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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

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The aim of this study was to determine and 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). The study was done on 105 milk samples obtained from bovine clinical mastitis and found that 101samples (96.2%) were positive for bacterial culture. CoNS species can be isolated from20 samples with a percentage 19.8%. Their ability to form biofilm as one of the most important virulence factors 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 6 CoNS isolates (50%). Also, six (100%) and three (50%) isolates were positive for *icaA* gene and *icaD* gene, respectively, and 3 isolates (50%) were positive for both *icaA* and *icaD* genes. In addition, one out of the six CoNS isolates (16.67%) was positive for the presence of *icaA, icaD* and *mecA* genes and also it has the ability to form biofilm. Conclusion, 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.

Key words: *Cows, clinical mastitis, coagulase-negative staphylococci, slime factor, mecA, icaA & icaD genes.*

INTRODUCTION

Mastitis in dairy cows is a serious problem as it an economically devasting disease causing immense economic losses in the dairy industry in Egypt (El-Damaty, 2013). 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 (Pyörälä and Taponen, 2009). 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 (Idriss *et al.*, 2013).

Coagulase negative staphylococci are always present on the udder skin and in teat canals; under favourable 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], (Bochniarz and Wawron, 2012).

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 (Oliveira *et al.*, 2006). Likewise, biofilm-producing isolates have been reported for many CoNS species, especially in *S. epidermidis* (Tormo *et al.*, 2005 and

Oliveira *et al.,* 2006). 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 (Cucarella *et al.,* 2004 and Melchior *et al.,* 2006). Biofilm consists of polysaccharide intercellular adhesion (PIA) encoded by the intercellular adhesion *icaADBC* operon (Stevens *et al.,* 2008).

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(Chambers, 1997).Incidence of methicillin resistance in CoNS is high, as well as, the accompanying antimicrobial resistance (Bogado *et al.,* 2001).

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 (Chambers, 1997). Methicillin resistance may result from a series of factors including high degree of intrinsic resistance, hyperproduction of *β* -lactamase or *mecA*-associated resistance. These factors may operate and interact in the same strain (De Lencastre *et al.,* 1991 and Unal *et al.,* 1992). 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* (Archer and Niemeyer, 1994).

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 (Bogado *et al.,* 2001).

Keeping in view the economic loss caused by the bovine mastitis and emergence of drug resistant CoNS, the present investigation was undertaken with the following objectives (i) assessment of prevalence of CoNS in clinical bovine mastitis in dairy cows and the antibiogram of the CoNS isolates (ii) 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).

MATERIALS and METHODS

A - Milk samples 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.

Amount of 0.01mL 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 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 Quinn *et al.* (2004).

B -*In vitro* antimicrobial susceptibility test:

It was evaluated by using the disc-diffusion method on the Mueller-Hinton agar according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, 2002). 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^{o}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) $\geq$ 18 mm; resistance (R) $\leq$ 17 mm (Quinn *et al.,* 2004).

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 was prepared as a concentrated aqueous solution and autoclaved (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 inoculated 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 (Freeman *et al.,* 1989).

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

1. Materials used for PCR:

1.1. Reagents used for agarose gel electrophoresis:

1.1.1. Agarose powder, Biotechnology grade (Bioshop^R, Canda inc. lot No: OE16323). It prepared in concentration 2% in 1× TAE buffer.

1.1.2. Tris acetate EDTA (TAE) electrophoresis buffer (50×liquid concentration) (Bioshop R, Canda inc. lot No: 9E11854). The solution diluted to 1× by adding 1 ml stock solution to 49 ml double dist. water to be used in the preparation of the gel or as a running buffer.

1.1.3. Ethidium bromide solution (stock solution) biotechnology grade (Bioshop ® Canda Inc, Lot No: 0A14667): The stock solution was diluted by 25µl /200ml double distilled water and stored covered at 4ºC. It was used for staining of PCR products that electrophoreses on agarose gel to be visualized by UV light.

1.2. Gel loading buffer (6×stock solution), (Fermentas, lot No: ooo56239).

The components were dissolved in sterile double distilled water and stored covered with aluminum foil at room temperature.

1.3. DNA ladder (molecular marker): 185 & 160 bp (Fermentas, lot No: 00052518).

1.4. 5X Taq master (Fermentas): Containing polymerase enzyme, Magnesium chloride (Mg Cl_2), Deoxy nucleotide triphosphate (dNTP) and PCR grade water.

1.5. Primer sequences of *Staphylococcus spp.* used for PCR identification system:

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.

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	**Martín *et al.* (2004)**
mecA (R)	5′ TTG CGA TCA ATG TTA CCG TAG ′3		
icaA (F)	5′ TCT CTT GCA GGA GCA ATC AA′3	188	**Shusheng *et al*. (2013)**
icaA (R)	5′ TCA GGC ACT AAC ATC CAG CA′3		
icaD (F)	5′ ATG GTC AAG CCC AGA CAG AG ′3	346	**Shusheng *et al.* (2013)**
icaD (R)	5′ CGT GTT TTC AAC ATT TAA TGC AA′3		

2. DNA preparation from bacterial culture (Iorio *et al.*, 2001):

After overnight culture on brain-heart infusion agar plates, one or two 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 (Jukes *et al.*, 2010):

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany) using 25 µl of PCR mixture containing 3 µl of boiled cell lysate, 250 µM of each desoxynucleotide triphosphate, 1.5 U of Taq DNA polymerase (Biotools, Madrid, Spain), buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl and 3 mM MgCl2, Biotools), 1 µM of the primers *mecA*-R, *mecA*-F; 0.8 µM of *icaA*-R, *icaA*-F and 0.8 µM of *icaD*-R, *icaD*-F. Amplification conditions were: denaturation for 3 min at 94°C, followed by 30 cycles of 94°C for 1min, 58°C for 1 min and 72°C for 1min, with final extension at 72°C for 5 min. Amplified products were analyzed by 1.5% of agarose gel electrophoresis stained with ethidium bromide and visualized and captured on UV transilluminator.

RESULTS

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 samples (96.2%) lead to positive culture. The isolated bacteria from 101 positive specimen culture were as shown in Fig. 1.

Among CoNS (n=17) isolates tested for slime production on CRA plates, 13 isolates (76.47%) were found to be slime producers [Five from 6 *Staph. epidermidis* isolates (83.3%) and 8 from 11 *Staph. saprophyticus* isolates (72.7%) were positive for slime production, Fig. 2].

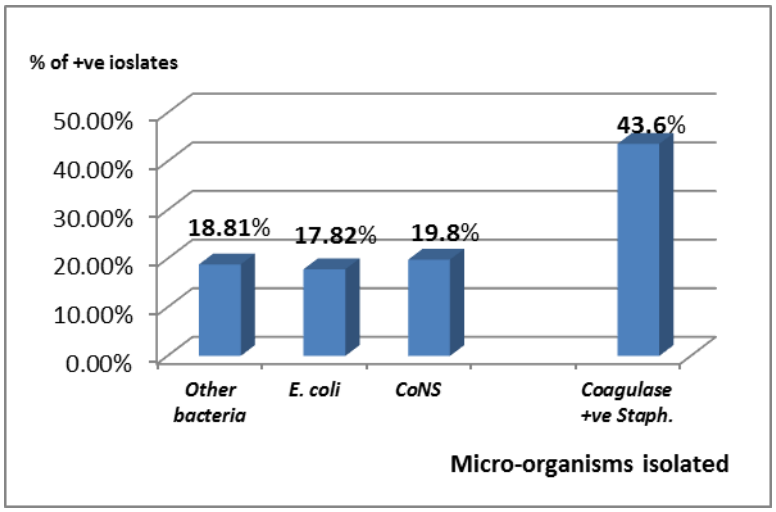

Fig. 1: The percentage of bacterial isolates from bovine clinical mastitis.

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

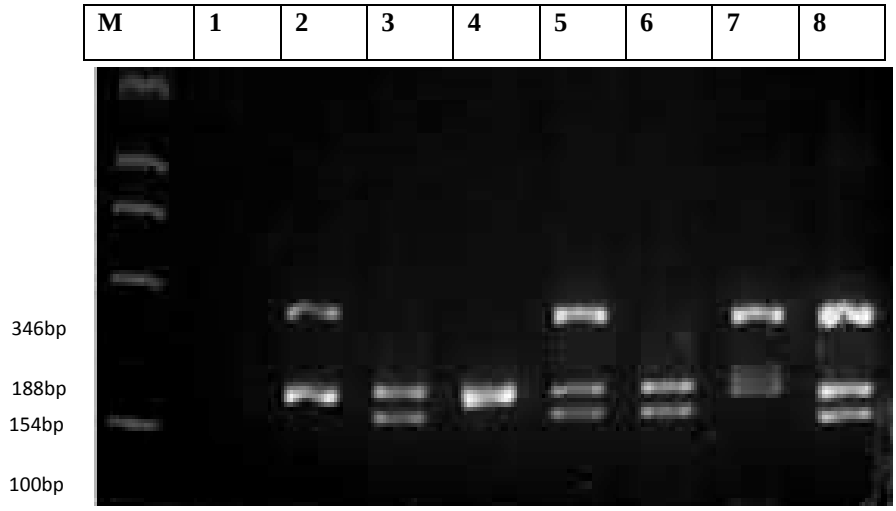

Fig. 3: Agarose gel electrophoresis of PCR of *mecA* (154 bp), *icaA* (188 bp) and *icaD* (346 bp) genes for characterization of *S. epidermidis* and *S. saprophyticus*. Lane M: 100 bp ladder as molecular size DNA marker. Lane 1: Control negative for *mecA,icaA* and *icaD* genes. Lane 8: Control positive 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.

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

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

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 (54.55%)	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 (94.12%)	1 (5.88%)
Trimethoprim – Sulflamethaxzole	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 (63.64%)	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 (94.12%)
Lincomycin	0 (0%)	11 (100%)	0 (0%)	6 (100%)	0 (0%)	17 (100%)

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

No.	Methicillin resistant coagulase – negative *staph. 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

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 (Pyörälä and Taponen, 2009 and Bochniarz *et al.,* 2013).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 19.8%, as shown in Fig. 1 & Table 2. Similar results of CoNS isolation were obtained byKudinha and Simango (2002); Kurjogi and Kaliwal (2011); Vasiľ *et al.* (2012); Bochniarz *et al.* (2013); Idriss *et al.* (2013) and El-Damaty (2013), (22.9; 18.91; 18.8; 23.3; 17.95 and 16.6%, respectively). The high percentage of CoNS 49.6% and 53.1% was detected byPitkälä *et al.* (2004) and Moniri *et al.* (2007), respectively. While, the low percentage 8.8% was found by Kalmus *et al.* (2006).

The CoNS consist of more than 50 species, and are the most frequently isolated pathogens from udder quarters (Larissa *et al.,* 2013). 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 Baba *et al.* (1980) and in Finland by Jarp (1991) was *S. epidermidis*; in Sweden by Birgersson *et al.* (1992) was *S. simulans*; in Poland by Malinowski *et al.* (2006) and Bochniarz *et al.* (2013) was *S. xylosus*. While in Korea, *Staph. simulans*, *Staph. haemolyticus*, *Staph. sciuri*, *Staph. xylosus*, *Staph. epidermidis* and *Staph. saprophyticus* isolates were identified by using biochemical tests from bovine mastitis milk (Moon *et al.,* 2007).

Determination of susceptibility or resistance of strains to antibiotics is very important from a clinical and economic point of view. Moreover, the public health of this issue is of great importance becauseantibiotic therapy of infectious diseases in animals poses the risk of selection of resistant strains and introduction of these strains into the food chain (Lee, 2003). The *in vitro* activities of CoNS against 11 selected antimicrobial agents are summarized in Table 3. The highest resistance rate of CoNS observed to Lincomycin (100%), followed by Cefotaxime (94.12%), 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 (94.12%). Present findings are comparable with the results provided by Moniri *et al.* (2007) 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 (Gianneechini *et al.*, 2002). Also, Bouman *et al.* (1999) and Moon *et al.* (2007) found that 58% and 60.2% of CoNS were resistant to Penicillin, respectively. Arslan and Özkardes (2007) recorded that currently, 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%. Idriss *et al.* (2014) found that CoNS showed complete sensitivity toTetradelta (100%) and higher sensitivity to Enrofloxacin (94.14%). Tetracycline more effective antibiotics against all bacteria isolated from bovine mastitis (Kurjogi and Kaliwal, 2011). In contrast to our findings, Kaliwal *et al.* (2011) havebeen reported that 79.41, 76.47, 73.52, 42.94 and 23.23% of CoNS isolates from bovine mastitis were susceptible toCefotaxime, Methicillin, Ciprofloxacin, Gentamycin and Penicillin, respectively. Idriss *et al.* (2014) found that 97.14% of CoNS isolates were sensitive to Lincomycin. Low resistant of CoNS strains to methicillin (2.4%) was reported by Moon *et al.* (2007). Penicillin- resistance found in our study is higher than that previously reported (10 and 5.71%) for CoNS by Kudinha and Simango (2002) and Kurjogi and Kaliwal (2011), 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 (Moon *et al.,* 2007). 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 (Nagappa and Singh, 2013). The frequency of methicillin-resistant strains in CoNS varies widely among different species, with resistance being predominant in *Staph. hominis*, *Staph. haemolyticus* and *Staph. epidermidis* and infrequent in *Staph. capitis* and *Staph. saprophyticus* (Bogado *et al.,* 2001).

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 (Odd and Maeland, 1997).

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 Bogado *et al.* (2001) and Moon *et al.* (2007), 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 (Moon *et al.,* 2007). In the present study, three (50%) CoNS strains were positive phenotypically by disc diffusion method and negative byPCR fordetection of methicillin resistance, (Table 4). The differences between molecular and phenotypic determinations of methicillin resistant was reported by Bogado *et al.* (2001). The isolates that did not carry *mecA* were phenotypically resistant to methicillin (Moon *et al.,* 2007). 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 (Chambers, 1997). 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 (Moon *et al.,* 2007).

Bacteria in a biofilm are more resistant to antibiotics than in their planktonic form (Melchior *et al.,* 2006).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 (Freeman *et al.,* 1989 & Türkyilmaz and Eskiizmirliler, 2006). In the present study, slime production was examined on Congo Red Agar, 13/17 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 Darwish and Asfour (2013). Slime production in CoNS isolates was 47.8% reported byTürkyilmaz and Eskiizmirliler (2006) and it was 48.7% in *S. epidermidis* has found by Mohan *et al.* (2002). 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 Arslan and Özkardes (2007) 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 (Vasudevan *et al.,* 2003). Among the *ica* genes, *icaA* and *icaD* have been reported to play a significant role in biofilm formation in *S. aureus* and *S. epidermidis*, (Gotz, 2002). 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. The prevalence rates of *icaA* and *icaD* genes were 5.9% and 47.1% in CoNS isolated from bovine subclinical mastitis, respectively (Darwish and Asfour, 2013). 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 (Tormo *et al.,* 2005). In the present study, all CoNS isolates which positive for slime production on CRA plates were also positive for detection of *icaA* gene andsome isolates were positive for presence of *icaD* gene by PCR (Table 4). Vasudevan *et al.* (2003) argued that a 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 strains.

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.

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الكشف عن المكور العنقودي السالب لإنزيم التجلط والمقاوم للميثاسلين والمفرز لعوامل الالتصاق المسبب لالتهاب الضرع الإكلينيكي في الأبقار باستخدام تفاعل البلمرة المتسلسل

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هدفت هذه الدراسة إلي التحقق من أنتاج البيوفيلم في المكورات العنقودية السالبة لإنزيم التجلط والمقاومة للميثاسلين باعتباره واحدا من عوامل الضراوة باختبار نموها معمليا على أطباق الكونغو الأحمر وكذلك الكشف عن الجينات *icaA, icaD* and *mecA* عن طريق تفاعل البلمرة المتسلسل. أجريت هذه الدراسة علي ١٠٥ عينات من اللبن لأبقار مصابة بالتهاب الضرع الإكلينيكي، وجد منها ١٠١ عينة (٩٦,٢٪) إيجابية للفحص البكتيريولجي. وأمكن عزلا لمكور العنقودي السالب لإنزيم التجلط من ٢٠ عينة بنسبة ١٩,٨٪. وكانت ١٣ عتره من ١٧ عترة (٧٦,٤٧٪) ايجابية لإفراز البيوفيلم معمليا. وباستخدام تفاعل البلمرة المتسلسل وجد ثلاث عترات من ست (٥٠٪) تحتوي علي الجين *mecA*. وكانت ست عترات (١٠٠٪) وثلاث عترات (٥٠٪) ايجابية للجين *icaA* والجين *icaD* ، علي الترتيب. كما وجد ثلاث عترات (٥٠٪) تحتوي علي كل من الجينين *icaD* and *icaA*. وكانت عترة واحدة (١٦,٦٧٪) مفرزة للبيوفيلم وتحتوي علي جميع الجينات *icaA, icaD* and *mecA*. خلصت هذه الدراسة علي قدرة المكور العنقودي السالب لإنزيم التجلط، المعزولة من حالات التهاب الضرع الإكلينيكي في الأبقار، علي تكوين البيوفيلم. ويجب اعتبار هذا الوضع ينذر بالخطر، ويجب الاهتمام لذلك نحو تنفيذ طرق جديدة للوقاية الفعالة، والسيطرة، وعلاج هذه العدوى في مزارع الألبان. وإن الاستخدام الرشيد للمضادات الحيوية والفحص السريع والمتواصل للكشف عن الميكروبات المقاومة ينبغي أن تكون أكثر تركيزا لمنع ظهور وانتشار المكور العنقودي السالب لإنزيم التجلط والمقاوم للميثاسلين، لأن هذه السلالات يمكن أن تسبب أضرارا بالغة في الأماكن المصابة ويمكن أن تكون واسعة الانتشار في البيئة.