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*Research article*

# ANTIBIOTIC RESISTANCE AND MOLECULAR STUDIES ON *SALMONELLA ENTERICA* SUBSPECIES *ENTERICA* SEROVAR INFANTIS ISOLATED IN HUMAN CASES AND BROILER CARCASSES

RAŠETA Mladen[1*], TEODOROVIĆ Vlado[2], BUNČIĆ Olivera[2], KATIĆ Vera[2], BRANKOVIĆ LAZIĆ Ivana[1], POLAČEK Vladimir[3], VIDANOVIĆ Dejan[3]

[1] Institute of Meat Hygiene and Technology, Kaćanskog 13, Belgrade, Serbia; [2] Faculty of Veterinary Medicine, University of Belgrade, Bulevar Oslobođenja 18, Belgrade, Serbia; [3] Veterinary Specialized Institute, "Kraljevo", Žička 34, Kraljevo, Serbia

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During the year 2012 a study was conducted on the hygiene of the production of broiler carcasses at three abattoirs in the Republic of Serbia. A total of 150 samples of broiler neck skin were examined and 17 salmonella isolates were recorded. Isolates were, by using the corresponding monovalent and polyvalent sera, determined according to type as *Salmonella enterica* subspecies *enterica* serovar Infantis (S. Infantis 6, 7, r, 1, 5). In the case of the disease in humans, 5 *Salmonella* samples of identical serovars were isolated. After that, 22 samples were tested for antibiotic resistance by the disk diffusion test. Isolates showed resistance to ampicillin and nalidixic acid (95.5%), tetracycline (91%), cefotaxime/clavulanic acid (68.2%), but not to ciprofloxacin, gentamicin, and trimethoprim/sulfamethoxazole. The degree of genetic similarity of isolates from diseased humans and broiler carcasses was determined at a molecular level. Cluster analysis revealed the presence of 7 profiles, while all isolates have 92% genetic similarity. Although there are differences in the antimicrobial resistance of isolates originating from diseased humans and neck skin of tested broilers, can not be excluded an epidemiological link, because in the dominant genotype SINFXB0001, established in 8 isolates from diseased humans (3 isolates), and the neck skin of broilers (5 isolates), a genetic similarity of 100% was recorded. Based on these results, the presence of *S*. Infantis on broiler carcasses can be considered a hazard to human health.

**Key words:** Salmonellosis, prevalence of *Salmonella* on broiler carcasses, *Salmonella* Infantis, antibiotic resistance, PFGE

## INTRODUCTION

A large number of foods, particularly meat and broiler meat products are the most important sources of human salmonella contamination [1-3]. At the local and global level due to a number of foods contaminated with salmonella serovars many patient

* Corresponding author: e-mail: mladen@inmesbgd.com

cases have been reported [4-6]. In the recent years, an additional health problem is represented by the emergence of multi-resistant strains of pathogenic bacteria, including salmonella especially in food samples [7]. Extensive and intensive use of antibiotics for preventive and therapeutic purposes in veterinary medicine, as well as growth promoters in animal feedstuffs, contributed to the emergence of resistant bacteria in animals, including zoonotic pathogenic microorganisms that may be transmitted in the food chain to humans [8,9].

European Food Safety Authority (EFSA) report released in year 2013 on the status and trends of zoonoses, zoonotic agents and foodborne agents in EU member states, disclosed that during 2011 were recorded 95,548 confirmed cases of salmonellosis in humans. The prevalence of salmonellosis in humans has been constantly declining by 5.4% compared to 2010 and by 37.9% compared to 2007. There is a downward trend in the occurrence of salmonellosis in the period from year 2008 to 2011. Many EU countries have reduced the presence of salmonella in animals and food of animal origin. In all tested food samples [10] *Salmonella* is usually identified in fresh broiler meat (5.9%).

In Serbia during year 2010 were recorded 2,636 confirmed cases of salmonellosis in humans, from which 2,536 were serotyped. The prevalence of salmonellosis in humans has been declining by 490 confirmed cases less than in year 2009 [11].

Infections caused by non-typhoid *Salmonella* serovars (NTS) are a major global health concern. Although NTS in humans predominantly lead to local gastrointestinal tract disorders (diarrhea), a focal and systemic infection can also develop [12]. S. Infantis is the third most common serovar isolated from humans in the EU since year 2006. Its presence gradually increased from 1% in year 2006 to 2.2% in year 2010 [10]. The most prevalent serovars of S. Enteritidis and S. Typhimuirum were the cause of about 90% of reported cases of salmonellosis, while serovar S. Infantis is responsible for about 5% of cases [13]. In Serbia *S*. Infantis was also the third most common serovar isolated from humans, with a prevalence of 2.5%. Most common isolated serovars from humans were *S*. Enteritidis (82.1%) and *S*. Typhimurium (5.4%) [11].

The aim of our study was to determine on the slaughter line the prevalence of salmonella on the neck skin of broiler carcasses. Thereon, to carry out the typisation of the isolates, and to examine their resistance to antibiotics, as well as to determine the degree of genetic similarity of the identified isolates with the same type serovars isolated from diseased humans, in order to determine the risk of the presence of *S*. Infantis in fresh broiler meat carcasses for human health.

## MATERIAL AND METHODS

In order to assess the hygiene of the production process of broiler chickens, at the slaughter line were taken 150 samples of broiler neck skin by consecutive sampling, i.e. five samples in a series of ten consecutive trials. In each series of tests, samples were

taken from 15 randomly selected carcasses. From each carcass at the slaughter line, 10 grams of the neck skin were sampled after cooling. Of the 15 samples 5 pooled samples were formed, each of which consisted of three neck skin samples (30 grams). From the so formed samples 25 g were taken for testing, which means that from 15 carcasses were formed 5 samples, 25 grams each.

From the Department of Public Health "Dr Milan Jovanović Batut", were obtained 5 samples originating from diseased people. Fecal samples were taken from affected patients, and isolates were frozen in cryogenic vials and stored at -18°C.

The sampled material was transported in a cold chain and delivered to the laboratory within a day. Samples were suspended with 250 ml of buffered peptone water, homogenized for 30 seconds, and after that were incubated at 37°C for 24 hours. After pre-enrichment and incubation, 0.1 ml of the slurry was passed down into 10 ml Rappaport - Vasilliadis medium (Biomerieux, France), which was then incubated at 42°C, for a period of 24-48 hours. After incubation, seeding was carried out on a differential substrate Rambach agar (Merck, Germany) and XLD agar (Oxoid, UK) which were incubated overnight at 37°C. Detection of Salmonella antigens in samples, was performed by using automated mini VIDAS system (Biomerieux, France). Positive samples were biochemically tested by using API 20E test kit (Biomerieux, France). Plastics strips holding twenty mini test tubes were inoculated with suspensions of the cultures according to manufacturer's directions. After 24 hours of incubation at 37°C, the color reactions were read. The data were analysed by the manufacturer's software and positive samples were confirmed as Salmonella. Biochemical test Colonies with typical growth and clearly differentiated were passed in cryogenic vials for further testing.

Serotyping of isolates was performed by agglutination method (glass microscopic plate) using polyclonal and monoclonal sera according to Kauffmann-White patterns [14,15]

Antibiotic resistance was performed by disk diffusion according to the National Committee for Clinical Laboratory Standards (NCCLS) recommendations using commercial discs and Mueller-Hinton agar (Bio-Rad, USA). The investigated isolates were first subcultured on trypticase soy agar (TSA) and incubated for 24 hours at 37°C. From grown salmonella colonies suspensions were prepared in physiological saline, which corresponded to the density of the 0.5 McFarland standard.

Salmonella suspensions were transferred by sterile swabs on Mueller-Hinton agar, followed by antibiotic discs application (automatic applicator, Oxoid, UK). The following antibiotic discs (Oxoid Ltd., Bashingstoke, UK) were used: nalidixic acid (quinolone) 30 mg, ampicillin (penicillin) 10 mg, cefotaxime/clavulanic acid (cephalosporin) 30 mg, ciprofloxacin (quinolone) 5 mg, gentamicin (aminoglycoside antibiotic) 10 mg, tetracycline (tetracycline) 30 mg, trimethoprim/sulfamethoxazole (inhibitors of folic acid) 30 μg. After 18 hours of incubation the growth inhibition zones were measured, and the results were interpreted according to NCCLS or Clinical

and Laboratory Standards Institute (CLSI) 2006 recommendations as sensitive, intermediate sensitive and resistant.

Electrophoresis in pulsed electric field (PGFE) is applied to discriminate within serovars and phagotypes of isolated samples. PFGE is based on the separation of DNA fragments after digestion with restriction enzymes, to give 10-20 linear DNA fragments of different lengths. These fragments are too large to be extracted by conventional agarose electrophoresis, thus the pulsed electric field technique is applied. In the electric field with changing direction and length the smaller fragments move faster through agarose, and with time they occupy a certain position in the gel [16]. During continuous electrophoresis, DNA fragments of more than 30 - 50 kb migrate at the same rate regardless of their size, which is observed as a diffuse band on the gel. However, if the DNA fragments are forced to change their direction of movement during electrophoresis, fragments of different size will be separated. With each new re-orientation of the electric field, the smaller DNA fragments will begin to move in a new direction faster than those of higher molecular weight. As a result, larger DNA fragments lag behind, providing separation from smaller DNA fragments [17]. Restriction enzymes provide 8-25 DNA bands containing from 40 to 600 pairs (kilobase-kb) [18]. With this method, for each *S*. Infantis isolate, a PFGE profile is obtained, these are then compared with each other, in order to determine the molecular genotype. Out of all molecular methods used in epidemiological studies, PFGE represents one of the most reliable methods for subtypisation of many microorganisms. The high resolving power of PFGE can be increased by using more restrictive enzymes, as well as by combining it with other subtypisation methods [17].

Pulse gel electrophoresis (PFGE) was carried out in accordance with the Pulsenet protocol [19]. Recording was performed with the GelDoc system, primary analysis of the gel was done with GelDoc software, and the analysis of the obtained fragments with FPQuest software (Bio-Rad). To generate the name and nomenclature of derived genotypes, recommendations of Tenover [24] were used. For the tabulation of the data Microfost Excel 2007 was used.

## RESULTS

The prevalence of *Salmonella* spp. isolates on the three observed slaughterhouses is given in Table 1.

Serological identification of the isolates of *Salmonella* spp., established the present serovariety on the broilers neck skin and patients fecal samples. The results are given in Table 2.

Serological tests have confirmed the presence of a *Salmonella enterica* serovars of subspecies *enterica* serovar Infantis (6, 7, r, 1, 5) in all 17 samples. Salmonella species isolated in the case of diseased humans have also been identified as the same serovars.

Confirmation of the identified serovar isolated from humans was performed by using monoclonal and polyclonal serum and PCR technique, thereon the obtained segments were subjected to examination in a pulsed electric field by the PFGE method.

**Table 1.** Prevalence of *Salmonella* spp. on broiler carcasses in three slaughterhouses in Serbia

| Location | Number of samples | Determined *Salmonella* spp. | |
|---|---|---|---|
| | | Number | % |
| Slaughterhouse 1 | 50 | 0 | 0 |
| Slaughterhouse 2 | 50 | 17 | 34 |
| Slaughterhouse 3 | 50 | 0 | 0 |
| Total | 150 | 17 | 11.3 |

**Table 2.** Results of serological typisation of *Salmonella* spp. isolated from the broiler neck skin and patients fecal samples

| Origin of *Salmonella* spp. | Number of isolates | Serovariety |
|---|---|---|
| Skin on the broiler nek | 17 | *S*. Infantis (6, 7 : r : 1,5) |
| Human feces | 5 | *S*. Infantis (6, 7 : r : 1,5) |
| Total | 22 | *S*. Infantis (6, 7 : r : 1,5) |

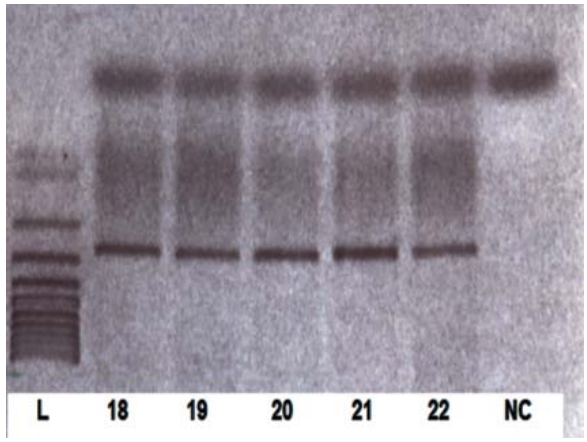


**Figure 1.** Agarose gel electrophoreses obtained by PCR technique (18-22 S. Infantis isolates originated from diseased humans)

The results of resistance to antibiotics was determined on 5 samples isolated from human cases and 17 from the neck skin of studied broilers were presented in Table 3.

**Table 3.** Salmonella resistance to antibiotics determined on samples isolated from human cases and from the neck skin of studied broilers

| Antibiotic | N° of isolates | Sensitive | | Intermediate | | Resistant | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| Ampicillin | 22 | 0 | 0 | 1 | 4.5 | 21 | 95.5 |
| Cefotaxime / clavulanic acid | 22 | 5 | 22.8 | 2 | 9.0 | 15 | 68.2 |
| Ciprofloxacine | 22 | 19 | 86.4 | 3 | 13.6 | 0 | 0 |
| Gentamicin | 22 | 18 | 81.8 | 4 | 18.2 | 0 | 0 |
| Nalidixic acid | 22 | 1 | 4.5 | 0 | 0 | 21 | 95.5 |
| Tetracycline | 22 | 2 | 9.0 | 0 | 0 | 20 | 91.0 |
| Trimethoprim /sulfamethoxazole | 22 | 22 | 100 | 0 | 0 | 0 | 0 |

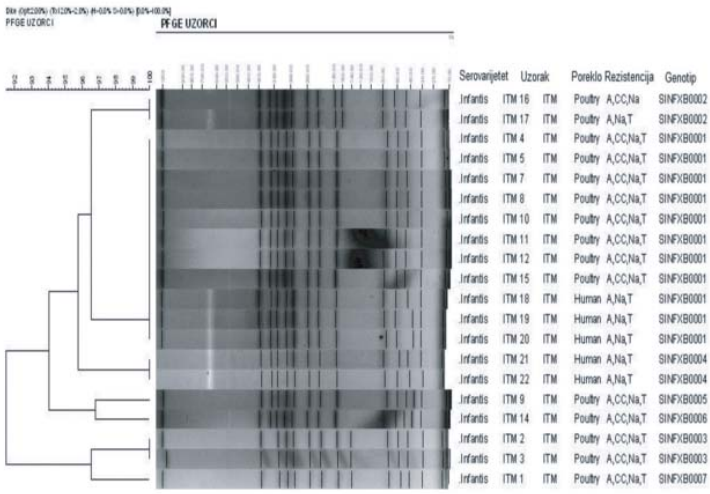


**Figure 2.** Dendrogram of the electrophoretic scheme obtained with the enzyme XbaI – broken down genome of DNA isolates of S. Infantis, derived from FPQuest program that shows similarity coefficients (Dice coefficient, UPGMA) between the tested isolates and antibiotic resistance of tested isolates (A - ampicillin, CC - cefotaxime / clavulanic acid, Na - nalidixic acid, T - tetracycline)

## DISCUSSION

As seen in Table 1 the prevalence of *Salmonella* spp. in samples from slaughterhouse 2 was 17 positive from a total of 50 tested samples.

Results of the determination of the present serovar are given in Table 2. By direct application of adequate monovalent and polyvalent sera the presence of one serovar was determined i.e. *S*. Infantis (6, 7, r, 1, 5). The presence of *S*. Infantis in broiler meat in the recent years is constantly increasing.

The profile of antibiotic resistance isolates from broiler carcasses from human cases did not differ as resistance to ampicillin and nalidixic acid (95.5%), tetracycline (91%) and cefotaxime (68.25%) was determined also. The isolated strains were sensitive (intermediate and complete) to trimetorpim/sulfamethoxazole, gentamicin and ciprofloxacin, which are the therapy drugs of choice. By comparing the additional antimicrobial profiles it was found that the isolates derived from poultry were resistant to ampicillin, cefotaxime/clavulanic acid, nalidixic acid and tetracycline, while isolates originating from human patients were resistant to ampicillin, tetracycline and nalidixic acid. Isolates originating from human cases did not show resistance to the combination of cefotaxime and clavulanic acid. Similar results were obtained in studies in Hungary [13], whereby the resistance of the isolated salmonella to nalidixic acid and tetracycline was determined. Studies carried out in Italy (2005-2006) are in agreement with the results obtained in our study as in both cases resistance to ampicillin and tetracycline was reported. However, the difference is reflected in the resistance to trimethoprim / sulfamethoxazole [20]. A study of bacterial resistance on farms in Hungary in 2006-2007 determined *S*. Infantis resistance to nalidixic acid, streptomycin, sulphonamides, and tetracycline [13]. In studies conducted in Italy from 2005 to 2006 were isolated 70 strains of *S*. Infantis from human and animal material. Strains were found to be resistant to ampicillin, chloramphenicol, streptomycin, sulfonamide, tetracycline, kanamycin, and trimethoprim/sulfamethoxazole [20]. Studies of antibiotic resistance of the isolated 106 serovars of *S*. Infantis, in Poland for the period 2008-2012 showed resistance to nalidixic acid (52.8%), tetracycline (32.1%), ampicillin (28.3%), streptomycin (28.35%), and sulfonamide (26.4%), while all tested isolates were sensitive to cefotaxime and ciprofloxacin. Of all tested S. infantis isolates, 73.3% were resistant to nalidixic acid, 66.7% to sulphonamide, 60% to tetracycline and streptomycin [21]. In studies conducted in Japan 135 isolates of *S*. Infantis isolated from broilers in Kagoshimi were tested for antibiotic resistance, and sulfamethoxazole resistance was determined in 95.6% samples, while only one isolate (0.7%) showed resistance to ampicillin [22].

Analysis of data obtained by PFGE electrophoresis was carried out with the aid of FPQuest software. The obtained by PFGE profiles were divided into 7 groups - genotypes, with mutual genetic similarity within groups of 100%. Profiles were assigned codes composed of the first letter of bacteria species, three letters of serovars, two letters of the used enzyme and a four digit number starting at 0001. Profile SINFXB0001 was detected in 11 isolates of which 8 were originally from the broiler neck skin (4, 5, 7, 8, 10, 11, 12, 15), and 3 were isolated in samples of human feces (18, 19, 20). Profiles SINFXB0002 (16, 17) and SINFXB0003 (2, 3) were found in isolates from broiler neck skin, a profile SINFXB0004 (21, 22) was detected in two isolates from human patients. Profiles SINFXB0005 (9), SINFXB0006 (14) and SINFXB0007 (1) were found in one isolate. The coefficient of similarity between genotypes was 92%. Samples No. 6 and 13 could not be typed.

By applying PFGE genotyping seven different profiles were determined with a genetic similarity to each other ≥ 92%. A similar study was conducted in Brazil, where in 35 isolates of S. Infantis derived from patients suffering from food poisoning, during the period from 1984 to 2009 a degree of similarity of ≥ 80.6% was determined, as well as 23 PFGE profiles [23].

The dominant profile SINFXB0001 was detected in 11 isolates (3 isolates from human fecal samples and 8 isolates from broiler neck skin). Despite not finding direct epidemiological evidence that the affected people ate contaminated broiler meat the identical PFGE genotyping profiles suggest this possibility [24]. By observing the dominant profile SINFXB0001 it can be concluded that different antimicrobial profiles are present in isolates which have a 100% genetic similarity. The antimicrobial activity of the microorganism of the profile present greatly depends on the drug selected for the treatment of broilers, more than does the drug of choice used in human medicine for the treatment of patients. SINFXB0002 profile was detected in the two isolates of the neck skin of broilers, where in one showed resistance to ampicillin, cefotaxime/clavulanic acid and nalidixic acid, and the other isolate was resistant to ampicillin, tetracycline and nalidixic acid. Profiles SINFXB0003, SINFXB0005, SINFXB0006 and SINFXB0007 originating from the neck skin of broilers showed identical antimicrobial resistance to ampicillin, cefotaxime/clavulanic acid, nalidixic acid and tetracycline. SINFXB0004 profile showed identical antimicrobial resistance, as did the predominant profile SINFXB0001 to ampicillin, nalidixic acid and tetracycline.

By comparing the genomes of the 22 strains of salmonella a degree of similarity of 92% was recorded (Figure 2). The same degree of similarity of the genome was determined by testing 76 isolates of *S*. Infantis isolated in the period from 2004 to 2009 from broiler meat and broiler feces [25]. The difference between our results compared to studies carried out in Hungary is reflected in the number of clusters in which the isolates were divided. In our study we found seven clusters, while in Hungary the isolates were divided into two clusters. Studies on the presence of S. Infantis on farms in Hungary (2006-2007) disclosed a series of 164 isolates, whereas the genomes showed a similarity of ≥ 88.7%. The obtained results showed that the same multiresistant clone of S. Infantis, spread from farms to slaughter lines and retail, where it appeared as the cause of sickness in people throughout the region, and was previously designated as the dominant clone characteristic of broilers and people across the country [13]. EFSA reported that in Hungary, the prevalence of *S*. Infantis in broiler flocks is 68% [26]. In an earlier study Nogrady with colleagues 2007 established the presence of multiresistant clones isolated in the period from 2004 to 2005 from the feces of diseased people, broilers, and broiler meat [27].

Research indicates that the most common serovars in humans, food and in poultry in Serbia are S. Enteritidis, *S*. Typhimurium and *S*. Infantis [28]. Scuderi with colleagues (2000) reported that every year from various sources collected in Italy about 10,000 of salmonella isolates are registered, with over 70% of human infections caused by serovars of *S*. Typhimuruim, *S*. Enteritidis and *S*. Infantis [29]. Prevalence of

salmonella in fresh meat is directly related to the findings in animals and, of course, depends on further technological processing in slaughterhouses [30,31].

## CONCLUSION

Our studies clearly indicate that salmonella isolated from poultry carcasses, pose a risk to the health of people in our country**.** The isolated salmonella from broiler farms is generally sensitive to most of the tested antibiotics. Multidrug resistance has occurred very rarely, while 20% of the isolates were resistant to quinolones. Resistance to fluoroquinolones was not detected. However, salmonella is very resistant to nalidixic acid, and showed reduced susceptibility to ciprofloxacin. Similar reports were obtained during testing of salmonella isolated from the feces of humans. The problem of antibiotic resistance will not be resolved by creating a number of novel antibacterial drugs because the micro-organisms will continue to constantly adapt to ambient conditions and endanger human health. Salmonella is easily transmitted through the flocks and is difficult to eliminate from farms and incubators.

The presence of salmonella on broiler carcasses poses a risk to human health. From broiler carcasses (neck skin) and human cases S. Infantis was isolated, and the degree of mutual genetic similarity was 92%. Although there are differences in the antimicrobial resistance of human isolates and isolates from broilers, an epidemiological link can not be excluded because there is a genetic similarity of 100% in the dominant genotype SINFXB0001, which was isolated from diseased humans and broiler neck skin.

Despite the development of new molecular methods, PFGE remains the preferred method for salmonella and other foodborne pathogens type determination.

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# ANTIBIOTSKA REZISTENCIJA I MOLEKULARNA ISPITIVANJA *SALMONELLA ENTERICA* SUBSPECIES *ENTERICA* SEROVAR INFANTIS IZOLOVANIH U SLUČAJEVIMA OBOLJENJA LJUDI I SA TRUPOVA BROJLERA

RAŠETA Mladen, TEODOROVIĆ Vlado, BUNČIĆ Olivera, KATIĆ Vera, BRANKOVIĆ LAZIĆ Ivana, POLAČEK Vladimir, VIDANOVIĆ Dejan

Tokom 2012. godine sprovedena su istraživanja higijene procesa proizvodnje trupova brojlera, na tri klanice u Republici Srbiji. Ispitano je 150 uzoraka kožica vrata brojlera i utvrđeno je 17 izolata salmonela. Izolati su, korišćenjem odgovarajućih monovalentnih i polivalentnih seruma, tipizovani kao *Salmonella enterica* subspecies *enterica* serovar infantis (*S*. Infantis 6, 7, r, 1, 5). U slučajevima oboljenja ljudi, izolovano je 5 uzoraka salmonele identičnog serovarijeteta. Nakon toga, na 22 uzorka, rađeno je ispitivanje antibiotske rezistencije izolata disk difuzionim testom. Izolati su pokazali rezistentnost prema: ampicilinu i nalidiksičnoj kiselini (95,5%), tetraciklinu (91%), cefotaksim/klavulanskoj kiselini (68,2%), ali ne i prema ciprofloksacinu, gentamicinu i trimetoprim/sulfametoksazolu (0%). Stepen genetske sličnosti izolata poreklom od obolelih ljudi i sa trupova brojlera je određen molekularnim metodama. Analiza klastera je pokazala prisustvo 7 profila, dok svi izolati imaju 92% genetske sličnosti. Iako postoje razlike u antimikrobnoj rezistenciji izolata poreklom od obolelih ljudi i sa kožica vrata brojlera, ne može se isključiti epidemiološka povezanost, jer kod dominantnog genotipa SINFXB0001, utvrđenog kod 8 izolata poreklom od obolelih ljudi (3 izolata) i sa kožica vrata brojlera (5 izolata), postoji genetska sličnost od 100%. Na osnovu dobijenih rezultata, istraživanje je pokazalo da prisustvo *S*. Infantis na trupovima brojlera predstavlja hazard po zdravlje ljudi.