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ORIGINAL ARTICLE

Impact of Biofilm Production in Methicillin Resistant *Staphylococcus aureus* among Diabetic Foot Patient

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ABSTRACT

Background: *Diabetic Foot Infection poses many problems in clinical practice. It is usually polymicrobial, and Staphylococcus aureus is the most common pathogen isolated.* ***Objective:*** *To determine the prevalence of methicillin resistant S. aureus (MRSA) and MRSA biofilm production among diabetic patients with chronic leg ulcers.* ***Methodology:*** *This study included 150 patients suffering from infected diabetic foot ulcers. We used VITEK 2 system to identify isolated bacteria. Colonies of S. aureus were screened for resistance to methicillin on Mueller–Hinton agar supplemented with oxacillin at 4 μg/mL Antibiotic sensitivity test was investigated using Kirby Bauer Disc Diffusion method. Investigation of biofilm formation was performed by tissue culture plate method. Detection of icaA and icaD genes was investigated by PCR.* ***Results:*** *S. aureus was isolated from 70 (46.6%) patients. Among the 70 S. aureus, 34 (22.6%) were (MRSA), Pseudomonas aeruginosa 36(24.0%), Klebseilla pneumoniae 25(16.6%) and E.coli were 19(12.6%). Twenty eight out of 34 tested MRSA (82.35%) were able to form biofilm. Twenty five isolates (73.3%) were strong biofilm former, 3 isolates (8.8%) were moderate biofilm producer and 6 isolates (17.6%) were non biofilm producers. Twenty two were positive for both icaA and icaD genes, On the other hand eight isolates were negative for both genes.* ***Conclusion:*** *A high prevalence of biofilm producing MRSA was detected in S. aureus isolated from patients with Diabetic foot.*

INTRODUCTION

Foot ulcer is very common in diabetic patients. Its prevalence ranges between 15% and 25% [1]. Infection of these ulcers is usually a frequent complication (40%–80%) which represent a major cause of mortality and morbidity [2]. It is reported to be the most common cause of amputation of lower-limb [3].

The pathophysiology of diabetic foot infection (DFI) is complex. The severity and prevalence are depending on pathogen-related factors (e.g., antibiotic-resistance, virulence and microbial organization) and host-related factors (e.g., immunopathy, arteriopathy and neuropathy) [4].

Diabetic Foot Infection poses many problems in clinical practice in terms of both management and diagnosis. Indeed, impairment of leukocyte functions and peripheral arterial disease reduce the local inflammatory response and classical symptoms and signs of local infection. Also, signs of toxicity (e.g., leukocytosis and fever) may be appear late, even in severe conditions [5,6].

Diabetic foot infections are polymicrobial, and *Staphylococcus aureus* is frequently isolated [7].

Staphylococcus aureus in diabetic patients started as an opportunistic agent then becomes a pathogen involved in distinctive manifestations, as diabetic foot ulcers. The same treatment for these infectious processes produces the selection of variants resistant to diverse antibiotics, generating multi resistant *S. aureus*, within them methicillin-resistant *S. aureus* (MRSA) stands out [8].

The isolation of (Methicillin Resistant *Staphylococcus aureus*- Small Colony Variant) MRSA-SCVs in patients with diabetic foot ulcers indicates that the treatment is more problematic and complex. Clinicians should be aware of identifying MRSA-SCVs to provide an efficient treatment, and prevent complications. Also to avoid the use of a great number of antibiotics without identifying the cause of infection[8].

Staphylococcus aureus that forms biofilm is difficult to be treated because many cells within the biofilm are dormant. The fibronectin-binding proteins (FnBPs) (FnBPA and FnBPB) stimulate biofilm formation by clinically relevant MRSA strains [9].

The intercellular adhesion (ica) locus formed of the genes icaADBC

Which encode the proteins mediating synthesis of PIA and PS/A in staphylococcal species**.**Tthe icaA and icaD have been reported to play the most significant role in biofilm formation among *S.aureus* and *S.epidermidis* [10].

Adaptation to attachment to surfaces in biofilms is accompanied by changes in gene and protein expression, also metabolic activity with resistance to antimicrobial agents and host mechanisms responsible of clearance [11].

The aim of this study was to investigate the prevalence of methicillin resistant *S. aureus* (MRSA) and MRSA biofilm production among diabetic patients with chronic leg ulcers

METHODOLOGY

A prospective study was performed on 150 patients with both type 2 diabetes mellitus and infected diabetic foot ulcers. The study was conducted from July 2015 to February 2016 at National Institute of Diabetes and Endocrinology, Cairo, Egypt, Diabetic foot Outpatient Clinic, Faculty of Medicine, Fayoum University and Faculty of Science, Cairo University.

- **Specimens collection, isolation and identification**:

Foot ulcers were cleaned and irrigated vigorously with sterile saline and antiseptic solution then were debrided afterward. The debridement was performed using sterile scalpels which remove unhealthy tissue from infected ulcers. Then foot ulcers were cleaned again and samples were obtained from the base of foot ulcers without touching the skin and other tissues using sterile swabs [12].

After collecting the samples, swabs were transported to the laboratory immediately and plated directly onto mannitol salt agar (Oxoid Ltd., Basingstoke, UK).incubated at 35°C with 5% CO_2 for 48 hours then identified by VITEK 2 system (bioMérieux, Craponne, France).

Gram positive cocci arranged in clusters that were mannitol-fermenting, catalase-positive, and coagulase-positive were screened for methicillin resistance on Mueller–Hinton agar supplemented with sodium chloride cations and oxacillin at 4 µg/mL (Oxoid Ltd., Basingstoke, UK). according to the Clinical Laboratory Standards Institute guidelines[13].

- **Antibiotic susceptibility testing:**

Antibiotic sensitivity test was studied using Kirby Bauer Disc Diffusion Susceptibility Test on Mueller-Hinton agar plate (Oxoid Ltd.,Basingstoke, UK) as described by Clinical and Laboratory Standards Institute (CLSI) [13]. Results were interpreted after 18 to 24 h incubation at 37ºC. Antibiotics used were Gentamicin (10µg), Oxycillin (1µg), Vancomycin (30µg), Linezolid (30µg), Erythromycin (15µg), Levolfloxacin (5µg), Clindamycin (2µg), Cefoxitin (30µg), Chloramphenicol (30 µg) (Oxoid Ltd.,Basingstoke, UK)**.**

- **Detection of biofilm production:**

Detection of biofilm formation was performed using tissue culture plate method (TCP) as described previously[14]. Interpretation of the results were as follows: non biofilm former when OD ≤ ODc, weak biofilm former when ODc < OD ≤2 ODc, moderate biofilm former when 2 ODc < OD ≤ 4 ODc, strong biofilm former when 4 ODc < OD, where ODc is the mean OD of the negative control and OD is the mean OD of the isolate.

- **Detection of of icaA and icaD Genes by Polymerase Chain Reaction (PCR):**

Genomic DNA was extracted from an overnight culture using Gene JET Genomic DNA Purification Kit (Axygen biosciences, USA) with the addition of Lysostaphin (Sigma-Aldrich) at final concentration of 100 µg/ml and incubation at 37°C for 1 h in the initial step. The presence of *icaA and icaD* were detected by polymerase chain reaction (PCR) as described by Bassyouni et al.[14].

RESULTS

A total of one hundred and fifty patients who were diagnosed to have diabetes mellitus with foot ulcers were included in this study. Out of 150 patients 82 (54.66%) patients were male and 68 (45.33%) patients were female. Table 1 summarizes the demographic and clinical characteristics of the patients. And Table 2 shows the characters of the foot ulcers.

Table 1: Demographic and clinical characteristics of the patients

Characteristic	**Total No.**
Age:/Years (mean)	53.3
Gender	
Male /Female	82/68
Duration of diabetes mellitus: years (mean)	11.7
Type of diabetes mellitus:	
Type I	35
Type II	105
Antidiabetic treatment	
Oral antidiabetic	38
Insulin	92
Both	15
None	5
Complication	
Retinopathy	41
Nephropathy	32
Neuropathy	15
Hypertension	62

Table 2: Characters of the foot ulcers.

Ulcer characters	**Mean**
Duration of the present ulcer/months	4.97
Size of the ulcer/cm²	9.77
Location of the ulcer:	
-Dorsal	72
-Planter	43
-Both	35

Table 3: Bacteria isolated from diabetic foot ulcers.

Organisms isolated	**No. (150)**	**(%)**
Staphylococcus aureus:	70	46.6
(MRSA)	(34)	22.6
Pseudomonas aeruginosa	36	24.0
Klebseilla pneumonia	25	16.6
E. coli	19	12.6

Table 3 shows the isolated bacteria recovered from diabetic foot ulcers. From the table it is evident that *Staphylococcus aureus* represents 46.6% of the isolates. Among them 22.6% were MRSA

Table 4 showed the antimicrobial susceptibility test results of 34 MRSA isolates. The 34 bacterial isolates were tested for the ability to form biofilm using microtitre plate technique. The results revealed that 28 out of 34 tested bacterial isolates (82.35%) had the ability to form biofilm.

Table 4: Antibiotic resistance pattern of isolated MRSA (34 strains).

	Sensitive		**Intermediate**		**Resistant**	
Antibiotics	No.	%	No.	%	No.	%
Oxicillin	0	0	0	0	34	100
Cefoxitin	0	0	0	0	34	100
Vancomycin	30	88.2	3	8.8	1	2.9
Clindamycin	20	58.8			14	41.1
Erythromycin	8	23.5			26	76.4
Chloramphenicol	15	44.1			19	55.8
Gentamicin	13	38.2			21	61.7
Levofloxacin	4	11.7			30	88.2
Linezolid	33	97.0	1	2.9	0	0

Screening of the extent of biofilm formation of the isolated MRSA by tissue culture assay reveled that twenty five isolates (73.3%) were strong biofilm producers, 3 isolates (8.8%) were moderate biofilm producer and 6 isolates (17.6%) were non biofilm producers (table 5).

Table 5: The extent of biofilm formation of the isolated MRSA

Micro-organisms	**Number of isolates**	**Biofilm formation (OD 570 nm)**					
		High (strong)		**Moderate**		**Non/- weak**	
		No	**(%)**	**No**	**(%)**	**No**	**(%)**
MRSA biofilm	34	25	73.3	3	8.8	6	17.6

Detection of genes *icaA* and *icaD* by Polymerase Chain Reaction (PCR) revealed that twenty two were positive for both genes and showed strong biofilm formation abilities, On the other hand eight isolates were negative for both genes and showed no or weak/moderate biofilm formation abilities, The remaining 4 isolates were positive for only *icaA* gene,

DISCUSSION

Many risk factors were implicated in development of diabetic foot ulcers, Zhang et al.[15], Al-Rubeaan et al[16] and Wu et al.[17] reported that the prevalence of foot complications in diabetic patients increased with age, diabetes duration and males more than females. Diabetic foot is more commonly seen among type 2

patients, diabetes duration ≥10 years, Charcot joints, , neuropathy, peripheral vascular disease, insulin use, nephropathy, retinopathy, age ≥45 years, poor glycemic control, cerebral vascular disease, coronary artery disease, smoking, male gender, and hypertension. These reports come in agreement with present results as the mean age of the patients was (53.3) years, the mean duration of diabetes was (11.7) years. Ninety two patients treated with insulin, 38 treated with oral antidiabetic, 15 were treated with both oral and insulin and 5 take no treatment, 62 were hypertensive, 41 had retinopathy, 32 had nephropathy, 15 had neuropathy

According to Cunha [18], the increased prevalence of diabetic mellitus is associated with the increase in the problem of infections among diabetic patients and diabetic foot infection accounts for 20% of hospital admission**.**

Diabetic foot infection is generally polymicrobial and both aerobic and anaerobic organisms were isolated from these infections [19, 20].

Gram-negative bacteria were then predominant isolates in our study (53.3%). In agreement to our results, Gram-negative bacteria were reported previously to be higher than Gram-positive bacteria in DFU [21].

In our study, from isolated Gram-negative bacteria, *P. aeroginosa* represents 24.0%, *Klebseilla pneumonia* (16.6%) and *E. coli* (12.6 %), nearly similar results were reported in a previous study as *P. aeroginosa*., *Escherichia coli* and *Proteus* spp. respectively had the highest frequencies [22].

Methicillin-resistant *Staphylococcus aureus* has emerged as a serious problem in patients with diabetic foot ulcers [23].

In present study, *S. aureus* was a frequently common bacterial pathogen (46.6%) followed by *P. aeruginosa* (24.0%). Almost similar rates were reported in other studies [19,24]. Among *S. aureus* isolated in the current study, methicillin resistant strains represent 22.6% our results go hand in hand with many previous studies [25-30].

Biofilm productions are known as a significant problem because biofilm formation protects pathogenic bacteria from the action of antibiotics and it's one of the main causes of development of chronic infections [31-33].

The ability of microbial species to form biofilm is responsible for chronic or persistent infections as biofilm protects microorganisms from host immune system and antimicrobial agents [34-36]. Biofilms make bacteria 1000 times more resistant to antibiotics therapy[37].

In the present study, the ability of 34 bacterial isolates to form biofilm was examined using microtitre plate assay. The results revealed that there were high prevalence of biofilm formation, Among MRSA isolates in this study biofilm formation was present in 82.35% of the isolates. By TCP method, twenty five of them (73.3%) were strong biofilm producers, three (8.8%) were moderate and six (17.6%) were non/weak producers which are close to results obtained by Eftekhar and Dadaei [38] and Cha et al.[39].

Bardiaue et al.[40] reported that biofilm formation ability was present in all MRSA isolates. Resistance to β-lactam antibiotics in MRSA is conferred by the acquisition of a mobile genetic element, carrying the *mecA* gene or the variant *mecC* [8].

Our results revealed that twenty two were positive for both genes and showed strong biofilm formation abilities, On the other hand eight isolates were negative for both genes and showed no or weak to moderate biofilm formation abilities, The remaining 4 isolates were positive for only *icaA gene*.

CONCLUSION

In conclusion a high prevalence of biofilm producing MRSA was detected in *S. aureus* isolated from patients with Diabetic foot. Identifying these variants is crucial to provide an efficient treatment, prevent complications, and to avoid the use of a great number of antibiotics without identifying the cause of infection.

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