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Prevalence and characterisation of quinolone resistance mechanisms in *Salmonella* spp.

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ABSTRACT

The study was focused on characterisation of quinolone resistance mechanisms in *Salmonella* isolated from animals, food, and feed between 2008 and 2011. Testing of Minimal Inhibitory Concentrations revealed 6.4% of 2680 isolates conferring ciprofloxacin resistance. Simultaneously 37.7% and 40.8% were accounted for, respectively, nalidixic acid and ciprofloxacin Non Wild-Type populations. Amplification and sequencing of quinolone resistance determining region of topoisomerases genes in 44 isolates identified multiple amino-acid substitutions in *gyrA* at positions Ser83 ($N = 22$; →Leu, →Phe, →Tyr), Asp87 ($N = 22$; →Asn, →Gly, →Tyr) and *parC* (Thr57Ser, $N = 23$; Ala141Ser, $N = 1$). No relevant mutations were identified in *gyrB* and *parE*. Twelve patterns combining one or two substitutions were related to neither serovar nor ciprofloxacin MIC. In 92 isolates suspected for plasmid mediated quinolone resistance two *qnr* alleles were found: *qnrS1* (or *qnrS3*; $N = 50$) and *qnrB19* (or *qnrB10*; $N = 24$). Additionally, two isolates with chromosomally encoded mechanisms carried *qnrS1* and *qnrS2*. All tested isolates were negative for *qnrA*, *qnrC*, *qnrD*, *qepA*, *aac(6′)-Ib-cr*. Both chromosomal and plasmid mediated quinolone resistance determinants were found in several *Salmonella* serovars and Pulsed Field Gel Electrophoresis was used to assess phylogenetic similarity of selected isolates ($N = 82$). *Salmonella* Newport was found to accumulate quinolone resistance determinants and the serovar was spreading clonally with either variable *gyrA* mutations, *qnrS1/S3*, or *qnrB10/B19*. Alternatively, various determinants are dispersed among related *S.* Enteritidis isolates. Antimicrobial selection pressure, multiple resistance determinants and scenarios for their acquisition and spread make extremely difficult to combat quinolone resistance.

1. Introduction

Fifty years have just passed since the introduction of quinolones into medical practice and decline in their therapeutic efficacy as well as emergence and spread of various resistance mechanisms are evoked (Poirel et al., 2012). The primal enthusiasm on perfect efficacy of fully synthetic bactericidal antimicrobials have been trimmed by discovery of chromosomal mechanisms involving spontaneous mutations (Hopkins et al., 2005; Jeong et al., 2011). Further limitations came along with the accumulation of genetic rearrangements leading to clinical resistance and the detection of transferable resistance mechanisms in late 1990-ties (Poirel et al., 2012). Due to efficacy of low dosage and favourable pharmacokinetics, fluoroquinolones are widely used in human and animals since 1980-ties (Hopkins et al., 2005; Fabrega et al., 2009). Being excreted during treatment mostly as active

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compounds their biological action is not limited to therapeutic site, but it is moved further as resistance selection pressure into environment (Kaplan et al., 2013).

Since all (fluoro)quinolones exert their antibacterial effect by inhibition of topoisomerases, the same mechanisms confer resistance to all clinically relevant molecules. Spontaneous point mutations leading to amino acid substitutions in quinolone resistance determining region (QRDR) of topoisomerases II (gyrase) and IV genes alter the quinolone binding site and result in failure of their antibacterial activity (Hopkins et al., 2005; Fabrega et al., 2009). As a step-wise process in *Salmonella*, single mutation in *gyr*A, *gyr*B, *parC*, or *parE* reduces susceptibility to fluoroquinolones and multiple substitutions are needed for clinical resistance (Hopkins et al., 2005; Yang et al., 2012). That scheme might be also interfered with non-specific mechanisms including outer membrane permeability or efflux pumps (Hopkins et al., 2005; Karczmarczyk et al., 2010; Akiyama and Khan, 2012; Rushdy et al., 2013). Plasmid mediated quinolone resistance (PMQR) confer lower susceptibility to fluoroquinolones and might be considered a background for selection of chromosome-encoded resistance. Three major mechanisms are involved in PMQR: qnr peptides protect topoisomerases from antimicrobial action, variant of aminoglycoside acetyltransferase (*aac(6′)-Ib-cr*) modifies ciprofloxacin molecule, and QepA protein modulates quinolone efflux pump (Poirel et al., 2012). The number of discovered *qnr* genes is currently approaching 100 alleles divided into 5 categories (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*) of which *qnrB* accounts up to 73 variants (http://www.lahey.org/qnrStudies) (Poirel et al., 2012).

Clinical salmonellosis in human is usually self-limiting food-borne disease that might be tracked back mainly to food obtained from subclinically infected animals. Neither animal carriers nor patients require antimicrobial therapy unless systemic infection occurs (Jeong et al., 2011). For that reasons antimicrobial resistance in *Salmonella* might be considered a side effect of fluoroquinolone selection pressure due to the other clinical indications and further spread of resistant strains along the food chain. Dissemination of quinolone resistant *Salmonella* clones among domestic animals, wildlife, and humans was described in Spain (Palomo et al., 2013). Previously we reported emergence of high level ciprofloxacin resistant *Salmonella enterica* subspecies *enterica* serovar (*S.*) Kentucky in turkeys and pet reptiles (Wasyl and Hoszowski, 2012; Zając et al., 2013) and occurrence of *S.* Stanley outbreak strain in Poland (Wasyl et al., 2013a). Transmission of resistant *Salmonella* via food (Cavaco et al., 2008b; Akiyama and Khan, 2012; Yang et al., 2012) or foreign travels (Weill et al., 2006; Hassing et al., 2011; Le Hello et al., 2013) may lead to serious clinical consequences (Jeong et al., 2011). The burden of quinolone resistant *Salmonella* is not limited to food-borne intoxications, but also confronted with human adopted serovars responsible for typhoid or paratyphoid fever and being often empirically treated with fluoroquinolones as drug of choice (Hassing et al., 2011).

For the above mentioned reasons the knowledge on epidemiology of resistant *Salmonella*, the genetic mechanisms behind as well as public awareness are needed. Therefore, based on a broad selection of *Salmonella* from animals, feeds, and foods available from antimicrobial resistance monitoring the current study aimed at the detection and characterisation of quinolone resistance mechanisms. Furthermore, epidemiological links between subset of those isolates were elucidated.

2. Material and methods

Minimal Inhibitory Concentration (MIC) of Nalidixic acid (Nal, dilution range 4–64 mg/L) and Ciprofloxacin (Cip, 0.008–8 mg/L) were tested in 2680 *Salmonella*. They were picked up from 9670 isolates from animals, food, and feed available between 2008 and 2011 according to resistance monitoring rules: no attention was paid for serovar, but one isolate per serovar and animal flock/herd or batch of food/feed, duplicates were excluded. They represented 136 *Salmonella* serovars and serological forms, ten of which contributed to 80.0% of study group: Enteritidis ($N = 1010$), Infantis ($N = 266$), Typhimurium ($N = 247$, including 21 monophasic variants), Mbandaka ($N = 153$), Newport ($N = 126$), Virchow ($N = 114$), Kentucky ($N = 79$), Hadar ($N = 55$), Agona ($N = 54$), Saintpaul ($N = 39$). Antimicrobial susceptibility testing was performed with Sensititre® EUMVS2 plate (Trek Diagnostic Systems, UK). For the purpose of current study MIC were interpreted according to European Committee on Antimicrobial Susceptibility Testing criteria (Table 1, Fig. 1), both epidemiological cut-offs and clinical breakpoint (not applicable for Nal).

Based on MIC distribution, a subset of isolates was selected for identification of quinolone resistance mechanisms. Forty-four *Salmonella* isolated in 2011 representing ciprofloxacin MIC ranging from 0.125 mg/L to 4 mg/L (Table 2) were used for identification of chromosomal mutations in QRDR of *gyrA*, *gyrB*, *parC*, and *parE*. Those isolates as well as all *Salmonella* with MIC_{Nal} ranging from 4 to 32 mg/L and $MIC_{Cip} \geq 0.125$ mg/L (Table 1) were tested for PMQR mechanisms: *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *aac(6′)-Ib-cr*. PCR assays were run in 25 μl amplification mixture composed of 12.5 μl of Maxima® Hot Start PCR Master Mix (2X) (Fermentas Life Sciences, Lithuania), 0.1 μl of each primer (Table S1), 1 μl of DNA template (boiling lysate of whole-cell suspension), and 11.4 μl of water. The conditions were: initial denaturation (95 °C for 5 min), 30 cycles of 30 s at 95 °C, 30 s in annealing temperature, elongation for 90 s at 72 °C, and final extension at 72 °C for 10 min. Duplex-PCR was applied for simultaneous detection of *qnrB* and *qnrS* genes, whereas all other assays focused on single targets. The obtained amplicons were analysed according to product size in 2% agarose gel (9 V/cm, 80 min). *S.* Typhimurium ATCC 14028 was used as quality control strain in assays targeting chromosomal genes whereas PMQR positive isolates or DNA were obtained from EURL-Antimicrobial Resistance (DTU, Kgs. Lyngby, Denmark). *qnrB19*-positive *S.* Newport and *qnrS1*-positive *S.* Saintpaul originated from previously described studies (Veldman et al., 2011).

Relevant amplicons were sequenced (Oligo, Institute of Biochemistry and Biophysics, Polish Academy of Sciences,