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Detection and Antimicrobial Susceptibility of *Staphylococcus aureus* Isolated from Cow's Milk Affected by Clinical Mastitis

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Abstract

THE CURRENT work aimed to isolate *Staphylococcus aureus* from milk of cows affected by clinical mastitis and to investigate the antimicrobial susceptibility phenotypes of the isolated strains. One-hundred and fifty milk samples were collected from cows with mastitis exhibiting clinical symptoms. Samples were obtained from small holders in the neighboring villages of Mansoura, Dakahalia governorates, Egypt. the bacteriological examination was carried out and The Suspected isolates were subjected for biochemical examination followed by PCR for species identification. *S. aureus* were then tested for its antimicrobial susceptibility against 18 antimicrobials commonly used for mastitis treatment in Egypt. *S. aureus* were recovered in a rate of 34% (51/150) from the total examined mastitic milk samples. Antimicrobial sensitivity indicated that the isolates had high susceptibility for imipenem, marbofloxacin and tetracycline (100% each), ciprofloxacin, vancomycin and ciprofloxacin (98% each),cefotaxime (94.12%), gentamicin (92.2%), cefotaxime (94.12%), ceftriaxone (84.4%), sulfamethoxazole trimethoprim (88.2%), streptomycin (66.7%), while, the recovered *S. aureus* isolates showed complete resistance (100%) against ceftiofur and oxacillin. While, erythromycin, penicillin, amoxicillin clavulinic and spiramycin showed 55%, 41.2% 39.2% and 39.2% rate of resistance respectively. Multi-drug resistance (MDR) was detected in 94.11% of the retrieved isolates. *S. aureus* retrieved in a high frequency from the present study and have demonstrated increased resistance to commonly used antimicrobials which considered a serious problem for humans and animals health. Thus, the obtained data on the resistance traits of *S. aureus* isolated from samples of cow's milk would enable source attribution, risk assessment, and the development of better treatment strategies.

Keywords: *Staphylococcus aureus*, cows, mastitis, Multi-drug resistance.

Introduction

Bovine mastitis caused by bacteria is the most common and economically important illness in dairy farms. It occurs in clinical or subclinical form [1]. Mastitis pathogens are categorized into two groups based on the mechanism of transmission and primary reservoir: environmental pathogens, which enter the host body from the environment, and contagious pathogens, which spread from one animal to another [2].The predominant infectious pathogens causing mastitis in dairy farms with a significantly low cure rate are staphylococcal infections. According to reports, *S. aureus* is the cause of up to 40.0% of mastitis cases in many countries [3]. The bacteria can withstand phagocytosis in the udder and frequently results in chronic infection [4].

During milking, intramammary infections (IMIs) by *S. aureus* are highly transmittable. *S. aureus* IMI has a great effect on both animal health and welfare. It results in significant economic losses as well as it has a public health importance due to contamination of raw dairy products [5]. *S. aureus* mastitis starts by colonization of the teat ends by staphylococci and then the infection spread into the intramammary space. *S. aureus* is attached to and then goes into the epithelial cells of the mammary alveolus. In the affected epithelial cells, the bacterium reproduces and causing a chronic infection [6], and in turn consuming such unpasteurized milk can pose a public health hazard owing to presence of pathogenic bacteria as a result of contamination. On dairy farms, *S. aureus* carries virulence and antibiotic resistance genes that facilitate the bacteria's successful

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colonization and spread across many environments and hosts [7].

Antimicrobial resistance is a major public health concern worldwide. The misuse and the overuse of antibiotics in treatment of bovine mastitis can results in development of antimicrobial resistance to the used antibiotics which may results in two significant features, delayed in treatment of mastitis and possible effects of resistant microorganisms spreading to people through the food chain. [8]. There has been a global rise in multidrug resistance, which is regarded as a hazard to public health. People who have multi-drug resistance bacteria have a high death rate. Antibiotic-resistant bacteria are predicted to be the cause of an additional 23,000 deaths annually in the United States and 25,000 deaths in the European Union [9]. Therefore, our purpose was to collect milk samples from mastitic cows from smallholders in different villages in Mansoura to demonstrate the prevalence of *S. aureus* in the collected samples and to investigate the phenotypic antimicrobial resistance profile of the isolated *S. aureus* strains

Material and Methods

Sampling

One hundred and fifty individual quarter milk samples were obtained from 150 cows suffering from clinical mastitis in the period between January to November 2022 from the neighbouring villages of Mansoura city, at Dakahlia Governorate, Egypt. Milk samples were obtained from cows showing abnormalities in the foremilk, such as blood, flakes, clots, or a watery appearance, as well as redness of the udder. All samples were taken before the cows receiving antibiotic treatment. Before sample collection, the teat tips were cleaned by using 75% alcohol-soaked cotton balls and the initial milk streams were thrown away. A sterile Falcon tube devoid of preservatives was used to collect 3 to 5 milliliters of milk from the afflicted quarter. The tubes were marked clearly and transported to the laboratory in a sterile cool container for bacteriological examination.

Bacteriological analysis

About 1ml from each milk sample was inoculation in 5 ml Trypton Soya Broth (TSB, (Oxoid Ltd, Hampshire, England) and incubated at 37°C for 24 h. A loopful of the incubated broth was then streaked onto the surface of Baird Parker agar media supplied with Egg Yolk Tellurite Emulsion (HiMedia, Mumbai, India). Milk samples were also streaked into Blood Agar plates (Oxoid Ltd, Hampshire, England) with 7% sheep blood. Colonies of suspected *S. aureus* were then purified on Trypton Soya Agar (TSA). The purified isolates of *S. aureus* were identified biochemically by performing catalase, coagulase, oxidation-fermentation, haemolysin detection, Mannitol fermentation and DNase tests [10].

Molecular identification of S. aureus

The suspected *S. aureus* were subjected to PCR for species identification. The thermonuclease gene (*nuc*) was targeted in this for *S. aureus* identification. A primer sequence designed by Oliveira et al. [11] obtained from was used with the following sequence F: GCGATTGATGGTGATACGGTT and R: AGCCAAGCCTTGACGAACTAAAG. PCRs were carried out in a total volume of 25 µL, with 12.5 µL of 2X PCR master mix (Promega, Madison, USA), 1 µL of each primer (ThermoFisher Scientific) comprising 20 pmol, and 5 µL of template DNA utilized in the reaction mixture. Nuclease-free water was used to complete the volume. Agarose gel electrophoresis was used to visualize approximately 5 µL of each PCR product, using 1.2% agarose prepared in TBE. The gel was visualized by a UV gel documentation device (Cleaver Scientific Ltd., ultra violet gel documentation system, Rugby, Warwickshire, UK).

Antibiotic susceptibility testing

The retrieved isolates were tested by Kirby-Bauer disc diffusion method [12] for detection their sensitivity to antimicrobial agents on Muller Hinton agar (Oxoid, UK). Eighteen antibiotic disks (Oxoid, UK) were used including penicillin (P, 10 iu), vancomycin (VA, 30 µg), , oxacillin (OX, 15 µg), sulfamethoxazole-trimethoprim (SXT, 25 µg), gentamicin (CN, 10 µg)cefuroxime (CXM, 30 µg), ampicillin (AM, 15 µg), cefotaxime (CTX, 30 µg), amoxicillin clavulinic (AMC, 10 µg), ciprofloxacin (CIP, 5 µg), cefetrixone (CRO, 30 µg), Erythromycin (E, 15 µg), streptomycin (S, 10 µg), tetracycline (TE, 30 µg), marbofloxacine (MAR, 5 µg), spiramycin (SP, 100 µg), ceftifuer (EFT, 30 µg), and imipenem (IMP, 10 µg). The antimicrobial discs were placed onto the surface of the media after spreading bacterial suspensions equivalent to the 0.5 McFarland standards turbidity. The plates were incubated at 37°C and the results were recorded after 24h according to recommendations of CLSI [12]. The isolates displayed resistance to three or more antimicrobials were categorized as MDR.

Statistical analysis

Microsoft Excel was used to enter and analyze descriptive data, such as percentages and frequency distributions, utilizing the Statistical Package for Social Sciences, version 16. A multiple antibiotic resistance (MAR) index was computed by dividing the total number of antimicrobial resistances found in

each isolate by the total number of antimicrobials tested [13].

Results

Prevalence of S. aureus

By streaking milk samples on the selected culture media, on Baird Parker agar plates, *S. aureus* colonies appear as black colonies encircled by a transparent halo zone as illustrated in Figure 1. Upon further incubation may produce lipolytic activity (opaque zone) due to an egg yolk–lecithinase reaction. On Blood agar plates, *S. aureus* produced complete zone of hemolysis (beta- hemolysis). After streaking of *S. aureus* on TSA, it appears as yellow golden colonies. The suspected colonies were confirmed by biochemical tests as above previously. The biochemically identified isolates were then subjected to PCR targeting *nuc* gene, *S. aureus* was successfully amplified at 270 bp from all biochemically confirmed isolates (Figure 2). Fifty – one *S. aureus* isolates (34%) were recovered from the total examined quarter milk samples. Geographical distribution of *S. aureus* is shown in Table 1, Fig. 3.

Susceptibility of Staphylococcus aureus to antibiotics

The results from this study demonstrated that the isolates were highly susceptible to imipenem, marbofloxacin and tetracycline (100% each), ciprofloxacin, vancomycin and ciprofloxacin (98% each), cefotaxime (94.12%), gentamicin (92.2%), cefotaxime (94.12%), Ceftriaxone (84.4%), sulfamethoxazole- trimethoprim (88.2%), Streptomycin (66.7%) while, the recovered *S. aureus* isolates were highly resistant (100%) against ceftiofur and oxacillin. Additionally, erythromycin, penicillin, amoxicillin clavulinic and spiramycin showed 55%, 41.2% 39.2% and 39.2% rate of resistance respectively against the tested isolates as displayed in Table 2. Interestingly *S. aureus* isolates displayed complete resistance to oxacillin. A 94.11% (48/51) of *S. aureus* isolates were categorized of as MDR strains. As it shown in Table 3 , 5.9% (3/51) showed resistance to two antimicrobial agents, 11.8% (6/51) showed resistance to three antimicrobial agents, 17.64% (9/51) showed resistance to four antimicrobial agents, 33.3% (17/51) showed resistance to five antimicrobial agents,17.64% (9/51) showed resistance to six antimicrobial agents, 9.8% (5/51) showed resistance to seven antimicrobial agents, 1.96% (1/51) indicated resistance to eight different antimicrobial agents, while 1.96% (1/51) shown resistance to 10 different antimicrobial agents.

Discussion

Several bacterial infections that afflict dairy animals can affect the yield of milk. One of the most significant and deadly illnesses affecting dairy herds is mastitis, in both its clinical and subclinical forms. *S. aureus* has been identified as the predominant bacterial agent of bovine mastitis in numerous countries [14]. *S. aureus* is considered a serious hazard to the safety of dairy products since it adversely affects their quality.

In the present study, 51 samples (34%) of the 150 mastitic milk samples were found infected by *S. aureus* which was similar to Sayed, [15] who examined an examination on 100 milk samples obtained from milking cows in Assiut Governorate, Egypt that were symptomatic mastitic, they found to be present in 34.65%. However, a greater prevalence percentage of *S. aureus* (71.4%) was reported by Abou-Zaid and El Sawalhy [16]. On the other hand, Ahmed et al. [17] and Sayed and Mohamed [18] reported a lower prevalence of *S. aureus* from mastitis (21.7 and 23.6%, respectively). In Bangladesh, Salauddin et al. [19] observed a greater frequency (100%). In Kashan, Iran: 21.9 % prevalence was detected by Moniri et al. [20] while, the distribution of *S. aurues* among the yielded organisms was 13.6% in the milk samples of lactating cows from three areas from southeastern US dairies [21]. Another study found that *S. aureus* infects 3% of the total investigated animals from7 dairy herds in New York State [22]. Generally, the obtained results from this study are within prevalence rate ranging from 8.0 to 64.0% recorded in previous investigations [23, 24, 25]. This difference could be explained by variations in the research areas' geographic distribution, biosecurity protocols, and immunological state relative to other studies. Since milkers' hands are the primary means of transmission for *S. aureus* during the milking process, inadequate hygiene procedures contribute to the pathogen's high occurrence [26]. Determining the most likely source or sources of IMI for understanding and in turn control *S. aureus* mastitis, which is most commonly transmitted primarily at milking time from cow to cow. When the infected cow is milked and the next cow is milked with the same unit, this action increases the risk of acquiring *S. aureus* mastitis.

One of the main ways that antibiotic-resistant bacterium infections spread to human populations is through the intentional use of antimicrobial drugs in veterinary medicine, thus may potentially lead to public health issues [27]. Resistance to a specific antibiotic may appear in a specific region as a result of its frequent and long-term use. In this study, 94.11% of *S. aureus* involved in cow's mastitis expressed a multiple antibiotic resistance phenotype, with 100% resistance against ceftiofur and oxacillin, followed by erythromycin and penicillin. Similarly, the results from the present study were go on line with a study conducted in China found that 77.3% of

S. aureus isolates tested showed resistance to the used antimicrobials [28]. MDR was also recorded at a different rate in other studies conducted in Denmark (75%), Brazil (55.1%), and Argentina (40%) [29, 30]. Hoque et al. [4] showed that most of the *S. aureus* strains were highly resistant to oxacillin and to a lesser extent to erythromycin and penicillin. Antimicrobial resistance of *S. aureus* isolates in the current investigation is marginally higher than findings in previous studies [31, 32, and 33].

In Canada, Naushad et al. [34] reported that 19% of isolates were resistant to beta-lactams and 7% of isolates showed resistance to sulfonamides, while resistance was uncommon against tetracycline, ceftiofur, and erythromycin and to the combination of penicillin and novobiocin (3, 3, 2, and 2% of all isolates, respectively). High morbidity and economic losses are related to infections with MDR S. *aureus*. The emergence of MDR strains of *S. aureus*, resulting in either clinical or subclinical mastitis has become a major public health importance in recent years. Similarly, in a previous study carried out by Salauddin et al. [19]. The findings of this study will help in selecting an effective and appropriate antimicrobial agent for controlling bovine mastitis in Egypt. The recognition of MDR-SA in mastitis milk is described as alarming for the health of human, as *S. aureus* has zoonotic potential as it signifies a great public health importance. Livestock may have the upper hand in the transmission of such bacteria to humans through milk [35], which may be described as a great threat to public health, veterinarians and farm-associated workers.

Conclusions

The study results revealed a significant frequency of *S. aureus* in dairy cows. The high prevalence of *S. aureus* may be contributed to the unhygienic milking process and inadequate farm management and the high prevalence of *S. aureus* contaminated milk that was resistant to widely used antibiotics, which presents a concern to public health. Therefore, it is advised that the animal treatments be based on antibiotic susceptibility testing. In addition, it is essential to create a successful control program to prevent *S. aureus* from infecting humans, especially those are MDR.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study was ethically approved by Research Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University, Egypt (Code No: M/93

TABLE 1. Geographical distribution of *S. aureus* in this study

Name of the area	Number of *S. aureus* isolates	Percentage (%)
Salaka	5	9.8%
Belgay	4	7.8%
Kom Ederby	7	13.7%
Shawa	6	11.8%
Neqita	5	9.8%
Tanah	3	5.9%
Baqlia	3	5.9%
Sandowb	1	1.9%
Mit Elakrad	3	5.9%
Telbana	6	11.8%
Menia Sandowb	5	9.8%
Ezba Rezkalla	3	5.9%
Total	**51**	**100%**

TABLE 2. Antimicrobial resistance of *S. aureus* isolates from cow mastitic milk

Antibiotics	Disc codes	Concentrations	Antimicrobial classes	Resistant		Sensitive	
				No.	%	No.	%
Amoxicillin clavulinic	AMC	10 µg	B-lactam	20	39.2	31	60.8
Sulfamethoxazole Trimethoprim	SXT	23.75/1.25 µg	Potentiated sulfonamides	6	11.8	45	88..2
Gentamicin	CN	10 µg	Aminoglycosides	4	7.8	47	92.2
Ciprofloxacin	CIP	5 µg	Quinolones	1	2	50	98
Cefuroxime	CXM	30 µg	B-lactam	9	17.65	21	82.35
Penicillin	P	10 IU	B-lactam	21	41.2	30	58.8
Ceftriaxone	CRO	30 µg	B-lactam	8	15.7	43	84.3
Ceftiofur	EF T	30 µg	B-lactam	51	100	0	0.00
Vancomycin	VA	30 µg	Glycopeptide	1	2	50	98
Streptomycin	S	10 µg	Aminoglycosides	17	33.3	34	66.7
Tetracycline	TE	30 µg	Tetracycline	0	0.00	51	100
Erythromycin	E	15 µg	Macrolides	28	55	23	45
Ampicillin	AM	15 µg	B-lactam	12	23.5	39	76.5
Oxacillin	OX	15 µg	B-lactam	51	100	0	0.00
Cefotaxime	CTX	30 µg	B-lactam	3	5.88	48	94.12
Imipenem	IMP	10 µg	B-lactam	0	0.00	51	100
Spiramycin	SP	100 µg	Macrolide	20	39.2	31	60.8
marbofloxacin	MARBO	5 µg	Fluoroquinolones	0	0.00	51	100

TABLE 3 Antibiogram-resistant patterns and MAR index of *S. aureus*

Antibiotypes	Resistance pattern	MAR index	Isolates no. (%)
1	AMC, AM, P, EFT, S, OX	0.33	1(1.96)
2	AMC, E, AM, EFT, S, OX	0.33	1(1.96)
3	E, P, EFT, S, OX	0.27	1(1.96)
4	AMC, E, P, CRO,EFT, OX	0.33	1(1.96)
5	AMC, EFT, OX	0.16	1(1.96)
6	P, EFT, S, OX, SP	0.27	1(1.96)
7	AMC, E, EFT, OX, SP	0.27	1(1.96)
8	AMC, P, CRO, EFT,S, OX, SP	0.38	1(1.96)
9	AMC, CXM, VA, E, AM, SXT,EFT, OX	0.44	1(1.96)
10	AM, P, EFT, S, OX, SP	0.33	1(1.96)
11	EFT, OX	0.11	3 (5.88)
12	E, EFT, OX	0.16	3 (5.88)
13	AMC, E, AM, EFT, OX	0.27	1(1.96)
14	AMC, AM, SXT, EFT,OX	0.27	1(1.96)
15	CXM, E, AM, P, EFT,OX, SP	0.38	1(1.96)
16	AMC, P, EFT, OX, SP	0.27	1(1.96)
17	CRO, EFT, OX, SP	0.22	1(1.96)
18	EFT, S, OX	0.16	2 (3.9)
19	EFT, S, OX, SP	0.22	1(1.96)
20	E, P, EFT, OX	0.22	1(1.96)
21	E, P, CRO,EFT, OX	0.27	1(1.96)
22	E, P, EFT, OX, SP	0.27	1(1.96)
23	AMC, CTX, AM, EFT, OX, SP	0.33	1(1.96)
24	AMC, CXM, CN, EFT, S, OX, SP	0.38	1(1.96)
25	CXM, EFT, S, OX	0.22	1(1.96)
26	CTX, CXM, E, EFT, S, OX	0.33	1(1.96)
27	CXM, E, SXT, EFT, OX	0.27	1(1.96)
28	AMC, CXM, E, SXT, P, EFT, OX	0.38	1(1.96)
29	E, SXT, P, EFT, OX	0.27	1(1.96)
30	AMC, E, AM, P, CRO,EFT, OX	0.38	1(1.96)
31	E, P, CRO, EFT, OX, SP	0.33	1(1.96)
32	E, EFT, OX, SP	0.22	2 (3.9)

Antibiotypes	Resistance pattern	MAR index	Isolates no. (%)
33	E, AM, EFT, OX, SP	0.27	2 (3.9)
34	AMC, CN, E, EFT, OX, SP	0.33	1(1.96)
35	AMC,CN, E, AM, P, CRO,EFT, S, OX, SP	0.55	1(1.96)
36	P, EFT, S, OX	0.22	2 (3.9)
37	AMC, E, P, EFT, OX	0.27	1(1.96)
38	CIP, AMC, CXM, E, EFT, OX	0.33	1(1.96)
39	AMC, CN, EFT, OX, SP	0.27	1(1.96)
40	AMC, SXT, P, EFT, OX	0.27	1(1.96)
41	P, CRO, EFT, S, OX	0.27	1(1.96)
42	E, EFT, S, OX	0.22	1(1.96)
43	CTX, CXM, EFT, OX, SP	0.27	1(1.96)

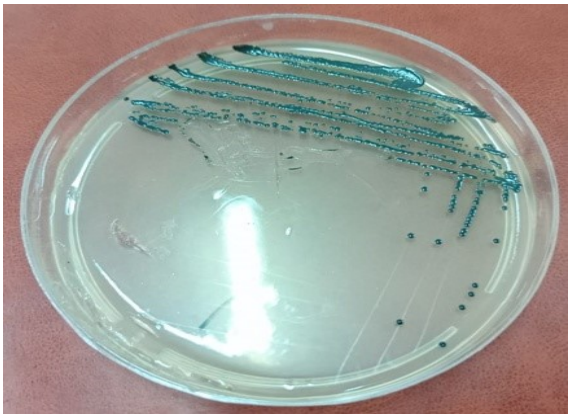

Fig. 1. Black jet colonies of *S. aureus* on Paired parker agar media

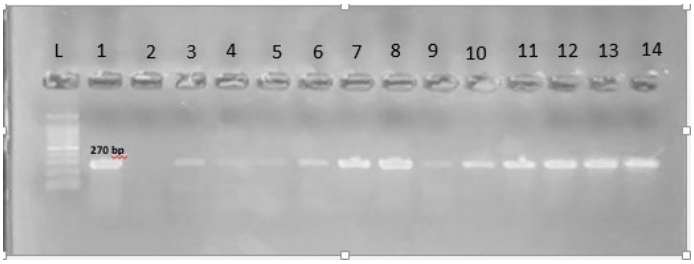

Fig. 2. Agarose gel electrophoresis showing amplification of *nuc* gene at 270bp

L: DNA ladder, 1: control positive, 2 : control negative

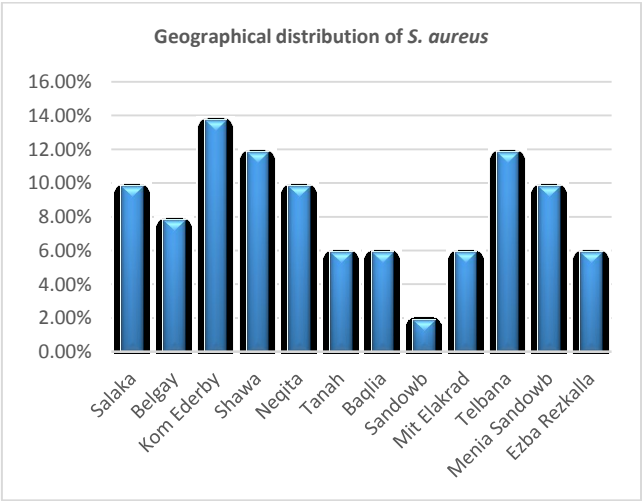

Fig. 3. Geographical distribution of *S. aureus* isolates recovered from this study

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الكشف عن تواجد المكور العنقودي الذهبي في ألبان الابقار المصابه بالتهاب الضرع الاكلينيكي مع تحديد الأنماط الظاهريه لمدي حساسيه المعزولات للمضادات الحيويه

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الملخص

يهدف العمل الحالي إلى عزل المكورات العنقودية الذهبية من حليب الأبقار المصابة بالتهاب الضرع ودراسة الأنماط الظاهرية للحساسية المضادة للميكروبات للسلالات المعزولة. تم جمع مائة وخمسين عينة حليب من الأبقار المصابة بالتهاب الضرع والتي تظهر عليها اعراض اكلينيكيه ظاهريه. تم الحصول على العينات من أصحاب الحيازات الصغيرة من القري المجاوره لمدينه المنصوره بمحافظة الدقهلية بمصر. تم فحص العينات المجمعة للتأكد من وجود بكتريا المكورات العنقودية الذهبية عن طريق زراعتها علي اوساط بكتيريه ثم إخضاع العزلات المشتبه بها للفحص الكيميائي الحيوي وبعدها إجراء تفاعل البلمرة المتسلسل (PCR) لتحديد الأنواع. تم بعد ذلك اختبار حساسيتها لمضادات الميكروبات ضد 18 نوعًا من مضادات الميكروبات المستخدمة عادةً لعلاج التهاب الضرع في مصر.اثبت النتائج وجود المكور العنقودي الذهبي بنسبة 34% (150/51) من إجمالي عينات اللبن التي تم فحصها. أشارت الحساسية للمضادات الميكروبية إلى أن العزلات لديها حساسية عالية لمضادات الإيميبينيم والماربوفلوكساسين والتتراسيكلين (100% لكل منهما)، والسيبروفلوكساسين والفانكومايسين والسيبروفلوكساسين (98% لكل منهما)، والسيفوتاكسيم (94.12%)، والجنتاميسين (92.2%)، والسيفوتاكسيم (94.12%)، سيفترياكسون (84.4%)، سلفاميثوكسازول تريميثوبريم (88.2%)، ستربتومايسين (66.7%)، بينما أظهرت عزلات المكورات العنقودية الذهبية المقاومة الكاملة (100%) ضد السيفتيوفور والأوكساسيللين. في حين أظهرت الاريثروميسين، البنسلين، الأموكسيسيلين كلافيولينك والسبيراميسين نسبة مقاومة 55%، 41.2%، 39.2% و 39.2% على التوالي. تم الكشف عن المقاومة للأدوية المتعددة (MDR) في 94.11% من العزلات. لذا فان وجود المكورات العنقودية الذهبية بنسبه مرتفعه في هذه الدراسه ومقاومتهاالمتزايدة لمضادات الميكروبات شائعة الاستخدام والتي تعتبر مشكلة خطيرة على صحة الإنسان والحيوان يوضح اهميه البيانات التي تم الحصول عليها عن المقاومة للمكورات العنقودية الذهبية المعزولة من عينات حليب البقر التي تساعد في تحديد المصدر، وتقييم المخاطر، وتطوير استراتيجيات علاجية أفضل.

الكلمات الداله: المكورات العنقودية الذهبية، الأبقار، التهاب الضرع، المقاومة المتعدده للمضادات الحيويه.