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Antimicrobial resistance and serotyping of *Salmonella enterica* subsp. *enterica* isolated from poultry in Croatia

Boris Habrun[1]*, Borka Šimpraga[2], Gordan Kompes[1], and Fani Krstulović[2]

[1]*Croatian Veterinary Institute, Department for Bacteriology and Parasitology, Zagreb, Croatia*

[2]*Croatian Veterinary Institute, Poultry Centre, Laboratory for Bacteriology, Zagreb, Croatia*

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ABSTRACT

During 2010, the minimum inhibition concentrations (MIC) for 158 isolates of *Salmonella enterica* subsp. *enterica* were determined. The highest number of isolates belonged to the serovars Enteritidis 54 (34%), Mbandaka 49 (31%) and Infantis 19 (12%). MICs were determined for ciprofloxacin, cefotaxim, trimethoprim, ampicillin, nalidixic acid, gentamicin, chloramphenicol, tetracycline, streptomycin and sulfamethoxazole. All tested isolates were sensitive to chloramphenicol and streptomycin. A total of 157 (99.3%) isolates were sensitive to gentamicin, 156 (98.7%) to cefotaxim, 154 (97.5%) to tetracycline, 151 (95.5%) to trimethoprim and ampicillin, 135 (85.4%) to ciprofloxacin, 128 (81%) to sulfamethoxazole and 92 (58%) to nalidixic acid. According to the number of antimicrobials to which individual isolates were resistant, 66 (41.7%) isolates were sensitive to all antimicrobials, 68 (43%) were resistant to one antimicrobial, 20 (12.7%) to two antimicrobials and 4 (2.6%) to three tested antimicrobials. In comparison with the level of resistence of *Salmonella* spp. in other European countries, it can be concluded that strains of *Salmonella* spp. isolated from poultry in Croatia have satisfactory sensitivity to antimicrobial drugs.

Key words: *Salmonella*, sensitivity, poultry

Introduction

Although the number of cases of *Salmonella* in humans has shown a significant decline over the past five years, according to a report by the European Food Safety Agency (ANONYM., 2010a) and the European Centre for Disease Prevention and Control (ANONYM., 2010c), they remain among the most common zoonotics. In 2008, a total of 131,468 cases of *Salmonella* infections in humans were reported in the European Union

*Corresponding author:

Boris Habrun, PhD, Croatian Veterinary Institute, Department for Bacteriology and Parasitology, Savska c. 143, 10000 Zagreb, Croatia, Phone: +385 1 6123 604; Fax: +385 1 6190 841; E-mail: habrun@veinst.hr

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Member States, dominated, as in previous years, by the two most common serovars, Enteritidis and Typhimurium. The incidence of infection caused by the serovar Enteritidis in humans dropped by 23% in 2008 compared to 2007, while the incidence of infection with the Typhimurium serovar increased by 18%. The most common sources of infection were fresh chicken (5.1%), turkey (5.6%) and pork (0.7%).

Implementation of the European Commission regulations to reduce specific salmonella serovars in flocks of laying hens of the species *Gallus gallus* (ANONYM., 2006a) in the National Programme led to a significant drop in the prevalence of infection of laying hens with the serovars Enteritidis and Typhimurium. This resulted in a reduction in the incidence of infection of humans with the Enteritidis serovar, which is most commonly found in eggs. Meanwhile, in 35.4% of all reported and confirmed cases of food poisoning (total of 5,332), the source was *Salmonella* spp., and the food poisoning was usually obtained from eggs (23.1%), pork and meat products (10.2%) and meals in restaurants (9.2%) (ANONYM., 2010a). Food poisoning caused by eggs, egg products and bakery products were linked to the Enteritidis serovar, while the Typhimurium serovar was most commonly found in pork meat.

The European Commission regulation on the prohibition of the use of antibiotics as a specific method of *Salmonella* control in poultry (ANONYM., 2006b) was also implemented in the National Programme. This regulation permits the use of antibiotics only in individual cases. In addition to mandatory collection of data on *Salmonella* as a causative agent of zoonosis, it is also mandatory to monitor the resistance of *Salmonella* isolates, as a source of food poisoning and in sensitive animal populations (ANONYM., 2009).

Due to the large economic damage from *Salmonella* infections, and the impact on human health, it is necessary to implement measures for the prevention, detection and control of *Salmonella* infections in all phases of poultry production, particularly in the primary phases (CARRIQUE-MAS and DAVIES, 2008; VAN IMMERSEEL et al., 2009; VANDEPLAS et al., 2010). These measures must include strict hygienic and biosafety measures (cleaning and disinfection, insect and rodent control), decontamination of water and feed, use of fatty acids and additivies in poultry feed and use of vaccines, prebiotics and probiotics (HAFEZ, 2008; VAN IMMERSEL et al., 2009).

Materials and methods

Isolating of Salmonella spp. Salmonella was isolated from organs according to the HRN EN ISO 6579:2003, and according to the OIE (ANONYM, 2008b). From fecal samples, shoe covers, transport beds with litter and drag swabs, *Salmonella* was isolated according to the ISO 6579: 2008, amendment 1: Annex D.

API biochemical gallery IC 32E (bioMérieux, France) was used for biochemical identification of *Salmonella*. The *Salmonella* serovar was determined by the slide agglutionation using properly antisera's (GRIMONTH and WEILL, 2007) by polyvalent and monovalent antisera (Immunology Institute, Croatia; Bio Rad, France and Statens Serum Institut, Denmark).

Determining the minimal inhibitory concentration (MIC). The minimal inhibitory concentration (MIC) was determined by the use of E-test (AB Biodisk, Sweden). The test is an expansion of the disk diffusion method with the same agar and inoculum preparation. The antibiotic content of the strip is graded, and the concentration is printed linearly along the strip. The test was performed according to the manufacturer's instructions. The MIC for the following antimicrobials was determined: amoxicillin, amoxicillin + clavulanic acid, cefotaxime, colistin, enrofloxacin, gentamicin, spectinomycin, streptomycin, tetracycline, and sulfamethoxazole + trimethoprim.

Antimicrobial agent concentrations ranged from 0.002 to 32 μg/mL (ciprofloxacin and thrimethoprim + sulfamethoxazole), from 0.016 to 256 μg/mL (amoxicillin, amoxicillin + clavulanic acid, cefotaxime, gentamicin, tetracycline and nalidixic acid), and from 0.064 to 1024 μg/mL (colistin, spectinomycin, and streptomycin). According to CLSI M31 A3 (ANONYM, 2008a), Mueller-Hinton agar was used as a culture medium (Merck 1.05435), and *E. coli* ATCC 25922 were used as the control strain.

For the interpretation of MICs (depending on the minimal inhibitory concentration achieved), the strain is interpreted as sensitive, moderately sensitive or insensitive to each individual antimicrobial. The data of EUCAST, as used by the EFSA, were used as criteria to ensure comparability with other EU studies.

Table 1 lists the values that were used as criteria for the interpretation of sensitivity readings and their sources.

Table 1. MIC cut-off values for the interpretation of sensitivity / resistance results

Antimicrobial	Border limit mg/L	International standard
Cefotaxim	≥0.5	EFSA
Nalidixic acid	≥16	EUCAST
Ciprofloxacin	≥0.125	EUCAST
Ampicillin	≥4	EFSA
Tetracycline	≥8	EFSA
Gentamicin	≥4	EUCAST
Chloramphenicol	≥16	EFSA
Streptomycin	≥32	EFSA
Trimethoprim	≥2	EFSA
Sulfamethoxazole	≥256	EFSA

Results

During 2010, the minimal inhibitory concentrations were determined for 158 strains of *Salmonella*, which was isolated from samples of farm chicks and laying hens and farm turkey. A total of 18 strains of *S.* Enteritidis and 3 strains of *S.* Typhimurium were isolated from shoe covers from farm chicks, while the dominant strain isolated from the C1 group was *S.* Mbandaka with a total of 42 strains and 31 other *Salmonella* strains. From samples of laying hens (pooled fecal samples, drag swabs, dust and organ samples), a total of 35 strains of *S.* Enteritidis, 5 strains of *S.* Typhimurium and 18 other salmonella strains were isolated. From samples of farm turkeys (shoe covers), 1 strain of *S.* Enteritidis, 1 strain of *S*. Typhimurium and 4 other salmonella strains were isolated.

Table 2. The minimal inhibition concetrations (MIC) of *Salmonella* Enteritidis (n = 54)

Antimicrobial	MIC_{50}	MIC_{90}	MIC Range
Cefotaxim	0.047	0.094	0.023 - 1.0
Nalidixic acid	3.0	4.0	2.0 - >256
Ciprioflоxacin	0.008	0.016	0.002 - 0.19
Ampicillin	0.75	1.0	0.5 - >256
Tetracycline	0.38	1.0	0.038 - 2.0
Gentamicin	0.19	0.38	0.064 - >256
Chloramphenicol	1.5	2.0	1.0 - 3.0
Streptomycin	1.0	3.0	0.38 - 32.0
Trimethoprim	0.25	0.38	0.094 - 4.0
Sulfamethoxazole	96	192	16.0 - >1024

Table 3. The minimal inhibition concentrations (MIC) of *Salmonella* Mbandaka (n = 49)

Antimicrobial	MIC_{50}	MIC_{90}	MIC Range
Cefotaxim	0.047	0.125	0.032 - 0.25
Nalidixic acid	> 256	> 256	1.5 - >256
Ciprioflоxacin	0.047	0.094	0.004 - 0.25
Ampicillin	0.75	1.0	0.25 - >256
Tetracycline	0.75	1.0	0.5 - 2.0
Gentamicin	0.38	0.5	0.125 - 0.75
Chloramphenicol	3.0	4.0	0.75 - 16.0
Streptomycin	4.0	6.0	1.5 - 16.0
Trimethoprim	0.25	0.5	0.19 - 6.0
Sulfamethoxazole	96	>1024	0.38 - >1024

Table 4. The minimal inhibition concentrations (MIC) for *Salmonella* Infantis n = 19

Antimicrobial	MIC_{50}	MIC_{90}	MIC range
Cefotaxim	0.125	0.19	0.032 - 0.25
Nalidixic acid	>256	>256	2.0 - >256
Ciprioflоxacin	0.38	0.5	0.008 - >256
Ampicillin	1.0	1.5	0.25 - 2.0
Tetracycline	2.0	3.0	0.75 - 4.0
Gentamicin	0.25	0.5	0.125 - 2.0
Chloramphenicol	4.0	6.0	2.0 - 8.0
Streptomycin	4.0	6.0	2.0 - 16.0
Trimethoprim	0.38	0.75	0.19 - 4.0
Sulfamethoxazole	32.0	64.0	0.032 - 96.0

Table 5. The minimal inhibition concentrations (MIC) for *Salmonella* Typhimurium (n = 9)

Antimicrobial	MIC_{50}	MIC_{90}	MIC range
Cefotaxim	0.032	0.047	0.032 - 0.094
Nalidixic acid	4.0	4.0	3.0 - >256
Ciprioflоxacin	0.012	0.023	0.006 - 0.19
Ampicillin	0.5	0.75	0.38 - 0.75
Tetracycline	0.75	1.0	0.38 - 2.0
Gentamicin	0.38	1.0	0.25 - 2.0
Chloramphenicol	1.5	2.0	1.0 - 2.0
Streptomycin	6.0	12.0	1.0 - 24.0
Trimethoprim	0.19	0.25	0.125 - 0.25
Sulfamethoxazole	32.0	48.0	24.0 - 48.0

Table 6. The minimal inhibition concentrations (MIC) for other serovars of *Salmonella* spp. (n = 27)

Antimicrobial	MIC_{50}	MIC_{90}	MIC Range
Cefotaxim	0.047	0.094	0.023 - 0.19
Nalidixic acid	3.0	>256	1.5 - >256
Ciprioflоxacin	0.012	0.25	0.004 - 0.38
Ampicillin	0.5	1.0	0.25 - 2.0
Tetracycline	1.0	3.0	0.5 - 48
Gentamicin	0.19	0.25	0.094 - 0.5
Chloramphenicol	2.0	3.0	1.0 - 6.0
Streptomycin	4.0	6.0	1.5 - 8.0
Trimethoprim	0.5	>32	0.25 - >32
Sulfamethoxazole	64.0	>1024	4.0 - >1024

Table 7. The minimal inhibition concentrations (MIC) for all isolates of *Salmonella* spp. (n = 158)

Antimicrobial	MIC_{50}	MIC_{90}	MIC range
Cefotaxim	0.047	0.19	0.023 - 1.0
Nalidixic acid	3.0	>256	1.5 - >256
Ciprioflоxacin	0.012	0.25	0.002 - >256
Ampicillin	0.75	2.0	0.25 - >256
Tetracycline	0.75	2.0	0.25 - 48
Gentamicin	0.19	0.5	0.064 - >256
Chloramphenicol	2.0	4.0	0.5 - 8.0
Streptomycin	2.0	6.0	0.38 - 32
Trimethoprim	0.25	0.75	0.094 - >32
Sulfamethoxazole	64	>1024	0.38 - >1024

Therefore, a total of 158 tested strains of *Salmonella* spp. belonged to the following serovars: *S.* Enteritidis 54 (34%), *S.* Mbandaka 49 (31%), *S.* Infantis 19 (12%), *S.* Typhimurium 9 (5.7%), *S.* Agona 4 (2.5%), *S.* Seftenberg 3 (1.9%), *S.* Chester 3 (1.9%), *S.* Trachau 2 (1.3%), *S.* Montevideo 2 (1.3%), *S.* Kastrup 2 (1.3%), *S.* Virchow 1 (0.6%), *S.* Agama 1 (0.6%), *S.* Ougadoago 1 (0.6%), *S.* Hayindogo 1 (0.6%), *S.* Staleywile 1 (0.6%), *S.* Isangi 1 and *S.* Lomita 1 (0.6%). Two of the isolates belonging to the C1 serological group and two to the E4 serological groups could not be typed.

The sensitivity results are shown separately for the serovars Enteritidis, Mbandaka, Typhimurium and Infantis in Tables 2 to 5, and for the other serovars of *Salmonella* spp in Table 6, while the results of all isolates pooled are shown in Table 7.

When the MIC results were interpreted, all the tested isolates of *S.* Enteritidis were sensitive to tetracycline, chloramphenicol and streptomycin, 53 isolates (98%) were sensitive to trimethoprim and gentamicin, 52 isolates (96%) to cefotaxim, ciprofloxacin and nalidixic acid, 49 (91%) to ampicillin and 48 (88%) to sulfamethoxazole.

Of 49 tested isolates of *S.* Mbandaka, all were sensitive to cefotaxim, tetracycline, gentamicin, chloramphenicol and streptomycin. A slightly lower sensitivity was established to ampicillin (47 isolates, 96%) and ciprofloxacin (45 isolates, 92%). A total of 36 isolates (73%) were sensitive to sulfamethoxazole, and only 8 isolates (16%) to nalidixic acid.

All 19 tested isolates of *S.* Infantis were sensitive to ceftiofur, ampicillin, tetracycline, gentamicin, chloramphenicol, streptomycin and sulfamethoxazole. A total of 17 isolates (89%) were sensitive to trimethoprim, while only 3 isolates were sensitive to ciprofloxacin (15.7%) and 2 isolates (10.5%) were sensitive to nalidixic acid.

Of the 9 tested isolates of *S*. Typhimurium, all were sensitive to ceftiofur, ampicillin, tetracycline, gentamicin, chloramphenicol, streptomycin, trimethoprim and sulfamethoxazole. A total of 8 isolates (89%) were sensitive to ciprofloxacin and nalidixic acid.

Of the 27 tested isolates of the other serovars of *Salmonella*, all were sensitive to ceftiofur, ampicillin, gentamicin, chloramphenicol and streptomycin. A total of 23 isolates (85%) were sensitive to ciprofloxacin, trimethoprim and tetracycline, 22 (81%) were sensitive to nalidixic acid and 16 (59%) were sensitive to sulfamethoxazole.

In considering all the tested isolates in 2010 (n = 158), all were sensitive to chloramphenicol and streptomycin. A total of 157 (99.3%) were sensitive to gentamicin, 156 to cefotaxim, 154 (97.5%) to tetracycline, 151 (95.5%) to trimethoprim and ampicillin, 135 (85.4%) to ciprofloxacin, 128 (81%) to sulfamethoxazole and 92 (58%) to nalidixic acid.

In terms of the number of antimicrobials to which individual isolates were resistent, 53 (33.5%) isolates were sensitive to all antimicrobials, 66 (41.7%) were resistent to one antimicrobial, 35 (22.2%) to two antimicrobials and 4 (2.6%) to three of the tested antimicrobials.

Discussion

From the appearance of the first data on bacterial resistance to antimicrobials, the need arose to standardize procedures that assess whether individual bacterial isolates are sensitive or resistant to a given antimicrobial. The gudelines of the Clinical and Laboratory Standards Institute, USA (ANONYM., 2008a) are used as standardized procedures for the determination of sensitivity in most countries. However, in Europe, the EUCAST (ANONYM., 2010d) standards are becoming increasingly common.

There are no longer any differences in the procedures for determining bacterial sensitivity (disc diffusion method, dilution method, E-test). Instead, the differences lie in the recommended interpretation of results, such that these countries do not apply the same values (growth inhibition zone in the disc diffusion method or MIC in the E-test and dilution method) in interpreting a sample as sensitive / intermediately sensitive / resistant. For some antimicrobials, CLSI does not provide criteria for the interpretation of results.

For example, differences arose for ciprofloxacin. When the results were interpreted according to the EUCAST criteria, strains having a MIC $\leq$0.125 mg/L are considered sensitive. The same MIC cut-off value was recommended by HAKANEN et al. (2001). With this interpretation, 3 isolates (15.7%) of *S.* Infantis were sensitive to ciprofloxacin. Using the EFSA (2010b) recommendations, which list cut-off value $\leq$ 0.06 mg/L, only 2 isolates

(10.5%) of *S.* Infantis would be considered sensitive. HAKANEN et al. (2001) reported reduced fluorqinolone susceptibily of *Salmonella*, especially in Europe.

In the present study, the majority of isolates were resistant to nalidixic acid (42%). The resistance was most common in serovars Infantis (89.5%) and Mbandaka (84%). Resistance to nalidixic acid in European countries ranged from 0% in Denmark and Finland, 31% in Italy, 36% in Poland, 39% in The Netherlands and up to 59% in Romania (ANONYM., 2010b).

Some guidelines and authors recommend that each isolate of the *Salmonella* spp. that is resistant to nalidixic acid is considered less sensitive or even resistant to fluoroquinolones (ANONYM., 2008a; LINDGREN et al., 2009). HAKANEN et al. (2005) describe the members of the genus *Salmonella* that are sensitive to nalidixic acid and ciprofloxacin, resistant to nalidixic acid and ciprofloxacin, and resistant to nalidixic acid but sensitive to ciprofloxacin, and interestingly, sensitive to nalidixic acid and resistant to ciprofloxacin.

LINDGREN et al.(2009) describe *Salmonella* isolates with reduced ciprofloxacin susceptibility and resistance to nalidixic acid (conventional quinolone resistance phenotype) and *Salmonella* isolates that showed reduced susceptibility to ciprofloxacin, but susceptible (MIC <32 mg/L) or only low-level resistant (MIC = 32 mg/L) to nalidixic acid (nonclassical quinolone resistance phenotype).

In terms of serovars, the serovar Enteritidis was most sensitive to nalidixic acid (MIC_{50} 3.0 mg/L and MIC_{90} 4.0 mg/L), while the serovars Mbandaka and Infantis MIC_{50} >256 mg/L (Tables 2, 3 and 4).

Tetracyclines are the most common antibiotics in veterinary medicine, and the incidence of resistance in Europe ranges from 0% in Sweden to 44% in Greece and 60% in Hungary (ANONYM., 2010b). In Croatia, 2.5% of strains are resistant, and therefore the incidence of resistance to tetracycline can be considered low. The MIC_{50} for tetracycline is 0.75, and the MIC_{90} is 2.0 mg/L (Table 7), which considering the long time use of tetracycline antibiotics in veterinary medicine is a satisfactory results. There were no significant deviations for individual *Salmonella* serovars (Tables 2 to 6).

In the present study, no strains were found to be resistant to chloramphenicol and streptomycin. The incidence of resistance to chloramphenicol in most European countries is less than 10%, with the exception of Greece, where in 2007, 40% of isolates of *Salmonella* spp. were resistant to this antimicrobial. In Croatia, 4.5% of *Salmonella* spp. were resistent to ampicillin in Croatia, while 14% of isolates were resistent in Austria and Greece, and 45% of isolates were resistant in Estonia (ANONYM., 2010b).

Only a few European countries have published data on the sensitivity of *Salmonella* spp. to cefotaxime, with negiglible rates of resistence, with the exception of Spain with 8% resistent isolates and The Netherlands with 13% resistant isolates.

In Croatia, 19% of isolates were resistent to sulfonamides, while the resistance rates were 38% in Italy, 41% in The Netherlands and 59% in Romania.

From the review of the achieved results, the level of sensitivity of *Salmonella* spp. isolates from poultry in Croatia is satisfactory, however, it is necessary to abide by the principles of justifiable and rational use of antimicrobials in animals, so as to avoid increasing bacterial resistance in animals, which in turn makes treatment more difficult and increases the possibility of transfer of resistant bacteria and the bacterial genes from animals to humans.

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SAŽETAK

Tijekom 2010. godine određene su minimalne inhibicijske koncentracije (MIK) za 158 izolata *Salmonella* spp. Najveći broj izolata pripadao je serovarovima Enteritidis 54 (34%), Mbandaka 49 (31%) i Infantis 19 (12%). Određivani su MIK-ovi na ciprofloksacin, cefotaksim, trimetoprim, ampicilin, nalidiksičnu kiselinu, gentamicin, kloramfenikol, tetraciklin, streptomicin i sulfametoksazol. Svi testirani izolati bili su osjetljivi na kloramfenikol i streptomicin. Na gentamicin je bilo osjetljivo 157 (99,3%) izolata, ciprofloksacin i cefotaksim 156 (98,7%), teraciklin 154 (97,5%), trimetoprim i ampicilin 151 (95,5%), sulfametoksazol 128 (81%) i nalidiksičnu kiselini 92 (58%). Prema broju antimikrobnih lijekova na koje je pojedini izolat bio otporan, 66 (41,7%) izolata bilo je osjetljivo na sve antimikrobne lijekove, 68 (43%) bilo je otporno na jedan antimikrobni lijek, 20 (12,7%) na dva antimikrobna lijeka i četiri (2,6%) na tri testirana antimikrobna lijeka. Usporedbom sa stupnjem rezistencije *Salmonella* spp. u drugim europskim zemljama, možemo zaključiti da sojevi *Salmonella* spp. izdvojeni iz peradi u Hrvatskoj imaju zadovoljavajuću osjetljivost na antimikrobne lijekove.

Ključne riječi: *Salmonella*, osjetljivost, perad