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BIOLOGICAL CHARACTERISTICS OF *STAPHYLOCOCCUS AUREUS* ISOLATES RECOVERED FROM MILK AND DAIRY PRODUCTS MANUFACTURED IN THE REPUBLIC OF CRIMEA

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SUMMARY

Staphylococci are one of the causes of food poisoning in many countries of the world. Intoxication occurs due to staphylococcal exotoxins entering the human body. One of the main sources of staphylococcal toxins is milk and dairy products contaminated with pathogenic staphylococci. *Staphylococcus aureus* has the greatest sanitary and hygienic importance. In 2016–2018 168 samples of ready-to-eat dairy products were tested for *Staphylococcus aureus* in the Food Safety Laboratory of the FGBI "ARRIAH" in the Republic of Crimea. The tests were performed according to GOST 30347-2016 "Milk and dairy products. Methods of *Staphylococcus aureus* detection". Biochemical properties of the recovered isolates were studied using Vitek 2 Compact analyzer. It was established that the following groups of products are contaminated with *Staphylococcus aureus* to the greatest extent: butter (20%), sour cream (9.09%), curd and curd products (4.55%), pasteurized milk in the consumer packaging (4.35%). The basic biological characteristics of the isolates have been studied and their antimicrobial resistance has been determined. All the isolated *Staphylococcus aureus* cultures demonstrated a 100% sensitivity to benzylpenicillin, oxacillin, imipenem, ticarcillin, meropenem, ciprofloxacin, ofloxacin, gentamicin, amikacin, doxycycline, tetracycline, rifampin, chloramphenicol, cefotaxime, ceftriaxone, trimethoprim and were 100% resistant to enrofloxacin. Resistance to streptomycin was determined in 28.6% of isolates, and 14.3% of isolates were resistant to vancomycin. Methicillin-resistant staphylococci were not detected among the bacteria.

Key words: foodborne toxicosis, *Staphylococcus aureus*, milk and dairy products, antimicrobial resistance.

INTRODUCTION

Among microorganisms causing foodborne toxicosis, a significant role belongs to toxigenic staphylococci [13–16]. Staphylococci were first isolated by Louis Pasteur in 1880 from the pus of a human abscess. There are saprophytic, opportunistic and pathogenic species. Saprophytic species are found in air, soil, water, on the surface of plants. Opportunistic and pathogenic species are observed in humans and animals: on the skin and mucous membranes [1].

Most often, the source of infection in the food industry is people with staphylococcal diseases of the skin and respiratory tract. Besides diseased people, healthy people in whom staphylococci are secreted from the surface of the mucous membranes, skin, and feces can also be carriers [1, 6, 15].

Staphylococci, including the enterotoxigenic ones, are isolated from many farm animals, more often if they demonstrate some pathological processes, for example, mastitis. Staphylococci are often detected in swabs from the equipment and tools of public catering and food industry establishments, especially when they are in poor sanitary condition [6, 11, 15].

Due to the wide distribution of staphylococci in nature, there is a high probability of contamination of various food products by them [6, 11, 15]. The most common causes of staphylococcal toxicosis are milk and dairy products. *Staphylococcus aureus* is of primary hygiene significance [1, 6, 7, 11, 15].

In the Customs Union Technical R 033/2013 "On safety of milk and dairy products", the admissible level of *S. aureus* content in processed products, when marketed, varies widely. So, in pasteurized milk, cream, sour cream, sour-milk and dry milk products, bacteria *S. aureus* should be absent in a volume of 1 cm^3; in cottage cheese, curd products, butter – in 0.1 g; in hard cheeses and cheese products – in 0.001 g [12].

Unlike the regulatory documents of the Russian Federation, international standards control the presence of

coagulase-positive staphylococci and not of *S. aureus*. Thus, Commission Regulation (EU) No. 1441/2007 of 05/05/2007 establishes the safety criteria for ready-to-eat foods, as well as hygienic indicators of the technological process for production of milk and dairy products. This document allows the presence of no more than 10 cells of coagulase-positive staphylococci per 1 g of product in dairy products [10].

Currently, *S. aureus* is isolated from milk and dairy products in the Russian Federation according to GOST 30347-2016 "Milk and dairy products. Methods for determination of *Staphylococcus aureus*", in which Baird-Parker agar, yolk-salt or milk-salt agar are used as selective media. On yolk-salt agar, colonies of microorganisms have the form of flat discs with a diameter of 2–4 mm and white, yellow, cream, lemon, golden color with smooth edges; around the colonies a rainbow ring and a hazy zone are formed. On Baird-Parker agar, growth of black shiny convex colonies, 1–1.5 mm diameter, surrounded by a clear zone of 1–3 mm wide, is observed [3].

When detecting the typical growth of colonies on solid nutrient media, further confirmation that isolated microorganisms belong to coagulase-positive staphylococci, namely *S. aureus*, is carried out. For this purpose Gram staining is performed, the ability of microorganisms to form catalase, coagulate rabbit blood plasma, form acetoin and ferment maltose under aerobic conditions is determined [2, 3].

S. aureus bacteria are gram-positive catalase-forming cocci, capable of coagulating blood plasma, forming acetoin and fermenting maltose. The main indicators of staphylococcal virulence are hemolytic activity, plasmocoagulase enzyme production and necrotoxicity [1, 3, 6, 11].

The determination of hemotoxin is carried out by direct culture inoculation on blood meat-peptone agar (MPA) containing 5–10% defibrinated rabbit or mutton blood. Determination of plasmocoagulase is carried out by adding a staphylococcus culture into a test tube with 5% rabbit citrate plasma [3].

Necrotoxin determination of is carried out by intradermal injection of 0.2 cm^3 suspension of daily agar staphylococcus culture in saline with a concentration of 2 billion cells to a rabbit. The animal is observed for 24–48 hours [1, 5, 6, 11].

Today, in medicine and science as a whole, the big problem is the rapid formation of resistance to antibiotics used against various microorganisms. Most scientists believe that one of the reasons for development of antimicrobial resistance in humans is ingestion of resistant microorganism strains with animal products, which, in turn, can cause development of resistance in microorganisms circulating in the human body. As a result, decrease or loss in the therapeutic efficacy of antimicrobial agents used in human medicine is observed [4].

The purpose of this work is to identify the presence of *S. aureus* bacteria in milk and dairy products produced in the Republic of Crimea and to study the biological properties of the recovered isolates.

MATERIALS AND METHODS

Studies on isolation of *S. aureus* bacteria from milk and dairy products were carried out on the basis of the food safety laboratory of the Laboratory Diagnostic Center of the FGBI "ARRIAH" Branch in the Republic of Crimea.

Test object. Samples of ready-to-eat dairy products delivered to the Laboratory Diagnostic Center of the FGBI "ARRIAH" Branch in the Republic of Crimea both from the Rosselkhoznadzor Territorial Administration in the Republic of Crimea and the city of Sevastopol were used as the test object in accordance with the plans for monitoring studies, and from individual customers.

Control strains: S. aureus ATCC 25923, *S. aureus* subsp. *aureus* ATCC 6538.

Nutrient media. To determine *S. aureus* in samples of dairy products in accordance with GOST 30347-2016, salt broth and yolk-salt agar were used (FBUN SSC PMB, Obolensk).

To identify the recovered cultures, we used a set of dyes for staining Gram smears, a 3% solution of hydrogen peroxide, rabbit plasma (NPO Mikrogen JSC, Moscow), a set for preparation of Clark's medium (NITsF LLC, St. Petersburg), Wednesday Gissa (FBUN SSC PMB, Obolensk), maltose.

Determination of S. aureus in dairy products according to GOST 30347-2016. A sample of the product or its corresponding dilution in a volume of 1 cm^3 was inoculated in test tubes with the corresponding volume of salt broth. The inoculations were placed in a thermostat with a temperature of (37 ± 1) °C and incubated for (24 ± 1) h. Next, reinoculation on Petri dishes with egg yolk-salt agar was performed. The inoculations were placed in a thermostat with a temperature of (37 ± 1) °C and incubated for 24–48 hours. The inoculation results were evaluated visually by the appearance of the colonies formed on the surface of yolk-salt agar. Flat disc-shaped colonies, with smooth edges, white, yellow, golden in color, around which a rainbow ring and a hazy zone formed, indicated that microorganisms belong to coagulase-positive staphylococci.

For further differentiation, isolated colonies with typical growth were subcultured onto the surface of slant agar in order to obtain one-day cultures. After that, Gram staining was performed, the ability of microorganisms to form catalase and the ability to coagulate rabbit blood plasma were determined.

Belonging of the identified coagulase-positive staphylococci to *S. aureus* was determined by the ability to form acetoin and the ability to ferment maltose.

Isolates forming flat disc-shaped colonies on egg yolk-salt agar with smooth edges, white, yellow, golden in color, around which a rainbow ring and a turbidity zone form; microscopy of which reveals gram-positive cocci; catalase forming; able to coagulate blood plasma; forming acetoin and fermenting maltose under aerobic conditions, were identified as *S. aureus*. Presence of hemolytic activity was assessed visually by the presence of the clear zone around the colony on 5% blood agar. The results were evaluated after (48 ± 1) h.

Biochemical properties of the recovered cultures were determined using a Vitek 2 Compact automatic microbiological analyzer (Biomerieux, France).

Determination of antimicrobial resistance. The sensitivity of *S. aureus* isolates to antimicrobial agents was determined on tryptone soy agar (TSA) by the disk diffusion method according to Methodical guidelines 4.2.1890-04. Evaluation of the results was carried out by the presence of inhibition zones around the disks. The diameter of inhibition zones, taking into account the diameter of the disk with the antibiotic, was measured using a ruler with an accuracy of 1 mm. The results were interpreted in accordance with MUK 4.2.1890-04 [8].

RESULTS AND DISCUSSION

In the period from 2016 to 2018, 168 samples of ready-to-eat dairy products were tested for *S. aureus* in the Food Safety Laboratory of the Laboratory and Diagnosis Center of the All-Russian Scientific Research Institute of Health and Nutrition Branch in the Republic of Crimea, 7 isolates were recovered (Table 1).

It was found that the following product groups were contaminated with these bacteria: butter (20%), sour cream (9.09%), cottage cheese and curd products (4.55%), pasteurized milk in consumer containers (4.35%) and fermented dairy products with a shelf life of more than 72 hours (2.63%). The obtained data may indicate an unsatisfactory sanitary conditions for production of dairy products.

The next stage was the study of biological properties of the obtained isolates, namely: Gram staining, catalase and lecithinase activity, plasma coagulation reaction, determination of hemolytic properties. The results are presented in Table 2.

As follows from Table 2, all the recovered isolates, gram-positive cocci, are able to form catalase, have lecithinase activity, are able to coagulate rabbit blood plasma, and cause β-hemolysis on 5% blood agar (Fig. 1), which confirms their belonging to *S. aureus.*

The next stage was to study biochemical characteristics of the isolates. The results are demonstrated in Table 3.

From table 3 it follows that 100% of the recovered isolates grow at a high concentration of NaCl, ferment D-galactose, D-maltose, D-mannitol, D-mannose, sucrose, D-trehalose and do not cleave D-raffinose and D-xylose. All studied isolates are characterized by the presence of enzymes phosphatase, lecithinase and absence of the enzyme beta-glucuronidase. The urease enzyme is present in 28.6% of the isolates. In accordance with Burgey's Manual they can be attributed to *S. aureus* [9].

In contrast to the Manual data, 42.9% of the isolates ferment lactose, and 28.6% – salicin. The enzyme arginine dihydrolase 2, according to Bergey's Manual, should be present in all isolates, however, its presence is observed only in 57.1% of the tested cultures. In our opinion, these discrepancies can be attributed to the biological characteristics of the isolated microorganisms.

Presence of enzymes is characteristic of all the tested isolates: arginine dihydrolase 1, L-pyrrolidonyl-arylomidase, L-lactate. Beta-galactosidase enzyme is present in 71.4% of microorganisms, alpha-glucosidase in 28.6%, and alaninarylamidase in 14.3%.

Table 1
Number of samples of ready-to-eat dairy products contaminated with *S. aureus*

Group number	Products	Number of tests	*S. aureus* detection	
			Number	%
1	Pasteurized drinking milk in consumer packaging	46	2	4.35
2	Pasteurized cream	2	0	0
3	Sour milk products with a shelf life of more than 72 hours	38	1	2.63
4	Sour cream	11	1	9.09
5	Curd, curd products	44	2	4.55
6	Cheese	3	0	0
7	Butter	5	1	20
8	Spread	1	0	0
9	Ice cream	18	0	0
	Total	168	7	4.17

Fig. 1. β-hemolytic activity of the isolate S. aureus No. 242/18 Crimea on 5% blood agar

85.7% of the isolates cleave D-ribose, methyl-B-D-glucopyranoside; 14.3% – D-sorbitol. All the tested bacteria (100%) cleave N-acetyl-D-glucosamine and do not decompose cyclodextrin and pullulan.

Table 2
Biological characteristics of *S. aureus* isolates

S. aureus isolate	Gram staining	Plasma coagulation reaction	RBC hemolysis	Catalase formation	Presence of lecithinase
1	G+	+++	β-hemolysis	+	+
2	G+	++++	β-hemolysis	+	+
3	G+	++++	β-hemolysis	+	+
4	G+	++++	β-hemolysis	+	+
5	G+	+++	β-hemolysis	+	+
6	G+	++++	β-hemolysis	+	+
7	G+	++++	β-hemolysis	+	+

«++++» – positive reaction, characterized by appearance of a dense clot in the reaction with 5% rabbit blood plasma;
«+++» – positive reaction, characterized by the appearance of a clot with a small partition in the reaction with 5% rabbit blood plasma.

Table 3
Biochemical characteristics of *S. aureus* isolates

No.	Test	*S. aureus* isolates							According to Bergey's manual
		1	2	3	4	5	6	7	
1	Arginine dihydrolase 2	–	–	+	+	+	+	–	+
2	Phosphatase	+	+	+	+	+	+	+	+
3	Beta glucuronidase	–	–	–	–	–	–	–	–
4	Lecithinase	+	+	+	+	+	+	+	+
5	Urease	+	+	+	+	+	–	–	+/–
6	D-galactose	+	+	+	+	+	+	+	+
7	Lactose	+	–	–	–	–	+	+	+
8	D-maltose	+	+	+	+	+	+	+	+
9	Growth at 6.5% NaCl	+	+	+	+	+	+	+	+
10	D-mannitol	+	+	+	+	+	+	+	+
11	D-mannose	+	+	+	+	+	+	+	+
12	D-melitose	–	–	–	–	–	–	–	–
13	Salicin	+	–	–	–	–	+	–	–
14	Sucrose	+	+	+	+	+	+	+	+
15	D-trehalose	+	+	+	+	+	+	+	+
16	D-xylose	–	–	–	–	–	–	–	–
17	Phosphatidylinositol phospholipase C	-	–	–	–	–	–	–	*
18	Arginine dihydrolase 1	+	+	+	+	+	+	+	*
19	Beta galactosidase	–	+	+	+	+	+	–	*
20	Alpha glucosidase	–	–	–	–	+	+	–	*
21	Ala-Phe-Pro-arylamidase	–	–	–	–	–	–	–	*
22	Cyclodextrin	–	–	–	–	–	–	–	*
23	L-aspartate amylidase	–	–	–	–	–	–	–	*
24	Beta galactopyranosidase	–	–	–	–	–	–	–	*
25	Alpha mannosidase	–	–	–	–	–	–	–	*
26	Leucinarilamidase	–	–	–	–	–	–	–	*
27	L-prolinarylamidase	–	–	–	–	–	–	–	*
28	Alpha galactosidase	–	–	–	–	–	–	–	*
29	L-pyrrolidonyl arylomidase	+	+	+	+	+	+	+	*
30	Alaninarylamidase	–	–	–	–	+	–	–	*
31	Tyrosinarylamidase	–	–	–	–	–	–	–	*
32	D-sorbitol	–	–	–	–	–	+	–	*
33	D-ribose	+	+	+	+	+	+	–	*
34	L-lactate, alkalization	+	+	+	+	+	+	+	*
35	N-Acetyl-D-Glucosamine	+	+	+	+	+	+	+	*
36	Methyl-B-D-glucopyranoside	+	+	+	+	+	+	–	*
37	Pullulan	–	–	–	–	–	–	–	*

«+» – positive reaction;
«–» – negative reaction;
«+/–» – both positive and negative reactions may be present;
«*» – no data available.

Table 4
Antimicrobial resistance of *S. aureus* isolates

Antibiotic	Inhibition zone diameter Methodical Guidance 4.2.1890-04 [8], mm			Number of isolates (%)		
	R ≤	I	S ≥	R ≤	I	S ≥
β- lactam preparations						
Benzylpenicillin	8	–	9			7 (100)
Oxacillin	10	11–12	13			7 (100)
Imipenem	13	14–17	18			7 (100)
Meropenem	12	13–19	20			7 (100)
Ticarcillin	11	12–16	17			7 (100)
Macrolides						
Erythromycin	13	14–22	23		2 (28.6)	5 (71.4)
Fluoroquinolones						
Ciprofloxacin	15	16–20	21			7 (100)
Ofloxacin	12	13–15	16			7 (100)
Enrofloxacin	13	14–16	17	7(100)		
Aminoglycosides						
Gentamicin	12	13–14	15			7 (100)
Amikacin	14	15–16	17			7 (100)
Kanamycin	13	14–17	18		2 (28.6)	5 (71.4)
Streptomycin	12	13–16	17	2 (28.6)	5 (71.4)	
Glycopeptides						
Vancomycin	14	–	15	1 (14.3)		6 (85.7)
Tetracyclines						
Doxycycline	12	13–15	16			7 (100)
Tetracycline	14	15–18	19			7 (100)
Rifampicin						
Rifampicin	16	17–19	20			7 (100)
Cephalosporins						
Cefotaxime	14	15–18	19			7 (100)
Ceftriaxone	13	14–17	18			7 (100)
Ceftazidime	12	14–16	18		4 (57.1)	3 (42.9)
Other						
Chloramphenicol	12	13–17	18			7 (100)
Trimethoprim	13	14–16	17			7 (100)

Phosphatidylinositol phospholipase C, Ala-Phe-Pro-arylamidase, L-aspartate arylamidase, beta-galactopyranosidase, alpha-mannosidase, leucinarylamidase, L-prolinerylamidase, alpha-galactosidase, tyrosinarylamidase enzymes are not found in the recovered isolates.

Wide spread of the resistant forms of pathogenic microorganisms and the decrease in the effectiveness of several antibiotics are currently one of the main problems of medicine and veterinary medicine. *S. aureus* is one of the first bacteria in which resistance to previously active antibiotics was found. Resistance to methicillin, coupled with resistance to the class of β-lactam antibiotics, gave the name methicillin-resistant staphylococci (MRSA) [4, 5].

Now methicillin is not used in clinical practice and in laboratory diagnostics, oxacillin is used instead, and the term "oxacillin resistance" has appeared, which is a complete synonym for the term "methicillin resistance" [4, 5].

In accordance with Methodical Guidlines 4.2.1890-04 determination of *Staphylococcus* spp. sensitivity to β-lactam antibacterial drugs should include performance of two sensitivity tests: to benzylpenicillin and oxacillin [8].

The final stage of the work was determination of antimicrobial resistance of the obtained isolates. In accordance with the methodology used, microorganisms were evaluated as sensitive (S), resistant (R) and intermediate (I). The results are presented in Table 4.

The following antibiotic groups were selected for the test: macrolides, fluoroquinolones, aminoglycosides, glycopeptides, tetracyclines, rifampicin, β-lactam drugs, cephalosporins, chloramphenicol – only twenty-two drugs.

All isolated cultures of *S. aureus* showed 100% sensitivity to benzylpenicillin, oxacillin, imipenem, ticarcillin, meropenem, ciprofloxacin, ofloxacin, gentamicin, amikacin, doxycycline, tetracycline, rifampicin, levomycetacitimetison, and cefot. Sensitivity to vancomycin was detected in 85.7% of isolates, to erythromycin and kanamycin – in 71.4%, to ceftazidime – in 42.9%. Intermediate resistance to kanamycin and erythromycin was observed in 28.6% of the tested cultures, to ceftazidime – in 57.1%, to streptomycin – in 71.4% of isolates.

All isolated cultures of *S. aureus* were 100% resistant to enrofloxacin. 28.6% of the isolates showed resistance to streptomycin, 14.3% of the isolates were resistant to vancomycin.

Methicillin-resistant (oxacillin-resistant) staphylococci were not detected in the recovered isolates.

CONCLUSION

In the period from 2016 to 2018, 168 samples of ready-to-eat dairy products were tested for *S. aureus* bacteria in the Food Safety laboratory of the FGBI "ARRIAH" branch in the Republic of Crimea. Seven *S. aureus* isolates were detected.

The following product groups were found to be most contaminated with these bacteria: butter (20%), sour cream (9.09%), curd and curd products (4.55%), pasteurized milk in consumer packaging (4.35%).

The basic cultural and biochemical properties of the detected isolates were studied. It has been established that the isolated microorganisms differ in the ability to ferment lactose (4 isolates), the ability to cleave salicin (2 isolates), and the presence of the enzyme arginine dihydrolase 2 (3 isolates) from the data of the Bergey's Manual.

The resistance of the tested cultures to 22 antibiotics was determined. Most of the isolates were sensitive to all antibiotics used, while all the tested isolates were resistant to enrofloxacin.

The recovered isolates did not possess methicillin resistance (oxacillin resistance) and were sensitive to other β-lactam antibiotics.

Conflict of interest. The author declares no conflict of interest.

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