Research Article

Molecular Detection of *mec*A Gene in *Staphylococcus* sp. Associated with Ruminant Mastitis

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Abstract | This study was aimed to detection the distribution of *mec*A gene among *Staphylococcus sp.* Isolated from mastitis cows, sheep and goats in Basrah/Iraq. A total of 26/70 (37.14%) was primarily isolated on fermented Mannitol salt agar (MSA) and examined by Gram stain. All suspected *Staphylococcus sp.* isolates were further detection by PCR and analysis the partial sequencing of the 16s rDNA gene and compared with those in the GenBank to find variances in the sequence using the BLAST tool (http://www.ncbi.nlm.nih.gov). The sequencing results of the 16s rDNA gene was shown that 9 (69.24%) bacterial isolates were diagnosed as *Staphylococcus aureus* while the 4 (30.76%) other samples were diagnosed as *Staphylococcus sciuri*, *Staphylococcus succinus, Staphylococcus equorum* and *Staphylococcus epidermidis.* On the other hand, the *mec*A gene was examined and detected in all isolates of *Staphylococcus sp.* using specific primer, and the sequence analysis results of this gene revealed that 11 isolates shown 100% homology to the *Staphylococcus aureus* cassette chromosome mec with ID number LC727174.1 and *Staphylococcus aureus* strain C249 chromosome with ID number CP127807.1. While one isolate was displayed 100% homology to the *mec*A gene that previously detected in *Staphylococcus epidermidis* strain 1FSE05 plasmid with ID: CP121525.1. We concluded that all this information may offer crucial understanding of the origin and persistence of current and possibly even future of *mec* A gene in *Staphylococcus* strains.

Keywords | *Staphylococcus* sp., *mec*A gene, *Staphylococcus aureus*, MRSA

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INTRODUCTION

Mastitis is an important global disease for the dairy industry, it is related with economic losses and decreasing milk production and quality, since it affects animal wellbeing (Sharun *et al.*, 2021; Yang *et al.*, 2020). Mastitis is an inflammation of the mammary gland caused mainly by a bacterial infection. It is characterized by a variety of local and systemic symptoms. It is a significant concern for both meat and milk producers as the infection can lead to considerable economic losses. This is due to the reduction in milk yield, decreased quality of milk, and treatment costs Sawant *et al.* (2009).

Many bacterial agents are causing animals mastitis. Among these bacteria are *Staphylococcus* sp. The genus *Staphylococcus* is a group of Gram-positive bacteria, and S. aureus in particular is one of the most harmful species of *Staphylococcus*. According to a report by Miles *et al.* (1992), *Staphylococcus aureus* is likely the most dangerous agent,

as it causes persistent and severe infections in mammary glands which are challenging to treat. *Staphylococcus* cause damage to the tissues lining the teat and gland cisterns then, move to the duct system and form deep– seated pockets of infection which are difficult to treat and may result in abscess formation. The body's defense mechanism walls-off the bacteria with scar tissue, which helps to isolate the infection but also lowers the effectiveness of antibiotics. As a result, the cure rates for this type of infection are generally poor (Philpot and Nickerson, 2000).

The virulence of *S. aureus* is believed to be multifactorial as it is caused by the combined effect of various virulence factors (Bien *et al*., 2011). It is a worldwide bacterium that causes subclinical and clinical mastitis infection in mammary glands of the domestic animals, that usually spreading through milking processing and leading to chronic illness, especially with antibiotics bacterial strain like Methicillin Resistant *Staphylococcus aureus* (MRSA) (Kumar *et al*., 2010; Zigo *et al*., 2021).

Additionally, methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogenic bacterium in veterinary medicine and public health, in addition to resistance to methicillin, most strains are also resistant to other β-lactam antibiotics (Nandhini *et al*., 2022). The methicillin resistance in *Staphylococcus* sp. is associated by *mec*A gene, that encodes the protein PBP 2A which is a low-affinity penicillin-binding (Stapleton and Taylor, 2002). The *mec*A gene is a 21-60 kb structural component that integrates into the staphylococcal large chromosome cassette (SCCmec). It is a mobile genetic element acquired by horizontal transfer from coagulase-negative staphylococcal species and may contain genetic constructs (Tn554, pUB110, pT181) that also encode resistance to non-lactam antibiotics (Becker *et al*., 2014; Ito and Hiramatsu, 1998).

At least five different types of SCCmec differing in gene sequence and size, have been reported. The types I to III of MRSA strains found in healthcare are usually large and multi-drug resistant. Types IV and V associated with community-acquired MRSA strains tend to be smaller and more sensitive to antibiotics other than β-lactams (Ahmad *et al*., 2009). However, the identification of the mecA gene as the primary mechanism behind MRSA isolates cause bovine mastitis led to the creation of quick molecular assays, first using DNA probes and later PCR, to differentiate between MRSA and methicillin-susceptible S. aureus (MSSA) colonies (Tokue *et al*., 1992). However, it proved more challenging to directly identify MRSA in clinical cases, since mecA can also be exist in methicillin-resistant strains of coagulase-negative staphylococci, which are also prevalent in a variety of clinical cases (Wang *et al*., 2014).

Nevertheless, the interest on methicillin-susceptible *S. aureus* (MSSA) has increased in recent years, given that they can also be implicated in important in human or animal infections This helps explain the onset and progression of infections and the different and successful MRSA lineages. Few data exist in the circulating genetic lineages of MSSA in food producing animals or in derived food products, as in the case of milk (Silva *et al*., 2013). Coagulase-positive and coagulase-negative staphylococci were recovered in a previous study from milk of local dairy farms with mastitis in Basrah government (de Freitas Guimarães *et al*., 2013; Hashosh *et al*., 2022a, b). Therefore, the recent study was aimed to the determine the genetic diversity of *mec*A gene among *Staphylococcus* sp. isolated from mastitis cows in Basrah/ Iraq.

MATERIALS AND METHODS

Samples collection and isolation of *Staphylococcus* sp.

A total of 70 milk samples were taken randomly from the following animals with clear signs of mastitis: 34 from cows, 19 from sheep, and 17 from goats in Basrah governorate. The examined animals were shown moderate to severe clinical signs that can included: Abnormalities and swelling the udder also heat, hardness, redness, or pain; and milk have a watery appearance, clots, or pus.

Before collecting the milk samples, the udder of the affected animals was cleaned from the dust, mud and dirt with water, then wipe with a cotton pad soaked in 70% alcohol and dried with a clean cloth. 10ml of milk were collected in sterile tubes and preservative in an icebox and transported as fast as possible to the microbiology laboratory. All milk samples were immediately plated on mannitol salt agar (MSA) and incubated at 37 °C for 24 h to identify *Staphylococcus* sp. Mannitol-fermenting colonies were subcultured on MSA medium and incubation at 37°C for 24-48h as performed previously by (Talan *et al*., 1989). Subsequently gram stain and the coagulase tube were further used according to (Cowan, 1993; Macfaddin, 2000).

Positive isolates were identified presumptively as *Staphylococcus* spp. DNA extraction and PCR analysis for confirmation.

DNA extraction and PCR analysis

Total genomic DNA was extracted using the Genomic DNA Purification Wizard® Kit according to the manufacturer's instructions (Promega, USA). Two sets of primer were used: The 16Sup primers used for amplification and detection of 16S rDNA gene of *Staphylococcus* sp., and mecup primer for detection of mecA gene in the *Staphylococcus* sp. isolates

(Poulsen *et al*., 2003). The oligonucleotides primers used for the 16S rDNA and mecA genes were (forward primer 5-GTG CCA GCA GCC GCG GTA A-3), (reverse primer 5-AGA CCC GGG AAC GTA TTC AC-3) and (forward primer 5-GGG ATC ATA GCG TCA TTA TTC-3), (reverse primer 5- AAC GAT TGT GAC ACG ATA GCC-3).

The PCR reaction was performed in 25 µl reaction mixtures with 1 µl (10 pmol/µl) for forward primer and reverse primer, 12.5 µl ready to use GoTaq® Hot Start Master Mixes (Promega, USA) and 5.5 µl nuclease-free water. Finally, the 5 µl DNA template was added to each reaction tube. For genes amplification, the PCR cycling and conditions was started with initial denaturation step for 5 min at 94°C, following by 30 cycles of 30 s at 94C, 30 s at 55C for annealing step and 30 s at 72C for elongation. The final elongation for 2 min at 72°C. The PCR products were then running on 1% agarose gel staining using ethidium bromide in order to assess the successful of amplification. Agarose gel was visualized using a gel documentation system. The DNA ladder from (SolGent™ 1 Kb Plus DNA Ladder) was used as a molecular mass marker and the fragment size of approximately 886 bp and 527bp was verified as positive for 16SrDNA gene of *Staphylococcus* sp., and mecup gene, respectively (Sambrook *et al*., 1989).

Genes sequencing and phylogenetic analysis

The PCR products of 16S rDNA and mecA genes were sequenced in (Macrogen Inc./ Seoul, South Korea Korea) by using the 16Sup and mecup primers for partial nucleotide sequence of the 16S rDNA and mecA genes respectively. The results of sequencing were analysis useing BLAST (Basic Local Alignment Search Tool) to compare and search for similarities in the National Center for Biological Information (NCBI) sequence database. The phylogenetic tree was constructed using the Neighbor Join method using the software MEGA-X (Kumar *et al*., 2018).

RESULTS AND DISCUSSION

Incidence of isolation and identification of *Staphylococcus* sp.

The emerging of *mec*A gene in *Staphylococcus* sp. bacteria pose a serious threat to human and animal health also stimulating the interest of researchers, as it could help bacteria to be resistant to antibiotics such as methicillin, penicillin and penicillin-like antibiotics. Based on previous studies, the *mec*A gene has been found to be present in all strains of MRSA (Berger-Bächi, 1999; Al-Deewan and Adlan, 2017). Methicillin resistance in *Staphylococcus aureus* occurs primarily due to the production of a unique penicillin-binding protein (PBP)-2a. This protein has a lower binding affinity for β-lactam antibiotics than other PBPs.

Figure 1: Cow (A) and goat (B) with clear signs of mastitis (swelling and redness of udder).

Table 1: A prevalence of mastitis milk samples taken from dairy animals with clear signs of mastitis.

Type of animals	Positive No. and (%) of isolates based on			
	Clinical signs	MSA and Gram stain	PCR and sequence of 16S rDNA	PCR and sequence of mecA
Cows	34	13	7	7
Sheep	19	8	4	4
Goats	17	5	2	2
Total	70	26 (37.14%)	13(50%)	13(50%)

PBP-2a requires a higher concentration of penicillin (2-10 times more than PBP-2 and 20 times more than PBP-1) to be neutralized (Kaszanyitzky *et al*., 2003). The aim of this study was to examine the distribution of *mec*A in *Staphylococcus aureus* and other CON populations of dairy animals. Two molecular methods were used to detect the *mec*A gene in bacterial isolates, PCR and sequences analysis. Therefore, the initial target step in this study was the detection and isolation of *Staphylococcus* sp. Seventy milk samples were collected from cows, sheep, and goats with clear signs of mastitis (Figure 1). In this study, it was found that animals with clinical mastitis have discolored milk and abnormal consistency ranging from watery to viscous. This variation is due to the presence of pus, blood, and milk clots (Biggs, 2009). Table 1 shows the results of bacterial culturing on MSA and examined by Gram stain in which 26 (37.14%) isolates out of 70 tested samples were suspected *Staphylococcus* sp. Additionally, all suspected colonies were appeared as a round, golden-yellow clusters on mannitol salt media (Figure 2). A study conducted in Iraq by Aboud and Khudaier (2018) found that 39.47 % of milk samples were positive for *Staphylococcus*, which is consistent with the results observed in the present study. In contrast, previous studies by Dehkordi *et al*. (2015) and Tibebu *et al*. (2021), reported lower isolation rates, where 25.53% and 24.8% of the milk samples tested positive for *Staphylococcus*, respectively. However, in Turkey, a higher isolation rate of *Staphylococcus* was observed, where 56% of raw milk and dairy products were positive to *Staphylococcus* (Gundogan and Avci, 2014). This highlights the importance of implementing effective mastitis control programs

and limiting the use of antibiotics in dairy projects. *Staphylococcus* is a common cause of mastitis, which can lead to persistent and recurring infections. Additionally, the cure rate after antibiotic therapy is usually low (Haag *et al.*, 2019). Furthermore, the PCR results demonstrated that 16S rDNA and mecA genes were detected in 13 (50%) of isolates (Table 1). The PCR-amplified DNA products with expected size of 16S rDNA (527bp) and *mec*A gene (886 bp) are shown in Figures 3 and 4.

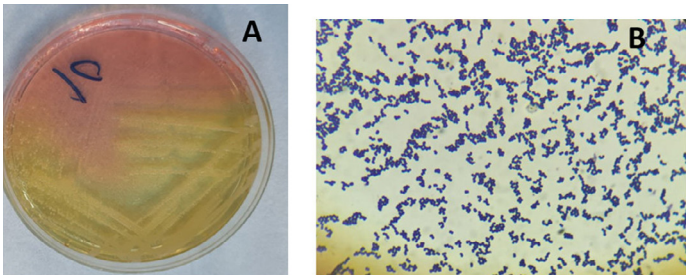

Figure 2: *Staphylococcus* cultured on MSA (A) showing round, golden-yellow clusters and Gram stain (B).

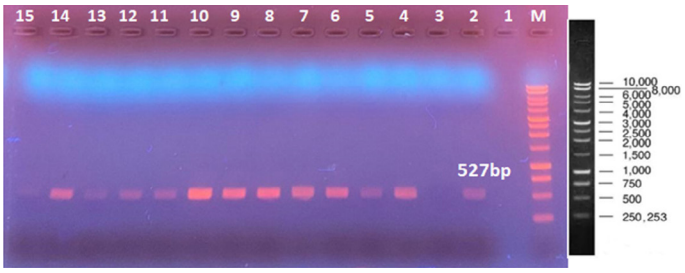

Figure 3: PCR amplification of 16S *rDNA* gene in 13 isolates of *Staphylococcus* sp. Lane 1 Negative control, Lane 3: *mec*A negative *Staphylococcus* sp. PCR product; Lanes 2 and 4-15: PCR products of *Staphylococcus* sp. isolates (527bp); M: 1kb DNA size marker.

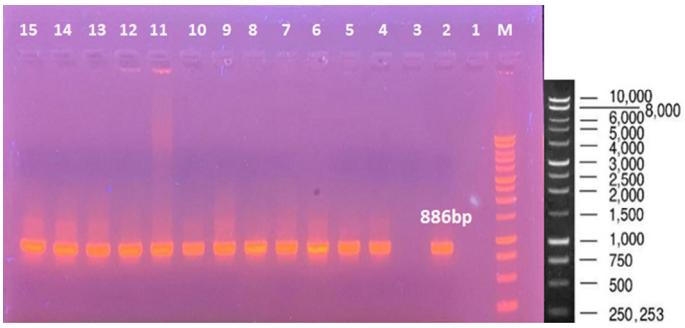

Figure 4: PCR amplification of *mec*A gene in 13 isolates of *Staphylococcus* sp.
Lane 1: Negative control, Lane 3: mecA negative *Staphylococcus* sp. PCR product; Lanes 2 and 4-15: PCR products of *mec*A gene (866 bp); M: 1kb DNA size marker.

In this study *Staphylococcus* sp. was identified by PCR and sequence analysis methods through used specific primers for the 16S rDNA gene that is located as a tandem repeat on the bacterial chromosome and shows significant variation among *Staphylococci* groups, confirmed by PCR and sequence analysis using the specific primers used. enabling for quick, highly sensitive, and specific identification of these species (Poulsen *et al.*, 2003).

Table 2: The A prevalence results of 13 isolates of *Staphylococcus* sp. based on 16S rDNA gene sequences.

***Staphylococcus* sp.**	**Positive No. based on sequence of 16S rDNA**	**(%) of isolates based on sequence of 16S rDNA**
S. aureus	9	69.24%
Other *Staphylococcus* sp.	4	30.76%
Total	13	100%

Table 3: The Nucleotides BLAST Analysis results of 13 isolates of *Staphylococcus* sp. based on 16S rDNA gene sequences.

Sample number	**Source**	**Identities**	**GenBank ID**
1	*Staphylococcus aureus*	96.31%	MN871924.1
2	*Staphylococcus sciuri*	97.96%	AB795259.1
3	*Staphylococcus aureus*	99.08%	KP295472.1
4	*Staphylococcus aureus*	98.57%	MN629267.1
5	*Staphylococcus succinus*	99.71%	LC216396.1
6	*Staphylococcus aureus*	99.85%	OR054191.1
7	*Staphylococcus aureus*	97.48%	LR134087.1
8	*Staphylococcus aureus*	99.85%	OR098500.1
9	*Staphylococcus equorum*	100.00%	MT409914.1
10	*Staphylococcus epidermidis*	97.29%	JF784023.1
11	*Staphylococcus aureus*	97.87%	MT628394.1
12	*Staphylococcus aureus*	99.86%	KP295472.1
13	*Staphylococcus aureus*	99.69%	MH429612.1

Furthermore, and in order to complete the genetic analysis of *Staphylococcus* sp., the PCR product result that exposed an amplicon size (527bp) of 16S rDNA gene was send for sequences analysis and all sequencing results were analyzed using the Basic Local Alignment Search Tool (BLAST) to compare the sequences to NCBI information and identify any discrepancies, as shown in Tables 2, 3, according to the sequencing results in Table 2 the prevalence results of 13 isolates of *Staphylococcus* sp. based on 16S rDNA gene sequences. only 9 (69.24%) local samples had similarity ranged between (96.31%-99.86%) to the *S. aureus* strains that previously recorded in the GenBank. However, the remaining of the local analysis samples 4 (30.76%) were diagnosed as *Staphylococcus sciuri, Staphylococcus succinus, Staphylococcus equorum and Staphylococcus epidermidis* at a homology ranged between 100%-96%. This percentage was lower than the research conducted by Khairullah *et al.* (2022), who isolated 80 (53.33%) of *S. aureus* from 150 milk samples, but agree with the result of another

study that conducted by Jahan *et al*. (2015) who isolated 12 (25.53%) of *S. aureus* from 47 milk samples regardless the number of *S. aureus* that could be isolated from milk samples the differences that occur could be attributed to variations in research design, such as residents and geographic distribution of samples, infection control procedures, the kind of antibiotic used for treatment, and poor hygiene procedure during milking, all of which can raise the risk of bacterial contamination in cow's milk (Tohoyessou *et al*., 2020; Omrani *et al*., 2020).

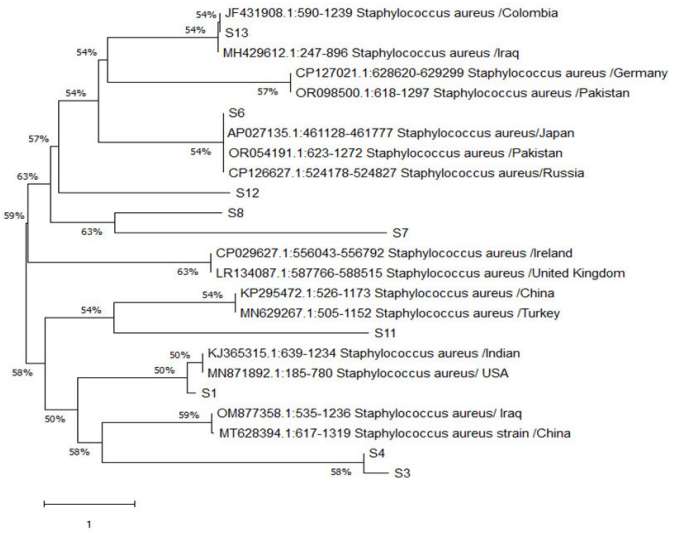

Figure 5: Phylogenetic tree of analysis of *Staphylococcus aureus*: The genetic relationship of Phylogeny showing 9 local isolates of *S. aureus* isolated from different sources in Basrah and with other international strains that extracted from GenBank database. S litter denote to sample.

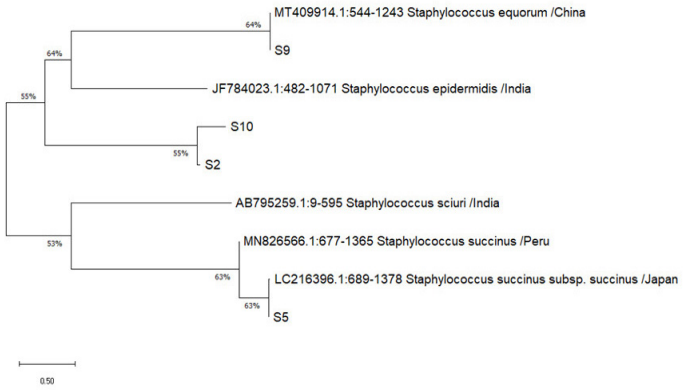

Figure 6: Phylogenetic tree of analysis of *Staphylococcus* sp.: The genetic relationship of Phylogeny showing 4 local isolates of *Staphylococcus* sp. bacteria isolated from different sources in Basrah and with other international strains that extracted from GenBank database. S litter denote to sample.

On the other hand, the phylogenetic tree based on *16SrDNA* gene was made by maximum likelihood method as in Figures 5, 6. In Figure 5 phylogeny of the *Staphylococcus aureus* was divided into two groups: The first group contained the S13, S6, S12, S8 and S7. In which S13 that shows extreme similarity to the samples from Colombia and Iraq while S6 show more relation to the samples from Japan, Pakistan and Russia.

Although S12 shares the same ancestor with S13 and S6 but it has the special branch. Additionally, both S7 and S8 arise from the same ancestor of groups one, but that had its evolution path. In contrast, groups two comprising S11, S1, S4 and S3. S11 shares the same ancestor with those from China and Turkey while S1 shows similarity with national strains from India and USA. Subsequently, both S3 and S4 were not national species, but shared a common ancestor with species from Iraq and China.

Alternatively, Figure 6 reveals the phylogeny of Iraqi isolates of other *Staphylococcus* sp., in this Figure 6, S9 shows extreme similarity with strain from China than those from India although it shares the same ancestor. While both S10 and S2 were lacks the similarity to the national isolates. Finally, S5 was shown more similarity with those strain from Japan than those came from Peru.

This indicates that *Staphylococcus* sp. Iraqi isolates are very similar to isolates from other countries and share the same 16s rDNA gene sequence. Because this gene is stable, has little mutation, and takes a long time to change, it is used to classify bacteria around the world, and lead to concluded that using of 16s rDNA in the clinical laboratory by sequence instead of phenotype can improve clinical microbiology technique for identifying bacteria and conducting taxonomic research (Clarridge, 2004).

Table 4: The nucleotides sequencing analysis results of *mecA* gene in 13 isolates of *Staphylococcus* sp.

Sample number	Source	Identities	GenBank ID
1	*Staphylococcus aureus*	100.00%	LC727174.1
2	*Staphylococcus aureus*	100.00%	CP127807.1
3	*Staphylococcus epidermidis*	100.00%	CP121525.1
4	*Staphylococcus aureus*	100.00%	LC727174.1
5	*Staphylococcus aureus*	100.00%	LC727174.1
6	*Staphylococcus aureus*	100.00%	LC727174.1
7	*Staphylococcus aureus*	100.00%	LC727174.1
8	*Staphylococcus aureus*	100.00%	CP127807.1
9	*Staphylococcus aureus*	100.00%	LC727174.1
10	*Staphylococcus aureus*	100.00%	CP127807.1
11	*Staphylococcus aureus*	100.00%	LC727174.1
12	*Staphylococcus aureus*	100.00%	CP121525.1
13	*Staphylococcus aureus*	100.00%	CP127807.1

On the other hand, the sequence analysis of *mec*A gene was also investigate using Blast algorithmic tool and listed in Table 4. In this Table 4 the sequencing results of *mec*A gene

was revealed 100% homology to the *Staphylococcus aureus* S286 DNA, *Staphylococcal* Cassette Chromosome mec with ID number LC727174.1 and *Staphylococcus aureus* strain C249 chromosome with ID number CP127807.1. While one isolate was displayed 100% homology to the *mec*A gene that previously detected in *Staphylococcus epidermidis* strain 1FSE05 plasmid with ID: CP121525.1.

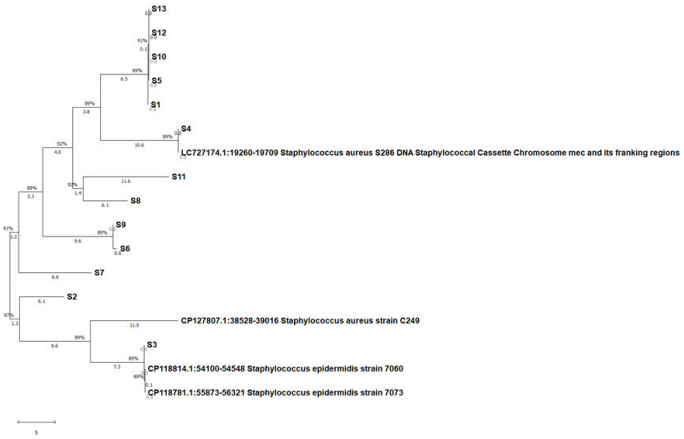

Figure 7: Phylogenetic tree of analysis of *mec*A gene in a local isolate: The sequence analysis of *mec*A genes in all 13 isolates were revealed 100% homology to the *Staphylococcus aureus* S286 DNA, *Staphylococcal* cassette chromosome mec with ID number LC727174.1 and *Staphylococcus aureus* strain C249 chromosome with ID number CP127807.1. While 1 isolate only was shown 100% homology to the *mec*A gene that previously detected in *Staphylococcus* epidermidis strain 1FSE05 plasmid with ID: CP121525.1. S litter denote to sample.

The phylogeny tree was also constructed in order to investigated the genetic distribution of *mec*A gene in local isolates and those listed and in GenBank as shown in Figure 7. The results of this Figure 7 give an adopt that the sources of *mec*A gene is mainly from *Staphylococcus aureus* which bring and share their Cassette Chromosome mec with other staphylococcal populations by horizontal gene transfer (Wielders *et al*., 2002). Additionally, it was recently discovered that using antibiotic treatment and or the abuse of antibiotics caused the gene to be transfer from *S. epidermidis* to *S. aureus* (Wielders *et al*., 2001), other similar investigations suggested that CoNS could also act as donors and source for the transmission of the *mec*A gene crossways the *Staphylococcaceae* family (Otto, 2013; John *et al*., 2019). On the other hand, it was recently discovered that using antibiotic in of treatment and or the abuse of antibiotics caused the gene to be transfer from *S. epidermidis* to *S. aureus* (Wielders *et al*., 2001). Moreover, the detection of *mec*A gene in the milk samples of mastitis cases have a significantly impact that there was a high degree of correlation between the presence of the *mec*A gene in *Staphylococcus* isolates and resistance to some common antibiotics such as cefoxitin and associated oxacillin and methicillin resistance (Wolk *et al*., 2009).

In this context, it is significantly notable that (70 to 75%) of all CoNS are currently methicillin-resistant (Diekema *et al*., 2001), creating a great potential reservoir of resistance.

CONCLUSION AND RECOMMENDATIONS

Regardless if the *S. aureus* or other CoNS is responsible for sharing and distribution of *mec*A gene, we concluded that all these information may offer crucial understanding of the origin and persistence of current and possibly even future of *mec*A in *Staphylococcus* strains. Additionally, it is advised to put control measures in place to prevent the establishment of resistance phenomena, such as a regular cleanliness regimen, routine MRSA screening, the use of antibiotic susceptibility testing before to treatment, or the random selection of antibiotics in field cases.

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NOVELTY STATEMENT

According to a recent study, it has been found that the majority of *staphylococci* strains, which cause mastitis in lactating animals, possess *mec*A gene. This gene plays a crucial role in the survival and persistence of *Staphylococcus* sp. as well as in their resistance to antibiotics. The study highlights the importance of following correct diagnostic procedures to identify the pathogen and using antimicrobials appropriately to prevent the development of antibiotic resistance.

AUTHOR'S CONTRIBUTION

HYJ: Clinical examination, sample collection and presumptive laboratory diagnosis. RMO: Molecular diagnosis and wrote the article. WMS: Microbiological diagnosis.

Conflict of interest

The authors have declared no conflict of interest.

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