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ORIGINAL ARTICLE

Characterization of Coagulase Positive and Negative Staphylococci Recovered from Mastitic Cows and Buffaloes

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Abstract
This study aimed to characterize the different *Staphylococci* recovered from mastitic cows and buffaloes. A total of 126 mastitis milk samples were aseptically collected from clinically mastitic animals including 87 cows and 39 buffaloes. Bacteriological examination and biochemical identification using VITEK-2-compact-SYSTEM revealed that a total of 94 *Staphylococcus* isolates (74.6%) were recovered; 56 isolates (59.6%) and 38 isolates (40%) from cows and buffaloes, respectively. *S. aureus* was the most predominant isolate (n=26; 15 from cows and 11 from buffaloes) with a percentage of 27.7%. Moreover, 68 coagulase-negative *staphylococci* (CNS) isolates (72.3%) were identified of which; 21 *S. epidermidis* (22.3%); all isolates were from cattle, followed by 18 *S. lentus* (19.1%); 8 and 10 from cows and buffaloes, respectively, 17 *S. simulans* (18%); 6 and 11 isolates, respectively, and finally 12 *S. hominis* (12.9%); 5 and 7 isolates, respectively. Antimicrobial susceptibility testing showed that all isolates were sensitive to ceftriaxone, ciprofloxacin, ofloxacin and sulfamethoxazole-trimethoprim. On the contrary, all isolates were resistant to penicillin and streptomycin. Multidrug resistance (MDR) was detected in 21 (22.3%) *Staphylococci* isolates. Biofilm formation capacity was phenotypically assessed on YESCA CR agar medium and showed that all *Staphylococci* isolates were curli-producing. Application of PCR technique revealed that *sed*, *seb* genes were the most prevalent genes in all isolates, followed by *fnb*A gene which was detected in 80% of the isolates, and then *mec*A, *bla*Z, and *ica*A with percentages of 60%, 40%, and 40%, respectively.

Keywords
Biofilm, Coagulase Negative *Staphylococci*, Mastitic Cows and Buffaloes, Resistance and Virulence Genes, *S. aureus*

1. Introduction
Worldwide bovine mastitis is the most common infectious disease affecting milk producing cows, rendering livestock unable to adequately produce milk, which results in heavy economic losses for the dairy industry **(Seegers et al., 2003)** compared with other diseases of dairy cattle **(Gillespie and Oliver, 2005).**

Identification of the bacterial pathogens in mastitic cows' milk is the definitive diagnosis. Also, it provides important information for prevention and control of the disease. In most of the clinical laboratories, identification methods are based on the microbiological culture of milk and biochemical identification of the recovered bacterial isolates. However, microbiological culture is limited by the dynamic nature of infections **(Phuektes et al., 2001).**

Staphylococcus species have been considered as one of the most significant causative pathogens of clinical and subclinical bovine mastitis **(Hassan et al., 2016).** *S. aureus*; a coagulase positive species was considered for a long time as the only important pathogen among *Staphylococcus species* responsible for clinical mastitis **(Darwish and Asfour, 2013)** and of foodborne intoxication importance in human **(D'amico and Donnelly, 2011; Elbargisy et al., 2016).** However, an increased worldwide attention has been paid to CNS **(Bal et al., 2010)** as CNS becomes the predominant pathogens associated with mastitis

(Tenhagen et al., 2006). They are currently considered as emerging pathogens of bovine mastitis **(Soares et al., 2012).** Mastitis caused by CNS usually remains mild clinically **(Taponen et al., 2007).** However, it was reported that the CNS mastitis could be aggravated to severe clinical cases **(Brînda et al., 2010).**

Biofilms are tightly packed populations of microbes that emerge on biological or inanimate surfaces and are surrounded by secreted polymers. Biofilm producing bacteria are characterized by slow growth and antibiotic-resistance, so they are difficult to be eradicated **(Flemming and Wingender, 2010).**

The antibiotic resistant bacteria can be transmitted from milk to human through the food chain causing serious problems on public health **(McDermott et al., 2002).**

Differences between the host biotypes are also reflected at the genotypic level as determined by macro-restriction analysis of the chromosome **(Hennekinne et al., 2003).** Due to the specificity of host-pathogen interactions needed to produce mastitis, it has been postulated that the nature of the virulence and the regulation of its expression are determining factors, when it comes to the ability of a strain to produce mastitis **(Vautor et al., 2008).**

The recent release of the complete genome sequence of *S. aureus* ET-3, a bovine isolate, provides new insight into the genomic basis of a putative host adaptation and the existence of host specific genetic traits in *S. aureus* isolated from bovine hosts **(Herron-Olson et al., 2007).**

The aim of the present study was directed for characterization of *S. aureus* and coagulase negative *Staphylococci* recovered from mastitic cows and buffaloes.

2. Materials and Methods

2.1. Animals

A total of 550 milk samples were collected from lactating animals; 310 cows and 240 buffaloes from different dairy farms and veterinary units at El-Fayoum Governorate. Animals were examined for evidences of clinical mastitis (swelling, hotness, redness and apparent milk change) during the period from May 2017 to November 2017. The udder of each animal was examined according to the guidelines of the National Mastitis Council before sampling for the presence of clinical signs of mastitis. This investigation was done in accordance with the recommendations in the updated guide for the care and use of animals. All procedures were approved by the Institutional Animal Care and Use Committee at Beni-Suef University, approval number (021-187).

2.2. Milk Samples Collection

A total of 126 mastitis milk samples were aseptically collected from clinically mastitic animals; 87 cows and 38 buffaloes. Sampling procedure was performed as recommended by **(Quinn et al., 2002)** for collection of milk samples aseptically. Before sampling the first few streams should be discarded then milk sample was collected into sterile screw capped McCartney bottle. The milkers' hands, the udder and tips of the teat were perfectly washed with water and soap and dried well with a towel. The teats were cleaned with 70% alcohol. Following sampling, milk samples were labelled, serialized and sent immediately to the lab for examination in ice container.

2.3. Bacteriological Examination of Milk Samples

Fresh Milk samples were centrifuged at 3000 rpm for 15 min. The cream layer and supernatant fluid were discarded. Loopful from the sediment was taken and cultivated into tryptone soya broth then incubated for 18-24 hrs at 37°C. Loopful from turbid tryptone soya broth was streaked onto Mannitol salt agar **(Oxoid)** and Baird-Parker agar **(Oxoid)** plates and incubated for 24-48 hrs at 37°C. *Staphylococcus spp*. were suspected based on phenotypic characters in culture media. Bacterial films were made from the colonies and stained with Gram's stain.

2.4. Morphological Examination (Quinn et al., 2002)

Suspected *Staphylococcus* isolates were identified primary as Gram-positive cocci occurring as singles, pairs or mostly as irregular clusters (bunches of grape like appearance). Pure isolates were subjected to further biochemical identification.

2.5. Biochemical Identification of the Isolates

These biochemical tests include oxidase, catalase, coagulase, urease and sugars; mannitol and maltose, fermentation tests beside other colonial characteristics; hemolysis on 5% sheep blood agar and pigmentation and sugar fermentation tests according to **Quinn et al., (2002)** and confirmed by VITEK-2-compact-SYSTEM.

2.6. Antimicrobial Susceptibility Testing of the Isolates

All isolates were examined for their antimicrobial sensitivity (AMS) to 14 different antimicrobial discs. The used antimicrobial discs (Oxoid, Basing Stoke, UK) were illustrated in **Table (1).** The disc diffusion technique was applied according to the Clinical and Laboratory Standards Institute **(CLSI, 2018).**

Table (1): interpretation values of growth inhibition zone of disc used in the antimicrobial susceptibility testing of the isolates.

Antimicrobial Class	Antimicrobial Type		Disc Content	Interpretation		
		Symbol	(µg)	Susceptible	Intermediate	Resistant
β–lactams	Penicillins	Ampicillin	AM	10	≥ 29		≤ 28
		Amoxicillin	AML	10	≥ 20	11-19	≤ 10
	β – lactamase stable	Amoxicillin – clavulanic acid	AMC	30	20		19
	Cephalosporines	Ceftriaxone	CTR	30	23	20-22	19
	Fluoroquinolones	Ciprofloxacin	CIP	5	21	16-20	15
		Ofloxacin	OFX	5	18	15-17	14
Lincosamides		Clindamycin	DA	2	21	15-20	14
Aminoglycosides		Gentamycin	GEN	10	15	13-14	12
		Kanamycin	K	30	18	14-17	13
		Apramycin	APR	15	17	15-16	14
		Streptomycin	S	10	15	12-14	11
Potentiated sulphonamides		sulfamethoxazole-trimethoprim	STX	1.25/23.75	16	11-15	10
Fosfomycin		Fosfomycin	FO	200	16	13-15	12
Tetracycline		Doxycycline	DO	30	16	13-15	12

2.7. Detection of Biofilm Formation on YESCA CR Agar Plate (Zhou et al., 2013)

Biofilm production was assessed YESCA Congo red agar (CRA). All isolates were streaked onto the YESCA CRA medium, incubated at 37°C for 24 hrs. The bacterial colonies were examined for the biofilm formation. The red color of bacterial colonies indicated positive biofilm formation, while the pink or white of colonies indicated negative biofilm formation.

2.8. Polymerase Chain Reaction for Detection of Some Selected Genes

PCR was done using Agarose gel electrophoreses and applied on 5 selected different *Staphylococcus* isolates on the basis of MDR to determine 6 genes; 2 enterotoxins producing genes (*sed* and *seb*), 2 biofilm associated genes (*ica*A and *fnb*A) and 2 resistance genes (*mec*A and *bla*Z). The primer sequences, size of generated products and cycling conditions used in PCR amplification were applied according to **Mehrotra et al., (2000); Ciftci et al., (2009); Vancraeynest et al., (2004); McClure et al., (2006)** and **Duran et al., (2012).**

2.9. Statistical Analysis

Data were statistically analyzed using Chi2-test for detection the significant of the incidences of the *S. aureus* among examined parameter. The statistical analysis was carried out using **(SAS, 2004).**

3. Results

3.1. Prevalence and Distribution of *Staphylococcus* Isolates in the Investigated Clinically Mastitic Dairy Animals

Out of 550 collected milk samples, a total of 94 (17.1%) *Staphylococcus spp*. were recovered with a total prevalence of 17.1%; distributed as 17.1 and 16.3% in cows and buffaloes, respectively. 26 of bacterial isolates (4.7%) were biochemically confirmed as *S. aureus* while 68 isolates (12.4%) were coagulase-negative *Staphylococci* **(Table, 2).** *S. aureus* represented in 15 and 11 isolates with 4.8% and 4.6% in cows and buffaloes, respectively. Meanwhile CNS represented in 40 (12.9%) and 28 isolates (11.7%) in cows and buffaloes, respectively **(Table, ٢).**

3.2. Identification and Distribution of CNS Isolates

CNS isolates (*n*=68) were identified using traditional methods including morphological, colonial and biochemical characteristics **(Table, 3)** and further identification by VITEK-2- COMPACT-SYSTEM®.

The results illustrated in **Table (4)** revealed that 4 different CNS species were identified. *S. epidermidis* was the most predominant species as 21 isolates (30.9%), followed by *S. lentus* as 18 isolates (26.5%), *S. simulans* (n=26; 17.25%), and finally *S. hominis* (n=12; 17.6%).

Table (2): Prevalence of Staphylococcus isolates in the clinically mastitic dairy animals.

Collected milk samples		Bacterial isolates					
		S. aureus		CNS		Total	
Examined animals	No. of samples	No.	%	No.	%	No.	%
Cattle	310	15	4.8	40	12.9	55	17.7
Buffaloes	240	11	4.6	28	11.7	39	16.3
Total	550	26	4.7	68	12.4	94	17.1

%: was calculated according to the corresponding number (No.) of samples.

Table (3): Results of colonial and biochemical identification of *Staphylococcus* isolates.

Staphylococcus *spp.*	*S. aureus*	*S. epidermidis*	*S. lentus*	*S. simulans*	*S. homins*
Catalase test	+	+	+	+	+
Oxidase test	-	-	-	-	-
Coagulase test	+	-	-	-	-
Hemolysis	+	+	-	+	+
Pigment production	+	-	-	-	-
Mannitol fermented	+	-	+	+	+
Maltose fermented	+	+	-	-	-

Table (4): Distribution of CNS among examined animals.

Host	Total No. of CNS	*S. epidermidis*		*S. lentus*		*S. simulans*		*S. hominis*	
		No	%	No.	%	No.	%	No.	%
Cow	40	21	52.5	8	20	6	15	5	12.5
Buffaloes	28	0	0	10	35.7	11	39.3	7	25
Total	68	21	30.9	18	26.5	17	25	12	17.6

%: was calculated according to the corresponding number (No.) of samples.

3.3. Results of Antimicrobial Susceptibility Test of Staphylococci Isolates Using Disc Diffusion Method

All isolates were tested for *in-vitro* susceptibility test against 14 antimicrobial agents of 9 different antimicrobial classes of important veterinary significance. The results demonstrated in **Table (5)** showed that all isolates were sensitive (100%) to ceftriaxone, ciprofloxacin, ofloxacin and sulfamethoxazole-trimethoprim. On the contrary, all isolates were resistant (100%) to penicillin and streptomycin. Multidrug resistance (MDR) was detected in 21 staphylococci isolates (22.3%).

Table (5): Antimicrobial susceptibility profile of *Staphylococcus* isolates from clinical mastitic animals.

Antimicrobial Class		Antimicrobial discs	Symbol	Disc Content (µg)	*S. aureus*				CNS			
					S		R		S		R	
					N	%	N	%	N	%	N	%
β –lactams	Penicillins	Ampicillin	AM	10	-	-	26	100	-	-	68	100
		Amoxicillin	AML	10	-	-	26	100	-	-	68	100
	β – lactamase stable	Amoxicillin – clavulanic acid	AMC	30	21	80.7	5	19.3	48	70.6	20	29.4
	Cephalosporines	Ceftriaxone	CTR	30	26	100	-	-	54	79	14	21
	Fluoroquinolones	Ciprofloxacin	CIP	5	26	100	-	-	68	100	-	-
		Ofloxacin	OFX	5	26	100	-	-	68	100	-	-
	Lincosamides	Clindamycin	DA	2	18	69.2	8	30.8	55	80.8	13	19
	Aminoglycosides	Kanamycin	K	30	21	80.7	5	19.3	51	75	17	25
		Gentamycin	GEN	10			26	100	11	17	57	83
		Apramycin	APR	15	8	31	18	69	20	30	48	70
		Streptomycin	S	10	-	-	26	100	-	-	68	100
	Potentiated Sulphonamides	Sulfamethoxazole-trimethoprim	STX	1.25/23.75	26	100	-	-	68	100	-	-
	Fosfomycin	Fosfomycin	FO	200	12	46	14	54	23	34	45	66
	Tetracycline	Doxycycline	DO	30	16	61.6	10	38.4	51	75	17	25

$Chi^2 = 15.24^{**}$ ** = Significant at (P < 0.01)

3.4. Prevalence of biofilm formation among isolated Staphylococci

All examined *S. aureus* (26) and *CNS* (68) isolates were 100% curli producing *Staphylococci* using YESCA CR agar medium **(Table, 6).**

Table (6): Results of biofilm formation among *Staphylococcus* isolates.

Bacterial isolates		No. of examined isolates	The percentage of biofilm-forming bacteria	
			No.	%
Staphylococcus spp.	*S. aureus*	26	26	100 %
	CNS	68	68	100 %
	Total	94	94	100%

$Chi^2 = 5.11^{**}$ ** = Significant at (P < 0.01)

3.5. Results of PCR for Detection of Virulence and Resistance Genes in Staphylococcus Isolates

The results of PCR revealed that *sed*, *seb* genes were the most prevalent genes as they were found in all isolates as shown in **Table (7)** and **Figs. (1 and 2),** followed by *fnb*A gene presented in 80% of the isolates then *mec*A, *bla*Z and *ica*A found in 60%, 40% and 40%, respectively as shown in **Table (7)** and **Figs (3 - 6).**

Table (7): Results of molecular detection of virulence and resistance genes in *Staphylococcus* isolates.

Staphylococcus Spp.	Enterotoxins				Biofilm				Resistance			
	sed		*seb*		*icaA*		*fnbA*		*mecA*		*blaZ*	
***S. aureus* (2)**	**2**	**100%**	**2**	**100%**	**1**	**50%**	**2**	**100%**	**0**	**0%**	**0**	**0%**
***S. epidermidis* (1)**	**1**	**100%**	**1**	**100%**	**0**	**0%**	**0**	**0%**	**1**	**100%**	**1**	**100%**
***S. lentus* (1)**	**1**	**100%**	**1**	**100%**	**0**	**0%**	**1**	**100%**	**1**	**100%**	**0**	**0%**
***S. simulans* (1)**	**1**	**100**	**1**	**100%**	**1**	**100**	**1**	**100%**	**1**	**100%**	**1**	**100**
Total (5)	**5**	**100%**	**5**	**100%**	**2**	**40%**	**4**	**80%**	**3**	**60%**	**2**	**40%**

%: was calculated according to the corresponding number of tested isolates. $Chi^2 = 12.40^{**}$ ** = Significant at (P < 0.01)

Fig. (1). Agarose gel electrophoresis showing the amplification of *sed* gene at amplicon of 278 bp. *LD: Molecular size ladder *Pos: Control positive *Lane (11-15): A positive result.

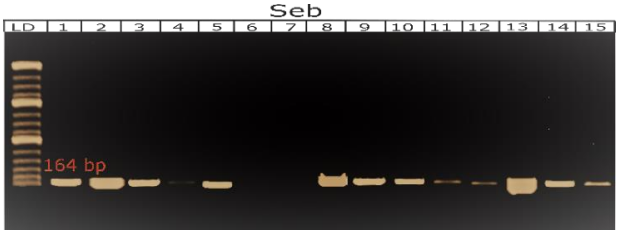

Fig. (2). Agarose gel electrophoresis showing the amplification of *Seb* gene at amplicon of 164 bp. *LD: Molecular size ladder *Pos: Control positive *Lane (11-15): A positive result.

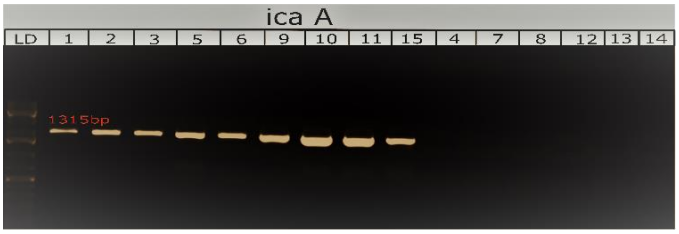

Fig. (3). Agarose gel electrophoresis showing the amplification of *ica A* gene at amplicon of 131 bp. *LD: Molecular size ladder *Pos: Control positive *Lane (11,15): A positive result

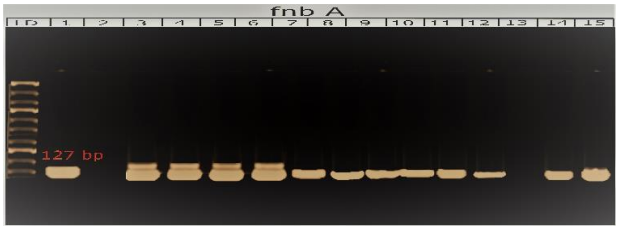

Fig. (4). Agarose gel electrophoresis showing the amplification of *fnb A* gene at amplicon of 127 bp. *LD: Molecular size ladder *Pos: Control positive *Lane (11, 12, 14, 15: A positive result.

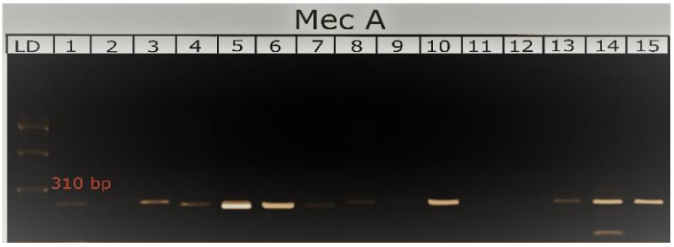

Fig. (5). Agarose gel electrophoresis showing the amplification of *mec A* gene at amplicon of 310 bp. *LD: Molecular size ladder *Pos: Control positive *Lane (13-15): A positive result

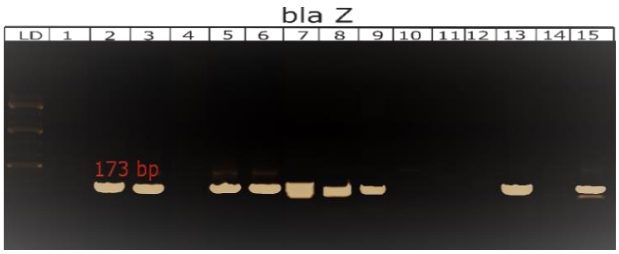

Fig. (6). Agarose gel electrophoresis showing the amplification of bla Z gene at amplicon of 173 bp. *LD: Molecular size ladder *Pos: Control positive *Lane (13-15): A positive result.

4. Discussion

Staphylococcus aureus is often recognized as the main infectious pathogen in bovine mastitis **(Feßler et al., 2010)** causing a great economic loss to milk industry through discarding of the contaminated milk **(Cohen et al., 2005; Bessen, 2009).** To date, more than 50 *Staphylococcus* species and subspecies have been involved in bovine staphylococcal mastitis **(El-jakee et al., 2013).** In the current study, phenotypic and genotypic methods were used to characterize the isolates of *Staphylococcus spp.* recovered from mastitic bovine milk samples. Results of the prevalence revealed a total prevalence of 17.1%; distributed as 17.1 % in cows and 16.3 in buffaloes. Out of 94 *Staphylococcus spp.* recovered, 26 (27.6%) isolates were identified as *S. aureus* and 68 (72.4%) isolates were CNS. This result is nearby with those of **Sumathi et al., (2008)** who recorded *S. aureus* as 24% while higher than those obtained in other studies as reported by **Normanno et al., (2007)** with 12.80% respectively and lower than those of **Waage et al., (1999)** who found *S. aureus* with (44.3%). Moreover, **Piepers et al., (2007)** reported that more than 50% of all intra-mammary infections was caused by CNS. In a related study, **Bal et al., (2010)** isolated 45.25% CNS species from milk samples.

The use of antimicrobials is still the cornerstone of treatment in mastitis control program **(Hassan et al., 2016; Srednik et al., 2017).** According to **CLSI (2018),** the obtained results of the antimicrobial susceptibility test of *Staphylococci* isolates using disc diffusion method cleared that, all isolates were completely sensitive to ceftriaxone, ciprofloxacin, ofloxacin and sulfamethoxazole-trimethoprim. On contrary, they were completely resistant to penicillin and streptomycin. Multidrug resistance (MDR); was represented by resistance to 3 or more different antimicrobial classes which were detected in 21 *Staphylococci* isolates (22.3%). These results were supported by **Sukanya et al., (2019)** and **Pitkälä et al., (2004)** who documented that the antimicrobial agents as ciprofloxacin can be used to control mastitis. In addition, **D'amico and Donnelly (2011)** and **Yang et al., (2017)** recorded high resistances of *Staphylococci spp*. against penicillin and other antimicrobial agents. Regarding MDR results, this incidence was close to that reported by **Klimiene et al., (2016)** who informed 21.9% while higher than those of **Soares et al., (2012)** and **Frey et al., (2013);** 15%, and lower than that of **Schmidt et al., (2015)** who recorded 39% MDR in *Staphylococcus* isolates from mastitic milk.

Biofilms enable microorganisms to resist harmful influences and to colonize the environment and they are also blamed for a slew of therapeutic failures since *Staphylococci* isolates developing biofilms are less vulnerable to antimicrobials typically used on dairy farms **(Nasr et al., 2012).** In this study biofilm production was studied among the recovered isolates by means of YESCA CRA medium; a phenotypic qualitative method fundamentally dependent on valuation of colony morphology on YESCA CRA. Our results cleared that, all the examined staphylococci isolates; including 26 *S. aureus* and 68 CNS, were biofilm producers (100%). Nearly more than half of the biofilms formed on CRA were of strong type which was confirmed by **Felipe et al., (2017),** but differed from **Darwish and Asfour (2013)** and **Srednik et al., (2017)** who reported 29.5 and 35.6% of isolates were strong biofilm producer, respectively.

Results of the molecular detection of some virulence and resistance genes in *Staphylococcus* isolates; by using PCR, showed that**,** *sed*, *seb* genes were found in all isolates (100%), while *fnb*A, *mec*A, *bla*Z and *ica*A genes were represented in 80, 60, 40 and 40% of the isolates, respectively. These results agreed with those of **El-Nomrousy (2014)** who used PCR technique for detection of *S. aureus* enterotoxin genes and showed that, *sed*, *seb* genes were the most prevalent genes found in all isolates meanwhile *mec*A gene was found in 28.5% of isolates. Moreover, **Awad et al., (2017)** reported *bla*Z and *mec*A genes in 95.7 and 50% of *S. aureus* isolates.

5. Conclusion

Out of totally examined mastitis milk samples, *S. aureus* was the most recovered isolate with a percentage of (27.7 %) followed by *S. epidermidis* (22.3%), *S. lentus* (19.1%), *S. simulans* (18%) and *S. hominis* (12.9%). Most of isolates were multidrug resistant. Biofilm formation capacity revealed that all *Staphylococci* isolates were curli producing. Using PCR, showed *sed* and *seb* virulence genes, followed with *ica*A and *fnb*A biofilm genes and resistance genes were *mec*A and *bla*Z genes.

6. Authors Contributions

All authors contributed equally to study design methodology, interpretation of results and preparing of the manuscript.

7. Conflict of interest

The authors declare no conflict of interest.

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