UDC 619:579.842.14:637:615.33

DOI 10.29326/2304-196X-2018-4-27-3-7

IDENTIFICATION AND ANTIMICROBIAL RESISTANCE OF *SALMONELLA* ISOLATES

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SUMMARY

The paper presents results of the *Salmonella* identification, testings of recovered isolates for their susceptibility to antibiotics and their serogroup and serovar distribution. In 2012–2017 13,774 tests of animal products were performed, 105 *Salmonella* contaminated samples were detected which is 0.76% of the total number of the tested samples. As a result, 31 isolates were recovered. It was established that 22 of them belonged to seven serovars: *S. enteritidis*, *S. infantis*, *S. nigeria*, *S. montevideo*, *S. typhimurium*, *S. derby*, *S. meleagridis*. *S. infantis* (38.7%) and *S. enteritidis* (16.1%) were identified as the most spread serovars. There was observed a trend of increase in contaminated samples: 1.13% in 2012 upto 2.84% in 2017. The performed tests for antimicrobial resistance demonstrated that all isolates were susceptible to the following antibiotics: carbpenemes (meropenem, imipenem), β-lactams (amoxicillin /clavulanate), aminoglycoside (amikacin, gentamycine), macrolides (azithromycin). Most of the isolates demonstrated susceptibility to β-lactam antibiotics (ceftriaxone, cefotaxime), fluoroquinolones (ciprofloxacin) and aminoglycosides (kanamycin). Resistance at least to one antibiotic was detected in 12.9% (4/31) of isolates. Resistance to at least three antibiotics was detected in 6.5% (2/31) of isolates. 58.1% (18/31) of isolates demonstrated multiple resistance (to four or more antibiotics).

Key words: *Salmonella*, isolates, products of animal origin, serovar, antimicrobial resistance.

INTRODUCTION

Salmonella spp. are mobile gram-negative rod-shaped bacteria belonging to *Enterobacteriaceae* family. According to the current nomenclature approved by the World Health Organization and American Society for Microbiology and based on DNA-DNA hybridization only two species of *Salmonella* genus are identified: *S. enterica*, which comprises six subspecies I–IV (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *S. enterica* subsp. *indica*), and *S. bongori*, containing one subspecies [13]. Most of the known serovars causing diseases in warm blooded animals and humans belong to *S. enterica* subsp. *enterica* [14]. They are zoonotic pathogens, and the range of animals carrying them is great. Nontyphoid *Salmonella* spp. *S. enterica*, such as *S. enterica* serovar *Typhimurium* and *S. enterica* serovar *Enteritidis*, are among the major agents of food-borne diseases in the world [12]. The cause of salmonellosis development in humans is consumption of contaminated water or food such as meat, meat products, and poultry products, especially eggs [15].

Despite considerable improvement of technologies and hygiene requirements during all stages of animal product manufacturing salmonellosis still presents a serious threat to human and animal health. Moreover, the number of *Salmonella* cases even in developed countries has lately increased. It is associated with emergence of *Salmonella* strains *S. typhimurium* and *S. enteritidis*, resistant to existing antibiotics and spread all over the world. Besides, such serovars as *S. panama*, *S. infantis*, *S. newport*, *S. agona*, *S. derby* and *S. london* also cause salmonellosis. Lately the European Legislation has required to control the usage of antimicrobials for productive animals. However, there have been observed greater resistance in salmonellas transmitted through the alimentary route [3, 4]. In veterinary practice antibiotics are used for therapeutic purposes, prevention as well as growth promotion (feed additives). Of particular concern is development of resistance to effectual drugs with broad spectrum of activity such as fluoroquinolones; more isolates demonstrate decrease in susceptibility to ciprofloxacin [9, 11] and β-lactams [5, 6, 8]. The number of isolates characterized by multiple drug resistance to seven or more antibiotics is also growing. Today more cases of nontyphoid salmonellosis characterized by multiple drug resistance are observed.

Our investigation was aimed at recovery of *Salmonella* spp. from animal product samples, determination of their serogroup and serovar distribution as well as studying antimicrobial resistance of the recovered isolates.

MATERIALS AND METHODS

Bacteria cultures: 31 *Salmonella* isolates recovered from raw material of animal origin were used for microbiological testing in 2017.

Escherichia coli No. 25922 (ATCC, USA) was used to control the quality of paper discs soaked with different antibiotics.

Microbiological tests of samples. The tests were performed according to GOST 31659-2012 [1]. The prepared product sample weighing (25 ± 0,1) g was added into a sterile bag containing 225 cm^3 of buffered peptone water. Then it was homogenized for 1 min and incubated at (37 ± 1) °C for (18 ± 1) h.

Reagents and nutrient media. The following reagents were used: BPW (HiMedia, India), Rappaport-Vassiliadis (RV) broth, RVS (Merck, Germany), Selenite enrichment broth (Merck, Germany), tryptic soya agar – TSA (Scharlau,

Spain), XLD agar (Obolensk, RF), bismuth-sulfite agar – VSA (Merck, Germany). All the nutrient media were prepared according to the manufacturer's instruction.

Salmonella serovars were determined using poly- and monovalent "Petsal" sera (RF).

After the first enrichment stage 1 cm^3 of the sample was inoculated into 10 cm^3 RVS and 10 cm^3 of selenite medium. Inoculated RVS was incubated at (41,5 ± 1) °C for (24 ± 1) h. Inoculated selenite medium was incubated at (37 ± 1) °C for (24 ± 1) h.

After incubation VSA and XLD were streak-inoculated from each tube using an inoculating loop. Incubation was performed at (37 ± 1) °C for (24 ± 1) h.

Biochemical tests API 20E (BioMerieux, France) and ELISA with mini VIDASanalyzer were used to confirm that the grown colony belonged to *Salmonella* spp. After identification the isolates were stored at –20 °C in casein soyabean digest broth containing 15% glicerine.

Preparation of suspension of the tested microorganisms for antimicrobial resistance determination. One-day culture of microorganisms, grown on dense MPA (meat peptone agar) medium was used to prepare the suspension. Several colonies were transferred to the saline solution using a loop adjusting the optical density of the suspension up to 0.5 MacFarland standard. The suspension was used within 15 minutes after preparation.

Antibiotics. Standard paper discs with the following antibiotics were used: azithromycin (15 µg/disk), kanamycin (30 µg/disk), nalidixic acid (30 µg/disk), streptomycin (10 µg/disk), tetracyclin (30 µg/disk), levofloxacin (5 µg/disk), amikacin (30 µg/disk), amoxicillin (25 µg/disk), sulfamethoxazole/trimethoprim (23,75/1,25 µg/disk), ampicillin (10 µg/disk), amoxicillin/clavulanate (20/10 µg/disk), gentamycine (10 µg/disk), doxycycline (30 µg/disk), ceftriaxone (30 µg/disk), cefotaxime (30 µg/disk), chloromycetin (30 µg/disk), imipenem (10 µg/disk), meropenem (10 µg/disk), ciprofloxacin (5 µg/disk) – made by Pasteur Institute of Epidemiology and Microbiology, RF.

Determination of antimicrobial resistance. Susceptibility of *Salmonella* spp. isolates to antibiotics was studied according to generally accepted and approved methodical recommendations [2, 7, 10]. 20 ml of the melted TSA were added to 100 mm Petri dishes. Prior to inoculation the medium surface was dried. 0.1 ml of the bacterial suspension was added to the agar surface and evenly spread using a pallete. Then disks with antibiotics were placed using sterile forceps. The effect of four antibiotics was tested in each dish. After disk application Petri dishes were incubated bottom up for 18–24 h at 37 °C. The results were assessed by the presence of inhibition zones around the disks. Test organism growth inhibition at 10 mm distance from the disc with the antibiotic was indicative of the strain's susceptibility. If the test organism developed close to the disk with antibiotics, this organism was determined as resistant to the antibiotic. The inhibition zone diameter including the disk diameter was measured to a precision of 1 mm [3].

RESULTS AND DISCUSSION

In 2012–2017 the FGBI "ARRIAH" laboratory for microbiological tests performed tests of animal products for safety criteria in the framework of the Residue Monitoring Plan, government orders and requests of raw material and food product manufacturers.

Detection of *Salmonella* spp. in such types of products as meat, meat products, poultry meat is regulated by the requirements of the Technical Regulations (TR) of the Customs Union (CU) – TR CU 034/2013, TR CU 021/2011. During the specified period 13,774 test for *Salmonella* spp. were performed and 105 positive samples were detected which is 0.76% of the total number of tests.

Table 1
Detection of *Salmonella* bacteria in samples of food products of animal origin in 2012–2017

Year	Number of tests	Number of positives	Percent (%) of positives
2012	2040	23	1.13
2013	3686	10	0.27
2014	2405	7	0.29
2015	2545	4	0.15
2016	2009	30	1.5
2017	1089	31	2.8
Всего	13 774	105	0.76

Analysis of data on *Salmonella* detection is shown in Table 1 which demonstrates the trend of increase in *Salmonella* spp. isolates obtained within the last three years. In 2017 *Salmonella* bacteria were detected in animal products 11 times more than in 2013–2015 and two time more than in 2016.

The majority of *Salmonella* isolates were detected in such products as poultry meat, pork, beef, poultry, and poultry offal. Most isolates (48.3%) were recovered when testing poultry meat preparations (Figure 1).

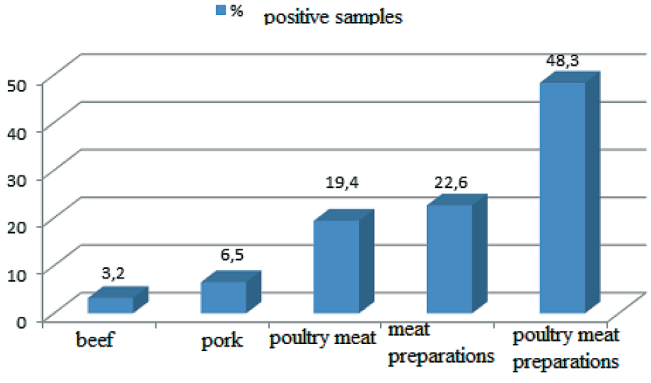
When determining the serogroup distribution of *Salmonella* isolates recovered in 2017 (Figure 2) it was observed that the majority of isolates belonged to group O_7. *Salmonella* spp. O_9 и O_4, O_5, were also detected in products of animal origin.

The most frequently detected serovars are *S. enterica* serovar *Infantis* – 12 (38.7%) and *S. enterica* serovar *Enteritidis* – 5 (16.1%). *S. typhimurium*, *S. nigeria*, *S. montevideo*, *S. derby*, *S. meleagridis* serovars were also detected. It was found out that 9 serovars (29%) were nontypeable.

Nowdays the major problem in veterinary science is wide spread of resistant forms of microorganisms and, consequently, less effective antibiotics.

The next stage of our research was determination of antimicrobial resistance of 31 *Salmonella* spp. isolates. According to the "Methodical instructions for determina-

Fig. 1. Frequency of Salmonella spp. detection in different animal products in 2017



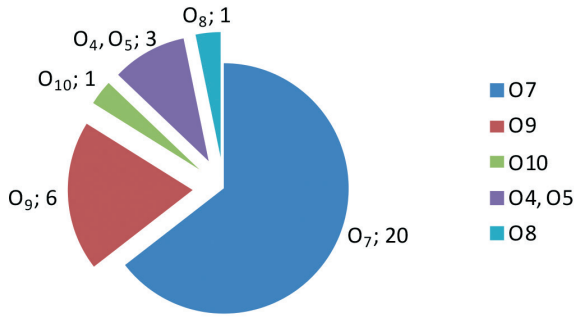


Fig. 2. Salmonella spp. serogroups

tion of microbial susceptibility to antimicrobials" the microorganisms were assessed as susceptible, resistant and intermediate by the presence of inhibition zones around discs [2]. The obtained results are demonstrated in Table 2.

Quinolones and their derivatives are synthetic antibiotics preventing bacterial DNA untwisting and replication. These preparations are often used for treatment of serious animal infections because of their low toxicity. Precisely because of that the development of resistance to new antibiotics is a real danger [13]. In our research we determined the susceptibility to three quinolones and it was demonstrated that a large group of isolates is resistant to nalidicsic acid (70.9%). Several *S. infantis* isolates (58.3%), *Salmonella* isolates of other serovars and non-typeable *Salmonella* spp. (42.9%) were resistant to levofloxacin. Herewith, all the isolates were susceptible to ciprofloxacin.

Chloramphenicol is a broad spectrum antibiotic active against gram-positive and gram-negative bacteria. For a long time it has been used for treating salmonellosis in humans and in animals. In the present time the use of this antibiotic is restricted because of *Salmonella* spp. resistance [13]. In our research all isolates demonstrated resistance to chloramphenicol except for *S. infantis*.

All isolates of *S. enterica* serovar *Enteritidis* were susceptible to all antibiotics used in the research except for nalidicsic acid. Four isolates demonstrated resistance to this antibiotic (80%).

Isolates of *S. enterica* serovar *Infantis* demonstrated resistance to streptomycin (Aminoglycosides) – 91.7%; doxycycline and tetracycline (tetracyclines) – 91.7%. Besides, there were demonstrated different levels of resistance to levofloxacin – 58.3% and nalidicsic acid – 83.3% (quinolones); trimethoprim/sulfamethoxazole – 33.3% (polymyxin). Single *Infantis* isolates demonstrated resistance to amoxicillin and ampicillin (β-lactam group) and chloramphenicol. At the same time isolates of this serovar were susceptible to carbapenems (meropenem, imipenem), β-lactams (amoxicillin/clavulanate, cefotaxime, ceftriaxone), aminoglycosides (amikacin, gentamycine, kanamycine), macrolides (azithromycin) and quinolones (ciprofloxacin) (Table 2).

Table 2
Antimicrobial resistance of *Salmonella* spp. isolates

Antibiotics	Inhibition zone diameter, mm			Number of isolates (%)								
				S. enteritidis (*n* = 5)			*S. infantis* (*n* = 12)			Other and nontypeable serotypes (*n* = 14)		
	R≤	I	S≥	R≤	I	S≥	R≤	I	S≥	R≤	I	S≥
Amikacin	14	14–16	17	–	–	5 (100)	–	–	12 (100)	–	–	14 (100)
Azithromycin	12	–	13	–	–	5 (100)	–	–	12 (100)	–	–	14 (100)
Amoxicillin	13	14–16	17	–	–	5 (100)	1 (8.3)	–	11 (91.7)	1 (7.1)	–	13 (92.9)
Ampicillin	13	14–16	17	–	–	5 (100)	1 (8.3)	–	11 (91.7)	1 (7.1)	–	13 (92.9)
Amoxycillin/clavulanate	13	14–17	18	–	–	5 (100)	–	–	12 (100)	–	–	14 (100)
Meropenem	13	14–15	16	–	–	5 (100)	–	–	12 (100)	–	–	14 (100)
Imipenem	13	14–15	16	–	–	5 (100)	–	–	12 (100)	–	–	14 (100)
Cefotaxime	14	15–22	23	–	–	5 (100)	–	–	12 (100)	–	1 (7.1)	13 (92.9)
Ceftriaxon	13	14–20	21	–	–	5 (100)	–	–	12 (100)	–	1 (7.1)	13 (92.9)
Gentamycine	12	13–14	15	–	–	5 (100)	–	–	12 (100)	–	–	14 (100)
Kanamycin	13	14–17	18	–	–	5 (100)	–	–	12 (100)	–	1 (7.1)	13 (92.9)
Chloramphenicol	12	13–17	18	–	–	5 (100)	1 (8.3)	–	11 (91.7)	–	–	14 (100)
Streptomycin	11	12–14	15	–	–	5 (100)	11 (91.7)	–	1 (9.1)	7 (50)	3 (20)	4 (30)
Ciprofloxacin	15	16–20	21	–	–	5 (100)	–	1 (8.3)	11 (91.7)	–	–	14 (100)
Tetracycline	11	12–14	15	–	–	5 (100)	11 (91.7)	–	1 (8.3)	8 (57.1)	–	6 (42.9)
Doxycycline	10	11–16	14	–	–	5 (100)	11 (91.7)	–	1 (8.3)	8 (57.1)		6 (42.9)
Nalidicsic acid	13	14–18	19	4 (80)	–	1 (20)	10 (83.3)	1 (8.3)	1 (8.3)	8 (57.1)	2 (14.2)	4 (26.7)
Levofloxacin	23	–	24	–	–	5 (100)	7 (58.3)	–	5 (41.7)	6 (42.9)	–	8 (57.1)
Trimethoprim/ sulfamethoxazole	10	11–15	16	–	–	5 (100)	4 (33.3)		8 (66.7)	1 (7.1)	–	13 (92.9)
Resistant to one antibiotic				4 (80)			–			–		
Resistant to four and more antibiotics				–			11 (91.7)			7 (50)		

R≤ – resistant; I – intermediate; S≥ – susceptible.

Salmonella isolates of other serovars and nontypeable *Salmonella* spp. demonstrated partial resistance to streptomycin (50%) and levofloxacin (42.9%). More than a half of isolates (57.1%) were resistant to tetracyclines: doxycycline and tetracyclin, as well as to nalidicsic acid, as it has been mentioned before.

It should be noted that most of the tested isolates (58.1%) are resistant to several (four or more) antibiotics demonstrating so called multiple resistance. Table 3 demonstrates different profiles of antimicrobial resistance of the recovered isolates.

Table 3 shows that 4 isolates of *S. enterica* serovar *Infantis* were resistant to six antibiotics (12.9%) and one isolate – to seven antibiotics (3.2%).

CONCLUSION

During the research 13,774 tests were performed and 105 (0.76%) samples of animal products contaminated with *Salmonella* spp. were detected. 31 *Salmonella* isolates were recovered.

When determining serogroups of *Salmonella* isolates it was found out that the majority of them belong to group O_{7}. It was also revealed that *Salmonella* spp. detected in products of animal origin belonged to groups O_{9}, O_{4} and O_{5}.

The recovered isolates belonged to seven serovars *S. enterica: Enteritidis*, *Infantis*, *Nigeria*, *Derby*, *Montevideo*, *Typhimurium*, and *Meleagridis*. Serovars *S. enterica Infantis* (38.7%) and *S. enterica Enteritidis* (16.1%) prevailed among others.

It was demonstrated that all the isolates were susceptible to gentamycin, amikacin, azithromycin, amoxicillin clavulanate, meropenem, and imipenem.

S. enterica serovar *Enteritidis* isolates demonstrated susceptibility almost to all antibiotics used in the research, except for nalidicsic acid (80%).

S. enterica serovar *Infantis* isolates were susceptible to carbapenems (meropenem, imipenem), β-lactams (amoxicillin/clavulanate, cefotaxime, ceftriaxone), aminoglycosides, (amikacin, gentamicin, kanamycine), macrolides (azithromycin). Four *S. enterica* serovar *Infantis* isolates were resistant to six antibiotics (12.9%) and one – to seven antibiotics (3.2%), thus demonstrating multiple resistance.

A large group of *Salmonella* isolates belonging to other serovars and nontypeable *Salmonella* spp. was resistant to tetracyclines (doxycycline and tetracycline), as well as to nalidicsic acid.

It is worth noting that most of the tested isolates (58.1%) demonstrated resistance to several (more than four) antibiotics.

Table 3
Resistance profiles of *Salmonella enterica* isolates

Resistance profile	Serotypes	Number of isolates
S	*Infantis, Nigeria, Derby*	13
Na	*Infantis, Enteritidis, Nigeri, Montevideo*	17
S T	*Infantis, Nigeria*	12
A T	*Infantis*	1
A S	*Infantis, Derby*	2
A S T	*Infantis*	1
A C S T	*Infantis*	1
Na S T	*Infantis, Nigeria*	12
Na S T Sxt	*Infantis*	4
Na S T Dx	*Infantis, Nigeria*	12
Na S T Dx Lvx	*Infantis, Nigeria*	7
Na S T Dx Lvx Sxt	*Infantis*	4
A C Na S T Dx Lvx	*Infantis*	1

A – ampicillin, C-chloramphenicol, Dx – doxycycline, Lvx – levofloxacin, Na – nalidicsic acid, S – streptomycin, Sxt – trimethoprim / sulfamethoxazole, T – tetracycline.

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Submitted on 05.06.18
Approved for publication on 20.11.18