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Improvement of allocation and identification of *Salmonella entericabacteria* of arizonae subspecies

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ABSTRACT

During the period between 2014 and 2015 we performed morphological, cultural, biochemical, genetic and biological studies of 623 salmonella strains of 270 different serovars, included in the Russian state collection of microorganism strains of FSBI «All-Russian State Research Institute for Control Standardization and Certification of Veterinary Preparations». During the investigation 16 museum bacteria strains of Salmonella genus, Arizonae sp. proved to be of great interest to us. Study of their biochemical properties revealed peculiarities enabling to improve indication and identification technique of the salmonella of this kind.

Keywords: Salmonella strains, *Salmonella arizonae*, biochemical identification, genetic identification.

INTRODUCTION

First *Salmonella arizonae* isolates were obtained in 1939 by Mary E. Caldwell and Dwight L. Ryerson, employees of the department of bacteriology and zoology of Arizona University. Heloderma suspectrum - Gila monster was the source on the agent. Further in literature there were references of the synonyms of this agent Paracolobactrum arizonae [1] and Arizona hinshawii [2] and only in 2002 it was labelled a single subspecies. The 9th edition of the Kauffman and White classification scheme published in 2007 by the International World Health Organization gives information on 99 existing serovars of this subspecies. However as opposed to the salmonella of the other subspecies of Salmonella Enterica, serovars of Arizonae and Diarizonae have no names and are labelled according

to the antigenic structure formula. Moreover, the issues of epizootic importance of serovariants and their identification within Arizonae subspecies have not been sufficiently studied.

The source and natural reservoir for the salmonella of these serovars are presented by reptiles (lizards, snakes and tortoises). It must also be stated that turkey poults, piglets, sheep [3], dogs, cats, monkeys and goats [4] are also susceptible to this agent.

Literary sources quite often give descriptions of clinical cases of human diseases caused by *Salmonella arizonae* agent with a substantially different form of disease manifestation, including diseases with the fatal outcome. Such forms of disease manifestation of the arizonous etiology are recorded: meningitis [5], including newborn meningitis [6], gastroenteritis in children with microcephaly [7], otitis, osteomyelitis [8], pleuritis, sinusitis, peritonitis, bacteriemia [7].

There is a described case of septic artritis of hip joint occurrence in a 10-month-old baby . There is also stated a possibility of the disease passing without the common for salmonella infection gastroenteritis [9].

Sporadic outbreaks of arizonosis among human beings are more commonly registered in the south-western part of the USA, inhabited by mostly Latin Americans, who often eat snake meat and make medical preparations using snake by-products [10]. Besides Latin America and USA, the problem is also common for South Asia and Great Britain [11].

Newborns and young babies are most susceptible to the disease. In accordance with a great number of clinical cases we can make a conclusion that the infectious process gets intensified and obtains its acute form because of low immune status. There is an opinion that human disease is connected with the increased permeability of the hematoencephalitic barrier, innate injuries, absence of the developed cellular immunity and is also conditioned by vertical contamination received from the mother. Patients who have had arizonosis frequently get neurologic complications, hearing loss, hydrocephalus, etc [5], afterwards. Antibiotic therapy within *Salmonella arizonae*-infection includes broad spectrum antibiotics - ampicillin, gentamicin, fluoroquinolones and third generation cephalosporins [7].

Within livestock and poultry farming the problem of Arizonae-infection is most urgent in turkey breeding. Turkey breeding is highly developing in Russia. Growth of production and consumption of turkey breeding requires development in the methods of veterinary-sanitary control and veterinary-sanitary expertise in accordance with the specificity of the product and the peculiarities of the agent [12]. According to the directives of the council 2009/158/EC from 30th November, 2009, the veterinary-sanitary conditions regulating market within the Community and importation of poultry and hatchable eggs from the third countries state that turkey hatchable eggs must necessarily go through inspection for the salmonellosis agent Arizonae subspecies [13]. Study of microbiological properties and genetic characteristics of salmonella strains Arizonae subspecies based on 16S rRNA gene will enrich the knowledge about the agent of the dangerous disease, that damages turkey breeding, and develop the methods of bacteriological investigation of Arizonae-infection.

The aim of the investigation lies within the study of the properties of Salmonella strains Arizonae subspecies and further development of the stages of complex indication and identification with differentiation of Arizonae-infection agent, based on routine microbiological methods and methods of genetic 16S rRNA gene sequencing.

MATERIALS AND METHODS

16 museum strains of *Salmonella arizonae* subspecies from the collection of FSBI «All-Russian State Research Institute for Control Standardization and Certification of Veterinary Preparations» were used as material of the study 63-z36; 44-67; 48:k:z53; 48:j:z; 1669/75; 107-36/71; 5992-52; S05; 4601/54; S0-50; 261-58; 3065-61-253; PC-123; Ni-14; 301-57/142; 4041. Reference cultures of Salmonella enteritidis13076 and Escherichia coli 25922 were used as test-cultures within the identification of enterobacteria with the help of commercial biochemical test-sets. For the research the following nutrient media were used: Endo agar, cromogenic medium for salmonella cm1007, bismuth-sulfite agar, beef-extract agar (BEA), beef-extract broth (BEB). For the biochemical identification test systems Microbact 12e, ENTERO test 24 N, API 20e were used.

In the work epizootiological, bacteriological, serological, statistical methods of investigation were used.

RESULTS

Study of the cultural, biochemical, biological properties of the strains and their genetic identification were performed on the premises of FSBI «All-Russian State Research Institute for Control Standardization and Certification of Veterinary Preparations». For the purpose of obtaining the first generation of salmonella bacterial bulk frozen-dried cultures of the studied strains' microorganisms were dissolved in 1 ml of beef-extract broth (BEB) with the following transfer of the material to the test tube with the same medium. 18-hour long cultivation in thermostat at 37^0C with the following reinoculation on BEB for obtaining the second generation within 5-hour cultivation was performed. Similar inoculations and cultivation at the same mode were performed with the strains of *Salmonella enteritidis* 13076 ATCC and *Escherichia coli* ATCC 25922, which were further used as reference cultures. After that the material was reinoculated on beef-extract agar (BEA), Endo agar, cromogenic medium for salmonella cm1007, bismuth-sulfite agar for the study of cultural properties.

After 24 hours of cultivating *Salmonella arizonae* strains on BEA growth of transparent 1-2 mm in diameter «S»-formed colonies was observed. After 24 hours of cultivation on cromogenic medium CM1007 11 studied strains showed blue colouring of the colonies, 5 strains had purple colouring. On bismuth-sulfite agar growth of black colonies with a glittering zone around them was observed, the medium at the same time becoming black-and-brown. After 48 hours of cultivation on Endo medium the colonies of 13 strains of *Salmonella arizonae* had metallic glitter and crimson colouring, colonies of three strains remained transparent (figure 1).

Attention must be paid to the fact that the given subspecies of Salmonella are of the slowly fermenting kind, that is why cultivation in Endo medium must last 48 hours. Metallic glitter observed within cultivating *S. arizonae* is typical for E. coli, that is why it is necessary to differentiate the agent of colibacillus from other coliforms when performing bacteriological investigation of pathologic material and objects of veterinary and sanitary-epidemiological supervision. Crimson colouring of Endo medium and metallic glitter in the case of *S. arizonae* growth is conditioned by the activity of β-galactosidase, that is why it will not be revealed at once, but by the 48-hour period of incubation [14].

In the figures1 and 2 growth of the strain cultures *S. arizonae* and S. Enterica subspecies (serovar enteritidis) is shown after 48 hours of incubation, where the cultural differences are evident.

Capability of the microorganisms to ferment lactose directly depends on β-galactosidase activity. As far as we know, organisms use two enzymes for lactose utilization. The first enzyme- permease is necessary for transferring lactose molecules to the cell. The second - β-galactosidase provides hydrolysis of the coming molecule with glucose and galactose formation. In true lactose-negative microorganisms (the ones that do not ferment lactose) both enzymes are absent. At the same time there are microbal species that lack only permease but have β-galactosidase. These are the so-called late fermenting species. *S. arizonae* belongs to this kind. Presence of β-galactosidase can be detected with the help of the test using galactopyranoside-ONPG, a colourless substance of the synthetic origin having structural similarity with lactose. β-galactosidase enzyme ferments ONPG with formation of glucose and ortho-nitrophenyl, a substance that colours the substrate yellow.

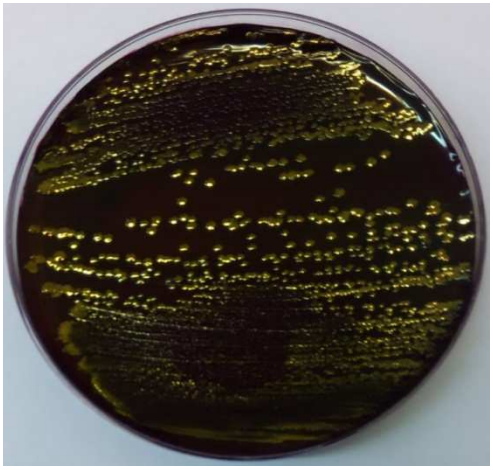

Figure 1 - Growth of *Salmonella arizonae* 44-67 strain colonies on Endo medium during 48 hours of incubation

Figure 2-Figure 2 - Growth of Salmonella Enterica strain colonies enteritidis serovar 13076 on Endo medium during 48 hours of incubation (control)

Besides when cultivating *Salmonella arizonae* on cromogenic medium cm1007produced by «OXOID» ambiguous results were obtained. On this medium eleven strains had blue colouring untypical for Salmonella (figures3,5), that is connected with β-glucuronidase activity.

β-glucuronidase (abbr. GUS) is an enzyme synthesised by certain bacteria. It catalyzes fermentation of all beta-glucuronides. Due to the fact that plants don't demonstrate this enzyme's activity, the bacterial gene coding this enzyme is widely used as a reporter gene in plant transgenesis. High levels of the bacterial enzyme β-glucuronidase can cause reactivation of carcinogens in the intestine and disintegration of bile acids.

Methods of GUS activity determination are based on release of phenolphthalein, nitro- or aminophenol, and fluorescent 4-methylumbelliferone from the corresponding synthetic beta- glucuronides under the enzyme's influence. As a result of the fermentation products' appearance the indicator colours the microbial colonies and the medium around, which helps to distinguish them from the colourless (achromatic) microorganism colonies that do not ferment this substrate. The differentiation within this approach is complicated by the fact that saccharolytic and proteolytic enzymes of microorganisms are highly diverse and universal (they are common for different species). This conditions relatively low differentiation properties of the traditional media. For the more precise culture differentiation one should identify their genus- and species-specific enzymes. In the end of the XX century the new generation differential media came into use in bacteriology: the chromogenic ones whose operating principle was based on detecting highly specific enzymes in the required organisms. To such enzymes the following ones are referred: beta-D-glucuronidase Escherichia coli or beta-D-glycosidase of enterococci. To detect the unique enzyme and thus to identify the microorganism one must inject the chromogenic substance (when this substance is fermented by this enzyme, coloured or fluorescent products are generated) into the medium. As a result the colonies within microbal growth get a certain colouring or acquire the ability to fluoresce in ultraviolet irradiation. As long as the chromogenic substance or their mixture is injected into the media structure (including selective ones) for primary inoculation, the result - selection of the pure culture and its identification - can be obtained during the first 24 hours of the study.

Figures3, 4 and 5 show the received growth of the culture colonies with the β-glucuronidase enzyme, identified consequently by sequencing as salmonella of Arizonae and S. Diarizonae subspecies, and cultures S.enteritidis (subspecies S. Enterica) that do not have this enzyme.

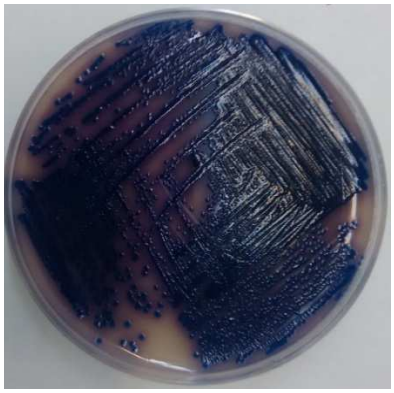

Figure 3-Figure 3 - Growth of *Salmonella arizonae* 44-67 strain colonies, having β-glucuronidase enzyme

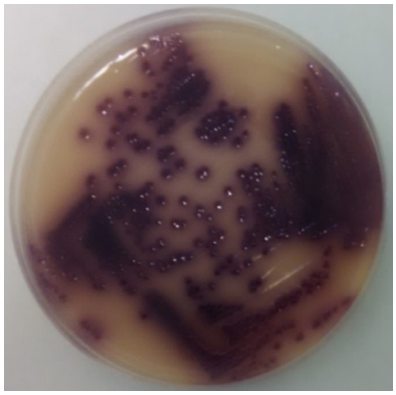

Figure 4-Growth of Salmonella enteritidis 13076 strain colonies, not having β-glucuronidase enzyme

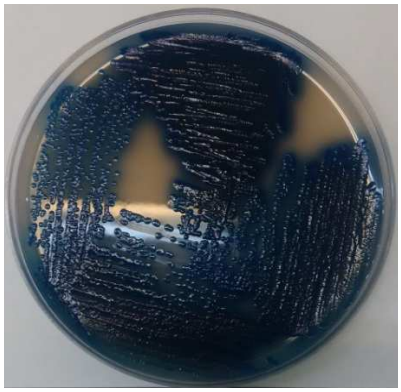

Figure 5-Growth of *Salmonella arizonae*1669/75 strain colonies (identified later as Salmonella Diarizonae), having β-glucuronidase enzyme

As it is seen in figures3,4,5 when chromogenic nutrient medium cm1007 is used, it is impossible to unambiguously assert the culture's belonging to a certain subspecies. The strains belonging to Arizonae subspecies must be differentiated from the other bacteria having positive β-glucuronidase activity including Salmonella Diarizonae, Salmonella Indiana, Escherichia coli, Enterobacter spp, Shigella dysenteriae etc.

Observation of the studied *Salmonella arizonae* strains' ability to utilize malonate showed that 15 strains have positive result in malonate test, while *S. arizonae* 63-z36 and S. enteritidis 13076 have negative results.

Biochemical identification. Use of biochemical sets considerably accelerates the process of bacterial identification. Testing the studied strains with the help of various sets for microorganism biochemical identification presents much interest.

We chose ENTERO test 24 N, Microbact 12e, API 20 E as test-systems for Gram-positive enterobacteria identification. Reaction staging was performed in accordance with the instructions for the mentioned test-systems. Biochemical data interpretation of the bacterial cultures obtained with ENTERO test 24N test system was performed with the help of the code book ENTERO test 24 N. Interpretation of the biochemical data obtained through using Microbact 12E test system was performed with the help of the Microbact 2009 software support. Interpretation of the biochemical data obtained through using API 20 E test system was performed with the help of web-interface Web Api. The results of the identification are given in the table №1.

Genetic identification. Further we performed genetic identification of the studied bacteria, basing on the methods given in «Guideline for bacterial genetic identification basing on analysis of 16S rRNA gene nucleotide sequence», developed by Federal State Budget Institution «All-Russian State Research Institute for Control Standardization and Certification of Veterinary Preparations». Data of the research results are also given in table 1 for correlation with the data of biochemical identification of *S. arizonae* strains.

Table 1.Results of biochemical and genetic identification of Salmonella strains Arizonae subspecies

№		ENTERO test 24 N	Microbact 12e	API 20 E	Results of genetic identification*
1	*S. arizonae 63-z36*	*S. enteritidis*	*S. arizonae*	*S. Enterica*	*Salmonella Bongori – 100% Genbank cp006692, fr877557, NR_116124.*
2	*S. arizonae 44-67*	*S. Diarizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Diarizonae – 100%. Genbank – AB273735, NR_044373.*
3	*S. arizonae 48:k:z53*	*S. Diarizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Diarizonae – 100%. Genbank – AB273735, NR_044373.*
4	*S. arizonae 48:j:z*	*S. Diarizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Diarizonae – 100%. Genbank – AB273735, NR_044373.*
5	*S. arizonae 1669/75*	*S. Diarizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Diarizonae – 100%. Genbank – AB273735, NR_044373.*
6	*S. arizonae 107-36/71*	*S. Diarizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Diarizonae – 100%. Genbank – AB273735, NR_044373.*
7	*S. arizonae 5992-52*	*S. Diarizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Diarizonae – 100%. Genbank – AB273735, NR_044373.*
8	*S. arizonae S05*	*S. arizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Arizonae – 100%. Genbank – cp006693.1, cp000880.*
9	*S. arizonae 4601/54*	*S. arizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Arizonae – 100%. Genbank – cp006693.1, cp000880.*
10	*S. arizonae S0-50*	*S. arizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Arizonae – 100%. Genbank – cp006693.1, cp000880.*
11	*S. arizonae 261-58*	*S. Diarizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Diarizonae – 100%. Genbank – AB273735, NR_044373.*
12	*S. arizonae 3065-61-253*	*S. Diarizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Diarizonae – 100%. Genbank – AB273735, NR_044373.*
13	*S. arizonae 4041*	*S. Diarizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Diarizonae – 100%. Genbank – AB273735, NR_044373.*
14	*S. arizonae PC-123*	*S. Diarizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Arizonae – 100%. Genbank – cp006693, cp000880.*
15	*S. arizonae Ni-14*	*S. arizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Arizonae – 100%. Genbank – cp006693, cp000880.*
16	*S. arizonae 301-57\142*	*S. Diarizonae*	*S. arizonae*	*S. arizonae*	*Salmonella Enterica subsp. Diarizonae – 100%. Genbank – AB273735, NR_044373.*
17	*S. Enteritidis 13076*	*S. Enteritidis*	*Salmonella spp.*	*S. Enteritidis*	*Salmonella Enterica subsp. Enterica – 100%. Genbank CP007222, CP007534.*

** In the column the data on genetic identification of the culture are given: name of the species, subspecies, percent of homogeneity with the strains from the gene bank, name of these strains.*

Summing up the received data given in table 1, we revealed some interesting aspects in the specificity of the diagnostic test-systems based on biochemical properties of bacterial cultures. Thus the system ENTERO test 24 N identified the museum strain *S. arizonae* 63-z36 as S. Enterica variant enteritidis, though genetic identification detected this strain's reference to Salmonella Bongori. At the same time when identified with the help of the test-system Microbact 12e a result indicating this strain's reference to *S. arizonae* was obtained. We did not manage to reliably identify the subspecies with the help of the test-system API 20 E, as well as with the two previous systems.
As it is well seen in the data given in table 1, the diagnostic specificity of the test-systems for enterobacteria identification is not uniform. 88,24% coincidence of the results obtained through genetic studies with the results of biochemical identification was recorded when using ENTERO test 24 N test-system, 35,29% coincidence when using Microbact 12e and API 20 E test-systems.

DISCUSSION

Studying the properties of the museum cultures of bacteria strains Salmonella genus Arizonae subspecies from the collection of FSBI «All-Russian State Research Institute for Control Standardization and Certification of Veterinary Preparations» made it possible for us to enlarge the knowledge of the microbiological peculiarities of these bacteria and review the data of their reference to subspecies. Thus ten strains of the sixteen studied cultures that were thought to belong to Arizonae subspecies were identified as Diarizonae subspecies, 1 strain belonged to S. Bongori, and only 5 of them belonged to Arizonae subspecies.

The study showed that biochemical properties of *Salmonella arizonae* subspecies, Diarizonae subspecies, and Salmonella Bongori species are analogous but for some peculiarities.

Salmonella Enterica subsp. Arizonae, Salmonella Enterica subsp. Diarizonae and Salmonella Enterica subsp. Salamae have the capability for malonate fermentation. In our study the capability for malonate fermentation was common for 15 strains, 5 of them belonging to Arizonae subspecies and 10 to Diarizonae subspecies. Salmonella Bongori does not ferment malonate. However, *S. arizonae* 63-z36 and S. enteritidis 13076 strains showed negative malonate test. We think that these results are conditioned by the fact that some bacteria of Salmonella genus are able

to use malonate as a source of carbons. Simultaneous utilization of sodium malonate and ammonium sulfate during the growth process leads to sodium hydroxide formation and thus increases alkalinity of the medium. In the forming medium the colour of the reagent changes from green to blue, that is conditioned by presence of PH indicator - bromothymol blue. This test is widely used for enterobacteria differentiation. Positive reaction is marked in the strains of Enterobacter and Klebsiella genera and Arizonae and Diarizonae subspecies Salmonella genera, while the other subspecies of this genus show negative reaction to this test.

It must be noted that this test was developed by E. Leifson in 1933 for easing the process of identifying colibacillus from Klebsiella. Later in1956 Shaw ascertained that the serovars of Arizonae subspecies Salmonella genus are malonate-positive, while all the other subspecies are malonate-negative (Shaw, 1965). The fact that during our study we identified a strain with a negative malonate test enriches the literary data available.

Positive ß-galactosidase activity is common for the bacteria of the species Salmonella Bongori, Salmonella Enterica subsp. Arizonae and Salmonella Enterica subsp. Diarizonae. In our case all the 16 strains showed ß-galactosidase activity.

Ability to ferment lactose is mostly typical for Salmonella Enterica subsp. Diarizonae, but there are serovars Salmonella Enterica subsp. Arizonae, that also possess this ability. Thus only five of the 16 studied strains showed this ability - 3 Diarizonae strains and 2 Arizonae strains.

Positive ß-glucuronidase activity is recorded in Salmonella Enterica subsp. Diarizonae and Salmonella Enterica subsp. Arizonae, and the serovars of this subspecies may demonstrate both positive and negative reaction. Among Diarizonae subspecies 9 of the studied strains showed positive ß-glucuronidase activity and one - negative. Among Arizonae subspecies this activity was detected in two of five strains. The strain referring to S. Bongori species has negative ß-glucuronidase activity.

We did not manage to perform exact identification to species reference for S. Bongori when conducting biochemical identification of the salmonella from the group under study with three commercial test-systems Microbact 12e, ENTERO test 24 N and API 20 E. This fact is essential in practical diagnostics because it actually determines the possibility to differentiate pathogenic variants of salmonella from humicular ones. Most accurate results in the detection of subspecies of Salmonella genus were obtained with the help of ENTERO test 24N, which was proved by genetic studies: coincidence of the results of genetic studies with the results of biochemical identification was recorded in 88,24% cases compared with 35,29% when using other test-systems.

Microbact 12e system for biochemical identification turned out to be ineffective for identification of the bacteria Salmonella genus Diarizonae subspecies and Bongori species. Positive accurate results were obtained in all cases of using this test-system for biochemical identification of *S. arizonae* cultures. API 20 E test system gave results similar to the results obtained when using Microbact 12e.

CONCLUSION

During the study of the properties of 16 museum strains which were previously referred to Salmonella genus Arizonae subspecies 16s rRNA gene sequencing helped us to define that the strain *S. arizonae* 63-z36 is a humicular strain belonging to Salmonella Bongori genus, and strains *S. arizonae* 44-67, 48:k:z53, 48:j:z, 1669/75, 107-36/71, 5992-52, 261-58, 3065-61-253, 4041, 301-57\142 according to the results of genetic identification belong to Diarizonae subspecies.

Highest effectiveness and reliability of the results received was obtained when employing 16 s rRNA gene sequencing. This way homogeneity with the base GenBank reached 100% in all the cases

Bacteria of Salmonella genus Arizonae subspecies have biochemical properties similar to Salmonella Diarizonae. That is why the strains of Diarizonae subspecies are major agents, one must differentiate *Salmonella arizonae* from them basing on biochemical properties.

When performing identification, one must pay special attention to the ability to ferment malonate. Salmonella Diarizonae subspecies and *Salmonella arizonae* subspecies have this ability in equal proportions.

Ability to ferment lactose is mostly common for S. Diarizonae, but there are serovars *S. arizonae*, which also have lactose-positive properties. In the present study 3 strains identified as Arizonae were lactose-negative and 2 strains were lactose-positive.

Variable reaction to the activity of ß- glucuronidase is peculiar only for two subspecies of Salmonella genus - Arizonae and Diarizonae. Among Diarizonae subspecies 9 of the studied strains showed positive ß-glucuronidase activity and 1 strain – negative one. Among Arizonae subspecies this activity was shown in 2 of 5 strains. When using commercial test-systems for identification of the studied bacteria according to their biochemical properties we concluded that ENTERO test 24N showed highest effectiveness. It managed to identify 88,24% studied samples. Test-systems API 20 E and Microbact 12e managed to equally well identify 35,29% studied samples. We managed to perform species identification of S. Bongori with neither of the three test-systems used. In all the cases we managed to reliably define gene reference of the bacteria with the applied systems for enterobacteria identification.

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