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 of 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). Interellular 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.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>icaA</td>
<td>F-CCT AAC TAA CGAAAG GTA G</td>
<td>1315</td>
<td>Ciftci <em>et al.</em>, 2009</td>
</tr>
<tr>
<td></td>
<td>R-AAG ATA TAG CGATAA GTG C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>icaD</td>
<td>F-AAA CGT AAG AGAGGT GG</td>
<td>381</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-GGC AAT ATG ATCAAG ATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bap</td>
<td>F-CCCTATATCGAA GGTGTAGAATTG</td>
<td>971</td>
<td>Cucarella <em>et al.</em>, 2001</td>
</tr>
<tr>
<td></td>
<td>R-GCTGTTGAAGT TA ATACTGTACCTGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.**

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
<td>No</td>
</tr>
<tr>
<td>Penicillin</td>
<td>4</td>
<td>17.4%</td>
<td>0</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>4</td>
<td>17.4%</td>
<td>14</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>12</td>
<td>52.2%</td>
<td>0</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>14</td>
<td>60.9%</td>
<td>6</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>16</td>
<td>69.6%</td>
<td>5</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>17</td>
<td>74%</td>
<td>3</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>17</td>
<td>74%</td>
<td>5</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>23</td>
<td>100%</td>
<td>0</td>
</tr>
</tbody>
</table>

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%).

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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 Klytman, 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 CO₂ (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

**Acknowledgment**: I would like to express my special thanks to Prof. Dr. Ismail Abd El-Hafeez Radwan, Prof. and head of Bacteriology, Mycology and Immunology Department, Faculty of Veterinary Medicine, Beni-Suef University and Prof. Dr. Walid Hamady Hassan, Prof. of Bacteriology, Mycology and Immunology, Beni-Suef University for their fruitful efforts and support throughout the work.

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(Received 29/7/2015; accepted 29/10/2015)

المقاومة لمضادات الميكروبات وتشكيل البيوفيلم في المستافيلوكوكس أوريس المعزولة من التهاب الضرع في الأبقار في بني سويف

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يعتبر المستافيلوكوكس أوريس هو أحد أكثر الأسباب شيوعاً لالتهاب الضرع الأبكرى والكبيرة لدى الأبقار. تم فحص 400 بقرة من الأبقار الحلوبة التي يتم تربيتها في خمس مزارع توجد في محافظة بني سويف بمصر وذلك لمعرفة وجود التهاب الضرع الأبكرى أو تحت الأكلينكى. وقد كشف الفحص عن وجود عدد 20 (5 بالمائة) و87 (21.8 بالمائة) من الحيوانات مصاباً بالتهاب الضرع الأبكرى أو تحت الأكلينكى. تم عزل 3 و23 S. عترة من المستافيلوكوكس أوريس من عدد 98 عينة من الحليب بنسبة انتشار 23.5٪.

وكشف اختبار الحساسية لمضادات الميكروبات مقبول 8 من المركبات عن وجود عترة واحدة فقط حساسة لمضادات الميكروبات الدهنية التي تم اختبارها بينما كانت نسبة عالية منهم (عدد ابعة عشر نسبة 60.9٪) مقاومة لأكثر من نوع من مضادات الميكروبات. وقد تم توثيق أعلى نسبة من المقاومة (82.6٪) ضد البنسلين.

وقد لوحظ المقاومة المتنوعة للأدوية في 26.1٪ من العترات المختبرة. بالإضافة إلى ذلك عدد ابعة عشر عرة (47.8٪) كان مقاومة للمستافيلوكوكس أوريس المقاومة للميثيسيلين وذلك تم تصنيفهم كمستافيلوكوكس أوريس المقاومة للميثيسيلين.

وقد تم زرع جميع العترات على مستوى الكوبون واعاج الأحمر لتقييم مدى قدرتهم على تكوين البيوفيلم. وقد تم التحقيق من العترات المعزولة عن وجود جينات icaA, icaD, bap. وقد أشار ذلك عن وجود جيني icaA, icaD, bap مشابه في عدد ابعة عشر عرة بنسبة 91.3٪ بينما كان اثنين آل icaA موجود في عرة واحدة بنسبة 4.3٪.