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Molecular characterization of *Staphylococcus aureus* isolated from mastitic cows in dairy farms

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Abstract

Mastitis is a major problem threat dairy production that caused by several pathogens, the most important one of them is *Staphylococcus aureus*. This study used diagnostic and molecular analysis of *Staphylococcus aureus* to determine the incidence of mastitis in cows. Mastitis was found in 43 of 150 total cows tested at random by (California Mastitis Test), 172 milk samples from quarters infected with mastitis were obtained for further culture and PCR. The prevalence of mastitis on farms, cows, and quarters was 73.3, 28.7, and 28.7 percent, respectively, with (SCM) being the most common kind in all instances. Incidence of mastitis caused by *Staph. aureus* on farm, cows, and quarter levels was 63.6, 67.4, and 18.02 percent, respectively, according to bacteriological testing, and this bacteria was largely related with clinical mastitis (CM). 31 *S. aureus* isolates were tested to antimicrobial susceptibility test; these revealed that, the highest sensitivity was to vancomycin (87.1%), enrofloxacin (77.4%) and linezolid (74.2%). Moderate sensitivity was noticed to gentamicin (54.8%), ciprofloxacin (48.4%), and cephalothin (45.2%), resistance rates were higher with kan- amycin and lincomycin (100%), followed by penicillin (96.8%), oxacillin (96.3%), nalidixic acid (90.3%), oxytetracycline (83.9%), erythromycin (80.6%) and trimethoprim/sulfamethoxazole (74.1%). Among the isolates examined in this study, *S. aureus* isolates were subjected for PCR of these virulence genes, coagulase and surface protein A (*coa and spa)*, enterotoxin A to E (*sea*, *seb, sec, sed, see*) and antimicrobial resistance genes *(mecA ,ermA , vanA , aac 6-aph 2*), methicillin resistance (*mec*A), erythromycin (*erm*A), gentamicin (*aac* 6- *aph* 2) and vancomycin *(van*A) of *S. aureus*. The results of PCR were as follows, all *S. aureus* isolates were positive for *coa* , *spa* genes, however *seb* enterotoxin gene was discovered in 50% of the examined strains, total finding ratios of *mec*A, *erm*A, *van*A, *aac (6)-aph (2)* genes were 25.0%, 56.25%, 12.5% and 43.75%, respectively, suggest that methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged and spread. Strains had numerous genes in common, they were shown to be more in SCM, and they had multiple drug resistant (MDR).

Keywords: mastitis, *S. aureus*, PCR, virulence and resistance genes

Introduction

Bovine mastitis is the most frequent disease in dairy cattle globally, prevalence rates of 30-50 percent recorded in many regions, reduction in milk production due to mastitis is generally known as primary reason of economic losses in the dairy industry **(Seegers *et al.*, 2003)**. Intra-mammary infection in dairy cows can be caused by a variety of things, especially during milking, around a third of all clinical- and subclinical mastitis cases globally is due to *Staphylococcus aureus* which is the most common and highly infected causative agent **(Holmes and Zadoks, 2011).**

The majority of dairy animals research regard this microbe as a real mastitis bacteria with significant virulence characteristics **(Hosseinzadeh and Saei 2014),** strong antibiotic resistance **(Frey *et al.*, 2013)**, and the propensity to cause chronic mastitis **(Gillespie *et al.*, 2009** and **Cervinkova *et al.*,**

2013).

The environment, in addition to the causal pathogenic bacteria, is a crucial role in mastitis, constituting a significant reservoir of infection. Correct antimicrobial medication remains the major strategy for controlling *staphylococcal* mastitis, despite the fact that environmental factors can be addressed by suitable management measures such as proper milking techniques and exclusion of chronic mastitis cases. Indiscriminate use of antibiotic treatment is typically associated with poor effectiveness, resulting in high execution rate **(Hendriksen *et al.,* 2008).** Additionally, because improper treatment of this organism causes significant harm to the glandular tissues of the udder and most antibiotics are unable to permeate affected tissues. **(Guler *et al.,* 2010) and (Pu *et al.,* 2014).**This microbe also inhibits phagocytosis and cell-mediated immunity, as well as producing an enzyme which renders most penicillin-based antibiotics ineffective. **(De Oliveira *et al.,* 2000)**. Multidrug-resistant *Staphylococcus aureus*, have emerged as a serious public health concern in the last years. **(Wang *et al.,* 2012).** In general, Despite the fact that *S. aureus* isolated from bovine infected with mastitis is responded to common antibiotics which used in animal husbandry, such as aminoglycosides, macrolides, lincosamides, and B-lactams **(De Oliveira *et al.,* 2000),** Increasing acquired resistance levels to penicillin G, lincomycin, streptomycin, gentamycin, and erythromycin have been observed (**(Kumar *et al.,* 2010**). Antibiotic-resistant bacteria can transmit resistance genes to humen's intestinal flora via foods, the commensally flora can serve as a store of resistance genes of these bacteria **(Aarestrup *et al.,* 2008).** In dairy farms, molecular diagnostic technologies such as mastitis diagnostic evaluation based on DNA, this has already been deployed on a regular usage. **(Koskinen *et al.,* 2009)**. Polymerase Chain Reaction has been a widely used molecular technology, for detecting and identifying the microbe that cause mastitis in milk by specifically genes on the DNA. **(Taponen *et al.,* 2009** and **Elsayed *et al.,* 2015)**. *Staphylococcal enterotoxins* (SEs) are classified based on their serological characteristics. into five categories. One or more of the five primary SEs (*SEA*, *SEB*, *SEC*, *SED*, and *SEE*) can be produced. In addition, coagulase, which is encoded by the *coa* gene, has been shown to be a virulence factor in intrammmary infection, this gene has a preserved and repetitive polymorphic domain which can be utilized to compare *S. aureus* strains. **(Reinoso, 2004).** Coagulase is a protein that may convert fibrinogen into fibrin strings through a method that differs from physiological clotting **(Palma *et al.,* 1999).** Numerous virulence factors are encoded by *Staphylococcus aureus*, which include surface IgG binding protein A (*spa*), which captures Fc region of immunoglobulin, preventing phagocytosis of bacteria by host's immune system **(Foster, 2005).**

The goal of this work was to look for the prevalence of genes responsible for multiple antibiotic resistance features and virulence genes characteristics in *S. aureus*, as well as their detection using polymerase chain reaction (PCR), obtained from both clinical and subclinical mastitic dairy cows.

Materials and methods

California mastitis test (CMT) and collecting of samples:

A number of 15 dairy farms with a past history of mastitis were chosen from the study locations (in Gharbia governorate), with an average number of 15 (10– 25). 150 dairy cows were randomly examined for mastitis (four quarters for each cow). CMT was utilized as a mastitis diagnostic test. It was used in accordance with the instructions provided by **(Hoque *et al.,* 2015).** Based on gel formation, the results of CMT were graded as 0 (negative), 1 (weak positivity), 2 (distinctive positivity), and 3 (strong positivity). CMT values of 0 were regarded negative, whereas scores of 1 and 2 indicated subclinical mastitis, and 3 indicated clinical mastitis. We considered the cow positive when at least one quarter with a CMT score of greater than one. Forty three of CMT positive cows from 172 quarters (70 clinical and 102 subclinical), (10–15 mL/sample) were gathered in an aseptic manner into

the sterile tubes and transferred to the laboratory in an ice-box.

Isolation and identification of *Staphylococcus aureus*:

Milk samples for CMT positive animals were induced for bacteriological examination to detect *S. aureus*. Cultivation onto Baird- Parker agar plates then placed in incubator for twenty four hours at 37°C. Isolates were identified by ordinary methods, colony appearance, Gram staining were used to identify isolates, tested for hemolysis, coagulase, catalase, and mannitol fermentation o **(Koneman *et al.*, 2001).**

Five representative colonies (shiny black with an opaque ring, encircled by a clear halo zone) were chosen for inoculation in tubes containing BHI and incubated for twenty four hours at 35 ±2 °C. 0.3 mL from each tube was transferred to test tubes containing rabbit plasma (0.5 mL), and incubated for six hours at 35°C ± 2°C. The existence of coagulates was confirmed using the following characteristics:

Small disordered coagulation is known as first interaction 1+.

Small structured coagulation is known as second interaction 2+.

Regular large coagulation in third interction 3+.

Fourth interaction 4+: the entire contents of the tube coagulate and when we flip it, it stays stuck in the tube. When the coagulation interaction was of third and fourth type, the presence of *Staphylococcus aureus* was verified. **(Brasil, 2003).**

Antimicrobial susceptibility testing:

S. aureus strains were routinely evaluated using the disc diffusion assay **(Bauer *et al.*, 1999)** on Mueller Hinton Agar plate for their susceptibility to a variety of antibacterial drugs (Oxoid, Milano, Italy). Antibacterial drugs includ: lincomycin (L 30 µg), nalidixic acid (NA 30 µg), ciprofloxacin (CIP 5 µg), cephaphalothin (CN 30), gentamycin (G 10 µg), vancomycin (V15 µg), kanamycin (K30 µg), linezolid (LZD) , enrofloxacin (5 µg), erythromycin (15 µg), penicillin G (10 IU), oxacillin (1 µg), oxytetracycline (30 µg) and sulfamethoxazole (25 µg). The test was performed, and the results were analyzed in accordance with the guidelines (CLSI, 2013).

PCR Procedue:

A-Extraction of DNA: The QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used to extract DNA from tested bacteria, by using specific changes depended on the manufacturer's instructions. For 10 minutes at 56°C, (two hundred µl) of bacterial suspension was treated with ten µl of proteinase K and (two hundred µl) of lysis buffer. After incubating, the lysate was given (two hundred µl) of 100 percent ethanol. According the manufacturer's instructions, the sample was washed and centrifuged. Elution of nucleic acid by (hundred µl) of kit's elution buffer.

B-Oligonucleotide Primer:

According to Metabion (Germany), the primers which used were shown in tables (1 and 2).

C-PCR amplification: These Primers were used 25- µl reaction that contain EmeraldAmp Max PCR Master Mix (12.5 µl) (Takara, Japan), 1µl of each primer with a concentration of 20 pmol, 5 µl of template of DNA, 5.5 µl of H_2O. An applied biosystem 2720 heat cycler was used to carry out the reaction. For multiplex PCR of virulence (*coa, spa, sea, seb, sec, sed, see*) and antimicrobial resistance *(mec*A ,*erm*A , *van*A , *aac 6-aph 2*) genes . Primers were used in a 50- µl reaction which contain 25 µl of EmeraldAmp Max PCR (Takara, Japan), one µl per primer of twenty pmol concentrations, nine µl of H_2O, and 6 µl of template of DNA.

D-Analysis of the PCR Products:

Using electrophoresis for separation of PCR products in 1x TBE buffer at normal temperature using 5V/cm gradients on a 1.5 percent agarose gel (Applichem, Germany, GmbH). Each gel hole was supplied with twenty µl of uniplex PCR products and Forty µl of multiplex PCR products for analysis of gel. Fragment sizes were assessed by a Gelpilot 100 base pare DNA ladder (Qiagen, Germany, GmbH) and (Fermentas, Thermo Scientific, Germany) Generular100 base pare ladder. A gel documented by (Alpha Innotech, Biometra) was used to photograph the gel, and the data was evaluated using software.

Table (1). Virulence genes with Sequences of their primers and cycling conditions phases.

Target gene	Primers sequences	Amplified segment (bp)	Primary denatur-ation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Sea	**F**.GGTTATCAATGT GCGGGTGG	102	94˚C for 5 minute.	94˚C for 30 second	57˚C for 40 second	72˚C for 45 second	72˚C for 10 minute	**(Mehrotra *et al.*, 2000)**
	R.CGGCACTTTTTT CTCTTCGG							
Seb	**F**.GTATGGTGGTGT AACTGAGC	164						
	R.CCAAATAGTGAC GAGTTAGG							
Sec	**F**.AGATGAAGTAGT TGATGTGTATGG	451						
	R.CACACTTTAGA ATCAACCG							
Sed	**F**.CCAATAATAGGA GAAAATAAAAG	278						
	R.ATTGGTATTTTT TTTCGTTC							
See	**F**.AGGTTTTTTCAC AGGTCATCC	209						
	R.CTTTTTTTTCTTC GGTCAATC							
Spa	**F**.TCA ACA AAG AAC AAC AAA ATG C	226	94˚C for 5 minute.	94˚C for 30 second	55˚C for 30 second	72˚C for 30 second	72˚C for 7 minute	**(Wada *et al*., 2010)**
	R. GCT TTC GGT GCT TGA GAT TC							
Coa	**F.** ATA GAG ATG CTG GTA CAG G	Four main sorts of bands can be detected .350 430 570 630	94˚Cfor 5 minute	94˚C for 30 second	55˚C for 45 second	72˚C for 45 second	72˚C for 10 minute	**(Iyer and Ku-mosani, 2011)**
	R. GCT TCC GAT TGT TCG ATG C							

Table (2). Antibiotic resistance genes with Sequences of their primers and cycling conditions phases.

Target gene	Primers sequences	Amplified segment (bp)	Prima-ry dena-turation	Amplification (35 cycles)			Final extension	Reference
				Secondary denatura-tion	An-nealing	Exten-sion		
***mec*A**	**F.**AAAATCGATGGTAAA GGTTGGC	533	94˚C for 5 minute	94˚C for 30 second	64˚C for 45 second	72˚C for 45second	72˚C for 10 minute	**(Buhlman n *et al.*, 2008)**
	R.AGTTCTGGAGTACCG GATTTGC							
***erm*A**	**F.**TATCTTATCGTTGAGA AGGGATT	139						**(Amghalia *et al.*, 2009)**
	R.CTACACTTGGCTTAG GATGAAA							
aac 6-*aph* 2	**F.**TTGGGAAGATGAAGT TTTTAGA	174						
	R.CCTTTACTCCAATAAT TTGGCT							
***van*A**	**F.**CATGAATAGAATAAAA GTTGCAATA	1030						
	R.CCCCTTTAACGCTAAT ACGATCAA							

Results and Discussion

Different Seasons, herd number, farm size, hygienic condition, farm management technique, geographic area, differences in identification technique, and variation in types of samples analyzed. All of these contributed to the difference in prevalence of *S. aureus* in milk in different regions.

Prevalence of mastitis, isolation of *Staphylococcus aureus*:

In the current study, the incidence of mastitis in dairy farm revealed that 73.3 percent of the farm. With consideration, it is difficult to compare our current data with other previous studies, this is due to dissimilarly in structured investigations .The total of each cow, quarter level mastitis was 28.7%. According to CMT and visual assessment, with 36.4 %, 37.2 % and 42.4% being clinical mastitis (CM) and 63.6 %, 62.8 % and 57.6 % being subclinical mastitis (SCM), respectively (Table 3). These findings are in agreement with in the range of the incidence rates of bovine mastitis cases (average from 8.0–64.0 percent) recorded by the majority of recently published research in the world **(Hoque *et al.*, 2015)** and **(Jha *et al.*, 2010).** Various previous studies and our results are greater than existing reports from other areas with dairy management practices that are more or less similar to these results, such as 63.8 percent subclinical mastitis in Thailand **(Jarassaeng *et al.*, 2012).** CM and SCM (18.2%, 33.7%) respectively in Pakistan **(Hameed *et al.*, 2012), (**30.6 to 33.7%) SCM (**Elango *et al.*, 2010**) and 16.0% CM in India **(Bangar *et al.*, 2016)**. In general ,this indicates the gravity of the crisis in the dairy sector , which requires immediate response.

By bacteriological examination, we confirmed overall 63.6% (7/11) of farm rate mastitis due to *Staphylococcus aureus*, of which 57.1% CM and 42.8% SCM, prevalence of cow and quarter rate of mastitis that caused by *Staphylococcus aureus* were 67.4% (58.6% CM, 41.3% SCM) and 18.02%%, (64.5% CM, 35.5% SCM) respectively as shown in (Table 4). This bacterium was more commonly in both cases, clinical and subclinical mastitis.

Staphylococcus aureus was shown to be the most common reason of mastitis in this investegation, with it was being detected in 63.6 percent of the farms evaluated.

These findings are backed up by the research developments of **(Abebe *et al.*, 2016)**, who identified this pathogen as the most common cause of bovine mastitis in Ethiopia. Numerous earlier studies have identified *Staphylococcus aureus* as the major causative pathogen of mastitis in many regions (**FAO 2014).** Percentage of bovine SCM which due to *Staphylococcus aureus* was observed in an average of 21.2 percent **(Nazneen *et al.*, 2014)**. Another study **(Tenhagen *et al.*, 2006)** found that 95% of clinical mastitis causes by a lot of microbes in dairy cattle and most of these microbes are *Staphylococcus aureus*. Previously working with bovine mastitis, (**Piepers *et al.*, 2007) and (Kirkan *et al.*, 2005)** found *Staphylococcus aureus* in 72.5 percent in Poland and 28.3 percent in Turkey, in samples of mastitic milk, respectively. *Staphylococcus aureus* and other pathogenic germ are most commonly found on the udder and teat surface of cows, they are the prime source of infection, which frequently occurs while milking. **(Abebe *et al.*, 2016).**

Table (3). Prevalence of mastitis (clinical and subclinical) in some dairy farms in Gharbia governorate (using CMT)

	Over all mastitis			Clinical mastitis		Subclinical mastitis	
	No. examination	No. positive	%	No.	%	No.	%
Farm level	15	11	73.3%	4	36.4%	7	63.6%
Cow level	150	43	28.7%	16	37.2%	27	62.8%
Quarter level	600	172	28.7%	73	42.4%	99	57.6%

Table (4). Prevalence of *Staphylococcus aureus* mastitis (clinical and subclinical) in some dairy farms in Gharbia governorate

	Mastitic cases caused by *St. aureus*			Clinical mastitis		Subclinical mastitis	
	No. examination	No. positive	%	No.	%	No.	%
Farm level	11	7	63.6%	4	57.1%	3	42.8%
Cow level	43	29	67.4%	17	58.6%	12	41.3%
Quarter level	172	31	18.02%	20	64.5%	11	35.5%

Antimicrobial susceptibility testing:

Antimicrobial sensitivity assay of the investigated isolates is depicted in (Table 5).The highest sensitivity was to vancomycin (87.1%), enrofloxacin (77.4%) and linezolid (74.2%). These findings somewhat agreed with **(Emmanuel and Magaji 2011)** who reported that *S. aureus* were sensitive to vancomycin which remained the most efficient treatment for *S. aureus* infection. such findings had been observed by **(Gucukoglu *et al.*, 2012 and Aydin *et al.*, 2011).** High activity of enrofloxacin, has been reported for *S. aureus* **(Sakwinska *et al.*, 2011) and (Jahan *et al.*, 2015).** Moderate sensitivity was noticed to gentamicin (54.8%), ciprofloxacin (48.4%) and cephalothin (45.2%). These agree with findings of previous research **(Oliveira *et al.*, 2012)**, moderet sensitivity rates to cephalosprins were observed in their study, and disagree with **(Sanders and Sanders 1986)** who reported that Cephalosporins have good to exceptional antibacterial action against Gram-positive bacteria. On the other hand, resistance rates were higher with kanamycin and lincomycin (100%), followed by penicillin (96.8%), oxacillin (96.3%), nalidixic acid (90.3%), oxytetracycline (83.9%), erythromycin (80.6%) and trimethoprim/sulfamethoxazole (74.1%). In previous research, comparable patterns of resistance against *S. aureus* have been documented. (**Muyiwa *et al.*, 2015** and **Kerouanton *et al.*, 2007).** Another study from China reported that antibacterial resistance was found in 77.3 percent of *Staphylococcus aureus* isolates **(Sudhan *et al.*, 2005),** While investigations in Denmark, Brazil and Argentina confirmed antimicrobial resistance rates of 75.0, 55.1 and 40.0 percent, respectively, **(Barkema *et al.*, 2006 and Schmid 2011).** Antimicrobial resistance was higher in our current study's *Staphylococcus aureus* isolates than in previous research **(Mehrotra *et al.*, 2000). Ben Said *et al.*, (2016)** reported that (62.8%) resistant to penicillin, also the high level of penicillin-resistance was detected by **(Nam *et al*. 2011).** The rates of erythromycin and lincomycin resistance identified in our study were significantly greater than those reported in other countries. **(Sakwinska *et al.*, 2011)**. Considering that 80.6% of the penicillin /lincomycin-resistant isolates were also resistant to macrolides (erythromycin), these results could indicate a risk of therapeutic failure for penicillin-resistant *Staph. aureus* which caused bovine mastitis. **(Constable *et al.*, 2008).** Macrolides and lincosamides (lincomycin) are the second-line antibacterial medicines in treatment of mastitis. Cross resistance between these classes

has been documented, it's because of the similarity of the mechanism of action on a subunit of the bacterial ribosome **(Matsuoka, 2000)**. High resistance rate of oxytetracycline in our study agrees with **(Kumar *et al.*, 2010** and **Kuang *et al.*, 2009)**. Who revealed that the tetracycline's low in vitro activity against *S. aureus*, combined with irreversible binding with milk constituents, lead to ineffective in treatment of bovine mastitis.

Table (5). Antimicrobial susceptibility of *S. aureus* strains isolated from bovine mastitis (subclinical and clinical mastitis) milk samples. (n=31).

Antimicrobial agent	Resistance		Sensitive	
	No.	%	No.	%
Kanamycin (K)	31	100	-	-
Lincomycin (L)	31	100	-	-
Oxacillin (OX)	30	96.3	1	6.5
Nalidixic acid (NA)	28	90.3	3	9.7
Penicillin (P)	30	96.8	1	6.5
Oxytetracycline (T)	26	83.9	5	16.1
Erythromycin (E)	25	80.6	6	19.4
Sulphamethoxazol (SXT)	23	74.1	8	25.8
Cephalothin (CN)	17	54.8	14	45.2
Ciprofloxacin (CP)	16	51.6	15	48.4
Gentamicin (G)	14	45.1	17	54.8
Linezolid (LZD)	8	25.8	23	74.2
Enrofloxacin (EN)	7	22.6	24	77.4
Vancomycin (V)	4	12.9	27	87.1

Table (6). Multidrug resistance (MDR) strain of *Staphylococcus aureus* isolates (n=31)

No. of Antibiotics	No. of Multidrug resistance strains	%
3	5	16.1
4	3	9.7
5	1	3.2
6	2	6.4
7	3	9.7

From the results in (table 6), 5 strains of *S. aureus* were resistant to 3 antibiotics out of 31 isolates (16.1%), 3 strains were resistant to 4 antibiotics **(9.7%)**, one strain was resistance to 5 antibiotics **(3.2**%) , 2 strains were resistant to 6 antibiotics (6.4%) and 3strains were resistant to 7 antibiotics **(9.7%)** . The remaining isolates were resistant to more than 7 antibiotics. These findings somewhat nearly agreed with **(Hoque, *et al.*, 2018)** who reported that 79.3% were resistant to at least one antimicrobial, while 49.0% to three or more antimicrobials. The acquisition of resistance (R. factor) that is plasmid-mediated may be to blame for the development of various antibiotic resistances amongst most of our isolates. **(Yamamoto *et al.*, 2013).**

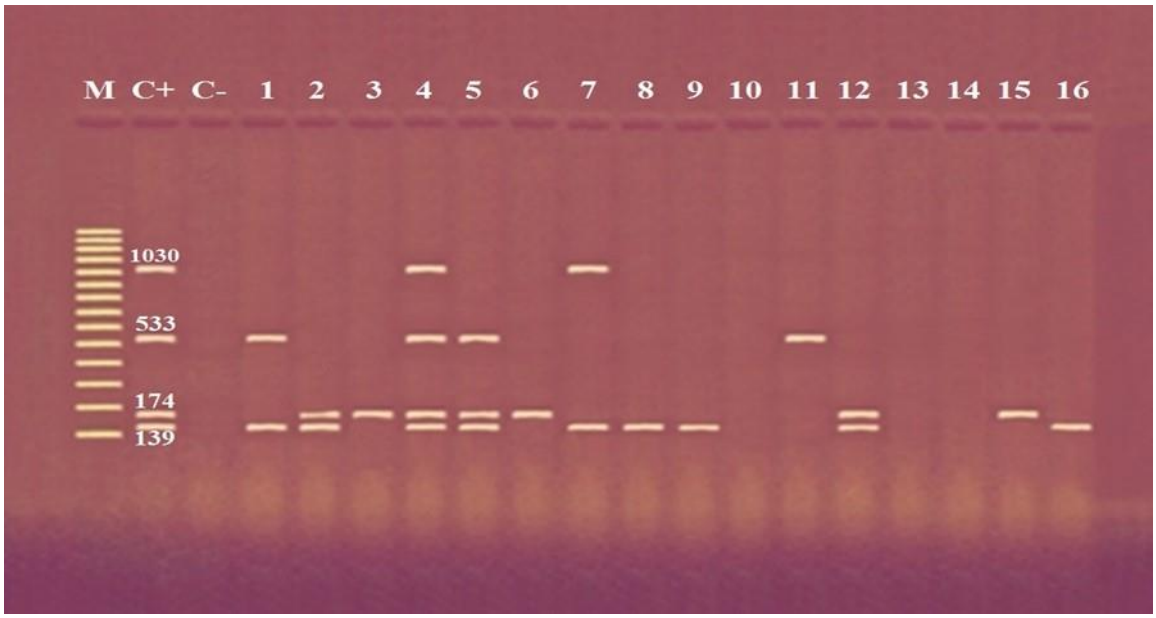

Fig. (1): Detection of *erm* A (139 bp), *aac* (6)-*aph* (2) (174 bp), *mec*A (533 bp) and *van*A (1030 bp) antibiotic resistance genes of *S. aureus* by agarose gel electrophoresis.

Lane M: marker of DNA (100 bp).
C+: Control positive for *erm*A, *aac* (6)-*aph* (2), *mec*A and *van*A genes. Lane
C-: Control negative.
Lane 1, 2, 4, 5, 7, 8, 9, 12 & 16: Positive for *erm*A gene.
Lane 2, 3, 4, 5, 6, 12 & 15: Positive for *aac* (6)-*aph* (2) gene.
Lane 1, 4, 5 & 11: Positive for *mec*A gene.
Lane 4 & 7: Positive for *van*A gene.
Lane 10, 13 & 14: Negative strains for *erm*A, *aac* (6)-*aph* (2), *mec*A and *van*A genes.

Table (7). Resistance genes in *Staphylococcus aureus* isolated from bovine mastitis milk samples

	No. of examined strains	**+ve *mec*Agene**		**+ve *van*Agene**		**+ve *erm*Agene**		**+ve *aac*(6)-*aph*(2)**	
		No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Subclinical mastitis	6	1	16,7%	0	0	4	66.7%	2	33.3%
Clinical mastitis	10	3	30%	2	20%	5	50%	5	50%
Total	16	4	25%	2	12.5%	9	56.25%	7	43.75%

(Table 7) (Fig.1). Detection rates of *mec*A, *erm*A, *van*A and *aac* (6)-*aph* (2) genes in the strains examined in this research were (25.0, 56.25, 12.5, and 43.75 percent, respectively), indicating a high incidence of methicillin resistant *Staph. aureus* (MRSA) isolated from mastitic cases. A 533-bp fragment denotes to *mec*A gene was recognized by 25%. MRSA (methicillin-resistant *Staph. aureus*) is a global threat, and *Staphylococcus aureus* that was isolated from cases of mastitis in cows had *mec*A gene **(Pajic *et al.*, 2014)**. Multiplex PCR indicated that 25 percent of MRSA isolates carried *mec*A genes, which agrees with the results of **(Turutoglu *et al.*, 2006)** who recorded prevalence rates of 20% and 17.5% of *mec*A genes. The existence of the *mec*A gene is widely believed to be the most documented way to detect methicillin resistance *staphylococci* (MRS), which are often resistant to the majority of antibiotics. Our findings revealed that 4 (25%) of the 16 oxacillin-resistant isolates had the *mec*A gene, indicating that they were MRSA. The absence of the *mec*A gene in the oxacillin-resistant strain makes us to believe that another mechanism of oxacillin resistance exists, such as hyper production of beta-lactamase or alteration of the penicillin binding protein (PBP). All penicillins are resistant against methicillin-resistant *staphylococci* (MRS), this resistance is imparted by an added penicillin-binding protein, which is absent in methicillin-sensitive strains **(Cloney *et al.*, 2001).** While using genotypic method to identify the existence of the *mec*A gene is regarded the gold standard, using a single phenotypic assay may result in false-negative or false-positive findings. **(Meucci *et al.*, 2010**). Amplification of a 174-bp fragment of the *aac*(6') *aph* (2") gene by PCR (aminoglycoside). Antibiotic sensitivity test to aminoglycoside (kanamycin) was determined by the disc diffusion assay, all tsted isolates were shown to be kanamycin resistant. After PCR test for the presence of *aac*(6') *aph*(2") gene, it was found that the *aac*(6') *aph*(2") gene was present in (43.75 percent) of the total sixteen isolates. The *aac* (6′) - *aph* (2") gene encodes a bifunctional enzyme that inactivates a wide variety of clinically relevant aminoglycosides, including gentamicin, tobramycin, netilmicin, and amikacin, **(Sandra et al., 2018)** detected *aac* (6′) - *aph*(2′) in *S. aureus* isolates by 20%. These results are consistent with our research, which found that all aminoglycoside-resistant bacteria carried aac(6′)- aph (2"). Amplification of (139 bp) fragment of *erm*A for erythromycin resistance isolates, they exhibited prevalence of 56.25% (9 out of 16 isolates). Our results almost agree with the results of **(Argudin *et al.*, 2012 and McCallum *et al.*, 2010),** who found resistance genes in 70% of erythromycin-resistant isolates (encoded by *erm*C, *erm*A, and *erm*B, alone or in combination). **Duran *et al.*, (2012)** reported that at least one of the erythromycin resistance genes (*erm*A, *erm*B and *erm*C,) was present in erythromycin-resistant isolates. The genes *erm*A and *erm*C were discovered by **Argudin *et al.*, (2012)** to be the cause of erythromycin resistance as well as stimulate clindamycin resistance. Two isolates (12.5 percent) tested positive for a 1030-bp fragment that corresponded to the *van*A gene. In furthermore, some investigations have observed the rise of vancomycin-resistant *S. aureus* **(Tenover *et al.*, 2004 and Ateba *et al.*,2010).**

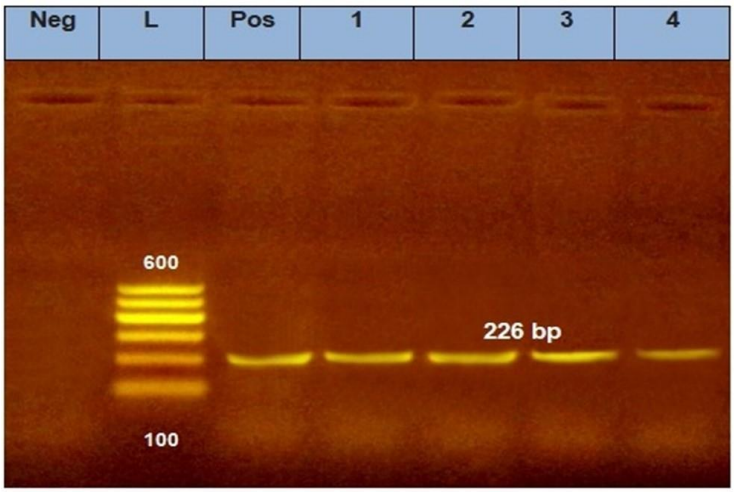

Fig. (2): Detection of *spa* gene at 226 bp by agarose gel electrophoresis. Lane L: marker of DNA (100 bp), (control positive: P, control negative: N). Lane (1, 2, 3, and 4): positive samples.

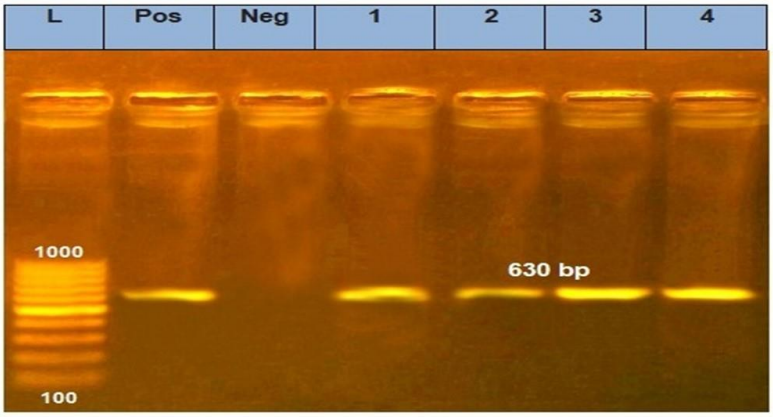

Fig. (3): Detection of *coa* gene at 630 bp by agarose gel electrophoresis. Lane L: marker of DNA (100 bp), (control positive: P, control negative: N). Lane (1, 2, 3, and 4) positive samples

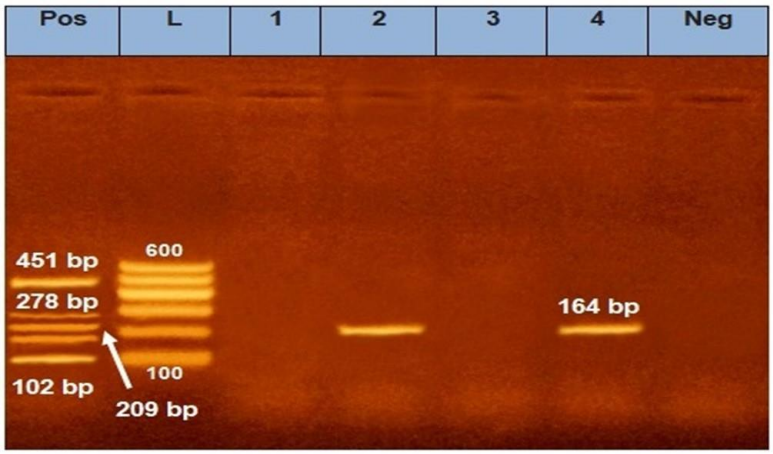

Fig. (4): Detection of enterotoxins *(sea,seb,sec,sed* and *see* gene at 102, 164, 451, 278 and 209 bp. by agarose gel electrophoresis. Lane L: marker of DNA (100 bp), (control positive: P, control negative: N). Lane (2, and 4) positive for *seb* only. Lane (1 and 3) negative for all enterotoxins.

The PCR findings indicate that our *S. aureus* strains were enterotoxigenic, positive for coagulase and *spa* gene. The gene encoding the IgG-binding region of protein (A) (*spa)* was amplified by PCR at 226-bp (100%) (Fig. 2).These findings were consistent with the results reported by **(Enany *et al.*, 2013)**. Some researchers have identified the protein A's immunoglobulin G binding region in different proportions as **(Mehndiratta, *et al.*, 2009**) (94.6%) and **(Bekhit *et al.*, 2010)** (32.4%). The amplification of the *coa* gene may be detected by four types of bands 430bp, 350 bp, 570 and 630 bp, amplification of *coa* gene at 630 bp for all strains (100%) as shown in (Fig. 3), these agree with **(Gharib *et al.*, 2013),** they observed that in bovine strains, an amplicon around 600 bp is prominent. On the other hand, from dairy cow *coa* gene of *S. aureus* isolates was found to be 650, 730, 810, and 1050 bp **(Schledgelova *et al.*, 2003),** between the isolates, the most prevalent class was 739 bp. *Staphylococcal* enterotoxins which are resistant to heat and gastrointestinal protease are another virulence factor to be concerned about, this explains its activity at high temperatures and multiplex PCR was used to detect the genes that code for these enterotoxins. *S. aureus* strains linked to bovine mastitis produce *sea, seb, sec, sed*, and *see*, according to **(Rall *et al.*, 2008).** Our results showed that *seb* gene was the predominant enterotoxigenic gene with percentage of (50%). It was possible to identify this gene at high rate in the strains isolated from subclinical cases, *sea*, *sed*, *sec*, and *see* genes were not present in any of the isolates (Fig. 4). Numerous toxin genes in *S. aureus* were previously thought to be uncommon **(Jorgensen *et al.*, 2005).** From country to country, the prevalent classical SE changed, *see* gene **(Sahebekhtiari *et al.*, 2011)** in Tehran; *see* and *sea* gene **(Mehrotra *et al.*, 2000)** in Canada; *sea* gene **(Rall *et al.*, 2014)** in Brazil; *sec* gene **(Khudor *et al.*, 2012)** and **(Neder *et al.*, 2011)** in Iraq and Argentina, respectively. *sed* gene is a gene that is found in Italy **(Carfora *et al.*, 2015)**, in Hungary, *seb* gene **(Zouharova and Rysanek, 2008).**

A previous study, (**Turutoglu *et al.*, 2006)** found 67.8% of *Staphylococcus aureus* isolates were positive for one or more enterotoxin genes, which is remarkably similar to our current results. In addition, according to our results, majority (50%) of our isolates were having one type of enterotoxin, and these were also resistant to a variety of medicines, this conclusion is backed up by the findings of **(Nazneen *et al.*, 2014),** who found 58.3 percent of *Staphylococcus aureus* had MDR characteristics. Even though the subclinical isolates had more enterotoxin genes than clinical, their antibacterial sensitivity profiles were not significantly different. As a result, it's possible that the presence or lack of enterotoxins has little effect on the resistance traits of *Staphylococcus aureus* isolates.

Conclusion and Recommendation

-Mastitis is the most dangerous infectious illness that affects dairy cattle worldwide, it continues to be a continual challenge in dairy products manufacturing.

-*Staphylococcus aureus* microbe is one of the most dangerous microbes that cause mastitis, most isolates have virulence genes which responsible for the pathogenicity of the organism.

-Also our study proved that a lot of isolates that have been isolated from mastitic milk was resistant to more than 3 antibiotics, we were able to detect the antibiotics resistant genes by PCR.

- High density of animals, high milk production and entry of dairy cows from other farms are among reasons for the spread of the infection

-Dairy farmers have the unavoidable responsibility for successful managing, prevention, and treatment of mastitis cases. Mastitis caused by *Staphylococcus aureus* is highly diverse and is influenced by strain-specific characteristics. Therefore, it has a priority to conduct extensive studies in molecular characterization.

-In addition to the importance of isolating this microbe and determining its spread and cause of mastitis

This requires strict management of farms and following the necessary health instructions and procedures during milking, storage, handling and transportation, these are important points for *Staphylococcus aureus* infection.

-In dairy herds, CMT is used to discover subclinical mastitis periodically to avoid conversion to clinical mastitis.

-The poor use of antibiotics in dairy farms is one of the most important factors that cause the emergence of antibiotic-resistant bacteria.

-Epidemiological studies are required to continuous monitoring of antibiotic resistance to *S. aureus* strains isolated from various sources in a specific area, as well as to apply trustable genetic studies which might enable the detection of the source contamination within the food chain. This would drastically minimize the spread of these bacteria to customers, as well as the prevalence of *staphylococcal* infections in humans.

Reference

Aarestrup F.M.; Wegener H.C. and Collignon, P. (2008). Resistance in bacteria of the food chain: Epidemiology and control strategies. Expert Rev. Anti. Infect. Ther., 6 (5): 733-750.

Abebe R.; Hatiya H.; Abera M.; Megersa B. and Asmare K. (2016). Bovine mastitis prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. BMC Vet Res 2016; 12: 270.

Amghalia, E.; AL-Haj, N.; Shamsu-din, M.; Radu, S.; Rosli, R.; Neela, V. and Rahim, R. (2009). Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in *Staphylococcus aureus* isolated from Malaysian hospitals. Res. J. Biol. Sci., 4 (4): 444-448.

Argudin, M.A.; Mendoza, M.C.; González-Hevia M.A.; Bances M.; Guerra B. and Rodicioa M.R. (2012). Genotypes, exotoxin gene content, and antimicrobial resis-tance of *Staphylococcus aureus* strains recovered from foods and food handlers. *Appl. Environ. Microbiol.*, 78(8): 2930-2935.

Ateba, C.N.; Mbewe, M.; Moneoang, M.S. and Bezuidenhout, C.C. (2010). Antibiotic-resistant *Staphylococcus aureus* isolated from milk in the Mafikeng Area, North West province, South Africa. *S. Afr. J. Sci.*, *106*, 1 –6.

Aydin A.; Sudagidan M.and Muratoglu K. (2011). Prevalence of *staphylococcal* entero-toxins, toxin genes and genetic-relatedness of foodborne *Staphylococcus aureus* strains isolated in the Marmara Region of Turkey. *Int. J. Food Microbiol.* 2011, *148*, 99–106.

Bangar Y.C.; Verma M.R.; Dohare A.K. and Reena Mukherjee R. (2016). Meta-analysis of prevalence of clinical mastitis in crossbred cows in India (1995–2014). J Anim Res 2016; 6: 933–8.

Barkema H.W.;Schukken Y.H.and Zadoks R.N. (2006). The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. J Dairy Sci 2006;89:1877–95.

Bauer A.W.; Kirby W.M.; Sherris J.C.and Turck M. (1999): Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol, 45(4): 493-496.

Bekhit M.M.S.; Muharram M.M.; Alhousiny I.M. and Hashem M.E.S.Y. (2010): Molecular detection of genes encoding virulence determinants in *Staphylococcus aureus* strains isolated from bovine mastitis. J. Appl. Sci. Res., 6(2): 121-128.

Ben Said M.; Abbassi1 M.S. ; Bianchini V.; Sghaier S.; Cremonesi P.; Roman A.; Gualdi V.; Hassen A. and Luini M.V. (2016): Genetic characterization and antimicrobial resistance of *Staphylococcus aureus* isolated from bovine milkApplied Microbiology 63, 473--481.

Brasil (2003): Ministério da Agr icultura, Pecuária e Abastecimento. Instrução Normativa, n 62 de 26 de agosto de. Métodos Analíticos Oficiais para Análises Microbiológicas para Controle de Produtos de Origem Animal e Água.

Buhlmann M.; K Bögli-Stuber K.; Droz S. and Muhlemann K. (2008): Rapid scr eening for carriage of methicillin-resistant *Staphylococcus aureus* by PCR and associated costs. J. Clin. Microbial, 46 (7): 2151–2154.

Carfora V.; Caprioli A.; Marri N.; Sagrafoli D.; Boselli C.; Giacinti, G.;Giangolini G., Sorbara L.; Dottarelli S.; Battisti A. and Amatiste S. (2015): Enterotoxin genes, enterotoxin production and methicillin resistance in *Staphylococcus aureus* isolated from milk and dairy products in Central Italy. International Dairy Journal,42: 12-15.

Cervinkova D.; Vlkova H.; Borodacova I.; Makovcova J.; Babak V. and Lorencova A. (2013): Prevalence of mastitis patho- gens in milk from clinically healthy cows. Vet Med ;58:567–75.

Clinical Laboratory Standards Institute (CLSI) (2013): Performance Standards for Antimicrobial SusceptibilityTesting; Twenty-third Informational Supplement; M100-S23.

Cloney L.; Marlowe C.; Wong A.; Chow R. and Bryan R. (2001): Rapid detection of *mecA* in methicillin resistant *Staphylococcus aureus* using cycling probe technology. *Mol. Cell. Probes* 13:191–197.

Constable P.D.; Pyörälä S.and Smith G.W. (2008): Guidelines for antimicrobial use in cattle. In: Guide to antimicrobial use in animals, Eds. Guardabassi L., Jensen L.B., Kruse H., 143-160, Blackwell Publishing, Oxford, UK.

De Oliveira A.P.; Watts J.L.; Salmon S.A. and Aarestrup F.M. (2000): Antimicr obial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Europe and United States. J Dairy Sci; 83:855–62.

Duran N.; Ozer B.; Duran G.G.; Onlen Y. and Demir C. (2012): Antibiotic resistance genes and susceptibility pat-terns in *staphylococci*. Indian J. Med. Res., 135(3): 389-396.

Elango A.; Doraisamy K. A.; Rajarajan G. and Kumaresan G. (2010): Bacteriology of sub-clinical mastitis and anti-biogram of isolates recovered from cross-bred cows. Indian J Anim Res ;44:280–4.

Elsayed M.S.; El-Bagoury A.M. and Dawoud MA. (2015): Phenotypic and genotypic detection of virulence factors of *Staphylococcus aureus* isolated from clinical and subclinical mastitis in cattle and water buffaloes from different farms of Sadat City in Egypt. Vet World; 8:1051–8.

Emmanuel Onwubiko Nwankwo and Magaji Sadiq Nasiru (2011): Antibiotic sensitivity pattern of *Staphylococcus aureus* from clinical isolates in a tertiary health institution in Kano, Northwestern Nigeria Pan Afr Med J. 2011; 8: 4.

Enany M.E.; Younes S.; A lgammal A.M.; Salem M. and El Dieb, H.A. (2013): Phenotypic and genotypic characterization of *Staphylococcus aureus* isolated from clinical and subclinical bovine mastitis. SCVMJ, XVIII, (1) 139-147.

Food and Agriculture Organization (FAO) (2014): Impact of mastitis in small scale dairyproduction systems. Animal Production and Health Working Paper. No. 13.

Foster T. (2005): Immune evasion by *staphylococci*. Nat. Rev. Microbiol., 3(12):948-958.

Frey Y.; Rodriguez J.P.; Thomann A.; Schwendener S.and Vincent P. (2013): Genetic characterization of antimicrobial resistance in coagulase-negative *Staphylococci* from bovine mastitis milk. J Dairy Sci; 96: 2247–57.

Gharib A.A.; Attia M.A.A. and Bendary M.M. (2013): Detection of the coa gene in *Staphylococcus aureus* from different sources by polymerase chain reaction. BendaryIntl. J. Microbiol. Res., 4 (1): 37-42.

Gillespie B.E.; Headrick S.I.; Boonyayatra S.and Oliver S.P. (2009): Prevalence and persistence of coagulase-negative *Staphylococcus* species in three dairy research herds. Vet Microb ;134:65–72.

Gucukoglu A.; Onur Kevenk T.; Uyanik T.; Çadirci Ö.; Terzi G.and Alişarli, M. (2012): Detection of enterotoxigenic *Staphylococcus aureus* in raw milk and dairy products by multiplex PCR. J. Food Sci., 77, M620–M623.

Guler L.; Ok Ü.; Gündüz K.; Gülcü Y. and Hadimli H.H. (2010): Antimicr obial susceptibility and coagulase gene typing of *Staphylococcus aureus* isolated from bovine clinical mastitis cases in Turkey. J Dairy Sci; 88:3149–54.

Hameed S.; Arshad M.; Ashraf M.; Avais M. and Shahid MA. (2012): Cross-sectional epidemiological studies on mastitis cattle and buffaloes of Tehsil Burewala, Pa-

kistan. J Anim Plant Sci; 22:371–6.

Hendriksen R.S.; Mevius D.J.; Schroeter A.; Teale C.; Meunier D. and Butaye P. (2008): Prevalence of antimicrobial resistance among bacterial pathogens isolated from cattle in different European countries: 2002–2004. Acta Vet Scand; 50:28.

Holmes M.A. and Zadoks R.N. (2011): Methicillin resistant S. aureus in human and bovine mastitis. J Mammary Gland Biol Neoplasia, 16: 3733-82.

Hoque M.N.; Das Z.C.; Rahman A.N.M.A; Haider M.G. and Islam M.A (2018): Molecular characterization of *Staphylococcus aureus* strains in bovine mastitis milk in Bangladesh. International Journal of Veterinary Science and Medicine 6 (2018) 53–60.

Hoque M.N.; Das Z.C.; Talukder A.K.; Alam M.S. and Rahman A.N.M.A. (2015): Different screening tests and milk somatic cell count for the prevalence of subclinical bovine mastitis in Bangladesh. Trop Anim Health Prod; 47:79–86.

Iyer A.P. and Kumosani T.A. (2011): PCR based detection of nosocomial infection causing MRSA (Methicillin resistant *Staphylococcus aureus*). 2nd International Conference on Biotechnology and Food Science IPCBEE vol.7 (2011) © (2011) IACSIT Press, Singapore.

Jahan Mueena; Marzia Rahman1; Md. Shafiullah Parvej; Shah Md. Ziqrul Haq Chowdhury; Md. Enamul Haque; Md. Abdul Khaleque Talukder and Sultan Ahmed.(2015): Isolation and characterization of *Staphylococcus aureus* from raw cow milk in Bangladesh. J. Adv. Vet. Anim. Res., 2(1): 49-55.

Jarassaeng C.; Aiumlamai S.; Wachirapakorn C.; Tchakumphu M.; Noordhuizen JPTM and Beynen A.C. (2012): Risk factors of sub-clinical mastitis in small-holder dairy cows in KhonKaen Province. Thai J Vet Med; 42:143–51.

Jha A.K.; Hoque M.N.; Kamal M.M.; Rahman M.M.; Bhuiyan M.M.U. and Shamsuddin M. (2010): Prevalence of mastitis and efficacy of different treatment regimens on clinical mastitis in cows. SAARC J Agric; 8:79–89.

Jorgensen H.J.; Mork T.; Hogasen H.R. and Rorvik L.M. (2005): Enterotoxigenic *Staphylococcus aureus* in bulk milk in Norway. J. Appl. Microbiol., 99: 158- 166.

Kerouanton A.; Hennekinne J.; Letertre C.; Petit L.; Chesneau O.; Brisabois A. and Buyser M. (2007): Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. Int. J. Food Microbiol. 2007, 115, 369–375.

Khudor M.H.; Abbas B.A. and Idbeis H.I. (2012): Detection of enterotoxin genes of *Staphylococcus aureus* isolates from raw milk. Bas. J. Vet. Res., 11(1): 254-264.

Kirkan S.; Goksoy E.O. and Kaya O. (2005): Identification and antimicrobial susceptibility of *Staphylococcus aureus* and coagulase negative *Staphylococci* from bovine mastitis in the Aydin Region of Turkey. Turk J Vet Anim Sci; 29:791–6.

Koneman E.W.; Allen S.D.; Janda W.M.; Schreckenberger M.P.C. and Winn Jr.W.C. (2001): DiagnósticoMicr obiológico - Texto e Atlas Colorido, 5ª ed. MEDSI, São Paulo.

Koskinen M.T.; Holopainen J.; Pyörälä S.; Bredbacka P.; Pitkälä A. and Barkema H.W. (2009): Analytical specificity and sensitivity of a real-time polymerase chain reaction assayfor identification of bovine mastitis pathogens. J Dairy Sci; 92:952–9.

Kumar R.; Yadav B.R. and Singh R.S. (2010): Genetic determinants of antibiotic resistance in *Staphylococcus aureus* isolates from milk of mastitic crossbred cattle. Curr Microbiol, 60: 379-386.

Matsuoka M. (2000): Study of macrolide, lincosamide, and streptogramin B antibiotics resistance in *Staphylococcus aureus*. Yakugaku Zasshi, 120: 374-386.

McCallum N.; Berger-Bachi B. and Senn M.M. (2010): Regulation of antibiotic resistance in *Staphylococcus aureus*. Int. J. Med. Microbiol., 300: 118-129.

Mehndiratta P.; Bhalla P.; Ahmed A. and Sharma, Y. (2009): Molecular typing of methicillin resistant *Staphylococcus aureus* strains by PCR-RFLP of SPA gene: a reference laboratory perspective, Indian Journal of Medical Microbiology,27(2):116-122.

Mehrotra M.; Wang G. and Johnson W.M. (2000): Multiplex PCR for Detection of

Genes for *Staphylococcus aureus* Enterotoxins, Exfoliative Toxins, Toxic Shock Syndrome Toxin 1, and Methicillin Resistance. Journal of clinical microbiology. Vol. 38, No.3.

Meucci V.; Vanni M.; Guardabassi L.; Moodley A. Soldani G.and Intorre L. (2010): Evaluation of methicillin resistance in *Staphylococcus intermedius* isolated from dogs. Vet Res Commun, 34: S79-S82.

Muyiwa Ajoke Akindolire ; Olubukola Oluranti Babalola and Collins Njie Ateba. (2015): Detection of Antibiotic Resistant *Staphylococcus aureus* from Milk: A Public Health Implication. Int. J. Environ. Res. Public Health 2015, 12, 10254-10275.

Nam H.M.; Lee A.L.; Jung S.C.; Kim, M.N.; Jang G.C.; Wee S.H. and Lim S.K. (2011): Antimicrobial susceptibility of *Staphylococcus aureus* and characterization of methicillin resistant Staphylococcus aureus isolated from bovine mastitis in Korea. Foodborne Pathog Dis 10, 231–238.

Neder V.E.; Canavesio V.R. and Calvinho L.F. (2011): Presence of enterotoxigenic *Staphylococcus aureus* in bulk tank milk from Argentine dairy farms.Revista Argentina de Microbiología, 43: 104- 106.

Nazneen N.I.; Farzana Z.; Chowdhury A.M.M.A. Mannan A. Kamaruddin K.M. and Siddiki A.M.A.M.Z. (2014): Characterization of bovine subclinical mastitis caused by *Staphylococcus aureus* in southern Bangladesh by bacteriological and molecular approaches. Asian J Biol Sci 2014; 7:1–12.

Oliveira L.; Langoni H.; Hulland C. and Ruegg P.L. (2012): Minimum inhibitory concentrations of *Staphylococcus aureus* recovered from clinical and subclinical cases of bovine mastitis. J Dairy Sci, 95: 1913-1920.

Pajic M,J. ; Rasic Z.B.; Velebit B.M.; Bobos S.F.; Mihajlovic M.M. and Radinovic M.Z. (2014): The prevalence of methicillin resistance and Panton-Valentine leukocidin synthesis genes in *Staphylococcus aureus* isolates of bovine and human origin. Vet Arch; 83: 205–14.

Palma M.; Haggar A. and Flock J. (1999): Adherence of *Staphylococcus aureus* is enhanced by an endogenous secreted protein with broad binding activity. J. Bacteriol., 181 (9):2840- 2845.

Piepers S.; DeMeulemeester L.; deKruif A.; Opsomer G.; Barkema H.W. and DeVliegher S. (2007): Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. J Dairy Res ;74:478–83.

Pu W.; Su Y.; Li J.; Li C.; Yang Z.and Deng H. (2014): High Incidence of Oxacillin-Susceptible mecA-Positive *Staphylococcus aureus* (OS-MRSA) Associated with bovine mastitis in China. PLoS ONE ;9:e88134.

Rall V.L.; Miranda E.S.; Castilho I.G.; Camargo C.H.; Langoni H.; Guimarães F.F.; Araújo Júnior J.P.and Fernandes Júnior, A. (2014): Diver sity of *Staphylococcus* species and prevalence of enterotoxin genes isolated from milk of healthy cows and cows with subclinical mastitis. J. Dairy Sci., 97 (2):829-37.

Rall V.L.M.; Viera F.P.; Rall, R., Vites R.L. Fernandes A. J.; Candeis J.M.G.; Cardoso K.F.G. and Araujo, J.P. J. (2008): PCR detection of *S. aureus* strains isolated from raw and pasteurized milk. Vet. Microbial., 132(3-4): 408-413.

Reinoso E.B. (2004): Análisis epidemiológicoy molecular de cepas de *Staphylococcus aureus* de distintosorígenes. Tese de Doutorado, Instituto de Microbiologia, Universidad Nacional de Rio Cuarto, Argentina. 199p.

Sahebekhtiari N.; Nochi Z.; Eslampour M.A.; Dabiri H.; Bolfion M.; Taherikalani M.,;Khoramian B.; Zali M.R. and Emaneini, M.(2011): Characterization of *S. aureus* strains isolated from raw milk of bovine subclinical mastitis in Tehran and Mashhad. Acta Microbial. Immunol. Hung., 58 (2):113-121.

Sakwinska O.; Morisset D.; Madec J.Y., Waldvogel A.; Moreillon P. and Haenni M. (2011): Link between genotype and antimicrobial resistance in bovine mastitis-related *Staphylococcus aureus* strains, determined by comparing Swiss and French isolates from the Rhône Valley. Appl Environ Microbiol, 77: 3428-3432.

Sambrook X.; Fritsch E.F. and Maniatis T. (1989): Molecular Cloninga Laboratory Manual. 2nd ed. Cold Spring Harbor, New York, Laboratory Press.

Sandra G.; Zárate M.; Luisa De la Cruz; Claure Raúl; Benito-Arenas ;Julia Revuelta ;Andrés G.; Santana and Agatha Bastida (2018): Overcoming Aminoglycoside Enzymatic Resistance: Design of Novel Antibiotics and Inhibitors. Molecules 2018, 23 (2), 284.

Sanders C.C. and Sanders W.E. (1986): The cephalosporins and cephamycins. In: The Antimicrobial Agents Annual 1, Eds. Peterson P.K., Verhoef J., 66-90, Elsevier Scientific Publishers, New York, N.Y.

Schmidt T. (2011): In vitro antimicrobial susceptibility of *Staphylococcus aureus* strains from dairy herds in KwaZulu-Natal. J South Afr Vet Assoc; 82:76–9.

Schlegelova J.; Dendis M.; Benedik J.; Babak, V. and Rysanek, D. (2003): *Staphylococcus aureus* isolates from dairy cow and human on a farm differ in coagulase genotype. J. Vet. Micr., 92: 327-334.

Seegers H.; Fourichon C. and Beaudeau F. (2003): Production effects related to mastitis and mastitis economics in dairy cattle herds. Vet Res, 34: 475-491.

Sudhan N.; Singh A.R.; Singh M. and Soodan J.S. (2005): Studies on prevalence, etiology, and diagnosis of sub clinical mastitis among cross breed cows. Indian J Anim Res ;39:127–30.

Taponen S.; Salmikivi L.; Simojoki H.; Koskinen T. and Pyörälä S. (2009): Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. J Dairy Sci; 92:2610–7.

Tenhagen B.A.; KoSter G.; Wallmann J. and Heuwieser W. (2006): Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. J Dairy Sci ; 89:2542–51.

Tenover F.C.; Weigel L.M.; Appelbaum P.C.; McDougal L.K.; Chaitram J.; McAllister S.; Clark N.; Killgore G.; O'Hara C.M. and Jevitt, L. (2004): Vancomycin-resistant *Staphylococcus aureus* isolate from a patient in Pennsylvania. Antimicrob. Agents and Chemother, 48, 275–280.

Turutoglu H.; Ercelik S. and Ozturk D. (2006): Antibiotic resistance of *Staphylococcus aureus* and coagulase-negative *Staphylococci* isolated from bovine mastitis. Bull Vet Inst Pulawy; 50:41–5.

Wada, M.; Lkhagvadorj, E.; Bian, L.; Wang, C.; Chiba, Y.; Nagata, S.; Shimizu, T.; Yamashiro, Y.; Asahara, T. and Nomoto, K. (2010): Quantitative rever se transcription-PCR assay for the rapid detection of methicillin-resistant *Staphylococcus aureus*. Journal of Applied Microbiology 108 (2010) 779–788.

Wang X.; Meng J.; Zhang J.; Zhou T.; Zhang Y. and Yang B. (2012): Characterization of *Staphylococcus aureus* isolated from powdered infant formula milk and infant ricecereal in China. Int. J Food Microb; 153:142–7.

Wayne P.A.; Elango A.; Doraisamy K.A.; Rajarajan G. and Kumaresan G. (2010): Bacteriology of sub-clinical mastitis and anti -biogram of isolates recovered from cross-bred cows. Indian J Anim Res 44:280–4.

Yamamoto T.; Hung W.C.; Takano T. and Nishiyama (2013): A. Genetic nature and virulence of community-associated methicillin-resistant *Staphylococcus aureus.* BioMedicine 2013, 3, 2–18.

Zouharova M. and Rysanek D. (2008): Multiplex PCR and RPLA identification of *Staphylococcus aureus* enterotoxigenic strains from bulk tank milk. Zoo. Pub. HIth., 55(6): 313-319.