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DETECTION OF PATHOGENIC AND ANTIBIOTIC GENES IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM MASTITIS IN THE MISAN PROVINCE, IRAQ

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Abstract

Staphylococcus aureus is one of the most important bovine mastitis related pathogens. The aim of this study was to assess the virulence and resistance gene profiles of 26 isolated *Staphylococcus aureus* from bovine mastitis milk in A-Amarah Region, Maysan, Iraq. PCR examined the presence of 4 virulence genes (*tst*, *sirB*, *icaA* and *hlg*) encoding toxic shock syndrome toxin, Siderophore compound transporter permease protein, polysaccharide intracellular adhesion and gama-hemolysin, respectively and 2 resistant genes (*mecA* and *femA*) encoding to resist Methicillin. It was found that the occurrence of *sirB* and *femA* genes was common (100%). The frequency of other genes was three (11.53%) of the isolate expressed in the *mecA* gene, four (15.38%) of the *hlg* gene, seven (26.92%) of the *tst* gene and 24 (92.30%) had *icaA* gene. The results indicate the variability in the presence of virulence genes in *Staphylococcus aureus* isolates that may cause mastitis in cows and PCR assays can be used as simple diagnostic tools to detect the Staphylococcus aureus virulence factors and antimicrobial susceptibility tests that help diagnose the severity of the infection. this study concluded that simple PCR assays can be used as fast and responsive diagnostic tools to detect virulence factors and *Staphylococcus aureus* antimicrobial susceptibility tests that help diagnose infection frequency, also our funding illustrates the incorrect management of AL-Amarah town's local bovine farms. It is therefore imperative to increase the awareness among farmers of using hygiene procedures in all dairy farms and must be strict hygienic and preventive measures are required between animals and humans and during food processing in order to avoid the colonization of MRSA isolates.

Key words: *tst* gene, *sirB* gene, *icaA* gene, *hlg* gene *mecA* gene and *femA* gene*, Staphylococcus aureus.*

Introduction

Mastitis is a mammalian gland inflammation with symptoms that sometimes lead to a systemic infection, it has a significant effect on the quality of animal welfare and milk (Maréchal *et al.,* 2011), resulting in substantial economic losses in milk production and the economy of the country (Gangwal *et al.,* 2017; Halasa *et al.,* 2009). Milk is an excellent culture medium for microorganism production and may be responsible for the transmission of harmful zoonoses (AMARAL *et al.,* 2004). The emergence and spread of pathogens, such as *Staphylococcus aureus*, is a major public health interest, so the identity of these strains' virulence and drug resistance profiles is of vital importance (Kozytska *et al.,* 2010; Monteiro *et al.,* 2019). *Staphylococcus aureus* is a pathogen identified by the International Dairy Federation (IDF) as the main etiological pathogen that causes intra-mammary infections (IMI) and is a major pathogen that can contribute to a large number of infections around the world, toxins and antimicrobial residues that can be transmitted by milk, which is of major concern in the epidemiology of foodborne diseases due to its high prevalence and potential risk during food production and significant changes in susceptibility to antimicrobials over the years; Staphylococcus aureus is a major pathogen that can contribute to a large number of infections around the world (Alreshidi, 2020; Zecconi and Hahn, 1999; Delgado *et al.,* 2011). It is responsible for more than 80% of subclinical bovine mastitis, which can result in a loss of about $300 per animal (Karahan *et al.,* 2009). *Staphylococcus aureus* ability to cause various infections and intoxication derives from the development of various extracellular and surface virulence factors involved in mastitis pathogenesis; such as adhesive

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properties targeting a variety of molecules (MSCRAMMs), invasions promoting tissue spread of bacteria, surface factors inhibiting phagocytic engulfment, Inherent and acquired antimicrobial resistance that makes it difficult to eradicate and toxins that damage the membrane such as hemolysins that lyses eukaryotic cell membranes and enzymes that can cause a variety of skin and soft tissue infections; Including intramammary mastitis and also the production of biofilms; thus preventing the action of antibiotics and the immune system by producing extracellular polysaccharide substances, creating clusters of bacterial cells in multilayer biofilms (Todar, 2019; Casey *et al.,* 2007; Momtaz *et al.,* 2010; Haveri *et al.,* 2008; Aung *et al.,* 2011). Contaminated milk, due to the presence of staphylococcal toxins, becomes a potential source of food poisoning once ingested raw and unprocessed. In addition, the detection of potential strains of formations of *Staphylococcus* biofilm may represent a toxic risk factor for consumers (Lim *et al.,* 2004). *S. aureus* may produce one or more additional exoproteins, including toxic shock syndrome toxin-1 (TSST-1), this toxin plays a prominent role in human and animal staphylococcal food poisoning and other infections (Elsayed *et al.,* 2015). The principal source of *S. aureus* tends to be the infected portion and infection typically occurs during milking from cow to cow. In bovine intramammary infections (IMI), the virulence of *S. aureus* differs between strains and no specific virulence factor or combination of factors was strongly associated with mastitis severity (Foster, 2005; Haveri *et al.,* 2005). Genes located on mobile genetic elements called pathogenicity islands (*i.e*., TSST and other enterotoxins) express certain virulence factors. In addition, *S. aureus* may also purchase the staphylococcal cassette chromosome SCCmec, resulting in a methicillin-resistant aureus (MRSA) (van Wamel *et al.,* 2006; Pantosti, 2012). In fact, the *mecA* gene's expression in *S. aureus* gives resistance to most B-lactams, drugs that are often used to treat mastitis (Sawant *et al.,* 2005). The determination of the origin of the *S.aureus* isolates involved in the etiology of bovine mastitis is epidemiologically very relevant. Over the past decades, a wide range of phenotyping and genotyping methods have been used or developed for *S.aureus* including, but not limited to, ribotyping, RAPD-typing, PFGE, MLST, spa-typing, RS-PCR based on PCR amplification of the 16S-23S rRNA intergenic spacer region, RFLP coagulase gene, MLVA, micro-arrays and entire genome comparisons; these studies were used as diagnostic tools to understand pathogen sources and mechanisms of transmission (Cremonesi *et al.,* 2015; Ikawaty *et al.,* 2009; Banoon *et al.,* 2019). *Staphylococcus aureus* has multiple properties that contribute to its virulence. In dairy cattle, *S. aureus* is one of the main causes of chronic and subclinical mastitis. The present study was carried out since there was limited data in Al-Amarah City, Maysan, Iraq on virulence factors of aureus with bovine mastitis milk. Thus, this study was aimed at identifying certain virulence factors and genes of resistance of *Staphylococcus aureus* isolates among the cow population with mastitis using PCR technique.

Materials and Methods

Staphylococcus aureus strains and the conditions for growth

This study included twenty-six strains of *S.aureus* isolated from cases of infectious mastitis that had been clinically diagnosed positive for the California Mastitis Test (CMT) according to (Markey *et al.,* 2013) from various local dairy farms in Maysan province, South Iraq. The isolates have been routinely grown on Mannitol salt agar (HiMedia, India) and incubated for 24 h., at 37^{o}C. (Quinn, 1994) and their identity was confirmed on the basis of numerous microbiological and biochemical tests (shape, gram-staining, catalase and coagulate) (MacFaddin, 2000).

Extraction of Bacterial Genomic DNA

Complete DNA of All isolates was collected from colonies grown on agar plates as per the Geneaid protocol of resto™ Mini gDNA Bacteria Kit, Taiwan. The spectrophotometric (NanoDrop) concentration of DNA was measured by measuring its optical density at 260 nm. The purity of the DNA solution is indicated by the ratio of OD260/OD280 in the range of 1.8±0.2 for pure DNA (Stephenson, 2003). The extracted DNA was then deposited at freezer in -20°C before PCR assay was performed.

Polymerase chain reaction (PCR)

The PCR assay was conducted to detect the Virulence and antibiotic genes as shown in (Table 1), were primers (*tst*, *mecA*, *femA* and *hgl*) synthesized by BioNEER, Korea while (*sirB* and *icA*) synthesized by IDT, USA. PCR screened the presence of various toxins, adhesives and other virulence factors including some genes of antibiotic resistance using the primers described in table 1. For the detection of these genes; monoplex PCR subjected the chromosomal DNA extracted from all isolates to primers. PCR mixture for each primer with 20 µl / reaction final volume and protocol used according to Master Mix instructions (AccuPower® PCR PreMix (Bioneer, Korea). The 2µl Forward Primer (10 picomole), 2µl Reverse Primer (10 picomole), 9µl De-ionized water

Table 1: Primers used in this study.

Gene	Product	Primers Sequence (5´ – 3´)	Amplicon size (bp)	Reference
tst	Toxic shock syndrome toxin	Fw: ATGGCAGCATCAGCTTGATA Rv: TTTCCAATAACCACCCGTTT	350	Akineden *et al.,* 2001
sirB	Siderophore compound transporter permease protein	Fw: CAGCTACGGCTACCGAAATA Rv: CATTTTTGGGGGCTATTGTTGT	399	Dale *et al.,* 2004
icaA	Polysaccharide Intracellular adhesion	Fw: GATTATGTAATGTGCTTGGA Rv: ACTACTGCTGCGTTAATAAT	770	Peacock *et al.,* 2002
mecA	Methicillin resistance	Fw: CGGTAACATTGATCGCAACG Rv: TTTGCCAACCTTTACCATCG	986	Stotts *et al.,* 2005
femA	Factor essential for methicillin resistance	Fw: AACAGCTAAAGAGTTTGGTGCC Rv:CATCACGATCAGCAAAAGCT	647	Stotts *et al.,* 2005
hlg	Gama-hemolysin	Fw: GCCAATCCGTTATTAGAAAATGC Rv:CCATAGACGTAGCAACGGAT	938	Kumar *et al.,* 2009

and 7µl DNA of the isolates were applied to the AccuPower® Taq PCR PreMix tubes that contain (*Taq* DNA polymerase, dNTPs, KCl, $MgCl_2$ and buffer). All PCR components were mounted in PCR tube and mixed for 10 seconds at 50 rcf (850 rpm) with a micro-centrifuge. All PCR reactions began with a 5-minute 95°C Initial Denaturation and ended with a 7-minute 72°C extension and a 4°C hold and the condition for further steps for each primer as described in table 2. Electrophoresis solved the PCR products in a 1.4% agarose gel stained with 0.5% staining of Ethidium bromide (Bio Basic, Canda) and then visualized by Gel Documentation (Vilber, France). The 100-bp DNA ladder (Bioneer, South Korea) used as the standard of reference (Sambrook and Russell, 2001).

Results and Discussion

PCR performed the isolates to identify the genes *tst*, *sirB*, *icaA*, *mecA*, *femA* and *hlg*. Three (11.53%) isolates expressed the *mecA* gene, four (15.38%) *hlg*, seven (26.92%) *tst* and 24 (92.30%) had *icaA* and 26 (100%) for *sirB* and *femA* genes as shown in table 3.

In this study, we record molecular analyzes carried out on animal-derived *S. aureus* isolates with mastitis collected from different farms in Al-Amarah city, South of Iraq, the presence of *sirB*, *icaA*, *tst* and *hlg* genes encoding for Siderophore compound transporter permease protein, Polysaccharide Intracellular adhesion, Toxic shock syndrome toxin and Gama-hemolysin as a virulence factors, while *mecA* and *femA* encoding for Methicillin resistance was determined by PCR. *SirB* gene presence in all isolates is in agreement with this (Delgado *et al.,* 2011). The ability of the bacterial siderophores to acquire iron from the host can facilitate infection. In *S.aureus* various siderophores were characterized, including iron-uptake ABC transporters (Dale *et al.,* 2004). Presence *icaA* gene agree with (Vasudevan *et al.,* 2003; Delgado *et al.,* 2011). *Staphylococcus aureus'* ability to form biofilms assists the bacterium in surviving aggressive conditions within the host and is considered responsible for chronic or recurrent infections (Costerton *et al.,* 1999). The presence of the *icaA* locus in all isolates of the *Staphylococcus aureus* mastitis confirms its potential role as a virulence factor in mastitis pathogenesis in ruminants (Vasudevan *et al.,* 2003). Several studies reported a higher frequency of distribution of the *icaA* locus in *Staphylococcus epidermidis* clinical isolates than in saprophytic strains, emphasizing its use as a virulence marker (Ziebuhr *et al.,* 1997; Arciola *et al.,* 2001). Moreover, the higher percentage (92.30%) containing the *icaA* locus is in accordance with a study carried out in Iran (Khoramian *et al.,* 2015) and (76.2%) by (Vitale *et al.,* 2019). This gene is associated with the development of biofilms, a well-known risk factor for chronic infection as it allows the long-term survival of bacteria in the mammary glands, which is an ideal environment for horizontal gene transfer (Vasudevan *et al.,* 2003; Gowrishankar *et al.,* 2016; Marques *et al.,* 2017; Madsen *et al*., 2012; Águila-Arcos *et al.,* 2017). This capacity is also correlated with reduced

Table 2: The PCR conditions used for amplification.

Primer name	Denaturation Temperature °/time	Annealing Temperature °/time	Extension Temperature °/time	Cycle Number
tst	95/2 min	55/2 min	72/1 min	35
sirB	95/1 min	61/1 min	72/2 min	30
icaA	95/1 min	50/1 min	72/2 min	30
mecA	95/45 sec	55/1 min	72/2 min	35
femA	95/1 min	60/1.5 min	72/2 min	35
hlg	95/1 min	55/1 min	72/1 min	35

antibiotic therapy efficacy and infection eradication problems (Melchior *et al.,* 2006). Contrary to our results, in 2017 by (Suvajdžić *et al.,* 2017) only 11 (25.4%) were positive for both *icaA* and *icaD* genes. Similar results were reported by (Ciftci *et al.,* 2009). The tests for the *mecA* gene show only three strains out of all positive *mecA* gene isolates and suggest these strain MRSA; MRSA is classified as *Staphylococcus aureus* isolates which are resistant to semi-synthetic *β-lactam* antibiotics; this bacteria is now a significant resistance-based threat to humanity. The rise of the methicillin-resistant *Staphylococcus aureus* (MRSA) reaffirmed its infection dominance (Dixit *et al.,* 2016). MRSA isolates usually have a gene (*mecA*) to inactivate a large number of antibiotics including *â-lactam* drugs. Although the presence of the *mecA* gene is closely related to *β-lactam* resistance, reports of *mecA*-negative MRSA are reported (Chambers, 1997). *Staphylococcus aureus'* resistance to various antibiotics is a worldwide vital concern, but the methicillin resistance is this agent's most well-known characteristic. MRSA usually spreads from human to human but it can also be transmitted from animal to human (Juhász-Kaszanyitzky *et al.,* 2007; Sakwinska *et al.,* 2011).

Though MRSA has been increasingly recognized in farm animal populations in recent years, there are limited data available regarding the prevalence of MRSA in the bovine mastitis milk sample in Iraq. Our findings suggest that there is a low prevalence of MRSA isolates in mastitis (11.53 %) (3 out of 26); this is consistent with studies of (Kumar *et al.,* 2011) in India, which reported that 9.3% of *Staphylococcus aureus* isolates were carried *mecA* gene from bovine mastitis milk. The percentage for this gene as the lowest in this study is in agreement with (Ahangari *et al.,* 2017; Stotts *et al.,* 2005) with only three isolates (1.3 %). Although (Vitale *et al.,* 2019; Öztürk *et al.,* 2019) related to the isolates derived from animals, none of them were positive for the *mecA* gene. Interestingly, in 14 of 20 MR-CNS isolates, the *mecA* gene was present (Klibi *et al.,* 2018). The PCR detection of *femA* gene for isolates of *Staphylococcus aureus* indicates that all of *Staphylococcus* aureus (n=26) had a 647-base Amplicon pair corresponding to the *femA* genetic marker, this study was consistent with (Stotts *et al.,* 2005; Xavier *et al.,* 2017; Gandhale *et al.,* 2017; Öztürk *et al.,* 2019), the *femA* gene is a marker used for genotypical identification at the level of *Staphylococcus aureus* isolates (Ardic *et al.,* 2006). The *mecA* and *femA* virulence genes were studied because they are believed to be major contributors to the resistance of methicillins (Maidhof *et al.,* 1991; Kuhl *et al.,* 1978). Therefore, the inconsistency between the content of the resistance genes may be due to variations in the expression of *mecA* and *femA* between strains (Stotts *et al.,* 2005). PCR tests for Gamma hemolysin toxin (*hlg*) gene amplification in *S. aureus* isolates showed that the *hlg* gene was amplified by 4 (15.38%) strains of 938 bp drug. Whereas other results (Memon *et al.,* 2012; Abdel-Tawab *et al.,* 2016) reported 7 (58.3%) by. This study contrasted with (Delgado *et al.,* 2011; Almeida *et al.,* 2013) whose results with this gene had been negative. Gamma toxin is a two-component exotoxin consisting of at least six different protein combinations, one of which is leukocidin that affects red blood cells in horses (Dinges *et al.,* 2000). In this study, the results of PCR for the detection of *mecA* gene and *hlg* gene for isolated strains from animal samples were 3 MRSA strains positive for the *mecA* gene and at the same time negative for the *hlg* gene and our findings were in accordance with this (Helal *et al.,* 2015). In this

Table 3: Distribution of aureus genes (n=26) for virulence and antibiotic resistance in this research.

No. of Isolate	virulence factor				antibiotic resistance	
	tst	*sirB*	*icaA*	*hlg*	*mecA*	*femA*
1	-	+	+	-	-	+
2	-	+	+	+	-	+
3	+	+	+	-	-	+
4	+	+	+	-	-	+
5	-	+	+	-	-	+
6	-	+	+	-	-	+
7	+	+	+	-	-	+
8	-	+	+	-	-	+
9	-	+	+	-	-	+
10	-	+	-	-	-	+
11	-	+	-	-	-	+
12	-	+	+	-	-	+
13	-	+	+	+	-	+
14	+	+	+	+	-	+
15	+	+	+	+	-	+
16	-	+	+	-	-	+
17	-	+	+	-	-	+
18	-	+	+	-	-	+
19	-	+	+	-	-	+
20	-	+	+	-	+	+
21	-	+	+	-	-	+
22	-	+	+	-	-	+
23	+	+	+	-	-	+
24	-	+	+	-	+	+
25	-	+	+	-	+	+
26	+	+	+	-	-	+
Total of positive (n) %	7 26.92 %	26 100 %	24 92.30 %	4 15.38 %	3 11.53 %	26 100 %

study, the *tst* gene was located in 7 isolates of *Staphylococcus aureus*. Although (Karahan *et al.,* 2009) found only three (3.3%) bovine subclinical mastitis *Staphylococcus aureus* positive for the *tst* gene in Turkey and (Kot *et al.,* 2016) harbored the *tst* gene by (2.4%) isolates in Poland. Unlike our findings; (Ahmed *et al.,* 2016) whose result was that all isolates were negative to the *tst* gene. Although (Klibi *et al.,* 2018) whose recorded tsst-1 virulence gene was observed in one strain of the MR-CNS. Assays for staphylococcal enterotoxin (*tst*) toxic shock toxin were included, because it can be transferred laterally among strains via bacteriophage infection (Ruzin *et al.,* 2001).

Conclusion

1. It is concluded that simple PCR assays can be used as fast and responsive diagnostic tools to detect virulence factors and *Staphylococcus aureus* antimicrobial susceptibility tests that help diagnose infection frequency, distribute and classify prevention and control measures, as well as modify appropriate therapy and prevent ineffective microbial infection treatments.

2. This analysis illustrates the incorrect management of AL-Amarah town's local bovine farms. It is therefore imperative to increase the awareness among farmers of using hygiene procedures in all dairy farms.

3. Strict hygienic and preventive measures are required between animals and humans and during food processing in order to avoid the colonization of MRSA isolates, as well as the importance of regulating the use of antibiotics in medical and veterinary practice and tracking the isolates extracted from animals as they establish greater resistance to the most common antibiotics.

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