Assiut University web-site: www.aun.edu.eg

DETECTION OF BIOFILM FORMATION AND ITS ASSOCIATED GENES IN COAGULASE NEGATIVE STAPHYLOCOCCI ISOLATED FROM MILK OF COWS AFFECTED WITH SUBCLINICAL MASTITIS

AMAL AWAD
Bacteriology, Mycology and Immunology Department, Faculty of Veterinary Medicine,
Mansoura University, Mansoura 35516, Egypt.

Received: 28 June 2017; **Accepted:** 30 July 2017

ABSTRACT

Staphylococcus pathogenicity is attributed to the presence of extracellular elements and invasive mechanisms including biofilms formation. The present study was designed to estimate the frequency of coagulase negative staphylococci (CNS) in cow's milk affected with subclinical mastitis, determine its ability to form biofilms on polyesterne surface, and for detection of biofilms associated genes (*bap, icaA and icaD*) using PCR. Accordingly, a total of 160 milk samples from cows affected with subclinical mastitis based on the results of California Mastitis Test (CMT) were collected from individual quarters of lactating cows. All collected milk samples were subjected to bacterial isolation procedures following the standard procedures. The overall prevalence of CNS was 69 (43.12%), *S. chromogenes* was the predominate CNS isolates followed by *S. epidermidis*. The phenotypic biofilms formation was tested using microtitre plate technique, 56(81.15%) strains were tested positive for biofilm formation of them, 53.57% (30/56) were strong, 32.14% (18/ 56) were moderate and 14.28% (8/56) were week biofilms producers. Among the CNS species, S. *chromogenes* was the predominated biofilm producing stains. Regarding the existence of biofilm associated genes, *icaA* was detected in 25(36.23%) isolates, *icaD* was harbored by 33(47.82%) CNS strains and *bap* gene couldn't be identified in all of the tested CNS isolates. *icaD* was correlated with biofilm formation on polysterne surface of microtitre plate. In conclusion, this study provides information about the frequency of CNS in bovine subclinical mastitis and its pathogenicity which is necessary to enterprise efficient approaches to control bovine mastitis.

Key words: *Bovine, Mastitis, Staphylococci, Coagulase negative, Biofilm formation.*

INTRODUCTION

Bovine mastitis is a multifactorial disease that affects dairy cows and causes serious economic losses in dairy animals worldwide (Bradley *et al*., 2002). Staphylococci are considered as a major cause of mastitis in dairy cows worldwide. Coagulase negative staphylococci are usually considered to be minor pathogens and this group is heterogenic which contains more than 50 species and subspecies. However, CNS have become the most common mastitis causing agents in many countries (Dieser *et al.*, 2014 and Vanderhaeghen *et al.,* 2014). CNS are found on normal skin of the udder teat and on milkers' hands which form part of the normal flora. This may cause opportunistic infection via penetrating secretory tissues. They usually isolated from subclinical mastitis but have also been found in clinical mastitis (Pyörälä and Taponen 2009; Vanderhaeghen *et al.,* 2014). This may be resulted in tissue damage and decrease in milk production. (Schukken *et al.,* 2009). Furthermore, CNS may cause insistent bovine intramammary infections that exist for long period especially in the absence of mediation (Gillespie *et al*., 2009).

A biofilm is an organized community of bacteria that are surrounded by a self-created, polymeric matrix that stick to either living or inorganic surface and creates a protected method of growth that permits its existence in a unfavorable condition of growth (Costerton *et al.,* 1999). Whereas biofilms might be not affect the severity of the disease (Tremblay *et al*. 2013 and Osman *et al.*, 2015), they may facilitate the adhesion and colonization of CNS on the mammary gland tissues, which may leads to persistent infections (Tremblay *et al.,* 2013). Moreover, biofilm-formation could possibly inhibit antimicrobial treatment as it makes CNS strains growing inside biofilms more resist to the frequently used antimicrobials. (Tremblay *et al.,* 2014).

Polysaccharide intercellular adhesin (PIA) is the primary determinant of the accumulation phase in biofilm formation. PIA Production is assisted by the

Corresponding author: Dr. AMAL AWAD
E-mail address: amalabdo@mans.edu.eg
Present address: Bacteriology, Mycology and Immunology Department, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt

ica ADBC operon, and strains contain this cluster are considered as biofilm producers (Cramton *et al.*, 1999). Biofilm associated protein (Bap) which is encoded by the bap gene may also responsible for formation of biofilms. Biofilm associated proteins have been recognized in staphylococcus species (Götz 2004). Bap shared in biofim formation as it involved in the primary attachment stage and responsible also for cell-to-cell aggregation with PIA (Cucarella *et al.,* 2001). The overall aims of this study, were to conduct a phenotypic characterization of coagulase negative staphylococci isolated from bovine mastitis, testing the ability of CNS to form biofilm in vitro in addition genotypic characterization of some biofilm associated genes (*icaA, icaD, bap*).

MATERIALS AND METHODS

Samples collection

160 individual quarter milk samples were collected from cows with subclinical mastitis during the first half of 2016 from two different dairy farms located in Dakahlyia provinces, Egypt. The numbers of the cows in each farm ranged from 300 to500 animals. California masttis test (CMT) was used for screening the milk samples for the existence of subclinical mastitis (CMT positive) following the protocol described by NMC (1990) and Quinn *et al.* (2002). All samples were collected following the standard milk sampling techniques (NMC 1990). In brief, hands were washed with clean water and detergents, udder and teats were cleaned with water and dried. The teat on the far side of the udder is cleaned first and then those of the near side (Nibret *et al.*, 2011). The teat ends subsequently were cleaned with a piece of cotton soaked in 70% ethyl alcohol. After discarding the first 2-3 milking streams, of milk (10mL) was collected from subclinical mastitic cases into sterile Falcon tubes. The samples were transferred in an icebox to the laboratory where they kept at 4ºC until examination within 24h.

Bacteriological examination

Bacterial examination was done according to Quinn *et al.* (2002). Briefly, loopful of milk sample was streaked on the surface of blood agar base supplemented with7% sheep blood (Oxoid, UK). The inoculated blood agar plates were incubated at 37 ºC for 24 hr under aerobic condition. The growing colonies were examined for their culture characters including, size, shape, color of colony, and their hemolytic activity. Staphylococcus presumptive colonies were subcultured onto the surface of nutrient agar plate (Oxoid) for farther investigation.

Identification of CNS

Gram stain was used for morphological characterization of the suspected colonies. Subsequently, the suspected staphylococcus colonies were subjected to biochemical tests including, catalase and coagulase tests. In addition, a group of biochemical reactions were used for differentiation of Staphylococci from Micrococci including, oxidation and fermentation of glucose, resistance to bacitracin (0.04 U) and susceptibility to furazolidone (100 µg) (Baker 1984). For CNS identification, A set of biochemical reaction were used following the reference method proposed by Kloos and Schleifer (1975) and Bannerman (2003), including, utilization of sugar (sucrose, mannitol, arabinose, lactose, fructose, ribose and mannose xylose, xylitol, maltose, trehalose), urease production, haemolysis on blood agar, nitrate reduction, ornithine decarboxylase and novobiocin resistance. Furthermore, staphylococcus isolates were confirmed by API Staph system (bioMérieux, Marcy l'Etoile - France).

Detection of biofilm formation in vitro

For detection of biofilm formation in vitro by CNS species using polysterne surface of microtiter plates, a 96 well microtiter plates were used following the protocol provided by Stepanović *et al.* (2007).

DNA extraction

For DNA extraction, QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used following the manufacturer's instruction with minor modification. In brief, 200 µl of the sample was incubated at 56^{O}C for 10 min with 200 µl of lysis buffer and 10 µl of proteinase K. Subsequently, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged and nucleic acid was eluted with 100 µl of elution buffer.

PCR on biofilm associated genes.

Biofilms associated genes *(icaA, icaD, bap*) were evaluated by PCR. Oligonucleotide primer sets (Metabion, Germany) used in this study are listed in table (1). PCR reaction was conducted in a final volume of 25µl consists of 12.5 µl of Dream Taq Green PCR Master Mix (2X) (Thermo Scientific), 5 µl of DNA template,1 µl of each primer of 20 pmol concentration and 5.5 µl of nuclease free water. PCR reactions were conducted in 96 well Applied Biosystem thermal cycler. PCR products were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH). The gel was visualized under UV and photographed.

RESULTS

In this study, a total of 160 quarter milk samples were collected from cows with subclincal mastitis after screening with CMT, the overall prevelance of CNS was 43.12% (69/160). The most frequently isolated species was *S. chromogenes* (17/69, 24.63%) followed by *S. epidermidis* (15/69, 21.72%) *S. xylosus* (14/69, 20.28%), *S.haemolyticus* (10/69, 14.49%), *S. hominis* (7/69, 10.14%), *S. simulans* (4/69, 5.79%) and *S. saprophyticus* (2/69, 2.89%) (Table 2).

The ability of CNS to form biofilms was evaluated by microtiter plate assay. The frequency of biofilm formation by CNS was 81.15% (56/ 69). Among CNS isolates, 53.57% (30/56) were strong, 32.14% (18/ 56) were moderate and 14.28% (8/56) were week biofilm producers. S. *chromogenes* had the highest ability to form biofilms (28.57%) followed by *S. epidermidis* and *S. xylosus* (19.64%), *S. haemolyticus* (16.07%), *S.hominis* (8.92%), *S. simulans* (5.35%) and *S. saprophyticus* (1.78%) (Table2). S. *chromogenes* was also the predominate strong biofilm producer (33.33%), followed By *S.epidermidis* (26.67%), *S.xylosus* (20%), *S.haemolyticus* (13.33%) and *S. homins* (6.67%).

Concerning the distribution of biofilms associated genes, the frequency of *icaA* was 36.23% (25/69) while, *icaD* frequency was 47.82% (33/69) and both of them were identified in 20 (28.98%) isolates. In the current study, all *icaD* positive genes were also able to produce biofilms in vitro. In addition, there are 7.25% (5/69) of *icaA*-positive failed to form biofilms. Finally, *bap* gene couldn't be identified in all CNS (Table 3).

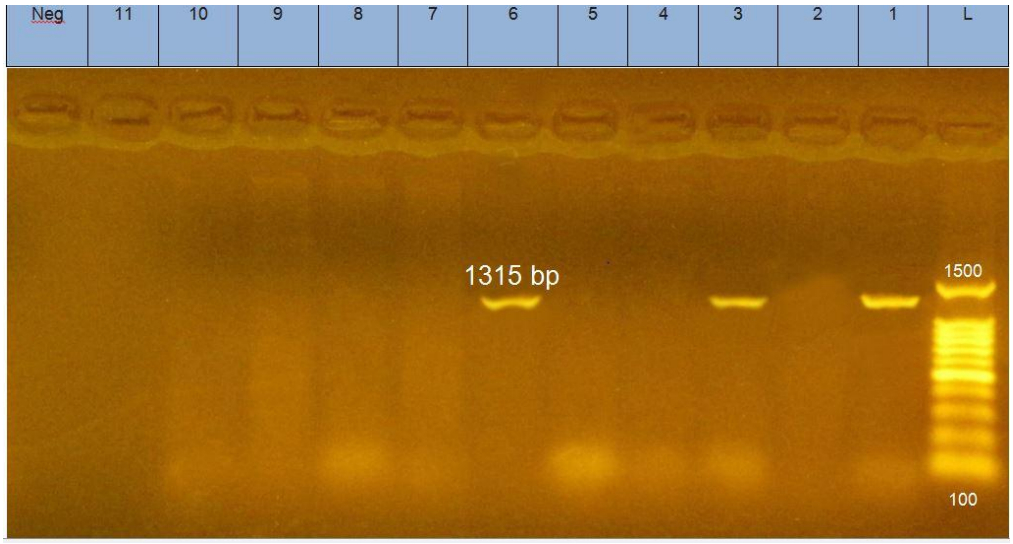

Photo 1: Agarose gel electrophoresis showing the amplification of *icaA* gene at 1315bp. Lane neg.: negative control. Lane L: 100 bp DNA ladder.

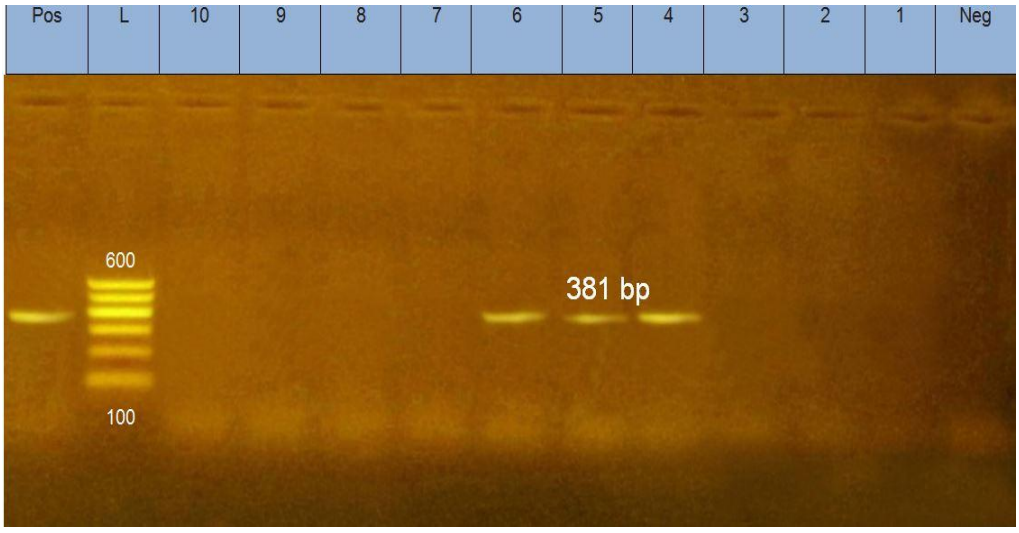

Photo 2: Agarose gel electrophoresis showing amplification of *icaD* gene at 381bp. Lane Neg. negative control, Lane Pos: positive control, Lane L: 100 bp DNA ladder.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Genes	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	References
				Secondary denaturation	Annealing	Extension		
icaA	CCT AAC TAA CGA AAG GTA G AAG ATA TAG CGATAA GTG C	1315	94˚C 5 min	94˚C 30 sec.	49˚C 1 min.	72˚C 1 min.	72˚C 12 min	Ciftci *et al*., 2009
icaD	AAA CGTAAG AGA GGT GG GGC AAT ATG ATC AAGATA	381	94˚C 5 min	94˚C 30 sec.	49˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
bap	CCCTATATCGAAG GTGTAGAATTG GCTGTTGAAGTTA ATACTGTACCTGC	971	94˚C 5 min	94˚C 30 sec.	58˚C 45 sec.	72˚C 50 sec	72˚C 10 min.	Cucarella *et al*., 2001

Table 2: Distribution of CNS species and their biofilms production.

CNS	No (%)	Total biofilm producers (%)			
		Strong	Moderate	Week	Total
S. chromogenes	17(24.63%)	10(33.33%)	4(22.22%)	2(25%)	16(28.57%)
S.epidermidis	15(21.73%)	8(26.67%)	3(16.67%)	0(0.00%)	11(19.64%)
S. xylosus	14(20.28%)	6(20%)	3(16.67%)	2(25%)	11(19.64%)
S. haemolyticus	10(14.49%)	4(13.33%)	3(16.67%)	2(25%)	9(16.07%)
S. hominis	7(10.14%)	2(6.67%)	1(5.56%)	2(25%)	5(8.92%)
S. simulans	4(5.79%)	0(0.00%)	3(16.67%)	0(0.00%)	3(5.35%)
S. saprophyticus	2(2.89%)	0(0.00%)	1(5.56%)	0(0.00%)	1(1.78%)
Total	69(43.12%)	30(53.57%)	18(32.14%)	8(14.28%)	56(81.15%)

Table 3: Patterns of in vitro biofilms formation and biofilms associated genes.

Patterns	Results				
	icaA	*icaD*	*bap*	In vitro biofilm	Total (%)
P1	+	-	-	-	5(7.25%)
P2	+	+	-	+	20(28.98%)
P3	-	+	-	+	13(18.84%)
P4	-	-	-	+	23(33.33%)
P5	-	-	-	-	8(11.59%)
Total	25	33	0	56	69

DISSCUSION

CNS are considered as the most commonly recovered bacteria from intramammary infections in several countries (Gentilini *et al.,* 2002; Sampimon *et al.,* 2009). For instance, In Argentina, CNS are the most prevalent pathogens (52.1%), followed by *S. aureus* (21.3%) (Dieser *et al.,* 2014). In this study, the prevalence rate for CNS was 43.12% (69/160). Compering to our findings, the prevalence rate for CNS in Eastern Ethiopia was 34.2% (Zeryehun and Abera, 2017) from mastitis. The most frequently isolated species was *S. chromogenes* (17/69, 24.63%) (Table 2). In agreement with the obtained results, *S. chromogenes* has been recorded as a predominant species in several studies conducted worldwide, in Canada (48.27%; Condas *et al.,* 2017), in Argentina (41.4%; Felipe *et al.* 2017 and 44.4%, Srednik *et al.* 2017), in Belgium (46.4%; Supré *et al*., 2011), USA (36.3%; Sawant *et al.*, 2009) and the Netherlands (36%; Sampimon *et al*., 2009).

Biofilm formation plays an important role in the protection of bacterial species from harsh environmental condition, which leads to their persistence, and results in long lasting infection. (Cucarella *et al.,* 2004). Biofilms protect the bacteria from the action of the immune system components and hindering the action of phagocytes (Fox *et al.,* 2005), as well as, biofilm acts as a barrier against antimicrobial agents (Stewart, 1996). In this study, out of 69 CNS isolates, 56 isolate able to form biofilms in vitro (81.15%). These findings were in agreement with several studies conducted worldwide (Tremblay *et al.,* 2013; Felipe *et al.,* 2017; Srednik *et al.,* 2017). While, a lower percentages (31.3%) were recorded by Simojoki *et al*. (2012).The diversity in the results of invitro biofilms formation by CNS may be attributed to the differences in the type of the used growth medium and its chemical composition as well as the condition of growth which might be influence the expression of bacterial genes and accordingly biofilm formation of staphylococci (Fredheim *et al.,* 2009). In this study, S. *chromogenes* had the highest ability to form biofilm and also S. *chromogenes* was the predominate strong biofilm producers (33.33%). The same results were recorded by Felipe *et al.* (2017), while, Srednik *et al.* (2017) found that *S. haemolyticus* was the most common biofilm producer strains, and Tremblay *et al*. (2013) found that *S. xylosus* had the highest ability to form biofilm. The diversity in the results may be attributed to the differences in the distribution of CNS species in different countries and the intraspecies variations (Tremblay *et al.,* 2013; Oliveira *et al*., 2015). Additionally, intraspecies variations in biofilm formation was explained by Ajitkumar *et al*. (2013) who identify three different genotypes within *S. chromogenes.*

Biofilm formation by *Staphylococcus* species has been associated with several genes (Simojoki *et al*., 2012; Tremblay *et al*., 2013). In this study, the frequency of *icaA and icaD* were 36.23% (25/69) and 47.82% (33/69) respectively, and both of them were identified in 20 (28.98%) isolates (Table 3). Compering to the obtained results, Simojoki *et al*. (2012) and Srednik *et al.* (2017) found very low frequency for *ica*A. While, Felipe *et al.* (2017) observed amplification for *ica*A and *ica*D in 73.2% of the isolates. In Egypt, in a study conducted by Darwish and Asfour (2013) on a total of 68 CNS isolates, the prevalence of *ica*A, *ica*D genes was 5.9%, 47.1% respectively. The percentage of biofilm positive/ *ica*- negative strains was 33.33% (23/69) (Table 3). However, it was very high (96.6%) with Srednik *et al.* (2017) and very low (13.8 %) with Osman *et al.* (2015). In this study, all *icaD* positive genes were also capable of producing biofilm in vitro which in agreement with Liberto *et al.* (2009) and its presence may confirm the role of *ica* in adhesion mechanism. However, in this study, there are 7.25% (5/69) of *icaA*-positive failed to form biofilm in vitro (Table 3). These finding were in agreement with Ruzicka *et al.* (2004) who demonstrated that 20% of strains with *ica* genes did not express invitro biofilm formation. The absence of phenotype inspite of the presence of *ica* could be attributed to several reasons which may affects *ica* expression such as, insertion of sequence elements or point mutations (Götz, 2004), or the action of the *ica R*repressor which inactivate the *ica* operon (Conlon *et al.,* 2002).

Regarding *bap* gene, non of CNS isolates harbored *bap* gene in this study which was in agreement with the findings of Arciola *et al.* (2003) and Serray *et al.* (2016). While, Srednik *et al.* (2017) identified *bap* gene in a few isolates (n=3), Felipe *et al.* (2017) identified bap gene in 13.4% (11/82) and Darwish and Asfour (2013) identified bap gene in 4.4% of the examined CNS isolates. Also, Tremblay *et al.* (2013) found that there was a difference in the distribution of the *bap* gene among CNS species, as they found that *bap* gene was confined to *S. xylosus* with a percentage of 92%. The discrepancies between studies may be attributed to geographical differences and the distribution of CNS species in the examined samples.

CONCLUSION

CNS species may persist in the mammary gland due to biofilm production and consequently, resulted in long lasting infection. In this study, 81.15% of CNS were capable of producing biofilms invitro and there is an association between biofilm-producing strains and presence of *ica* genes which suggests that *ica* genes plays an important role in the pathogenesis of infection and as a result revealed their important role of *ica* genes as a virulence determinates for staphylococci.

CONFLICT OF INTERESTS

No conflict of interests is declared.

REFERENCES

Ajitkumar, P.; Barkema, HW.; Zadoks, RN.; Morck, DW.; Van Der Meer, FJ. and De Buck, J. (2013): High-resolution melt analysis for species identification of coagulase-negative staphylococci derived from bovine milk. Diagn. Microbiol. Infect. Dis., 75: 227-234.

Arciola, CR.; Campoccia, D.; Gamberini, S.; Donati, ME.; Baldassarri, L. and Montanaro, L. (2003): Occurrence of *ica* genes for slime synthesis in a collection of *Staphylococcus epidermidis* strains from orthopedic prosthesis infections. Acta. Orthop. Scand., 74: 617-621.

Baker, JS. (1984): Comparison of various methods for differentiation of staphylococci and micrococci. J. Clin. Microbiol., 19: 875-879.

Bannerman, TL. (2003): Staphylococcus, Micrococcus, and other catalase-positive cocci that grow aerobically. In PR Murray, EJ Baron, JH Jorgensen, MA Pfaller, RH Yolken (eds), Manual of Clinical Microbiology, American Society Microbiology, Washington, p. 384-404.

Bradley, A. (2002): Bovine mastitis: an evolving disease. Vet. J., 164: 116-128.

Condas, LAZ.; De Buck, J.; Nobrega, DB.; Carson, DA.; Roy, JP.; Keefe, GP.; DeVries, TJ.; Middleton, JR.; Dufour, S. and Barkema, HW. (2017): Distribution of non-aureus staphylococci species in udder quarters with low and high somatic cell count, and clinical mastitis. J. Dairy Sci., 100:1–15.

Conlon, KM.; Humphreys, H. and O'Gara, JP. (2002): Ica Rencodes a transcriptional repressor involved in environmental regulation of ica operon expression and biofilm formation in *Staphylococcus epidermidis*. J. Bacteriol., 184: 4400-4408.

Costerton, JW.; Stewart, PS. and Greenberg, EP. (1999): Bacterial biofilms: a common cause of persistent infections. Science, 284: 5427–5433

Cramton, SE.; Gerke, C.; Scnell, NF.; Nichols, WW. and Go¨tz, F. (1999): The intercellular adhesion (ica) locus is present in Staphylococcus aureus and is required for biofilm formation. Infect. Immun. 67: 5427–5433.

Cucarella, MA.; Tormo, C.; Ubeda, MP.; Trotonda, M. and Monzo C, Peris (2004): Role of Biofilm-Associated Protein bap in the pathogenesis of bovine *Staphylococcus aureus*, Infect. Immun., 72:2177–2185.

Cucarella, C.; Solano, C.; Valle, J.; Amorena, B.; Lasa, I. and Penades, JR. (2001): Bap, a Staphylococcus aureus surface protein involved in biofilm formation. J. Bacterio., 183: 2888-2896.

Darwish, SF. and Asfour, HAE. (2013): Investigation of biofilm forming ability in *Staphylococci* causing bovine mastitis using phenotypic and genotypic assays, Sci. World J. 2013, 378492. doi:10.1155/2013/378492.

Dieser, SA.; Vissio, C.; Lasagno, MC.; Bogni, CI. and Larriestra, AJ. (2014): Prevalence of pathogens causing subcinical mastitis in Argentinean dairy herds, Pak. Vet. J., 34:124–126.

Felipe, V.; Morgante, CA.; Somale, PS.; Varroni, F.; Zingaretti, ML.; Bachetti, RA.; Correa, SG. and Porporatto, C. (2017): Evaluation of the biofilm forming ability and its associated genes in *Staphylococcus* 1 species. isolates from bovine mastitis in Argentinean dairy farms. Microb. Pathog., 104: 278-286.

Fox, LK.; Zadoks, RN. and Gaskins, CT. (2005): Biofilm production by *Staphylococcus aureus* associated with intramammary infection, Vet. Microbiol., 107: 295–9.

Fredheim, EGA.; Klingenberg, C. and Rodhe, H. (2009): Biofilm formation by *Staphylococcus haemolyticus*. J. Clin. Microbiol., 47: 1172-1180.

Gentilini, E.; Denamiel, G.; Bentancor, A.; Rebuelto, M.; Rodriguez Fermepin, M. and De Torres, RA. (2002): Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitis in Argentina. J. Dairy Sci., 85: 1913-1917.

Gillespie, BE.; Headrick, SI.; Boonyayatra, S. and Oliver, SP. (2009): Prevalence of coagulase-negative *Staphylococcus* species from three dairy research herds. Vet. Microbiol.,134: 65-72.

Götz, F. (2004): Staphylococci in colonization and disease: prospective targets for drugs and vaccines. Curr. Opin. Microbiol., 7: 477-487.

Kloos, WE. and Schleifer, KH. (1975): Simplified scheme for routineidentification of human *Staphylococcus* species. J. Clin. Microbiol., 1: 82-88.

Liberto, MC. Matera G, Quirino A, Lamberti AG, Capicotto R G, Puccio GS, Barreca E, Foca A, Cascio (2009): Phenotypic and genotypic evaluation of slime production by conventional and molecular microbiological techniques. Microbiol. Res., 164: 522-528.

Nibret, M.; Yilikal, A. and Kelay, B. (2011): "A cross sectional study on the prevalence of subclinical mastitis and associated risk factors in and around Gondar, Northern Ethiopia," Intern. J. Animal Vet. Advan., 3(6): 455–459.

NMC (National Mastitis Council) (1990): Microbiological Procedures for the Diagnosis of Bovine Udder Infection, National Mastitis Council Inc., Arlington, Va, USA, 3rd edition.

Oliveira, F.; Lima, CA.; Brás, S.; França, Â. and Cerca, N. (2015): Evidence for inter- and intraspecies biofilm formation variability among a small group of coagulase-negative staphylococci. FEMS Microbiol.Lett.,362 doi: 10.1093/femsle/fnv175.

Osman, KM.; Abd El-Razik, KA.; Marie, HSH. and Arafa, A. (2015): Relevance of biofilm formation and virulence of different species of coagulase-negative staphylococci to public health. Eur. J. Vlin. Microbiol. Infect. *Dis.*, 34: 2009-16.

Pyörälä, S. and Taponen, S. (2009): Coagulase-negative staphylococci emerging mastitis pathogens. *Vet. Microbiol.*,134: 3-8.

Quinn, PJ.; Carter, B.; Markey, BK. and Carter, GR. (2002): Clin. Vet. Microbiol., Har Court publishers, Arlington, Va, USA.

Růžička, F.; Holá, V.; Votava, M.; Tejkalová, R.; Horvát, R.; Heroldová, M. and Woznicová, V. (2004): Biofilm detection and the clinical

significance of *Staphylococcus epidermidis* isolates. Folia. Microbiol., 49: 596-600.

Sampimon, OC.; Barkema, HW.; Berends, IM.; Sol, J. and Lam, TJ. (2009): Prevalence and herd level risk factors for intramammary infection with coagulase-negative staphylococci in Duch dairy herds. Vet. Microbiol.,134: 37-44.

Sawant, AA.; Gillespie, BE. and Oliver, SP. (2009): Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk. Vet. Microbiol.,134: 73-81.

Schukken, YH.; González, RN. and Tikofsky, LL. (2009): CNS mastitis: nothing to worry about? Vet. Microbiol.,134: 9-14.

Serray, B.; Oufrid, S.; Hannaoui, I.; Bourjilate, F.; Soraa, N.; Mliji, M.; Sobh, M.; Hammoumi, A.; Timinouni, M. and El Azhari, M. (2016): Genes encoding adhesion factors and biofilm formation in methicillin-resistant *Staphylococcus aureus*in Morocco. J. Infect. Dev. Ctries.,10(8): 863-869.

Simojoki, H.; Hyvönen, P.; Plumed Ferrer, C.; Taponem, S. and Pyörälä, S. (2012): Is the biofilm formation and slime producing ability of coagulase-negative staphylococci associated with the persistence and severity of intrammamary infection? Vet. Microbiol., 158: 344-352.

Srednik, ME.; Tremblay, YD.; Labrie, J.; Archambault, M.; Jacques, M.; Alicia, FC. and Gentilini, ER. (2017): Biofilm formation and antimicrobial resistance genes of coagulase-negative staphylococci isolated from cows with mastitis in Argentina. FEMS Microbio lLett. pii: fnx001. doi: 10.1093/ femsle/fnx001

Stepanović, S.; Vuković, D.; Hola, V.; Bonaventura, G.; Djukić S.; Ćirković, I. and Ruzicka, F. (2007): Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS, 115: 891–899.

Stewart, PS. (1996): Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrob. Agents Chemother., 40:2517–2522.

Supré, K.; Haesebrouck, F.; Zadoks, RN.; Vaneechoutte, M.; Piepers, S. and De Vliegher, S. (2011): Some coagulase-negative *Staphylococcus* species are affecting udder health more than others. J. Dairy Sci., 94: 2329-2340.

Tremblay, YDN.; Lamarche, D.; Chever, P.; Haine, D.; Messier, S. and Jacques, M. (2013): Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. *J. Dairy Sci.*, 96: 234-246.

Tremblay, YDN.; Caron, V.; Blondeau, C.; Messier, S. and Jacques, M. (2014): Biofilm formation by coagulase-negative staphylococci: Impact on the efficacy of antimicrobials and disinfectants commonly used on dairy farms. Vet. Microbiol.,172: 511–518.

Vanderhaeghen, W.; Piepers, S.; Leroy, F.; Van Coillie, E.; Haesebrouck, F. and De Vliegher, S. (2014): Invited review: effect, persistence, and virulence of coagulase negative *Staphylococcus* species associated with ruminant udder health. J. Dairy Sci., 97: 5275–93.

Zeryehun, T. and Abera, G. (2017): Prevalence and bacterial isolates of mastitis in dairy farms in selected districts of Eastern Harrarghe Zone, J. Vet. Med., 6498618. doi: 10.1155/2017/ 6498618.

الكشف عن تكوين الاغشيه الحيويه والجينات المصاحبه لها في المكورات العنقوديه السالبه لاختبار تلزن البلازما والمعزوله من لبن أبقار مصابه بإلتهاب الضرع الغير ظاهري

أمـل عـوض

Email:amalabdo@mans.edu.eg Assiut University web-site: www.aun.edu.eg

تعزي أليه تكوين المرض في المكورات العنقودية الي عوامل ضراوة تفرز خارج الخليه الي جانب غزوها والتصاقها بالانسجه مثل قدرتها علي تكوين اغشيه حيويه. ولذا صممت هذه الدراسه للكشف عن مدي تواجد المكورات العنقوديه السالبه لاختبار تلزن البلازما في ألبان الأبقار المصابه بإلتهاب الضرع الغير ظاهري ، إلي جانب قدرتها علي تكوين أغشيه حيويه وأيضا الكشف عن الجينات المصاحبه لتكوين الأغشيه الحيويه بإستخدام تفاعل البلمره المتسلسل. وفقا لهذا، فقد تم تجميع عدد ١٦٠ عينه لبن من أربع مزارع مختلفه من أضرعة أبقار مصابه بإلتهاب الضرع الغير ظاهري بعد إختبارها بإختبار الكشف عن التهاب الضرع (كاليفورنيا). وبإجراء خطوات العزل البكتيري علي العينات فقد أوضحت النتائج تواجد المكورات العنقوديه السالبه لإختبار تلزن البلازما بنسبه ٤٢.١٢% وكانت أكثر الأنواع إنتشار هي كروموجينز (S.chromogenes) . وقد تم الكشف عن مدي تواجد الأغشيه الحيويه ظاهريا علي أسطح البوليسترن في عدد ٨١.١٥% والتي تقسم إلي أغشيه حيويه قوية متمثله في ٥٣.٥٧% ومتوسطه في ٣٢.١٤% و ضعيفه في ١٤.٢٧%. كما أتضح ان المكورات العنقوديه من النوع الكروموجينز هي الاكثر قدره علي تكوين تلك الأغشيه. وبإجراء تفاعل البلمره المتسلسل للكشف عن الجينات المصاحبه لتكوين الاغشيه الحيويه فقد تم الكشف عن جين icaA (٣٦,٢٣%) وجين icaD (٤٧,٨٢%) ولم يتم التعرف علي جين bap في كل العينات. واخيرا تفيد نتائج هذه الدراسه في رفع الكفاءه والقدره علي السيطره والحد من اضرار هذا النوع من المكروبات التي تتسبب في اصابه الابقار بالتهاب الضرع.