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Mastitis caused by methicillin resistant *staphylococcus aureus* (MRSA)

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ABSTRACT

Mastitis is one of the most common diseases in dairy cattle and cause great economic loss to the dairy industry', this study will shine a spotlight on *Staphylococcus aureus* isolates with particular reference to MRSA strains from milk of mastitic cows, as well as its in-vitro antimicrobial sensitivity. And biochemical blood changes. Our result indicates that out of 300 samples of quarter milk from cows showing signs of mastitis, 50 blood samples from mastitic cows and 10 blood samples from apparently healthy contact cows in Giza Provence; the results show that S. aureus was isolated from 100/300 samples with an incidence of 33.3%. of which 36/100 strains are Antibiotic resistant to at least one antibiotic was obtained through the results of antibiotic sensitivity tests. 10/100 strains of S. aureus were multi-drug resistant to more than three Antibiotics. 8/100 of strains are confirmed methicillin resistant Staphylococcus aureus (MRSA) by Vitek 2 Compact System's declaration and the presence of the resistance MecA gene in MRSA strains was confirmed by PCR results. Significant increases in WBCs, neutrophils, lymphocytes, malondialdehyde, total protein, and globulin, AST, ALT, and ALP are also caused by mastitis. Mastitis also causes a significant decrease in RBCs, Hb, PCV percent albumin/globulin (A/G ratio), catalase, and super oxide dismutase. It is possible to draw the conclusion that mastitis has a negative impact on the hemogram, liver, and kidney functions of cattle in a number of ways.

INTRODUCTION

Staphylococcus aureus (S. aureus) is one of the leading sources of intra-mammary infections in dairy cows **(Dufour et al. 2012; Zecconi and Scali, 2013).** It is reported that 10–40% of the mastitis cases are caused by S. aureus (**Kateete et al. 2013; Basanisi et al. 2017; Liu et al. 2017).** Mastitis is a global challenge that it can result in financial losses for the dairy industry and the economy due to the substandard quality of milk, treatment costs, and causing

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subsequent new infection of other cows **(Schroeder, 2012).**

Contaminated raw milk at farm level, may lead to subsequent problems further along the food chain giving rise to S. Aureus associated food contamination **(Jakobsen et al. 2011; Rola et al. 2016).**

Nowadays antibiotics are widely used in feed to promote growth in animals which led to Antibiotic resistance in bacteria the main issues affecting public health **(Oniciuc et al. 2017).**

Due to a modified penicillin binding protein (PBP 2a) with decreasing affinity for beta lactams, the resistance to methicillin and other beta lactams was caused. The staphylococcal cassette chromosome mec (SCCmec) contains the mecA gene, which encodes this protein **(Paterson et al. 2014).** Since MRSA is the primary pathogen in livestock animals that can infect humans, it has become a major public health concern **(Doulgeraki et al. 2017 Nam et al. 2011).** which first revealed in 2012 that the bacteria were isolated from dairy cows **(Paterson et al. 2012).**

Because it can spread methicillin resistance to people through milk or food, MRSA is extremely important for public health. **(Lozano et al. 2016; Tenhagen et al. 2018).**

This study sought to evaluate some S. aureus and antibiotic resistance factors found in raw mastitic milk samples and obtained through bacteriological and molecular methods with regard to MRSA strains. The following procedures were therefore planned as S. aureus isolation from raw milk samples in order to fulfill the study's objectives. Isolate identification through biochemistry. Isolates of S. aureus were tested for antibiogram. Antibiotic resistant strains are identified using the conventional diffusion method, which conforms to the VITEK® 2 compact system, and is followed by the detection of MRSA strains using PCR by detecting resistance genes in tested isolates in addition to effect mastitis hematobiochemical parameters.

MATERIAL AND METHOD

A total of 300 samples of mastitic cow milk (quarter milk sample) and 50 blood samples of mastitic cow were collected, 10 blood samples from apparently healthy contact cows.2 blood sample of each cow. To obtain clear serum for the estimation of serum hematobiochemical parameters sample was taken in a centrifuge tube but blood samples for RBCs, Hb, PCV, were taken in tubes containing EDTA, At the Giza Governorate, samples were taken from individual households and farms. Immediately after collection, samples were transported in an ice box to the lab for bacteriological analysis.

Isolation and identification of S. aureus.

Milk samples were centrifuged at 3000 rpm for 10 minutes to isolate S. aureus, after which the sediments were cultured on Baird-Parker agar (BPA) and Mannitol salt agar (MSA) (Oxoid, England) and were incubated at 37°C in an aerobically for 24-48 hours. The morphological characteristics, Gram-staining, coagulase test, and catalase test results helped identify the isolates as S. aureus **(APHA, 1992).**

Identifying some S. aureus isolate virulence factors.

The formation of biofilms and hemolytic activity in S. aureus isolates were all examined in this study. Based on Boerlin et al.'s explanation, (2003). hemolytic activity was measured. According to **Buxton (2005).**

The Congo red agar (CRA) test, as described by **Freeman et al. (1989).,** was used to assess the isolates' capacity for biofilm formation and the growth of rough, black colonies point to slime forming strains .

Testing the susceptibility of S. aureus isolates to various antibiotics.

Oxacillin (ox) (1ug) Penicillin (p) (10 ug) Amoxicillin/clavulanic acid (A/c) (30ug) Erythromycin (E) (15 ug), gentamicin (GN) (10 ug), ciprofloxacin (CIP) (5 ug), tetracycline (TE) (30 ug), chloramphenicol (C) (30 ug) and Vancomycin (V) (30 ug) were tested against S. aureus isolates using the Kirby-

Bauer disk diffusion method (Oxoid, UK). An aliquot of each tested isolate's suspension (0.5 McFarland) was spread plated onto Mueller-Hinton agar and the inoculated plate was then incubated for 24 hours under aerobic conditions at 37°C. The diameters of the inhibition zones were then measured and interpreted in accordance with CLSI (2017). MDR was defined by **Dai et al. (2019)** as resistance to three or more antibiotics. The more recent VITEK 2 Compact method, which is used in low to middle-level clinical laboratories, was used to further identify isolates and determine their antibiotic sensitivity. The 21 CFR 11 compliance and a colorimetric reagent card (BCL) for identifying spore-forming Gram-positive bacilli like Bacillus and related species are features for industrial microbiology. The other BCLs (YST, GN, and GP) were used in all system formats for clinical and industrial applications.

Molecular identification of some antibiotic resistance and virulence Factors in some MRSA isolates:

All of the examined MRSA isolates (n=10) were genotypically identified as S. aureus through identification of the 16s rRNA gene of S. aureus, as shown in Fig. (1) In addition, it was discovered that all of the MRSA isolates under examination carry (80%) of them have the mecA gene Figs. (2). The phenotypic and genotypic characteristics of the MRSA isolates under investigation were compiled in Table (4)

Table 1. Target genes in the study, primers sequences and PCR conditions used:

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
aacC	GGCGCGATCAAC-GAATTTAT-CCGA CCATTCGATGCCGAAGGA AACGAT	448	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Lynne et al., (2008)
bla ctx-m	ATGTGCAGCACCAGTAAA GT ACCGCGATATCGTTGGTG G	545	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Mendonça et al., (2007)
tet	GCYRTVGGSATHGGCYTK RTYATGC AC- MGCMCCWGTVGCBCCKG TGAT	293	94˚C 5 min.	94˚C 30 sec.	60˚C 45 sec.	72˚C 30 sec.	72˚C 10 min.	Schnabel et al., (1999)

RESULTS

Prevalence of S. aureus among the examined raw cow's milk samples:

Out of 300 samples examined, 100 samples were positive for *S. aureus* on MSA and BPA with percentage of (33.3%). *S. aureus* isolates produced yellow colonies on MSA while produced black colonies surrounded by opaque zone on BPA. They were catalase and coagulase positive and they appeared as gram positive cocci arranged in clusters resemble bunches of grapes on microscopical examination.

Results of determination some virulence factors of S. aureus isolates:

On blood agar media, (68.7%) of *S. aureus* isolates produced β-hemolysis while (31.3%) of them produced α-hemolysis. On the other hand, (46.3%) of *S. aureus* isolates showed CRB activity.

Antimicrobial susceptibility testing and MDR profiles of S. aureus isolates:

Antimicrobial susceptibility testing of *S. aureus* isolates (n=100) revealed the highest sensitivity to vancomycin (100%), followed by amoxicillin/clavulanic acid and gentamycin (97% for

each), ciprofloxacin (92%) and erythromycin (82%), while the highest resistance to oxacillin (71%) followed by tetracycline (67%), chloramphenicol (60%) and penicillin (48%) (Table 2).

On the other hand, it was found that (36%) of *S. aureus* isolates were resistant to 3 antibiotics or more (MDR) and that 27 (75%) of these MDR isolates were MRSA, (Resistant to Oxacillin with other 2 antibiotics at least) So that we select 10 strains of suspected MRSA strains for molecular identification by PCR we found that 8 strains only contain MecA gen. in table (4). While 9 (25%) of MDR strains of S. aureus were MSSA (but still Resistant to Chloramphenicol, ***Ciprofloxacin, Penicillin, Tetracycline and Erythromycin)*** shaded in table (3)

Table (2) Results of antimicrobial susceptibility of S. aureus isolates.

Antibiotic	Disc conc.	Result					
		Sensitive		Intermediate		Resistance	
		NO.	%	NO.	%	NO.	%
Oxacillin	1 µg	29	29.0%	0	0.0%	71	71.0%
Penicillin	10 µg	52	52%	0	0.0%	48	48.0%
Amoxicillin / clavulanicacid	30 µg	97	97.0%	0	0.0%	3	3.0%
Tetracycline	30 µg	31	31.0%	2	2.0%	67	67.0%
Erythromycin	15 µg	55	55.0%	27	27.0%	18	18.0%
Ciprofloxacin	5 µg	85	85.0%	7	7.0%	8	8.0%
Chloramphenicol	30 µg	31	31.0%	9	9.0%	60	60.0%
Gentamycin	10 µg	97	97.0%	3	3.0%	0	0.0%
Vancomycin	30 µg	100	100.0%	0	0.0%	0	0.0%

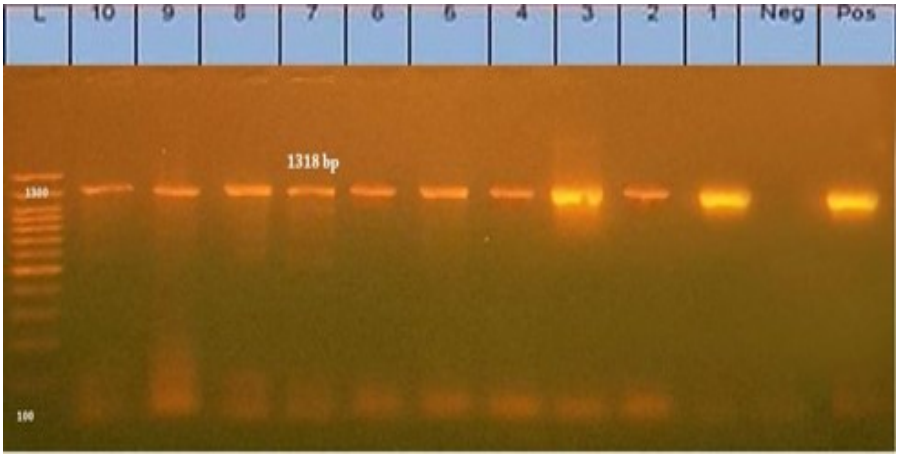
Table 3. MDR patterns of S. aureus isolates

MDR pattern	No. of resistance antibiotics	No. of resistance antibiotics classes	MDR isolates	
			No.	%*
OX-TE-C	3	3	4	4%
OX-TE-E	3	3	4	4%
P-TE-C	3	3	3	3%
OX-CIP-C	3	3	2	2%
P-E-CIP	3	3	2	2%
P-TE-E	3	3	2	2%
OX-P-TE-C	4	3	8	8%
OX-P-TE-E	4	3	3	3%
OX-AMC-TE-E	4	3	2	2%
OX-P-TE-CIP	4	3	2	2%
P-TE-E-C	4	4	2	2%
OX-P-TE-E-CIP	5	4	2	2%
Total			36	36%

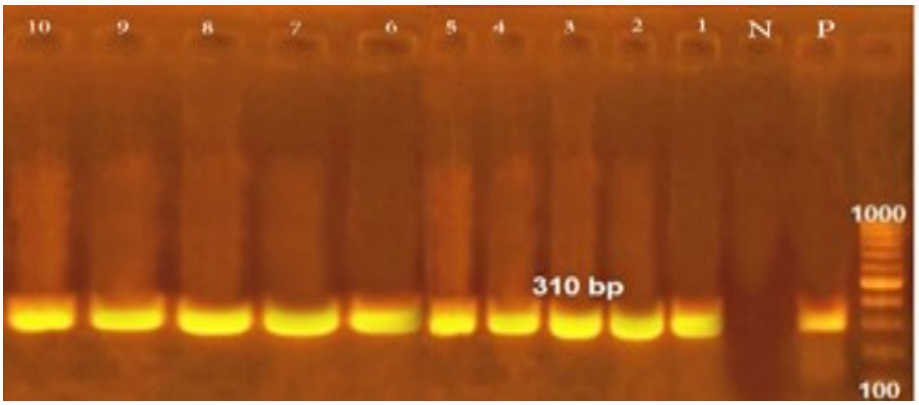
* Percentage was calculated according to the total number of *S. aureus* isolates (n=100).
AMC=Amoxicillin/Clavulanic acid, C=Chloramphenicol, CIP=Ciprofloxacin, CN=Gentamycin,E=Erythromycin, OX=Oxacillin, P=Penicillin, TE=Tetracycline and VA=Vancomycin

Table 4. Phenotypic and genotypic characters of the examined MRSA isolates by PCR.

StrainNo.	Virulence factors			MDR pattern	Antibiotic resistance gene
	Coagulase	Hemolysin (β or α)	Biofilmactivity		*mec*A
S1	+	+	+	OX- P-TE-C	+
S2	+	+	+	OX-P-TE-C	+
S3	+	+	+	OX-TE-E	+
S4	+	+	+	OX-P-TE-E-CIP	-
S5	+	+	+	OX-AMC-TE-E	+
S6	+	+	+	OX-P-TE-CIP	-
S7	+	+	+	OX-P-TE-E	+
S8	+	+	+	OX-TE	+
S9	+	+	+	OX-P-TE-C	+
S10	+	+	+	OX-P-TE	+



The 16s rRNA gene in the examined MRSA isolates is the target of agar gel electrophoresis for PCR products using specific primers. Lane L: a 100 bp molecular weight marker, Lane Pos: a positive control, Lane Neg: a negative control, and Lanes 1-10: DNA extracted from the tested isolates (S1-S10, respectively), showing positive bands at 1318 bp in all tested isolates. (fig. 1)



Mec A gene is targeted in the examined MRSA isolates by agar gel electrophoresis for PCR products using particular primers. Lanes 1-10 show DNA extracted from the examined isolates (S1-S10, respectively), with positive bands at 310 - bp in all but 4 and 6 of the examined isolates. Lane L is a 100 bp molecular weight marker. Lane Pos is a positive control. Lane Neg is a negative control. (fig. 2)

Hematobiochemical parameters

Mastitis in cattle induce significant decrease in RBCs, Hb, PCV% albumin A/G, catalase, super oxide dismutase and significant increase in WBCs, neutrophil, lymphocyte, total protein, globulin, malondialdehyde AST, ALT& ALP,

Table 5. Effect of mastitis in blood picture (n=10/50)

parameter			Healthy cattle	Mastitic cattle
Erythrogram	HB (g m %)		9.13±1.02	6.17±0.87**
	PCV (%)		31.17±1.06	26.09±0.83**
	RBcS (10^6/ml)		7.98±0.89	4.53±0.58**
	Blood indices Pg/dl	M.C. V	45.16±0.94	47.42±0.99
		M.C.H	15.42±0.73	16.83±0.54
		M.C. HC	29.48±0.79	27.32±049
Leukogram	Total WBcS. (10^3/ml)		8.89±0.93	12.21±0.92**
	Differenial count%	Neutrophils	46.5±0.55	49.12±0.87*
		Lymphocyt	44.5±0.45	46.02±0.58*
		Eosinophils	3.20±0.21	2.09±0.31
		Basophils	2.07±0.15	1.87±0.54
		Monocytes	3.71±0.21	2.04±0.75

*Significant at P < 0.05 ** Significant at P < 0.01

Table 6. Effect of mastitis in some biochemical parameters (n=10/50)

parameter			Healthy cattle	Mastitic cattle
Liver function	Liver enzyme (U/L)	ALT	49.56±0.87	59.12±0.93**
		AST	21 56±0.79	26.23±0.69**
		ALP	47.48±0.93	56.13±0.99**
	Protein picture (gm/dl)	T. protein	7.34±0.79	4.54±0.44*
		albumin	4.35±0.67	2.20±0.35*
		Globulin	2.99±0.79	2.14±0.62
		A/G Ratio	1.45±0.43	1.03±0.37
malondialdehyde (nmol/ml)			12.12±056	17.22±0.37*
Antioxidant (U/ml)	Catalase		30.56±0.36	26.35±0.43*
	SOD		95.24±0.43	89.12±0.45*

*Significant at P < 0.05 ** Significant at P < 0.01

DISCUSSION

Our results out of 300 mastitic milk samples found staphylococci. 100 (33.3 %0). Low prevelance of staphylococci were reported by **Kumar and Prasad (2010)** isolate staphylococci in milk sample in percentage 26%. In other study reported by **Thaker et al. (2013**) who isolate staphylococci in milk sample in percentage of 6.25%. Hight prevelance were reported by **Sarkar, et al. (2014)** isolate staphylococci aureus in 74.5% of the milk samples. **Patel et al. (2007)** that showed the prevalence of S. aureus isolated from raw milk samples in India is 10.16 %.

Lingathurai and Vellathurai (2011) reported 61.7% of prevalence of S. aureus from raw milk samples that are higher than our

study. However lower prevalence has been previously reported by **Fagundes et al., (2010)** (10.8%) from **Brazil, Ayano et al., (2013)** (13.8%) from Ethiopia. Also, these results are nearly similar to results of other study in Turkey conducted by **Erhan, et al., (2020)** who reported 37.32% of S. aureus from isolates and Results of this study also, differ from results of other study conducted by **Abou-Khadra et al., (2020)** found that 20% of raw milk samples from Sharkia Governorate were found contaminated with S. aureus. This rate of prevalence has previously been documented by a number of other studies **(Ammar et al. 2016**) found analyzed samples contaminated with S. aureus in a varying rate of percentages that ranged from 17.34% to 18.80%. Some other studies reported significantly higher levels of contamination ranging from 40% to 61.7 % **(Zakary et al. 2011)** This is not surprising because milk can be contaminated internally through the production of milk from a diseased animal or externally by infected person or the surrounding .

Environment. These prevalence results varied from place to place and regions to regions around the world due to sample size, use of antibiotics in animal husbandry, and hygiene practices among dairy cows. The high incidence of S. aureus is an indication of poor hygienic during production, distribution and handling. **Vyas et al. (2015).**

In the recent study mecA gene (figure 18) has been identified in all tested isolates that is recognized as MRSA. A lot of studies have been conducted to demonstrate and discuss genetics of S. aureus that includes genes responsible for antibiotic resistance with genes responsible for virulence. **Rong et al. (2017).** The mecA gene was an important mark gene in detection of methicillin resistance, however many studies now a days discussed the failure of mecA gene in detection of MRSA. **Elhassan et al., (2015).** Many previous studies have reported MRSA in mastitis cases 48.3% by **Guimaraes et al. (2017),** 15.5% by **Wang et al. (2015),** 11.6% by **Jamali et al. (2015),** and 2.5% by **Moon et al. (2007).** Transmission of MRSA to humans occur by consuming mastitic milk or direct contact with dairy cows that lead to serious problems to food safety and public health. These results are differed from several other studies which report the presence of 10.3% MRSA isolates. **Guimarães et al. (2017**) and other study conducted by **Gücükoğlu et al. (2012)** which report 9.1% MRSA isolates and other study which detect only 1.8% of MRSA in the sample tested. The source of MRSA transmission is due to direct contact with humans or transport animals, where cows infected with MRSA act as a reservoir and then transmit to other animals or humans. **Decline et al. (2020).** Colonization of MRSA in cows can be a risk factor for people who are in close contact with MRSA infected cows such as milkers, farmers, workers in slaughter-houses and veterinarians. **Zakary et al. (2011).** The detection of MRSA in milk is of high concern and need strict farm management practices as well as proper sanitation procedures in handling, storage and transportation. Over the last few decades, the prevalence of MRSA is increased exponentially and cause fatal infections and this is reported by clinical and laboratory standards institute (2017).

In this study antibiotic resistance gene (mecA) was detected using PCR as shown in figure (2).

The present work revealed that, mastitic cattle revealed significant reduction in RBCs, Hb PCV% coupled with significant increase in WBCs, neutrophil and lymphocyte beside insignificant effect in MCV, MH and MCHC in infected cattle when compared with control cattle. This change in blood picture may be due to bacterial infection and inflammatory reactions **(Coles, 1986).** Another explanation for changes in blood picture of mastitic cattle come from **Workineh, et al. (2002)** stated that reduction in RBCs and Hb may be due to effects of bacterial toxins but increase in leucocyte, neutrophil and decrease in lymphocyte due to body defense to infection and stress factors. Similar results were reported by **Mosallam, et al. (2014)** mentioned that cattle suffering from mastitis showed significant increase in leucocyte, neutrophil and decrease in RBCs, Hb PCV% and lymphocyte Mastitic cattle showed significant reduction in RBCs, Hb PCV% beside significant increase in WBCs, neutrophil and lymphocyte associated with insignificant effect in MCV, MH and

MCHC in infected cattle **(Mohanned, et al. 2015).** Same results were recorded by **Hussein and Ahmed (2019)** recorded that mastitis induce reduction in RBCs, Hb, PCV% beside increase in WBCs, same changes in blood picture were reported by **Garba B, et al. (2019**) in mastitic goats.

The present investigation declared significant increase serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase in mastitic cattle. Similar results were observed by **Ismail and Hatem, (1998)** who attributed increase in liver enzymes may be due to damage of hepatic tissues in acute mastitis. This study is agreed with **Asadi et al. (2009)** stated that liver enzyme increased in liver enzyme. This finding fitted closely with those of **Anirban, et al. (2012)** in mastitic buffalo. Same results were reported by **Garba B, et al. (2019) and Hussein and Ahmed (2019)** stated that mastitis induce increase in liver enzymes.

In the current work obtained data indicated mastitis induces significant increase in total protein and globulin beside decrease in albumin. This reduction in albumin may be due to infiltration of albumin from blood to milk due to increase permeability of blood vessels as a result of inflammation **(Honkanen-Buzalski, et al. 1995).** In addition, **Godden, et al. (2002)** stated that bacterial infection and its toxins induce damage of hepatic parenchyma resulting in failure of liver to produce albumin. Same change in total protein, albumin and globulin was recorded in mastitic cattle by **Darwish, et al. (2003).** These finding were similar to that reported by **Mosallam, et al. (2014)** found that mastitis induced decrease in albumin and increase in globulin. Our results were agreed with results of **Santos, et al. (2018)** stated that mastitis induce significant decrease in serum albumin in ewes. These findings are in accordance with **Garba B, et al. (2019)** who detected a significant decrease in serum decrease in albumin and increase in globulin in mastitic goats and they attributed that to endotoxin release from pathogenic bacteria which damaged liver cells.

The present investigation declared a significant increase serum malondialdehyde beside significant decrease in catalase and super oxide dismutase in the mastitic cattle when compared with the healthy control cattle. These findings are in accordance with **Hayrettin et al., (2005)** reported that mastitis induce significant increase in malondialdehyde in mastitic ewes. This finding fitted closely with those of **Feng et al., (2011)** found that bacterial infections induce increase in malondialdehyde beside decrease in Catalase and super oxide dismutase in mastitic cattle. This finding fitted closely with those of **Ranjan, et al. (2005)** stated that mastitis increased malondialdehyde and decrease in catalase and .in dairy cows. this finding was similar to that reported by **Jhambh, et al. (2013**) found that mastitis in dairy cows showed increase in malondialdehyde and decrease in antioxidant enzymes (catalase and super oxide dismutase.).

CONCLUSSION

MRSA can be transmitted from infected cows to humans who come into contact with them, such as farmers and veterinarians. This can pose a significant risk to human health, as MRSA infections can be difficult to treat and can lead to severe complications, such as sepsis and pneumonia.

Preventing the spread of MRSA in dairy herds is therefore essential to protect both animal and human health. Good management practices, such as regular cleaning and disinfection of milking equipment and proper hygiene during milking, can help reduce the risk of infection.

In addition, selective use of antibiotics and implementation of infection control measures can also help prevent the spread of MRSA in dairy herds. Vaccination against S. aureus is another potential strategy for reducing the incidence of mastitis caused by this bacterium.

Overall, MRSA infections in cows with mastitis represent a significant challenge for the dairy industry and public health. Further research is needed to better understand the epidemiology and transmission of MRSA in dairy herds and to develop effective strategies

for preventing and treating these infections.

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