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RESEARCH ARTICLE

Prevalence of *Staphylococcus aureus* and *Streptococcus agalactiae* isolated from Raw Milk in Dakahlia Governorate, Egypt

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Abstract

This study was carried out on 200 random milk samples collected from different areas at Dakahlia Governorate for detection of prevalence and antimicrobial resistance of some zoonotic bacteria in milk as *Staphylococcus aureus* and Streptococci by using biochemical tests, antibiotic sensitivity test, PCR for confirmation and detection of some resistance and virulence genes. *Staphylococcus aureus* (*S. aureus*) was detected in 63%, 76%, and 42%, while *Streptococcus agalactiae* (*St. agalactiae)* was found in 8%, 10% and 4% of examined individual milk samples, retail milk samples, and milk of Bulk tank, respectively. *S. aureus* isolates revealed high levels of resistance to ampicillin (100%), nalidxic acid (90%), oxacillin (85%), penicillin (85%), cefoxitin (75%), rifampin (25%), tetracycline (20%) and erythromycin and sulphamethoxazole/ trimethoprim (10%). Whereas, *St. agalactiae* show high resistance to tetracycline (90%), ampicillin (80%), rifampin (60%), sulphamethoxazole/ trimethoprim (50%), gentamycin (45%), erythromycin and nalidxic acid (40%)**,** choloramphenicol (30%), and streptomycin (25%). PCR results revealed that 4 out of 5 (80%) methicillin-resistant (MRSA) isolates had *mecA* and 2 (40%) had *mecC,* while 1 (20%) had *Sea* enterotoxin. Three isolates (100%) of *St. agalactiae* had *sul*1 gene and one out of three (33.3%) had *tetK* genes, while *dfrA* could not be detected. The main outcome of the current work is that milk can cause severe public health hazards to people because it had a variety of microorganisms. It is important to ensure using good hygienic practices in farms and prevent the haphazard abuse of antibiotics.

Keywords: Raw milk samples, *S. aureus, St. agalactiae,* Antibiotic resistant, Resistance genes

Introduction

Milk is considered an essential food commodity for humans. Milk contains essential elements for the human body such as protein, glucose, minerals, and vitamins. Moreover, milk is considered the cheapest source of animal protein [1].

The presence of food-borne pathogens in milk may be due to direct contact with contaminated sources in the dairy farm environment and excretion from the udder of an infected animal [2]. Milk and its products can cause severe public health hazards to people as they are highly susceptible to a variety of microorganisms because of their high nutritive value [3].

Staphylococcus aureus (*S. aureus*) is a common cause of food-borne disease worldwide which produces heat-stable enterotoxins that cause gastroenteritis, this causes an estimated 241,000 illnesses per year in the United States [4, 5]. *S. aureus* is considered one of the most common agents causing food poisoning [6]. *S. aureus* produces several virulence factors, including enterotoxins (SEG to SEQ and SEA to SEE), and other toxins, such as toxic shock syndrome toxin (TSST-1) and exfoliative toxin A and B

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[7]. Methicillin-resistant (MRSA) isolates could produce one or more staphylococcal enterotoxins (SEs) which are part of the main virulence factors of the pathogen. Members of these SEs play a vital role in outbreaks of food poisoning and other infections that are septic-related [8]. *Staphylococcal enterotoxins* are heat stable which able to survive high temperatures and able to thrive and maintain their activity in food previously contaminated with the pathogen [9].

Streptococci are one of the major mastitis pathogens which have a significant effect on dairy animals wellbeing, quality, and productivity of milk [10]. Streptococcus is the main cause of pharyngitis and tonsillitis in human especially in children and is the main bacteria transmitted from the milk to human and also can transmitted from the human to animals [11].

Streptococcus agalactiae (*St. agalactiae*) is associated with skin and soft tissue infections (SSTI), bacteremia, and urinary tract infections (UTI) and occasionally with arthritis, necrotizing fasciitis, toxic shock syndrome, endocarditis, or meningitis in adults [12]. *St. agalactiae* were detected in 4% of 100 bulk tank milk samples of cattle origin, collected from different dairy farms in Sharkia Governorate, Egypt [13].

Antibiotic resistance is a type of drug resistance where a microorganism is able to survive exposure to an antibiotic. Infections of human by resistant microorganisms often fail to respond to conventional treatment and lead to prolonged illness and greater risk of death [14]. Therefore, this study was designed to investigate the public health hazard of milk contaminated with zoonotic bacteria and to detect the resistance phenotype and antibiotic resistance genes of the isolated bacteria.

Material and methods

Milk samples:

This study included two hundred random raw milk samples (100 buffalo milk samples collected from owners in different localities, 50 raw milk samples from dairy cow farms, and 50 buffalo raw milk samples collected from different Markets, Dakhlia Governorate, Egypt. The collected samples were directly transferred to the laboratory under refrigeration and hygienic conditions with minimum delay.

Total Staphylococcus aureus count

By using the surface plating technique, one-tenth (0.1) mL of prepared dilutions of each sample was dispended onto a dry surface of mannitol salt agar (Oxoid, UK) plates and evenly distributed. Inoculated plates were incubated at 37ºC for 24-48h. The plates were examined for the presence of typical yellow colonies [15].

Total count of Streptococci

One tenth mL of prepared dilutions of each sample was dispended onto modified Edward's medium (Oxoid, UK) plates and incubated at 35ºC for 24h. *St. agalactiae* were identified as esculin negative (purple colonies) of 0.5 mm diameter and surrounded by a hemolytic zone, while group D streptococci (aesculin positive) appear as black colonies [16, 17].

Identification of the isolated staphylococci and streptococci

Microscopical examination:

Films were made from the pure culture of the suspected colonies and examined by light microscope at 1000X magnification after staining by Gram's stain for the presence of Gram-positive cocci arranged in grapes like clusters (*S. aureus*) or chains (Streptococci) [15].

Biochemical reactions:

Staphylococcus aureus:

Staphylococcus aureus isolates were subjected to catalase test, coagulase test, and growth at 10% NaCl [16].

Streptococci species

Streptococcus species were classified with CAMP test [17], oxidase test [16], bile esculin test [16], growth at 6.5% NaCl [16], detection of arginine decarboxylase (ADH) [16], hippurate hydrolysis test, hemolysis [16], and sugar fermentation test [16].

Antimicrobial susceptibility testing of the recovered isolates

By using the disc diffusion method [18] the antibiotic susceptibility of the isolates was determined following the guidelines of the Clinical Laboratory Standards Institute (CLSI) [19]. Antibiotic discs (Oxoid, UK) that were used in antibiotic sensitivity test of Staphylococci were ampicillin (10 mg), erythromycin (15 mg), oxacillin (1 mg), cefoxitin (30 mg),chloramphenicol (30 mg), streptomycin (10 mg), penicillin (10 mg), rifampin (5 mg), gentamycin (10 mg), tetracycline (30 mg), nalidxicacid (30 mg), and sulphamethoxazole / trimethoprim (25 mg). The inhibition zone diameters were read and interpreted according to the CLSI [19].

Molecular detection of antibiotic resistance and virulence genes

The recovered isolates were screened for some antibiotic resistance genes and virulence genes using oligonucleotide primers as previously described. Polymerase chain reaction amplification (PCR) of *Staphylococcus* resistance genes *MecA* [20], MecC [21], and *MecI* [22], and the *Sea* [23], *Sec, Sed* [26] virulence genes were performed. Streptococcus *tetK*, *sul*1 [24], and *dfrA* genes [25] were examined. The amplified products were separated on 1.5% agarose gel containing ethidium bromide 0.5µg/mL [26].

Results and Discussion

Table (1) illustrated that the total *S. aureus* count in the examined individual milk samples ranged from 2.0×10^2 to 3.46×10^6 CFU/g with a mean value of $1.30\times10^5 \pm 5.58\times10^4$ CFU/g, while in retail milk ranged from 4.0×10^2 to 6.0×10^5 with a mean value of $1.22\times10^5 \pm 2.54\times10^4$ CFU/g, but it ranged from 2.9×10^2 to 1.42×10^6 in bulk tank with a mean value of $9.33\times10^4 \pm 3.36\times10^4$ CFU/g. These findings agreed with Rall and coauthors [27] who found that *S. aureus* was detected in 70.4% of raw milk samples with maximum count of *S. aureus* were 8.9 x 10^5, While lower results were detected in a previous study [28] in which *S. aureus* was detected in 41.66% of the collected raw milk samples from different localities in Iran, while mean counts of *S. aureus* was at a range of $1.4\times10^2 \pm 78$ to $2.3\times10^3 \pm 109$. In a previous research, El-Leboudy and coauthors [29] showed that mean value of *S. aureus* counts in raw milk samples collected from governmental and private farms at Alexandria Governorate, Egypt were 5.26 $\times10^2 \pm 8.1 \times 10$ and $2.95 \times 10^2 \pm 7.7 \times 10$, respectively. *Staphylococcus aureus* is an important human pathogen which found in upper respiratory tract and skin wound of human [30]. Most staphylococcal foodborne intoxications are due to food contamination by food-handlers during food processing [31]. As presented in **Table (1)**, *S. aureus* was detected at high percent in retail milk samples (76%) then individual milk samples (63%) and the lowest percent (42%) was found in bulk tank milk which gives indication on the sanitary condition. These findings agreed with Rall *et al.* [27] who found that *S. aureus* were detected in 70.4% of raw milk samples at concentrations of up to 8.9 x 10^5. Whereas, Haque and coworkers [32] reported that *S. aureus* was detected in 79.16% of raw cow's milk samples.

The demonstrated results in **Table (1)** showed that total streptococci count of examined individual milk samples ranged from 2.0×10^2 to 5.0×10^4, while in retail milk ranged from 6.0×10^2 to 8.0×10^6, but it ranges from 2.9×10^2 to 1.4×10^6 in the bulk tank. Streptococci were detected in 80%, 90%, and 86% with mean value $9.66\times10^3 \pm 1.17\times10^3$, $1.12\times10^6 \pm 3.16\times10^5$, and $5.59\times10^5 \pm 1.88\times10^5$ in individual milk samples, retail milk samples, and bulk tank milk. Our results were higher than a previous study [33] in which the mean values of total streptococci counts in milk samples from three farms in Gharbia Governorate were 28.65 x 10^3 + 5.75 x 10^3, 22 x 10^3 + 5.7 x 10^3, and 27.1 x 10^3 + 14.2 x 10^3 CFU/mL. The *streptococci* count in raw milk samples collected from El-Behera Governorate markets were 26 %, with mean values of $4.5x10^3 \pm 0.7x10^3$ CFU/mL [34].

Table (1): Total count of *Staphylococcus aureus* and Streptococci in the examined milk samples

Examined samples (No.)	Positive samples for *S. aureus*		Count /mL				Positive samples for Streptococci		Count /mL			
	No.	**%**	Min.	Max.	mean	± S.E	**No.**	**%**	Min.	Max.	mean	± S.E
Individual milk (n=100)	63	63	2.0×10^{2}	3.46×10^{6}	1.30×10^{5}	5.58×10^{4}	80	80	2.0×10^{2}	5.0×10^{4}	9.66×10^{3}	1.17×10^{3}
Retail milk (n=50)	38	76	4.0×10^{2}	6.0×10^{5}	1.22×10^{5}	2.54×10^{4}	45	90	6.0×10^{2}	8.0×10^{6}	1.12×10^{6}	3.16×10^{5}
Bulk tank (n=50)	21	42	2.9×10^{2}	1.42×10^{6}	9.33×10^{4}	3.36×10^{4}	43	86	2.9×10^{2}	1.4×10^{6}	5.59×10^{5}	1.88×10^{5}

Table (2) showed that individual milk samples were detected in 8, 13, 9, 15, 4, 47, and 7% as *St. agalactiae, St. dysagalactiae, St. pyogenes, St. uberis, St. pneumoniea, E. fecalis* and *E. facieum*, respectively. While in retail milk were detected in 10, 32, 16, 18, 4, 58 and 8% as *St.agalactiae, St.dysagalactiae, St.pyogenes, St.uberis, St.pneumoniea, E.fecalis* and *E.facieum* respectively and in bulk tank were detected in 4, 32, 6, 4, 0, 56 and 6% as *St.agalactiae, St.dysagalactiae, St.pyogenes, St.uberis, St.pneumoniea, E.fecalis* and *E.facieum* respectively. the present study in accordance with previous study of Citak [35] who found *E. faecalis* and *E.* faecium in 54.2% and 29.0% of milk samples, respectively. *St. agalactiae* were detected in 4% of milk samples in Sharkia Province, Egypt [13]. Moreover, *St. agalactiae* and *St. uberis* were detected in 22.5%, 21.88% and 7.5%, 3.13% from smallholder farms and supermarkets in Ismailia city, respectively [36]. However, *St. agalactia* and *St. pyogenes* were detected at percent of 70% and 30%, respectively from random samples of buffalo milk in different areas at Behera Governorate [37].

Enterococci especially *E. faecalis* and *E. faecium* can contaminate milk from human or animal feces, water sources, the farm environment, or from milking equipment, bulk storage tanks, and equipment used during milk harvesting or local processing [30].

Table (3) presented the antibiogram of 20 *S. aureus* isolates. High levels of resistance were observed to ampicillin (100%), nalidxic acid (90%), oxacillin (85%), penicillin (85%), cefoxitin (75%), rifampin (25%), tetracycline (20%), erythromycin, and sulphamethoxazole / trimethoprim (10%). These findings were in harmony with previous research [38] in which high rate of resistance was recorded to ampicillin (100%). The susceptibility of MRSA isolates to trimethoprim-sulphamethazole and rifampin was low [39]. *S. aureus* showed high resistance toward penicillin G (86.04%), ampicillin (74.42%), and tetracycline (13.95%) [40]. Whereas in another study, *S. aureus* showed high resistance rate to ampicillin (95.2%) and penicillin (83.3%) [41]. Hoque and coauthors [42] reported that 8.2% of *S. aureus* isolates were resistant to erythromycin.

Table (2): Prevalence of *Streptococcus species* in the examined milk samples.

Species	Individual milk (n=100)		Retail milk (n=50)		Bulk tank milk (n=50)	
	No.	%	No.	%	No.	%
St. agalactiae	8	8	5	10	2	4
St. dysagalactiae	13	13	16	32	16	32
St. pyogenes	9	9	8	16	3	6
St. uberis	15	15	9	18	2	4
St. pneumoniea	4	4	2	4	0	0
E. fecalis	47	47	29	58	28	56
E. facieum	7	7	4	8	3	6

Table (3): Phenotypic antimicrobial susceptibility of *S. aureus* isolates from milk samples.

Antimicrobial agent	Profile					
	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Oxacillin	2	10	1	5	17	85
Cefoxitin	5	25	ND	ND	15	75
ampicillin	ND	ND	ND	ND	20	100
Erythromycin	13	65	5	25	2	10
Tetracycline	13	65	3	15	4	20
Gentamycin	20	100	ND	ND	ND	ND
Penicillin	3	15	ND	ND	17	85
Rifampin	7	35	8	40	5	25
Nalidxic acid	ND	ND	2	10	18	90
Sulphamethoxazole / Trimethoprim	17	85	1	5	2	10

ND: not determined

Table (4) showed that tetracycline was the least effective antibiotic on *St.agalactia* since the resistance rate was 90%, followed by ampicillin (80%), rifampin (60%), sulphamethoxazole/trimethoprim (50%), gentamycin (45%), erythromycin and nalidxic acid (40%), choloramphenicol (30%), and finally streptomycin (25%). Our findings were in accordance with the results of Jain *et al*. [43] 33.3% of *St. agalactiae* isolates were resistant to erythromycin. While, 94.5 % of isolates were resistant to tetracycline and 24.9 % were resistant to chloramphenicol [44].

Figures 1 A and **1 B** showed that 4 out of 5 (80%) MRSA isolates had *mecA* and 2 out of 5 (40%) carried *mecC* gene. While, 20% (1 out of 5) of isolates were positive to *sea* enterotoxin gene at 102 bp (**Figure 2**); *sec* and *sed* gene could not be detected. Our results were in accordance with the previous study of Cikman [45] who mentioned that *mecA* gene was found in 315 out of 494 (63.8%) isolates. However, 20% (29/145) of the tested MRSA isolates carried *mecA* gene [32].

Table (4): Antimicrobial sensitivity pattern of *Streptococcus agalactia* isolated from raw milk samples

Antimicrobial agent	Profile					
	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Ampicillin	4	20	ND	ND	16	80
Erythromycin	8	40	4	20	8	40
Tetracycline	2	10	ND	ND	18	90
Gentamycin	8	40	3	15	9	45
Sulphamethoxazole/trimethoprim	4	20	6	30	10	50
Rifampin	8	40	ND	ND	12	60
Nalidxicacid	12	60	ND	ND	8	40
Streptomycin	12	60	3	15	5	25
Choloramphenicol	13	65	1	5	6	30

ND: not determined

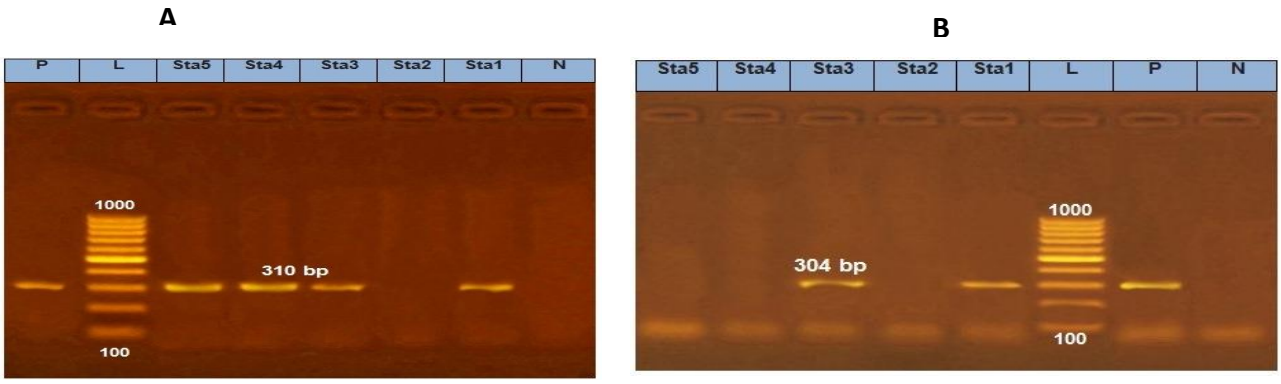

Figure (1): Agarose gel electrophoresis of PCR amplified products from *S. aureus* isolates. Lane L: molecular size marker (size range 100-1000 bp), lanes 1, 3, 4, 5 showed positive results for the presence of *mecA* gene at 310 bp (A) and *mecC* gene at 304bp (B). Lane P: positive control, lane N: negative control.

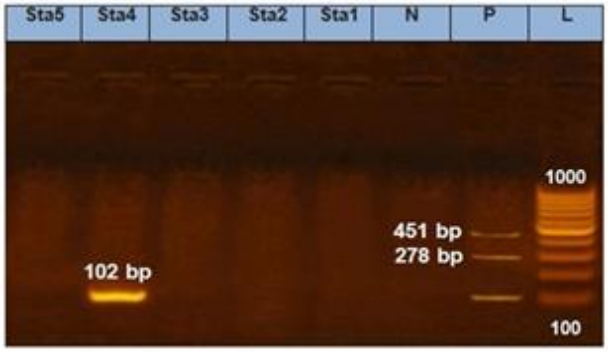

Figure (2): Agarose gel electrophoresis of PCR amplified products from *S. aureus* isolates showed positive results in isolate 4 for the presence of *sea* gene at 102bp and absence of *sec* and *sed* genes. Lane L: molecular size marker (size range 100-1000 bp), lane P: positive control, lane N: negative control.

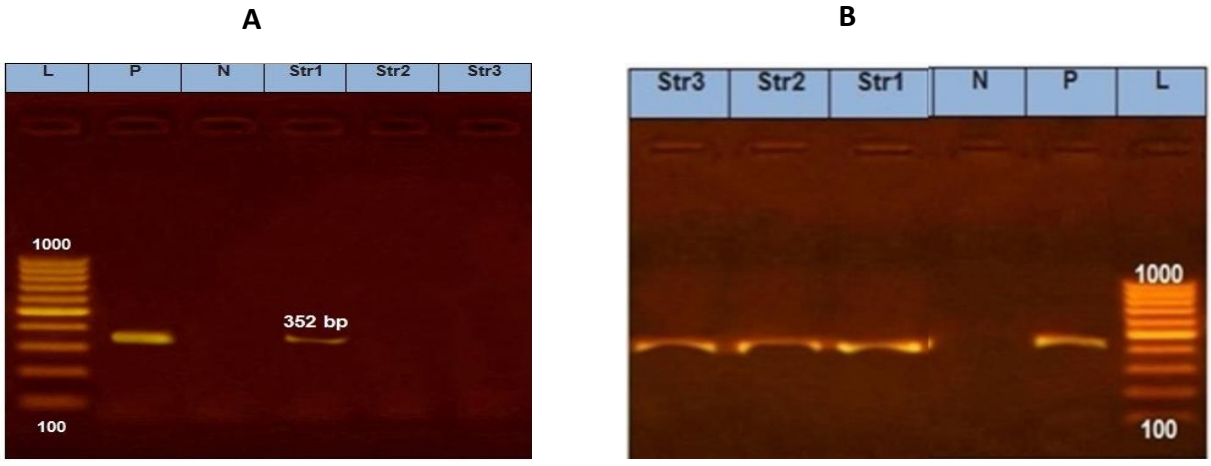

Figure (3): Agarose gel electrophoresis of PCR amplified products from *St. agalactiae* isolates. Lane L: molecular size marker (size range 100-1000 bp), Lane 1 showed positive results for the presence of *tetK* gene at 352 bp (A) and *Sul1* gene at 433 bp (B), lane P: positive control, lane N: negative control.

Staphylococci pathogen was blamable for a huge scale of infections in human because of their invention of secreted and other cell-surface related virulence factors that regulate by various genes. The common serotypes are SEA, SEB, SEC, and SED. It is well acknowledged that bacterial components and products, including the capsule, surface-associated adhesins, secreted proteins and exotoxins, play a role in the process such as coagulase, hemolysins (encoded by hl genes), exfoliative toxin (ET, et genes) , toxin of toxic shock syndrome 1 (TSST-1, tst gene), bicomponentleukotoxins (LukS–LukF, encoded by luk genes), enterotoxin-like toxins (SEls, sel genes) and enterotoxins (SEs, se genes) [46]

As revealed in **Figures (3 A** and **B)** 3 out of 3 (100%) *St. agalactiae* isolates had *sul1* gene and one out of three (33.3%) had *tetK* genes, while *dfrA* could not be detected. However, Emaneini *et al.* [47] reported that *tetK* gene was detected in 16% of *St. agalactiae* isolates. This difference could be due to variations in the use of antimicrobials in the area of study. It has been mentioned that sensitive strains refuge antibiotic resistance genes might express this resistance and generate strains that are likely to be resistant to those antibiotics [48].

Conclusion

Although milk is considered an essential food for humans due to its high nutritive value, it can cause severe public health hazards to people because it had a variety of microorganisms. Milk is considered the main cause of transmission of some zoonotic bacteria as *S. aureus* and *St. agalactiae* and increases the resistance of antibiotics between animals and humans who consume the milk of these animals. It is important to ensure using good hygienic practices in farms and prevent the haphazard use of antibiotics.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربي

معدل انتشار الميكروب العنقودي الذهبي واستربتكوكس أجلاكتيا فى الحليب الخام في محافظة الدقهلية ، مصر

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أجريت هذه الدراسة على 200 عينة حليب عشوائية تم جمعها من مناطق مختلفة بمحافظة الدقهلية للكشف عن انتشار ومقاومة مضادات الميكروبات لبعض البكتيريا حيوانية المصدر في الحليب مثل المكورات العنقودية الذهبية والعقدية باستخدام الاختبارات البيوكيميائية ، واختبار الحساسية للمضادات الحيوية ، واختبار تفاعل البلمره المتسلسل (PCR) لتأكيد واكتشاف الجينات المقاومة والضراوة. استنتجت نتائجنا أن المكورات العنقودية الذهبية تم اكتشافها في 63٪ و 76٪ و 42٪ ، بينما تم اكتشاف الاستربت الاجلاكتيا 8% و 10% و 4٪ من عينات اللبن الفردية المفحوصة وعينات الحليب بالتجزئة وعينات الحليب المعزولة من الخزان السائب على التوالي. أظهرت سلالات المكورات العنقودية الذهبية وجود مستويات عالية من المقاومة للأمبيسيلين (100٪) ، حمض النااليدكسيك (90٪) ، أوكساسيلين (85٪) ، البنسيللين (85٪) ، سيفوكسيتين (75٪) ، ريفامبين (25٪) ، تتراسيكلين(20٪) وإريثروميسين وسلفاميثوكسازول / تريميثوبريم (10٪) وأظهرت الاستربت اجلاكتيا مقاوه عاليه للتتراسيكلين (90٪) ، أمبيسيلين (80٪) ، ريفامبين (60٪) ، سلفاميثوكسازول / تريميثوبريم (50٪) ، جنتاميسين (45٪) ، إريثروميسين ونااليد يكسيسك (40٪) ، كولورامفينيكول (30٪). والستربتوميسين (25٪). أكداختبار البلمره المتسلسل ان 4 معزولات من 5(80 ٪) (المكورات العنقودية الذهبية المقاومة للميثيسيلين) تحتوي علي (*mecA*) جين و 2 من 5 (4٪) كان لديهم (*mecC*) جين ، في حين أن 1 من 5 (20 ٪) كان لديه (*Sea*) جينبينما استربت اجلاكتيا 3 من أصل 3 (100٪) تحتوي علي جين(*Sul1*) و 1 من اصل 3 (33.3%) يحتوي علي جين (*TetK*) بينما لم يتم الكشف عن جينات (*dfrA*). النتيجة الرئيسية للعمل الحالي هو أن الحليب ممكن ان يسبب مخاطر صحية عامة شديدة للناس لأنه يحتوي على مجموعة متنوعة من الكائنات الحية الدقيقة. من المهم التأكد من استخدام الممارسات الصحية الجيدة في المزارع ومنع سوء الاستخدام العشوائي للمضادات الحيوية.