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Bacteriological study on staphylococcal bovine clinical mastitis with reference to methicillin-resistant *Staph. aureus* (MRSA)

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ABSTRACT

This descriptive study was done on 101 milk samples obtained from clinically mastitic dairy cows in Assiut Governorate, Egypt. Staphylococcus aureus (*S. aureus*) was the main causative agent of clinical mastitis (34.65%) followed by *S. saprophyticus* (10.89%), *S. intermedius* and *S. epidermidis* (8.91%, for each). The other causative agents (non *Staph. Spp.*) were identified. Sensitivity test of *S. aureus* isolates was performed against 11 antimicrobial agents, where found that 21 S. aureus strains (60%) were methicillin resistant *S. aureus* (MRSA). Ten MRSA strains were subjected for: I- slime-producing factor on Congo Red Agar (CRA) plates phenotypically, as 6 isolates (60%) were positive for slime production. II- PCR which was optimized targeting mecA, icaA and icaD genes, where 5 isolates (50%) were positive for mecA gene. Six isolates (60%) and 8 isolates (80%) were positive for icaA and icaD genes, respectively. Five strains (50%) were positive for both icaA and icaD genes. Also 3 strains (30%) were positive for all mecA, icaA and icaD genes. Conclusion, it was concluded that bovine staphylococcal mastitis was the most predominant issue where *S. aureus* was the main cause. Detection of mecA gene in *S. aureus* isolates indicating that several cases suffering from *S. aureus* mastitis have an MRSA problem. Genotypic determination of mecA gene proved the most reliable method for detection of methicillin-resistant *S. aureus*. The present work paid an attention to the 3 MRSA strains (30%) were positive to all tested genes rather than slime production as the worst isolated strains all over this study (

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multidrug resistant, slime producing as well as carrying mecA, icaA and icaD genes). In vitro Enrofloxacin, Gentamicin and Doxycycline the most effective drugs for *Staph. spp.* clinical mastitis and should be recommended for treatment of such cases of bovine mastitis.

Keywords:

Cows, clinical mastitis, methicillin-resistant *Staphylococcus aureus, mecA, icaA, icaD,* slime factor

Introduction

Mastitis is one of the major challenges of the dairy industry. *Staphylococcus aureus* [*S. aureus*] is one of the most important pathogens causing mastitis in dairy cattle [1,2]. Methicillin resistant *Staphylococcus aureus* [MRSA] has been recovered from dairy cattle in Korea [1-3]; Turkey [4]; Netherland [5]; Iran [6] and Uganda [7]. Several efforts to remove this pathogen from farms are hampered by some factors, where one of these factors is antibiotic resistance. One of the major mechanisms of resistance to β-lactam antibiotics is β-lactamase producing by staphylococci. This enzyme hydrolyzes the β- lactam ring and causes inactivation of β-lactams. In the early 1950s, it has been aware of the effectiveness of penicillin in treatment of *S. aureus* infections because of β-lactamase producing plasmids. In 1959, methicillin, synthetic, penicillinase –resistant penicillin, was introduced and solved problems in clinical practice, for a time. However, by 1960, *S. aureus* strains were found to be resistant to the new semisynthetic β-lactams [methicillin, oxacillin, flucloxacillin], and became known as methicillin-resistant *S. aureus* [MRSA]. This type of resistance was termed “intrinsic resistance” because it was not due to destruction of the antibiotic by β-lactamase [8]. Methicillin resistance in *S. aureus* is mediated by the production of an altered penicillin-binding protein [PBP2a], a transpeptidase. *mecA* encodes this enzyme involved in cell wall peptidoglycan synthesis. Unlike conventional PBPs of *S. aureus*, PBP2a does not bind to β-lactam antibiotics with high affinity [9].

It is considered that the first step in mastitis progress is adhesion of *S. aureus* to mammary epithelial cells and slime factor plays an important role for adhesion and colonization [10]. Production of slime factor also plays an important role in antibiotic resistance and it has been reported that slime producing strains are more resistant to antibiotics than non-slime producing strains [11]. Intercellular adhesion is encoded in the *ica* locus containing *icaA, icaB, icaC, icaD* genes in *S. aureus* strains [12]. *icaA* gene encodes N-acetylglucosaminyl transferase, further, *icaD* plays an important role in expression of this enzyme. *icaA* and *icaD* were found to be in high prevalence among *S. aureus* mastitis isolates and this finding confirms that *ica* locus has a potential role as a virulence factor in the pathogenesis of mastitis in ruminants [10].

This study was undertaken to determine the bovine mastitis *Staph. Spp*., their resistance to antimicrobial agents approved for its control and to determine the methicillin resistance and slime factor production of *S. aureus* in bovine mastitis phenotypically and genotypically for *mecA, icaA and icaD* genes.

Materials and Methods

Milk samples

A total of 101 milk samples were collected from 101 cows, at various private farms in Assiut, Egypt, showing clinical signs of mastitis. All samples were taken under aseptic conditions and transferred in ice box to laboratory as soon as possible.

Isolation and identification of bacterial isolates

Amount of 0.01 ml of each milk samples was cultured on blood agar with 5% sheep blood, Mannitol salt agar [BBL], Baird-Parker medium [Oxoid] and MacConkey agar [Biomark Lab. India] which incubated at 37°c for 48 h. The suspected colonies were identified: morphologically, by Gram’s stain and biochemically confirmed by using catalase activity, coagulase test as well as Novobiocin [5 µg] and polymixin-ß sulphate [300 U] sensitivity tests, according to [13].

Phenotypic detection of methicillin resistance

Disc diffusion sensitivity testing was performed according to the Kirby-Bauer method, as described in the guidelines of the National Committee for Laboratory Standards [14], using discs [Bioanalyse-Turkey] containing Oxacillin [OX] 1 µg, Ampicilin [AM] 10 µg, Cefotaxime [CTX] 30 µg, Cloxacillin [CX] 1 µg, Doxycycline [DO] 30 µg, Enrofloxacin [ENR] 5 µg,

Table 1 Primers sequences, target genes, amplicon sizes.

Primer	Target gene	Primer sequence (5'-3')	Length of amplified product (bp)	Reference
mecA-1	mecA	GTA GAA ATG ACT GAA CGT CCG ATA A	310 bp	(15)
mecA-2		CCA ATT CCA CAT TGT TTC GGT CTA A		
IcaA- AF	IcaA	CCT AAC TAA CGA AAG GTA G	1315 bp	(4)
IcaA- AR		AAG ATA TAG CGA TAA GTG C		
IcaD- DF	IcaD	AAA CGT AAG AGA GGT GG	381 bp	
IcaD- DR		GGC AAT ATG ATC AAG ATA		

Table 2 Isolated micro-organisms from mastitic milk samples.

Isolates		No.	%
Staph. spp	Coagulase + ve *Staph. spp.*		
	1- *S. aureus*	35	34.65
	2- *S. intermedius*	9	8.91
	Coagulase -ve *Staph. spp.*		
	1- *S. saprophyticus*	11	10.89
	2- *S. epidermidis*	9	8.91
Non *Staph. spp*	Gram –ve bacilli	32	31.68
	Corynebacterium spp.	4	3.96
	Strept. pyogenes	1	0.99
	Total	101	100

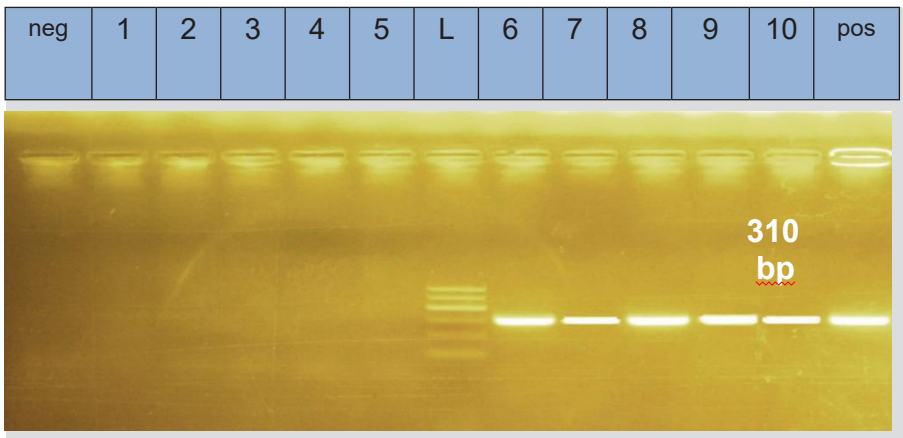

Fig. 1 PCR results for mecA gene showing amplification of 310 bp. (L): 100 bp ladder (QIAGEN, Gmbh) (100-600 bp), 1-10 lanes for the 10 strains respectively.

Gentamicin [CN] 10 µg, Lincomycin [L] 2 µg, Oxytetracycline [T] 30 µg, Penicillin [P] 10 µ and Trimethoprim – Sulflamethaxzole [SXT] 25 µg. For Oxacillin susceptibility determinations, inhibition zones around the disc were measured after 24 and 48 h using the following breakpoints: susceptible [S] ≥ 18 mm; resistance [R] ≤ 17 mm [13].

Slime production assay

Slime production assay was performed by cultivation of ten *S. aureus* strains, which were methicillin resistant by phenotypic test, on Congo Red Agar [CRA] plates containing 0.8 g of Congo Red dye, 21 g Mueller-Hinton broth, 15 g granulated agar and 36 g sucrose per Liter distilled water. Strains were inoculated on CRA plates and incubated for 24-72 h at 37ºC. Slime producing strains and non-slime producing strains constitutes rough black colonies and red colonies on CRA, respectively [12].

PCR for detection of mecA, icaA and icaD genes

Detection of *mecA, icaA* and *icaD* genes was performed on those ten *S. aureus* isolates, which were methicillin resistant by phenotypic test, as follows:

I- DNA extraction: DNA extraction from samples was performed using the QIAamp DNA Mini kit [Qiagen, Germany, GmbH] with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

II- Oligonucleotide Primers: Primers encoding for *mecA, icaA* and *icaD* genes were supplied from [Metabion, Germany] are listed in Table [1].

III- PCR amplification: Primers were utilized in a 25-µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix [Takara, Japan], 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of template. The reaction was performed in a Biometra thermal cycler. For *mecA* gene PCR, a primary denaturation step was done at 95 ^{O}C for 5 min, followed by 35 cycles of 95^{O}C for 45 sec., 50^{O}C for 45 sec. and 72^{O}C for 45 sec. A final extension step was done at 72^{O}C for 10 min, according to [15]. However for the *icaA* and *icaD* genes, the cycles consisted of 95ºC for 1 min, 49º C for 1 min and 72º C for 1 min, according to [4].

IV- Analysis of the PCR Products: The products of PCR were separated by electrophoresis on 1.5% agarose gel [Applichem, Germany, GmbH] in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A 100 bp DNA Ladder [Qiagen, Germany, GmbH] and 100 bp plus DNA Ladder [Fermentas, Cat.No. SM 0323] were used to determine the fragment sizes. The gel was photographed by a gel documentation system [Alpha Innotech, Biometra] and the data was analyzed through computer software.

Results

Results are in tables 1-4 and figures 1-3.

Discussion

Mastitis is an important and a persistent infection producing high economies losses due to poor milk quality, reduced milk yield and increased expenditure on treatment especially staphylococcal mastitis [16] which resembled 63.37% through the current study and bacteriological examination shows that *S. aureus* was the main causative agent of clinical mastitis in cows [34.65%], followed by *S. saprophyticus* [10.89%], *S. intermedius* and *S. epidermidis* [8.91%, for each], as shown in Table [2]. This result of *S. aureus* in close agreement with previous findings; 30, 30 and 36%, by [17]; [18]; [19], respectively. However, the findings [71.4%] of *S. aureus* by [20] are much higher than the present report. The lower prevalence reported by [21]; [22]; [23] were 21.7; 21.9 and 23.6%, respectively. High prevalence of *S. aureus* points to poor milking hygiene as this pathogen is mainly spread during milking via milkers' hands and towels [24].

In vitro activities of *Staph. spp.* against 11 antimicrobial agents are summarized in Table [3]. In the present work the highest rate of resistant *S. aureus* exhibited to Lincomycin followed by Cefotaxime, Ampicilin and Penicillin [91.43, 88.57, 68.86 and 57.14%, respectively] and MRSA resembled 60% of these isolates. The highest rate of sensitivity to Enrofloxacin & Gentamicin followed by Doxycycline [100, 100 and 91.43%, respectively], Table [3]. Enrofloxacin the most effective drugs against *S. aureus* [16]. The highest resistance rate of *S. aureus* against Penicillin [66; 47.6; 47.6 and 56.5%] was reported by [2, 25, 26, 27],

Table 3 *In vitro* antimicrobial susceptibility test of isolated *Staph. spp*. From bovine clinical mastitis according to the agar disc diffusion method.

Antimicrobial agentS	*S. aureus* (n. = 35)		*S. intermedius* (n.= 9)		*S. saprophyticus* (n.= 11)		*S. epidermidis* (n.= 9)		Total (n.= 64)	
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
Oxacillin	14 (40%)	21 (60%)	6 (66.67%)	3 (33.33%)	4 (36.36%)	7 (63.64%)	5 (55.56%)	4 (44.44%)	29 (45.31%)	35 (54.69%)
Enrofloxacin	35 (100%)	0 (0%)	9 (100%)	0 (0%)	11 (100%)	0 (0%)	9 (100%)	0 (0%)	64 (100%)	0 (0%)
Gentamicin	35 (100%)	0 (0%)	9 (100%)	0 (0%)	11 (100%)	0 (0%)	9 (100%)	0 (0%)	64 (100%)	0 (0%)
Doxycycline	32 (91.43%)	3 (8.57%)	9 (100%)	0 (0%)	10 (90.91%)	1 (9.09%)	9 (100%)	0 (0%)	60 (93.75%)	4 (6.25%)
Trimethoprim –Sulfla-methaxzole	18 (51.43%)	17 (48.57%)	7 (77.77%)	2 (22.22%)	5 (45.45%)	6 (54.55%)	7 (77.77%)	2 (22.22%)	37 (57.81%)	27 (42.19%)
Oxytetracycline	18 (51.43%)	17 (48.57%)	6 (66.67%)	3 (33.33%)	7 (63.64%)	4 (36.36%)	6 (66.67%)	3 (33.33%)	37 (57.81%)	27 (42.19%)
Penicillin	15 (42.86%)	20 (57.14%)	5 (55.56%)	4 (44.45%)	7 (63.64%)	4 (36.36%)	5 (55.56%)	4 (44.45%)	32 (50%)	32 (50%)
Ampicilin	13 (37.43%)	22 (68.86%)	7 (63.64%)	2 (22.22%)	7 (77.77%)	4 (36.36%)	3 (33.33%)	6 (66.67%)	30 (46.88)	34 (53.12%)
Cloxacillin	14 (40%)	21 (60%)	6 (66.67%)	3 (33.33%)	4 (36.36%)	7 (63.64%)	5 (55.56%)	4 44.45%)	29 (45.31%)	35 (54.69%)
Cefotaxime	4 (11.43%)	31 (88.57%)	3 (33.33%)	6 (66.67%)	1 (9.09%)	10 (90.915%)	0 (0%)	9 (100%)	8 (12.5%)	56 (87.5%)
Lincomycin	3 (8.57%)	32 (91.43%)	3 (33.33%)	6 (66.67%)	0 (0%)	11 (100%)	0 (0%)	9 (100%)	6 (9.38%)	58 (90.63%)

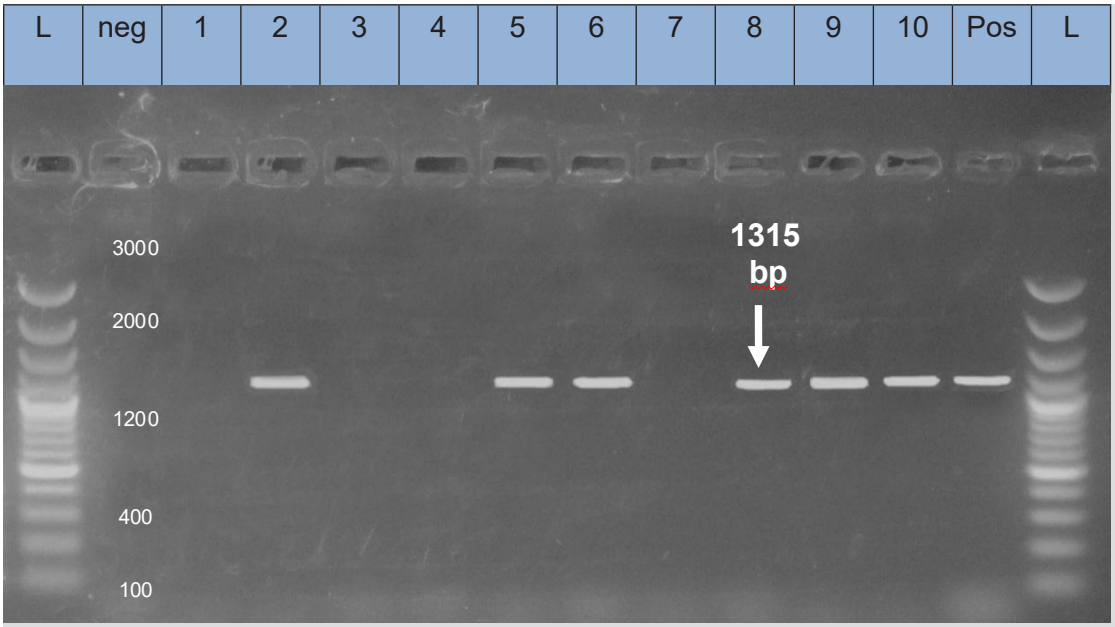

Fig. 2 PCR results for icaA gene showing amplification of 1315 bp. (L): 100 bp plus ladder (Fermentas, Cat. no. SM 0323) (100-3000 bp), 1-10 lanes for the 10 strains respectively.

respectively. An unusual high prevalence of MRSA in Belgian cases of subclinical and clinical *S. aureus* mastitis in cows [28]. *S. aureus* can adapt rapidly to the selective pressure of antibiotics and this resulted in emergence and spread of MRSA [29].

Extracellular polysaccharides, slime factor, are considered to be significant virulence factors for some staphylococci. Slime layer surrounding the *S. aureus* strains help in adherence and colonization of these microorganisms on the mammary gland epithelium. It is reported that slime factor production in *S. aureus* isolates from mastitis cause antibiotic resistance which is due to the decreased diffusion of antibiotics through the biofilm matrix and decreased metabolic activity of bacteria [11, 12].

In the present study, ten MRSA strains were subjected for slime production on Congo Red Agar [CRA] plates and PCR study targeting *mecA*, *icaA* and *icaD* genes. Among the tested MRSA strains only five isolates [50%] were positive for *mecA* gene genotypically, Table [4] and Fig. [1]. MRSA were positive for *mecA* gene with a percentage of 61.9 and 30.7% [1, 4], respectively. MRSA resistance to methicillin and other ß-lactam antibiotics is caused by the action of *mecA* gene [29]. The discrepant results between disc diffusion methods and PCR for detection of methicillin resistance may be due to another resistance mechanism such as hyperproduction of beta-lactamase, also MRSA strains show a heterogeneous character with the level of resistance varying according to the culture conditions and β-lactam antibiotic being used. Because of this heterogeneous resistance, and time consuming the detection of MRSA by phenotypic methods becomes problematic [4]. However, PCR-based methods have shown to be a rapid and reliable approach for the identification and genotypic characterization of MRSA. Detection of *mecA*- based PCR methods has accepted as “gold standard” [30].

The present work found that six isolates [60%] of the tested MRSA strains were slime producing positive on CRA plates *in vitro*, Table [4]. Slime-producing *S. aureus* isolates from different clinical origins such as bovine mastitis [4, 10], wound infection [12] and clinical cases [31] has been detected *in vitro* by using Congo Red Agar plates in percentages of 37.2, 91.4, 52 and 53.3%, respectively. Phenotype on CRA was found to be an unreliable indicator of slime-forming capacity among clinical isolates of *S. aureus* [32]. Therefore, although CRA methods may be easier to perform than a molecular analysis of the genes implicated in biofilm production and could be performed easily in a diagnostic laboratory, it may be a poor method for determining the slime producing capacity of clinical isolates in the diagnostic laboratory [33].

PCR methods provided a direct evidence of the genetic basis of slime production complementary to the CRA test. The *ica* locus consists *ica A, D, B, C* genes. Slime synthesis is controlled by the ica [intercellular adhesion] operon. Coexpression of *icaA* with *icaD* leads to a significant increase in activity and is related to phenotypic expression of the capsular polysaccharide [34]. In this study, slime factor production of MRSA isolates were detected by PCR targeting *icaA* and *icaD* genes and found that 5 [50%] of the tested MRSA strains were positive for both *icaA* and *icaD* genes. Six [60%] and eight [80%] isolates were positive for *icaA* gene and *icaD* gene, respectively as shown in Table [4] and Fig. [2 & 3]. Fifteen [25.42%] out of 59 *S. aureus* strains were positive for both *icaA* and *icaD* genes, in addition 16 [27.12%] and 38 [64.41%] out of the 59 strains were positive for *icaA* and *icaD* gene, respectively [4]. Also [31] found that 75% of MRSA carried *ica* operon. The *icaAD* gene was detected in 32% of *Staphylococcal spp*. [35]. While [34] found that all strains which were positive for *icaA* gene were also positive for *icaD* gene. In addition [10, 12] have reported that all *S. aureus* isolates possessed the *ica* locus, *icaA* and *icaD* genes. *icaA* and *icaD* genes were not be together in some isolates may due to some mutations on *icaA* gene, although coexpression on *icaA* and *icaD* is necessary for slime production it was considered that other genes in *ica* locus play role in controlling slime expression [4].

In present study, among four isolates which were negative for slime production on CRA plate *in vitro,* one isolate was positive for both *icaA* and *icaD* genes, two isolates were positive for *icaD* gene and the last one was negative for both *icaA* and *icaD* genes, Table [4]. Among 37 *S. aureus* strains which did not produce slime factor on CRA plate *in vitro,* only 7 strains [18.9%] were positive for both *icaA* and *icaD* genes [4], they suggest that some environmental conditions or presence of accessory genes can influence the phenotypic behavior on the Congo red agar plate, giving colonies which did not fully express the *ica* genes.

In this work, six isolates [60%] were positive for both methicillin resistance and slime production phenotypically and three strains [30%] were positive for all *mecA*, *icaA* and *icaD* genes, Table [4]. Only 2 [3.39%]

Table 4 Methicillin resistant *S. aureus* strains tested for their phenotypic and genotypic characteristics and slime factor production

MRSA strains	Congo red agar test	Genes		
		mecA	icaA	icaD
1	-ve	-ve	-ve	+ve
2	-ve	-ve	+ve	+ve
3	-ve	-ve	-ve	-ve
4	+ve	-ve	-ve	+ve
5	+ve	-ve	+ve	+ve
6	+ve	+ve	+ve	+ve
7	-ve	+ve	-ve	+ve
8	+ve	+ve	+ve	+ve
9	+ve	+ve	+ve	+ve
10	+ve	+ve	+ve	-ve

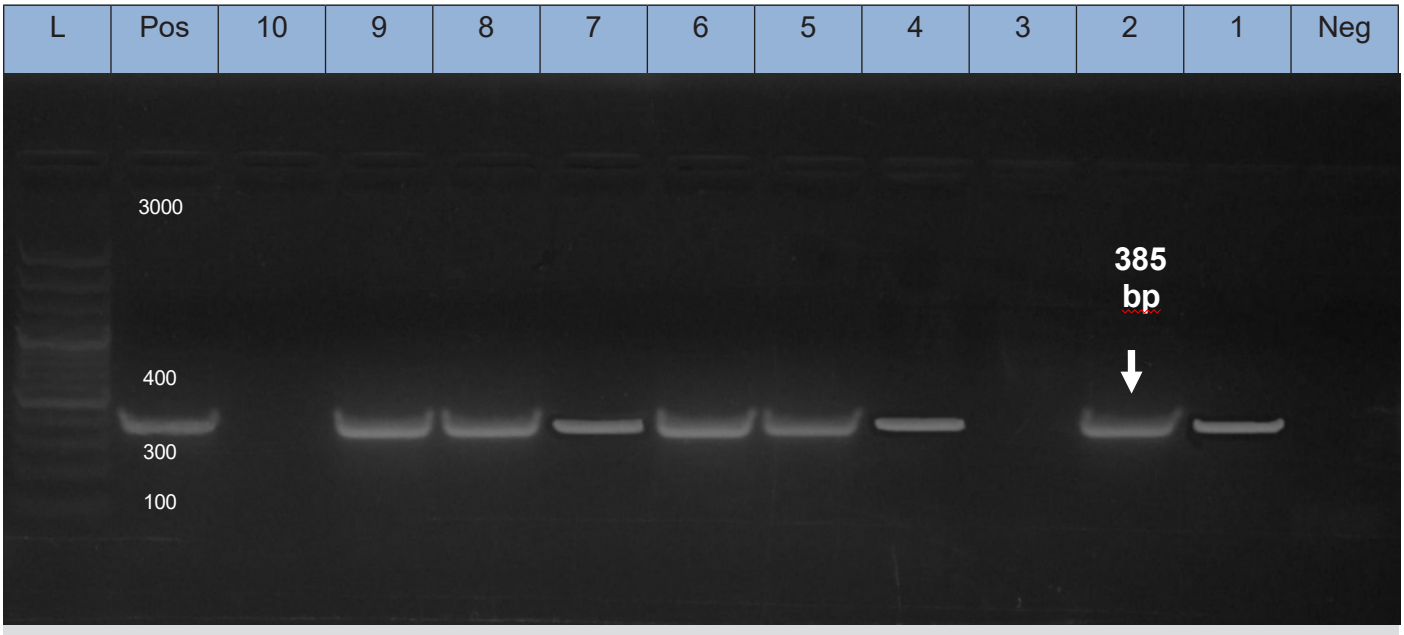

Fig. 3 PCR results for icaD gene showing amplification of 385 bp. (L): 100 bp plus ladder (Fermentas, Cat. no. SM 0323) (100-3000 bp), 1-10 lanes for the 10 strains respectively.

of 59 *S. aureus* strains were positive for both methicillin resistance and slime producing, phenotypically [4].

In conclusion, it was showed that detection of *mecA* gene in *S. aureus* isolates indicating that several cases suffering from *S. aureus* mastitis have an MRSA problem. Genotypic determination of *mecA* gene proved the most reliable method for detection of methicillin resistance. The present work paid an attention to the 3 MRSA strains [30%] were positive to all tested genes rather than slime production as the worst isolated stains all over this study [multidrug resistant, slime producing as well as carrying *mecA, icaA* and *icaD* genes]. *In vitro* Enrofloxacin, Gentamicin and Doxycycline are the most effective drugs for *Staph. spp.* clinical mastitis.

Conflict of interest : None.

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