates was higher (8%) in Vilnius than in Huddinge (3%) [18].

In the present study, 9% of Lithuanian isolates of *S. enterica* of animal origin and 4% of isolates from clinical samples were found to be resistant to nalidixic acid (Table 1). The level of nalidixic acid-resistant *Salmonella* from veterinary is comparable to that recently registered in other European countries such as Poland, England [19]. *Salmonella* isolates were susceptible to norfloxacin and ciprofloxacin. This observation indicates that *Salmo-*

Table 1. **Quinolone resistance of Lithuanian veterinary and clinical *S. enterica* and *E. coli* isolates**

	Animal isolates				Human isolates			
	Number of strains tested	Nalidixic acid	Ciprofloxacin	Norfloxacin	Number of strains tested	Nalidixic acid	Ciprofloxacin	Norfloxacin
S. enterica	63	9%	0%	0%	73	4%	0%	ND
E. coli	5	40% (pigs)	20% (pigs)	20% (pigs)	140	10%	9%	ND
	18	22% (calves)	0% (calves)	0% (calves)				

ND – not determined.

Table 2. ***gyrA* and *parC* gene mutations in Lithuanian *S. enterica* and *E. coli* isolates**

Isolates	Source	Resistance	Resistance mutations	
			gyrA	*parC*
S. enteritidis 33				
S. enteritidis 14	animal	NA	83 Ser→Phe	ND
S. glostrup 6130				
S. enteritidis 6377	human	NA	87 Asp→Asn	ND
S. enteritidis 6380				
S. enteritidis 6382				
E. coli EG02	animal	NA, NOR, CIP	83 Ser→Leu 87 Asp→Gly	84 Glu→Lys
E. coli EG04	animal	NA	83 Ser→Leu	-
E. coli EK14	animal	NA	87 Asp→Tyr	-
E. coli 8441				
E. coli 9142				
E. coli 11240	human	NA, CIP	83 Ser→Leu 87 Asp→Asn	80 Ser→Ile 84 Glu→Val 192 Ala→Val
E. coli 13022	human	NA, CIP	83 Ser→Leu 87 Asp→Asn	80 Ser→Ile 84 Glu→Lys
E. coli 23538	human	NA, CIP	83 Ser→Leu 87 Asp→Asn	80 Ser→Arg 84 Glu→Lys
E. coli 6935				
E. coli 5512				
E. coli 8609	human	NA, CIP	83 Ser→Leu 87 Asp→Asn	80 Ser→Ile
E. coli 9580				
E. coli 9942				
E. coli 12096				
E. coli 13485				
E. coli 9586	human	NA	83 Ser→Leu	-

NA – nalidixic acid, CIP – ciprofloxacin, NOR – norfloxacin.
ND – not determined.

nella resistance to first generation quinolones has increased during the last 10 years, while resistance to the second generation of quinolones remains low.

10% of clinical isolates of *E. coli* were resistant to nalidixic acid and 9% were resistant to ciprofloxacin (Table 1). The resistance to quinolones of veterinary isolates of *E. coli* was notably higher: 22% of isolates from calves and 40% of isolates from pigs were resistant to nalidixic acid, the later being considerably higher as compared with other European countries [19]. 20% of *E. coli* isolates from pigs were resistant to ciprofloxacin and norfloxacin. A high level of *E. coli* resistance to quinolones and fluoroquinolones observed in livestock could reflect an intensive use of these antimicrobials in veterinary.

Quinolone-resistant isolates were analysed for mutations clustered within QRDR regions of *gyrA* and *parC* genes. The corresponding DNA fragments were amplified and sequenced as described in Materials and Methods. Resistance to quinolones in *Salmonella* occurs by chromosomal point mutations in QRDR region of *gyrA gene* between amino acids 67 and 106 [20]. All analysed human *Salmonella* isolates carried a single 87 codon mutation Asp→Asn within QRDR of *gyrA* gene (Table 2). Two *Salmonella* isolates of animal origin con-

tained a single *gyrA* 83 codon mutation Ser→Phe. Amino acid changes at 83Ser (to Phe, Tyr, or Ala) or at 87Asp (to Gly, Asn, or Tyr) in GyrA subunit of DNA gyrase are the most frequently observed mutations in nalidixic acid resistant *Salmonella* strains [20]. They appear to be the first mutations developed in quinolone-resistant *Salmonella.* Double mutations at both residues 83 and 87 show a high-level resistance to fluoroquinolones [20]. Less frequently, amino acid substitutions at 67Ala (to Pro), 81Gly (to Ser), and 119Ala (to Glu) have been described in quinolone-resistant *Salmonella* strains [21]. Unlike in *E. coli*, mutations within the subunit of topoisomerase IV ParC are rare in *Salmonella* isolated from animals [22].

E. coli isolates were analyzed for mutations in *gyrA* and *parC* QRDR. The most favored residues in *gyrA* at which alteration leads to resistance are the same as in *Salmonella* (83Ser and 87Asp) [23]. One or two substitutions in the QRDR of *parC* at 80Ser and 84Glu are most often accumulated [23]. Mutations in *gyrB* and *parE* are much rarer than mutations in *gyrA* or *parC* [23].

Nalidixic acid resistant *E. coli* isolates from animals (EK14) had a single mutation 87Asp→Tyr, and EG04 had a single mutation 83Ser→Leu within QRDR of *gyrA* but both had no mutations in *parC* gene (Table 2). The nalidixic acid, norfloxacin and ciprofloxacin resistant *E. coli* isolate EG02 carried a double mutation in *gyrA* 83Ser→Leu, 87Asp→Gly and a single mutation within QRDR of *parC* at codon 84Glu→Lys. Experimental evidence from other studies with *E. coli* have shown that single mutations in *gyrA* are typically associated with high-level quinolone and low-level fluoroquinolone resistance. The high-level fluoroquinolone resistance is induced by the accumulation of several mutations in different genes [23].

Nalidixic acid and ciprofloxacin resistant *E. coli* isolates from humans carried multiple mutations within QRDR of *gyrA* and *parC* (Table 2). All isolates had *gyrA* mutations at codons 83 (Ser→Leu) and 87 (Asp→Asn). Only nalidixic acid resistant isolates had a single *gyrA* mutation at codon 83 (Ser→Leu). Ciproflaxacin-resistant isolates had a various spectrum of mutations within QRDR of *parC* at codons 80 (Ser) and 84 (Glu). These mutations have been associated with a high-level resistance to fluoroquinolones in similar studies [23, 24]. 80Ser was substituted for Ile (five isolates) or Arg (one isolate). 80Ser→Ile substitution was found to occur more often in *E. coli* from patients with urinary tract infections in Sweden also [23]. 84Glu was substituted for Val (three isolates) or Lys (two isolates). 192Ala/Val mutation was determined in some isolates within *parC* outside of QRDR (QRDR of *parC* involves amino acids between 64 and 103). We have no data on whether the mutation Ala/Val could affect the resistance of bacteria to quinolones or fluoroquinolones.

The first study on the frequency and genetic determinants of quinolone-resistant clinical and veterinary *E. coli* and *Salmonella enterica* strains in Lithuania provides background information on the present epidemiological situation of quinolone-resistant *E. coli* and *S. enterica* strains in the country. Further studies are underway to obtain more phenotypic and genetic information.

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LIETUVOJE IŠSKIRTŲ *ESCHERICHIA COLI* IR *SALMONELLA ENTERICA* PADERMIŲ ATSPARUMAS CHINOLONAMS

Santrauka

Šiame darbe ištirtas Lietuvoje iš gyvūnų ir žmonių klinikinių šaltinių išskirtų *Escherichia coli* ir *Salmonella enterica* padermių atsparumas chinolonams bei fluorochinolonams ir nustatytos šį atsparumą lemiančios mutacijos. 9% išskirtų iš gyvūnų ir 4% išskirtų iš žmonių *S. enterica* padermių buvo atsparios nalidikso rūgščiai, bet buvo jautrios fluorochinolonams. Nustatyta, kad gyvūnų *S. enterica* izoliatai turėjo pavienę *gyrA* geno 83 kodono mutaciją (Ser→Phe), o žmonių – pavienę *gyrA* geno 87 kodono mutaciją (Asp→Asn). 10% žmonių *E. coli* izoliatų buvo atsparūs nalidikso rūgščiai, 9% – ciprofloksacinui. 22% *E. coli* padermių, išskirtų iš galvijų, buvo atsparios tik pirmosios kartos chinolonui – nalidikso rūgščiai. 40% *E. coli* padermių, išskirtų iš kiaulių, buvo atsparios nalidikso rūgščiai, o 20% šių padermių buvo atsparios ir fluorochinolonams. Tirtose *E. coli* padermėse iš gyvūnų ir žmonių klinikinių šaltinių, atspariose nalidikso rūgščiai, nustatytos pavienės *gyrA* geno 87 kodono (Asp→Tyr) arba 83 kodono (Ser→Leu) mutacijos. Fluorochinolonams atsparios *E. coli* padermės, išskirtos iš galvijų ir sergančių žmonių, turėjo daugybines *gyrA* ir *parC* genų mutacijas: *gyrA* (83Ser→Leu, 87Asp→Gly arba Asn) ir *parC* (80Ser→Ile arba Arg, 84Glu→Val arba Lys). Gauti rezultatai liudija, kad bakterijos mutuoja įgaudamos atsparumą naujos kartos fluorochinolonams. Tai gali būti susiję su gausiu antimikrobinių medžiagų, ypač fluorochinolonų, naudojimu veterinarijoje.