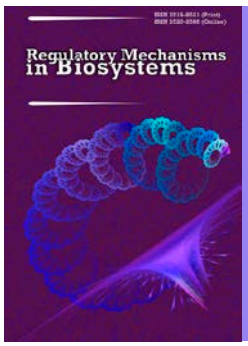

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Characteristics of antibiotic sensitivity of *Staphylococcus aureus* isolated from dairy farms in Ukraine

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Staphylococcus aureus is one of the most important microorganism in the process of raw milk production and has significance for people's health as it causes dangerous microbial contamination of dairy production. Furthermore, raw milk and the environment of livestock farms may be potential vehicles for distribution of antibiotic-resistant strains of *S. aureus*. The aim of the present study was to establish antibiotic sensitivity profiles of *S. aureus* depending on its origin from dairy farms, with a special focus on methicillin-resistant isolates. A total of 165 samples were collected for investigation in the period 2014–2016 from 5 dairy farms in Ukraine. All samples were analyzed for the presence of *S. aureus* using the Baird Parker Agar with Egg Yolk Tellurite Emulsion. Typical staphylococcal colonies were examined morphologically and for presence of coagulase and hemolysin activities. From these, positive samples for *S. aureus* were 62 (37.6%): 4 (6.5%) raw milk, 17 (77.4%) swabs of udder skin, 18 (29.0%) milk from cows with subclinical mastitis and 21 (33.9%) environmental samples. The standard disk diffusion method was used to determine sensitivity of *S. aureus* isolates to 10 antibiotics. The antimicrobial sensitivity profiles of *S. aureus* isolates showed differences between them, which depends on the origin of the isolates. Our results demonstrated that most of *S. aureus* isolates were resistant to penicillin, oxacillin and vancomycin. Of the 62 *S.aureus* isolates, 20 (32.3%) and 5 (8.1%) were found to be multiresistant to 3 different antibiotics, 6 (9.8%) isolates to 4 antibiotics, 12 (19.4%) and 3 (4.8%) to 5 antibiotics (P^{10}, OX^{1}, VA^{5}, L^{10}, TE^{30} and P^{10}, OX^{1}, VA^{5}, CIP^{10}, TE^{30} respectively). All isolates resistant to penicillin and oxacillin were typed by mec A gene in PCR with two primers (MecA147-F and MecA147-R). The results show that 66.7% of these isolates yielded a mecA product. The information obtained from this study is useful for understanding the prevalence of *S. aureus* and its antibiotic sensitivity in dairy farms and can be useful for local and national monitoring or for designing specific control programs of methicillin- and multiresistance isolates present in the food chain of milk production.

Keywords: raw milk; objects of dairy farms; antibiotic sensitivity profiles; MRSA; mecA gene

Introduction

Staphylococcus aureus is an opportunistic pathogen of human or animal skin and mucosal surfaces. It is well known that this microorganism can cause a wide range of infections such as skin infection (abscesses, carbuncle, furuncle and impetigo), pneumonia, osteomyelitis, endocarditis, sepsis and toxic shock syndrome. Also, it is common knowledge that *S. aureus* can be an agente responsible for food poisoning in people with severe vomiting and diarrhea. Most often poisoning is caused by the enterotoxins of *S. aureus* that are accumulated in milk or dairy products as result of multiplication of this type of microorganism (Bhunia, 2008; Wang et al., 2014; Brennan et al., 2016).

Therefore *S. aureus* has become an important problem in raw milk production and the dairy industry all over the world (Li et al., 2009; Ateba et al., 2010; Szweda et al., 2014; Carfora et al., 2015). There are many ways in which staphylococci enter raw milk and dairy products during their production. First of all, *S. aureus* can live on the surface of the skin of cow udders or even in the teat canals and, under certain conditions, causes subclinical and clinical mastitis in dairy cows. In this case, infected mammary glands are the main reservoir of contamination of raw cows' milk, dairy equipment and the dairy farm environment by these microorganisms (Febler et al., 2010; Basanisi et al., 2017; Hamid et al., 2017). Secondly, *S. aureus* can survive and persist in these objects for a long time and contaminate raw milk again. Few researchers have published information about biofilm formation by *S. aureus* on dairy equipment, from which bacteria penetrate raw milk, reducing the time of its shelf life and also cause poisoning of consumers (Kirmusaoglu, 2017; Kukhtyn et al., 2017).

But, the big problem of *S. aureus* in milk production is aggravated by the fact that the microorganism can quickly adapt to environmental conditions including antibiotics, which are used in livestock for treatment or as additives for animal feed. The long-term use of antibiotics on dairy farms has increased *S. aureus* resistance to antibiotics and particular to β-lactams (MRSA – methicillin-resistant *S. aureus*) (Keefe, 2012; Al-Ashmawy et al., 2016; Doulgeraki et al., 2017). The methicillin resistance characteristic in *S. aureus* is due to the presence of altered

penicillin binding protein (PBP2a) in the cell wall that has a reduced binding affinity to β-lactam antibiotics. PBP2a is encoded by mecA gene that is located in the large chromosomal cassette called staphylococcal chromosome cassette mec element (SCCmec) (Zhang and McClure, 2005; Ganai et al., 2016; Rahim et al., 2017).

Epidemiologically MRSA which were isolated from livestock animals, from workers in animal farms or the food production chain equipment and from the products of animal origin indicate as LA-MRSA (livestock-associated MRSA). In recent years, many studies have been published on the isolation and distribution of antibiotic resistance among *S. aureus* isolates from different sources of dairy farms depending on the geographical localization where each investigation was conducted. According to this data, wide variation in MRSA prevalence has been observed (Pu et al., 2014; Mehli et al., 2017; Sergelidis and Angelidis, 2017).

Moreover, MRSA has the ability to resist different antibiotics and become a multidrug-resistant microorganism (Thaker et al., 2013; Ateba et al., 2016). Thus, this pathogen has become a major concern in the livestock industry as well as a public health hazard (Jihasz-Kaszanytzky et al., 2007; Sergelidis and Angelidis, 2017).

However, there has been little discussion in the literature about antibiotic sensitivity of *S. aureus* isolated from raw milk and dairy farms environments in Ukraine. Against this background, the present paper aims to identify the prevalence of *S. aureus* on dairy farm facilities and milk samples from raw cows, to establish their antimicrobial sensitivity profiles with a special focus on methicillin-resistant isolates. The following tasks were pursued in this study: (i) isolation of *S. aureus* from samples (ii) study of their antimicrobial sensitivity depending on their origin (iii) typing methicillin-resistant isolates by mec A gene in PCR.

Materials and methods

Samples collection. A total of 165 samples: 32 samples of raw milk, 38 swabs of udder skin, 38 samples of milk from cows with subclinical mastitis and 57 environmental samples (10 swabs of milking machines, 13 swabs of milk tanks, 10 samples of animal feed, 24 swabs from floors of farm buildings) were collected for investigation in the period from 2014 till 2016 from 5 dairy farms in Sumy Region, Ukraine. The herd structure was characterized by medium-holder state-owned dairy farms with lactating cows from 1,500 to 1,000 per herd. All farms had similar breeding and milking systems. The cows were milked twice a day using milking machine.

The raw milk samples to the amount of 250 ml were taken in sterile bottles directly from milk tanks. The milk from cows with subclinical mastitis was collected after indicating it by using the Rapid California Mastitis Test (CMT, DeLaval). Swabs from the surface of udder skin of cows and swabs from milking machines, milk tanks and from floors were taken using cotton-tipped swabs. All samples were transported to the laboratory in containers with ice and then were immediately analyzed within 24 hours.

Methods of isolation and identification *S. aureus* from samples. For detection of staphylococci, all samples were inoculated onto Baird-Parker Agar with Egg Yolk Tellurite Emulsion (Himedia, India) and cultivated at 35–37 ºC for 24–48 h aerobically. Typical staphylococcal colonies (black, convex, shiny colonies surrounded by clear zones) were used for microscopic examination, and the coagulase test with rabbit plasma and for production of hemolysin on Blood Agar to distinguish *S. aureus* from other staphylococci. On microscopic examination, all the *S. aureus* isolates were found to be Gram positive, non-spore forming, nonmotile cocci, giving a clustered bunch of grape (Bhunia, 2008).

Antimicrobial susceptibility testing. Ten antimicrobial agents (Oxoid, UK): penicillin (10 IU/disk), oxacillin (1 μg/disk), gentamicin (10 μg/disk), streptomycin (10 μg/disk), vancomycin (5 μg/disk), enrofloxacin (10 μg/disk), ciprofloxacin (10 μg/disk), lincomycin (10 μg), erythromycin (15 mkg/disk), tetracycline (30 μg/disk) were tested in this study. Antibiotic sensitivity of *S. aureus* isolates was tested by the standard disk diffusion method on Mueller – Hinton Agar (Oxoid, UK) according to the guide. Based on the inhibition zone size, results were recorded as "Susceptible," "Intermediate," or "Resistant" according to the interpretive criteria specified in CLSI (Performance Standards for Antimicrobial Disk Susceptibility Tests, 2012).

mec A PCR typing. The mecA PCR typing was carried out with two primers according to technique described by Zhang and McClure (2005). MecA147-F (GTG AAG ATA TAC CAA GTG ATT) and MecA147-R (ATG CGC TAT AGA TTG AAA GGA T) were used for it. In brief, DNA isolation of *S. aureus* was performed by the following steps: a colony of pure culture was suspended in a test tube with 0.5 cm^3 of sterile deionized water, then was heated for 10 minutes at 99 °C, after centrifugation at 30,000 g for 1 min, 2 μl of the supernatant was used as template in a 25 μl PCR. Polymerase chain reaction was performed in termocycles "Tertsyk" (DNA technology, Russia) and "T1" (Biometra, Germany). Thermal cycling parameters were as follows: 95 °C for 4 min (1 cycle), followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s; and a terminal extension step of 72 °C for 7 min.

Results

Isolation of *S. aureus* from samples. A total of 62 (37.6%) *S. aureus* isolates from 165 investigated samples were detected. As can be seen in Table 1, the highest percentage of samples, from which *S. aureus* was isolated, were from farm 4 (58.1% positive samples) and from farm 2 (51.5% positive samples). And conversely, the smallest number of positive samples was determined from farms 3 and 5 (21.6% and 29.0% respectively). The results also show that *S. aureus* was isolated from 2.4% (4/165) samples of raw milk, from 10.3% (17/165) of samples of swabs from the surface of udder skin and from 10.9% (18/165) of samples of milk from cows with subclinical mastitis. A total of 57 environmental samples were examined and 21 were positive (12.7% from all investigated samples).

Antibiotic susceptibility testing of *S. aureus* isolates. Antibiotic susceptibility testing of 62 *S. aureus* isolates from 5 farms was done by the disk diffusion method on Mueller – Hinton Agar using disks of 10 antibiotics. The results of the testing of all *S. aureus* isolates are shown in Table 2. It has been found that the level of antimicrobial sensitivity of the investigated *S. aureus* isolates was different in each farm. But, interestingly, that a significaly higher number of resistant *S. aureus* isolates were detected from farm 2. Twelve *S. aureus* isolates from 17 investigated (82.4%) were resistant to 5 (P^{10}, OX^{1}, VA^{5}, L^{10}, TE^{30}) of 10 tested antibiotics. Interestingly, that the same situation was found with isolates from farm 4, but relating to resistance to other antibiotics (P^{10}, OX^{1}, VA^{5}, CIP^{10}, TE^{30}). And conversely, a low number of samples with resistant *S. aureus* were found from farms 1 and 3. Isolates showed resistance to 2–3 antibiotics.

Table 1
Results of *S. aureus* isolates detection from samples (n = 165)

Samples	Farm 1			Farm 2			Farm 3			Farm 4			Farm 5		
	N	n	%	N	n	%	N	n	%	N	n	%	N	n	%
Raw milk	5	0	0.0	7	2	28.6	5	0	0.0	8	2	25.0	7	0	0.0
Swabs of udder skin	8	3	37.5	8	4	37.5	10	2	20.0	5	4	80.0	7	4	57.1
Milk from cows with subclinical mastitis	8	3	37.5	8	5	62.5	10	3	30.0	7	5	71.4	5	2	40.0
Environmental samples	12	4	33.3	10	6	60.0	12	3	25.0	11	5	45.6	12	3	25.0
Total	33	10	30.3	33	17	51.5	37	8	21.6	31	18	58.1	31	9	29.0

Note: N – number of investigated samples, n – number of positive samples, % – percentages of positive samples.

Table 2
Antibiotic sensitivity of *S. aureus* isolates from dairy farms (n = 62)

Antibiotics	No. (%) of *S. aureus* isolates from														
	Farm 1 (n = 10)			Farm 2 (n = 17)			Farm 3 (n = 8)			Farm 4 (n = 18)			Farm 5 (n = 9)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
P^{10}	0	2 (20.0)	8 (80.0)	0	0	17 (100.0)	0	0	8 (100.0)	2 (11.2)	8 (44.4)	8 (44.4)	0	0	9 (100.0)
OX^{1}	3 (30.0)	1 (10.0)	6 (60.0)	0	5 (29.4)	12 (70.6)	0	2 (25.0)	6 (75.0)	12 (66.6)	3 (16.7)	3 (16.7)	3 (33.3)	0	6 (66.7)
GEN^{10}	10 (100.0)	0	0	12 (70.6)	3 (17.6)	2 (11.8)	8 (100.0)	0	0	18 (100.0)	0	0	9 (100.0)	0	0
S^{10}	7 (70.0)	2 (20.0)	0	11 (64.8)	3 (17.6)	3 (17.6)	8 (100.0)	0	0	15 (83.3)	3 (16.7)	0	6 (66.7)	3 (33.3)	0
VA^{5}	4 (40.0)	1 (10.0)	5 (50.0)	2 (11.8)	3 (17.6)	12 (70.6)	5 (62.5)	3 (37.5)	0	15 (83.3)	0	3 (16.7)	9 (100.0)	0	0
EX^{10}	8 (80.0)	2 (20.0)	0	13 (76.6)	2 (11.8)	2 (11.8)	8 (100.0)	0	0	18 (100.0)	0	0	9 (100.0)	0	0
CIP^{10}	9 (90.0)	1 (10.0)	0	12 (70.6)	3 (17.6)	2 (11.8)	9 (87.5)	1 (12.5)	0	12 (66.6)	3 (16.7)	3 (16.7)	6 (66.7)	3 (33.3)	0
L^{10}	8 (80.0)	2 (20.0)	0	3 (17.6)	2 (11.8)	12 (70.6)	5 (62.5)	3 (37.5)	0	15 (83.3)	3 (16.7)	0	5 (55.6)	0	4 (44.4)
E^{15}	10 (100.0)	0	0	5 (29.6)	12 (70.6)	0	5 (62.5)	3 (37.5)	0	14 (77.8)	4 (22.2)	0	7 (77.8)		2 (22.2)
TE^{30}	6 (60.0)	4 (40.0)	0	2 (11.8)	3 (17.6)	12 (70.6)	5 (62.5)	3 (37.5)	0	10 (55.6)	2 (11.1)	6 (33.3)	9 (100.0)	0	0

Note: P^{10} – penicillin (10 IU/disk), OX^{1} – oxacillin (1 μg/disk), GEN^{10} – gentamicin (10 μg/disk), S^{10} – streptomycin (10 μg/disk), VA^{5} – vancomycin (5 μg/disk), EX^{10} – enrofloxacin (10 μg/disk), CIP^{10} – ciprofloxacin (10 μg/disk), L^{10} – lincomycin (10 μg), E^{15} – erythromycin (15 mkg/disk), TE^{30} – tetracycline (30 μg/disk); interpretive criteria for antimicrobial sensitivity of *S. aureus* isolates: S – susceptible, I – intermediate, R – resistant.

The highest percentage of resistance among investigated isolates was observed in the case of β-lactam antibiotics: penicillin (n = 50, 80.6%) and oxacillin (n = 33, 53.2%). Despite this, 20 isolates (32.3%) that were resistant to penicillin and oxacillin were also resistant to vancomycin. It has been found that, the high resistance was also observed in the case of lincomycin (n = 16, 25.8%) and tetracycline (n = 18, 29.0%). The rates of resistance to ciprofloxacin and streptomycin were 9.9% (6/62) and 4.8% (3/62), respectively. Gentamicin, enrofloxacin and erythromycin were active against most of tested isolates.

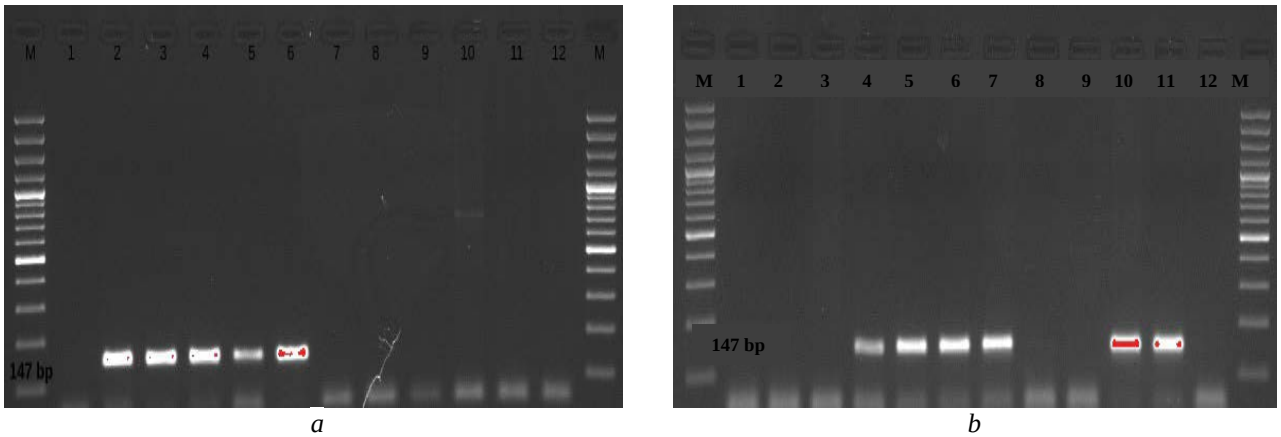
Antimicrobial resistance varied among *S. aureus* isolates, isolated from the different sources. The antimicrobial resistance of *S. aureus* isolates from swabs of udder skin from cows and samples of milk from cows with subclinical mastitis were higher in comparison to other samples. A significant difference in antimicrobial resistance was observed between the abovementioned isolates and isolates obtained from environmental samples.

Table 3 demonstrates antibiotic resistance profiles of isolates. Of the 62 *S. aureus* isolates, 20 (32.3%) and 5 (8.1%) were found as multiresistant to 3 antibiotics. Also, the analysis indicates that 6 (9.8%) isolates were resistant to 4 antibiotics. As mentioned above, 12 *S. aureus* isolates were resistant to 5 antibiotics (P^{10}, OX^{1}, VA^{5}, L^{10}, TE^{30}) and the 3 isolates were resistant to another 5 antibiotics (P^{10}, OX^{1}, VA^{5}, CIP^{10}, TE^{30}).

Table 3
The antibiotic resistance profiles of *S. aureus* isolates (n = 62)

Combination of antibiotic	Number of isolates	Percentage of isolates, %
P^{10},OX^{1}	33	53.2
P^{10},OX^{1}, VA^{5}	20	32.3
P^{10},OX^{1}, L^{10}	5	8.1
P^{10},OX^{1}, TE^{30}	6	9.8
P^{10},OX^{1}, VA^{5}, TE^{30}	6	9.8
P^{10},OX^{1}, L^{10}, TE^{30}	7	11.3
P^{10},OX^{1}, VA^{5}, L^{10}, TE^{30}	12	19.4
P^{10},OX^{1}, VA^{5}, CIP^{10}, TE^{30}	3	4.8

PCR typing. Initially, 15 isolates (3 from raw milk, 3 from swabs of udder skin and 9 from environmental samples) were identified as suspected isolates with typical cultural and antibiotic resistance (to β-lactams) properties. Conclusive statement concerning methicillin-resistant isolates was performed by detection of mecA gene in isolates by PCR method (Fig. 1).



a *b*

Fig. 1. PCR amplification of the mecA gene in selected *S. aureus* isolates: *a*: M – 100 bp molecular weight marker, 1 – negative control, 2 – positive control, 3–6 – amplified product of 147 bp of mecA gene, 7–12 – no amplified products of mecA gene; *b*: M – 100 bp molecular weight marker, 4–7, 10, 11 – amplified product of 147 bp of mecA gene, 1–3, 8, 9, 12 – no amplified products of mecA gene

The results show that 10 (66.7%) from 15 isolates of *S. aureus* yielded a mecA product.

Discussion

In the last few decades the number of publications about antibiotic resistance of microorganisms has increased and it is one of the most serious problem for human and animal health in the world (Normanno et al., 2007; Li et al., 2009; Gopal and Divya, 2017). In this paper we study antimicrobial sensitivity profiles of *Staphylococcus aureus* from 5 dairy farms in Ukraine with a special focus on methicillin-resistant isolates. Despite the fact that similar studies have been conducted by other authors in different regions of the world (İkiz et al., 2013; Szweda et al., 2014; Abebe et al., 2016), this is the first study in Ukraine concerning antimicrobial sensitivity profiles of *S. aureus* isolates from dairy farms, genetic typing of methicillin-resistant isolates by mec A gene in PCR and identifying of multiresistance.

We started our investigation with isolation of *S. aureus* from 165 collected samples. *S. aureus* isolates were detected in 37.6% of these. But we indicated 2 farms with the highest percentage (58.1% and

51.5%) of samples from which *S. aureus* was isolated and 1 farm with the smallest number of positive samples (21.6%). Our results also show that the higher prevalence of *S. aureus* was found from environmental samples (12.7%) and the lowest in raw milk (2.4%). Other studies report different rates of raw milk contamination with *S. aureus*: 35.0% in Egypt (Al-Ashmawy et al., 2016), 17.9–35.4% in South Africa (Ateba et al., 2010), 12.9% in South Italy (Basanisi et al., 2017). But other potential sources of contamination of raw milk with *S. aureus* also can be the surface of udder skin (10.3% positive samples) and milk from cows with subclinical mastitis (10.9% positive samples). These results can be found in other studies (İkiz et al., 2013; Bardiau et al., 2013; Pu et al., 2014; Szweda et al., 2014).

But, the main task of this study was to characterize of antibiotic sensitivity of *S. aureus* isolates. In this study, 10 antibiotics for testing were selected based on 3 main factors: (i) state recommendation of use in veterinary practice, (ii) after establishing their real use on each farm and (iii) use in medicine important for humans (penicillin, oxacillin, vancomycin). The results show a resistance of *S. aureus* isolates to a variety of antibiotics (vancomycin, tetracycline, lincomycin), and most for β-lactams (penicillin and oxacillin). Similar to studies of other authors from different regions of the world, the highest rate of resistance was detected for β-lactam antibiotics. The resistance to other tested agents was less common, which is also in agreement with the general trend observed worldwide (Li et al., 2009; Abebe et al., 2016; Kirmusaoglu, 2017).

In the last few years, there has been a growing interest in multiresistance (resistant to other antibiotics as well as β-lactams) of *S. aureus* (Brennan et al., 2016; Ganai et al., 2016; Gopal et al., 2017). It has been found that multiresistance of tested *S. aureus* isolates was to between 3 to 5 antimicrobial agents. Twenty isolates (32.3%) that were resistant to penicillin and oxacillin were also resistant to vancomycin, twelve isolates (19.4%) were resistant to 5 antibiotics (P^{10}, OX^{1}, VA^{5}, L^{10}, TE^{30}) and 3 isolates (4.8%) were resistant to another 5 antibiotics (P^{10}, OX^{1}, VA^{5}, CIP^{10}, TE^{30}). We associate these results with the level of use of antimicrobial agents on each farm.

According to the scientific literature, MRSA is primarily mediated by the mecA gene carried on a mobile genetic element (MGE), the staphylococcal cassette chromosome mec (SCCmec) (Zhang et al., 2005). Several scientific studies conducted in several countries have shown the wide distribution of mecA gene in the world (Pu et al., 2014; Ganai et al., 2016). Our results show that 10 (66.7%) from 15 isolates of *S. aureus* yielded a mecA product.

Conclusion

The information obtained from this study is useful for understanding the prevalence of *S. aureus* and its antibiotic sensitivity in dairy farms and can be useful for local and national monitoring or for designing specific control programs of methicillin-resistant and multiresistance isolates present in the food chain of milk production. Also, study of antibiotic resistance among *S. aureus* isolates on each farm is very important, especially for the successful treatment of staphylococcal infections of animals. The presence of isolates resistant to antibiotics, including MRSA, in the raw milk of cows and on dairy farms can be a potential risk in the food chain. The improving of hygienic conditions on dairy farms may reduce the high level of *S. aureus* in environments of farms and in raw milk.

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