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Resistance to Antimicrobials and Biofilm Formation in *Staphylococcus aureus* Isolated from Bovine Mastitis in Beni-Suef Governorate

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STAPHYLOCOCCUS (S.) aureus is one of the most prevalent causes most prevalent causes of clinical and subclinical bovine mastitis. A bovine mastitis. A total of 400 lactating cows housed in 5 farms in Beni-Suef governorate, Egypt, were examined for presence of either clinical or subclinical mastitis. The examination revealed that 20 (5%) and 78 (21.8%) of animals showed clinical and subclinical mastitis criteria, respectively. Twenty three *S. aureus* isolates were recovered from 98 milk samples in a prevalence of 23.5%. Antimicrobial susceptibility testing of them against 8 compounds revealed that only one isolate was susceptible to all the tested antibiotics while high percentage (n=14, 60.9%) were resistant to more than one antibiotic. The highest percentage of resistance (82.6%) was documented against penicillin. Multiple drug resistance was observed in 26.1% of the tested isolates. Additionally 11(47.8%) isolates were resistant to cefoxitin so they were categorized phenotypically as methicillin-resistant *S.aureus*. All the recovered isolates were seeded on congo red agar to evaluate their biofilm forming ability and 18 (78.3%) of them were recorded as biofilm producers. Investigation of *icaA*, *icaD* and *bap* genes among the recovered isolates revealed that *icaA* and *icaD* were coexisted in 21 isolates (91.3%) while *bap* gene was existed in only one isolate (4.3 %).

Keywords: Mastitis, *Staph. aureus*, Biofilm, Resistance.

Bovine mastitis is defined as an inflammation of the mammary gland which is still the most predominant and costly disease in the dairy industry (Thompson-Crispi *et al.,* 2014). *Staphylococcus (S.) aureus* is one of the major causes of either clinical or subclinical bovine mastitis (Bergonier *et al.,* 2014). *S. aureus* mastitis is usually chronic in nature that makes it difficult to cure with high recurrent infection rate (Cucarella *et al.,* 2004 and Melchior, 2006).

Antimicrobial treatment is critical in the control of *S. aureus* mastitis however; resistance against multiple antimicrobials especially to beta-lactams favors treatment failures and its persistence in the herd (Kumar *et al.,* 2010).

An additional common reason for the persistence of *S. aureus* in the udder is the biofilm formation, biofilms are bacterial communities aggregate in an exopolysaccharide slime matrix of their own synthesis that adhered to surfaces (Costerton *et al.,* 1999 and Vancraeynest *et al.,* 2004).

Bacteria in biofilm are less invasive but resistant to the host's defense mechanisms and to most of therapeutic interference, however at any time biofilm is capable to shed planktonic cells (free floating) that grow rapidly and occupy other surfaces (Melchior, 2006).

Biofilm formation involves two consecutive steps: adhesion of cells to a surface shadowed by cell-cell adhesion, creating several layers of cells (Cramton *et al.*, 1999). Intercellular adhesion requires the polysaccharide intercellular adhesin (PIA) or polymeric N-acetyl-glucosamine (PNAG) which encoded by the ica operon (icaABCD) (Götz, 2002), among them *icaA* and *icaD* genes have been reported to play a major role in the biofilm formation in *S. aureus* isolated from bovine mastitis (Vasudevan *et al.*, 2003).

Additionally some proteins named as biofilm associated proteins (Bap) which encoded by *bap* gene are known to contribute in the formation of *S. aureus* communities (Latasa *et al.*, 2006).

Since antimicrobial susceptibility and biofilm forming ability of *S. aureus* are of crucial concern all over the world, the purpose of this study was to determine the antimicrobial susceptibility behavior, biofilm forming ability on congo red agar, presence of *icaA*, *icaD* and *bap* genes in a collection of *S. aureus* isolates of intramammary origin in Beni-Suef Governorate, Egypt.

Material and Methods

A total of 400 lactating cows housed in 5 different farms were examined for presence of udder inflammation signs or alterations in milk (clinical mastitis). The subclinical mastitis was detected in animals that showed absence of signs or alterations on milk but revealed positive results on California Mastitis Test (CMT) (Schalm and Noorlander, 1957).

An average of 5 mL of milk was collected under aseptic conditions from each animal (showed either clinical or subclinical mastitis).

10 µl of milk were plated on Baird Parker agar, incubated at 37°C for 48h. Black colonies with a clear halo zone were considered presumptive of *S. aureus*. These presumptive colonies were examined by Gram's staining and catalase test.

Complete biochemical identification of *S. aureus* was carried out in accordance to Collee *et al.* (1996).

Antimicrobial susceptibility testing

All the isolates were tested for their antimicrobial susceptibility using the disk-diffusion method on Mueller-Hinton agar (Oxoid, UK) according to the Clinical and Laboratory Standards Institute (CLSI, 2013). Disks impregnated with the following antibiotics were used: penicillin G (P 10 U), gentamicin (CN 10µg),

cefoxitin (FOX 30 µg), ciprofloxacin (CIP 5 µg), doxycycline (DO 30 µg), rifampicin (RD 5 µg), spectinomycin (SH 100 µg) and vancomycin (VA 30 µg).

Resistance against cefoxitin disk indicated methicillin resistant *S. aureus* (MRSA) phenotype according to the CLSI (2013).

Phenotypic detection of biofilm production on congo red agar

Biofilm production in *S. aureus* strains was performed by cultivation on congo red agar (CRA) as previously described (Freeman *et al.,* 1989). Strains producing black colonies with a rough, dry and crystalline consistency were considered biofilm producers. Strains producing red colonies with rough, dry and crystalline consistency or smooth colonies were classified as biofilm non-producers.

DNA extraction and PCR reaction for detection of *icaA, icaD* and *bap* genes.

S.aureus isolates were inoculated on Triptycase Soy Agar. After incubation period, fresh colonies were suspended in 500 µl sterile saline. DNA was extracted from the suspension using a QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen).

PCR reaction was carried out to detect *icaA* and *icaD* genes as previously described by Ciftci *et al.* (2009) while for detection of *bap* gene the conditions was previously described by Cucarella *et al.* (2001).

The sequences of primers are listed in Table 1. The amplified products were visualized by electrophoresis on 1.5% agarose gel.

TABLE 1. List of primers used for detection of *icaA, icaD* and *bap* genes.

Primer	Sequence (5'-3')	Product (bp)	Reference
icaA	F-CCT AAC TAA CGAAAG GTA G	1315	**Ciftci *et al*., 2009**
	R-AAG ATA TAG CGATAA GTG C		
icaD	F-AAA CGT AAG AGAGGT GG	381	
	R-GGC AAT ATG ATCAAG ATA		
Bap	F-CCCTATATCGAA GGTGTAGAATTG	971	**Cucarella *et al*., 2001**
	R-GCTGTTGAAGTTA ATACTGTACCTGC		

Results

Out of 400 lactating cows, 20 (5%) and 78 (21.8%) animals showed clinical and subclinical mastitis criteria respectively.

Twenty three *S. aureus* isolates were recovered from 98 milk samples in a prevalence of 23.5%, of them 5 and 18 isolates were recovered from clinical and subclinical cases in prevalence of 25 and 23.1% respectively.

Antimicrobial susceptibility

Antimicrobial susceptibility testing of 23 *S.aureus* isolates against 8 compounds revealed that only one isolate was susceptible to all the tested antibiotics while high percentage of them (n=14, 60.9%) were resistant to more than one antibiotic. The remaining isolates revealed resistance in a variable prevalence. The highest percentage of resistance (82.6%) was documented against penicillin followed by cefoxitin and spectinomycin in a prevalence of 47.8 and 21.7% respectively (Table 2).

All the tested isolates were susceptible to vancomycin. Multiple drug resistance (MDR) was observed in 26.1% of the tested isolates. Additionally 11(47.8%) isolates were resistant to cefoxitin so they were categorized phenotypically as methicillin-resistant *S.aureus* (MRSA).

TABLE 2. Antimicrobial susceptibility pattern of *S. aureus* isolates recovered from milk samples.

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Penicillin	4	17.4	0	0	19	82.6
Spectinomycin	4	17.4	14	60.9	5	21.7
Cefoxitin	12	52.2	0	0	11	47.8
Rifampicin	14	60.9	6	26.1	3	13
Gentamicin	16	69.6	5	21.7	2	8.7
Doxycycline	17	74	3	13	3	13
Ciprofloxacin	17	74	5	21.7	1	4.3
Vancomycin	23	100	0	0	0	0

No: number of isolates.
%: percentage in relation to the total isolates.

Determination of biofilm production on congo red agar (Fig. 1)

All the recovered isolates were seeded on CRA to evaluate their biofilm forming ability and 18 (78.3%) of them were recorded as biofilm producers.

Biofilm Related Genes (Fig. 2)

Investigation of *icaA*, *icaD* and *bap* genes among the recovered isolates revealed that *icaA* and *icaD* were coexisted in 21 isolates (91.3%) while *bap* gene was existed in only one isolate (4.3 %).

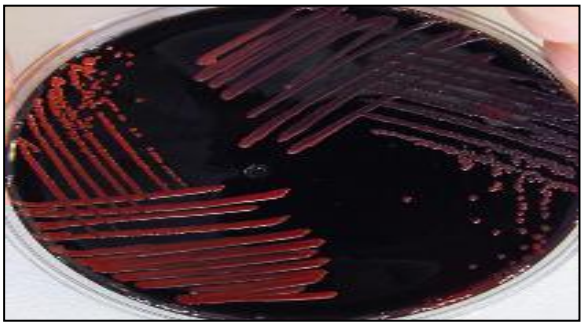

Fig. 1. Showed biofilm producer (black colonies) and non-biofilm producer (red colonies) on congo red agar.

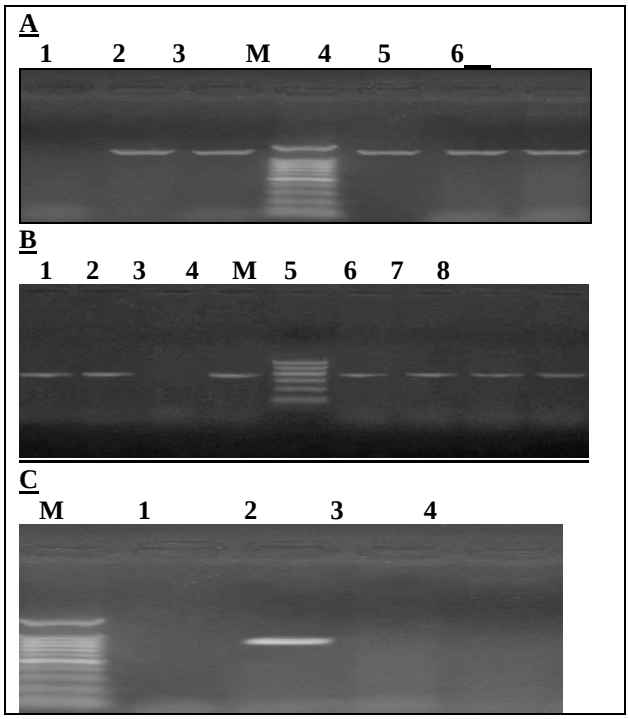

Fig. 2. Agarose gel electrophoresis of PCR products stained with ethidium bromide.
(**A**) *icaA* gene (1315bp), M: 100 bp plus ladder (Size range: 100-1500 bp) positive samples: lane 2, 3, 4, 5, 6: negative samples: lane 1
(**B**) *icaD* gene (381 bp), M: 100 bp ladder (Size range: 100-1500 bp): positive samples: 1, 2,4,5,6,7,8: negative samples: lane 2
(**C**) *bap* gene (971 bp), M: 100 bp plus ladder (Size range: 100-1500 bp) positive samples: lane 2 negative samples: lane 1,3,4.

Discussion

The present study was carried on 400 lactating cows reared in five farms located in Beni-Suef governorate which is situated in the center of Egypt. Out of them, 20 (5%) and 78 (21.8%) animals showed clinical and subclinical mastitis criteria respectively.

Twenty three *S. aureus* isolates were recovered from the cases of mastitis in a prevalence of 23.5%. This high proportion concurs with that of previous studies in Egypt and worldwide (Gianneechini *et al.,* 2002 and Amin *et al.,* 2011).

All the recovered isolates were tested for their antimicrobial susceptibility against 8 antimicrobial agents using disk diffusion method and a high percentage of these isolates (60.9%) showed resistance against more than one antibiotic besides MDR among 26.1% of them. Several reports all over the world have described MDR against *S. aureus* (Kumar *et al.,* 2010 and Shi *et al.,* 2010)

Among the investigated isolates, the highest percentage of resistance (82.6%) was detected against penicillin that could be attributed to the long-term use in agricultural and healthcare settings (Moon *et al.,* 2007).

Resistance of *S. aureus* to penicillins is a well-known phenomenon worldwide but varies in its rate depending on the geographical location (Vintov *et al.,* 2003).

The recorded percentage is higher than those identified by Rajala-Schultz *et al.* (2004) and Alian *et al.* (2012) while higher rates were noted by Pu *et al.* (2014) and Jamali *et al.* (2014).

Cefoxitin DD test was employed in this study for phenotypic characterization of MRSA. This test is able to foretell the presence of *mecA* gene in *S. aureus* with a high degree of sensitivity and specificity (Swenson *et al.,* 2005 and CLSI, 2013).

Nearly 47.8% of the total isolates were considered as MRSA. Literatures showed that the prevalence of MRSA among *S. aureus* isolates was as high as 52% between 2003 and 2005 in Egypt (Falagas *et al.,* 2013).

Methicillin resistance in *S. aureus* is principally mediated by *mecA* gene, which located on a mobile genetic element and encodes an altered penicillin-binding protein (PBP2a) with an extremely low affinity to beta-lactam antibiotics (Hiramatsu *et al.,* 2001). Regarding MRSA as a critically important human pathogen (Verkade and Kluytmans, 2014), detection of MRSA in milk of dairy cattle could represent a source of zoonotic transmission between livestock and humans (Harrison *et al.,* 2013 and Petersen *et al.,* 2013).

Biofilm formation has an essential role in the virulence of *S. aureus* isolated from bovine intramammary infections (Vasudevan *et al.,* 2003 and Fox *et al.,* 2005). Literature recommended both phenotypic and genotypic methods for investigating biofilm forming ability in *S. aureus* (Vasudevan *et al.,* 2003 and De Castro Melo *et al*., 2013).

All the tested isolates were seeded on CRA to evaluate their biofilm forming ability as a phenotypic method and 18 (78.3%) of them were recorded as biofilm producers.

Nearer results were mentioned by Darwish and Asfour (2013) and De Castro Melo *et al.*(2013) while lower percentages were observed by Fox *et al.* (2005) and Krukowski *et al.* (2008).

On the other hand, the biofilm related genes, *icaA* and *icaD* were detected simultaneously in 91.3% of the total isolates. The percentage is comparable to that of Vasudevan *et al.* (2003) and De Castro Melo *et al.* (2013).

The total isolates were also investigated for the presence of *bap* gene that encoding the biofilm associated protein and it was detected in only one isolate (4.3%).This very low prevalence was correlated to that reported by Cucarella *et al.* (2001) and Darwish and Asfour (2013). Other authors did not detect the *bap* gene in their *S. aureus* isolates of intramammary origin (Vautor *et al.,* 2008, Melchior *et al.,* 2009 and Szweda *et al.,* 2012).

Detection of ica locus in some of the tested isolates although they failed to produce biofilm on CRA may be due to the high sensitivity of these isolates to the growth conditions as previously suggested by Cramton *et al.* (1999) or could be due to some capsular exopolysaccharides that required in the biofilm production are not well expressed in the presence of oxygen but they require Co_2 (Gotz, 2002).

It can be concluded that, some of the investigated isolates showed multiple drug resistance to the most commonly used antibiotics, high percentage of them was categorized as MRSA with high capability for biofilm formation a fact representing a hazard to public health

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المقاومـــة لمضـــادات الميكروبـــات وتشكيـــل البيوفيلـــم في الستافيلوكوكس اوريس المعزولة من التهاب الضرع في الأبقار في بني سويف

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يعتبر الستافيلوكوكس اوريس هو واحد من أكثر الأسباب شيوعا لألتهاب الضرع الأكلينيكى وتحت الأكلينيكى فى الأبقار. تم فحص اربعمائة بقرة من الأبقار الحلابة التى يتم تربيتها فى خمس مزارع توجد بمحافظة بنى سويف بمصر وذلك لمعرفة وجود التهاب الضرع الأكلينيكى أو تحت الأكلينيكى. ولقد كشف الفحص عن وجود عدد ٢٠ (٥ بالمائة) و٨٧ (٢١,٨ بالمائة) من الحيوانات مصابا بألتهاب التهاب الضرع الأكلينيكى و تحت الأكلينيكى على التوالى. تم عزل ثلاثة وعشرين .Sعترة من الستافيلوكوكس اوريس من عدد ٩٨ عينة من الحليب بنسبة انتشار ٢٣,٥٪.

وكشف اختبار الحساسية لمضادات الميكروبات مقابل ٨ من المركبات عن وجود عترة واحدة فقط حساسة لجميع المضادات الحيوية التى تم اختبارها بينما كانت نسبة عالية منهم (عدد ابعة عشر بنسبة ٦٠,٩٪) مقاومين لأكثر من نوع من المضادات الحيوية .وقد تم توثيق أعلى نسبة من المقاومة (٨٢,٦٪) ضد البنسلين.

وقد لوحظ المقاومة المتعددة للأدوية في ٢٦,١٪ من العترات المختبرة . بالإضافة إلى ذلك عدد احدى عشرة عترة (٤٧,٨٪ كان مقاوما للسيفوكسيتين ولذلك تم تصنيفهم كستافيلوكوكس اوريس المقاومة للميثيسيلين .

وقد تم زرع جميع العترات على مستنبت الكونغو أجار الأحمر لتقييم مدى قدرتهم على تكوين البيوفيلم . وقد تم التحقيق مابين العترات المعزولة عن وجود جينات *icaA, icaD, bap* وقد اسفر ذلك عن وجود جينى *icaA, icaD* بنسبة متساوية فى عدد احدى وعشرون عترة بنسبة ٩١,٣٪ بينما كان جين ال *bap* موجود فى عترة واحدة بنسبة ٤,٣ ٪.