

SEROLOGICAL CHARACTERISTICS AND ANTIMICROBIAL RESISTANCE OF *SALMONELLA* ISOLATES, RECOVERED FROM ANIMAL RAW MATERIAL

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SUMMARY

Salmonella continues to be the primary cause of foodborne intestinal infections in many countries around the world. According to the official data, 47% of the infection outbreaks in the world are associated with salmonellosis, while chicken meat (34%) plays a significant role in the infection transmission to humans through food. Since the early 90's of the last century, with the massive use of antibiotics, *Salmonella* strains resistant to a number of antimicrobials began to appear and currently pose a serious public health problem. Resistant strains persistent in animals can be transmitted to humans through the food chain. The paper presents results of studies of morphological, biochemical, serological properties of *Salmonella* bacteria recovered from animal raw material: beef, pork, poultry meat, tallow, offal derived from broiler chickens and pig slaughter products. In 2018 the FGBI "ARRIAH" Microbiological Laboratory performed 1,204 tests of animal raw material for *Salmonella* bacteria and recovered 45 *Salmonella* isolates. *Salmonella* bacteria were isolated in accordance with GOST 31659-2012 (ISO 6579:2002). Most *Salmonella* isolates (56%) were recovered from poultry meat. Biological properties of all the studied isolates were quite typical: they formed hydrogen sulfide, fermented glucose and mannitol with the formation of gas and acid, did not utilize sucrose, lactose and urea; reaction to indole was negative. It was established that the recovered *Salmonella* isolates belong to serogroups O₇, O₉, O₃, O₄. The frequency of recovering *Salmonella* group B was 8.9%, group C – 51.1%, group D – 40.0%. Among *Salmonella* group B, *S. derby* (4.4%) and *S. typhimurium* (2.2%) were more common; group C – *S. infantis* (29.0%), *S. virchow* (17.8%); group D – *S. enteritidis* (40.0%). Isolated cases of *S. reading* (2.2%) and *S. oranienburg* (4.4%) were observed. All *Salmonella* isolates recovered from raw material of animal origin demonstrated sensitivity to ciprofloxacin, chloramphenicol, amoxicillin, amikacin, azithromycin, meropenem, gentamicin, ceftriaxone, kanamycin; were less sensitive to cefotaxime, ampicillin, levofloxacin and had low sensitivity to nalidixic acid, doxycycline, streptomycin, tetracycline. The phenomenon of multiresistance is characteristic of 44.4% of the isolated *Salmonella* isolates.

Key words: *Salmonella*, zoonoses, serovar, isolates, raw materials of animal origin, chromogenic nutrient media, antimicrobial sensitivity.

INTRODUCTION

Currently, *Salmonellosis* is widely spread in many countries around the world. It is one of the major infectious diseases and is of a great veterinary and medical concern due to the risk of infection transmission to humans from sick animals and through food. *Salmonellosis* is an infectious disease caused by numerous serotypes of bacteria of the genus *Salmonella*, characterized by a variety of clinical manifestations from asymptomatic carrier and mild forms of gastroenteritis to severe generalized forms of the disease, occurring with pronounced intoxication and prolonged fever [1, 2].

Phylogenetic analysis shows that *Salmonella* belongs to the family of enterobacteria (*Enterobacteriaceae*), the γ class of *Proteobacteria*, the genus *Salmonella*, which consists of phenotypically and genotypically related

microorganisms. Based on the genomic analysis, two species are distinguished in modern classification – *S. bongori* and *S. enterica*. *S. bongori* is small and is composed of only 10 rarely encountered serovars (serotypes); *S. enterica* includes about 2,500 serovars. Each *Salmonella* serovar is further classified into biovars and phage types. Herewith, new *Salmonella* serotypes are isolated annually in national reference centers (40–60 per year) and their epidemiology is studied [4, 18].

Divergence in the nucleotide sequence of orthologous genes ranges between 3.8 and 4.6% and differences in their deduced amino acid sequences range between 0.7 and 1.3%. This close DNA relatedness among *Salmonella* serotypes is evidence for their clonal origin, and based on the degree of sequence divergence, it can

be estimated that a common ancestor of the genus existed about 25 to 40 million years ago [4, 13].

Salmonella species are facultative intracellular parasites capable of penetrating (invading) and surviving within different cell types, escaping from the destructive power of phagocytosis, and spreading throughout the body via the systemic circulation. After phagocytosis by neutrophils and macrophages, *Salmonella* survive and replicate within special vacuoles. Most *Salmonella* serovars do not contain virulence plasmids, while the most medically important ones (including *Typhimurium*, *Enteritidis*, and *Choleraesuis*) do [13].

Salmonella has factors of adhesion and colonization, factors of invasion; they have endotoxin and *S. typhimurium* and some other serotypes can synthesize two types of exotoxins: heat-labile (LT) and heat-stable (ST) enterotoxins, shiga-like cytotoxins. A specific feature of toxins is intracellular localization and isolation after the destruction of bacterial cells [6].

Adhesion is mediated by fimbriae (pili) found on the outer membrane of bacteria. *Salmonella* genome encodes acid shock proteins that are important for survival at low pH values, so that *Salmonella* remains viable in the acidic environment of the stomach, before reaching the areas in the gastrointestinal tract suitable for colonization. *Salmonella* causes three forms of food poisoning in humans: gastroenteric, cholera-like, and flu-like. Thus, human is the only natural host and reservoir for *Typhi* and *Paratyphi A* serovars. These serovars cause systemic infections in humans – typhoid and paratyphoid. The *Gallinarum* and *Pullorum* serovars are isolated from birds; the *Dublin* serovar causes severe systemic infection in cattle and can cause illness in humans. The situation is similar with the *Choleraesuis* and *Typhisuis* serovars isolated from pigs, and with the *Abortusovis* serovar – the causative agent of sheep *Salmonellosis*. The factors that contribute to the establishment of the carrier have not been studied much, but their dependence on the serovar is observed. From the total number of typhoid fever cases not treated with antibiotics, 10% of patients secrete *S. typhi* with feces for 1–3 months and 2–5% of patients become chronic carriers of *Salmonella*. Non-typhoid serovars persist in the gastrointestinal tract of warm-blooded animals for an average of 1.5–3.0 months, however, carriers are detected only in 0.1% of cases. A characteristic feature of the outbreaks epidemiologically related to poultry products is that the pathogen belongs to the “avian” serovars *Pullorum* and *Gallinarum* [8, 13].

Of a particular concern is that *Salmonellosis* often causes latent infection in poultry. However, meat and other products from infected poultry are a source of *Salmonella* and can pose a risk to human health [5].

According to the Reference Centre for *Salmonellosis* Monitoring and WHO's global ten-year monitoring of food-borne infections, 47% of outbreaks worldwide are related to *Salmonellosis*, with chicken meat playing a significant role in the infection transmission to humans through food (34%). In the Russian Federation, *Salmonellosis* associated food products include: meat and meat products – 63%, chicken – 28%, eggs – 5.5%. In 49.6% of cases *Salmonella* strains isolated from animals are found in birds [12].

Salmonella continues to be the primary cause of food-borne intestinal infections in many countries around the world. In the United States alone, 1.4 million people get

infected with *Salmonellosis* every year, of which about 400 cases are fatal [10].

Analysis of data published by the World Health Organization on the detection of pathogens of this acute intestinal infection in 2009–2011 showed that the most common cause of human disease in different regions of the world (Europe, North and South America, Asia, Africa, Oceania) is *S. enteritidis*, *S. typhimurium*, *S. virchow*, *S. panama*. Data for 2012–2013 confirmed this trend [7].

According to the Reference Centre for *Salmonellosis* Monitoring, the etiological structure of *Salmonella* in humans and animals continues to be dominated by *S. enteritidis* – 80.6% of *Salmonella* is isolated from humans, and 26.8% from animals. In 2011, in contrast to 2010, *S. typhimurium* held the leading position in the serovariant diversity of *Salmonella* isolated in food products (31.9%). The percentage of *S. infantis* isolates isolated from food is quite significant and is 14.6% [15].

The following strains are of major significance in animal *Salmonellosis* etiology in the Russian Federation: *S. enteritidis* (35.9%), *S. typhimurium* (13.7%), *S. dublin* (11.2%), *S. choleraesuis* (10.1%), *S. gallinarum* and *S. pullorum* (8.0%). *S. enteritidis* was detected in cattle, pigs, poultry and humans, *S. typhimurium* – in cattle and pigs, and *S. choleraesuis* in pigs and humans [8].

Salmonella is mainly transmitted through such food products as meat, milk, and eggs. The peculiarity of *Salmonella*-infected products is the absence of sensory changes: their appearance, color, smell, and taste remain unchanged [17].

In the Russian Federation, the absence of *Salmonella* bacteria in raw animal materials is regulated by the Technical Regulations of the Customs Union “On food safety” (TR CU 021/2011). Safety control of poultry meat and products thereof is carried out in accordance with SanPiN 2.3.2.1078-01. According to this regulation, the presence of *Salmonella* in meat (25 g from deep layers) as well as in mechanically deboned poultry meat and other meat products is not admissible [11, 14, 16].

Since the early 90's of XX century with mass use of antibiotics *Salmonella* strains resistant to a number of antimicrobial drugs have emerged. Today they pose a serious problem for public health. Resistant strains that persist in animals can be transmitted to humans by alimentary route through the entire food chain [3].

Purpose of work: to study biological properties of *Salmonella* isolates recovered from raw animal materials at the microbiological laboratory of the FGBI “ARRIAH” in 2018.

MATERIALS AND METHODS

The following raw animal materials were studied: beef, pork, poultry meat, raw fat, offal from broiler chicken, and pig by-products. The total number of samples was 1,204, the number of recovered isolates – 45.

Salmonella was isolated according to GOST 31659-2012 (ISO 6579:2002) “Food Products. Method for *Salmonella* detection”.

25 g sample was added to 225 cm³ of buffered peptone water, homogenized for 1 min and incubated at (37 ± 1) °C for (18 ± 1) hours. After the initial enrichment stage, 1 cm³ of the suspension was added to 10 ml of Rappaport-Vassiliadis (MSRV) medium and Selenite cystine medium, incubated at (41.5 ± 1) °C for (24 ± 1) hours, and transferred onto two media: xylose-lysine-deoxycholate (XLD) and bismuth-sulphite (BSA) agars.

The cultural properties of the isolates were studied in nutrient broth (FPH-broth), nutrient agar (FPH-agar), Endo medium, and semi-liquid agar. The culture was incubated at $(37 \pm 1)^\circ\text{C}$ for (24 ± 1) hours.

The tinctorial properties of *Salmonella* isolates were determined by microscopic examination of gram-stained 24 h culture smears (100×1.25 immersion lens magnification).

For biochemical and serological identification and determination of antibiotic resistance, pure cultures of *Salmonella* bacteria obtained during incubation of typical colonies on FPH slant agar were used.

Biochemical identification was performed in accordance with GOST 31659-2012 (ISO 6579: 2002) "Food Products. Method for *Salmonella* detection" and GOST 54354-2011 "Meat and meat products. General requirements and methods for microbiological analysis" using semi-liquid GISS media with glucose, lactose, mannitol, sucrose, maltose, xylose. Additionally, chromogenic nutrient media were used: Rambach-agar and Coliform Agar ES (enhanced selectivity).

The ability to decompose urea was determined by streaking the urea agar (Christensen agar) slant surface. To detect indole, the test cultures were introduced into test tubes containing nutrient broth with L-tryptophan using a loop. 1 cm^3 of Kovacs reagent was added to the test tubes with 24 h broth culture. No later than 5 minutes after that, test results were read based on the color of the ring formed in the medium.

The serogroup and serovariant of the obtained isolates was determined by slide agglutination test using polyvalent serum for detection of *Salmonella* of ABCDE groups and monoreceptor O- and H-agglutinating sera.

Antibiotic resistance of *Salmonella* isolates was determined by disc diffusion method using paper disks produced by the Saint Petersburg Pasteur Research Institute of Epidemiology and Microbiology (Russian Federation) according to Methodical Guidelines 4.2.1890-04 [9].

Sensitivity was determined to the following drugs: azithromycin, meropenem, kanamycin, nalidixic acid, streptomycin, tetracycline, amikacin, levofloxacin, amoxicillin, ampicillin, gentamicin, doxycycline, ceftriaxone, cefotaxime, levomecetin, ciprofloxacin.

RESULTS AND DISCUSSION

During the research in 2018, 45 *Salmonella* isolates were recovered from 1,204 samples of animal raw materials. The largest number of isolates was recovered from poultry, beef and pork (Fig. 1).

When studying the morphological properties of *Salmonella* bacteria, it was found that they are small, straight gram-negative rods with rounded edges. All isolates showed similar cultural and biochemical properties: smooth convex semitransparent rounded colonies with a diameter of 1–3 mm were formed on the nutrient agar. Uniform turbidity of the medium and gray-white sediment were observed in test tubes with nutrient broth.

In semi-solid agar medium motile bacteria gave a diffuse spreading growth throughout the agar column; on XLD agar the colonies had a black centre and a lightly transparent zone of the pinkish color around the colonies; on Endo agar – round, translucent, slightly pinkish colonies; on bismuth-sulfite agar – black rounded colonies with metallic sheen and colouring of the medium under the colonies; on Rambach agar *Salmonella* stock cultures produced a crimson-colored growth.

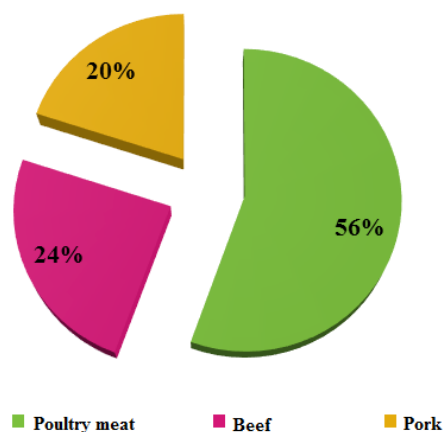


Fig. 1. Detection of *Salmonella* bacteria in various raw animal materials in 2018

On Coliform Agar ES (Enhanced Selectivity) the colonies of all the recovered isolates appeared as colorless, which means that *Salmonella* bacteria do not have β -galactosidase enzyme.

Salmonella isolates exhibited typical biochemical properties: they produced hydrogen sulfide, fermented glucose and mannitol with the production of gas and acid, did not utilize sucrose, lactose and urea; showed negative reaction to indole.

Based on the agglutination test results, the largest number of isolates belonged to the O₇ group. In addition to that, *Salmonella* from groups O₉, O₅, and O₄ were present in the studied samples (Fig. 2).

The following *Salmonella* groups were most commonly isolated: group B – 8.9%, group C – 51.1%, and group D – 40.0%. The most common serotypes in group B were *S. derby* (4.4%) and *S. typhimurium* (2.2%); in group C – *S. infantis* (29.0%), *S. virchow* (17.8%); and in group D – *S. enteritidis* (40.0%). There were few cases of *S. reading* (2.2%) and *S. oranienburg* (4.4%) (Fig. 3).

Antibiotic susceptibility was determined by measuring the diameter of the zones of bacterial inhibition around the antibiotic disks and comparing the diameter with disk diffusion interpretive criteria. Based on the zone diameter measurement results, the strains were classified as sensitive, intermediate, and resistant (Table).



Fig. 2. Serogroup identification of 45 *Salmonella* isolates recovered from raw animal materials

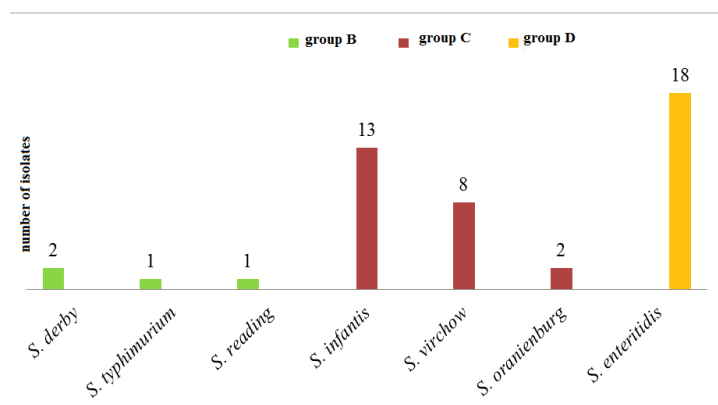


Fig. 3. Serovariants of 45 *Salmonella* isolates recovered from raw animal materials

The recovered isolates showed high sensitivity to meropenem (100%), azithromycin (97.8%), ceftriaxone (97.7%), amikacin (95.6%), gentamicin (95.6%), ciprofloxacin, amoxicillin, and levomycetin (93.3% each). The highest resistance was shown to nalidixic acid (82.0%), tetracycline (55.6%), and doxycycline (53.3%) (Fig. 4).

As a result of the conducted research, 44.4% of the recovered *Salmonella* isolates were found to be multiresistant. 92.3% of *S. infantis* isolates demonstrated resistance to two groups of antibiotics at the same time: fluoroquinolones

(nalidixic acid) and tetracyclines (tetracycline). All the studied isolates of *S. virchow* are resistant to cephalosporins (cefotaxime), penicillins (ampicillin), and aminoglycosides (streptomycin). 76.9% of *S. infantis* isolates showed high resistance to aminoglycosides (streptomycin) and 87.5% of *S. virchow* isolates – to fluoroquinolones (nalidixic acid).

88.9% of *S. enteritidis* isolates are resistant to fluoroquinolones (nalidixic acid) and 27.8% are resistant to tetracyclines (doxycycline, tetracycline).

66.6% of other *Salmonella* isolates are resistant to tetracyclines (doxycycline) and aminoglycosides (streptomycin).

CONCLUSION

A large number of tests (1,240 tests) for *Salmonella* was performed to assess the microbiological safety of raw animal materials. As a result, 45 *Salmonella* isolates were recovered.

All isolates showed identical morphological, cultural, and biochemical properties, typical for the genus *Salmonella*.

When determining the serogroup of isolates, it was found that the largest number of them belonged to the O₇ group. *Salmonella* groups O₉, O₃, and O₄ were also identified in the studied samples of raw animal materials. The prevailing serotypes were *S. enteritidis* (40.0%) and *S. infantis* (29.0%).

Table
Antibiotic resistance of the recovered *Salmonella* isolates

Antibiotic	Breakpoints zone diameter growth suppression (mm)			Number of isolates											
				<i>S. infantis</i> n = 13			<i>S. virchow</i> n = 8			<i>S. enteritidis</i> n = 18			Other <i>Salmonella</i> serotypes n = 6		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Levomycetinum	12	13–17	18	2	–	11	–	1	7	–	–	18	–	–	6
Amoxicillin	13	14–16	17	1	1	11	–	–	8	–	1	17	–	–	6
Amikacin	14	14–16	17	–	–	13	–	2	6	–	–	18	–	–	6
Azithromycin	12	–	13	–	–	13	–	–	8	–	1	17	–	–	6
Meropenem	13	14–15	16	–	–	13	–	–	8	–	–	18	–	–	6
Ciprofloxacin	15	16–20	21	–	1	12	1	1	6	–	–	18	–	–	6
Gentamicin	12	13–14	15	–	–	13	–	–	8	–	1	17	–	1	5
Kanamycin	13	14–17	18	–	1	12	–	2	6	2	1	15	1	2	3
Ceftriaxone	13	14–20	21	–	–	13	–	1	7	–	–	18	–	–	6
Cefotaxime	14	15–22	23	–	–	13	8	–	–	–	–	18	1	1	4
Ampicillin	13	14–16	17	1	–	12	8	–	–	1	1	16	2	1	3
Nalidixic acid	13	14–18	19	12	1	–	7	–	1	16	–	2	2	3	1
Doxycycline	10	11–16	14	11	–	2	4	–	4	5	2	11	4	–	2
Streptomycin	11	12–14	15	10	3	–	8	–	–	1	6	11	4	1	1
Tetracycline	11	12–14	15	12	–	1	6	1	1	4	–	14	3	2	1
Levofloxacin	23	–	24	8	–	5	6	1	1	3	–	15	2	–	4

R – resistant; I – intermediate; S – sensitive isolates.

All *Salmonella* isolates recovered from raw animal materials showed sensitivity to ciprofloxacin, levomycetin, amoxicillin, amikacin, azithromycin, meropenem, gentamicin, ceftriaxone, kanamycin, lower sensitivity to cefotaxime, ampicillin, levofloxacin, and low sensitivity to nalidixic acid, doxycycline, streptomycin, and other substances, tetracycline. The recovered isolates showed high sensitivity to meropenem (100%), azithromycin (97.8%), ceftriaxone (97.7%), amikacin (95.6%), gentamicin (95.6%), ciprofloxacin, amoxicillin, and levomycetin (93.3% each). The highest rate of resistance was found for nalidixic acid (82.2%), doxycycline (53.3%), and tetracycline (55.6%). The phenomenon of multiresistance is characteristic of 44.4% of the recovered *Salmonella* isolates.

Conflict of interests. The authors declare no conflict of interest.

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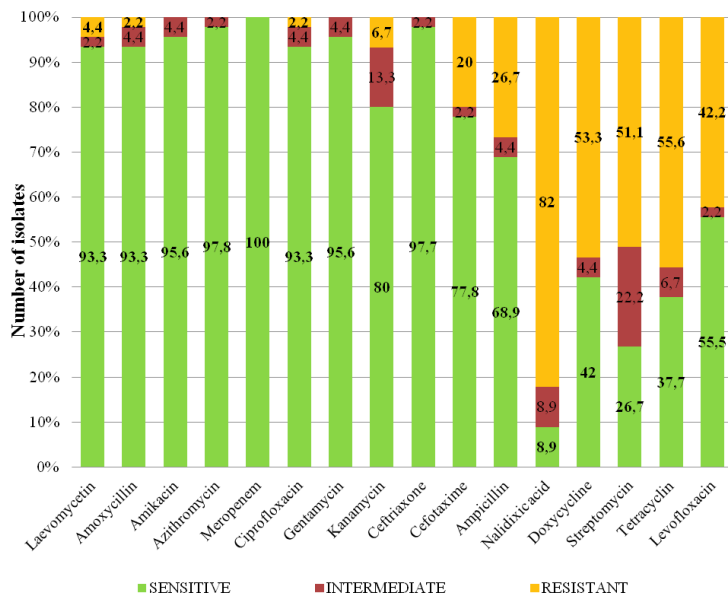


Fig. 4. Antibiotic sensitivity of *Salmonella* isolates recovered from raw animal materials

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