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Detection of Methicillin-Resistant *Staphylococcus aureus* (MRSA) from bovine subclinical mastitis in Egypt using Real-Time PCR

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ABSTRACT

Mastitis caused by methicillin resistance *Staphylococcus aureus* (MRSA) is a severe infection of milk-producing animals. Consequently, it is critical to recognize the genetic elements that contribute to methicillin resistance in *S. aureus*. This research was designed to evaluate the incidence of MRSA in bovine with subclinical mastitis, histopathological examination of udder tissues exhibiting mastitis, and detection of MRSA strains by Taqman probe-based real-time PCR. Additionally, our study assessed the phylogenetic relationship of MRSA strains recovered from Egypt to illuminate the potential genetic connection between isolates from other sources and locations. Based on our research, out of 62 *S. aureus* isolates recovered from subclinical mastitis, 39 isolates were resistant to methicillin (62.9%) phenotypically by antimicrobial susceptibility test. All 39 *S. aureus* isolates were confirmed genotypically by Probe-based real-time PCR. Ten positive probe-based RT-PCR samples (MRSA) were subjected to conventional PCR before being submitted to DNA sequencing. Ten PCR products were grouped into two groups (EGY-*mecA* 1-5 and EGY-*mecA* 6-10) according to the nucleotide sequence. The alignment of multiple sequences and construction of a phylogenetic tree for the mecA gene revealed a high similarity (100%) with MRSA isolates recovered from the milk of cattle and goats in Egypt. The histopathological examination of the cattle mammary glands tissue sample with mastitis shows severe destruction of secretory acini, with aggregation of mononuclear inflammatory cells infiltration among acini, mainly lymphocytes. Our investigation will help protect human and animal health by reducing instances of infection caused by *S. aureus*. Probe-based real-time PCR successfully identified *S. aureus* strains in subclinical mastitis within a few hours, demonstrating high sensitivity and specificity. This technique is helpful for effectively treating and controlling *S. aureus* mastitis and quickly screening MRSA isolates.

Key words: MRSA; Mastitis; *mec*A; *S. aureus;* Subclinical mastitis; Real-Time PCR and bovine.

INTRODUCTION

Mastitis, defined as the inflammation of the mammary gland, is one of dairy animals' most common and economically significant diseases (El-Demerdash et al., 2023; Rifatbegovi'c et al. 2024). Mastitis-related economic losses can be characterized as decreased output caused by the disease and the advantages that would otherwise accrue without mastitis. Costs are divided into two categories: direct costs (extra labor requirements, wasted milk while giving medication, and veterinary services) and indirect costs (premature culling, milk yield reduction, and decrease of milk quality premiums) (Azooz et al. 2020; Kovaˇcevi'c et al. 2022; Meçaj et al. 2023). In Egypt, Azooz et al. (2020) assessed the economic consequences of mastitis. This investigation included several factors. Lower expenses were computed once clinical mastitis instances existed ($6536); milk output decreased annually linked to subclinical mastitis was $411,274. The losses from the quality premium increased to $27,392. The two other significant expenses were milk thrown out ($2) and early culling ($14,720).

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Bovine mastitis is distinguished chiefly based on clinical (or subclinical) characteristics and etiology (noninfectious or infectious). Infectious reasons are the most widespread and in some situations, infections caused by bacteria constitute a widespread appearance among livestock. Additionally, bacterial pathogens are divided into three groups: opportunistic, environmental, and contagious bacteria (Ndahetuye et al. 2019). Many investigations have shown that mastitis is commonly caused by *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus spp.*, *Klebsiella pneumoniae*, and less commonly by *Mycobacterium spp*., *Mycoplasma spp*., and *Pseudomonas aeruginosa* (Ardicli et al. 2022; Shoaib et al. 2022; Abdalhamed et al. 2024).

Staphylococcus aureus is usually abundant in the teat microbiota of udder skin. It is the primary pathogen linked with intramammary infection (IMI). *S. aureus* can penetrate and settle inside the udder via the teat tips and duct (Moreira et al. 2019). Recently, Preziuso et al. (2024) investigated that *S. aureus* has been linked to other severe human health risks and is the cause of mastitis, especially in light of the emergence of methicillin-resistant *S. aureus* (MRSA).

Furthermore, bacterial resistance has emerged as a growing problem as resistance mechanisms propagate worldwide. To date, standard antibiotic treatments easily lead to the emergence of resistant bacteria (Peng et al. 2022). Many reports have been published about resistance to antimicrobials, including gentamicin, erythromycin, amoxicillin, penicillin, amikacin, and tetracycline. Recent investigation still indicates that resistance to newly developed antimicrobial drugs has grown; bacterial profiling revealed the resistance to cefquinome, colistin, piperacillin, tigecycline, ceftazidime, and vancomycin. Moreover, drug residue has become a more severe problem (Monistero et al. 2021; Campos et al. 2022; Vidal et al. 2022; Abd El-Razik et al. 2023a, b; Bonardi et al. 2023). Acquiring the *mec*A gene results in methicillin resistance. This gene produces PBP2A, an alternative penicillin-binding protein with a restricted affinity for beta-lactam antibiotics (Vanderhaeghen et al. 2010a). The *mec*A gene belongs to the Staphylococcal Cassette Chromosome mec (SCCmec), a critical mobile genetic component. Rapid diagnosis of MRSA is essential regarding therapy and infection prevention approaches.

Additionally, real-time PCR provides 100% specificity and sensitivity regarding these target genes compared to standard and traditional approaches (Galia et al. 2019). Despite the long-established substantial harm caused by MRSA in dairy cattle, no effective therapies or preventive strategies have been proposed. This can be attributed to both the fast-changing genetic variety of the pathogens and our ignorance of the connection between the bacteria and the host. This investigation aimed to determine the incidence of MRSA in bovines with subclinical mastitis, histopathological examination of udder tissues exhibiting mastitis, and detection of MRSA strains using Taqman probe-based real-time PCR. Furthermore, our investigation assessed the phylogenetic relationship of MRSA isolates recovered from Egypt to provide light on the potential genetic connection between isolates from other sources and locations.

MATERIALS AND METHODS

Ethical approval

The NRC's Medical Research Ethics Committee (permission no. 231712012023) approved this investigation.

Bacterial isolates

A total of 62 strains of *S. aureus* were recovered and identified phenotypically and genotypically in a previous study which differentiated as 21 isolates of *S. aureus* were obtained from 33 bulk tank milk of sub-clinically mastitic cattle and buffalo and 41 isolates of *S. aureus* recovered from cattle and buffalo with subclinical mastitis from privately owned smallholders in Kafr El-Sheikh, Fayoum, Kalyobia, Giza and Cairo governorates of Egypt (Abd El-Razik et al. 2023a).

Antimicrobial susceptibility test (phenotypic detection of MRSA)

All *S. aureus* isolates were examined for antibiotic susceptibility via disk diffusion test, as the Clinical Laboratory Standards Institute (CLSI) mentioned. Isolates were inoculated into Mueller–Hinton broth (Oxoid) and incubated overnight at 37°C. The suspension turbidity was adjusted to a 0.5 McFarland standard and then streaked onto Mueller–Hinton agar (Oxoid) plates. To detect MRSA, the antimicrobial disk (Oxoid) contained oxacillin (5µg) was added to the plates, and they were incubated aerobically for 16–18 h at 37°C. We measured the inhibitory zone diameter, and the results were classified as sensitive, moderate, or resistant. Using CLSI's zone diameter interpretation standards (CLSI 2017), resistance was measured by measuring growth suppression around the antimicrobial disk.

DNA extraction

The QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used to extract DNA from bacterial cultures following the manufacturer's instructions. In brief, 200µL of PBS was used for the re-suspension of the bacterial pellets, and they were then treated at 56°C for 10min in a mixture of 20µL proteinase K and 200µL lysis buffer. 200µL of 100% ethanol was added to the lysate following incubation. The sample was then centrifuged and cleaned. An elution buffer (50µL) was used to extract the nucleic acid.

Genotypic identification of MRSA using Real-time PCR

For thermocycling and fluorescence detection with the qTOWER 3G (AnalytikJena, Germany), a PCR TaqMan test was run with the MRSA target. Twenty µl was the total volume used for the real-time PCR amplification. This included 10µL of 2X Topreal Taqman Probe quantitative PCR mixture (Cat RT600, Enzynomics), prepared in compliance with the manufacturer's guidelines, 2µL of template DNA, 0.2µL of each primer(10µm), and 0.4µL of TaqMan probe mixture(10µm). Double Distilled water (DDW) was added to complete the 20µL. Table 1 lists the precise primers and probes used to identify the MRSA *mec*A gene, which is responsible for methicillin resistance. Table 2 contains a list of the cycling conditions.

Table 1: Sequence of *mecA* genes primers and probes

Gene	Sequence (5´-3´)	Amplicon size (bp)	Reference
MecA1	AACAGGTGAATTATTAGCACTTGTAAG ATTGCTGTTAATATTTTTTGAGTTGAA	174	Martineau et al. (2000)
MecA2	GTAGAAATGACTGAACGTCCGATAA CCAATTCCACATTGTTTCGGTCTAA	310	Spanu et al. (2004)
MecA3	GCTCAAATTTCAAACAAAAATTTAGATAATG TGAAAGGATCTGTACTGGGTTAATCAGT HEX-Probe AGCTGATTCAGGTTACGGACAAGGTGA	--------	Wang et al. (2014)

Table 2: Cycling conditions for *mecA* genes

Gene	Init. Denat.	Denat.	Anneal.	Extension	Final Extension	Cycles
mecA1	95°C/2min	95°C/20s	50°C/30s	72°C/45s	72°C/10min	35
MecA2	95°C/2min	95°C/20s	55°C/30s	72°C/45s	72°C/10min	35
MecA3 (Q-PCR)	95°C/10min	95°C/10s	52°C/20s	60°C/40s	----------	40

Genotypic identification using conventional Polymerase Chain Reaction (PCR)

For identification of MRSA (*mecA* gene), PCR reactions were carried out using GS-96 gradient thermocycler (Hercuvan, Malaysia) in a final volume of 25µL reaction that included 1µL of sample DNA, 0.5µL of each primer (10µM) (Vivantis, Malaysia), and 12.5µL of 2x COSMO PCR RED Master Mix (Cat. W1020300X, Willofort Co., UK). After electrophoresis on a 1.5% agarose gel to separate the examined PCR products, analysis, and photos were taken using the InGenius3 gel documentation system (Syngene, UK). Tables 1 and 2 listed the primers (*mec*A1 and *mec*A2) utilized and the cycling conditions.

DNA sequencing

The MACROGEN Company (Korea) sequenced the *mec*A gene using 3730XL sequencers (Applied Biosystems, USA) after ten positive PCR products were purified by the GeneJETTM Gel Extraction Kit (K0691, Thermo Fisher, USA).

Phylogenetic relationship and tree construction

Ten PCR products of MRSA isolates were purified using GeneJET™ Gel Extraction Kit (K0691, Thermo Fisher, USA), then subjected to sequencing by MACROGEN Company (Korea) on 3730XL sequencer (Applied Biosystems, USA). The precision of the data was confirmed by bidirectional sequencing using PCR's forward and reverse primers. The nucleotide sequences obtained during this research were analyzed with the BioEdit 7.0.4.1 and Muscle programs (EMBL's European Bioinformatics Institute, 2020). The following sequences were matched with reference sequences in GenBank using a neighbor-joining analysis of the aligned sequences via CLC Genomics Workbench 3.

Histopathological examination

According to Abd El-Razik et al. 2023a, 12 udder tissue samples collected randomly from slaughterhouses were preserved in a 10% formalin solution from the same governments at which milk samples had been collected; the preserved tissue was subsequently embedded in paraffin wax after being dehydrated using some alcohol solutions. Once the tissue had been thinly sectioned, it was arranged on glass slides using a microtome. After deparaffinization and rehydration, the Hematoxylin and eosin (H and E) staining was applied to the slides. The slides underwent staining, dehydration, clearing, and coverslip mounting before being examined under a microscope (Bancroft and Stevens 1996).

RESULTS

Incidence of Methicillin-Resistant *S. aureus* (MRSA)

Methicillin susceptibility testing showed that 47.6% (10/21) of the isolated *S. aureus* isolates were MRSA, with 50% (6/12) from cattle and 44.4% (4/9) from buffaloes, as shown in Table 3.

Table 3: Recovery rates of MRSA among the recovered *S. aureus* isolates from bulk tank milk samples

	Governorate	Cattle	Buffalo	Total
1	Kafr El-Sheikh	1/4 (25)	1/6 (16.7)	2/10 (20)
2	Fayouma	3/4 (75)	2/2 (100)	5/6 (83.3)
3	Kalyobia	0/1 (0)	0	0/1 (0)
4	Giza	1/2 (50)	1/1 (100)	2/3 (66.7)
5	Cairo	1/ 1 (100)	-	1/1 (100)
	Total	6/12 (50)	4/9 (44.4)	10/21 (47.6)

Values in parenthesis are %.

Forty-one isolates of *S. aureus* were isolated, where 20 and 21 isolates were from cattle and buffalo, respectively, as shown in Table 4. Twenty-nine MRSA isolates were identified from the 41 *S. aureus* isolates (70.7%), whereas 15 and 14 MRSA isolates were from cattle and buffalo, respectively.

Table 4: Recovery rates of MRSA among the recovered *S. aureus* isolates (Small holders)

	Governorate	Cattle	Buffalo	Total
1	Cairo	1/1 (100)	2/4 (50)	3/5 (60)
2	Giza	2/3 (66.7)	2/6 (33.3)	4/9 (44.4)
3	Kalyobia	3/5 (60)	3/4 (75)	6/9 (66.7)
4	Fayoum	3/4 (75)	4/4 (100)	7/8 (87.5)
5	Kafr El-Sheikh	6/7 (85.7)	3/3 (100)	9/10 (90)
	Total	15/20 (75)	14/ 21 (66.7)	29/41 (70.7)

Values in parenthesis are %.

Genotypic identification using Real-time PCR

A total of 39 MRSA isolates from the milk of cattle and buffaloes with subclinical mastitis were isolated and identified bacteriologically. Using Taqman Probe-based Real-time PCR, all isolates were molecularly verified with an incidence of 100%.

Genotypic identification using conventional Polymerase Chain Reaction (PCR)

Ten positive samples of probe-based RT-PCR (MRSA) were subjected to standard PCR reactions for DNA sequencing. Conventional PCR was used on all samples; the results are presented in Fig. 1.

Phylogenetic Analysis of *mecA* Gene (penicillin-binding protein PBP2 encoding)

Ten PCR products were grouped into two groups (EGY-*mecA* 1-5 and EGY-*mecA* 6-10) according to the nucleotide sequence. The alignment of multiple sequences and construction of a phylogenetic tree for the *mecA* gene revealed a high similarity (100%) with other MRSA isolates recovered from the milk of cattle and goats in Egypt (KX668407, KY467024, MW256759), as shown in Fig. 2 and 3.

Histopathological examination

The histopathological examination of cattle udder tissue samples with mastitis shows severe destruction of secretory acini, with aggregation of mononuclear inflammatory cells infiltration among acini, mainly lymphocytes (Fig. 4). Furthermore, congestion was observed in the affected tissue. Some other tissue samples show mild fibrous connective tissue proliferation between some acini.

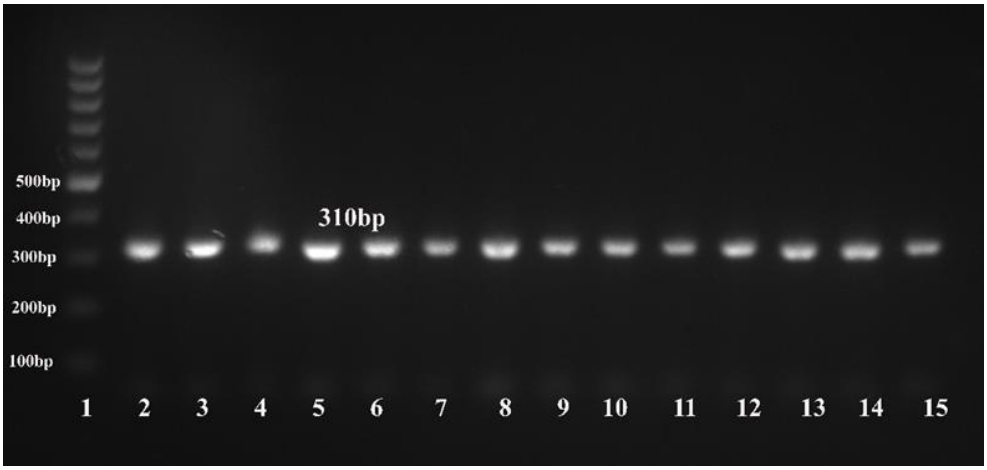

Fig. 1: Agarose gel electrophoresis of *mec*A gene (310 bp). Lane 1: 100bp DNA ladder; Lanes 2-11: positive samples of MRSA.

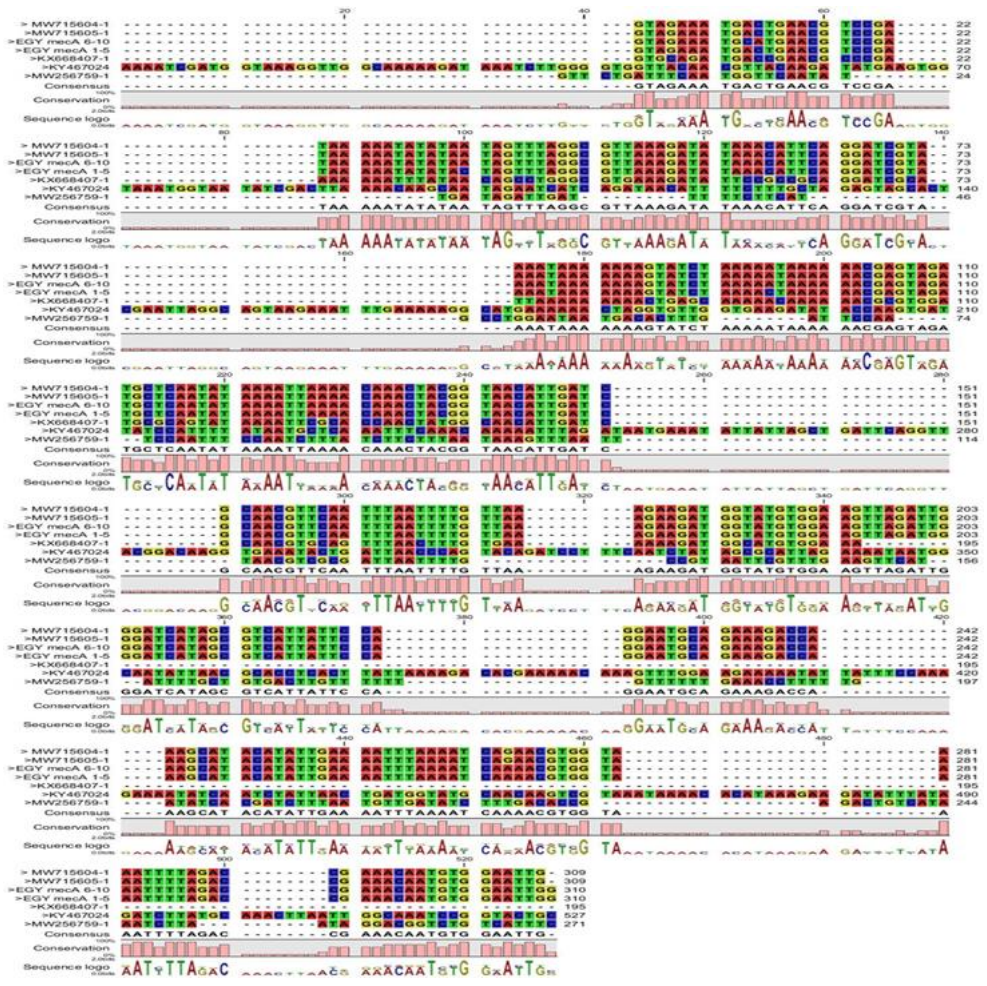

Fig. 2: Multiple sequence alignment of *mecA* gene detected in bovine milk (EGY-*mecA* 1-5 and EGY-*mecA* 6-10) and their corresponding reference sequences. Only variable sites are shown with different color. Dashes in the middle indicate gaps.

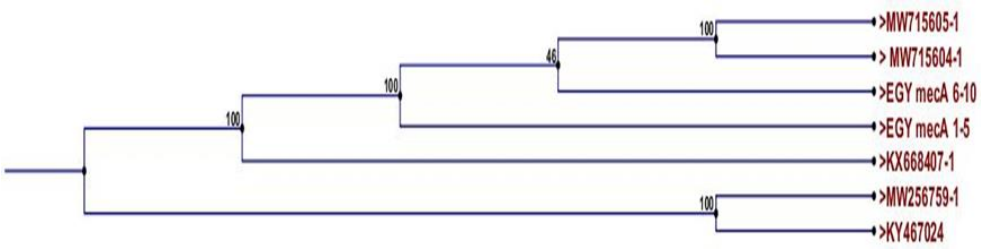

Fig. 3: Phylogenetic tree of selected isolates of *MRSA*, based on the *mecA* gene.

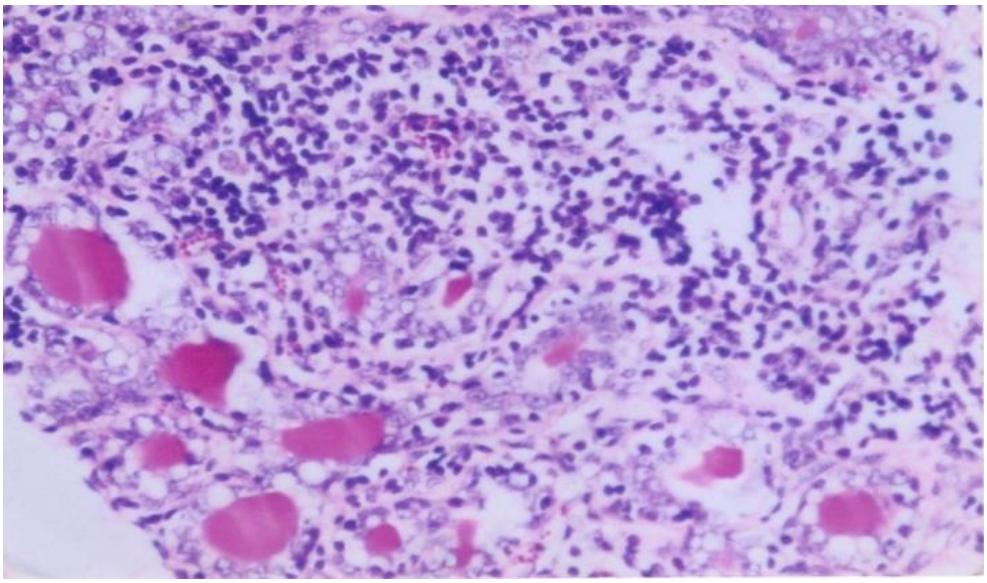

Fig. 4: Udder tissue of cattle shows lymphocytic mastitis characterized by complete destruction of acini and severe considerable accumulation of mononuclear inflammatory cell infiltrations, mainly lymphocytes (H&E stain; X100).

DISCUSSION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major hazard to public health. MRSA strains are *S. aureus* isolates that are resistant to β-lactam drugs. As a community pathogen, MRSA has drawn more attention. Many reports have identified MRSA as an emerging concern in animal healthcare (Tesfaye et al. 2021).

Our study investigated that 47.6% (10/21) of *S. aureus* isolates were MRSA, with 50% (6/12) from cattle and 44.4% (4/9) from buffaloes by antimicrobial sensitivity testing. Other investigations on mastitis in Egypt varied incidences of methicillin-resistant *S. aureus* strains to be 32.4% (Tesfaye et al. (2021), 42.1% (Abdalhamed et al. 2022), and 60% (Sayed 2014) which are similar to the incidence we found, these findings further suggested a correlation between mastitis and MRSA. Another study conducted in Egypt found that 57.14% of the Staphylococcal strains resistant to antibiotics were found in food products such as pasteurized milk, raw milk, pasteurized yogurt, minced meat, and beef burgers (Arafa et al. 2016). Additionally, according to Khazaie and Ahmadi (2021), 11 (11.57%) of the 95 *S. aureus* isolates from Iran were identified as MRSA strains. According to a recent Brazilian investigation, 177 out of 191 *S. aureus* isolates (92.7%) had beta-lactam resistance (Silva et al. 2023).

Furthermore, Japan (39.74%) (Hata 2016), Bangladesh (29%) (Hoque et al. 2018), Isfahan, Iran (20%) (Havaei et al. 2015), the Northwest of Iran (15.51%) (Ahmadi et al. 2020), India (13.1%) (Kumar et al. 2011), Thailand (8.33%) (Pumipuntu et al. 2019), and Belgium (4.4% and 10%) (Vanderhaeghen et al. 2010b; Bardiau et al. 2013), have reported higher frequencies of mastitis-associated MRSA than Finland (1.6%) (Gindonis et al. 2013) and Ahvaz, Iran (1.3%) (Ahangari et al. 2017). It gives evidence of the spread of MRSA and sheds light on the study of its epidemiology.

mecA gene, a modified penicillin-binding protein that provides resistance to methicillin and other penicillin derivatives, is the gene responsible for beta-lactam antibiotic resistance. This mobile gene element is known as the "staphylococcal cassette chromosome-mec" (SCC-mec) (Khoramrooz et al. 2017; Mišić et al. 2017; Parthasarathy and Chougale 2021; Sasaki et al. 2021). From 16 MRSA isolates identified phenotypically from ruminants with mastitis, Abdalhamed et al. (2022) discovered that 14 bacteria (36.8%) possessed *mec*A gene. According to Arafa et al. (2016), 7 of *S. aureus* isolates had a resistance rate of 57.14% (4/7) to penicillin and methicillin. They also carried the *bla*Z gene at 100% (7/7) and the *mec*A gene at 85.7% (6/7). They also discovered that coagulase-negative staphylococci had a 33.3% resistance rate to penicillin and methicillin. Also, they detected *mec*A gene in *S. sciuri* isolates. Following the development of beta-lactam antibiotics, the incidence of MRSA infections rose gradually (Rothenburger et al. 2018). The pervasive usage of antibiotics in the past few years has contributed to developing the present problem (Pyatov et al. 2017).

Rapid MRSA diagnosis is critical for implementing infection control programs and treatment to avoid disease transmission and recurrence (Calfee et al. 2014). Rapid MRSA identification has been achieved using molecular approaches such as real-time PCR and polymerase chain reaction (Al-Talib et al. 2014). Fast and precise diagnosis of methicillin resistance and *S. aureus* are essential for initiating effective antibiotic treatment immediately

(Llarrull et al. 2009), lowering morbidity and mortality rates (Teng et al. 2009). Using Taqman Probe-Based Real-time PCR, we ensured that all 39 (100%) traditionally identified strains showed amplification plots and threshold cycles. Triplex Real-time PCR assay was developed by Galia et al. (2019) in 2019 and is suitable for use in regular microbiology laboratories. Compared to traditional methods and culture, the Triplex Real-Time PCR showed 100% specificity and sensitivity for these target genes. The study conducted by Kim et al. (2013) reported introducing a multiplex real-time PCR test in a region with significant MRSA infection endemicity, which allows for the simultaneous identification of *mec*A. The assay was evaluated utilizing 444 staphylococcal strains as a direct MRSA molecular detection system.

Juhász-Kaszanyitzky et al. (2007) concluded that MRSA was the source of multiple instances of bovine's subclinical mastitis in Hungary farms, and these strains were identical to MRSA recovered from a carrier who was in close contact with the cows. This implies that these isolates are transferred from humans to cows, although whether the transfer occurs from person to person or from cow to human is unclear. In our study, the phylogenetic relationship of MRSA strains recovered from Egypt was investigated, 10 positive PCR products of MRSA were sequenced, and the alignment of multiple sequences and construction of a phylogenetic tree for *mec*A gene exhibited a high similarity (100%) with that of MRSA isolates recovered from the milk of cattle and goats in Egypt.

Histopathological investigation is critical for identifying mastitis and assessing the severity of the disease. One of the most noticeable histological findings is extensive damage of secretory acini, which can significantly impact milk output in affected cattle. This damage is frequently followed by interstitial fibrosis, which might worsen mammary gland function. These findings are in agreement with Ebtsam et al. (2020). Another common histological feature seen is the aggregation of mononuclear inflammatory cells within acini. These cells indicate an active immunological response in the affected glands. These pathological alterations are reliable, as shown by the findings of Ibrahim et al. (2016) and Abba et al. (2014) on similar histological findings in bovine mammary gland infections. Overall, histological analysis revealed various alterations caused by bacterial infection, including secretory acini destruction and inflammatory cell infiltration. These alterations indicate the bacterial infections inflammatory and immunological responses and tissue damage.

Conclusion

Based on the potential public health risk posed by MRSA isolates and the relevance of *S. aureus*-caused mastitis in the dairy industry, our findings may aid in developing effective control approaches. Our data revealed a high frequency of MRSA in raw milk. Thus, designing treatment approaches depending on the diverse seasonal and regional factors associated with the incidence of resistance in *S. aureus* isolates is critical. This investigation will help health protection of human and animal by reducing infections in lactating animals which caused by *S. aureus*. Probe-based real-time PCR successfully identified *S. aureus* strains in subclinical mastitic milk samples within 3 hours, demonstrating high sensitivity and specificity. This technique is definitely helpful for effectively treating and controlling *S. aureus* mastitis as well as for quickly screening MRSA isolates.

Author's contribution

K.A.A., A.A.A. and E.A.F. participated in the study design, carried out the PCR, genetic markers of antibiotic resistance. A.H.S. participated in the pathological section of the study. D.S. was responsible for antimicrobial resistance phenotypically and DNA extraction. The final manuscript was reviewed by all authors and approved.

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