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Original article

Association between methicillin susceptibility and biofilm production by *Staphylococcus aureus* colonizing atopic dermatitis patients and their impact on disease severity

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ABSTRACT

Background: Atopic dermatitis (AD) is a major public health problem worldwide characterized by significant colonization of *Staphylococcus aureus (S. aureus)* in the skin lesions. This study focused on investigating the association between methicillin susceptibility and biofilm production by *S. aureus* isolates from AD patients and correlating them with the severity of the disease. **Methods:** Out of 108 AD patients, sixty- six *S. aureus* isolates were isolated and subjected for detection of biofilm production by microtiter plate method. PCR was performed to detect *mec A, ica A,* and *ica D* genes. **Results:** Biofilm production among *S. aureus* isolates was 83.3%. There was a high statistically significant association between the severity of AD and biofilm production (p<0.001). 86.4% of isolates were confirmed as methicillin resistant *Staphylococcus aureus* (MRSA) by *mec A* gene detection. There was statistically significant association between methicillin resistance and biofilm production and *ica A* and *D* genes expression (p <0.001), as well as the severity of AD and positive *ica A* and *D* genes (p=0.003 for each). No significant association was detected between the severe form of AD and *mec A* gene expression (p=0.07). **Conclusion:** Severity of AD correlates with *S. aureus* ability to produce biofilm. Although MRSA was found in a high percentage colonizing AD patient especially in the severe form, it does not affect the severity of the disease.

Introduction

Atopic dermatitis (AD) is considered a major public health issue. It is a chronic relapsing skin disease which is characterized by inflammatory skin lesions, weakened epidermal barrier function, and significant colonization by *S. aureus* in the lesions [1].

Patients are usually presented mainly by severe pruritus with itchy skin, xerosis, eczema lesions in flexural areas, and recurrent skin

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infections. AD is a part of the atopy triad together with asthma and allergic rhinitis. Affected personnel usually have a family history of one or more of the three conditions [2]. The severity of AD is assessed by the SCORAD index, which is a scoring system combining extent, severity, and subjective symptoms of AD [3].

Patients with AD are described to have *S. aureus* on their skin lesions at rates ranging from 30–100% [4]. *Staphylococcus aureus* exacerbates inflammation in the skin lesions by a number of elements that modulate host immunity or disturb barrier function in the skin such as exogenous proteases, α-toxin, protein A, superantigens, and biofilm formation [5].

Methicillin-resistant S. aureus (MRSA) is a serious public health problem which gives rise to considerable morbidity and mortality worldwide. The key factor for verifying methicillin resistance in *S. aureus* is the *mec* complex which consists of *mec A* gene, together with two regulatory genes, *mecI* and *mecR1* [6].

Mature biofilm formation in *Staphylococcus* begins with bacterial adhesion to a primary surface, followed by cell accumulation through intracellular adhesion pathways, and eventually the formation of a mature biofilm [7].

Cellular accumulation together with biofilm formation are mediated by genes *icaADB* and *C*, that encode the crucial needed proteins for polysaccharide intercellular adhesion (PIA) and capsular polysaccharide/adhesion (PS/A) synthesis in *Staphylococci* [8].

Clinical improvement in AD correlates with a reduction in *S. aureus* colonization. However, although antibiotics and antiseptics can reduce *S. aureus* colonization, recolonization occurs frequently within a few weeks, leading to limited clinical improvement and relapses of pathologic manifestations [9].

This study was designed to investigate the association between methicillin resistance and biofilm production by *S. aureus* isolates from AD patients and to correlate that with the severity of the disease.

Methods

Study design and settings

This cross-sectional study was conducted in Medical Microbiology and Immunology Department and Dermatology, Venereology and Andrology Department, Faculty of Medicine, Zagazig University, Egypt, during the period from December 2019 to June 2021. Institutional Review Board (IRB) of Zagazig University approved the study protocol (IRB#:5718-17-11-2019). We followed the guidelines of the STROBE statement for observational studies [10]. A written informed consent was obtained earlier from every patient included in the study.

Sample size was calculated to be 92; assuming that attendance rate to the outpatient dermatology clinic through 6 months is 120 patients and prevalence of *S. aureus* on the lesional skin of AD patients was found to be 53.1% (11), non-response rate is 20%, so the total sample was 108. At CI 95%, power of the test 80%. The sample was calculated using open epi program.

Sampling, identification and testing biofilm production

One hundred and eight patients with AD presented to the Dermatology outpatient clinic in Zagazig University Hospitals of any age and sex were recruited for analysis. We excluded patients receiving systemic antibiotic treatment in the last 4 weeks or topical in the last 2 weeks, having immunological skin diseases other than atopic dermatitis, with a severe fungal infection, or patients with the concomitant presence of systemic infection.

Baseline demographic and clinical data were obtained from all patients and severity was assessed by SCORAD index [3]. Samples were collected from an open, excoriated, or crusted eczema lesion using sterile cotton swabs. *Staphylococcus aureus* isolation and identification were done by standard microbiological techniques [12]. Evaluation of *S. aureus* biofilm production was done by microtiter plate method [13]; 200 µl of adjusted 0.5 McFarland turbidity in Trypticase soy broth (TSB) of tested isolate were added to wells of tissue culture microtiter plate. Each strain was tested in triplicate and three wells in each plate were used as negative control (200 µL of TSB), then the plates were covered and incubated for 24 hours at 37°C. the wells were washed three times with phosphate buffered saline. 200 µl of 99% methanol were added to the wells for twenty minutes to fix adherent bacteria. The plates were emptied and air dried then stained with 200 µl crystal violet (1%) for 15 minutes at room temperature then washed by sterile distilled water. After air drying, the attached stain was eluted by aliquots of 150 µl of 95% ethanol. The

plate was covered and left at room temperature for 30 min. The optical densities of the stained adherent films were measured with ELISA reader at 630 nm (Biotek, USA). the average optical density values were calculated for all tested isolates & negative controls, the cut off value (ODc) was detected.

According to the absorbance values, strains were divided into non-biofilm producers, weak, moderate, and strong biofilm producers.

Non biofilm producer = OD ≤ ODc

Weak biofilm producer = ODc < OD ≤ 2×ODc

Moderate biofilm producer = 2×ODc < OD ≤ 4×ODc

Strong biofilm producer = 4×ODc <OD

PCR assay

Molecular characterization of MRSA and biofilm production were done by detection of *mec A* gene, *ica A*, and *D* genes respectively using PCR assay. DNA extraction was carried out using the Intron extraction kit (i-genomic BYF DNA extraction mini kit. Biovision, USA). DNA amplification was performed using Thermo Scientific Phusion high-fidelity DNA polymerase (Thermo Scientific Company, Lithuania) and primers targeting *mec A* gene, *ica A*, and *D* genes (Invitrogen, UK) are demonstrated in **table (1).** The gene segments were amplified using DNA thermal cycler (Applied Biosystems, USA) by touchdown PCR **(Table 2)** and finally visualized by agarose gel electrophoresis using a marker with molecular weight 100-1000 bp **(Figure 1).**

Statistical analysis

Statistical packages (EPI-info Version 6.04 and SPSS Version 20 inc. Chicago, USA) were used to analyze collected data. The Chi-square test ($\chi 2$) was used to compare proportions as appropriate. *P* values for calculated statistics tests were obtained. A *p*-value <0.05 was considered to be statistically significant at a 95% confidence interval.

Table 1. Primer sets for amplification of *mec A, ica A,* and *D* genes.

Gene	Primer sequence (5′→ 3′)	Bp	Reference
mec A	F: GTGAAGATATACCAAGTGATT R: ATCAGTATTTCACCTTGTCCG	112 bp	14
ica A	F: ACACTTGCTGGCGCAGTCAA R: TCTGGAACCAACATCCAACA	188 bp	15
ica D	F: ATGGTCAAGCCCAGACAGAG R: AGTATTTTCAATGTTTAAAGCAA	198 bp	15

Table 2. Thermal profile for *mec A, ica A,* and *D* genes (16).

Temperature	Time	Stage	No. of cycles
98º C	30 sec	(Stage1) Polymerase activation	1
98º C 61º C 72º C	10 sec 30 sec 15 sec	(Stage2) 1.Denaturation 2. Annealing 3.Extension	10
98º C 55º C 72º C	10 sec 30 sec 20 sec	(Stage3) 1.Denaturation 2. Annealing 3.Extension	30
72º C	5 min	(Stage4) Final extension	1

Results

The age of the studied patients ranged from 4 to 33 years (10.2 ± 6.54). male to female ratio was 52/56. SCORAD score revealed 54 patients (50%) with mild severity, 40 patients (37%) with moderate disease and 14 patients (13%) with severe disease. 40 patients (37%) had prominent lesions in leg and thigh,34 patients (31.5%) with arm & forearm lesions, 21 patients (19.4) with lesions in abdomen and 13 patients (12%) with prominent neck lesions.

Among 108 AD patients, 66 patients (61.1%) were confirmed to have *S. aureus* colonization in their skin lesions. 16.7% of our *S. aureus* isolates (11/66) were non-biofilm producers, while 40.9% of isolates were weak biofilm producers (27/66), 28.8% were moderate biofilm producers (19/66) and 13.6% were strong biofilm producers (9/66). **Table 3** demonstrated association between severity of the disease and biofilm production (*p*-value<0.001).

The frequency of *mec A, ica A,* and *ica D* genes among our *S. aureus* isolates were 86.4% (n=57) of *mec A* gene and 83.3% (n=55) for both *ica A* and *D* expression.

There was statistically significant association between MRSA isolates and positive *ica A* and *D* genes expression (*p*-value<0.001); 93% of MRSA isolates showed positive *ica A* and *D* gene expression (n=53) versus 22.2% of MSSA isolates (n=2) for each gene **(Table 4).**

Out of 9 methicillin susceptible strains, 7 isolates were non biofilm producers (77.8%) and one isolate for each mild and moderate producers' group (11.1%) with none for strong biofilm grade. So, there was a high statistically significant association between methicillin resistance, and biofilm production (77.8% [7/9] of *mec A* negative gene isolates gave no reaction versus 7% [4/57] of *mec A* positive gene isolates) **(Table 4).**

A statistically significant association was observed between severe AD and positive *ica A* and *D* genes expression, however, there was no statistically significant association between severe AD and *mec A* gene expression **(Table 5).**

Table 3. Association between biofilm production among *S. aureus* isolates and severity of disease.

Disease severity / ***Biofilm grade***	***Mild N=29***		***Moderate N=25***		***Severe N=12***		***X^2***	***P-value***
	N	***%***	***N***	***%***	***N***	***%***		
No Biofilm	*10*	*34.5*	*1*	*4.0*	*0*	*0.0*	*8.4*	*0.01*
Weak	*15*	*51.7*	*12*	*48*	*0*	*0.0*	*6.7*	*0.03*
Moderate	*4*	*13.8*	*11*	*44*	*4*	*33.3*	*6.12*	*0.04*
Strong	*0*	*0.0*	*1*	*4.0*	*8*	*66.7*	*24.8*	*<0.001*
X^2	*50.7*							
P-value	*<0.001*							

HS: P-value<0.001 is high significant

Table 4. Association between methicillin resistance, *ica A* and *D* genes expression and biofilm production among *S. aureus* isolates.

Gene expression & Biofilm grade / **MRSA N=57**	**Gene expression**	**Biofilm grade**			
	Ica A and *Ica* D genes	No	Mild	Moderate	Strong
No.	53	4	26	18	9
%	93	7	45.6	31.6	15.8
X^2	*28.1*	21.5	3.82	1.58	0.05
		28.2			
***P*-value**	*<0.001*	*<0.001*	*0.05*	*0.2*	*0.8*
		<0.001			

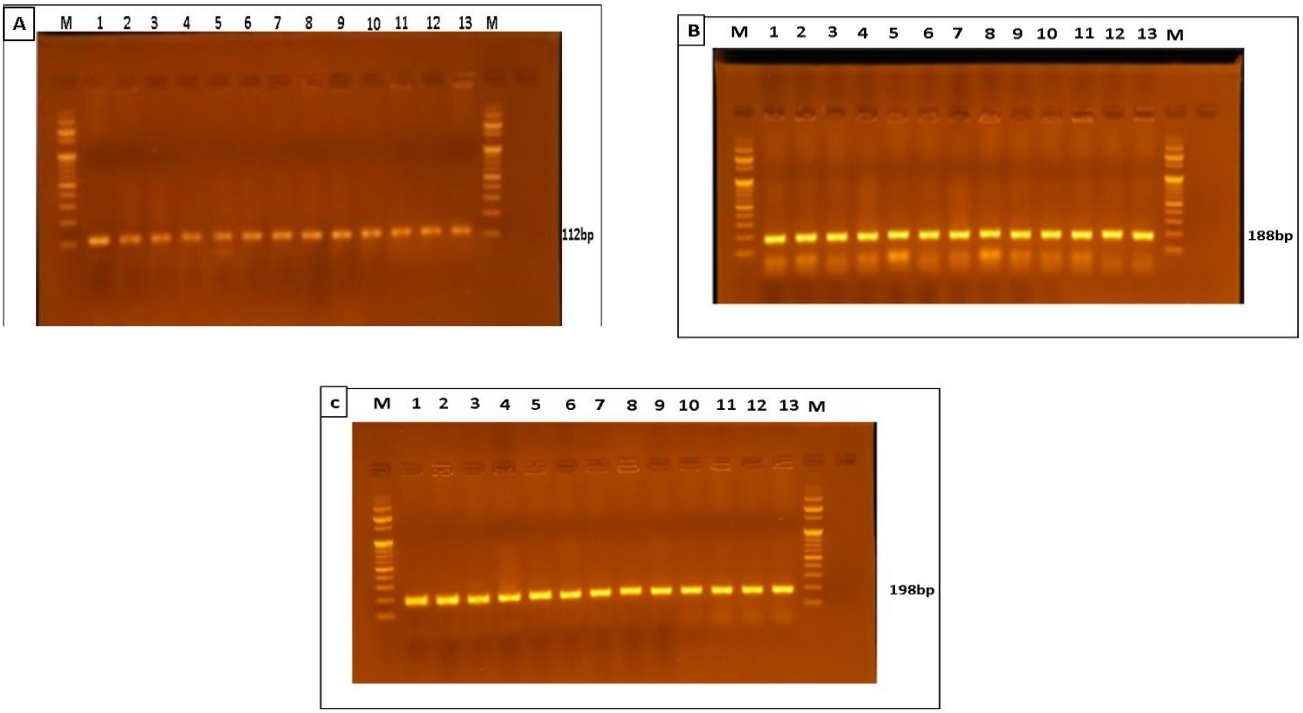
HS: P-value<0.001 is high significant

Table 5. Association between gene expression and severity of disease among patients colonized by *S. aureus.*

Severity of disease / Gene expression	Mild N=29		Moderate N=25		Severe N=12		X^2	*P*-value
	N	%	N	%	N	%		
Mec A								
Positive	22	75.9	23	92.0	12	100	5.29	0.07
Negative	7	24.1	2	8.0	0	0.0		
Ica A								
Positive	19	65.5	24	96.0	12	100	11.9	0.003
Negative	10	34.5	1	4.0	0	0.0		
Ica D								
Positive	19	65.5	24	96.0	12	100	11.9	0.003
Negative	10	34.5	1	4.0	0	0.0		

NS: P-value>0.05 is not significant *S: P-value<0.05 is significant*

Figure 1. PCR amplification: lane (M) shows 100 bp Mwt marker, lanes (1-13) show positive isolates; (A) *mec A* gene (112bp), (B) *Ica A* gene (188bp), and (C) *Ica D* gene (198bp).



Discussion

Atopic dermatitis is a common, chronic skin disease which impairs the quality of life of individuals as well as their families. Previous studies declared that around (30-100%) of AD patients are colonized with *S. aureus*. *Staphylococcus aureus* possesses the ability to produce biofilms leading to recurrent, hard-to-treat infections [4].

Age of our studied group ranged from 4 to 33 years with a mean age 10.2 and SD ± 6.54; 52 were males and 56 were females. This was in agreement with a similar study on Egyptian patients carried out by **Nada et al.** [12] who found the age of their studied group ranged between 5 and 26 years, with a mean age of 10 years and SD ±4 and male/ female ratio was 16/14. **Tanei** [17] and **Raznatovic et al.** [18] supported us and declared that atopic eczematous rash could occur in all ages, although the onset is usually in childhood but also is common in adolescents and adults. **Atar-Snir** [19] reported

that gender did not influence prevalence of AD until ages 10–18 years, and dermatitis became more prevalent among females, which might suggest a role for pubertal factors.

Fifty percent of our patients had mild disease by SCORAD index, 37% had moderate disease and 13% had severe disease. This comes in accordance with **Celakovska et al.** [20] who reported about 9% only had severe disease. **Chu et al.** [21] declared that severity score increased as the age of the patients increased, also onset of disease affects severity; pure adult onset tended to be less severe and that severity varied during different months of the year. The most common sites of swabbing with prominent lesion in our studied patients were leg and thigh (37%) and the least was the neck (12%). However, **Chu et al.** [21] found different distributions in their studied group; the head and neck area was the most prominent and involved in 35% of their patients, this could be explained that their studied group had a predominance of adult patients more than 19 years old where the infection mostly occur in face and neck [19].

In our study, sixty-six patients (61.1%) were found to be colonized with *S. aureus* in their skin lesions. In two Egyptian studies; one carried out by **Nada et al.** [12] who found 87% of AD patients were colonized with *S. aureus* and the other by **Ali et al. [**22] who reported *S. aureus* in 60% of their skin swabs. different isolation rates had been described by another previous studies; **Ogonowska et al.** [23] declared 56–96.2% isolation rate on lesional skin of AD patients, 28-39% on non-lesional skin, 46.1-64.1 % colonizing nose which may cause auto transmission to skin area and only 10.1% isolation rate among healthy control group colonized on skin. **Nada et al.** [12] described that the variation in the prevalence of *S. aureus* in lesional skin of AD patients could be due to the fact that individuals might be colonized only intermittently by *S. aureus* so it is not always found at the time of examination, or due to eczema severity which was found to be directly correlated with *S. aureus* colonization.

Our biofilm results included that 55 isolates (83.3%) were identified as biofilm producers; 40.9% were mild, 28.8% were moderate, and 13.6% were strong producers, however, only 11 *S. aureus* isolates (16.7%) were non-biofilm producers. These were consistent with the results of **Gonzalez et al.** [24] who revealed that 78% of *Staphylococcal* isolates had the ability to form adherent biofilms. On the contrary, a previous study conducted in Brazil by **Cavalcante et al.** [25] found that (74.5%) were non-biofilm producers. This difference could be due to different localities with different running strains or due to some immunological factors.

Significant association between severity of AD disease and biofilm production in *S. aureus* isolates (p <0.001) was detected; 66.7% with the more severe form of the disease were constantly colonized by strong biofilm producers. The previous data suggests that biofilm is considered an essential key element for the persistence of *S. aureus* colonization in AD lesions. A previous study carried out by **Di Domenico et al.** [11] supported our findings (P < 0.01). In addition, **Di Domenico et al.** [26] reported 74% of high SCORAD AD patients with severe biofilm reaction and 25% of them gave a moderate reaction.

We were interested in studying methicillin resistance among our isolates and we identified 57 isolates (86.4%) with positive *mec A* gene (MRSA). Our results were slightly higher than **Ali et al. [**22] who reported 51.9 % MRSA isolated from atopic dermatitis lesions in a study carried out in Mansoura, Egypt. In addition**, Algammal et al.** [27] studied epidemiology of MRSA in Egypt and declared that MRSA was characterized in 11.5% of community acquired *S. aureus* strains. Frequent use of topical and systemic antibiotics could explain the high isolation rate of MRSA in our studied group. **Lo et al.** [28] found 60% of their isolates (12/20) as MRSA from AD children. Unfortunately, we didn't study the isolation rate in the first few years of life which is a limitation in our study.

On the other hand, an Italian study carried out by **Di Domenico et al.** [11] who reported that there was a very low prevalence of (MRSA) isolated from AD patients (6.8%). The difference in the prevalence of MRSA could be due to the hygienic status of patients, intermittent colonization, and geographical characteristics of different countries.

In the present study, there were 83.3% of our isolates express *ica A* and *D* genes. Concordant with our study, **Allen et al.** [29] had found that 93% of isolates expressed *ica* genes. Moreover, **Di Domenico et al.** [11] reported that all the *S. aureus* isolates from AD patients harbored *ica A and D* genes. On the contrary, **Keikhaie et al.** [30] found

only 30% had *ica A* gene and 20% had *ica D* gene. The difference in results may be due to the distinct inclusion criteria between the two studies. That study collected samples from hospitalized infected males of any age and did not exclude other skin infections while we allocated AD patients of any age or sex.

Our study revealed a significant association between methicillin resistance and *ica A* and *D* genes expression (p <0.001) (93% of MRSA cases showed positive genes expression versus 22.2% of methicillin-sensitive cases). Compatible with what we found, a Sudanese study carried out by **Elboshra et al.** [31] and reported a significant association of the *ica* genes in methicillin-resistant strains. However, **Ghasemian et al.** [32] found that MSSA and MRSA strains did not show significant differences regarding *ica A* and *ica D* genes presence. However, the same study reported that all MRSA isolates harbored both *ica A* and *D* genes.

The current study highlighted an important statistically significant association between methicillin resistance and biofilm production (p <0.001); 93 % of MRSA isolates were biofilm producers while 77.8% of methicillin sensitive strains were unable to form biofilm. This finding was in keeping with the results declared by **Bimanand et al.** [33] who found that biofilm formation in MRSA isolates had reached 96%. In addition, **Piechota et al.** [15] reported that MRSA strains possessed a significantly higher ability of biofilm production than MSSA strains (p <0.001). Also, **Cha et al.** [34] reported the ability to 86 out of 126 MRSA isolates to form a biofilm (68.3%).

The present study revealed a statistically significant association between severe atopic dermatitis and positive *ica A* and *D* genes expression (p=0.003); all severe disease cases showed positive *ica A* and *D* genes expression and 96% of moderate cases were also positive for *ica A* and *D* genes. In accordance with our study, comparing patients having a more severe form of the disease to those with a mild form, **Pascolini et al.** [35] reported that genes encoding biofilm *(ica A* gene) were more frequent in a significant manner (p<0.001 and p = 0.002, sequentially).

Conclusions

Higher incidence of *S. aureus* colonization especially MRSA strains among AD patients. The severity of disease correlates with biofilm production. Methicillin resistance associated with biofilm production, but doesn't affect severity of the disease.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding statement

No funding was used for the study.

Limitations of study

Mixing the different age groups in results.

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