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PHENOTYPIC RESISTANCE TO ANTIBIOTICS OF *Staphylococcus aureus* STRAINS ISOLATED FROM COW MILK

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Abstract

The emergence of multiresistant strains has sharply escalated in recent decades due to the wide use of antimicrobials. The infections caused by *Staphylococcus aureus* are most common in highly productive dairy animals. The aim of our work was to assess the phenotypic resistance of *S. aureus* strains isolated from cow milk to the main antibacterial drugs used to treat various forms of mastitis in the Russian Federation, as well as to determine their minimum inhibitory concentrations (MIC, MIC_{50} and MIC_{90}). From January to December 2018 milk samples were taken from cows of black-motley Holstein breed at the farms of the Central region of the Russian Federation. A total of 314 milk samples were examined and 447 potential staphylococci were isolated. All isolates were evaluated by conventional phenotypic methods. Only 103 isolates met all the identification criteria and were used for further analysis. The disk diffusion method (DDM) was used to test the susceptibility of the isolated strains to the following antibiotics: penicillin (PEN, 10 U), oxacillin (OX, 10 μg), gentamicin (GN, 10 μg), erythromycin (ER, 15 μg), and lincomycin (LN, 15 mcg), rifampicin (RF, 5 mcg), ciprofloxacin (CP, 5 mcg), vancomycin (VA, 30 μg), fusidine (FZ, 10 μg) (Pharmacotherapy Research Center, Russia; Mueller Hinton Agar, HiMedia Laboratories Pvt. Ltd., India). A method of double (Log_2) serial dilutions of antibiotics (penicillin, erythromycin, gentamicin, and ciprofloxacin) in TSB medium (from 64 to 0.125 mg/l) with 5×10^5 CFU/ml inoculum was used to determine the minimum inhibitory concentrations (MIC, MIC_{50}, MIC_{90}) and LD_{50}. The incubation was carried out at 37 °C for 20 hours (220 rpm). The bacterial growth in TSB medium with antibiotics was evaluated by plating on Baird Parker Agar medium (HiMedia Laboratories Pvt. Ltd., India). The interpretation of the results was carried out according to the recommendation of The European Committee on Antimicrobial Susceptibility Testing (EUCAST). The highest degree of resistance was observed to erythromycin (82.5 %) and fusidine (75.7 %). Seven of 103 strains tested were susceptible to all studied antibiotics, 96 isolates were resistant to at least one of them. When assessing multiple resistance (MAR), 65 (63.1 %) strains were resistant to four or more antibiotics. The predominant phenotypes for the isolates were ER + LN + FZ and ER + CP (58.3 % and 47.6 %, respectively). A total of 31 strains of the 47 isolates DDM-resistant to penicillin grew at ≥ 64 mg/l penicillin, while in the concentration range of ≤ 1.0 mg/l, the growth was observed in 33.0 % of the strains. Fifteen of the 85 erythromycin-resistant isolates showed growth at MIC ≥ 64 mg/l; only 7 strains out of 18 DDM-susceptible ones were in the MIC ≤ 0.5 mg/l range. A total of 29 (28.2 %) of the 103 studied strains were DDM-resistant to gentamicin, 72 (60.2 %) showed growth at MIC ≥ 0.5 mg/l, and 5 strains at MIC ≥ 64.0 mg/l. Only 2 of the 49 DDM-resistant strains grew at MIC ≥ 64 mg/l. It is important to point out that the MIC_{50} was not in the susceptible range for any of the studied antibiotics, and the MIC_{90} showed the susceptible range for penicillins, aminoglycosides, and fluoroquinolones (> 0.5, > 0.25, and > 0.25 mg/l, respectively). In our study the high phenotypic resistance indices of *S. aureus* isolates from cow milk emphasizes the importance of routine screening of *S. aureus* isolates for inducible phenotype resistance.

Keywords: mastitis, *Staphylococcus aureus*, antibiotics, antimicrobial susceptibility, MIC,

Although emergence and spread of resistant bacteria is a natural and an inevitable process, its rate is directly related to selective pressure of antibiotics, which expression, in turn, is associated with rate of their application both in medicine and in agriculture (animal production and health) [1]. The infections caused by *Staphylococcus aureus* are most common in highly productive dairy animals. The emergence of multiresistant strains has sharply escalated in recent decades due to the constant use of antimicrobials, increasing the relevance of antibiotic treatment of mastitis.

Macrolide, aminoglycoside, and penicillin groups, including such antibiotics as benzyl-penicillin, erythromycin, amoxicillin, and gentamicin are often used to treat mastitis in cows in the Russian Federation [2-4]. Findings of recovery studies in vitro are an important tool at selection of the most effective antimicrobial agent for therapeutic and preventative interference. Subinhibitory concentrations (sub-MIC) of antibiotics result in broad transcriptional changes in a bacterial cell, affecting the adhesive ability and development of antibiotic resistance [5, 6]. In such a case, antibiotics are acting as signal molecules and are able to modulate bacterial phenotypes. It is known that sub-MIC of antibiotics, underlying mechanism of which lies in DNA damage, reinforce mutations in bacteria. Gene expression controlling formation of bio-film (BF) is induced in *S. aureus* in presence of macrolides (erythromycin, clarithromycin, and azithromycin) and β-lactam antibiotics (penicillin, oxacillin, cephalexin, cephalothin, and vancomycin) [7-9]. Cephalosporins initiate protein synthesis but do not influence on gene expression of Quorum Sensing (QS) system modulating BF formation [10, 11]. Formed bio-films are resistant to wide spread antimicrobials, which calls for more effective approach and development of BF control strategies in medicine and animal health fields.

Availability of representative sample during the microbiological analysis of milk is of paramount importance for making a correct diagnosis [12]. Mechanisms for developing antibiotic resistance vary from spontaneous producing of enzyme and antimicrobial selectivity of a cell wall to efflux-systems [13, 14]. Traits of resistance to antimicrobial agents are encoded by genes, which may be transferred on bacterial chromosome, plasmids, transposone or on gene cassettes included in integrons [15-17]. Resistance genes *rpoB*, *blaZ*, *mecA* [18], *aacA-D*, *tetK*, *tetM*, *ermA* [19, 20], *msrA* (21), *linA*, *vatA*, *vatB*, *dfrA*, *gyrA*, *grlA*, *cat1* to antibiotics of ansamycin, penicillin, aminoglycoside, tetracycline, macrolide-lincosamide-streptogramin B, lincosamide, streptogramin, and fluoroquinolone groups were found in *S. aureus* strains [22, 23]. Penicillin resistant isolates bear plasmid-located gene *blaZ*, which encodes β-lactam enzyme called penicillinase [24, 25].

Emergence and spreading of penicillinase resistance in *S. aureus* is called the first wave of resistance, whilst development of methicillin resistance is called the second wave. The latter is mediated by presence of *mecA* gene encoding low affine protein binding penicillin (PBP2a) [26]. Quinolone resistance in *S. aureus* emerges stepwise due to point mutation (mainly, in topoisomerase IV subunit GrlA gene and helicase subunit GyrA gene), as well as expression of *norA* gene encoding NorA protein with main pumping function (NorA efflux pump). *S. aureus* isolates resistant to aminoglycosides produce three types of aminoglycoside-modifying enzymes (AME) [27] — aminoglycoside-3″-O-phosphoryltransferase III [aph(3′)-III] [28], aminoglycoside-4′-O-phosphoryltransferase I [ant(4′)-I] [29], and aminoglycoside-6′-N-acetyltrans-ferase/2″O-phosphoryltransferase

[aac(6')/aph(2″)] [30], which accordingly modify aminoglycosides kanamycin, tobramycin, and gentamicin [31, 32]. Study of macrolide resistance in *S. aureus* shows that *erm* encoding ribosomal methylase are the most common genes determining the erythromycin resistance [33, 34].

Current work for the first time describes daily activity dynamics of *S. aureus* strains isolated from milk of highly productive cows in the environment with high concentrations of antimicrobials. Direct correlation between the strain resistances was found at evaluation by disk diffusion method (DDM) and by minimum inhibitory concentrations. This work introduces information on combined antibiotic resistance in *S. aureus* strains found in cow milk in the Central region of Russia.

The aim of our work was to assess the phenotypic resistance of *Staphylococcus aureus* strains from cow's milk to the main antibacterial drugs used to treat various forms of mastitis in the Russian Federation, as well as to determine their minimum inhibitory concentrations (MIC, MIC_{50} and MIC_{90}).

Techniques. From January to December 2018 milk samples were collrcted from cows of black-motley Holstein breed *(Bos taurus taurus)* at the farms of the Central region of the Russian Federation. Samples were taken aseptically in milking period (from each cow in 60-70 days after calving). Samples were delivered to the laboratory in 2 hours at temperature of +4 °C or in frozen state at temperature of –20 °C.

All isolates were assessed by commonly accepted phenotypic methods: microscopy evaluation of colonies by Gram coloration; growth in Baird Parker Agar medium (HiMedia Laboratories Pvt. Ltd., India); hemolysis in Azide Blood Agar Pronadisa medium (Conda, Spain); coagulase positive take (CJSC ECOlab, Russia); biochemical identification with the use of microbiological mediums and API 20 STAPH panel (BioMerieux, France). Strains for further studies were kept in vials with Trypticase Soy Broth (TSB, Merck, Germany) with 30 % sterile glycerin (Sigma, USA) at –18 °C.

Strain sensitivity to penicillin (PEN, 10 U), oxacillin (OX, 10 μg), gentamicin (GN, 10 μg), erythromycin (ER, 15 μg), and lincomycin (LN, 15 μg), rifampicin (RF, 5 μg), ciprofloxacin (CP, 5 μg), vancomycin (VA, 30 μg), fusidine (FZ, 10 μg) (Pharmacotherapy Research Center, Russia) was tested by disk diffusion method (DDM) on Mueller Hinton agar medium (HiMedia Laboratories Pvt. Ltd., India). Screening of methycillin-resistant isolates of *S. aureus* (MRSA) was performed using disks with cefoxitin (CF, 30 μg) [35].

Minimum inhibitory concentrations (MIC, MIC_{50}, MIC_{90}) and LD_{50} of antibiotics were determined by method of serial dilutions in TSB medium. To that effect, inoculum of tested *S. aureus* strains with density of 0.5 based on McFarland Standard was made from agar culture. Antibiotics were diluted in physiological solution. Double (Log_2) serial dilutions of antibiotics (penicillin, erythromycin, gentamicin, and ciprofloxacin) in concentrations from 64 to 0.125 mg/l were made according to methodological guidelines [36]. Approximately 5×10^5 CFU/ml of tested microorganism was tested in 1 ml of each dilution of antimicrobial drug (AMD). The incubation was carried out at nutator at 37 °C for 20 hours (220 rpm). The bacterial growth in TSB medium with antibiotics was evaluated by plating on Baird Parker Agar medium. The results were interpreted based on recommendation of The European Committee on Antimicrobial Susceptibility Testing (EUCAST) [37].

Biometrical processing was carried out with the use of Microsoft Excel 2010, SAS version 6.12 (SAS Institute, USA).

Results. A total of 314 milk samples from high productive cows were examined and 447 potential staphylococci were isolated, of which only 103 met all identification criteria and were used for further analysis. Such level of contamination is similar to that noted during previous studies in Russia [37, 38] and other countries [39-41].

The highest degree of *S. aureus* isolate resistance in vitro was observed in case of erythromycin (82.5 %) and fusidine (75.7 %) (Table 1). Sensitivity to all studied antibiotics was found only in 7 isolated strains (6.8 %), whilst 96 isolates were resistant to at least one of them. Other authors found evident development of *S. aureus* resistance to penicillin, ampicillin, and erythromycin [40, 42, 43].

1. Antibiotic sensitivity of *Staphylococcus aureus* strains isolated from cows of black-motley Holstein breed at the farms of the Central region of the Russian Federation (2018)

Antibiotic	Concentration	MSSA (n = 47)				Total strain number (n = 103)			
		resistant		susceptible		resistant		susceptible	
		n	%	*n*	%	*n*	%	*n*	%
Pen	10 U	47	100	0	0	47	45.6	56	54.4
OX	10 μg	0	0	47	100	0	0	103	100
GN	10 μg	20	42.6	27	57.4	29	28.2	74	71.8
ER	15 μg	41	87.2	6	12.8	85	82.5	18	17.5
LN	15 μg	31	66.0	16	34.0	61	59.2	42	40.8
RF	5 μg	30	63.8	17	36.2	49	47.6	54	52.4
CP	5 μg	37	78.7	10	21.3	49	47.6	54	52.4
VA	30 μg	0	0	47	100	0	0	103	100
FZ	10 μg	42	89.4	5	10.6	78	75.7	25	24.3
CF	30 μg	0	0	47	100	0	0	103	100

Note. Pen — penicillin, OX — oxacillin, GN — gentamicin, ER - erythromycin, LN — lincomycin, RF — rifampicin, CP — ciprofloxacin, VA — vancomycin, FZ — fusidine, CF — cefoxitin; MSSA — methycillin-susceptible *S. aureus*.

Frequency of staphylococci resistance related to production of β-lactamase comprises from 55.7 to 92.6 % worldwide [44]. In our studies, phenotype traits of resistance to β-lactam antibiotics were found in 47 out of 103 isolates. All 47 (45.6 %) strains were resistant to penicillin (100 %), which is consistent with findings of Shi et al. [45] that had tested 206 strains of *S. aureus* in Mongolia, but which is inconsistent with findings of Aarestrup et al. [46] denoting low frequency (10 %) of benzyl-penicillin resistance in Denmark, Northway, and Sweden. Share of penicillin resistant isolates in other part of Europe varied from 23 to 69 % [47].

We had not found resistance to oxacillin and cefoxitin in all tested strains, making them methycillin-susceptible *S. aureus* MSSA. In case of MSSA, it is assumed that penicillin surpasses oxacillin by its effect, but only if strains do not produce penicillinase [48]. A total of 12 out of 47 strains (25.5 %) had simultaneous resistance to 7 antibiotics (gentamicin, erythromycin, lincomycin, rifampicin, ciprofloxacin, and fusidine).

It was found that 100 % strains isolated from cow's milk in the Central region of the Russian Federation were sensitive to vancomycin. Such drug is not used in veterinary medicine anymore in many countries, including Russia, which may explain the presented findings.

Multiple antibiotic resistance (MAR) phenotypes were identified for 103 strains of *S. aureus* (Table 2). Hence, 65 (63.1 %) strains were resistant to four and more antibiotics. Maximum frequency of combined resistance was observed in case of erythromycin + lincomycin + fusidine) (58.3 %) and erythromycin + ciprofloxacin (47.6 %). Penicillin resistant strains had higher phenotype resistance that resistance to other antibiotics. Multiple resistances were developed in 60 out of 103 strains.

2. Combined resistance to various antibiotics in *Staphylococcus aureus* strains isolated from cows of black-motley Holstein breed at the farms of the Central region of the Russian Federation (n = 103, 2018)

Antibiotic	Susceptible strains per sample	
	n	%
Pen + GN	20	19.4
Pen + ER	41	39.8
Pen + LN	31	30.1
Pen + CP	37	35.9
Pen + FZ	42	40.8
ER + CP	49	47.6
Pen + GN + ER	20	19.4
Pen + GN + ER + LN	16	15.5
Pen + GN + ER + LN + RF + CP + FZ	12	11.7
ER + LN + FZ	60	58.3

N o t e . Pen — penicillin, 10 U; OX — oxacillin, 10 μg; GN — gentamicin, 10 μg; ER - erythromycin, 15 μg; LN — lincomycin, 15 μg; RF — rifampicin, 5 μg; CP — ciprofloxacin, 5 μg; VA — vancomycin, 30 μg; FZ — fusidine, 10 μg; CF — cefoxitin, 10 μg.

3. Minimum inhibitory concentrations (MIC, mg/ml) **for coagulose-positive *Staphylococcus aureus* strains isolated from cows of black-motley Holstein breed at the farms of the Central region of the Russian Federation** (n = 103, 2018)

Antibiotic	MIC		Suppressed isolates for different MIC										MIC_{50}	MIC_{90}
	TV	PV	≥ 64	32	16	8	4	2	1	0,5	0,25	≤ 0,125		
ER	0.5	0.25-1.0	15	10	27	14	10	9	11	3	4	0	> 16.0	> 1.0
Pen	0.25-0.5	0.125-1.0	31	10	7	6	8	7	14	12	8	0	> 8.0	> 0.5
GN	0.25	0.125-0.5	5	13	14	13	11	6	10	13	10	8	> 4.0	> 0.25
CP	0.5-1.0	0.25-2.0	2	8	12	12	13	10	12	14	11	9	> 2.0	> 0.25

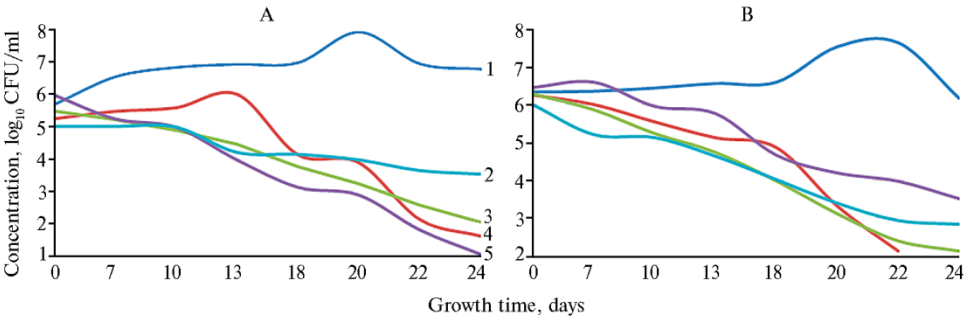
N o t e . TV — target values, AV — permitted values; ER erythromycin, Pen — penicillin, GN — gentamicin, CP — ciprofloxacin; $MIC_{50\ and}$ MIC_{90} — concentrations at which 50 and 90% strains were killed, accordingly. Target values were calculated by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) [49]. Permitted values are consistent to ISO 20776-1: 2006 (as updated based on the latest version of CLSI M100).

MIC values for coagulate positive *S. aureus* strains are provided in Table 3. Due to insignificant difference (2-3 %) in specificity and sensitivity of *S. aureus* strains to cefoxitin at assessment by MIC and DDM methods, which reliably predicts the resistance to methycillin, MIC studies for such antibiotic were not carried out [50]. A total of 31 strains of the 47 isolates DDM-resistant to penicillin grew at ≥ 64 mg/l penicillin, while in the concentration range of ≤ 1.0 mg/l, the growth was observed in 33.0 % of the strains. MIC_{50} and MIC_{90} for penicillin comprised > 8.0 and > 0.5 mg/l, much higher than the established standard for susceptible strains. High penicillin resistance among *S. aureus* strains could be due to the use of intramammary drugs containing combinations of various broad-spectrum antibiotics and antimicrobials [51].

A total of 15 out of the 85 erythromycin-resistant isolates showed growth at MIC ≥ 64 mg/l; only 7 strains out of 18 DDM-susceptible ones were in the MIC ≤ 0.5 mg/l range. MIC_{50} and MIC_{90} for erythromycin were maximal as compared to all studied antibiotics (see Table 3). A total of 29 (28.2 %) of the 103 studied *S. aureus* strains were DDM-resistant to gentamicin, 72 (60.2 %) showed growth at MIC ≥ 0.5 mg/l, and 5 strains at MIC ≥ 4.0 mg/l. Ciprofloxatin was the most effective regarding the isolated *S. aureus* strains. Thus, only 2 of the 49 DDM-resistant strains grew at MIC ≥ 64 mg/l; MIC_{50} and MIC_{90} for such antibiotic comprised > 2.0 and > 0.25 mg/l, respectively. Finally, it is important to point out that the MIC_{50} was not in the susceptible range for any of the studied antibiotics, and the MIC_{90} showed the susceptible range for penicillins, aminoglycosides, and fluoroquinolones.

A total of 12 multiresistant *S. aureus* strains, susceptibility of which was described above, were selected out of 103 strains as samples to study the antibiotic effect as anti-growth factor. Active growth at average for all strains began by the 7th hour of culturing (Fig.). Antibiotics in the medium suppressed or inhibit-

ed growth. The observed effect depended upon the time of culture growth and type of antibiotics. Penicillin in concentration of 64 mg/l resulted in decrease of the number of viable cells in 83.3 % cases, whilst complete suppression of growth by 20 hour was observed only in two cultures. Gentamicin, ciprofloxatin, and erythromycin in concentration of 64 mg/l did not render full suppressive effect on any of 12 strains. After 24 hours, number of *S. aureus* cells in the medium with gentamicin was not lower than 2.84 $\log_{10}$ CFU/ml, and with erythromycin not lower than 2.97 $\log_{10}$ CFU/ml. Viability of *S. aureus* in ciprofloxatin-containing medium comprised from 18.5 to 84.0% for various strains, which implies a presence of cells with genes resistant to such group of antibiotics. Similar dependence in antibiotic effect we observed in all 12 studied strains.



Growth of *Staphylococcus aureus* strains 615 (A) **and 1839** (B) **isolated from cows of black-motley Holstein breed at the farms of the Central region of the Russian Federation depending on time and type of antibiotics:** 1 — control, 2 — ciprofloxatin, 64 mg/l, 3 — gentamicin, 64 mg/l, 4 — penicillin, 64 mg/l, 5 — erythromycin, 64 mg/l.

In our work, high phenotype resistance of *S. aureus* isolates from cow's milk emphasizes the importance of routine screening of *S. aureus* isolates for identification of inducible resistance of phenotypes. Observations of changes in count of viable cells exposed to high antibiotic concentrations during 24 hours allow tracing development of the resistance.

Therefore, 23.0% of all isolates from milk of cows of black-motley Holstein breed at the farms of the Central region of the Russian Federation were classified as *Staphylococcus aureus*, of which 63.1 % strains were resistant to four and more antibiotics. MIC_{50} and MIC_{90} for penicillin comprised > 8.0 and > 0.5 mg/l, much higher than the established standard for susceptible strains. Out of DDM-resistant *Staphylococcus aureus* to penicillin, erythromycin, gentamicin, and ciprofloxatin at MIC ≥ 64.0 mg/l growth potential was confirmed in 65.96; 17.65; 17.24, and 4.08 % respectively. Antibiotics in maximum dosages (64 mg/l) did not render complete suppressive effect in viable strains: cell count for all 12 studied isolates was not lower than 2.84 $\log_{10}$ CFU/ml in gentamicin medium and 2.97 $\log_{10}$ CFU/ml in erythromycin medium. Count of viable cells comprised 16.7 % for penicillin and 84.0 % for ciprofloxatin. Practical use of phenotypic estimates makes it possible to identify antibiotic resistance in mastitis etiologic agent in high productive cows. This findings allowed us to select strains for further studies aimed at searching resistance genes.

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