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Short communication

CHARACTERIZATION OF TETRACYCLINE RESISTANCE OF *SALMONELLA ENTERICA* SUBSPECIES *ENTERICA* SEROVAR INFANTIS ISOLATED FROM POULTRY IN THE NORTHERN PART OF SERBIA

TODOROVIĆ Dalibor[1], VELHNER Maja[1*], MILANOV Dubravka[1], VIDANOVIĆ Dejan[2], SUVAJDŽIĆ Ljiljana[3], STOJANOV Igor[1], KRNJAIĆ Dejan[4]

[1]Scientific Veterinary Institute „Novi Sad", Novi Sad, Republic of Serbia; [2]Veterinary Institute Kraljevo, Kraljevo, Republic of Serbia; [3]Faculty of Medicine, Department of Pharmacy, University of Novi Sad, Republic of Serbia; [4]Faculty of Veterinary Medicine Belgrade, University of Belgrade, Republic of Serbia

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Resistance to tetracycline was studied in *Salmonella* Infantis isolated from 28 poultry farms in the Northern part of Serbia (The Autonomous Province of Vojvodina). A total of 18 isolates were resistant to nalidixic acid (NAL) and tetracycline (TET). The minimal inhibitory concentration (MIC) to TET, ranged from 1-256 mg/L. Namely, 13 isolates exhibited MIC to TET at 256 mg/L, in four of the isolates, the MIC was 128 mg/L and one isolate had MIC 64 mg/L. Ten isolates were exhibiting a MIC of 1mg/L. It was evident that *Salmonella* Infantis had also spread to breeders and layers. In this work, we detected the *tetA* gene and the corresponding *tet*R gene (encoding the repressor protein) as well as the truncated transposon Tn*1721,* which are responsible for the resistance to TET. The presence of the non conjugative transposons from the conjugative plasmid has facilitated the spread of resistance to TET in *Salmonella.* It was concluded that higher biosecurity practice in poultry farming presents the best option to eliminate infections caused by *Salmonella* spp. from poultry flocks in Serbia. A rational use of antimicrobials is necessary to prevent any further spread of *Salmonella* Infantis resistant clones.

Key words: poultry, *Salmonella* Infantis, tetracycline, *tetA* gene, *tet*R gene, transposone Tn*1721*

INTRODUCTION

Salmonella is one of the most important food borne pathogens worldwide. The occurrence of *Salmonella* Infantis (*S*. Infantis) in poultry production in Serbia is important. By applying pulse field gel electrophoresis, it was determined that strains resistant to NAL and TET are indistinguishable [1,2]. The clones resistant to (fluoro)

*Corresponding author: e-mail: maja@niv.ns.ac.rs

quinolones and TET were recently found in all types of commercial chickens i.e. broiler breeders, layer breeders, broilers and layer chickens [3]. Ultimately, the incidence of *S.* Infantis has to be minimized in poultry farms in Serbia. The mechanism of resistance to (fluoro) quinolones is attributed to the mutations on the topoisomerase genes encoding GyraseA and the topoisomerase IV enzymes. A single mutation on the *gyrA* gene Ser83→Tyr was found in the nalidixic acid resistant strains, while in isolates with an increased MIC to ciprofloxacin (CIP) of 2 mg/L, additional mutations were found on *parC* gene at Ser80→Arg [2].

The genes responsible for the resistance to TET were not elucidated. Tetracycline inhibits protein synthesis by preventing the association of the aminoacyl tRNA to the A site of the ribosome. Hence, the resistance is encoded by the family of *tet* genes. According to amino acid sequence identity, the *tet* genes encoding the membrane bound proteins, are divided in 6 groups. The first group consisted of the following genes: *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(I), *tet*(J), *tet*(Z) (the *tetZ* gene is also found in Gram positive bacteria), *tet*(30) and *tet*(31). The second group consisted of the *tet*(K) and *tet*(L) genes. In the third group, the *otr*(B) and *tcr*3 genes were classified. In groups IV and V the *tet*(P) and *tet*(V) genes were found, respectively. Group VI was represented by an unnamed determinant from *Corynebacterium striatum*. Ribosomal protection proteins confer the resistance to doxycycline and minocycline. The genes encoding this type of resistance are so far defined as: *tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(Q) *tet*(T), *otr*(A), *tetB*(P) and some genes without the (*tet*) designation have also been identified. A less abundant mechanism of resistance is the enzymatic inactivation of tetracycline, encoded by the *tetX* gene. The *tet* genes are associated with mobile genetic elements - the transposons, which are integrated into conjugative or non conjugative plasmids of different incompatibility groups [4]. In *Salmonella,* the *tet* genes are detected on chromosomes or less frequently on plasmids. The *tetA* gene is found in the truncated non-conjugative transposons Tn*1721* inserted into the conjugative plasmid [5-7].

The aim of this study was to isolate *S.* Infantis from poultry farms in the Autonomous Province of Vojvodina during the year 2014 and to determine the antimicrobial susceptibility profiles, as well as TET resistance genes in the antibiotic-resistant isolates.

MATERIAL AND METHODS

Bacterial isolates, isolation, identification and storage

During the year 2014, regular monitoring of the *Salmonella* status was carried out in 55 poultry farms located in the Autonomous Province of Vojvodina. *Salmonella* Enteritidis was isolated from 26 farms and in one farm *Salmonella* Typhimuirum was detected. *S.* Infantis was isolated from 28 poultry farms, comprising of 22 broiler farms, one broiler breeder farm and five farms of layer chickens.

The detection of *Salmonella* spp. in animal feces and in environmental samples from the primary production stage was done as prescribed in the EN ISO 6579:2008, Annex D [8] by applying: pre enrichment in buffered peptone water (BPW), selective enrichment on modified semi-solid Rappaport-Vassiliadis agar; isolation on a selective medium Xylose Lysine Deoxycholate (XLD) agar. All the media were from Biokar diagnostics (Bequvais, CEDEX-FRANCE). *Salmonella* differential agar (HIMEDIA, Mumbai, India) was also used for selection of *Salmonella* colonies.

The identity of isolates was confirmed by biochemical testing which included: triple sugar agar, urea agar (Christensen), Lysine decarboxylase broth, the detection of β-galactosidase (ONPG disc), and the Voges-Proskauer (VP) test [8]. The serotyping was done with sera against somatic (O:6,7) and flagellar (H phase 1:r and phase 2:1,5) antigens [9]. Up until testing, the strains were stored in Tryptone soya broth (TSB) (CM0129, Oxoid, Basingstoke, UK) with 15% glycerol at -20°C. Overnight cultures, cultivated on XLD agar at 37°C, were used for the examination.

Antimicrobial susceptibility testing and the determination of minimal inhibitory concentration (MIC)

Antimicrobial susceptibility test methods were performed according to The Clinical Laboratory Standard Institute documents [10,11]. A reference strain, *Escherichia coli* ATCC 25922, was used for quality control purposes. *S*. Infantis isolates were investigated for antibiotic susceptibility by applying the disc diffusion method on Mueller-Hinton agar (CM0337, Oxoid, Basingstoke, UK) while using the following antibiotic disc: Ampicilin 10 μg (AMP), Amoksicilin/clavulanic acid 20 μg + 10 μg (AMC), Chloramphenicol 30 μg (CAP), Ciprofloxacin 5 μg (CIP), Gentamycin 10 μg (GEN), Nalidixic acid 30 μg (NAL), Streptomycin 10 μg (STR), Sulphonamides 300 μg (SA), Tetracycline 30 μg (TET), Trimethoprim/sulfamethoxazole 1.25 μg + 23.75 μg (SXT), Trimethoprim 5 μg (TMP), Cefpodoxime 10 μg (CPD), Cefotaxime 30 μg (CTX), Ceftazidime 30 μg (CAZ), (BioRad, Marnes-la-Coquette, France). Resistance to tetracycline was interpreted at MIC $\geq$ 16 mg/L. The tetracycline powder was from Sigma Aldrich (München, Germany).

Polymerase chain reaction protocols

The polymerase chain reaction (PCR) was conducted to detect, *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG* genes, the transposon Tn*1721*, the left and the right arm of the Tn*1721* and also the repressor gene *tet*(R). DNA was isolated using the boiling procedure (100°C for 5 minutes) and the master mix was prepared from the HotStarTaq master Mix kit according to the manufacturer instruction (Qiagen, Hilden, Germany). The PCR was performed by using the Thermal cycler TECHNE (Bibby Scientific LTD, UK) under the following conditions for the *tet* genes: denaturation at 95°C 15 min, 30 cycles at 94°C 1 min, 55°C 1 min, 72°C 1 min, extension at 72°C 10 min. The cycling conditions for the detection of the Tn*1721* and the left arm of the transposon were: 95°C, 15

min, 30 cycles at 94°C, 30s, 53°C, 1 min, 72°C 1 min, and the final step at 72°C, for 7 min. For the detection of the right arm of the Tn*1721* and the *tet*R gene the cycling conditions were, 15 min at 95°C, 30 cycles at 94°C for 30 sec, annealing at 55°C for 1 min, and 72°C, 1 min, the final extension was 72°C for 7 min. The sequences of the primers, the amplicon size and the references used in this work are presented in Table 1. PCR products were analyzed on a 2% gel (AppliChem, Darmstadt, Germany).

Table 1. The sequence of primers (5'-3'), annealing temperature, fragment size and references used in the study

Primer name	Primer sequence	Anneal. temp. °C	Fragment size bp	Reference
tetAF	GCT ACA TCC TGC TTG CCT TC	55	210	12
tetAR	CAT AGA TCG CCG TGA AGA GG			
tetBF	TTG GTT AGG GGC AAG TTT TG	55	659	12
tetBR	GTA ATG GGC CAA TAA CAC CG			
tetCF	CTT GAG AGC CTT CAA CCC AG	55	888	12
tetCR	ATG GTC GTC ATC TAC CTG CC			
tetDF	AAA CCA TTA CGG CAT TCT GC	55	variable	12
tetDR	GAC CGG ATA CAC CAT CCA TC			
tetEF	AAA CCA CAT CCT CCA TAC GC	55	variable	12
tetER	AAA TAG GCC ACA ACC GTC AG			
tetGF	CAG CTT TCG GAT TCT TAC GG	55	variable	12
tetGR	GAT TGG TGA GGC TCG TTA GC			
TAF	GTA ATT CTG AGC ACT GTC GC	53	1199	6
tetAR3	GGC ATA GGC CTA TCG TTT CCA			
LAF	GTT CGG GTC AGC AGC TTT GAC	53	509	6
LAR	GAG GGT TTC CCG GCT GAT GT			
TRF	CGT ATG ATT CTC CGC CAG CA	55	736	7
TAR2	CGA CCA TCC CGA ACC CGA A			
TAF2	CTT CTT CAT CAT GCA ACT TGT	55	912	7
Tn1721R	CGT TCC AGT AGC TTT AGT GT			

RESULTS AND DISCUSSION

Resistance to TET in strains of *S*. Infantis from Serbia is the prominent property of the clones. The reason why this research was conducted was to determine the genes responsible for resistance to TET.

S. Infantis was isolated from 28 poultry farms out of 55 in the Northern part of Serbia. Three resistotypes were detected but the most prevalent resistance was to NAL and TET in 18 of the isolates. Two isolates were resistant only to NAL and eight isolates were susceptible to antibiotics. The MIC distribution is presented in Table 2. Most of the strains exhibited high MIC to TET, ranging from 64 - 256 mg/L. In 10 out of 28 isolates the MIC to TET was 1 mg/L.

Table 2.

No of the sample	Poultry flock	Resistotype	MIC-CIP mg/L	MIC-TET mg/L	tetA*	Tn1721	Left Arm Tn1721	tet(R)	Right Arm Tn1721R
3947	Broilers	/	0,031	1	-	-	-	-	ND**
4143	Broilers	NAL	0,5	1	-	-	-	-	ND
4682	Broilers	NAL, TET	2	256	+	+	-	+	+
4716/1	Broilers	NAL, TET	2	256	+	+	-	+	+
4855	Broilers	NAL, TET	2	256	+	+	-	+	+
5580	Broilers	NAL, TET	4	256	+	+	-	+	+
5582	Broilers	NAL, TET	2	256	+	+	-	+	+
5656	Broilers	NAL, TET	0,25	128	+	+	-	+	+
5736/1	Broilers	NAL, TET	1	256	+	+	-	+	+
7639/3	Broilers	NAL, TET	1	256	+	+	-	+	+
9643	Broilers	NAL, TET	1	256	+	+	-	+	+
9784	Broilers	/	0,031	1	-	-	-	+	+
9849/2	Broilers	/	0,007	1	-	-	-	-	ND
10175	Broilers	/	0,015	1	-	-	-	-	ND
10101	Broilers	NAL, TET	2	256	+	+	-	+	+
9699	Broilers	NAL, TET	0,25	64	+	+	-	+	+
11420/2	Broilers	NAL, TET	1	256	+	+	-	+	+
11288	Broilers	NAL, TET	2	256	+	+	-	+	+
10596	Broilers	NAL, TET	2	256	+	+	-	+	+
12913	Broilers	NAL/TET	1	128	+	+	-	+	+
12140	Broilers	NAL, TET	2	256	+	+	-	+	+
13220/1	Broilers	NAL, TET	0,5	128	+	+	-	+	+
5653	Layers	NAL, TET	0,25	128	+	+	-	+	+
6597	Layers	/	0,031	1	-	-	-	-	ND
9815/2	Layers	NAL	0,5	1	-	-	-	-	ND
12777	Layers	/	0,015	1	-	-	-	-	ND
12473	Layers	/	0,007	1	-	-	-	-	ND
8761	Broiler Breeder	/	0,015	1	-	-	-	-	ND

**tetA* gene, Tn*1721* (transposon), Left arm Tn*1721* (left arm of the transposon), tet(R), the repressor gene; Right Arm Tn*1721* (right arm of the Tn*1721* transposon); ** Not done

The genes which encode resistance to TET are found on mobile genetic elements, the transposons. In this work we detected the *tetA* gene and the truncated non conjugative transposon Tn*1721* in *S*. Infantis, isolated from poultry flocks in the Autonomous Province of Vojvodina. None of the *S*. Infantis isolates contained the *tetB*, *tetC*, *tetD*, *tetE* and *tetG* genes. We have also identified the *tetR* gene which is responsible for encoding the repressor protein and we have detected the right arm of the truncated transposon. In isolates that are not exhibiting resistance to tetracycline, the *tetA* gene, the Tn*1721* and the *tetR* genes were not found (Fig 1). The results of our experiments are in good correlation with the evidence that the *tet* genes are arranged in different conjugative and non conjugative transposons [4]. The functional *tetA* gene in the truncated transposon Tn*1721* was detected for the first time in *Salmonella* Typhimurium var. Copenhagen DT002. It was elucidated that the Tn*1721* is located in the 9.5 kbp plasmid designated pGFT4. Additionally, the *Escherichia coli* transformant JM107:pGFT4 was highly resistant to TET [5]. In *S*. Typhimurium, isolated from the irrigation water in Culiacan Valley Mexico, the *tetA* gene and truncated Tn*1721* transposon were identified. The *tetA* gene in *S*. Typhimurium has high sequence homology to the *tet* gene of the plasmid RP1 [7]. In each case, the non conjugative transposons (Tn*1721*) were located on the conjugative plasmid, facilitating a wide distribution of the *tet* genes in nature.

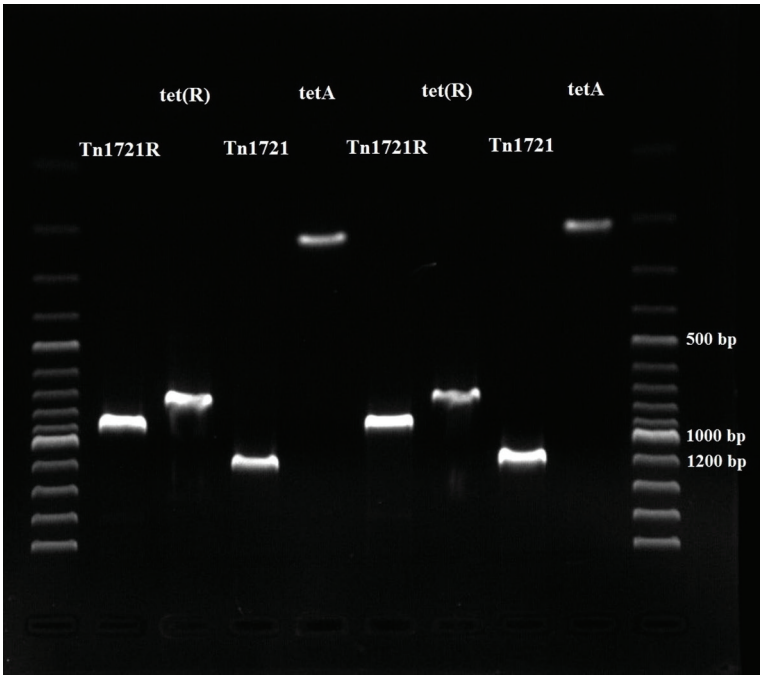

Figure 1. Detection of *tetA* gene, the repressor gene *tetR* and truncated transposon Tn*1721* from *S*. Infantis resistant to NAL and TET

To this day, several types of *tet* genes were reported in *S*. Infanits. In Japan and Israel the *tetA* gene was found in isolates from poultry [13,14]. In the epidemiologically unrelated *S*. Infantis, distributed evenly in two clusters obtained by pulsed field gel electrophoresis, the *tetA* and *tetB* genes were found in Italy [15]. The *tetD* gene was identified in hospital isolates from Rio de Janeiro, Brazil, [16]. Therefore, *tet* genes of different hybridization classes were found in *Salmonella* [17].

It is evident that *S*. Infantis is widespread among poultry flocks and also causes gastrointestinal infections in humans. These facts emphasize the need for strict control of *Salmonella* infections in the poultry industry especially because *S*. Infantis typically found in broilers is also infecting layer chickens. It is plausible that different types of chickens (possibly broilers and layers) are hatched in the same hatchery cabinet and such management practice increases the possibility of clonal spread of *S*. Infantis.

In conclusion, three resistotypes of *S*. Infantis were detected in poultry flocks. Eighteen isolates were resistant to NAL and TET, two isolates were resistant only to NAL and eight isolates were susceptible to all antibiotics used in the study. We have determined that the *tetA* gene is responsible for the resistance to tetracycline in poultry isolates from *S*. Infantis in the Vojvodina Province. It is plausible that the truncated tranposon Tn*1721* is detected in resistant clones of *S*. Infantis. The occurrence of TET resistant clones is attributed to transferable plasmids in *Salmonella*. Adequate farm management practice is necessary to minimize the occurrence of *Salmonella* spp. in poultry farms in Serbia.

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KARAKTERIZACIJA REZISTENCIJE NA TETRACIKLINE KOD *SALMONELLA ENTERICA* SUBSPECIES *ENTERICA* SEROVAR INFANTIS IZOLOVANE OD ŽIVINE U SEVERNOM DELU SRBIJE

TODOROVIĆ Dalibor, VELHNER Maja, MILANOV Dubravka, VIDANOVIĆ Dejan, SUVAJDŽIĆ Ljiljana, STOJANOV Igor, KRNJAIĆ Dejan

Ispitivana je rezistencija na tetraciklin kod *Salmonella* Infantis izolovanih sa 28 farmi živine u severnom delu Srbije (Autonomna pokrajina Vojvodina). Ukupno 18 izolata je bilo rezistentno na nalidiksinsku kiselinu (NAL) i tetraciklin (TET). Minimalna inhibitorna koncentracija (MIC) na TET iznosila je od 1-256 mg/L. Trinaest izolata pokazalo je MIC na TET od 256 mg/L, kod četiri izolata utvrđen je MIC od 128 mg/L, a jedan izolat je imao MIC na TET 64 mg/L. Deset izolata je pokazalo MIC od 1 mg/L. Evidentno je da se *Salmonella* Infantis sa brojlerskih pilića prenela i na nosilje. Detektovan je *tetA* gen i pripadajući *tetR* gen (koji kodira represorski protein) kao i skraćeni transpozon Tn*1721*, koji su odgovorni za rezistenciju na tetraciklin. Pojava nekonjugabilnog transpozona na konjugabilnom plazmidu doprinosi širenju rezistencije na tetraciklin kod salmonela. Zaključeno je da se primenom boljih biosigurnosnih mera mogu eliminisati infekcije izazvane salmonelama na farmama živine u Srbiji. Kako bi se sprečilo dalje širenje rezistentnog klona *Salmonella* Infantis potrebno je da upotreba antibiotika bude racionalnija.