Prevalence of *Salmonella enterica* in slaughtered pigs in Serbia: Serotyping, PFGE-genotyping and antimicrobial resistance

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**Abstract**

Introduction: The aim of this study was to determine the prevalence of *Salmonella* along the slaughter line and to identify possible critical control points in one slaughterhouse facility located in the city of Belgrade.

Methodology: In total, 700 samples were tested: two swabs from both sides of carcass were taken from each of 100 pigs. In this way, 200 pig skin swab samples were taken after stunning, 200 after processing and 200 after chilling. Additional 100 samples of ileal contents were also taken from the same pigs to obtain a collection of 270 isolates. All samples were analyzed using standard culture methods and serotyping. PFGE was performed for 27 isolates. Determination of antimicrobial resistance was performed by E-test.

Results: In total, 47 (23.5%) swab samples were positive for the presence of *Salmonella* after stunning. After processing, *Salmonella* was isolated in two swab samples (1%), whereas all samples which were collected after chilling were negative for *Salmonella*. The sampling of ileal contents was positive for five *Salmonella* isolates (5%). The most frequently isolated serotypes were *S. Derby* (90.74%), *S. Infantis* (5.56%) and *S. Typhimurium* (3.7%). All tested isolates were resistant to tetracycline. Resistance was recorded to nalidixic acid (23.3%), ciprofloxacin (20%), ampicillin (10%) and chloramphenicol (14.4%), as well. The PFGE results indicated that isolates had a high genetic similarity.

Conclusions: The investigation has confirmed that bacteriological examinations of carcass swabs, as well as ileal content, could be used to assess the carriage of salmonellae in pigs at the time of slaughter.

**Key words:** pigs; *Salmonella*; antimicrobial resistance; PFGE.

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**Introduction**

Non-typhoidal *Salmonella* remains the leading cause of bacterial food-borne infections and continues to be a major problem, in terms of both morbidity and economic costs. Extensive use of antibiotics for preventive and therapeutic purposes in veterinary medicine or as growth promoters in animal feedstuffs, contributed to the emergence of resistant bacteria in animals, including the zoonotic and pathogenic microorganisms that may be transmitted via the food chain to humans [1,2].

The most common *Salmonella* serotypes in pigs are *S. Typhimurium* and *S. Derby* [3,4]. Control of this pathogen is essential in all production stages, in order to decrease the contamination levels in the final product [5]. Genetically similar isolates of *S. Typhimurium* and *S. Derby* were found in slaughterhouses and retail markets, implicating a common genetic background of the isolates and their spread through the food chain [6]. In Serbia, the percentage and prevalence of *Salmonella* serotypes isolated from different surfaces in lairage, stunning boxes and from pork carcasses were as follows: *Salmonella Typhimurium* 68.6% (48/70), *Salmonella Mbandaka* 17.1% (12/70), *Salmonella Senftenberg* 8.6% (6/70), *Salmonella Bredeney* 4.3% (3/70) and *Salmonella Menston* 1.4% (1/70) [7]. However, in Serbia, *Salmonella Enteritidis* considered the most significant foodborne *Salmonella* causing illness in humans, followed by *Salmonella Typhimurium* [8].

As no recent data are available about the prevalence of *Salmonella* in pigs after slaughter in Serbia, in order to overcome carcass contamination, it is crucial to identify the sources of contamination throughout the slaughter process.
The aim of this study was to determine the prevalence of *Salmonella* along the pig slaughter line and to identify possible critical control points for carcass contamination. In order for this aim to be achieved, the investigation encompassed *Salmonella* isolation from pig carcasses after stunning, processing and chilling, as well as from the ileal content. Serotyping, PFGE-genotyping and examination of antimicrobial susceptibility patterns were performed to reach the objectives.

**Methodology**

**Slaughter conditions**

The investigation was carried out in one pig slaughterhouse in the area of the city of Belgrade, where pigs from farms located in the different regions of Serbia are slaughtered. The slaughterhouse processes approximately 110 pigs per hour. Scalding was done by complete immersion of pigs in a scalding tank containing water heated at 61°C. Gas was used for a flaming and it was followed by polishing with rubber beaters and rotating brushes. After bung dropping, evisceration and veterinary inspection took place. During evisceration, no plastic bags were used to seal off the rectum. Splitting the carcasses was done by automatic carcass splitters.

**Sampling**

A total of 600 swabs from the slaughterhouse were collected from 100 pigs, during 10 weeks in summer period, covering 10 slaughter days. Two swabs from both sides of the carcass were taken from each pig from an area of approximately 10 cm × 100 cm. In this way, 200 pig skin swab samples were taken after stunning, 200 after processing and 200 after chilling. In addition, 100 samples of ileal contents were taken from the same pigs, aseptically collected immediately after evisceration and placed into separate sterile plastic bags.

Dry biocide-free 3.8 × 7.6 cm sponges in sample bags (3M Food Safety, Neuss, Germany) were used for sampling. Shortly before the taking of the samples, the sponges were moistened with 10-mL Maximum Recovery Diluent (MRD). In the laboratory, 90 mL buffered peptone water (Oxoid, Hampshire, UK) was added into the stomacher bag. The sponge was mixed in the stomacher bag for 2 minutes and incubated at 36°C (±1°C) overnight (16-20 hours).

**Salmonella isolation procedure and serotyping**

Horizontal method for the detection of *Salmonella* spp. according to the ISO 6579:2008 standard was used for *Salmonella* isolation [9]. From the same positive sample, five colonies were taken to make a collection of 270 isolates, which were subjected to biochemical and serological confirmation. Biochemical confirmation was done using API 20 E (bioMérieux, Marcy l’Etoile, France). All of the *Salmonella* isolates were identified by agglutination method according to the White Kauffman Le Minor [10] by the *Salmonella* Reference Laboratory at the Institute of Veterinary Medicine of Serbia. Commercial available antisera were used for serotyping (Institute of Public Health of Serbia “Dr Milan Jovanović Batut”, Belgrade, Serbia and Statens Serum Institute, Copenhagen, Denmark).

**Antimicrobial susceptibility testing**

In order to determine the Minimum Inhibitory Concentration (MIC), antimicrobial resistance was performed by E-test according to EUCAST (European Commission on Antimicrobial susceptibility testing) [6] recommendations using commercial E-tests (bioMérieux, Marcy l’Etoile, France) and Mueller-Hinton agar (Becton, Dickinson and Co, New Jersey, USA). The following E-tests were used: nalidixic acid (NAL) 0.016-256 µg/mL, ceftazidime (CAZ) 0.016-256 µg/mL, ciprofloxacin (CIP) 0.002-32 µg/mL, trimethoprim (TMP) 0.002-32 µg/mL, ampicillin (AMP) 0.016-256 µg/mL, chloramphenicol (CAP) 0.016-256 µg/mL, meropenem (MER) 0.002-32µg/mL, gentamicin (GEN) 0.016-256 µg/mL, tetracycline (TET) 0.016-256 µg/mL (bioMérieux, Marcy l’Etoile, France). The results were interpreted according to European Commission on Antimicrobial Susceptibility Testing Standards Institute (Version 5.0, 2015) recommendations as sensitive or resistant [11].

**PFGE**

Twenty isolates of S. Derby, three of S. Infantis and four of S. Typhimurium were genotyped. PFGE was carried out in 1% SeaKem Gold Agarose gel (Lonza, Rockland, USA) after the digestion of genomic DNA with the restriction enzyme *XbaI* (Fermentas, Vilnius, Lithuania) according to the Pulse-Net protocol [12]. Fragment patterns were documented with the GelDoc system and analyzed with GelDoc and with FPQuest software (Bio-Rad, California, USA). To generate the name and nomenclature of the derived genotypes, recommendations by Tenover et al. were used [13]. Briefly, the profiles were assigned codes which were 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. For data tabulation Microsoft Excel 2007 was used.
Results

In total, 47 (23.5%) swab samples from the 200 tested were positive for the presence of *Salmonella* after stunning. After processing, *Salmonella* was isolated in two swab samples (1%), whereas all samples which were collected after chilling were negative for *Salmonella*. Five *Salmonella* isolates were obtained from ileal contents (5%).

The incidence of isolated *Salmonella* serotypes in pig samples is shown in Table 1. Only three serotypes of *Salmonella* were determined in the slaughterhouse. The most frequently isolated serotype was *S*. Derby (90.74%). Other serotypes that were isolated from the pig samples were *S*. Infantis (5.56%) and *S*. Typhimurium (3.7%) (Table 1).

Resistotyping was determined for 30 isolates from the examined samples. Accordingly, the results are presented in Table 2. All tested isolates were resistant to tetracycline. Resistance was recorded to nalidixic acid (23.3%), ciprofloxacin (20%), chloramphenicol (14.4%) and ampicillin (10%) according to the epidemiological breakdowns.

In the case of *S*. Derby, two PFGE profiles were detected with mutual similarity of 98%. The first profile SDERXB0001 to which isolates 13, 31, 46, 55, 65, 79, 111, 116, 125, 137, 142, 151, 155, 159, 164, 168, 171, 178 belonged and the second profile SDERXB0002 to which isolates 4 and 10 belonged with 100% genetic similarity between isolates. All three isolates (101, 22, 43) of *S*. Infantis belonged to one profile i.e. SINFXB0001. In the case of *S*. Typhimurium, one profile i.e. STYPXB0001 was observed to which all four isolates belonged (Figure 1).

Discussion

Accurate detection of *Salmonella* spp. in food provides an opportunity to prevent the contaminated food from entering the food supply. In this study, *Salmonella* was isolated from 47 (23.5%) swab samples after stunning. In the investigation done by Karabasil *et al.* [14], 46.7% of examined pig carcasses were positive on for the presence of *Salmonella* after stunning as well. These results suggest the possibility that many pigs had became contaminated during the slaughter process by cross contamination which is especially noticeable in bad hygienic conditions in slaughterhouse lairage. A significantly lower number of carcasses detected to be positive for *Salmonella* after processing, indicate the importance of using good hygiene and manufacturing practices in slaughterhouses. The occurrence of *Salmonella* in pig carcasses after processing is different from country to country. The reported percentage of the occurrence was 6% in Italy [15], 0.2% in Switzerland [16], 5.3% in Great Britain [17] and 4.7% in Germany [18] during the period from 2000 to 2003.

After 24h of chilling no *Salmonella* were found on the examined carcasses in this study. This can be attributed to low temperature and decreased water activity due to the air flow in the cooling room. Unlike minced meat, which contains fat and can protect *Salmonella* from low temperatures, skin surface dries quickly, which is not favorable for *Salmonella*[19].

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Table 1. Results of serological typing of *Salmonella* spp.

| Serotype            | Total No. of samples | Total No. of isolates | No. of isolates after stunning | No. of isolates after processing | No. of isolates after cooling | No. of isolates from ileal content |
|---------------------|----------------------|-----------------------|-------------------------------|----------------------------------|-------------------------------|-----------------------------------|
|                     | n        | %     | n   | %     | n   | %     | n   | %     | n   | %     |
| *S*. Derby          | 49       |       | 245 | 90.74 | 220 | 81.48 | 10  | 3.7   | 0   | 0     | 15  | 5.56  |
| *S*. Infantis       | 3        |       | 15  | 5.56  | 15  | 5.56  | 0   | 0     | 0   | 0     | 0   | 0     |
| *S*. Typhimurium    | 2        |       | 10  | 3.70  | 0   | 0     | 0   | 0     | 0   | 0     | 10  | 3.7   |

Table 2. Resistance of *Salmonella* to antibiotics.

| Antibiotic                  | No. of tested isolates | Sensitive isolates | Resistant isolates |
|-----------------------------|------------------------|--------------------|-------------------|
|                             | No. | (%)   | No. | (%)   |
| Nalidixic acid 0.016-256 mg/L | 30  | 23    | 7   | 23,3  |
| Ceftazidime 0.016-256 µg/mL  | 30  | 30    | 100 | 0     |
| Ciprofloxacin 0.002-32 µg/mL  | 30  | 24    | 80  | 6     | 20   |
| Trimethoprim 0.002-32 µg/mL  | 30  | 30    | 100 | 0     |
| Ampicillin 0.016-256 µg/mL   | 30  | 27    | 90  | 3     | 10   |
| Chloramphenicol 0.016-256 µg/mL | 30  | 26    | 86,6| 4     | 14,4 |
| Meropenem 0.002-32µg/mL      | 30  | 30    | 100 | 0     |
| Gentamicin 0.016-256 µg/mL   | 30  | 30    | 100 | 0     |
| Tetracycline 0.016-256 µg/mL | 30  | 0     | 0   | 30    | 100  |
Salmonella was found in five samples of the ileal content in our investigation. In such cases inapparent carriers can contaminate surfaces in lairage, thus becoming a source for infection to other animals.

In total, three serotypes of Salmonella were determined in the slaughterhouse in this study. S. Derby was isolated most frequently (90.74%) and thereafter S. Infantis (5.56%) and S. Typhimurium (3.7%). In the European Union, the most frequent serotype in pigs is S. Typhimurium (57%), and then S. Derby (10.4%), S. Bovismorbificans (3.2%), S. Infantis (2.9%) and S. Branderburg (2%) [20].

As the use of numerous antibiotics both in human and veterinary medicine (gentamicin, ampicillin, amoxicillin) as well as the use of growth promoters in intensive pig farming and poultry production has favored the development of resistance in some bacteria, the European Union Regulation (EC) No. 1831/2003 prohibits the use of antimicrobial agents such as additives for animal feed, since January 2006 [21].

In our investigation, all isolates were resistant to tetracycline. The resistance was recorded to nalidixic acid (23.3%), ciprofloxacin (20%), ampicillin (10%) and chloramphenicol (14.4%). In another investigation in Serbia, 25% Salmonella isolated from pigs carcasses have shown resistance to amoxicillin and sulfamethoxazole [22]. There are also data about the clonal spread of S. Infantis in Serbia where mutations in the topoisomerase genes the gyrA and parC lead to increased resistance to fluoroquinolones [23]. The antibiotic resistance patterns in Salmonella isolates from food producing animals in Austria have shown that 42% of isolates were resistant to fluoroquinolones, 33% to tetracycline, 27% to streptomycin and 17% to ampicillin[24]. However, there has been a trend towards the emergence of multiple resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, sulfonamides, and tetracyclines in certain serotypes in recent years [25], and such resistance patterns were partially confirmed in our investigation.

In our investigation, S. Derby was the most frequently isolated serotype and two PFGE profiles were observed. It is known that S. Derby is one of the most prevalent serovars in pigs in Europe and the U.S. and ranks among the top 10 serovars in humans. S. Derby clone is often isolated from pigs and humans in Germany and contaminated pork was identified as a

Figure 1. PFGE patterns obtained by XbaI restriction enzyme of S. Derby, S. Infantis and S. Typhimurium derived from FPQuest program that shows similarity coefficients (Dice coefficient, UPGMA) between the tested isolates.
possible vehicle for the transmission from animals to humans [26].

Conclusion
In this study, no Salmonella were isolated on pig carcasses after chilling, which is very important from the aspect of food safety for the consumers. The Salmonella status of pigs at the time of slaughter and the associated risk of dissemination of Salmonella organisms can be assessed by bacteriological examinations which may, according to the results of this study, include carcass swabs as well as ileal content. Also, all tested isolates were resistant to tetracycline. Multiple resistance was confirmed to nalidixic acid, ciprofloxacin, ampicillin and chloramphenicol in three isolates. The PFGE results indicated that tested isolates had high genetic similarity which suggests that the lairage area and/or the transportation vehicle are a primary source of Salmonella contamination in slaughter pigs.

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References
1. Tollefson L, Miller MA (2000) Antibiotic use of food animals: controlling the human health impact. J AOAC Int 83: 245–254.
2. Su LH, Chiu CH, Chu C, Ou JT (2004) Antimicrobial resistance in nontyphoidal Salmonellaserotypes: A global challenge. Clin Infect Dis 39: 546–551.
3. De Busser EV, Maes D, Houf K, Dewulf J, Imberechts H, Bertrand S, De Zutter L (2011) Detection and characterization of Salmonella in lairage, on pig carcasses and intestines in five slaughterhouses. Int J Food Microbiol 145: 279–286.
4. European Food Safety Authority (2015) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014, EFSA Journal 13: 4329.
5. Sheridan JJ (1998) Sources of contamination during slaughter and measures for control. J Food Saf 18: 321–339.
6. Cai Y, Tao J, Jiao Y, Fei X, Zhou L, Wang Y, Zheng H, Pan Z, Jiao X (2016) Phenotypic characteristics and genotypic correlation between Salmonella isolates from a slaughterhouse and retail markets in Yangzhou, China. Int J Food Microbiol 222: 56-64.
7. Karabasil N, Dimitrijević M, Kilibarda N, Gašić N, Petrović J (2012a) Salmonellaserotype prevalence in two pig slaughterhouses. Proceedings of the International Conference: Biological Food safety & Quality, BFSQ 2012, Belgrade, Serbia, 4-5 October 2012, 64-67.
8. The Institute of Public health of Serbia “Dr Milan Jovanovic Batut” (2011) Health statistical yearbook of Republic of Serbia 2010. Available: http://www.batut.org.rs/download/publikacije/pub2010.pdf. Accessed on 10 October 2011.
9. Microbiology of food and animal feeding stuffs - Horizontal method for the detection of Salmonella spp. EN ISO 6579: 2008.
10. Popoff MY (2001) Antigenic formulas of the Salmonella serovars, 8th revision. Paris: Institute Pasteur 166 p.
11. European Committee on Antimicrobial Susceptibility Testing (2015) Breakpoint Tables for Interpretation of MICs and Zone Diameters, Version 5.0. Available: http://www.eucast.org. Accessed on 1 January 2015.
12. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, Barrett TJ (2006) Standardization of pulsed-field gel electrophoresis protocols for the subtyping of Escherichia coli O157:H7, Salmonella and Shigella for PulseNet. Foodborne Pathog Dis 3: 59–67.
13. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B (1995) Interpreting chromosomal DNA restriction patterns produced, by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. J Clin Microbiol 33: 2233–2239.
14. Karabasil N, Pavličević N, Gašić N, Dimitrijević M, Lončina J, Ivanović J, Baltić M (2012b) Salmonella on pig carcasses during slaughter and processing. Vet glasnik 66: 377-386.
15. Bonardi S, Brindani F, Pizzin G, Lucidi L, Incau MD, Liebana Morabito S (2003) Detection of Salmonella spp., Yersinia enterocolitica and Verocytotoxin Escherichia coli O 157 in pigs at slaughter in Italy. Int J Food Microbiol 85: 101-110.
16. Sauli I, Danuser J, Wenk C, Stähr K (2003) Evaluation of the safety assurance level for Salmonella spp. throughout the food production chain in Switzerland. J Food Prot 66: 1139-1145.
17. Davis R, Paiba G, Evans S, Dalziel B (2000) Surveys for Salmonella in pigs, cattle and sheep at slaughter in Great Britain. Vet Rec 147: 695.
18. Káshbohrer A, Prozt D, Helmuth R, Nöckler K, Blaha T, Conraths FJ, Geue L (2000) Salmonella slaughtered pigs of German origin: an epidemiological study. Eur J Epidemiol 16: 141-146.
19. Karabasil N, Theodorović V, Dimitrijević M, Pavličević N, Kurelujić J, Đurić S, Sočo I, Savčić Radovanović R (2013) Behavior of Salmonella Typhimurium in pork minced meat and pork skin at different storage temperatures. Acta Vet 63: 655-663.
20. EC (European Commission) (2002) Trends and sources of zoonotic agents in animals, feedstuffs, food and man in the European Union and Norway to the European Commission in accordance with Article 5 of the Directive 92/117/EEC, prepared by the Community Reference Laboratory on the Epidemiology of Zoonoses, BgVV, Berlin, Germany. Working document SANCO/927/2002: 1: 45-122.
21. European Union (2003) Regulation (EC) No. 1831/2003 of the European Parliament and the council of 22 September 2003 on additives for use in animal nutrition. Off J Eur Union 268: 29-43.
22. Petrović J, Milanov D, Ratajac R (2008) Contemporary food safety trends: antimicrobial resistance in zoonotic pathogens. Vet glasnik 62: 373-382.
23. Velhner M, Kozoderović G, Grego E, Gašić N, Stojanov I, Jelesić Z, Krehnenberg C (2014) Clonal spread of Salmonella enterica serovar Infantis in Serbia: Acquisition of mutations in the topoisomerase Genes gyrA and parC leads to increased resistance to fluoroquinolones. Zoonoses Public Health 61: 364-370.
24. Mayrhofer S, Paulsen P, Smulders F, Hilbert F (2004): Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, pork and poultry. Int J of Food Microbiol 97: 23-29.

25. Gebreyes WA, Altier C (2002) Molecular characterization of multidrug-resistant Salmonella enterica subsp. enterica serovar Typhimurium isolates from swine. J Clin Microbiol 40: 2813–2822.

26. Hauser E, Hebner F, Tietze E, Helmuth R, Junker E, Prager R, Schroeter A, Rabsch W, Fruth A, Malorny B (2011) Diversity of Salmonella enterica serovar Derby isolated from pig, pork and humans in Germany. Int J Food Microbiol, 151: 141-149.

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