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Original Article

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 frompork carcasses were as follows: *Salmonella* Typhimurium68.6% (48/70), *Salmonella* Mbandaka17.1% (12/70), *Salmonella* Senftenberg8.6% (6/70), *Salmonella* Bredeney 4.3% (3/70) and *Salmonella* Menston 1.4%(1/70) [7]. However, in Serbia, *Salmonella* Enteritidisis 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) 0.016-256µg/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.

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

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 *Salmonell*a 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 breakpoints.

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].

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	76,7	7	23,3
Ceftazidime 0.016-256 µg/mL	30	30	100	0	0
Ciprofloxacin 0.002-32 µg/mL	30	24	80	6	20
Trimethoprim 0.002-32 µg/mL	30	30	100	0	0
Ampicillin 0.016-256 µg/mL	30	27	90	3	10
Chloramphenicol 0.016-256µg/mL	30	26	86,6	4	14,4
Meropenem 0.002-32µg/mL	30	30	100	0	0
Gentamicin 0.016-256 µg/mL	30	30	100	0	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.

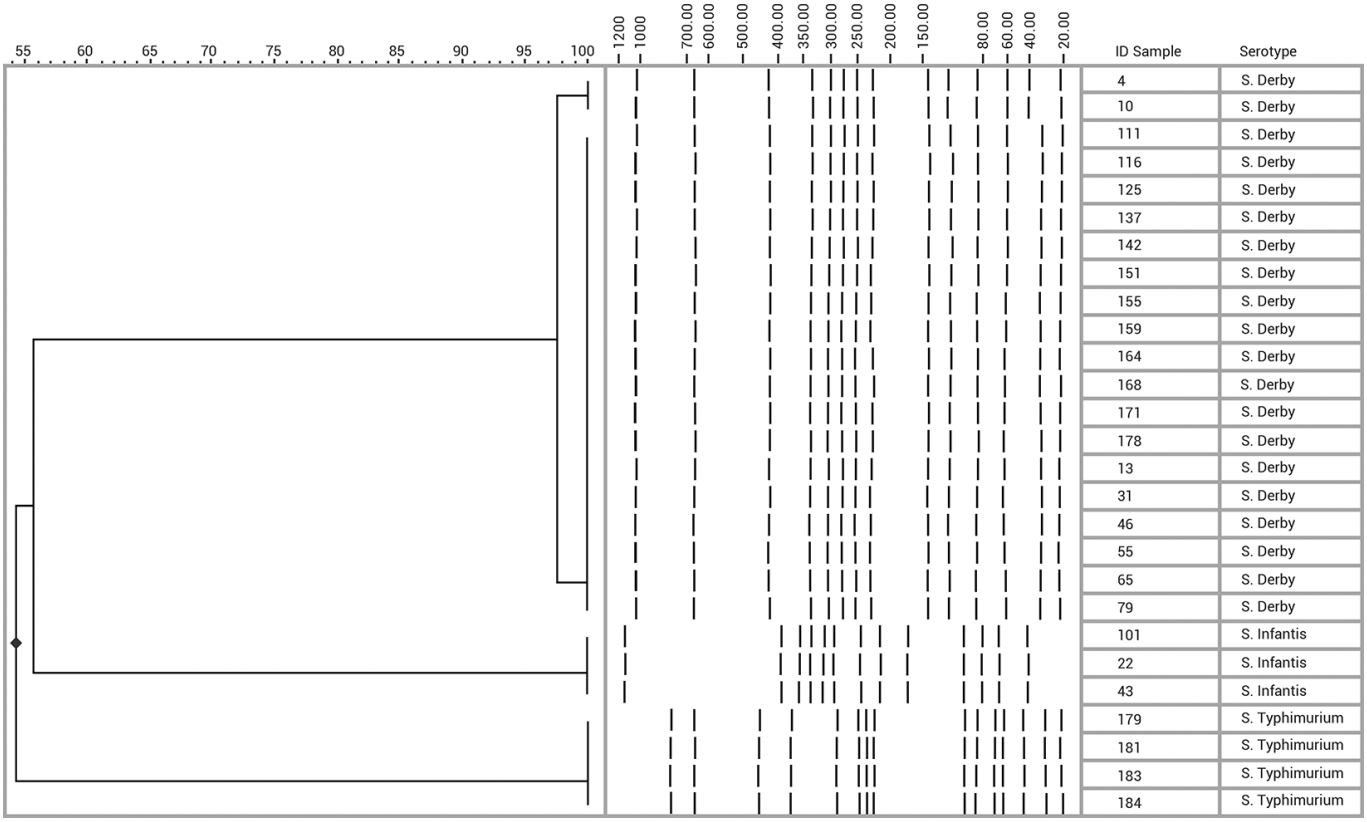
In total, three serotypes of *Salmonella* were determined in the slaughterhouse in this study. *S.* Derby was isolated most frequently (90.74%) and thereafter *S.* Infantis (5.56%) and *S.* Typhimurium (3.7%). In the European Union, the most frequent serotype in pigs is *S.* Typhimurium (57%), and then *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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