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# Bacteriological studies on methicillin -resistance *Staphylococcus aureus* (MRSA) isolated from cow's mastitis milk.

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**ABSTRACT**

An enormous problem reported worldwide namely bovine mastitis caused by MDR (multi-drug resistant*) S. aureus* results in prolonged antibiotic treatment and livestock death. Mastitis caused by pathogenic *S. aureus* strains led to a focused study on surveillance of antibiotic susceptibility along with phenotypic and genotypic characterization. The isolation of Staphylococcus *aureus* to the determination of antibiotic susceptibility pattern from obtained cow's milk samples was the aim of the current study. Cows affected by mastitis from different farms in Kalyuobia Governorate, one hundred milk samples were conducted for *S. aureus* isolation with a prevalence of 30% where 20% isolates were coagulase positive (CoPS) and 10% isolates were coagulase negative CoNS (Gram staining, oxidase, catalase, DNase, hemolysis, and the coagulase test were employed for bacterial identification). The majority of the strains (COPS) n=20 was profiled revealing antimicrobial sensitivity to be multidrug resistant. The occurrence of oxacillin susceptible *mec*A strains positive strains (OS-MRSA) was revealed in 6 strains for the first time. Identification of MRSA strain by detection of *mec*A gene in the PCR.

## 1. INTRODUCTION

Staphylococcus aureus is a pathogenic gram-positive, facultative anaerobe bacterium that represents worldwide threat to man and animals (Nemeghaire et al., 2014). staphylococcus aureus infections in cattle can result in several disease conditions including, mastitis, skin and GIT infections, osteomyelitis, meningitis, pneumonia, endocarditis and toxic shock syndrome (Pal et al., 2020). Staphylococcus aureus -induced mastitis represents a significant infectious disease resulting in severe economic losses to the dairy industry worldwide; moreover, economic losses resulting from Staphylococcal mastitis were estimated at around €300 per cow annually (Fluit, 2012).

Antibiotic resistance is a challenging threat to human and animal health (Silva et al., 2020). Indeed, S. aureus is extraordinarily capable of developing resistance to various antimicrobial drugs (Foster 2017). Moreover, the massive use of antibiotics in veterinary medicine is a critical aspect for the dissemination of antibiotic resistant bacteria to humans that poses a significant risk for public health (Salauddin et al., 2020). Penicillin was first discovered in 1929 and it showed antibacterial action against S. aureus (Fleming 1929). By 1940, penicillin was introduced into clinic as an effective antibiotic against infectious diseases including S. aureus and by the massive usage of penicillin in treatment in 1950; penicillin resistant S. aureus stains had emerged that were capable of producing penicillinase, which hydrolyze beta-lactam ring of penicillin. Afterwards in 1959, methicillin antibiotics were administrated for treatment of infections caused by penicillin resistant S. aureus; however, Methicillin resistant S. aureus strains emerged in 1961 (Guo et al., 2020). Methicillin resistance is encoded mecA and mecC genes that encode for penicillin-binding protein 2a (PBP2a), these genes are carried on a mobile genetic element called Staphylococcal chromosomal cassette mec (SCCmec) (Khairullah et al., 2020).

The emergence of multi-drug resistant S. aureus (MDR) and methicillin resistant S. aureus (MRSA) is a rising public health concern; therefore, this study was designed to monitor the prevalence of Staphylococcus aureus in cow's mastitis milk with determination of the resistance profile of Staphylococcus aureus isolates to different antibiotics to identify the MDR S. aureus and MRSA strain. Polymerase chain Reaction through mecA gene.

## 2. MATERIAL AND METHODS

*2.1. Milk samples:*

Collection of one hundred milk samples(n=100) from Egyptian cows suffering from mastitis in Kalyuobia Governorate in between April to October 2020.

The samples were collected aseptically from cows with clinical signs of mastitis including inflammation of the udder, milk discoloration and decreased milk production and immediately transported to the laboratory in an ice container (Hogan et al., 1999).

*2.2. Isolation of S. aureus from mastitis milk using conventional cultural method (Quinn et al., 2002 and Arora 2003):*

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One ml of milk was inoculated into nutrient broth (Oxoid CM0001), incubated aerobically at 37°c for 12hrs. A loopful from the incubated nutrient broth was streaked onto nutrient agar (Oxoid CM0003) and incubated for 24 h at 37°c for 24 hrs. After that, the suspected colony were subculture on Baird Parker agar (Oxoid CM1127) supplemented with Egg Yolk Tellurite Emulsion (Oxoid SR0054), mannitol salt agar (Oxoid CM0085) and 5% sheep blood agar (CM0055) and incubated at 37°c for 24-48hrs. The suspected colonies (black colonies with yellow halo around them on the Baird Parker agar, yellow colonies surrounded by halo zone on mannitol salt agar and white or yellow, smooth round, hemolytic and shiny colonies on blood agar) were picked up and kept in semi-solid agar for biochemical identification.

*2.3. Biochemical identification of S. aureus isolates (Quinn et al., 2002 and Arora, 2003):*

It was performed by application of Gram staining, catalase, coagulase, oxidase, sugar fermentation, indole, triple sugar iron, Methyl red, Voges-Proskauer and Urease tests.

*2.4. Anti- microbial resistance profile of S. aureus isolates:*

*Staphylococcus aureus* isolates were submitted to antimicrobial susceptibility testing by using disc diffusion method CLSI (2017) for 9 antibiotics; cefoxitin (F0X, 30µg), norfloxacin (Nor, 10µg), sulphamethoxazole-trimethoprime (SXT, 25µg), doxycycline (Do, 30µg), clindamycin (DA, 2µg), amoxicillin (AX, 25µg), ofloxacin (Ofx, 5µg), gentamicin (CN, 2µg), oxacillin (Ox, 1µg). (Table 1).

Sterile Mueller- Hinton Agar plates (Oxoid CM0337) were swabbed with bacterial suspension equivalent to 0.5 McFarland then placement of the antimicrobial discs onto the agar was done using sterile forceps and to ensure the complete contact with the agar surface, it was press gently and incubated at 35°c for 24hr. The diameter of the inhibition zone around the antimicrobial discs were measured and interpreted according to Nccls (2007).

*2.4.1 Identification of multi-Drug resistant (MDR) S. aureus isolates:*

*Staphylococcus aureus* were identified as isolates showing resistance to three or more antimicrobial drugs from different categories (Rota., 1996). Staphylococcus aureus were screened by susceptibility testing for cefoxitin 30µg and oxacillin 1µg and isolates showing inhibition zone with diameter ≤ 23 mm and 10 mm for cefoxitin 30µg and oxacillin 1µg respectively, were identified as MRSA.

*2.5. PCR for identification of mecA gene for resistance of MRSA:*

Ten (n=10) *S. aureus* isolates including 5 phenotypic MRSA (2 fox,3ox) and 5 MDR *S. aureus* selected and submitted to PCR targeting mecA gene of mecithillin resistance *S. aureus* using forward primer F mecA1 5' GTAGAAATGACTGAACGTCCGATAA3' and reverse primer R mecA2 5' CCAATTCCACATTGTTTCGGTCTAA with 310 bp amplicon size (McClure et al., 2006).

*2.5.1. Extraction of genomic DNA from S. aureus isolates:*

It was performed using QIAamp DNA mini kit instructions (Qiagen, cat no. 51304) according to manufacturer's instructions.

*2.5.2. Amplification and cycling conditions of PCR targeting mecA gene of S. aureus isolates:*

performed according to McClure et al., (2006) and (Emerald Amp GT PCR master mix 2x premix (Takara code no. RR310A). PCR amplification was carried out on T3 thermal cycler (Biometra) using 25 µl reaction volume containing 12.5 µl master mix (Takara code no. RR310A), 1µl of each forward and reverse primer, 4.5 µl molecular biology grade water and 6 µl test DNA at a thermal profile of 1 cycle at 94c for 10 min: 35 cycles of at 94°c for 45s, 50°c for 45s, 72°c for 45s and 1 cycle of final extension at 72°c for 10 min. Then the amplicons were detected by agarose gel electrophoreses on 1.5% agarose gel stained by ethidium bromide and examined by gel documentation system (bio-rad) (Sambrook et al., 1989).

## 3. RESULTS

*3.1. Isolation of S. aureus from cow's mastitis milk:*

After screening one hundred of milk samples of Egyptian cows suffered from mastitis that were isolated on mannitol salt agar 30*S. aureus* with yellow color surrounded by yellow halo (ferment mannitol) and appear circular, smooth, and opaque with B-hemolysis on blood agar. Growth of those isolates on Baired Parker medium showed black small 1mm colonies after 24 hours incubation and large 2.5mm after 48 hours incubation surrounded by an opalescent ring and a clear zone. *The S. aureus* isolates showed positive results for gram staining with the characteristic grape like clusters by oil immersion oil).

*3.2. Biochemical reactions:*

*Staphylococcus aureus* isolates were positive for catalase, coagulase, V-P test and negative oxidase testes. The results revealed that 30 out of 100 raw milk samples (30%) were positive for *staphylococcus aureus*. Out of those 30 samples, 20(66.6%) were coagulase positive *Staphylococcus aureus* (COPS). while 10(33%) were coagulase negative *Staphylococci aureus* (CONS).

*3.3. In-vitro antimicrobial test (antibiogram activity):*

In our study, S. aureus isolates were showed variable resistant against antimicrobial agents. The highest sensitivity towards ofloxacin (100%), Do (100%) followed by FOX (90%), amoxicillin (85%), gentamicin (75%), Nor (70%), sxI (65%), DA (55%), oxacillin (35%) were showed among the S. aureus isolates. This study presents S. aureus that the overall high percent of S. aureus isolate resistant to OX (60%), DA (30%), SXI (20%), Nor (20%), FOX (10%), CN (5%), DO (0%), OFX (0) However, low numbers of isolates showed intermediate resistance for DA (15%), SXI (15%), CN (20%).

Most of S. aureus isolates were verified as multi-drug resistant S. aureus (MDR-SA), where 5/20 (25%) isolates were resistant to three or more antimicrobial agents from different groups of antimicrobials as shown in (Table 2).

It was found that fluroquinolone members: norfloxacin, tetracycline (DO) and ofloxacin showed the lowest resistance ratio of 5/20 (25%), 0/20(0%), and0/20 (0%), respectively, while Oxacillin, clindamycin displayed the highest resistant in ratios of 65%, 30% respectively.

*3.4.(MRSA screening test):*

The effect of 30-µg cefoxitin,1- µg oxacillin discs on *S. aureus* isolate and the MRSA isolates was assessed *Staphylococcus aureus* strain (n = 18) was found to be sensitive to cefoxitin (30 µg) and the zone of inhibition was estimated, while the MRSA isolates (2/20) 10% were resistant and not affected by cefoxitin. On the other side MRSA isolates 13/20 (65%) were oxacillin resistant (table 1).

*3.5. Result of PCR for mecA gene detectionSSS:*

The PCR amplification of the 10 phenotypically MRSA isolates (2 resistant to fox,3resistant to ox and 5 MDR) was revealed that 6/10 (60%) of them were found to carry*mec* A gene, which amplified at 310 bp. (figure 1).

Table 1 results of screening test

| Isolate | Oxacillin (1µg) | Cefoxitin (30µg) |
|---|---|---|
| 1 | R | S |
| 2 | R | S |
| 3 | R | S |
| 4 | R | R |
| 5 | S | S |
| 6 | S | S |
| 7 | S | S |
| 8 | R | S |
| 9 | R | S |
| 10 | R | S |
| 11 | S | R |
| 12 | S | S |
| 13 | R | S |
| 14 | R | S |
| 15 | R | S |
| 16 | S | S |
| 17 | R | S |
| 18 | R | S |
| 19 | R | S |
| 20 | S | S |

R: Resistant, S:Sensitive

Table 2 Staphylococcus aureus resistance against different Antimicrobial Agents norfloxacin (Nor), sulphamethoxazole-trimethoprime (SXT), doxycycline(D), clindamycin (DA), amoxicillin (AX) , ofloxacin (Ofx ), gentamicin (CN)

| Antimicrobial | Disk | Sensitive | | Intermediate | | Resistant | |
|---|---|---|---|---|---|---|---|
| Agent | Conc. | No. | % | No. | % | No. | % |
| OFX | 5 µg | 20 | 100 | 0 | 0 | 0 | 0 |
| AX | 25 µg | 16 | 85 | 2 | 10 | 2 | 10 |
| CN | 10 µg | 15 | 75 | 4 | 20 | 1 | 5 |
| DO | 30 µg | 20 | 100 | 0 | 0 | 0 | 0 |
| NOR | 10 µg | 14 | 70 | 2 | 10 | 4 | 20 |
| SXT | 25 µg | 13 | 65 | 3 | 15 | 4 | 20 |
| DA | 2 µg | 11 | 55 | 3 | 15 | 6 | 30 |
| OX | 1 µg | 8 | 35 | 0 | 0 | 12 | 60 |
| FOX | 30 µg | 18 | 90 | 0 | 0 | 2 | 10 |

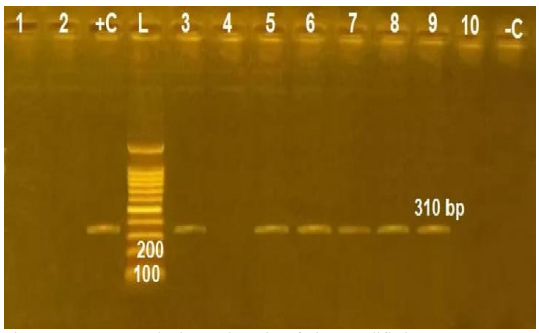


Figure 1 Agarose gel electrophoresis of the amplified mecA gene PCR product (310 bp) for the five methicillin-resistant *S. aureus* (MRSA). Lane L:100-600bp DNA Ladder. C-: Neg. control, C+: Pos. control (S.aureus reference ATCC 25923), Lane 3:5:6:7:8 &9:Positive strains for mecA gene at 310bp.

## 4. DISCUSSION

*Staphylococcus aureus* is one of the most widespread pathogens in the globe. *S. aureus* is able to make infection through which different virulence factor. Firstly, bacteria invade the host then established and escape from destruction by the immune system beyond enzymes, toxin, adhesion molecules (Kuroda et al.2001; Langley et al. 2017).

Around this study, *Staphylococcus aureus* isolates were Gram positive cocci arranged like grape aerobic growing, coagulase positive. The morphological and biochemical characters of *S. aureus* isolates were similar to Harmon et al., (1991), Howard & Kloss (1993) and Wladimir et al., (2000). The bacteriological results of 100 samples of cow's mastitis milk cleared that 20 *S. aureus* isolates (20%). The results of *S. aureus* isolation were nearly similar to those recorded by El-kholyetal (1994), Fabre et al. (1997), Baudet & chieze (1994), Andrade(2001), Janosi & Baltay (2004)and Saini et al.,(1994) who reported that *S. aureus* isolates were (22.9%), (29%), (30%), (30.2%), (32.5%) and (34%) respectively. But our results were disagreed with those obtained by Gianneechini et al. (2002) who isolated *S. aureus* with higher incidence reached (62.8%).

The variations between the isolation rate of *S. aureus* were due to geographic distribution, biosecurity practices in the study area as well as difference in immunological status These factors might cause decrease in the isolation rate of S. aureus infection.

The results of antibiogram for the isolated *S. aureus* ( n=20) were revealed that, highest sensitivity towards ofx (100%), Do (100%) followed by FOX(90%), amoxicillin (85%), gentamicin (75%), Nor (70%), sxI (65%), DA (55%), oxacillin( 35%), This study presents high percent of *S. aureus* isolate resistant to OX (60%), DA (30%), SXI (20%) ,Nor(20%), FOX (10%), CN(5%), DO (0%), OFX(0), Nearly similar to Corti et al. (2003) recorded that (91%) of the *S. aureus* strains were sensitive to all antimicrobial, while the isolated strains of S .aureus were resistant to pencillin G and ampicillin with percentage (9%) and (7) respectively. Nearly similar to Andrada et al. (2000) and Chowdhury et al. (2002). While Gentilini et al. (2000) recorded resistance to gentamicin (3.4%). Moreover, the current results proved that MDR are widely spread among *S. aureus n strains* and the misuse of antimicrobials agent s in the bovine farms in Egypt might be the main cause of multidrug resistance appearance for S. aureus.

Application of MRSA screening test showed that 13/20 S. aureus isolates resist to oxacillin and 2/20 resist for cefoxitin. This test revealed that 6/20 of cops isolate were methicillin resistant *staphylococcus aureus* (MRSA). The current findings were agreed with Tenhagen et al., (2009).

The PCR technique is used for *mec*A gene detection in 10 MRSA isolates from cows' mastitis milk which were MDR. The PCR results showed 6/10 MDR *S. aureus* strains positive for *mec*A gene at 310 bp. This result was agreed with (Nunes et al., 2007), Virgin et al.,2009). On the other side, Alian et al. (2012) were disagreed with our results.

## 5. CONCLUSION

This study concluded that staphylococcus aureus represents high prevalence in cow's milk in Kalyuobia governorate. The increase in common antimicrobial agents, resistance used in the treatment against bovine mastitis led to the potential risk of MRSA transmission from animal to human.

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