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Detection of methicillin resistant and slime factor production of coagulase negative *Staphylococcus spp.* in bovine clinical mastitis by using PCR

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ABSTRACT

This study aims to investigate the slime production of Coagulase negative staphylococci (CoNS) isolates by phenotypic method on Congo Red Agar plates (CRA) and Genotypic detection of icaA, icaD and mecA genes by polymerase chain reaction (PCR). Out of 105 milk samples obtained from clinical bovine mastitis, 101samples (96.2%) were positive for bacterial growth. CoNS isolates was detected in 20 isolates with a percentage of 19.8%. Their ability to form biofilm as one of the most important virulence factors of the organisms using Congo Red Agar (CRA) method was investigated in which 13 out of 17 CoNS isolates (76.47%) were found to be slime producers. By PCR, mecA gene was found in threeout of 6 CoNS isolates (50%). Also six (100%) and three (50%) isolates were positive for icaA gene and icaD gene, respectively. In addition one isolate out of the six CoNS isolates (16.67%) was positive for the presence of icaA, icaD and mecA genes and also has the ability to form biofilm. The in vitro activities of CoNS against 11 selected antimicrobial agents referred that the highest resistance rate of CoNS observed to Lincomycin (100%), followed by Cefotaxime (94.41%), Oxacillin (58.82%), Ampicillin (47.06%) and Penicillin (41.18%), while the highest rate of sensitivity observed to Enrofloxacin and Gentamicin (100%, for each), followed by Doxycycline (94.11%).Conclusion, the findings of the present study demonstrated the ability of CoNS isolated from bovine clinical mastitis to form biofilms. This

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must be considered as an alarming situation, and so attention must be paid toward implementation of new ways for effective prophylaxis, control, and treatment of such infections in the dairy farms. The prudent use of antibiotics and rapid and continuous screening for resistant microorganisms should be more focused to prevent the emergence and spread methicillin resistant coagulase negative staphylococci, because these strains can cause severe damage to infected sites and may be widespread in the environment.

Keywords: Cows, clinical mastitis, coagulase-negative staphylococci, slime factor, mecA, icaA, icaD genes.

Introduction

Mastitis in dairy cows is a serious problem as it is an economically devasting disease causing immense economic losses in the dairy industry in Egypt[1]. During recent years, coagulase negative staphylococci [CoNS] have become the most common bovine mastitis isolates in many countries and are regarded as emerging mastitis pathogens [2]. The impact of CoNS is increasing, probably because prevalence of major pathogens is decreasing. Otherwise, the high frequency of CoNS and *E. coli* occurrence indicated insufficient hygiene of housing and milking causing the risk of environmental mastitis[3].

Coagulase negative staphylococci are always present on the udder skin and in teat canals; under favorable conditions they permeate the galactogenic pathway to the quarter. The pathogenic mechanisms of CoNS are expressed by two parameters: invasiveness [ability to permeate through protective barriers, to adhere to host cells and to form a biofilm] and toxicity [capacity to produce enzymes and toxins, including haemolysins and proteases],[4].

Biofilm is an exopolysaccharide, a slime matrix around multiple layers of cells. The ability of Staphylococci to form biofilms is one of the virulence factors that facilitate the adherence and colonization of Staphylococci on the mammary gland epithelium, also contributing to the evasion of the immunological defenses and to the difficulty of pathogen eradication, leading to recurrent or persistent infections[5]. Biofilm-producing isolates have been reported for many CoNS species, especially in *S. epidermidis*[5, 6]. Biofilm prohibits host immune defense by impairing phagocytosis and production of antimicrobial peptides by epithelial cells and neutrophils, it also protect bacteria from antimicrobial therapy[7, 8]. Biofilm consists of polysaccharide intercellular adhesion [PIA] encoded by the intercellular adhesion *icaADBC* operon[9].

CoNS strains have become a serious problem as they express methicillin resistance, which involves all *β*-lactam antibiotics and leads to a significant limitation in therapeutic options. Methicillin resistance is associated with the presence of the *mecA* gene which encodes a penicillin-binding protein [PBP2a] with altered properties responsible for the observed resistance[10]. Incidence of methicillin resistance in CoNS is high, as well as the accompanying antimicrobial resistance[11].

Resistance to *β*-lactamase-resistant penicillins, or methicillin resistance, depends on a complex expression mechanism of the *mecA* gene, which is often species-idiosyncratic among staphylococci [10]. Methicillin resistance may result from a series of factors including high degree of intrinsic resistance, hyperproductionof *β* -lactamase or *mecA*-associated resistance. These factors may operate and interact in the same strain[12, 13].The *mecA* gene encoding methicillin resistance is widely disseminated among various *Staphylococcus species*. This widespread distribution of *mecA* might be due to the horizontal transmission between CoNS isolates and *Staph. aureus*[14].

In CoNS, which display a complex regulation of methicillin resistance, PCR amplification provides the most reliable test for identification of methicillin resistant coagulase negative staphylococci [MRCoNS]. Although the use of PCR-based determinations represents a significant increase in reagent costs relative to phenotypic reagents, their reliability, as well as considerations of the time and labour, make these molecular methods increasingly recommendable for early detection of methicillin resistance[11].

Keeping in view the economic loss caused by the bovine mastitis and emergence of drug resistant CoNS, the present investigation was undertaken with the

Table 1: Primers sequences, target genes, amplicon sizes.

Target gene	Oligonucleotide sequence (5′ → 3′)	Amplicon length (bp)	Reference
mecA (F)	5′ TAG AAA TGA CTG AAC GTC CG ′3	154	(18)
mecA (R)	5′ TTG CGA TCA ATG TTA CCG TAG ′3		
icaA (F)	5′ TCT CTT GCA GGA GCA ATC AA′3	188	(19)
icaA (R)	5′ TCA GGC ACT AAC ATC CAG CA′3		
icaD (F)	5′ ATG GTC AAG CCC AGA CAG AG ′3	346	(19)
icaD (R)	5′ CGT GTT TTC AAC ATT TAA TGC AA′3		

Table 2: Proportions of CoNS species in bovine clinical mastitis milk samples (n= 101).

Spices of CoNS**	Staph. epider-midis	Staph. saprophyticus	Staph. chromogenes	Staph. simulans	Total
Number (%)	6 (5.94%)	11 (10.89%)	2 (1.98%)	1 (0.99%)	20 (19.8%)

**Highly significant statistical variations $\chi^2 = 16.53$ $P < 0.01$

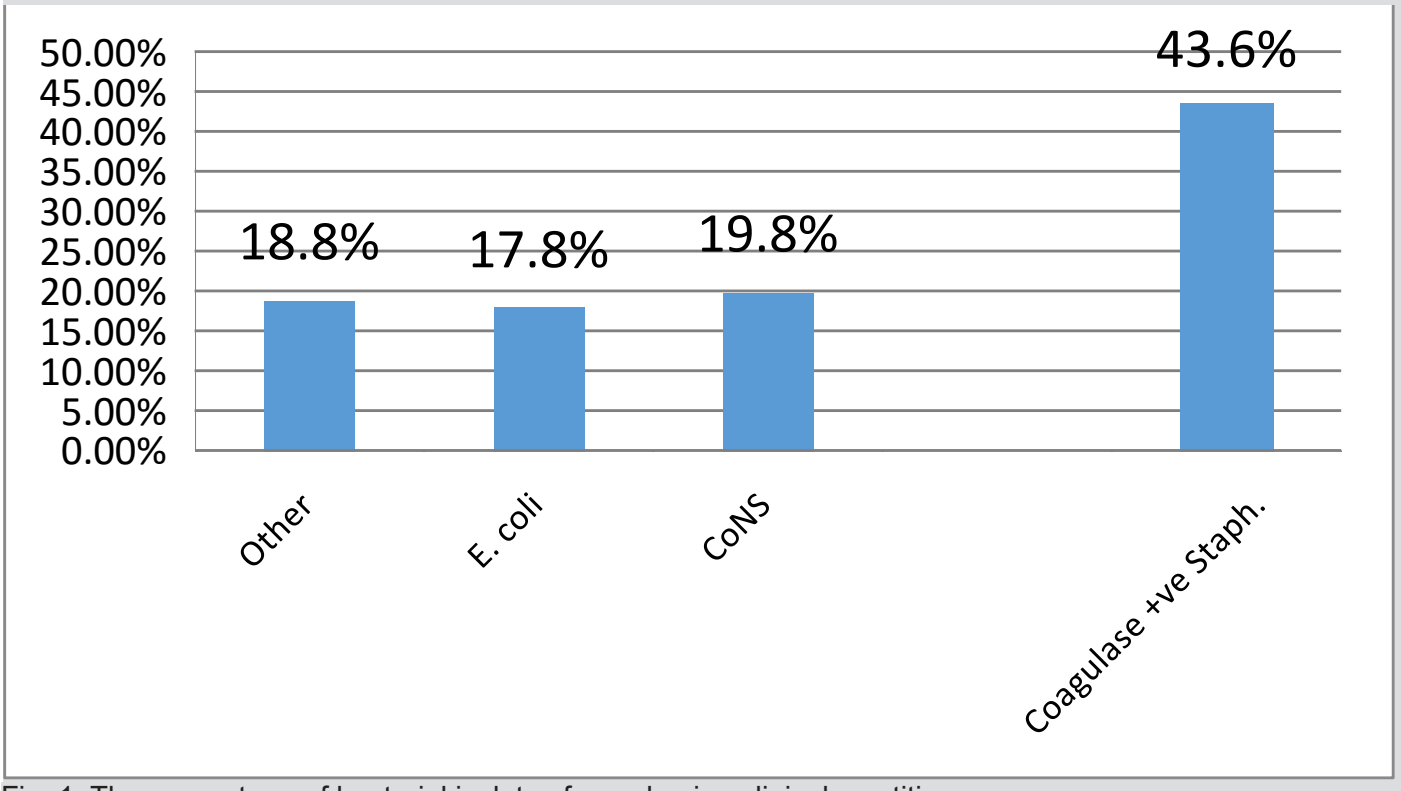

Fig. 1: The percentage of bacterial isolates from bovine clinical mastitis.

objective [i] to assessment of prevalence of CoNS in clinical bovine mastitis in dairy cows and the antibiogram of their isolates [ii] to investigate the slime production of CoNS isolates by phenotypic method on Congo Red Agar plates [CRA] [iii] Genotypic detection of *mecA,icaA and icaD* genes by polymerase chain reaction [PCR].

Material and Methods

A. Milk sample collection and laboratory analysis:

This study was done on 105 dairy cows with clinical mastitis admitted to Veterinary Clinic, in Assiut, Egypt. After physical examination and conformation of clinical mastitis, 20 ml milk samples was taken aseptically from all quarters of animals suffering from clinical mastitis and immediately transferred cool to the laboratory.

Mill samples incubated for 24 h. at 37°C, centrifuged, and then 0.01mL of sediment of each milk samples was cultured on blood agar with 5% sheep blood, Mannitol salt agar [BBL] and MacConkey agar [Biomark Lab. India] which incubated at 37°C for 48 h. The growing surface colonies were identified by cultural, morphological and biochemical characters according to[15], as well as coagulase test to detect coagulase negative isolates.

B. In vitro antimicrobial susceptibility test:

It was evaluated using the disc-diffusion method on the Mueller-Hinton agar according to the guidelines of the National Committee for Clinical Laboratory Standards[16]**.** Kirby- Bauer's disc diffusion technique was adapted for antibiogram. The CoNS strains susceptibility to the following antimicrobial [Bioanalyse-Turkey] was tested: Oxacillin [OX] 1 µg, Ampicillin [AM] 10 µg, Cefotaxime [CTX] 30 µg, Cloxacillin [CX] 1 µg, Doxycycline [DO] 30 µg, Enrofloxacin [ENR] 5 µg, Gentamicin [CN] 10 µg, Lincomycin [L] 2 µg, Oxytetracycline [T] 30 µg, Penicillin [P] 10 µ and Trimethoprim–Sulflamethaxzole [SXT] 25 µg.]. Plates with discs were left at room temperature for 30 minutes and incubated at 35°C for 24 h. 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[15]**.**

C. Detection of slime production on Congo Red Agar medium:

The medium was composed of brain heart infusion broth [Oxoid Ltd, Basingstoke, England] 37 g/l, sucrose 50 g/L, agar No 1 [Oxoid] 10 g/L and Congo red 0.8 g/L Congo red stain [prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes separately from the other medium constituents] and was then added when the agar had cooled to 55°C. Plates of the medium were cultured and incubated aerobically for 24 hours at 37°C. A positive result was indicated by black colonies with a dry crystalline consistency. Non-slime producers usually remained pink[17]**.**

D. PCR for detection *of mecA, icaA* and *icaD* genes:

Application of PCR for identification of *mecA, icaA and icaD* genes of *Staphylococcus spp.* was performed essentially by using Primers [Pharmacia Biotech] as shown in the following Table [1]:

2. DNA extraction from bacterial culture [20]:

After overnight culture on brain-heart infusion agar plates, the bacterial colonies were suspended in 20 ml of sterile distilled water, and the suspension was then heated at 100ºC for 20 minutes. From this suspension, a 5 µl aliquot was directly used as a template for PCR amplification.

3. DNA amplification reaction of Staphylococci:

The amplification was performed on a programmable thermal Cycler [Biometra] using total volum of 25 µl of PCR mixture consists of 5 µl of jena biosciences mix[Lot:111.816], 10 PM of each primer set [mecA,*icaA, icaD*],2 µl of extracted DNA and 15 µl of dist. water . The PCR programis: initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1min, annealing at 58°C for 1 min and extension at 72°C for 1min, with final extension at 72°C for 5 min. Amplified products [154,188 and 346 bp respectively] were analyzed by 1.5% of agarose gel electrophoresis stained with ethidium bromide and visualized and captured on UV transilluminator.

Statistical analysis was done using Chi-square by SPSS, 2005 program [Statistical Package for Social Sciences for Windows Release 14.0.0.].

Results

Table 3: In vitro antimicrobial susceptibility of CoNS isolated from bovine clinical mastitis (n= 17)

Antimicrobial agents**	S. saprophyticus (n.= 11)		S. epidermidis (n.= 6)		Total (n.= 17)	
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
Oxacillin	6 (54.54%)	5 (45.45%)	1 (16.67%)	5 (83.33%)	7(41.18%)	10 (58.82%)
Enrofloxacin	11 (100%)	0 (0%)	6 (100%)	0 (0%)	17 (100%)	0 (0%)
Gentamicin	11 (100%)	0 (0%)	6 (100%)	0 (0%)	17 (100%)	0 (0%)
Doxycycline	10 (90.91%)	1 (9.09%)	6 (100%)	0 (0%)	16 (94.11%)	1 (5.88%)
Trimethoprim –Sulflamethax-zole	5 (45.45%)	6 (54.55%)	5 (83.33%)	1 (16.67%)	10 (58.82%)	7 (41.18%)
Oxytetracycline	7 (63.64%)	4 (36.36%)	4 (66.67%)	2 (33.33%)	11 (64.71%)	6 (35.29%)
Penicillin	7 (63.64%)	4 (36.36%)	3 (50%)	3 (50%)	10 (58.82%)	7 (41.18%)
Ampicillin	7 (77.77%)	4 (36.36%)	2 (33.33%)	4 (66.67%)	9 (52.94%)	8 (47.06%)
Cloxacillin	4 (36.36%)	7 (63.64%)	3 (50%)	3 (50%)	7 (41.18%)	10 (58.82%)
Cefotaxime	1 (9.09%)	10 (90.91%)	0 (0%)	6 (100%)	1 (5.88%)	16 (94.11%)
Lincomycin	0 (0%)	11 (100%)	0 (0%)	6 (100%)	0 (0%)	17 (100%)

**Highly significant statistical variations between different antimicrobial agents; χ^2= 49.87 for S. saprophyticus; χ^2= 36.42 for S. epidermidis $P < 0.001$ for both.

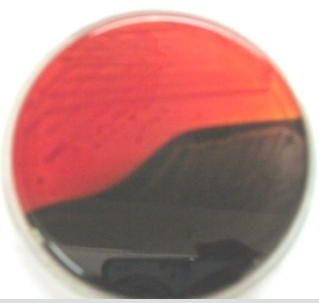

Fig. (2): Congo Red binding test. Above: Non slime producing CoNS isolate (pink colonies). Below: Slime producing CoNS isolate (black colonies).

Detailed obtained results were illustrated in Tables [2 -4].

In this study, from a total of 105 milk samples collected from clinical mastitis cases, 101 [96.2%] were positive for bacterial growth. The isolated bacteria from 101 positive specimen culture were as shown in Fig. 1.

Among the17 CoNS tested for slime production on CRA plates, 13 isolates [76.47%] were positive [5 [83.3%] & 8 [72.7%] isolates out of [6] and [11] *Staph. epidermidis* and *Staph. saprophyticus*, respectively, Fig. 2].

Discussion

For many decades, coagulase-negative staphylococci [CoNS], widely spread in the natural environment and colonizing the skin and mucosa of animals and humans, have been considered non-pathogenic. At present, they are the predominant aetiological factor of bovine mastitis in many countries[2,21]. Our findings and literature data reveal that the highest incidence of mastitis is caused by bacteria, including coagulase-negative staphylococci prevalent in many countries, according to this study the percentage of CoNS species isolated from milk of cows with clinical mastitis was 19.80%, as shown in Fig. 1 & Table 2. Similar results of CoNS isolation were obtained [16.6; 17.95; 23.3; 22.9; 18.91 and 18.8 %, respectively] [1, 3, 21, 22, 23, 24].The high percentage of CoNS 49.6% and 53.1% was detected by[25, 26], respectively. While the low percentage 8.8% was found by [27].

The CoNS consist of more than 50 species, and are the most frequently isolated pathogens from udder quarters[28]. The present work showed that *Staph. Saprophyticus* [10.89%] and *Staph. epidermidis* [5.94%] constituted the highest percentage of CoNS species isolated from the milk of cows with clinical mastitis followed by *Staph. chromogenes* [1.98%] and *Staph. simulans* [0.99%], Table 2. The results regarding other countries were slightly different. The highest percentage of CoNS species isolated from the milk of cows with mastitis in Japan by[29]and inFinland by[30]was *S. epidermidis*; in Sweden by [31]was*S. simulans*; in Poland by[21, 32]was *S. xylosus*. While in Korea *Staphylococcus simulans*, *Staphylococcus haemolyticus*, *Staphylococcus sciuri*, *Staphylococcus xylosus*, *Staph. epidermidis*, and *Staphylococcus saprophyticus* isolates were identified by using biochemical tests from bovine mastitis milk[33].

Determination of susceptibility or resistance of the isolates to antibiotics is very important from a clinical and economic point of view. Moreover, the public health of this issue is of great importance because antibiotic therapy of infectious diseases in animals poses the risk of selection of resistant strains and introduction of these strains into the food chain[34]. The *in vitro* activities of CoNS against 11 selected antimicrobial agents are summarized in Table 3. There are highly significant statistical variation between different antimicrobial agents [p < 0.001] in the effect on different isolated spices. The highest resistance rate of CoNS observed to Lincomycin [100%], followed by Cefotaxime [94.41%], Oxacillin [58.82%], Ampicillin [47.06%] and Penicillin [41.18%], while the highest rate of sensitivity observed to Enrofloxacin and Gentamicin [100%, for each], followed by Doxycycline [94.11%].Present findings are comparable with the results provided by[26] that CoNS species were sensitive to Enrofloxacin [100%] followed by Kanamycin [92.2%], and resistance to Penicillin was 56.6%. CoNS bacteria were not Gentamicin-resistant [35]. Also[33, 36]found that 58% and 60.2% of CoNS were resistant to Penicillin, respectively. More than 70% of the CoNS isolates worldwide are resistant to methicillin or oxacillin and in their study found that CoNS clinical isolates were resistant to oxacillin with a percentage 62.1%[37]. Coagulase negative staphylococci[CoNS] showed complete sensitivity to Tetradelta [100%] and higher sensitivity to Enrofloxacin [94.14%],[38]. Tetracycline more effective antibiotics against all bacteria isolated from bovine mastitis [23]. In contrast to our findings,[39]have been reported that 79.41%, 76.47, 73.52, 42.94 and 23.23% of CoNS isolates from bovine mastitis were susceptible to Cefotaxime, Methicillin, Ciprofloxacin, Gentamycin and Penicillin, respectively.97.14% of CoNS isolates were sensitive to Lincomycin[38]. Low resistant of CoNS strains to methicillin [2.4%] was reported by[33]. Penicillin- resistance found in our study is higher than that previously reported [10 and 5.71%] for CoNS by[22, 23], respectively. Coagulase negative staphylococci [CoNS] are capable of causing opportunistic bovine mastitis, many of these strains are resistant to Penicillin or Ampicillin because of the long-term use of β-lactam antibiotics in agricultural and healthcare settings[33]. Indiscriminate use of the antibiotics in the farm animal practice coupled with the increasing pathogenicity of the CoNS was suspected to be the issue of major concern [40]. The frequency of methicillin-resistant strains in CoNS varies widely among

Table 4: Methicillin resistant CoNS strains tested for their phenotypic(slime production) and genotypic characteristics.

No.	Methicillin resistant coagulase – negativestaph. spp.(MRCoNS)	Result on CRA	PCR results		
			mecA	icaA	icaD
1	Staphyl. saprophyticus	+ve	-ve	+ve	+ve
2	Staphyl. saprophyticus	+ve	+ve	+ve	-ve
3	Staphyl. saprophyticus	-ve	-ve	+ve	-ve
4	Staphyl. epidermidis	+ve	+ve	+ve	+ve
5	Staphyl. epidermidis	+ve	+ve	+ve	-ve
6	Staphyl. epidermidis	+ve	-ve	+ve	+ve

No significant statistical variations $\chi^2 = 1.5$

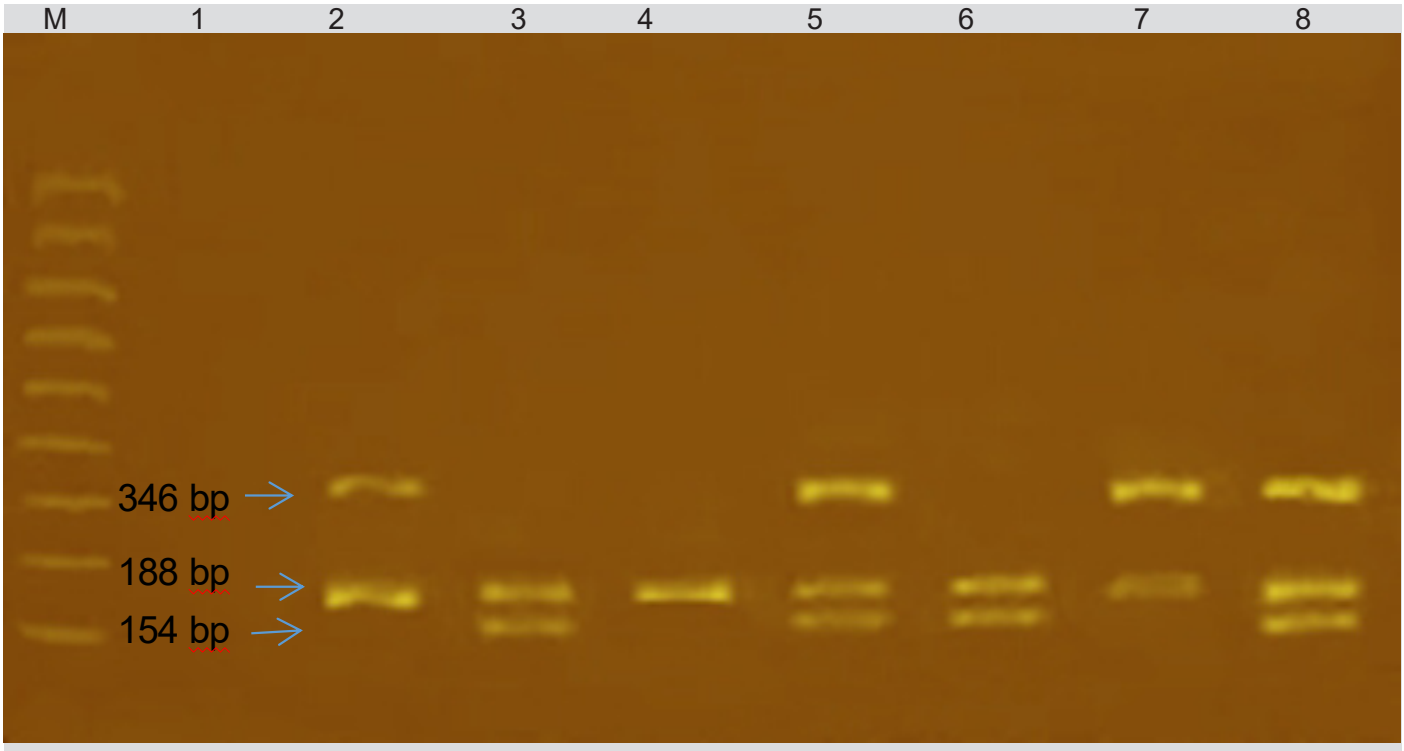

Fig.(3): 1.5% Agarose gel electrophoresis of multiplex PCR of mec A (154 bp), icaA (188 bp) and icaD (346 bp) genesfor characterization of S.epidermidis and S.saprophyticus. Lane M: 100 bp ladder as molecular size DNA marker. Lane 1: Control negative for mecA, icaA and icaD genes. Lane 8: Control positive for mecA, icaA and icaD genes. Lane 2: Positive S.saprophyticus for icaA and icaD genes. Lane 3: Positive S.saprophyticus for mecA and icaA genes. Lane 4: Positive S.saprophyticus for icaA gene. Lane 5: Positive S.epidermidis for mecA, icaA and icaD genes. Lane 6: Positive S.epidermidis for mecA and icaA genes. Lane 7: Positive S.epidermidis for icaA and icaD genes.

different species, with resistance being predominant in *Staph. hominis*, *Staph. haemolyticus* and *Staph. epidermidis* and infrequent in *Staph. capitis*and and *Staph. saprophyticus*[11].

β-Lactam antibiotics are frequently used in intramammary infusion therapy. Bacterial β-lactam resistance mechanisms include production of β-lactamases and low-affinity penicillin-binding protein 2a [PBP2a]. The latter, designated for methicillin resistance, precludes therapy with any of the currently available β-lactam antibiotics, and may predict resistance to several classes of antibiotics other than β-lactams[41].

In the present work the presence of the *mecA* gene was investigated by PCR, the incidence of methicillin resistance in the tested MRCoNS was 50% [3/6] by the presence of *mecA* gene, as shown in Table [4] and Fig. [3]. The positive detection rates of *mecA* in MRCoNS were 79% and 63.2% by[11,33], respectively. In recent years, increased numbers of *β*- lactamase- producing CoNS and *mecA*- gene positive CoNS [MRCoNS] resistant to all groups of β-lactam antibiotics have been observed [33].In the present study, three [50%] CoNS strains were positive phenotypically by disc diffusion method and negative byPCR for detection of methicillin resistance, Table [4]. The differences between molecular and phenotypic determinations of methicillin resistant was reported by [11]. The isolates that did not carry *mecA* were phenotypically resistant to methicillin[33]. These strains appeared to be β-lactamase hyper-producing strains. The phenotypic expression of resistance can vary depending on the growth conditions [e.g., the temperature or osmolarity of the medium], making susceptibility testing of MRS by standard microbiological methods potentially difficult [10]. PCR method detecting the *mecA* gene from staphylococci isolated rapidly and provides a definitive answer for the presence of the *mecA* gene, whereas the phenotypic tests do not[33].

Bacteria in a biofilm are more resistant to antibiotics than in their planktonic form[8]. The Congo Red method is rapid, sensitive, practical and reproducible for the detection of slime production in *Staphylococcus spp.* and has the advantage that colonies remain viable on the medium[17, 42]. In the present study, slime production was examined on Congo Red Agar, 13 CoNS isolates [76.47%] were found to be slime production positive result was indicated by black colonies Fig. 2. These results agreed with that reported [72.1%]by[43]. Slime production in CoNS isolates was 47.8% reported by[42] and it was 48.7% in *S. epidermidis* has found by[44]. The data reported here indicate an important role of slime production as a virulence marker for *S. epidermidis,* where 83.3% of the isolated *S. epidermidis* were slime producer. These results similar to those reported by[37]who found that clinical CoNS isolates had a high frequency of slime production and drug resistance, particularly *S. epidermidis* strains.

Combination of phenotypic and genotypic methods recommended for identifying biofilm producing strains. The intercellular adhesion [*ica*] locus, consisting of the genes *icaADBC*, has been reported to have a potential role as a virulence factor in the pathogenesis of mastitis in ruminants,[45]. Among the *ica* genes, *icaA* and *icaD* have been reported to play a significant role in biofilm formation in *S. aureus* and *S. epidermidis*,[46] In this study, slime factor production of methicillin resistant coagulase – negative *staph. spp.* [MRCoNS] isolates were detected by PCR targeting *icaA* and *icaD* genes and found that 3 [50%] of the tested MRCoNS strains were positive for both *icaA* and *icaD* genes. Six [100%] and three [50%] isolates were positive for *icaA* gene and *icaD* gene, respectively. In addition one isolates out the six CoNS isolates [16.67%] was positive for the presence of *icaA, icaD* and *mecA* genes and also has the ability to produce slime as one of the most important virulence factor, as shown in Table [4] and Fig. 3. While the prevalence rates of *icaA* and *icaD* genes were 5.9% and 47.1% in CoNS isolated from bovine subclinical mastitis, respectively[43]. This difference in the prevalence rates can be attributed to variation in DNA sequences which may lead to failed amplification of the gene in some isolates leading to false negative results [6]. The better methodology for biofilm detection is to screen strains for *ica* genes in addition to CRA or MTP methods not to miss the genotypically positive phenotypically negative strain[45].

Conclusion

The findings of the present study demonstrated the ability of CoNS isolated from bovine clinical mastitis to form biofilms. This must be considered as an alarming situation, and so attention must be paid toward implementation of new ways for effective prophylaxis, control, and treatment of such infections in the dairy farms. The prudent use of antibiotics and rapid and continuous screening for resistant microorganisms should be more focused to prevent the

emergence and spread methicillin resistant coagulase negative staphylococci, because these strains can cause severe damage to infected sites and may be widespread in the environment.

Conflict of interest

None

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