

## Phenotypic and genotypic profiling of Methicillin-resistant *Staphylococcus aureus* isolates from human and bovine milk

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### ABSTRACT

The current work aimed to track the spread of methicillin-resistant *Staphylococcus aureus* (MRSA) in human and bovine milk in Fayoum city, Egypt. 74% of isolates obtained from a human with septic sore throat and 40% of bovine isolates from bulk milk tank were found positive for *S. aureus*. The typical characteristics of MRSA including resistance to oxacillin and meca positivity were detected in 8% and 7% of the human and bovine isolates respectively. The antibiotic resistance profile of human and bovine MRSA isolates have shown a high degree of similarity with complete resistance to beta-lactam antibiotics associated with variable resistance to other antibiotic classes. Nevertheless, 99-100% identity has been detected between meca gene of the human and bovine isolates and multiple MRSA strains in Genebank. The identified phenotypic and genotypic similarity provides clear evidence of potential epidemiological relation between the human and bovine MRSA isolates. However, further study is required to identify the mode of transmission of MRSA isolates between human and different livestock animals on wide-scale and expand the study to include wild animals.

**Keywords:** Methicillin-resistant *Staphylococcus aureus*, septic sore throat, meca gene, Bulk milk tank.

### INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major public health threat worldwide. However, MRSA infections have only begun in the health care community, a high rate of MRSA was recently observed among people in different communities and have no risk factors for acquiring MRSA infections. This might be attributed to emergence of such infections in livestock and food chain animals. Previous reports provided shreds of evidence for the capability of MRSA to transmit between equines and humans especially veterinarians and workers at veterinary hospitals. The study found that people in contact with horses infected with MRSA showed skin infections caused by horse strain (Weese *et al.*, 2006 and Schwaber *et al.*, 2013). Various companion animals including pets, pigs, rabbits and sheep have been found to host MRSA of human origin, revealing that animals that come into contact with infected personnel may serve as effective reservoirs for spreading the infection widely (Medhus *et al.*, 2013., Haenni *et al.*, 2012, Vincze *et al.*, 2014 and Walther *et al.*, 2012).

*Staphylococcus aureus* is the major cause of infections in cattle and is associated with significant economic losses in dairy business. The staphylococcal infection has been complicated by reporting first case of MRSA in bovine mastitis in the 1970s, and to date, MRSA isolates have been reported in cattle and milk all over the world (Bardiau *et al.*, 2013). The growing spread of MRSA in cattle and dissemination of this organism in milk highlights the significant importance of MRSA as an emerging zoonotic pathogen and points that further evaluation of interspecies transmission of MRSA is highly required. High phenotypic and genotypic similarities have been previously reported in MRSA isolates from cattle and humans suggesting the possibility of direct transmission of MRSA from cattle to humans and vice versa (Juhász *et al.*, 2007). The current work was undertaken to monitor the prevalence of MRSA in human and bovine milk (bulk milk tank), and to study the phenotypic and genotypic features of bovine and human isolates to track the spread of MRSA strains in Fayoum city, Egypt.

### MATERIALS AND METHODS

#### 1. Isolation and characterization of *S. aureus*:

**1.1. Sampling:** The bovine milk samples were collected from bulk tank milk in dairy farms and throat swabs were collected from human admitted to a private bacteriological laboratory. The throat swabs were collected voluntarily from human with septic sore throats during routine sampling for bacteriological analysis.

**1.2. Bacteriological analysis and characterization (Kümmel *et al.*, 2016):** Milk samples and throat swabs were enriched in peptone water (1 ml milk in 9 ml sterile PW) for 48 hr at 37 °C, followed by streaking on selective media (Baird parker) for 24 hrs at 37°C. The suspected black colonies were then streaked on nutrient agar for 24 hr at 37°C and then the primary isolates were analyzed by gram staining. Furtherly, the isolated strains were examined for blood hemolysis and submitted to biochemical characterization including catalase, urease, mannitol and coagulase tests.

#### 2. Antimicrobial susceptibility testing:

all the *S. aureus* isolates including human and bovine isolates were examined by disk diffusion method using oxacillin. Then, oxacillin resistant *S. aureus* were further tested against 16 antibiotics. Briefly, the inoculum suspension was prepared by transferring few colonies from the nutrient agar to sterile saline and the inoculum turbidity was adjusted to a 0.5 McFarland standard. Then, lawn culture of the tested bacterial suspension was

prepared by spreading few microliters of inoculum on Muller Hinton agar plates. Antibiotic discs were then applied on the cultured agar plate. After overnight incubation at 37°C, the inhibition zone was measured, and the results were interpreted according to CLSI (Clinical and Laboratory Standards Institute 2009).

### 3. Molecular characterization of MRSA using PCR (McClure et al., 2006):

**3.1. DNA extraction.** To extract the DNA, the QIAamp DNA Mini kit is used with slight modifications from the manufacturer's instructions (Qiagen, Germany, GmbH). Briefly, 200 µl of the sample suspension was incubated for 10 minutes with 10 µl of proteinase K plus 200 µl of lysis buffer at 56°C. Following the incubation, 200 µl of 100% ethanol was added to the lysate. The sample mixture was then washed and centrifuged according to the manufacturer's method. The elution of nucleic acid was performed using 100 µl of elution buffer.

**3.2. PCR amplification.** The Oligonucleotide primer (Metabion, Germany), listed in [Table \(1\)](#), were used in a 25 µl reaction composed of 12.5 µl EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer (20 pmol), 4.5 µl H<sub>2</sub>O, and 6 µl DNA template. The amplification reaction was conducted in an Applied biosystem 2720 thermal cyclor.

**3.3. Analysis of the PCR Products.** The PCR products were fractionated by electrophoresis using 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products was loaded in each gel slot. A generuler 100 bp DNA ladder (Fermentas, Thermo, Germany) was also loaded to detect the fragment sizes. The gel was imaged by a gel documentation system (Alpha Innotech, Biometra) and the data were then subjected to analysis using computer software.

**Table 1:** Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Denaturation	Annealing	Extension		
<i>meca</i>	GTA GAA ATG ACT GAA CGT CCG ATA A	310	94°C 5 minutes.	94°C 30 second.	50°C 30 second.	72°C 30 second.	72°C 7 minutes.	McClure <i>et al.</i> , <a href="#">2006</a>
	CCA ATT CCA CAT TGT TTC GGT CTA A							

### 4. Nucleotide sequencing:

PCR products were purified using QIAquick PCR Product extraction kit. (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit(Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisesp spin column. DNA sequences were obtained by Applied Biosystems3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990) was initially performed to establish sequence identity to GenBank accessions. The phylogenetic tree was created by the MegAlign module of LasergeneDNAStar version 12.1 Thompson *et al.*, (1994) and Phylogenetic analyses were done using maximum likelihood, neighbour-joining and maximum parsimony in MEGA6 (Tamura *et al.*, 2013).

## RESULTS

### Isolation and Characterization of MRSA:

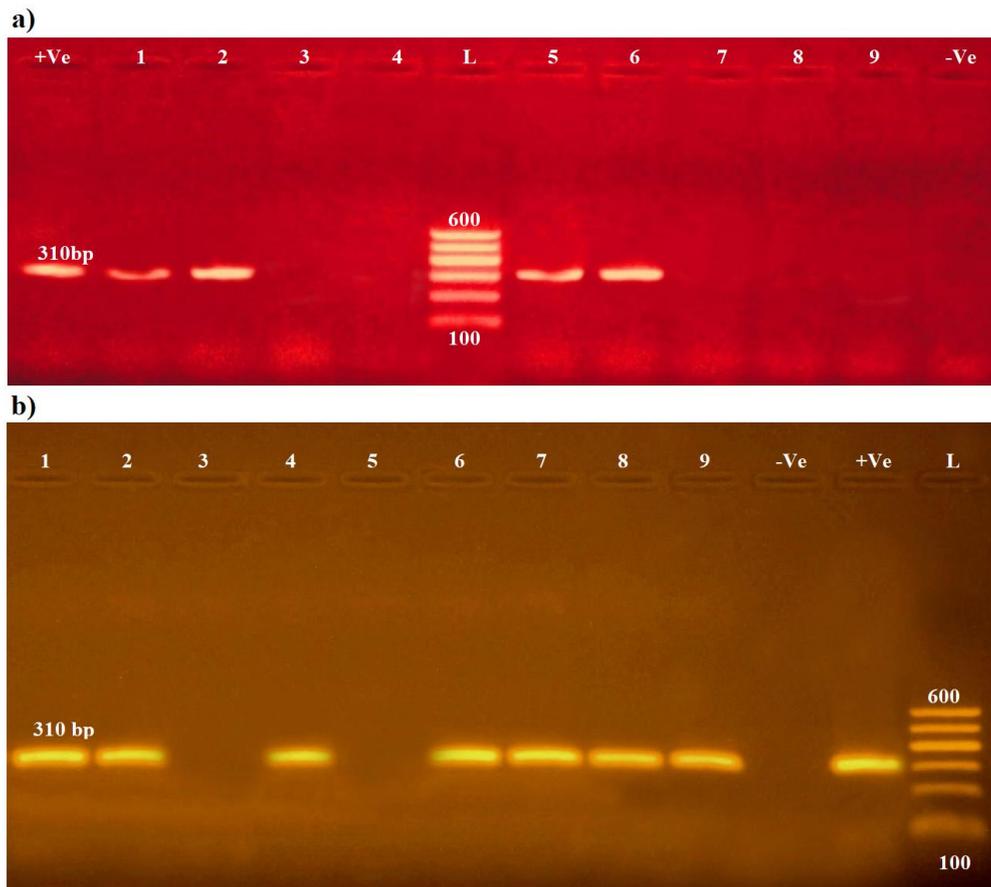
50 samples from bulk milk tank and 100 throat swabs from human with septic sore throat, were collected from different villages in Fayoum city during 2017 and 2018. The human with septic sore throat were voluntarily sampled during attending private bacteriological laboratories. Both milk samples and throat swabs were firstly enriched in peptone water then plated on Baird parker. The culture-positive Staphylococcus isolates were furtherly characterized via biochemical tests and hemolysis on blood agar. The *S. aureus* isolates were characterized as gram-positive cocci, with positive catalase test, positive coagulase tests and showing beta-hemolysis on blood agar.

To detect MRSA strains, the *S. aureus* isolates were first screened by oxacillin disk diffusion method and the result showed that 9 (24.3%) of *S. aureus* isolates recovered from bovine milk and 9 (22.5%) of human isolates exhibited resistance to oxacillin. The molecular method using PCR demonstrates that 4 bovine isolate and 7 human isolates harboring *mec A* gene and showing the expected band at 310 bp ([Figure 1](#)).

As shown in table 1, MRSA prevalence in bovine milk and human samples based on phenotypic identification, was approximately 18% and 9% respectively. However, lower incidence rate in (8%) bovine milk and (7%) human samples were maintained the typical characteristics of MRSA including resistance to oxacillin and *mecA* positivity ([Table 2](#)).

**Table 2:** The prevalence of MRSA in human and bovine milk based on oxacillin resistance and harboring of *mec A* gene.

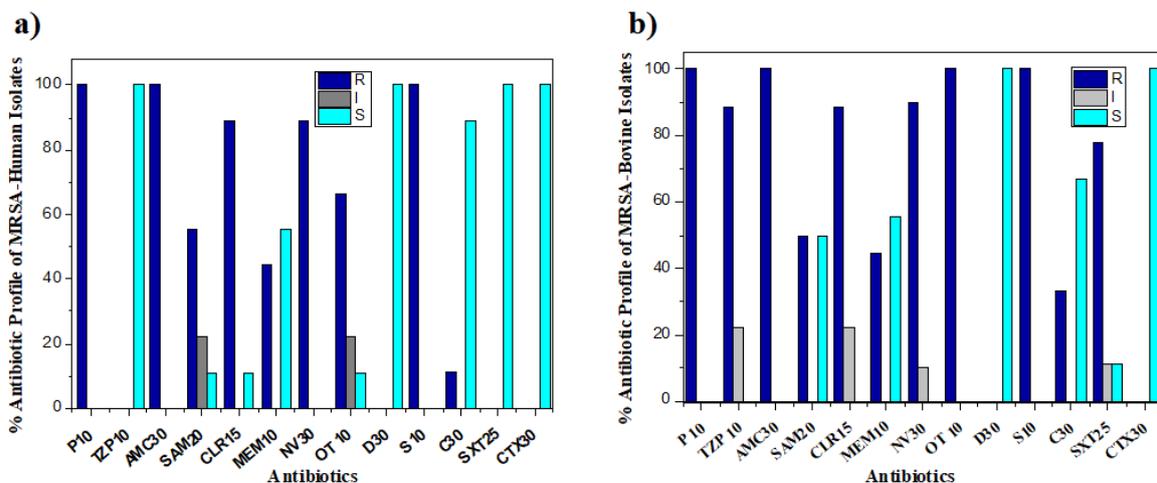
Source	<i>S. aureus</i>		MRSA			
	No Prevalence		Oxacillin-resistant No Percentage		<i>mec A</i> gene positive No Percentage	
Bovine milk	37	74 %	9	18 %	4	8 %
Human	40	40 %	9	9 %	7	7 %



**Fig1:** PCR amplicon of *mec A* gene in MRSA isolates with positive band at 310 bp. a) MRSA isolates from bovine milk: lane1, 2, 5 and 6 are positive, lane 3, 4, 7, 8, and 9 are negative. b) MRSA isolates from human samples (throat swabs): lane 1, 2, 4, 6, 7, 8 and 9 are positive, while lane 3 and 5 are negative. Lane +Ve is control positive, lane -Ve is control negative and lane L is DNA ladder.

**The antibiotic susceptibility profile of MRSA isolates:**

To determine the antibiotic susceptibility profile of the phenotypically identified MRSA isolates through oxacillin disk diffusion method, the isolates were tested against 13 antibiotics as shown in **Figure 2**. The results showed that both human and bovine isolates had pronounced resistance to beta-lactam antibiotics, with 100 % resistance to amoxicillin-clavulanic acid and penicillin. Resistance to ampicillin/Sulbactam was observed in 55.5 % of human isolates and 50% of bovine isolates.



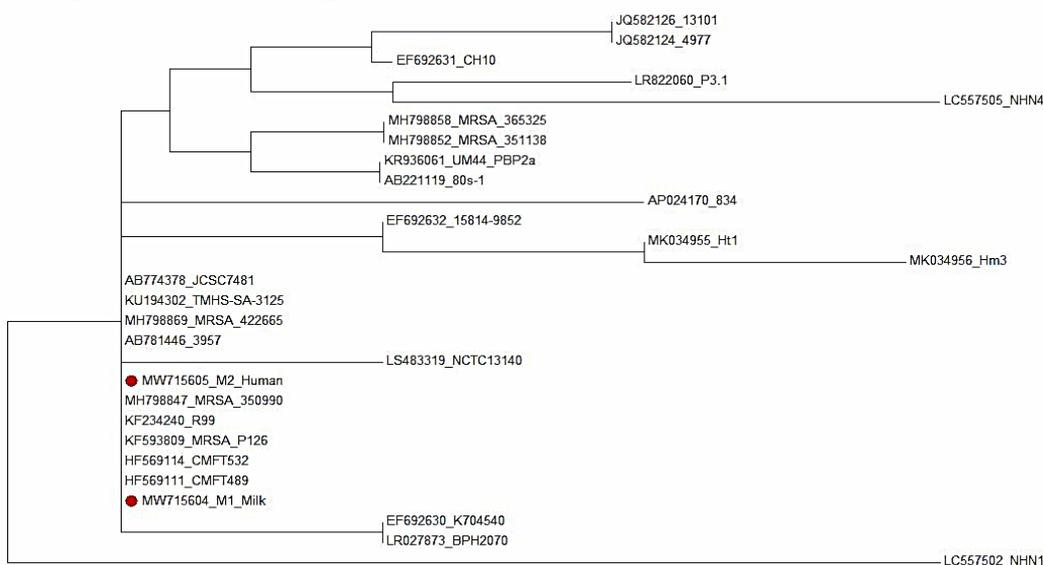
**Fig. 2:** The antibiotic susceptibility profile of MRSA isolates obtained from a) human, b) bovine milk. The isolates were tested against AMC, amoxicillin-clavulanic acid; SAM20, ampicillin/Sulbactam; C 30, chloramphenicol; NOR10, norfloxacin; NV30, novobiocin; CTX30, Cefotaxime; CLR15, Clarithromycin; S10, streptomycin; MEM10, Meropenem; DA, doxycycline; OT30, tetracycline; SXT25, Trimethoprim/Sulfamethoxazole; R, resistant; I, intermediate; S, susceptible.

Furthermore, high similarities in resistance of human and bovine isolates were also found with streptomycin (100%), clarithromycin (88.8%) and meropenem (44.4%). No resistance was observed against doxycycline and cefotaxime among bovine and human isolates. A similar high resistance rate was reported with novobiocin among human isolates (88.8%) and bovine (90%). The variable resistance of human isolates (66.6%) and bovine isolates (100%) against oxytetracycline was also detected, in addition to, low resistance ranged from 11.1% in human isolates to 33.3 in bovine isolates against chloramphenicol. However, bovine isolates showed 77% and 88.8% resistance against trimethoprim + sulfamethoxazole and piperacillin/tazobactam respectively, on the contrary, human isolates showed 100% sensitivity to the same antibiotic.

### Nucleotide sequencing:

The *mecA* gene amplicons (310 bp) of human and bovine MRSA isolates were subjected to partial sequence analysis. The partial *mecA* gene sequences of both human and bovine isolates were submitted to the Genbank database with accession numbers MW715605\_M2\_Human and W715604\_Milk respectively. Partial nucleotide sequences of MRSA *mecA* genes that generated from the study isolates were firstly compared with published MRSA sequences using BLAST search within the EMBL/GenBank database. The analysis revealed that *mecA* gene of both human and bovine MRSA isolates had 100% nucleotide identity with each other and shared 99-100 % nucleotide identity with multiple references MRSA strains of human and animal origin in Genbank database. Nucleotide sequence alignments also revealed that the arrangement patterns of study isolates were identical to various reference MRSA strains. Reference strains used for comparisons comprised of isolates identified previously in Italy (MRSA 350990, 624/I, QXIBV and B1648) as well as MRSA from other countries (e.g. China, USA and Australia).

The phylogenetic analysis of *mecA* gene sequences generated from both human and bovine milk isolates are typically clustered with MRSA strains in Genbank database including MH798847\_350990, KF593809\_MRSA\_P126, KF234240\_R99, HF569114\_CMFT532 and HF569111\_CMFT489 (Figure 3).



**Fig. 3.** Phylogenetic tree of study isolates including human MRSA isolate (MW715605\_M2\_Human) and bovine isolate (MW715604\_Milk) based on a 310bp fragment of *mecA* gene sequence.

## DISCUSSION

The growing importance of MRSA is attracting significant epidemiological interest in tracing such strains to picture the distribution of infections in humans and animals and to understand the dynamics of their spread. Here, to describe the prevalence of MRSA and epidemiological relation between isolates from humans and animals in Fayoum city. 50 samples were collected from bulk milk tanks from some villages of Fayoum city and 100 throat swabs from human infected with septic sore throat who live in the same villages. bovine milk and throat swabs were subjected to bacteriological and biochemical analysis. The result detected *S. aureus* in 74 % (37 isolates) of analyzed bovine milk and 40% (40 isolates) of human samples respectively. The high prevalence of *S. aureus* in bulk milk tanks, provides evidence of the milked cows were in a phase of subclinical mastitis (da Costa et al., 2016).

Further, the *S. aureus* isolates were subjected to phenotypic and genotypic examination to detect MRSA isolates. Oxacillin disk diffusion method is the major phenotypic method used for MRSA identification and detection of *mecA* gene or its product (penicillin-binding protein 2a) by molecular methods such as PCR is a golden standard method for confirmation of MRSA isolates (Skov et al., 2006). The result indicated that detection of MRSA using oxacillin disk diffusion method had low sensitivity and is not correlate with the molecular method, which under former studies (Anand et al., 2009; Koupahi et al., 2016). Our study obtained high incidence of MRSA in bovine milk, which is in agreement with various Egyptian studies that documented the MRSA incidence in cattle. For example, El-Ashker et al. (2015) recorded prevalence rate of MRSA (26.5 %) in clinical and subclinical bovine mastitis in Dakahlia city. With regard to incidence of MRSA in human, our data also recorded a high incidence rate in communities other than healthcare communities. Although, MRSA infection has been reported

previously in Egypt with higher rate in healthcare community than in other communities (Ahmed *et al.*, 2014). Colonization of human with *S. aureus* and MRSA has been demonstrated worldwide. In United States, a national survey study during 2003–2004 detected high nasal colonization in human with (29%) *S. aureus* and (1.5%) MRSA (Rachel *et al.*, 2008).

The antibiotics profile of both human and bovine MRSA isolates has shown high degree of similarity, especially in developing resistance to beta-lactams. The beta-lactam antibiotics kill bacteria by disrupting penicillin-binding proteins (PBPs), which are important for synthesis of the bacterial cell wall. PBPs help generate the bacterial cell wall polymer peptidoglycan (PGN), and bind to the glycan filaments in a process called transpeptidation. However, the MRSA *mecA* gene conferred resistance by encoding peptidoglycan transpeptidase and the penicillin-binding protein 2a, which has a poor affinity for the beta-lactam antibiotic than the original PBPs targeted by these antibiotics (Baek *et al.*, 2014). The observed resistance profile of MRSA strains including resistance to beta-lactams and other antibiotics classes is agreed with Gajdác who reported inactivity of MRSA strains to tetracyclines, fluoroquinolones, aminoglycosides and macrolides besides resistance to beta-lactam antibiotics (Gajdác, 2019).

In addition to significant similarity in antibiotic profile, the sequence and phylogenetic analysis of *mecA* gene produced from human and bovine MRSA isolates revealed high identity. Suggesting that unidirectional transmission of MRSA from human to animal or animal to human. Since the tested humans declared that they had no livestock direct contact, we hypothesized that indirect contact might have happened as they live in villages with high densities of livestock and they may interact indirectly with animals. Our alternative hypothesis suggests that human might be colonized with MRSA due to consumption of raw milk harboring MRSA bacteria. The first hypothesis is in agreement with what already reported in a recent study, demonstrating that adults without professional livestock contacts were found colonized with livestock-associated MRSA strains (Anouk *et al.*, 2020). Another recent study also highlighted the high incidence of MRSA in Shrines environment, where human and primates frequently interact and share antibiotic-resistant bacteria, antibiotic-resistant genes, and diseases (Charu Arjyal *et al.*, 2020).

The power of MRSA strains to adapt to environment and create reservoirs from any host, facilitate their dissemination in a wide scale to include people with no risk for developing infections. Multiple studies isolate MRSA strains from insect and wild animals which have no reasons for antibiotic exposure (Silva *et al.*, 2020). A survey study analyzed stool samples of 2855 wild animals from Germany, Sweden and Austria. The study identified a great diversity of methicillin susceptible and resistant strains from wildlife, with clonal lineages related to humans and animals. Wild birds have been found to carry MRSA strains in Austria (Monecke *et al.*, 2013). Loncaric *et al.* examined 54 samples from a population of a migratory rook and 102 samples collected from a non-migratory population. The authors reported MRSA strain with *mecA* gene positivity in five samples of migratory birds, also carrying resistance to different antibiotics classes including ciprofloxacin, tetracycline, aminoglycosides and macrolides (Loncaric *et al.*, 2014). Another study conducted in Austria examined samples from 66 *Rattus norvegicus* and isolated only one strain harboring *mecA* gene (Desvars *et al.*, 2016). Insects were also found colonized with MRSA as reported by (Schaumburg *et al.*, 2016). Therefore, the most possible hypothesis is both human and animals acquire infection from different hosts in the environment such as insects or rats. This hypothesis might explain the existence of similar MRSA strains in bovine milk and human with no direct contact with animals.

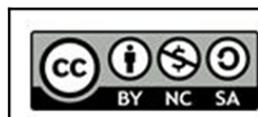
## CONCLUSION

The present work revealed a significant similarity between *mecA* gene encoding methicillin resistance in *S. aureus* of human origin and *mecA* gene responsible for methicillin resistance in *S. aureus* isolated from bovine milk. In addition, there is a high degree of similarity in antibiotic profiles among isolates from both origins. The data suggesting unidirectional transmission of MRSA between humans and cattle in village of Fayoum city which is an area with dense livestock, where both human and livestock are frequently interacted and may share the antibiotic-resistant bacteria and diseases. Further study is required to expand the investigation to include more villages and to spotlight on the mode of MRSA transmission in the environment, companion animals, livestock and wildlife.

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## النمط الظاهرية والجينية بين معزولات المكورات العنقودية الذهبية المقاومة للميثيسيلين من الإنسان و حليب الأبقار

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يهدف العمل الحالي إلى تتبع انتشار المكورات العنقودية المقاومة للميثيسيلين في الإنسان و حليب الأبقار في مدينة الفيوم ، مصر. تم العثور على 74% من العزلات المأخوذة من إنسان مصاب بالتهاب الحلق و 40% من عزلات الأبقار من خزان الحليب السائب إيجابية لبكتريا الاستافيلو كوكاس اورييس. تم الكشف عن الخصائص النموذجية لجرثومة المرسا بما في ذلك المقاومة للأوكساسيلين وإيجابية الميكا جين في 8% و 7% من عزلات الإنسان والأبقار على التوالي.وقد أظهرت مقاومة المضادات الحيوية لعزلات المرسا البشرية والأبقار درجة عالية من التشابه بما في ذلك المقاومة الكاملة للمضادات الحيوية بيتا لاكتام مع المقاومة لفئات مختلفة من المضادات الحيوية الأخرى. ومع ذلك ، تم الكشف عن تشابه بنسبة 99-100% بين جين الميكا البشرية والأبقار وسلالات متعددة في بنك الجينات.ولذلك يعتبر التشابه الظاهري والجيني المحدد دليلاً واضحاً على العلاقة الوبائية المحتملة بين عزلات المرسا البشرية والأبقار. ومع ذلك ، هناك حاجة إلى مزيد من الدراسة لتحديد طريقة انتقال المرسا بين الإنسان وحيوانات الماشية المختلفة على نطاق واسع وتوسيع الدراسة لتشمل الحيوانات البرية.

**الكلمات المفتاحية:** المكورات العنقودية المقاومة للميثيسيلين ، التهاب الحلق ، جين الميكا ، خزان الحليب السائب.