*Brief Original Article*

# Occurrence and antimicrobial susceptibility of *Salmonella* isolates recovered from the pig slaughter process in Romania

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## Abstract

Introduction: Reported human salmonellosis cases have increased in Romania. Antibiotic susceptibility testing of *Salmonella* strains isolated from pork and chicken meat indicate a worrying multidrug resistance pattern. This study aimed to investigate the occurrence of *Salmonella* and to evaluate the antibiotic resistance of *Salmonella* strains in a pig slaughterhouse-processing complex, which receives animals from 30% of the large industrialized swine farms in Romania.

Methodology: A total of 108 samples, including pork (n = 47), packaged pork products (n = 44), scald water sludge (n = 8), and detritus from the hair removal machine of the slaughterhouse (n = 9) were examined for the presence of *Salmonella* through standard methods. The antibiotic susceptibility of the isolated strains to 17 antibiotics was tested using the Vitek 2 system.

Results: Twenty-six (24.1%) samples were found to be *Salmonella* positive; this included 25.5% of meat samples and 15.9% of packaged products, as well as samples from two different points of the slaughter (41.2%). Resistance was observed against tetracycline (61.5%), ampicillin (50%), piperacillin (50%), trimethoprim-sulfamethoxazole (34.6%), amoxicillin/clavulanic acid (26.9%), nitrofurantion (23.1%), cefazolin (15.4%), piperacillin/tazobactam (7.7%), imipenem (3.8%), ciprofloxacin (3.8%), and norfloxacin (3.8%). No resistance towards cefoxitin, cefotaxime, ceftazidime, cefepime, amikacin, and gentamicin was found.

Conclusions: Our study demonstrated the occurrence of multidrug-resistant *Salmonella* strains in the investigated pork production complex and highlighted it as a potential source of human infections. The results demonstrate the seriousness of antibiotic resistance of *Salmonella* in Romania, while providing a useful insight for the treatment of human salmonellosis by specialists.

**Key words:** *Salmonella*; slaughterhouse; pig meat; multidrug resistance.

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## Introduction

Members of the genus *Salmonella* are recognized to be one of the most important zoonotic pathogens, causing serious foodborne illnesses in humans worldwide. Human salmonellosis outbreaks are related to the consumption of contaminated foodstuffs of animal origin, and the disease is characterized by acute gastroenteritis, fever, abdominal pain, and nausea [1,2].

Based on a recent European Union summary report, *Salmonella* has been the leading cause (26.6%) of reported foodborne outbreaks in 2011, with 95,548 confirmed human clinical cases [2]. The results of several *Salmonella* surveys, carried out in pig slaughtering and processing plants, have shown that fresh pig meat and pork products thereof can be considered an important vehicle for the transmission of *Salmonella* to humans [3-6]. The application of misused and prolonged antibiotic treatments in farm animals with therapeutic and prophylactic purposes can lead to the development of antimicrobial-resistant commensals and/or zoonotic foodborne bacterial pathogens, including *Salmonella* [7]. This undesired consequence is frequently associated with the transmission of resistant bacteria populations to humans, which may pose significant therapeutic challenges for clinicians treating diseases. Therefore, the continuous surveillance of the antimicrobial resistance of zoonotic bacterial strains such as *Salmonella* isolated from food and production environments can contribute greatly to a successful control program.

Swine breeding under an intensive production system is considered to be the most important economic sector of the pig industry in Romania. The survey of the occurrence and antibiotic resistance

pattern of *Salmonella* strains isolated from pork is limited to only one study carried out in various regions of the country [6]. Therefore, additional studies in this regard are still needed.

The aim of this study was to investigate the occurrence of *Salmonella* and to evaluate the antibiotic resistance of *Salmonella* strains isolated in a pig slaughterhouse-processing plant complex, which receives animals from 30% of the large industrialized swine farms in Romania.

## Methodology

In the first 24 hours after slaughter, randomly selected pig meat samples provided from different carcass regions (n = 37), including haunches (n=4), sow flaps (n = 4), and tongues (n = 2) in the cutting and deboning room of the slaughterhouse were collected during several sampling days. In addition, packaged products were also provided by the processing plant, which included minced meat (n = 14), traditional Romanian peasant sausages (n = 15), and mici pasta (n = 15). Sampling was performed weekly for a period of six months. Sludge from the water used in the scalding process (n = 8) (a vertical scalding operation point with regular changing of the scald water) and detritus from a hair-removing machine (n = 9) of the slaughterhouse were also collected on different sampling days and further processed for *Salmonella* detection.

The samples were individually sterile packed, stored under refrigeration and transported to the laboratory of Food Hygiene of the Faculty of Veterinary Medicine Timişoara, Romania. All samples were processed on the day of arrival and *Salmonella* strains were isolated according to the ISO 6579/2006 standard.

Briefly, for pre-enrichment, 25 g of each sample was homogenized in a Stomacher (bioMérieux, Marcy l'Etoile, France) (90 seconds) with 225 mL of peptone buffered solution (PBS) and incubated at 37°C for 18 hours. Next, 0.1 mL aliquot from the pre-enriched culture was transferred to 10 mL of Rappaport-Vassiliadis (Biokar Diagnostics, Beauvais, France) broth and incubated at 41.5°C for 24 hours. At the same time, 1 mL of the pre-enriched culture in PBS was further cultured on Müller-Kauffmann Tetrathionate Novobiocin Broth (MKTTn; Biokar Diagnostics) selective enrichment medium at 37°C for 24 hours. Next, the samples were streaked onto solid xylose lysine deoxycholate (XLD; Biokar Diagnostics) and Rambach (Biokar Diagnostics) mediums and incubated again at 37°C for 24 hours. *Salmonella* colonies from each positive plate were selected, transferred and cultured on brain-heart infusion (Biokar Diagnostics) agar at 37°C for 24 hours. After growth, the isolates were confirmed to be *Salmonella* on the basis of their biochemical properties including indole, hydrogen sulphide and urease production, the fermentation of glucose and lactose, and lysine decarboxylation. The serological properties were tested using the *Salmonella* antisera O poli group (Statens Serum Institut, Copenhagen, Denmark).

Additionally, all strains were molecularly tested for the presence of the *Salmonella*-specific *invA* gene (~285 bp) through the polymerase chain reaction (PCR) protocol according to Rahn *et al.* (1992) with slight modifications [8]. Amplification products were analyzed on 1.5% agarose gel stained with ethidium bromide.

Antimicrobial susceptibility of the isolated *Salmonella* strainsto 17 antibiotics from eight classes of antibiotics including penicillins, cephalosporins, aminoglycosides, carbapenem, quinolones, tetracyclines, nitrofurans, and sulfonamides was determined using the Vitek 2 automated system (bioMérieux, Marcy-l'Etoile, France). The AST-GN27 card was used. The tested antibiotics were ampicillin (minimum inhibitory concentration [MIC] range 2–32 μg/mLl), amoxicillin/clavulanic acid (1–32 μg/mL), piperacillin (4–128 μg/mL), piperacillin/tazobactam (1–32 μg/mL), cefazolin (4–64 μg/mL), cefoxitin (4–64 μg/mL), cefotaxime (1–64 μg/mL), ceftazidime (1–64 μg/mL), cefepime (1–64 μg/mL), amikacin (2–64 μg/mL), gentamicin (1–16 μg/mL), imipenem (0.25–16 μg/mL), ciprofloxacin (0.25–4 μg/mL), norfloxacin (16–512 μg/mL), tetracycline (1–16 μg/mL), nitrofurantion (0.5–16 μg/mL), and trimethoprim/sulfamethoxazole (20–320 μg/mL). After being automatically processed by the Vitek 2 system, the isolates were categorized as sensitive, intermediate, or resistant to the tested drugs.

Statistical analyses were performed using the online version of VassarStats software (http://vassarstats.net/prop1.html).

## Results

Overall, 26 (24.1%; 95% confidence interval [CI] 16.5%–33.4%) of the 108 collected samples were found to be *Salmonella* positive through standard examination methods. All isolated bacterial strains were successfully amplified targeting the *invA* gene, confirming the results of the standard method. The obtained PCR products showed typical profiles for *Salmonella* genus in the 1.5% agarose gel. The

distribution of the isolated *Salmonella* strains according to their origin is summarized in Table 1. *Salmonella* was recovered from pork samples provided from tongues (1/2; 50%), carcasses (10/37; 27%), and sow flaps (1/4; 25%), but no bacteria were found in haunches. From the packaged meat products, *Salmonella* was isolated from peasant sausages (4/15; 26.6%) and minced meat (3/14; 21.4%), while the mici pasta was found to be *Salmonella*-free. With regards to sludge and detritus collected from two different operation points of the slaughterhouse, *Salmonella* was isolated from both scalding (1/8; 12.5%) and hair removal (6/9; 66.6%) points.

The results of antibiotic resistance and susceptibility patterns of the isolated *Salmonella* strains are presented in Table 2. The Vitek 2 system proved to be an accurate, rapid (18 hour), and relatively easy test to use for the determination of antimicrobial susceptibility of the bacterial strains. Multidrug resistance was a common finding; 22/26 (84.6%; CI 64.3–94.7) bacterial strains showed resistance towards at least two tested antimicrobials. Drug resistance was observed in descending order towards tetracycline (16/26 isolates; 61.5%; CI 40.7–79.1), ampicillin (13/26; 50%; CI 30.4–69.6), piperacillin (13/26; 50%; CI 30.4–69.6), trimethoprim-sulfamethoxazole (9/26; 34.6%; CI 17.9–55.6), amoxicillin/clavulanic acid (7/26; 26.9%; CI 12.3–48.1), nitrofurantion (6/26; 23.1%; CI 9.7–44.1), cefazolin (4/26; 15.4%; CI 5.0–35.7), piperacillin/tazobactam (2/26; 7.7%; CI 1.3–26.6), imipenem (1/26; 3.8%; CI 0.2–21.6), ciprofloxacin (1/26; 3.8%; CI 0.2–21.6), and norfloxacin (1/26; 3.8%; CI 0.2–21.6). No resistance was found towards cefoxitin, cefotaxime, ceftazidime, cefepime, amikacin, and gentamicin. In addition, intermediate resistance was observed towards nitrofurantion (12/26; 46.1%; CI 27.1–66.2), piperacillin/tazobactam (5/26; 19.2%; CI 7.3–39.9), tetracycline (3/26; 11.5%; CI 3.0–31.3), and cefoxitin (2/26; 7.7%; CI 1.3–26.6).

## Discussion

This is the first report about the occurrence and antibiotic susceptibility testing of *Salmonella* isolates recovered from the slaughter process in Romania. The current survey confirmed the presence of *Salmonella* strains in pork samples (12/47; 25.5%), packaged meat products (7/44; 15.9%), and at two different points along the swine slaughter line (7/17; 41.2%). The relatively high contamination rate (19/91; 20.9%; CI 13.3–30.9) with *Salmonella* of pig meat and products recorded in the current survey pointed out that these foodstuffs can be considered an important vehiclefor *Salmonella* transfer from animals to humans.

Results of other studies that aimed to detect *Salmonella* in fresh pig meat and pork products provided by slaughterhouses and processing plants have largely varied, depending on the study design, number of enrolled samples, or sensitivity of the diagnostic tests used. In accordance with results obtained in the current survey, data reported in the most recent European Union surveillance program showed the presence of *Salmonella* in fresh pig meat provided by slaughterhouses in Slovakia (3.3%) and Spain (7.5%). Also, *Salmonella* surveys conducted at cutting and processing plants yielded positive findings in Hungary (3.0%), Portugal (5.0%), Italy (0.7%) and Estonia (0.4% and 0.9%). In contrast, no *Salmonella*-positive samples were detected in pig meat provided from a slaughterhouse in Bulgaria and from cutting and processing plants in Finland [2].

**Table 1.** Distribution of the isolated *Salmonella* strains according to their origin

| Sample origin | Isolation source | No. of enrolled samples | No. of *Salmonella*-positive samples (%) | 95% CI |
|---|---|---|---|---|
| Fresh meat | Carcass | 37 | 10 (27.0) | 14.3–44.4 |
| | Haunch | 4 | 0 | 0.0–60.4 |
| | Sow flaps | 4 | 1 (25.0) | 1.3–78.1 |
| | Tongue | 2 | 1 (50.0) | 2.6–97.3 |
| Packaged meat products | Minced meat | 14 | 3 (21.4) | 5.7–51.2 |
| | Peasant sausages | 15 | 4 (26.6) | 8.9–55.2 |
| | Mici pasta | 15 | 0 | 0.0–25.4 |
| Scalding point | Sludge | 8 | 1 (12.5) | 0.7–53.3 |
| Hair removal point | Detritus | 9 | 6 (66.6) | 30.9–91.0 |
| **Total** | | **108** | **26 (24.1)** | **16.6–33.4** |

**Table 2.** Antimicrobial resistance and susceptibility pattern of the isolated *Salmonella* strains

| Antimicrobial | | No. of the tested strains | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Group** | **Agent** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **1** | **2** | **3** | **4** | **5** | **1** | **2** | **3** |
| Penicillins | Ampicillin | R | S | R | R | R | R | S | R | R | R | R | R | R | S | S | S | S | S | R | S | S | S | S | R | S | S |
| | Amoxicillin/ clavulanic acid | S | S | R | R | R | S | S | R | R | R | S | S | S | S | S | S | S | S | R | S | S | S | S | S | S | S |
| | Piperacillin | R | S | R | R | R | R | S | R | R | R | R | R | R | S | S | S | S | S | R | S | S | S | S | R | S | S |
| | Piperacillin/ tazobactam | S | S | R | R | I | I | S | S | S | S | I | I | I | S | S | S | S | S | S | S | S | S | S | S | S | S |
| Cephalosporins | Cefazolin | S | S | S | S | S | S | S | R | R | R | S | S | S | S | S | S | S | S | R | S | S | S | S | S | S | S |
| | Cefoxitin | S | S | S | S | S | S | S | S | S | I | S | S | S | S | S | S | S | S | I | S | S | S | S | S | S | S |
| | Cefotaxime | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| | Ceftazidime | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| | Cefepime | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| Aminoglycosides | Amikacin | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| | Gentamicin | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| Carbapenem | Imipenem | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R |
| Quinolones | Ciprofloxacin | S | S | S | S | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| | Norfloxacin | S | S | S | S | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| Tetracyclines | Tetracycline | S | R | R | R | R | R | R | I | I | I | R | R | R | R | R | R | R | R | S | S | R | S | S | S | S | R |
| Nitrofurans | Nitrofurantion | S | I | R | R | R | I | S | I | S | S | I | I | I | I | S | I | I | S | R | S | I | S | I | R | I | R |
| Sulfonamides | Trimethoprim-sulfamethoxazole | S | R | S | S | S | S | R | S | S | S | S | S | S | R | R | R | R | R | I | S | R | S | R | S | S | S |

R: resistant; S: susceptible; I: intermediate

In Romania, in a study conducted in various regions by Mihaiu *et al.* (2014), 23.7% (48/208) of the tested pork samples provided by production units and retail markets were found to be *Salmonella* positive, but without any information regarding the territorial distribution of the positive samples [6]. Also, occurrence of *Salmonella* in pork was reported by Gomes-Neves *et al.* (2012) in 14/100 samples (14%) provided from cutting and deboning rooms of abattoirs in Portugal [5], and by Delhalle *et al.* (2009) in cutting plants (90/379; 23.7%) in Belgium [9]. In our study, in accordance with the current microbiological EU standards [10,11], unacceptable *Salmonella*-positive findings were recorded in minced meat (3/14; 21.4%) and traditional Romanian peasant sausages (4/15; 26.6%). Compared to our results, in a survey conducted by Modzelewska-Kapituła and Maj-Sobotka (2014) in Poland, no *Salmonella*-positive samples were recorded in porcine minced meat, but the bacteria were detected in 0.7% (2/305) of the fresh pork sausages examined [12]. Also, Delhalle *et al*. (2009) reported the presence of *Salmonella* ranging from 0.3% to 4.3% in porcine minced meat from Belgium [9], and Mürmann *et al*. (2009) reported that *Salmonella* was found in 24.4% (82/336) of fresh pork sausages from Brazil [13]. These results highlight the need to improve the quality of raw materials provided by pork slaughterhouses in a screened complex, with particular emphasis on the most important parameters and procedures in each stages, such as time-temperature couples, handling, or general hygiene [9,14].

Considering that *Salmonella* on the skin of pigs entering the slaughter line is a common finding [3,15], the low *Salmonella* isolation rate recorded at the scalding point of the investigated slaughterhouse suggests the efficiency of scalding in *Salmonella* reduction, as was previously demonstrated by Davies *et al.* (1999). On the other hand, contrary to what would be expected, a relatively high frequency isolation rate of *Salmonella* was observed in the detritus from the hair removal equipment. As described by various authors [1,16], carcass contamination during the hair removal process can occur, especially through the squeezing of feces from the anus, followed by bacterial proliferation in the detritus and subsequent contamination of carcasses. Regarding this issue, significant improvement of the processing technology at this level should be encouraged and implemented.

Antimicrobial susceptibility testing showed multidrug resistance towards at least two tested antimicrobials for 22/26 (84.6%) isolated and tested *Salmonella* strains. Only three strains were susceptible to all tested antibiotics and another strain showed resistance towards one drug (Table 2). Antimicrobial resistance towards 11 tested drugs was observed, with a variable proportion from 3.8% to 61.5%. High resistance levels to tetracycline (61.5% of the isolates), ampicillin (50%), piperacillin (50%) and trimethoprim-sulfamethoxazole (34.6%) were found. In agreement with our results, the highest level of resistance to tetracycline has been reported in Portugal (70%; 17), Italy (56%; 18) and Brazil (70.7%; 13) for *Salmonella* strains isolated from different products and levels of the pre- and post-harvest pig meat chain. Also, high resistance frequency was obtained in various studies for ampicillin [7,17,19] and sulfonamides compounds [13,17-19]. Moreover, marked resistance of pig meat origin *Salmonella* isolates towards ampicillin (56.2%), sulfonamides (54.5%), and tetracyclines (52.8%) was published by the European Food Safety Authority (EFSA) in its most recent summary report [20]. It is important to mention that in Romania, in a previous study conducted by Mihaiu *et al.* (2014), similar to our findings, high resistance levels were obtained for tetracycline, sulphonamides, ampicillin, and trimethoprim [6]. These results suggest the possible over-usage of these drugs in the pig breeding industry in Romania. On the other hand, the lack of the resistance to cephalosporins (cefoxitin, cefotaxime, ceftazidime, cefepime) and aminoglicosides (amikacin, gentamicin) can provide useful insight for the treatment of human salmonellosis by public health specialists.

Although the recorded resistance rates of the isolated *Salmonella* strains to amoxicillin/clavulanic acid (26.9%), nitrofurantion (23.1%), cefazolin (15.4%), piperacillin/tazobactam (7.7%), imipenem (3.8%), ciprofloxacin (3.8%), and norfloxacin (3.8%) were moderate or relatively low, it is noteworthy that the range of drugs to which resistance was acquired is wide and worrying enough, showing the seriousness of the emergence of this pathogen's antibiotic resistance.

## Conclusions

Multidrug-resistant *Salmonella* strains in the slaughterhouse and processing phases of the pig meat chain of the investigated meat production complex were found to be a potential source for human infections. The antimicrobial resistance pattern showed increased resistance to some antibiotics, but decreased or a lack of resistance to others, suggesting

some value in the treatment of salmonellosis in humans. However, taking into account the methodological limitations of the current survey and in order to have a more comprehensive understanding of the epidemiology of *Salmonella* infections, including the complexity of transmission or the presence of clinically relevant strains, further studies based on serotyping on a larger scale are still needed. On the other hand, even if the number of enrolled samples is limited, the results of the present study indirectly support the increasing trend of reported human salmonellosis cases since 2007 in Romania [2].

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