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Detection of Biofilm in Multidrug Resistant *Staphylococcus* Strains Isolated from Chicken

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ABSTRACT

Staphylococcosis infections are common in poultry worldwide because of the causative bacteria resisting a wide range of commonly used antibiotics. The formation of biofilm is the hallmark characteristic of staph infection. Biofilms constitute reservoir of pathogens and are associated with resistance to antimicrobial agent and chronic infections. In this study 90 multidrug resistant *Staphylococcus* strains (61 coagulase- negative *Staphylococcus* (CNS) and 29 *S. aureus*) were screened by tissue culture plate method for biofilm formation and presence of *mecA*, *icaA*, and *icaD* genes by PCR technique. 38 (42.2%) isolates were strongly positive for biofilm production, 49 (54.4%) were moderate biofilm producers and 3 (3.4%) were weak or negative for biofilm formation. All biofilm producing strains were positive for *icaA* and *icaD* genes, and all biofilm negative strains were negative for *icaA* gene. Biofilm production was higher in methicillin resistant strains as compared to the methicillin sensitive strains of *Staphylococcus* species. From this study attention should be given in treatment of *Staphylococcus* because *Staphylococci* isolated showed a high extent of biofilm production. All biofilm producing *Staphylococci* are positive for *icaA* and *icaD* genes, which indicates the important role of ica genes as virulence markers in staphylococcal infections.

Introduction

Staphylococcus genus is a member of the family Staphylococcaceae, the species in the genus are classified based on the production of coagulase enzyme into two major groups: coagulase positive (CPS) and coagulase negative microorganisms (CNS) as reported by Cunha(2009). It is considered a disturbing issue in the poultry industry due to its impact on public health and a challenge to the medical and veterinary officials worldwide (Ruban and Fairoze, 2011). Chicken meat is one of the popular foods items that consumed worldwide and commonly contaminated by antibiotic resistant strains of *S. aureus*, which pose a great risk in the food web. *S. aureus* are usually present in the intestinal epithelium and skin of humans and animals and may contaminate meat during slaughtering of animal. (Abdalrahman *et al*., 2015). It is a serious pathogen that can give rise to several lesions in poultry causing 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),

Staphylococcus aureus is a virulent organism that is resistant to most of the conventionally available antibiotics. This is attributed to the fact that they are capable of biofilms formation (Taj *et al*., 2011; Jacques *et al*., 2010). It can adhere to and develop biofilms on food contact surfaces, thereby affecting the quality and safety of food products (Srey *et al*., 2013). Biofilms increase bacterial resistance to environmental stresses including cleaning, disinfection, and inhibition, enabling these microorganisms to persist on surfaces (Bridier *et al*., 2015). Several tests are available to detect slime production by *Staphylococci*, which include quantitative methods such as tissue culture plate (TCP), which is considered as the gold-standard method for biofilm detection (Christensen *et al*., 1985; Hassan *et al*., 2011).

Biofilm formation is regulated by expression of polysaccharide intracellular adhesion (PIA), which mediates cell to cell adhesion and is the gene product of *icaADBC* (Ammendolia *et al*., 1999). Among ica genes, *icaA* and *icaD* have been reported to play a significant role in biofilm formation in *S. aureus* and *S. epidermidis* (Yazdani *et al*., 2006). The *icaA* gene encodes N-acetylglucosaminyltransferase, the enzyme involved in the synthesis of N-acetylglucosamine oligomers from UDP-N-acetylglucosamine. Further, *icaD* has been re-

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ported to play a role in the maximal expression of N-acetylglucosaminyltransferase, leading to the phenotypic expression of the capsular polysaccharide (Arciola *et al*., 2001).

Although CNS have emerged as important pathogens, little is known about the virulence factors of these bacteria. The most important virulence factor of CNS is assumed to be the capacity for biofilm formation, which could be a useful marker for the pathogenicity of CNS (Stepanovic *et al*., 2001). Testing for the formation of biofilm is important in deciding the pathogenicity of CNS and should be routinely performed in diagnostic laboratories (Izano *et al*., 2008).

The aim of this study was to determine the biofilm-forming capacity of staphylococcal strains isolated from chicken and the occurrence of *icaA* and *icaD* genes in biofilm-producing strains.

Materials and methods

Bacterial strains

Ninety multidrug resistant *Staphylococcus* isolates from poultry origin (8 from table eggs, 14 unhatched eggs, 8 baby chicks, 13 broilers and 47 from chicken meat) were used to test their biofilm -forming capacity, the genetic characterization of all strains has been previously identified based on biochemical and PCR (Ahmed *et al*., 2016; El-Nagar *et al*., 2017).

Strains were transferred from freeze-dried cultures (in 25% glycerol, 80°C), to Baird Parker (BP) agar plates (oxoid), followed by incubation for 48 h at 37°C (Oniciuc *et al*., 2016).

Detection of biofilm formation

Tissue culture plate method (TCP)

This quantitative test described by Christensen *et al*. (1985) is considered the gold-standard method for biofilm detection (Mathur *et al*., 2006), where isolated organisms from fresh agar plates were inoculated in 10 mL of trypticase soy broth with 1% glucose. Broths were incubated at 37oC for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates were filled with 200 µL of the diluted cultures. Negative control wells contained inoculated sterile broth. The plates were incubated at 37oC for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 200 µL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro-ELISA auto reader (infinite f50) at wavelength 570 nm.

The experiment was performed in triplicate. The interpretation of biofilm production was done according to the criteria of Stepanovic *et al*. (2001). The amount of biofilm formed was scored as weak/none (OD≤ 0.120), Moderate (OD>0.120-0.240) and high/strong (OD> 0.240), according to Thilakavathy *et al*. (2015).

Detection of Mec A, icaA and icaD Genes by Polymerase chain reaction (PCR)

Extraction

DNA extraction from bacterial isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer

Primers used were supplied from Metabion (Germany) are listed in Table 1.

PCR amplification

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. Gelpilot 100 bp plus ladder (Qiagen, Germany, GmbH) and a generuler 100 bp ladder (Fermentas, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Results

Out of total 90 multidrug resistant *Staphylococcus* isolates tested for biofilm formation, 87(96.7%) were found to be susceptible to biofilm formation. 38 (42.2%) of isolates were strongly positive for biofilm production 3 (10.3%) for CNS and 35 (57.4%) for *S. aureus*, 49 (54.4%) were moderate biofilm producers 25 (86%) for CNS and 24 (39.3%) for *S. aureus* whereas 3(3.4%) were negative for biofilm formation1(3.5%) for CNS and 2(3.3%) for *S. aureus* as shown in Fig. 1.

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

Target gene	Primers sequences	Amplified segment (bp)	Amplification (35 cycles)				Final extension	Reference
			Primary denaturation	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 10 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 40 sec.	72˚C 10 min.	
mecA	GTA GAA ATG ACT GAA CGT CCG ATA A CCA ATT CCA CAT TGT TTC GGT CTA A	310	94˚C 5 min.	94˚C 30 sec	50˚C 30 sec.	72˚C 45 sec	72˚C 7 min.	McClure et al. (2006)

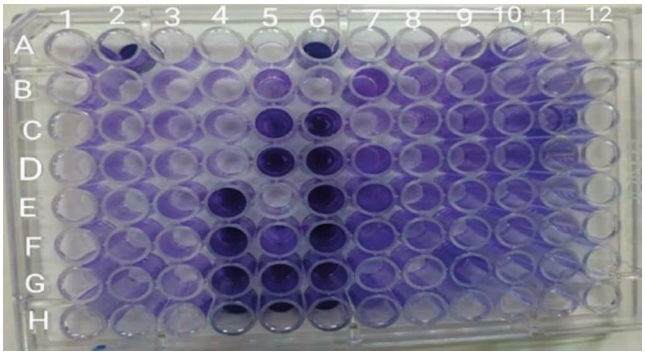

Fig. 1. Tissue culture plate showing different biofilm intensities.; C1: Non-biofilm producer; C2: moderate biofilm producer; C5: strong biofilm producer.

45 out of 61(73.7%) of *S. aureus* were methicillin resistant. and 15 out of 29(51.7%) of CNS were methicillin resistant, biofilm production was detected in 96.6% of MRS (97.7% of MRSA, 93.3% of MRCNS) and 96.6% of MSS were biofilm producers as shown in Table 2.

PCR was done to identify *icaA* and *icaD* genes in 50 *Staphylococcus* isolates (25 CNS and 25 CPS). All isolates, which gave moderate and strong biofilm formation were found to be positive for both genes, giving a 1315-bp band for *icaA*, and a 381-bp band for *icaD* genes and two strains, which revealed non biofilm formation were negative for *icaA* gene and positive for *icaD* gene (1 from CNS and 1from CPS). It was also found that all strains, which were positive for *icaA* were also positive for *icaD* except two strains positive to *icaD* gene and negative for ica A gene. On the other hand, all non biofilm producing strains were negative for *icaA* gene but positive for *icaD* gene as shown in Figs. 2,3, 4.

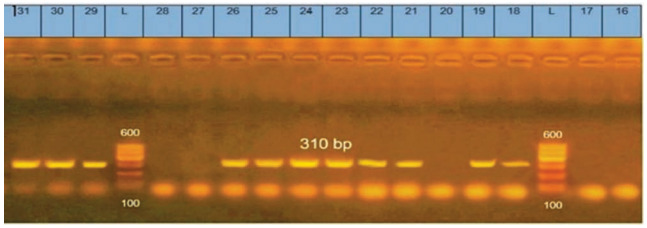

Fig. 2. PCR result of *mecA* gene among *Staphylococcus* isolates.; Lane L: ladder, lane Pos: control positive, lane Neg: control negative, lane 18, 19, 21-26,29- 31 (+ve *mecA*). lane 16, 17, 20, 27 and 28 (-ve *mecA*).

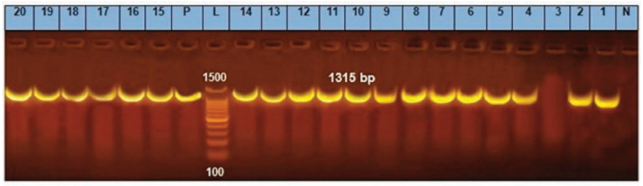

Fig. 3. PCR result of *icaA* gene among *Staphylococcus* isolates. Lane L: ladder, lane P: control positive, lane N: control negative. lane 3 (-ve *icaA*) other lanes (+ve *icaA*).

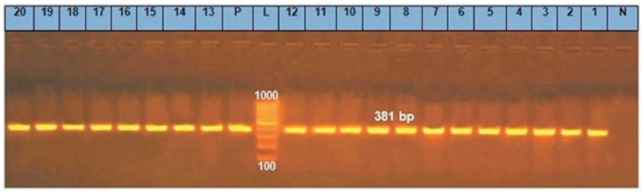

Fig. 4. PCR result of *icaD* gene among *Staphylococcus* isolates. Lane L: ladder, lane P: control positive, lane N: control negative. Other lanes (+ve *icaD*).

Discussion

Biofilm formation is an important characteristic of all staphylococcal species (O,Gara and Humphrey, 2001), polysaccharide intercellular adhesion (PIA) plays an important role in the pathogenesis as it mediate the contact of bacterial cells with each other, resulting in the accumulation of a multilayered biofilm (Chaudhary *et al*., 2009). Biofilms constitute reservoir of pathogens and are associated with resistance to antimicrobial agents and chronic infections.

In present study, the percentage and degrees of biofilm formation agree with Johannes *et al*. (2002), who found that 57.1% of the *S. aureus* strains displayed a biofilm-positive phenotype under optimized conditions in the Tissue culture plate (TCP) test. Fatima *et al*. (2011) detected 38 (14.51%) isolates that were strong biofilm producers by TCP method, 132 (50.38%) were moderate biofilm producers and 92 (35.11%) strains were non producers of biofilm. Furthermore, Gamal *et al*. (2009) classified Staphylococcal strains as high (56.6%), moderate (30.2%) and non biofilm producers (13.2%). Mathur *et al*. (2006) classified the strains based on TCP method as high 22 (14.47 %) and moderate 60 (39.4 %), while in 70 (46.0 %) isolates weak or no biofilm was detected and he concluded from his study that The TCP method was found to be most sensitive, accurate and reproducible screening method for detection of biofilm formation by *Staphylococci* and has the advantage of being a quantitative model to study the adherence of *Staphylococci* on biomedical devices. On the other hand, BOSE *et al*. (2009) reported that in TCP method, biofilm formation was observed in 97 (54.19%) isolates and non-biofilm producers were 82 (45.81%) and his study showed that TCP is the better screening test for biofilm production than Congo Red Agar (CRA) and Tube Method (TM)). The test is easy to perform and can be assessed both qualitatively and quantitatively. Hassan *et al*. (2011) recorded that from the total of 110 *Staphylococcus* isolates, TCP method detected 22.7% as high, 41% moderate and 36.3% as weak or non-biofilm producers. They observed higher antibiotic resistance in biofilm producing bacteria than non-biofilm producers, also concluded from their study that the TCP method is a more quantitative and reliable method for the detection of biofilm forming microorganisms as compared to TM and CRA method, and it can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

In this study, out of the 90 isolates, 60 (66.6%) were me-

Table 2. Detection of biofilm formation in staphylococci in relation to methicillin susceptibility by tissue culture plate method.

	S. aureus (no.61)		CNS (no.29)		Total (no.90)	
	MRSA (no.=45)	MSSA (no.16)	MRCNS (no.15)	MSCNS (no.14)	MRS (no.60)	MSS (no. 30)
Strong (%)	20 (44.4%)	9 (56.3%)	4 (26.7%)	0	24 (40%)	9 (30%)
Moderate (%)	24 (53.3%)	6 (37.5%)	11 (73.3%)	13 (92.9%)	35 (58.3%)	19 (63.3%)
Non/weak (%)	1 (2.2%)	1(6.3%)	0	1 (7.1%)	1 (1.7%)	2 (6.67%)

MRSA: Methicillin-Resistant *Staphylococcus aureus*; MSSA: Methicillin-Sensitive *Staphylococcus aureus*; MRCNS: Methicillin-Resistant Coagulase negative *Staphylococci*; MSCNS: Methicillin Sensitive Coagulase negative *Staphylococci*; MRS: Methicillin-Resistant *Staphylococci*; MSS: Methicillin Sensitive *Staphylococci*.

thicillin resistant *Staphylococcus* (MRS) and 30 (33.3%) were methicillin sensitive *Staphylococcus* (MSS). Out of 60 MRS, 24 (40%) were strong biofilm formation, 35 (58.3%) were moderate biofilm formation and 1 (1.7%) was weak or non biofilm formation, while, out of 30 MSS, 9 (30%) were strong biofilm formation,19 (63.3%) were moderate biofilm formation and 2 (6.67%) were weak or non biofilm formation. David *et al*. (2018) confirmed in their results that methicillin resistant *Staphylococcus aureus* (MRSA) isolates from foods of animal origin have significant capacity for forming biofilms with a high protein content. In the present study, it was found that biofilm production was higher in MRSA (98%) as compared to MSS (93%), this agree with Fatima *et al*. (2011) and O,Neil *et al*. (2007), who noted that methicillin resistant strains of *S. aureus* were more prone to biofilm formation as compared to the methicillin sensitive strains of *S. aureus*.

In the current study, polymerase chain reaction (PCR) was carried out to identify *icaA* and *icaD* genes in 50 *Staphylococcus* isolates (25 CNS and 25 CPS). All isolates, which showed moderate and strong biofilm formation were found to be positive for both genes and two strains, which exhibited non biofilm formation were negative for *icaA* gene and positive for *icaD* gene (1 from CNS and 1from CPS). It was also found that all strains, which were positive for *icaA* were also positive for *icaD* except two strains positive to *icaD* gene and negative for ica A gene. On the other hand, all the isolated non biofilm producing strains were negative for *icaA* gene but positive for *icaD* gene, this agree with Gamal *et al*. (2009), who found that all biofilm producing strains were positive for *icaA* and *icaD* genes, and all biofilm negative strains were negative for both genes. Also, Thilakavathy *et al*. (2015) reported that 39.58% of CNS isolates were biofilm producers, ica gene was identified by PCR in 36.45% of isolates. In addition, Myrella *et al*. (2016) stated that biofilm-producing frequencies in CNS were 45.4% and 43.7% for *S. aureus*. all *S. aureus* isolates were positive for *icaD*. Moreover, Roberta *et al*. (2018) found that 42% of CNS isolates produced biofilms, 11.4% expressed *icaA*D. However, Shrestha *et al*. (2018) detected biofilm formation in 71.8% of CNS isolates.

Most of the *S. aureus* strains formed the biofilm in an ica-dependent. this finding is consistent with results reported by Tang *et al*. (2013), who detected *icaA*D in 87.5% of *S. aureus* strains isolated from several sources. Gutiŕerrez *et al*. (2012) also recorded that 100% of *S. aureus* strains were positive for the *icaA* and *icaD* genes. while results obtained by Szczuka *et al*. (2103) revealed that, out of 74 biofilm-positive strains, 56 carried the *icaA* (76%) gene. Sarah *et al*. (1999) found that all strains of *Staphylococcus aureus* tested contain the ica locus and can form biofilms in vitro. Sequence comparison with the *S. epidermidis* ica genes revealed 59 to 78% amino acid identity. Deletion of the ica locus results in a loss of the ability to form biofilms, produce PIA, or mediate N-acetylglucosaminyltransferase activity in vitro. Cross-species hybridization experiments revealed the presence of *icaA* in several other *Staphylococcus* species, suggesting that cell-cell adhesion and the potential to form biofilms is conserved within this genus.

Conclusion

Most strains that are positive for *icaA* are also positive for *icaD*. On the other hand, all non biofilm producing strains are negative for *icaA* gene and positive for *icaD* gene.

Conflict of interest

Authors declare no conflict of interest exists.

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