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Biofilm-Producing *Staphylococcus aureus* Screening in Poultry Farms and Abattoirs

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Abstract: Surveillance on *Staphylococcus aureus* in poultry farms and poultry abattoirs in 2015 in Egypt was applied for determining the biofilm formation, an important virulence and antibiotic resistance determinant. Sixty-seven (68.3%) out of 98 poultry and environmental samples collected in this study were positive for *S. aureus*. For phenotypic analysis of biofilm formation, Microtitre Plate (MTP) and Congo Red Agar (CRA) tests revealed 63 (94.02%) and 59 (88.05%) positive samples, respectively. A high correlation (94.02%) between MTP and CRA tests was encountered. The PCR showed variable results for genes encoding Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) and for those encoding Polysaccharide Intercellular Adhesion/Poly-N-Succinyl-β-1-6-Glucosamine (PIA/PNSG). Partial DNA sequencing of 606 nucleotides of the *icaR-icaA* fragment in 21 selected strains showed 98.2%-100% identity with two clearly distinct phylogroups. This study indicated that biofilm producing *S. aureus* are widely distributed in poultry and poultry abattoirs in Egypt.

Key words: *S. aureus*, poultry, biofilm, abattoirs, PCR, sequence, MTP, CRA

INTRODUCTION

Staph aureus is a serious pathogen that can give rise to several lesions in poultry causeing severe economic losses in poultry industry (Wladyka *et al*., 2011). Those lesions include osteomyelitis, pododermatitis and arthritis, where it is mostly isolated from the joints, tendon sheaths and bones of infected poultry (Andreasen, 2003). As well, *S. aureus* has been frequently isolated from poultry environment including litter, water and feed (Nasrin *et al*., 2007).

A population of bacterial cells sticking to each other and gathering on a certain surface where it is embedded within a self-produced matrix of Extracellular Polymeric Substance (EPS) is a bacterial biofilm (Stepanovic *et al*., 2004). Bacteria within a biofilm are more resistant than planktonic bacteria to antibiotics (Fux *et al*., 2005), disinfectant (Stewart and Costerton, 2001) and dynamic environments (Chen *et al*., 1998). Biofilm also supports bacterial resistance to host immune response which results in chronic infections (Cramton *et al*., 2001).

S. aureus Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) which are adhesive surface proteins that augment the bacterial virulence through mediating its attachment to various surfaces (Wagner *et al*., 2011). Based on their modes of action, those adhesive molecules involve many types such as *FnbA* and *FnbB* (Fibronectin-binding proteins) (Jonsson *et al*., 1991), *ClfA*, *ClfB* and *Efb* (fibrinogen-binding proteins) (Cheung *et al*., 1995), *Can* (Collagen-binding protein) (Patti *et al*., 1992), *EbpS* (Elastin-binding protein) (Gillaspy *et al*., 1998), *eno* (encoding laminin binding protein) (Tristan *et al*., 2003) and *Bbp* (Bone sialoprotein-binding protein) (Tung *et al*., 2000).

Biofilm production by *S. aureus* has been partially dependant on the secretion of two surface proteins: the first one is encoded by the *icaADBC* operon and participates in the synthesis of Polysaccharide Intercellular Adhesion/Poly-N-Succinyl-β-1-6-Glucosamine (PIA/PNSG) (Cramton *et al*., 1999). The second protein is a Biofilm-associated protein (Bap) that is not only supports intercellular adhesion as PIA/PNSG but it also has a role in the attachment to solid surfaces (Cucarella *et al*., 2001).

Microtiter Plate test (MtP) (Cucarella *et al*., 2001) and Congo Red Agar (CRA) test (Freeman *et al*., 1989) are commonly used for phenotypic identification of cbiofilm-producing bacteria. On the other hand, molecular techniques have latterly been used for the detection of the genes responsible for the production of MSCRAMMs (Arciola *et al*., 2005) and/or PIA proteins (Castro*et al*., 2013).

Early detection of pathogenic *staphylococci* is an essential step to achieve prevention. Here, we assessed the current diagnostic methods for the detection of biofilm-forming *S. aureus* in poultry and poultry environments in Egypt in 2015.

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MATERIALS AND METHODS

Collection of samples: Ninety eight samples were collected aseptically from 34 poultry farms and abattoirs in summer 2015 from birds showing decreased egg production, decreased body weight and/or lameness. Birds were sacrificed and the lung, liver, oviducts and joints were collected aseptically. Environmental samples were collected from feeders, drinkers, cages and poultry abattoir processing utensils. Samples were transported aseptically to the lab for further investigations.

Bacterial cultural conditions: Based on Geidam *et al.* (2012) method, *S. aureus* was isolated and identified through cultivation on mannitol salt agar (Oxoid, UK). Confirmation was done by gram staining, microscopy, catalase, coagulase, oxidase and sugar fermentation tests.

Phenotypic characterization of biofilm

Microtitre Plate (MTP) test: Method was done according to Cucarella *et al.* (2001) and Castro *et al.* (2013). Briefly, overnight cultures were diluted 1:200 with trypticase soy broth (Difco, MI, USA) containing 0.25% D(+)-glucose (Merck, Germany) and 200 μ well^{-1} were distributed in sterile 96-well polystyrene cell culture plates (Greiner Bio-One) at 37°C for 18 h. Biofilm was fixed with 200 μL methanol for 15 min. Methanol was removed and wells were washed three times by 0.9% phosphate buffered saline solution (to remove planktonic ''free-floating'' bacteria) then wells were dried at 60°C for 60 min. The adhered cells were stained with 0.5% crystal violet (Fluka) for one minute. Wells were then rinsed three times with distilled water and then dried at room temperature. The absorbance of the adherent biofilm was measured at 570 nm in an automated microplate reader (Tecan sunrise, Jencons, UK). Wells containing only TSB with glucose acted as blanks. Interpretation of the results was applied in respect to the pattern mentioned by (Stepanovic *et al.*, 2000) as follows: Nonbiofilm producers (OD = Odc[a]), weak biofilm producers (Odc<OD = 2×ODc), moderate biofilm producers (2×Odc<OD<4×ODc) and strong biofilm producers (4×ODc<OD). The isolates were tested for biofilm production in duplicates and the assay was repeated three times. The "a" ODc is set as three standard deviations above the mean OD of the negative control.

Congo Red Agar (CRA) test: According to (Mathur *et al.*, 2006), *S. aureus* isolates were grown on Congo Red Agar (CRA) plates which were prepared using tryptic Soy agar containing 0.08% Congo red (Applichem, USA). The inoculated plates were then incubated aerobically at 37°C for 24 h. Phenotypes were interpreted according to Dubravka *et al.* (2010) as follows:

- Positive biofilm producers: black colonies of dry consistency and rough surface and edges
- Intermediate biofilm producers: either black colonies with smooth, round and shiny surface or red colonies of dry consistency and rough edges and surface
- Non-biofilm producers: red colonies with smooth, round and shiny surface

Detection of PIA and MSCRAMMs related genes by PCR: Bacterial DNA was purified from pure cultures using QIAamp DNA Mini kit (Qiagen, Germany, GmbH). Specific PCR primers used are listed in Table 1 and were

Table 1: Oligonucleotide primers used in this study

Target gene	Oligonucleotide sequence (5′- 3′)	Annealing temperature (°C)	Amplified product (bp)	Reference
bab	CCCTATATCGAAGGTGTAGAATTG GCTGTTGAAGTTAATACTGTACCTGC	62	971	Cucarella *et al.* (2001)
bbp	AACTACATCTAGTACTCAACAACAG ATGTGCTTGAATAACACCATCATCT	55	575	Tristan *et al.* (2003)
cna	GTCAAGCAGTTATTAACACCAGAC AATCAGTAATTGCACTTTGTCCACTG		423	
eno	ACGTGCAGCAGCTGACT CAACAGCATYCTTCAGTACCTTC		302	
fnbB	GTAACAGCTAATGGTCGAATTGATACT CAAGTTCGATAGGAGTACTATGTTC		524	
Fib	CTACAACTACAATTGCCGTCAACAG GCTCTTGTAAGACCATTTTCTTCAC		404	
clfA	ATTGGCGTGGCTTCAGTGCT CGTTTCTTCCGTAGTTGCATTTG		292	
clfB	ACATCAGTAATAGTAGGGGGCAAC TTCGCACTGTTTGTGTTTGCAC		205	
ebpS	CATCCAGAACCAATCGAAGAC CTTAACAGTTACATCATCATGTTTATCTTTG		186	
fnbA	GTGAAGTTTTAGAAGGTGGAAAGATTAG GCTCTTGTAAGACCATTTTTCTTCAC		643	
icaA	CCTAACTAACGAAAGGTAG AAGATATAGCGATAAGTGC	49	1315	Ciftci *et al.* (2009)
icaD	AAACGTAAGAGAGGTGG GGCAATATGATCAAGATAC		381	

supplied from Metabion (Germany). Each PCR reaction was carried out in a 25 μL master mix containing 12.5 μL of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 μL of 20 pmol conc. of each primer, 4.5 μL of water and 6 μL of template DNA. Cycling was performed in Biometra T3 thermal cyclers.

DNA sequencing: Forward primer (5'TTTAGCTATATC ATCAAGTGTTGTACCG3') and reverse Primer (5'ATG CCTTGATTGAGTGCGTTG3') were designed according to the GenBank accession number AP004831 using PrimerQuest® design tool. Those primers were used for partial sequencing of icaR-icaA fragments from 21 representative strains. Sequences were aligned and the identity matrices were automatically determined using BioEdit software. For phylogenetic analysis, sequences were retrieved from the GenBank database and the best fit model was selected using jModelTest v.2 (Darriba *et al*., 2012). A mid-point tree was generated using Markov Chain Monte Carlo tree-sampling methods based on 2 runs consisting of 4 heated chains of 1,000,000 with a burn-in of 10% as implemented in MrBayes 3.2.2 (Ronquist *et al*., 2012).

RESULTS AND DISCUSSION

Bacterial isolation and identification: Out of the 98 poultry and environmental samples, 67 (68.3%) samples showed typical *S. aureus* colonies. liver samples possessed the greatest number of isolates (n = 28/67; 41.8%) from poultry while poultry processing utensils in poultry abattoirs revealed the greatest no. of isolates from inanimate objects (n = 21/67; 31.3%). All types of samples showed positive results for *S. aureus* isolation with variable proportions.

Phenotypic characterization of biofilm: As shown in Table 2, MTP test revealed positive results for 94.02% of the *S. aureus* isolates, while 88.05% of the isolates showed either moderate or strong positive biofilm patterns using CRA test.

Polymerase chain reaction: The PCR revealed high prevalence for those genes where 3 (4.47%), 0 (0%), 34 (50.74%), 65 (97.01%), 65(97.01%), 63 (94.02%), 43 (64.17%), 55 (82.08%), 67 (100%), 67 (100%), 45 (67.16%) and 67 (100%) were positive for *bab*, *bbp*, *cna*, *ebps*, *eno*, *fnbA*, *fnbB*, *fib*, *clfA*, *clfB*, *icaA* and *icaD* genes, respectively.

DNA sequence: About 606 nucleotides of *icaR-icaA* fragment (68 nucleotides of *icaR* ORF and 121 nucleotides of the intergenic region and 417 nucleotides of *icaA* ORF) were generated. Partial DNA sequences were assigned GenBank accession numbers (KU314663- KU314683). The nucleotide identity among the Egyptian strains ranged from 98.2-100% while the strains showed a 98.3-99.8% maximum identity to the *S. aureus* strain MW2 (GenBank accession number AP004831). Fourteen different point mutations were detected collectively in the sequenced strains from which first record of 3 different mutations was encountered. As shown in Fig. 1, sequences were generated in the phylogenetic tree for the 21 representative strains and 39 strains from the public database.

Isolation of *S. aureus* with high incidence was accomplished from different biological organs while poultry processing utensils in poultry abattoirs were frequently contaminated representing a major source for isolation of *S. aureus* with a public health significance.

While biofilm production grades varied, MTP revealed 94.02% positive results (Table 2). 62.68% of the samples were strong biofilm producer which affirm the very high detection limit of the MTP test. However, Stepanovic *et al.* (2000) claimed that the specificity of MTP to determine the PIA secretion is lower than its sensitivity. Contrarily, Mathur *et al.* (2006) recommended the use of MTP test due to its high specificity, sensitivity and positive predictive value.

The correlation between MTP and CRA tests was 94.02%. Therefore, CRA test was an alternative method to MTP for biofilm producing staphylococcal isolates detection (Jain and Agarwal, 2009). Nevertheless, our results contradict those results obtained by Mathur *et al.* (2006) and Nasr *et al.* (2012) who reported up to 20% correlation between MTP and CRA tests for detection of biofilm-producing isolates.

Discrepancy in the colony morphology patterns according to which the positive and negative results were determined may be the cause of that variation between the researchers' thoughts about this test. This discrepancy was cleared by Freeman *et al.* (1989) who reported that the test itself was not originally designed for *S. aureus* isolates. PCR was applied to detect genes responsible for the synthesis of MSCRAMMs and PIA where, it revealed high prevalence for those genes.

Table 2: Biofilm detection in *S. aureus* using MTP and CRA tests

	No. of isolates produced biofilm (%*)				
Test	Nonbiofilm producer	Weak producer	Moderate producer	Strong producer	Total positive
MTP	4 (5.97)	9 (13.43)	12 (17.91)	42 (62.68)	63 (94.02)
CRA	8 (11.94)	-	18 (26.86)	41 (61.19)	59 (88.05)

* Percentage to total no. of isolated strains

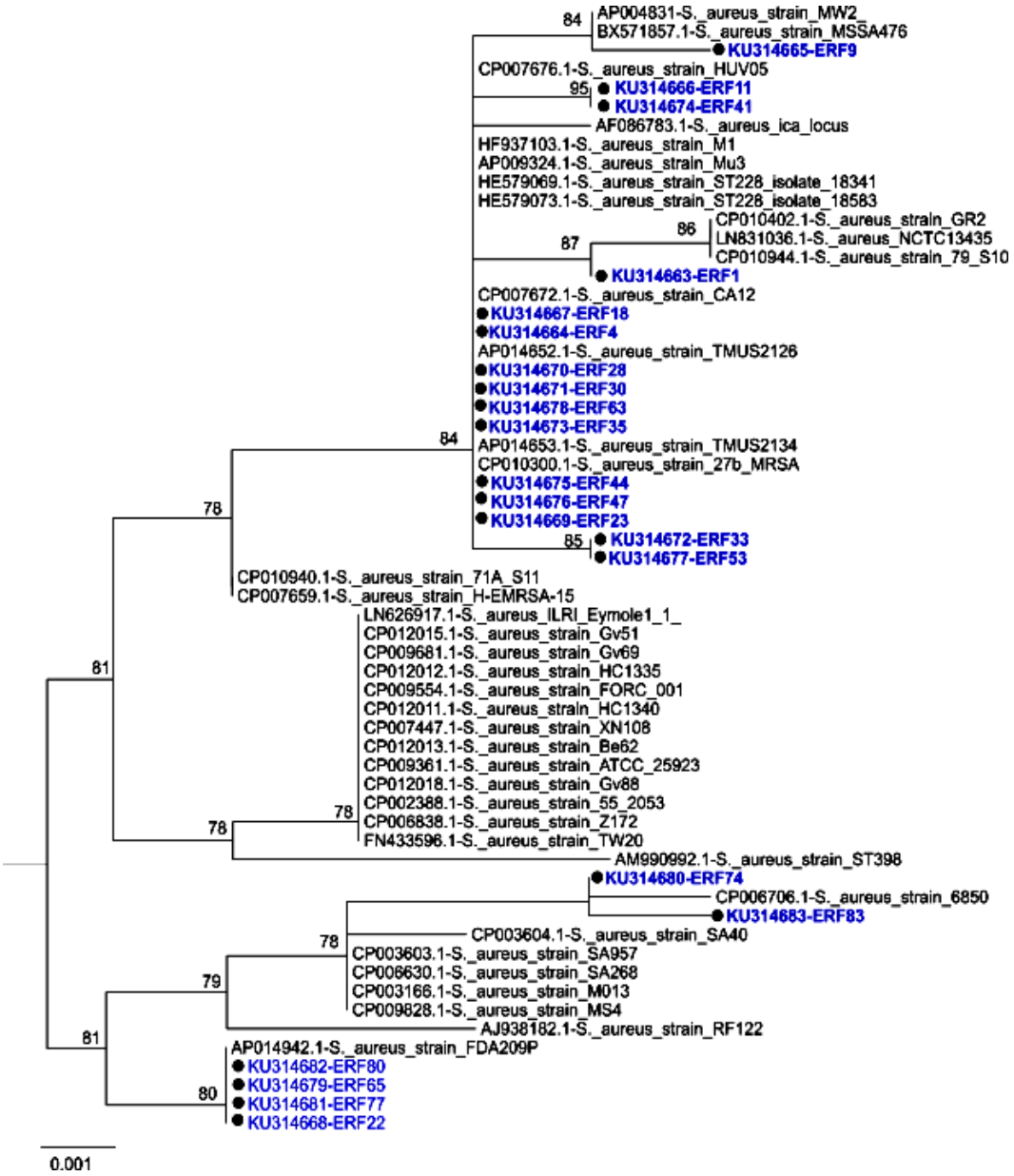

Fig. 1: Mid-point phylogenetic tree for icaR-icaA partial sequences was generated after selection of the best fit model using j model test v.2 (Oliveira and Lourdes, 2010) as implemented in MrBayes (Patti *et al*., 1992). Sequences generated in this study are illustrated in blue using Inkscape free software

Results showed that *icaA* gene was detected only in 67.16% of the isolates which prove that *icaA* result may not correlate will with phenotypic test. This was explained by Liberto *et al*. (2009) who reported that an *ica* gene independent control of slime production/adhesion mechanism may exist. Oliveira Lourdes (2010) recommended that ica-positive isolates should be considered to be potential biofilm producers irrespective of ica genes expression.

Results showed also that 100% of the isolates were *icaD* positive resulting in 5.97% of the samples with uncorrelated results obtained by MTP. Previously, Fenton *et al*. (2013) reported new clones possessing *ica* gene without formation of biofilm. Thus, the use of both ica PCR in addition to MTP and CRA to screen biofilm formation which may avoid missing the genotypically positive phenotypically negative strains was recommended by Vasudevan *et al*. (2003).

The positive results encountered for MSCRAMM genes (*bap*, *can*, *ebps*, *eno*, *fnbA*, *fnbB*, *fib*, *clfA* and *clfB*) indicate that the isolates have a good genetic capacity for adherence. The very high incidence of *ebps*, *eno*, *fnbA*, *clfA* and *clfB* genes, very low incidence of *bap* gene and the negative result for *bbp* gene were in agreement with the results obtained by Nemati *et al.* (2009) who recorded 100% prevalence for *ebps*, *eno*, *fnbA*, *clfA* and *clfB* genes and 0% for both *bap* and *bbp* gene in the recent poultry *S. aureus* isolates. Likewise, *bap* gene was not detected in *S. aureus* isolates examined by Simojoki *et al.* (2012), whereas only 5% of *S. aureus* were positive in samples examined by Cucarella *et al.* (2001).

Jefferson *et al.* (2003) reported that deleting the five nucleotides "TATTT" in *S. aureus* MN8m strain resulted in PIA/PNAG overproduction. However, all the sequenced strains harbored those five nucleotides and possessed strong positive biofilm using MTP and CRA and revealed positive result for most of the tested genes by PCR.

Although, the DNA sequencing revealed very high similarity between the isolates (98.2-100%), 14 different point mutations were detected from which three mutations were firstly recorded. Thirteen mutations were silent (including the three firstly recorded). However, amino acid substitutions were observed (one resulted in stop codon, the other was missense conservative and the third one was missense non conservative).

Although, DNA sequencing revealed many mutations, none of them can be claimed to change the phenotypic character in MTP or CRA tests. Even for the three firstly recorded mutations (One mutation in strain ERF9 (Accession KU314665), another one recorded in strains ERF11 and ERF41 (Accessions KU314666 and KU314674) and the third one in strain ERF83 (Accession KU314683)) that were found to be located in the *icaADBC* transcription start site bordered by Jefferson *et al.* (2003) (starting from nucleotide 190 to nucleotide 231 in the sequences generated in this study), they didn't show any change in the phenotypic pattern of the three strains. This can be accepted as all the three mutations were silent.

Also, the *icaR* fragment showed one mutation in 2 strains (ERF74 (Accession KU314680) and ERF83 (Accession KU314683)), this also didn't show any effect on the biofilm pattern of those strains. Conlon *et al.* (2002) reported that *icaR* gene doesn't regulate the expression of its own self.

The present study described the high incidence of biofilm producing *S. aureus* in biological organs and inanimate surfaces in different poultry farms and poultry processing plants. MTP test showed high sensitivity and specificity in accordance to PCR while CRA test can be used as a supporting test. Both MSCRAMMs and PIA/PNSG encoding genes are good candidates for detection of biofilm producing *S. aureus*. *icaR* and *icaADBC* genes are highly recommended to be studied. However many queries still exist, this study sheds some light on the biofilm producing *S. aureus* and its diagnosis in poultry fields in Egypt.

CONCLUSION

In the present study, biofilm producing *S. aureus* screening in poultry farms and abattoirs was applied when phenotypic tests (MTP and CRA) were highly correlating. Genes encoding MSCRAMMs and PIA were tested using PCR which showed variable results for those genes. DNA sequencing of 606 nucleotides of the *icaR-icaA* fragments in 21 representative strains showed very high identity showing two clearly distinct phylogroups.

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