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Occurrence and Zoonotic Importance of Methicillin-Resistant *Staphylococcus aureus* in raw Milk and Some Dairy Products at Ismailia City, Egypt

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Abstract

This study was undertaken to determine the occurrence and the zoonotic importance of methicillin resistant *Staphylococcus aureus* (MRSA) in raw milk of sheep, goats and buffaloes, some dairy products and dairy workers at Ismailia City, Egypt. A total of 150 samples were collected randomly and cultured on Baird Parker agar and CHROMagar MRSA. Culturing on Baird Parker agar, the results revealed that 46.7 % of raw goat milk, 40% of raw sheep milk, 40% of raw buffaloes' milk, 80% of yogurt, 36.7% of ice cream, 63.3% of Kareish cheese and 63.3% of human swabs' samples were contaminated by coagulase positive *Staphylococcus aureus.* The isolation rates of MRSA on CHROMagar MRSA in relation to the number of the examined samples and the number of *S. aureus* isolates were (33.3 and 71.4%), (20 and 50%), (13.3 and 33.3%), (40 and 50%), (13.3 and 36.4%), (33.3 and 52.6%) and (13.3 and 21.1%) from the examined milk samples (goat, sheep, and buffaloes), yogurt, ice cream, Kareish cheese and human swabs' samples, respectively. PCR results showed that all the isolates that were classified as MRSA on CHROMagar contained *mec*A gene. Results of the disk diffusion test revealed that the resistance rates of MRSA strains to penicillin, gentamycin, vancomycin, clindamycin, amikacin, erythromycin and oxacillin were 91.2%, 67.6%, 14.7%, 94.1%, 91.2%, 82.4% and 100%, respectively. The effectiveness of some hand cleansing agents against the selected MRSA isolates was assessed. It was found that hand gel rub based on alcohol and triclosan together was the most effective agent. The findings of the present study necessitate exerting more efforts for effective control of MRSA in dairy products.

Keywords: MRSA, Milk, Dairy products, Humans, Antibiotic, Antiseptic

Introduction

Staphylococcus aureus (*S. aureus*) is one of the most important food poisoning pathogens. It is frequently present on the skin, nose and mouth of humans without causing illness [1]. It can cause diseases such as septic wounds, abscesses, pneumonia, meningitis, endocarditis and septicaemia. *S. aureus* can access milk through direct excretion from udders of animals affected with staphylococcal mastitis and by bad hygiene during the milk handling and processing [2].

Milk and dairy products, especially the traditionally and manually produced ones are good substrates for *S. aureus* causing staphylococcal food poisoning.

Staphylococcal food poisoning results from consumption of food containing 20 to < 1000 ng of staphylococcal toxin. It can cause gastrointestinal symptoms such as nausea, explosive vomition, abdominal cramps, and diarrhea in humans [2].

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Methicillin resistant *S. aureus* (MRSA) constitutes an emerging worldwide antimicrobial resistance problem, both in human and veterinary practices [3]. The widespread use of antibiotics in hospitals led to evolution of MRSA strains that are resistant to virtually all available beta (β)-lactam antimicrobials. MRSA is resistant to all penicillins, cephalosporins and carbapenems and the hyper-production of β-lactamase is supposed to be the resistance mechanism which is mediated by the *mec*A gene [4]. Presence of *S. aureus* in food could be attributed to contamination from infected humans or from colonization in food-producing animals. The first food borne outbreak of MRSA that caused death of 5 out of 21 patients was reported by Kluytmans *et al.* [5]. Therefore, food examination especially milk and dairy products is essential for detecting MRSA [6].

The study aimed to determine the occurrence of MRSA in raw milk of sheep, goats and buffaloes, some dairy products and dairy workers at Ismailia City, Egypt, using bacteriological and molecular methods. The antimicrobial resistance profile and the susceptibility of MRSA isolates to some hand cleansing agents were also assessed.

Material and Methods

Sample collection and preparation

A total of 150 samples comprised of raw buffalos' milk (15), sheep milk (15), goat milk (15), yogurt (15), Kareish cheese (30) and ice-cream (30) were collected randomly from retail markets, street vendors and free range (reared) sheep and goat flocks at Ismailia City, Egypt. Generally, milk samples were collected in sterile containers, Kareish cheese samples were collected in sterile plastic bags, while, yogurt and ice-cream samples were collected in their manufacturing containers. Thirty human swabs (from anterior nares and pyogenic hand lesions) were collected from dairy workers in sterile tubes containing 10 ml peptone buffer. All samples were transferred in an ice-box at 4°C to the lab for microbiological examination. The samples were prepared according to APHA [7].

Isolation and identification of S. aureus

Isolation of *S. aureus* was performed according to ICMSF [8] by plating 0.5 ml from the prepared dilution onto Baird-Parker agar (CM0275- Oxoid, Hampshire, England) with egg yolk tellurite emulsion (SR0054 Oxoid) and mannitol-salt agar (LAB B007). Inoculated plates were incubated at 37°C for 48 h. Suspected coagulase-positive *S. aureus* colonies were identified by biochemical tests and Gram stain according to Iurlina and Fritz [9].

Isolation and identification of MRSA

Primary enrichment of the samples was carried out as follows: Twenty-five grams or ml were inoculated into 225 ml bacto ™ tryptic soy broth (211825- Becton, Dickinson and Company, USA) , thoroughly mixed and incubated at 37°C for 24 hours. A loopful from the aforementioned broth was streaked onto CHROMagar™ MRSA (MR500), supplemented with CHROMagar MRSA supplement (SU620) (CHROMagar Microbiology, Paris, France) and incubated at 37°C for 48 hours. Typical MRSA colonies that were rose to mauve in color were identified biochemically according to Bennett and Lancette [10].

Antibiogram assay

The antibiotic susceptibility testing of the biochemically suspected MRSA isolates was carried out using disc diffusion method according to the British Society for Antimicrobial Chemotherapy (BSAC) standard method [11]. Columbia agar (Oxoid CM331) supplemented with 2% NaCl was used for performing the assay using Penicillin (10 IU), Gentamycin (10 µg), Vancomycin (30 µg), Clindamycin (2 µg), Amikacin (30 µg), Erythromycin (15 µg) and Oxacillin (1 µg) (Oxoid).

Molecular identification of MRSA

A single colony was picked and suspended in 100 µl of MilliQ water. The suspension was then heated at 95°C for 15 minutes. After centrifugation for one minute at 20,800 g, the

clear supernatant was used as a template for PCR [12].

Suspected MRSA isolates were confirmed by PCR using pair of primers targeting *mecA* gene (Mec A1 5'-GCA ATC GCT AAA GAA CTA AG-3' and Mec A2 5'-GGG ACC AAC ATA ACC TAA TA-3') according to Smyth *et al*. [13]. The primers amplify a region of 222 bp length.

Sensitivity of selected MRSA isolates to some hand cleansing agents

The following agents and their dilutions were used for the assessment; Dettol conc. (Chloroxylenol B.P, 4.8% w/v), hydrogen peroxide (10%), povidone iodine (5 and 10%), savlon conc. (chlorhexidine gluconate, 0.3% w/v and cetrimide B.P, 3% w/v), ethyl alcohol (70%), hand gel rub type 1 (ethanol and triclosan), hand gel rub type 2 (ethanol 70% v/v), hand gel rub type 3 (alcohol denat), liquid antiseptic soap types (1, 2 and 3) and plain liquid soap (non- antimicrobial). The sensitivity of the aforementioned agents was evaluated according to Saha *et al.* [14]. Briefly, tryptic soy broth tubes were inoculated with the tested strains and incubated at 37°C for 24 hours. Bacterial lawn was prepared on Columbia agar plates supplemented with 2 % NaCl, by spreading 1ml of the incubated broth uniformly on the aforementioned medium using sterile glass spreader. The plate was air dried for few minutes.

On the bottom of the plate, small circles were drawn and numbered sequentially to indicate position of disks. The sterile disks (6 mm) were saturated with the aforementioned hand cleansing agents or with sterile distilled water that was used as negative control. The excess fluid was shaked off. The individual disks were placed above the specified circles using sterile forceps (the forceps was immersed in sterile water after each application and dipped in ethyl alcohol and flamed). The plates were incubated at 37^{o}C for 18-24 h. The diameter of the zone of inhibition was measured in millimeters.

Statistical analysis

The Chi square test and Fisher exact test were analyzed with IBM SPSS software version 20 (Armonk, NY: IBM Corp.) and p value ≤ 0.05 was considered significant.

Table 1: Occurrence of *S. aureus* and MRSA among the examined milk, dairy products and human samples

Type of sample	Coagulase + ve *S. aureus*		MRSA		
	No	%	No	%[a]	%[b]
Goat milk (n=15)	7	46.7	5	33.3	71.4
Sheep milk (n=15)	6	40	3	20	50
Buffalo milk (n=15)	6	40	2	13.3	33.3
Yogurt (n=15)	12	80	6	40	50
Ice cream (n=30)	11	36.7	4	13.3	36.4
Kareish cheese (n=30)	19	63.3	10	33.3	52.6
Human swab (n=30)	19	63.3	4	13.3	21.1

χ^2=12.46, p≤ .05 considered significant, χ^2= 9.28, p=0.158 considered non-significant
N: number of examined samples, % [a]: out of total samples % [b]: out of *S. aureus* isolates

Results and Discussion

Milk and dairy foods are an important source of calcium, protein, vitamins A and D and other nutrients. On the other hand, they can present a health hazard due to possible contamination with pathogenic bacteria [2].

The cultured samples on Baird Parker agar indicated that 46.7 % of raw goat milk, 40% of raw sheep milk, 40% of raw buffaloes' milk, 80% of yogurt, 36.7% of ice cream, 63.3% of Kareish cheese and 63.3% of human swabs' samples were contaminated by coagulase positive *S. aureus*. The difference between the examined samples was significant (χ^2=12.46, p≤ 0.05) (Table 1).

The morbidity rate of Methicillin-resistant *S. aureus* (MRSA) is 100-fold higher than that of tuberculosis (TB), while, its annual mortality rate exceeds that of HIV-AIDS [15]. Detection of MRSA from clinical samples is usually accomplished with the use of conventional non specific media. The disadvantage of such media is that confirmatory tests are necessary to differentiate *S. aureus* from other staphylococci. The use of chromogenic media can differentiate between MRSA and methicillin-susceptible *S. aureus* (MSSA) and reduce the number of confirmatory tests to achieve isolation and presumptive identification in a single step [16].

Out of 150 samples, 34 were positive for MRSA. All the isolates that were classified on CHROMagar MRSA as suspected MRSA were positive for *mecA* gene (Figure 1). In agreement, Taguchi *et al.* [17] reported that CHROMagar MRSA has a sensitivity and specificity of 100% for MRSA.

The isolation rates of MRSA on CHROMagar MRSA in relation to the number of the examined samples and the number of *S. aureus* isolates were (33.3 and 71.4 %), (20 and 50 %), (13.3 and 33.3%), (40 and 50 %), (13.3 and 36.4%), (33.3 and 52.6%) and (13.3 and 21.1%) from the examined goat milk, sheep milk, buffaloes' milk, yogurt, ice cream, Kareish cheese and human swabs' samples, respectively.

The difference was considered non-significant (χ^2= 9.28, p=0.158) (Table 1). These results were supported by other studies that detected MRSA in 0.16- 28% of the examined milk and dairy products' samples [18-20]. Moreover, 2 (50%) of pasteurized milk isolates and 2 (22%) of traditional cheese isolates were contaminated by MRSA using *mecA* gene primers [21]. As well as, MRSA was detected in 26.7% and 40% of raw milk and soft cheese samples, respectively [22].

A much lower percentage of isolation was recorded by Kamal *et al*. [23] who isolated MRSA from 8.6%, 3.3% and 3.3% of the examined raw milk, kareish cheese and ice cream samples, respectively. However, MRSA could not be detected among *S. aureus* isolates from cows, ewes and goats' milk samples and raw milk cheese [19,24].

Previous studies reported that MRSA was found in 7 of the 9 tested herds personnel (nasal and oropharyngeal swabs) [25] and 3% of 133 veterinarians (nasal swabs) [19]. On the contrary, MRSA could not be detected in any of the examined hand swab samples from dairy workers [23].

Table 2: Sensitivity percentages of MRSA isolates from the examined milk, dairy products and human samples to antimicrobials

Samples	Penicillin (10 IU) S (%) R (%)	Gentamycin (10 µg) S (%) R (%)	Vancomycin (30 µg) S (%) R (%)	Clindamycin (2 µg) S (%) R (%)	Amikacin (30 µg) S (%) R (%)	Erythromycin (15 µg) S (%) R (%)	Oxacillin (1 µg) S (%) R (%)
Goat milk (n=5)	- 5(100)	3(60) 2(40)	5(100) -	- 5(100)	- 5(100)	2(40) 3(60)	- 5(100)
Sheep milk (n=3)	- 3(100)	- 3(100)	3(100) -	- 3(100)	- 3(100)	- 3(100)	- 3(100)
Buffalo milk (n=2)	- 2(100)	1(50) 1(50)	2(100) -	- 2(100)	- 2(100)	- 2(100)	- 2(100)
Yogurt (n=6)	2(33.3) 4 (66.7)	2(33.3) 4 (66.7)	3(50) 3 (50)	- 6(100)	- 6(100)	4 (66.7) 2(33.3)	- 6(100)
Ice cream (n=4)	1(25) 3(75)	2(50) 2(50)	4(100) -	- 4(100)	- 4(100)	- 4(100)	- 4(100)
Kareish cheese (n=10)	- 10(100)	- 10 (100)	8(80) 2(20)	- 10 (100)	3(30) 7(70)	- 10(100)	- 10(100)
Human swab (n=4)	- 4(100)	3(75) 1(25)	4(100) -	2(50) 2(50)	- 4(100)	- 4(100)	- 4(100)
Total (n=34)	3(8.8) 31(91.2)	11(32.4) 23(67.6)	29(85.3) 5 (14.7)	2(5.9) 32(94.1)	3(8.8) 31(91.2)	6(17.6) 28 (82.4)	- 34(100)

x^2= 46.73, p≤ 0.01 considered significant n: refers to number of MRSA isolates

The differences in MRSA isolation rate of our study compared to previous studies could be attributed to different animal production systems among various countries and differential distribution of this gene in different places or to the method of determining them. Different national antimicrobial policies and regulations and presence of multiple animal species in the same area that facilitate transfer of genetic material between *S. aureus* strains may contribute to these differences [25].

Results of disk diffusion test in Table (2) revealed that the highest rate of resistance was recorded for oxacillin (100%), followed by clindamycin (94.1%), penicillin and amikacin (91.2%, each), erythromycin (82.4%) and gentamycin (67.6%). The difference among the isolates was considered significant (x^2= 46.73, p≤ 0.01). Ten MRSA phenotypes were identified on the basis of penicillin, gentamycin, vancomycin, clindamycin, amikacin, erythromycin, and oxacillin susceptibility (data not shown). One phenotype was resistant to all tested antibiotics.

The use of antimicrobial agents in dairy farms as well as in other food animal production has led to the emergence of resistant zoonotic bacterial pathogens [26]. These results were supported by data reported by Al-Ashmawy and Khalid [27] who examined the antimicrobial susceptibility of MRSA against 13 antimicrobial drugs and found that the least effective drugs were penicillin, cloxacillin, tetracycline and amoxicillin with resistance percentage 87.9%, 75.9%, 65.2% and 52.6%, respectively. They found that the most effective antimicrobials against MRSA isolates were vancomycin, sulphmethazole/timethoprim, ciproflaxin, netilmicin and gentamycin. However, one out of five MRSA isolates showed resistance against vancomycin and oxacillin, but other strains showed resistance against oxacillin only [23].

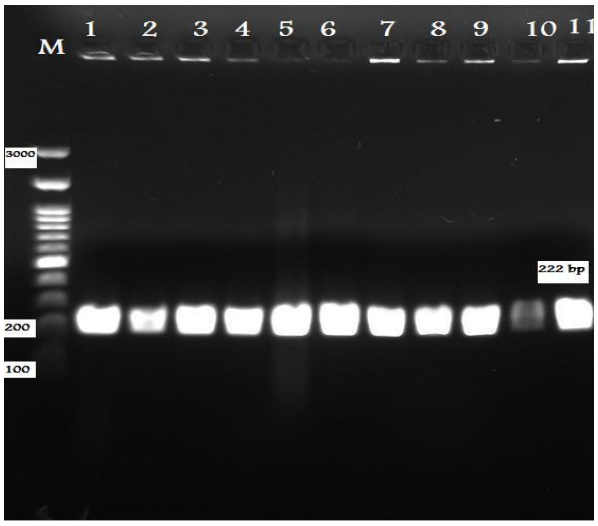

Figure 1: Agarose gel electrophoresis of PCR products (*mecA* gene); Lane M: molecular marker (100 bp), Lanes 1-11: positive test samples showing the 222 bp gene product.

Vancomycin was the most effective drug against the tested MRSA isolates (susceptibility, 85.3%). It is noteworthy to mention that five MRSA strains that were isolated from yogurt and Kareish cheese were resistant to vancomycin. Vancomycin and other glycopeptides antimicrobials are used to treat severe infections caused by staphylococci in certain hospitalized patients [28].

Table 3: Sensitivity of selected MRSA isolates to some hand cleansing agents

Antiseptic	Range of zone of inhibition in mm	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7	Strain 8	Mean ±SE
Dettol (conc.)	18-40	18	30	30	40	22	30	30	30	28.75±2.30
Dettol (diluted)	12-30	13	24	20	30	20	20	12	20	19.88±2.02
Hydrogen peroxide(10%)	2-50	2	40	40	40	40	50	40	40	36.5±5.08
Povidone iodine (10%)	10-24	14	20	10	24	22	20	20	16	18.25±1.62
Povidone iodine (5%)	0-16	0	10	5	12	16	10	7	6	8.25±1.72
Savlon (conc.)	24-50	40	40	40	24	28	30	28	50	35±3.12
Savlon (dil.)	8-22	18	22	10	18	16	16	8	14	15.25±1.60
Ethyl alcohol 70%	0-24	0	0	24	0	20	24	23	0	11.38±4.32
Hand gel 1	30-50	30	40	36	40	30	34	38	50	37.25±2.30
Hand gel 2	0	0	0	0	0	0	0	0	0	0
Hand gel 3	0-18	0	0	0	8	0	18	0	0	3.25±2.33
Antiseptic soap 1	0-14	0	10	0	0	0	0	10	14	4.25±2.12
Antiseptic soap 2	0	0	0	0	0	0	0	0	0	0
Antiseptic soap 3	0	0	0	0	0	0	0	0	0	0
Plain soap (non-antimicrobial)	0	0	0	0	0	0	0	0	0	0
Control (distilled water)	0	0	0	0	0	0	0	0	0	0

Strains 1-2: from sheep and goat. Strain 3: from buffalo. Strains 4-5: from man. Strain 6: from ice-cream Strain 7: from Kareish cheese Strain 8: from yogurt

F= 34.391 P< .0001 considered extremely significant

These antimicrobial agents are listed as "Critically Important Antimicrobials" by WHO. However, vancomycin-resistant *S. aureus* strains (VRSA) have increasingly been reported, thereby causing public health concern [29]. The primary route of zoonotic transmission of MRSA is considered to be the occupational contact of livestock professionals with colonized animals [30].

Generally, bacteria can access milk through colonization of the teat canal or udder of infected dairy animal or through contamination from the animal, milker, dirt or unclean process water [31].

The high isolation rate of coagulase positive *S. aureus* and MRSA in yogurt and Kareish cheese might be attributed to different reasons. Locally manufactured Kareish cheese is produced mostly by villagers. This type of cheese is manufactured from raw milk and is subjected to various sources of contamination during manufacture, storage and handling. Plain yogurt is also produced from raw milk without heat treatment and can be exposed to unhygienic practices during processing and storage. Overall, MRSA may contaminate raw milk and traditional dairy products. Insufficiently hygienic handling of these contaminated foods may lead to transmission of MRSA to human with possible colonization in nostrils, skin, and gastrointestinal tract [21].

The experimental evaluation of the efficacy of some hand cleansing agents against selected MRSA strains showed that hand gel type (1) was the most effective (mean: 37.25 mm ± 2.3), followed by H_2O_2 (mean: 36.5 mm ±5.08), then conc. savlon (mean: 35mm ±3.12) (Table 3). These findings agreed with WHO guidelines [32] which stated that alcohol based products are superior to plain soap and water, or antimicrobial soaps for reducing bacterial counts on hands. Moreover, the addition of another antiseptic agent as chlorhexidine and triclosan increases persistence [32].

A moderate activity against the tested MRSA strains was shown by conc. dettol, diluted dettol, povidone iodine (10%), diluted savlon, and ethyl alcohol (70%), where the means of zones of inhibition in millimeters ±SE were 28.75 ± 2.30, 19.88 ± 2.02, 18.25 ±1.62, 15.25 ±1.60 ,and 11.38 ±4.32, respectively (Table 3). The least effective hand cleansing agents were povidone iodine (5%; mean 8.25 mm± 1.72), antiseptic soap 1 (4.25 mm ± 2.12) and hand gel rub type 3 (3.25 mm ± 2.33). On the other hand, antiseptic soap types (2 and 3), hand gel rub type (2) and plain liquid soap had no germicidal effect against MRSA strains. The aforementioned results indicated that the use of antiseptic soap should not be considered as a safeguard against MRSA. This finding agreed with Rotter [33] who reported that the non-aqueous use of ethanol or propanols has various advantages over washing hands with either unmedicated or medicated soap. It was recorded that 10% povidone-iodine liquid soap (PVP-I) and 70% ethyl alcohol were the most effective hand-cleansing agents (compared to chlorhexidine gluconate (4%) detergent and plain liquid soap for removing MRSA isolates from either lightly or heavily contaminated hands [34].

Conclusion

In conclusion, high isolation rates of MRSA were detected among the tested dairy workers, milk and dairy products. Hand gel rubs varied considerably in their effectiveness against tested MRSA isolates.

Hand gel rub based on alcohol and triclosan together was most effective against MRSA isolates compared to alcohol based gels and antimicrobial soaps. Therefore, the information from this study highlights the necessity of monitoring MRSA in dairy animals as well as in dairy workers and food handlers. Strict hygienic measures and formulation of a definite antimicrobial policy for reducing the incidence of MRSA infection should be adopted.

Conflict of interest

None of the authors have any conflict of interest to declare.

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الملخص العربى

نسبة تواجد وأهمية ميكروب MRSA فى الألبان الخام و منتجاتها فى مدينة الاسماعيلية بجمهورية مصر العربية

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تعد بكتيريا المكور العنقودى الذهبى واحدة من أهم الميكروبات المسببة للتسمم الغذائى فى الانسان . ويعد اللبن ومنتجاته أحد مصادر العدوى بهذا الميكروب. وقد يسبب الميكروب أيضا أمراضا أخرى منها تقيح الجروح، الالتهاب الرئوى والتسمم الدموى. يشكل نشوء سلالات من هذه البكتريا مقاومة للمضادات الحيوية (MRSA) خطورة كبيرة على صحة الانسان و الحيوان. لذا أجريت هذه الدراسة لتقييم مدى تواجد ميكروب (MRSA) فى اللبن ومنتجاته وكذلك فى العمال المخالطين للحيوانات الحلابة بمدينة الاسماعيلية. وعليه تم تجميع (١٥٠) عينة موزعة كالتالى: لبن الماعز (١٥)، لبن الأغنام (١٥)، لبن الجاموس الخام (١٥)، اللبن الزبادى (١٥)، الأيس كريم (٣٠)، الجبن القريش (٣٠) ومسحات من الأنف والجروح المتقيحة لعمال مزارع الألبان (٣٠). كما تم زرع العينات على مستنبتى CHROMagar MRSA و Baird-Parker agar) وبلغت نسبة عزل (MRSA) من الألبان الخام (للماعز، الأغنام والجاموس)، اللبن الزبادى، الأيس كريم، الجبن القريش ومسحات عمال مزارع الألبان (٣٣.٣%، ٢٠% ، ١٣.٣%،٤٠%، ١٣.٣% ، ٣٣.٣ % و١٣.٣%) على التوالى. وقد أوضح تفاعل البلمرة المتسلسل وجود جين (*mecA*) فى جميع العزلات المصنفة (MRSA) على مستنبت (CHROMagar MRSA). كما أوضح اختبار حساسية عزلات (MRSA) للمضادات الحيوية أن الفانكوميسين كان الأكثر كفاءة فى تأثيره على الميكروب. وبلغت نسبة الحساسية و المقاومة للبنسلين (٨.٨ و ٩١.٢ %) ، الجنتاميسين (٣٢.٤ و٦٧.٦%) ،الفانكوميسين (٨٥.٣ و١٤.٧%)، الكلنداميسين (٥.٩ و٩٤.١%)، الأميكاسين (٨.٨ و٩١.٢%)، الارثروميسين (١٧.٦ و٨٢.٤%) و الأكساسللين (٠ و ١٠٠%) على التوالى. و أوضح اختبار تأثير المطهرات و الصابون المستخدم فى العناية بالبشرة أن الجيل المحتوى على الكحول و التريكلوسان معا كان الأكثر كفاءة ضد بكتريا (MRSA). وخلصت الدراسة الحالية إلى أن تواجد بكتريا (MRSA) بنسب عالية فى عينات الألبان الخام و منتجاتها المختبرة؛ الأمر الذى قد يشكل خطورة على صحة المستهلكين لتلك الأغذية. لذا يجب اختبار الحيوانات الحلابة دوريا لتقييم تواجد الميكروب بها و كذلك عدم الاسراف فى استخدام المضادات الحيوية لتفادى نشوء الأنواع المقاومة للمضادات الحيوية.