Methicillin Resistant *Staphylococcus aureus* and its Biofilm in Persistent Diabetic Foot Ulcer in Qassim Region.

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Abstract: Background: Diabetic foot ulcer is a disastrous complication of diabetes mellitus that may end up with leg amputation. MRSA in diabetic foot disease is increasing worldwide to develop into a problem of healthcare provision. MRSA biofilm is a serious threat as it is considered responsible for chronic or persistent infections and may cause therapeutic failure with regular antibacterial therapy. **Objectives:** To evaluate the prevalence of MRSA and MRSA biofilm production among diabetic patients with chronic leg ulcers and to clarify risk factors related to this infection in resistant diabetic foot ulcers. **Subjects and methods:** Three hundred and eighty four (384) patients with persistent diabetic foot ulcers was involved in this study. Samples obtained from ulcers were directly plated on MacConkey, blood and mannitol salt agar,incubated aerobically, and anaerobic culture on GN and NS media was done, all colonies appeared were examined macroscopically and different pathogens identified by gram stain and the available biochemical reactions.Coagulase, catalase tests and APIStaph system for *Staphylococcus aureus* (*S. aureus*) identification were done. All *S.aureus* isolates were examined by polymerase chain reaction (PCR) for MRSA mecA gene detection, then MRSA strains examined for biofilm formation via PCR detection of icaA and D gene and quantitative tissue culture plate method (TCP). **Results:** A total of 293 isolates were detected from 384 patients and 414 persistent diabetic foot ulcer specimens. The most frequent isolated organism was 106(36.2%) *Staphylococcus aureus*.Among the 106 *Staphylococcus aureus*, 55 (51.9%) were (MRSA) and among them 31 (56.4%) were biofilm producers.Screening of the extent of biofilm formation of the isolated MRSA by tissue culture assay (TCP) revealed that 18/31(58.1%) were strong adherent, 7(22.6%) were moderate adherent and 6(19.3%) were also non/weak adherent. Regarding the demographic and clinical characteristics of patients with MRSA and non MRSA infected diabetic foot ulcers, the risk for MRSA isolation was significantly increase with older age, longer duration of diabetes mellitus, larger size of the ulcer as well as presence of osteomyelitis ($p \leq 0.05$). Regarding risk factors for MRSA and MRSA biofilm infections; the risk for MRSA isolation was significantly increase with older age while biofilm formation increase significantly with previous antibiotic treatment and previous colonization with MRSA. **Conclusion:** The high rate of MRSA infections in diabetic foot that have the ability of biofilm formation was alarming for public health. Biofilm-based wound care is "a significant shift in the whole approach to wound healing". Early identification and discrimination between MRSA and MRSA biofilm can be one of the essential steps towards the prevention and treatment of the most serious diabetic foot ulcer infections.
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1. Introduction:

Diabetes mellitus is a disease as old as mankind itself and is a major health care challenge. [(1)]. Magnitude of diabetes mellitus is increasing globally at an alarming rate. About 150-170 million populations are suffering from this diseases worldwide. [(2)] Diabetes mellitus confers a special vulnerability to infection due to defects in both cell mediated and humoral immunity, probably due to hyperglycemia.

Once these infections occur, they are more difficult to treat and pose a great threat to the diabetic than to a healthy person [(3)]. Diabetic foot ulcer is an important complication among diabetes mellitus [(2),]People with diabetes are 25 times more likely to have a leg amputated than those without the condition, according to the International Diabetes Federation[(4)].

Sensory neuropathy, atherosclerotic vascular disease and uncontrolled hyperglycemia are the favoring factors of skin and soft tissue infections [(2)].

Staphylococcus aureus (*S.aureus*) is found to be the commonest pathogen present in diabetic foot infections[(5,6)]. Almost 50% of *S. aureus* isolates are methicillin-resistant *S. aureus* (MRSA) and several studies have found its emergence in as many as 15–30% of diabetic wounds[(7)]. Methicillin -resistant *Staphylococcus aureus* (MRSA) is associated with serious infections. Having the ability of biofilm-formation decrease their susceptibility to antibiotics[(8)]. Biofilms are the population of bacteria growing on the

biotic and abiotic surfaces and embed themselves in a self-produced extracellular matrix of exopolysaccharide (EPS), proteins and some micro molecules such as DNA [9,10].

There are various definitions for biofilm but all of them enumerate three major ingredients for it: microbes, slime exopolysaccharide and surface, removing any of them can stop developing biofilm [11]. The intercellular adhesion (ica) locus consisting of the genes icaADBC encodes the proteins mediating the synthesis of PIA and PS/A in staphylococcal species**.** Among ica genes, the icaA and icaD have been reported to play a significant role in biofilm formation of *S.aureus* and *S.epidermidis*[12,13]**.** Adaptation to surface attached growth within a biofilm is accompanied by significant changes in gene and protein expression, as well as metabolic activity which confers resistance to antimicrobial therapy and host mechanisms of clearance [14--16]**.**

Thus, both systemic and topical antibiotics alone are unable to eradicate biofilm infections[13]). There is increasing interest in their aetiological role. As such, there is an increasing clinical need to identify biofilms in these wounds[17].

Potential opportunities exist that include prevention of bacterial attachment, prevention of biofilm formation, disruption of the biofilm to allow penetration of topical antimicrobial agents, interference with quorum sensing, and enhancement of bacteria dispersion from biofilms to a more easily destroyed planktonic state[18]**.**

The aim of this study was to predict the prevalence of MRSA and MRSA biofilm production among diabetic patients with chronic leg ulcers and to clarify risk factors related to this infections in resistant diabetic foot ulcers.

2. Subjects and methods:

Three hundred and eighty four (384) patients with persistent diabetic foot ulcers (267 males and 117 females) seeking treatment at Diabetic Foot Center, King Saud Hospital, Qassim were included in this study. Infection was diagnosed according to the criteria proposed by the international consensus on the diabetic foot[19]. Peripheral vascular disease was diagnosed when patients had an ankle–brachial pressure index (ABI) < 0.9, as determined with a portable Doppler machine or when they had a history of intermittent claudication or of re-vascularization procedures[20]. None of the study patients had infections at other body sites. Informed consent from each patient was obtained.

Samples:

From curettage of the diabetic ulcer base and skin biopsies from the ulcer edge and web swabs. Cotton swabs were moistened with 0.9% saline [21]. Curette samples were placed in 10 ml trypticase soy broth (Himedia lab. PVT. Ltd. India), sonicated for one minute and vortexed for 15 seconds then 0.1 ml was cultured. All samples were plated directly on MacConkey agar, blood agar and mannitol salt agar, incubated aerobically at 37°C for 24 hours**.** Also, anaerobic culture on GN and NS media (bioMerieux, Inc) was done.

All colonies appeared on any culture were examined macroscopically and different microbial pathogens were identified by gram stain, and the available biochemical reactions. Catalase and coagulase tests was done for all Staphylococcus strain then APIStaph system (bioMerieux, France) was applied for identification. *Staphylococcus aureus* isolates were maintained in trypticase soy broth, to which 15% glycerol was added, at -80°C. *Staphylococcus aureus* strains was examined by PCR for MRSA mecA gene detection, then MRSA isolates examined for biofilm formation via PCR detection of icaA/D gene and quantitative tissue culture plate method (TCP).

PCR for detection of MRSA mecA and biofilm icaA and D genes:

Three standard steps of PCR was done; DNA extraction, amplification and detection.

DNA extraction: DNA extraction kit (Axygen biosciences, USA). Samples were processed according to the instructions of the manufacturer from isolates on trypticase soy agar plates after thawing the samples. DNA extracted from staphylococci isolates for mecA gene detection and from MRSA isolates for icaA and D gene detection.

PCR amplification: Taq Master / high yield (Jena Bioscience GmbH, Germany) was used as ready to use mixes which contain all reagents required for PCR except template and primers in a premixed 5x concentrated ready to use solution. For 50 uL PCR assay; 10uL 5x Taq Master Mix, to which 50 pmol each primer, 200ng template DNA was added and completed up to 50uL with PCR grade water.

Amplification thermal program for MRSA mecA and biofilm icaA and D genes:

PCR cycling was carried out in PerkinElmer thermal cycler 9700 as follow: an initial denaturing step at 94°C for 5 minutes, followed by 40 cycles for mecA and 50 cycles for icaA and D genes,each consists of three steps: 94°C for 30 seconds (denaturation), 55.5°C for 30 seconds (annealing), 72°C for 30 seconds for mecA and 1minute for icaA and D genes for (extension), These were followed by a final extension step at 72ºC for 5 min.

Primers were supplied from (Jena Bioscience GmbH, Germany) (table1). The amplified products were mixed with gel loading buffer and run on a 2% agarose gel in Tris-borate buffer. DNA marker was used (50 bp, Promega, USA).

Detection of biofilm formation by tissue culture plate method (TCP)[22]

Strains were subcultured in brain heart infusion broth (Himedia lab. PVT. Ltd. India) at 37°C for 8 hours then plated on trypticase soy agar plates. Isolates from fresh agar plates were inoculated in trypticase soy broth with 1% glucose and incubated for 24 hours at 37°C then diluted (1 in 100) with fresh medium. Individual wells of sterile, polystyrene, flat-bottom tissue culture plates were filled with 0.2 ml aliquots of the diluted cultures, and only broth served as control to check sterility and non-specific binding of media. The tissue culture plates were incubated for 24 hours at 37°C**.** After incubation, the content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free- floating planktonic bacteria; then 25 µl of 1% solution of crystal violet was added to each well (this dye stains the cells but not the polystyrene) plates. The plates were incubated at room temperature for 15 minutes, rinsed thoroughly and repeatedly with saline. Adherent cells, which usually formed biofilm on all side wells, were uniformly stained with crystal violet. Crystal violet-stained biofilm was solubilized in 200 µL of 95 % ethanol (to extract the violet color), of which 125 µl were transferred to a new polystyrene microtiter dish, then Optical densities (OD) at wavelength 570 nm with ethanol as blank were determined via Da Vinci (bioMérieux, France) microplate reader, as an index of bacteria adhering to surface and forming biofilms. According to (OD_{570} nm) Biofilm production is considered; Non/weak < 0.120, moderately 0.120-0.240 and High/Strong > 0.240.

Statistical analysis:

Statistical analysis was done using SPSS version 10.0. Data are represented as Mean±SD. Unpaired student t-test, fisher exact probability test and chi-square test, were used when appropriate. $P<0.05$ considered to be statistically significant in all tests.

3. Results:

Three hundred and eighty four (384) patients with persistent diabetic leg ulcers (267 males and 117 females; 283 with type II and 101 with type I diabetes mellitus) were included in the study and random blood glucose level was≥200 mg/dl in 228 patients at time of admission.

The mean age of the patients was (53 ± 10.2) years, the mean duration of diabetes was (11.1± 3.9) years. Two hundred and forty five patients treated with oral antidiabetic, 102 treated with insulin, 32 were treated with both oral and insulin and 5 take no treatment. One hundred eighty three were hypertensive, 164 had retinopathy,64 had nephropathy, 201 had neuropathy, 133 had peripheral vascular disease and osteomyelitis were present in 46 patients (Table 2).

As regard characters of foot ulcers**;** the Mean ±SD of duration for the present ulcers/months was 6.2±1.9, size of the patients had dorsal located ulcers,145 planter and 28 patients had both dorsal and planter ulcers (Table 3).

A total of 293 isolates were detected from 414 ulcer specimens. The most frequent isolated organism was 106(36.2%) *Staphylococcus aureus*, 47(16%) *Pseudomonas aeruginosa*, Proteus spp.44 (15%), *E.coli* were 20(6.8%), *Klebseilla pneumoniae* 18 (6.1%), coagulase negative staphylococci 17(5.8%), *Serratia marcescens* 8(2.7%) and finally 29(9.9%) anaerobes and fungal 4(1.4%) (Table 4).

Among the 106 *Staphylococcus aureus*, 55 (51.9%) were (MRSA) and among them 31 (56.4%) were biofilm producers. Screening of the extent of biofilm formation of the isolated MRSA by tissue culture assay (TCP) revealed that 18/31(58.1%) were strong adherent, 7(22.6%) were moderate adherent and 6(19.3%) were also non/weak adherent (Tables 5 and 6). Regarding the demographic and clinical characteristics of patients with MRSA and non MRSA infected diabetic foot ulcers, the risk for MRSA isolation was significantly increase with older age, longer duration of diabetes mellitus, larger size of the ulcer as well as presence of osteomyelitis ($p\leq0.05$) (Table7).

Regarding risk factors for MRSA and MRSA biofilm infections; the risk for MRSA isolation was significantly increase with older age while biofilm formation increase significantly with previous antibiotic treatment and previous colonization with MRSA (Table 8).

Table (1): Oligonucleotide primers used for PCR of MRSA and MRSA biofilm.

Primer	Primer sequence(5`-3`)	Amplicon size(bP)
mecA[23,24]	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTTGC	**533**
icaA[25,26]	ACACTTGCTGGCGCAGTCAA TCTGGAACCAACATCCAACA	**188**
icaD[25,26]	ATGGTCAAGCCCAGACAGAG AGTATTTTCAATGTTTAAAGCAA	**198**

Table (2): Demographic and clinical characteristics of the patients.

Characteristic	**Total number**
Age/Years (Mean ±SD)	53 ± 10.2
Gender	
Male /Female	267/117
Duration of diabetes mellitus (years)	11.1± 3.9
Type of diabetes mellitus	
Type I	101
Type II	283
Antidiabetic treatment	
Oral antidiabetic	245
Insulin	102
Both	32
None	5
Comorbidities	
Retinopathy	164
Nephropathy	64
Neuropathy	201
Associated diseases	
Hypertension	183
Peripheral vascular disease	133
Osteomyelitis	46
Rondom blood glucose level:≥200mg/dl	228
(at time of admission**)** **<200mg/dl**	156

Table (3): Characters of the foot ulcers.

Ulcer characters	**(Mean ±SD)**
Duration of the present ulcer/months	6.2± 1.9
Size of the ulcer/cm²	4. 1±3.2
Location of the ulcer:	**Total number**
	414
- Dorsal	241
-Planter	145
- Both	28

Table (4): Prevalence of different micro-organisms isolated from chronic diabetic foot ulcers.

Organisms isolated	**No. (293)**	**(%)**
Aerobes		
Staphylococci spp.		
-*Staphylococcus aureus*	106	36.2
(MRSA)	*(55)*	*(51.9)*
-coagulase negative staphylococci	17	5.8
Pseudomonas aeruginosa	47	16
Proteus spp.	44	15
E. coli	20	6.8
Klebseilla pneumoniae	18	6.1
Serratia marcescens	8	2.7
Anaerobes	29	9.9
Fungal	4	1.4

Table (5): Prevalence of MRSA and its biofilm among Staphylococcus aureus isolates.

Micro-organisms	Total No (%)	MRSA		MRSA biofilm	
		No	%	No	%
Staphylococcus aureus	106(36.2%)	55/106	51.9	31/55	56.4

Table (6): Screening of the extent of biofilm formation of the isolated MRSA by tissue culture assay (TCP).

Micro-organisms	Number of isolates	Biofilm formation (OD_{570} nm)					
		High (strong)		Moderate		Non/weak	
		No	%	No	%	No	%
MRSA biofilm	31	18	58.1	7	22.6	6	19.3

Table (7): Comparison between risk factors of patients with MRSA and non MRSA infected diabetic foot ulcers as regard demographic and clinical characteristics.

Characteristic	**MRSA** (No:55)	**Infections Other than MRSA** (No:238)	***P***
Age (Years)	60.2 ± 6.4	57.1±10.1	0.03*
Gender			
Male /Female	35/20	149/89	1
Duration of diabetes mellitus (years)	12.9± 3.1	6.05±4.1	0.0001*
Type of diabetes mellitus			
Type I	21	68	0.2
Type II	34	170	
Duration of the present ulcer (months)	6.9± 1.9	6.5±1.7	0.13
Size of the ulcer (cm²)	5.7±3.3	3.2±1.9	0.0001*
Antibiotic treatment (3 months ago)	47	198	0.8
Complications			
Hypertension	32	161	0.2
Retinopathy	29	135	0.7
Nephropathy	15	49	0.3
Neuropathy	37	164	0.9
Peripheral vascular disease	20	113	0.2
Osteomyelitis	15	31	0.01*

*Significant difference ≤ 0.05.

Table (8): Comparison between risk factors of patients with MRSA and MRSA biofilm infected diabetic foot ulcers as regard demographic and clinical characteristics.

Characteristic	**MRSA** (No:24)	**MRSA biofilm** (No:31)	***P***
Age (Years)	60.1 ± 10.2	54.2±8.2	0.02*
Gender			
Male /Female	17/7	20/11	0.8
Antibiotic treatment (3 months ago)	16	29	0.01*
Hospitalization	10	12	1
Colonization	5	15	0.04*

*Significant difference ≤ 0.05.

4. Discussion:

Diabetic foot infection is a common problem in Saudi Arabia due to complications of diabetic disease. The role of foot infection in diabetic foot ulceration is well documented [(27)].

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a serious and common problem in patients with diabetic foot ulcers [(28,29)] in addition MRSA infections are life-threatening due to emergence of multidrug resistance strains and also occurrence of isolates that are able to form strong biofilms [(8)].

In the present study, *Staphylococcus aureus* was the most frequent isolated organism (36.2%). Detection of mecA gene by PCR revealed that among the *Staphylococcus aureus* isolates, MRSA was high

prevalent strain (51.9%). Our results were consistent with that reported in many studies [30, 31]. Although Predominance of Gram negative bacteria was documented in other studies too [32,33]. Zubair *et al.*[34] and Hena & Growther[35] explained the high prevalence of MRSA in such studies by the indiscriminate use of broad spectrum antibiotics, resulting in a pathogen-selective survival advantage.

Biofilms are defined as communities of bacteria encased in a self-synthesized extracellular polymeric matrix that attaches to a biotic or abiotic surface and biofilm-forming staphylococci including *Staphylococcus aureus* especially MRSA has been one of the major cause of chronic polymer-associated infection[36-38] mediated by a polysaccharide intercellular adhesin (PIA) and encoded by the ica operon[39].

Among MRSA isolates in this study, biofilm ica A and D genes which detect potential ability for biofilm formation was present in 56.4% of the isolates. By TCP method, eighteen of them (58.1%) were strong adherent, 7 (22.6%) were moderate and 6 (19.3%) were non/weak adherent. Eftekhar & Dadaei[39] reported that MRSA biofilm from clinical isolates were 53.3% but weak biofilm production was observed in 57.8% of them, that can be explained by variability of the samples. Khan *et al.*[40] and Cha *et al.*, [41] reported high tendency of MRSA for biofilm formation.

In the current study, Regarding the demographic and clinical characteristics of patients with MRSA and non MRSA infected diabetic foot ulcers, the risk for MRSA isolation was significantly increase with older age, longer duration of diabetes mellitus, larger size of the ulcer as well as presence of osteomyelitis. These results were compatible with many other reports[42,43]. While Alizargar *et al.*, [44] reported no association of MRSA infection with older age, gender, previous antibiotic treatment.

Various risk factors are suggested to affect MRSA biofilm formation preferable environments to a greater or lesser extent than MRSA infection. Our results showed that significant risk factors were the previous antibiotic treatment and previous colonization (Table 8).

Cha *et al.*, [41] mentioned that the presence of invasive devices and prior hospitalization were significant risk factors for MRSA biofilm. While proportions of patients with prior antibiotic use and prior MRSA colonization were higher in biofilm-forming isolates than nonforming isolates not significant statistically.

The threat of MRSA infections results from not only the occurrence of multidrug resistance but also the emergence of bacteria that form strong biofilms[8]. Biofilm infections are important clinically because bacteria in biofilms exhibit recalcitrance to antimicrobial compounds and persistence in spite of sustained host defenses[45].

In conclusion, the high rate of MRSA infections in diabetic foot that have the ability of biofilm formation was alarming for public health. Biofilm-based wound care is “a significant shift in the whole approach to wound healing”. The size of the ulcer, the duration of diabetes and osteomyelitis were independent predictors of MRSA infection. The age of patients, prior antibiotic treatment and previous colonization were predisposing factors for MRSA biofilm formation.

Early identification and discrimination between MRSA and MRSA biofilm can be one of the essential steps towards the prevention and treatment of the most serious diabetic foot ulcer infections.

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References :

1. Rani K. Bacteriological profile of Diabetic Foot Ulcer. IJPRBS, 2013; 2(2): 36-45.
2. Pappu A, Sinha A, Arvind J. Microbiological profile of Diabetic Foot Ulcer. Calicut Medical Journal, 2011;9(3):e2.
3. Khairul k, MuppidiS, Ketan P.Bacteriological study of diabetic foot infections. Brunei Med J., 2012;8(1):19-26.
4. Lau TW, Sahota DS, Lau CH, *et al.* An *in vivo* Investigation on the Wound- Healing Effect of two Medicinal Herbs Using an Animal Model with Foot Ulcer. Eur Surg Res 2008; 41:15-23.
5. Lima AF, Costa LB, da Silva JL, *et al.* Interventions for wound healing among diabetic patients infected with *Staphylococcus aureus*: a systematic review. Sao Paulo Med J., 2011; 129(3):165-70.
6. Kandemir O, Akbay E, Sahin E, *et al.* Risk factors of infection of the diabetic foot with multi-antibiotic resistant microorganisms.J infect., 2007; 54: 439-445.
7. Wang S, Yuan Y, Sun Z, *et al.* Meticillin-resistant *Staphylococcus aureus* isolated from foot ulcers in diabetic patients in a Chinese care hospital: risk factors for infection and prevalence. J Med Microbiol., 2010; 59: 1219–1224.
8. Rezaei M, Moniri R, Abbas Mousavi S *et al.* Prevalence of Biofilm Formation Among

Methicillin Resistance *Staphylococcus aureus* Isolated From Nasal Carriers.Jundishapur J Microbiol. 2013;6(6):e9601.
9. Gowrishankar S, Duncun Mosioma N, Karutha Pandian S.Coral-Associated Bacteria as a Promising Antibiofilm Agent against Methicillin-Resistant and -Susceptible *Staphylococcus aureus* Biofilms. Evid Based Complement Alternat Med., 2012; 2012:ID 862374 pages16.
10. El-Feky MA, El-Rehewy MS, Hassan MA *et al.* Effect of ciprofloxacin and N-acetylcysteine on bacterial adherence and biofilm formation on ureteral stent surfaces. Pol J Microbiol., 2009; 58(3):261-7.
11. Murugan K, Usha M, Malathi P *et al.* Biofilm forming multi drug resistant Staphylococcus spp. among patients with conjunctivitis. Pol J Microbiol. 2010; 59(4):233-9.
12. Gotz F. Staphylococcus and biofilms. Mol Microbiol., 2002; 43:1367-78.
13. Arciola CR, Baldassari L, Montanaro L. Presence of icaA and icaD genes and slime production in a collection of Staphylococcal strains from catheter-associated infections. J Clin Microbiol., 2001;39: 2151–56.
14. Anderson GG,O'Toole GA. Innate and induced resistance mechanisms of bacterial biofilms. Curr Top Microbiol Immunol 2008; 322:85–105.
15. Thurlow LR, Hanke ML, Fritz T,etal. *Staphylococcus aureus* biofilms prevent macrophage phagocytosis and attenuate inflammation *in vivo*. J Immunol., 2011; 186:6585–6596.
16. Carlos J,Sanchez Jr, Katrin M, etal. Biofilm formation by clinical isolates and the implications in chronic infections. BMC Infectious Diseases 2013; 13:47.
17. Angela O, Frank L, Andrew J, *et al.* The Visualization of Biofilms in Chronic Diabetic Foot Wounds Using Routine Diagnostic Microscopy Methods. Journal of Diabetes Research. 2014; Article ID 153586- 8.
18. Davis SC, Martinez L, Kirsner R. The diabetic foot: the importance of biofilms and wound bed preparation. Curr Diab Rep 2006;6(6):439-45.
19. Lipsky BA, Berendt AR, Embil J, *et al.* Diagnosing and treating diabetic foot infections. Diabetes Metab Res Rev 2004;20(suppl 1):56–64.
20. Tentolouris N, Petrikkos G, Vallianou N, *et al.* Prevalence of methicillin-resistant *Staphylococcus aureus* in infected and uninfected diabetic foot ulcers. Clin Microbiol Infect., 2006; 12: 186–189.
21. National Institute for health and clinical excellence. NICE clinical guideline119: inpatient management of diabetic foot problems. London: National Institute for health and clinical excellence; 2011.
22. Christensen GD, Simpson WA, Younger JA, *et al.* Adherence of coagulase negative Staphylococci to plastic tissue cultures: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 1985;22: 996-1006.
23. Del Vecchio VG, Petroziello JM, Gress MJ, *et al.* Molecular genotyping of methicillin resistant *Staphylococcus aureus* via fluorophore-enhanced repetitive sequence PCR. J Clin Microbiol., 1995; 33: 2141-4.
24. Murakami K, Minamide W, Wada K, *et al.* Identification of methicillin-resistant strains of Staphylococci by polymerase chain reaction. J Clin Microbiol., 1991; 29: 2240-44.
25. Gad GF, El-Feky MA, El-Rehewy MS, *et al.* Detection of icaA, icaD genes and biofilm production by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from urinary tract catheterized patients. J Infect Dev Ctries 2009; 3(5):342-351.
26. Mariana NS, Salman SA, Neela V, *et al.* Evaluation of modified Congo red agar for detection of biofilm produced by clinical isolates of methicillin–resistance *Staphylococcus aureus*. African Journal of Microbiology Research 2009; 3(6): 330-338.
27. Samira A, Amer A S. Diabetic foot infection and prevention measures: A literature review. Nat Sci., 2012;10(9):26-34].
28. Game F, Jeffcoate W. MRSA and osteomyelitis of the foot in diabetes. Diabet Med., 2004; 21:16–19.
29. Tentolouris N, Jude E, Smirnof I, *et al.* Methicillin-resistant *Staphylococcus aureus*: an increasing problem in a diabetic foot clinic. Diabet Med 1999; 16: 767–771.
30. James G.,Swogger E.,Wolcott R.,etal.Biofilms in Chronic wounds.Wound Repair and regeneration 2008;16:37-44.
31. Mustafa F, Thomas B, aus Kirketerp-Moller K, etal. Nonrandom distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in chronic wounds. J Clin Microbiol 2009;47(12):4084-4089.
32. Shankar E, Mohan V,PremlathaG, *et al.* Bacterial etiology of diabetic foot infections in South india. Eur J Intern Med., 2005:16:567-570.
33. Gadepalli R, Dhawan B, Sreenivas V, et al. Clinico-Microbiological study of Diabetic foot

ulcers in an Indian tertiary care hospital. Diabetes care 2006;29:1727-1732.
34. Zubair M, Malik A, Ahmad J. Clinico-microbiological study and antimicrobial drug resistance profile of diabetic foot infections in North India. Foot (Edinb) 2011;21(1):6–14.
35. Hena JV, Growther L. Studies on bacterial infections of diabetic foot ulcer. Afr J Cln Exper Microbiol., 2010;11(3): 146-149.
36. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999; 284(5418): 1318e22.
37. Cucarella C, Solano C, Valle J, *et al.* Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol 2001; 183(9):2888e96.
38. O'Neill E, Pozzi C, Houston P, *et al.* Association between methicillin susceptibility and biofilm regulation in *Staphylococcus aureus* isolates from device-related infections. J Clin Microbiol., 2007; (5):1379e88.
39. Eftekhar F, Dadaei T. Biofilm Formation and Detection of IcaAB Genes in Clinical Isolates of Methicillin Resistant *Staphylococcus aureus*. Iranian Journal of Basic Medical Sciences 2011;14(2) :132-136.
40. Khan F, Shukla I, Rizvi M, *et al.* Detection of Biofilm Formation in *Staphylococcus aureus*. Does it have a role in Treatment of MRSA Infections? Trends in Medical Research 2011; 6 (2): 116-123.
41. Cha J, Jae Il, Sik Yoo J.Investigation of Biofilm Formation and its Association with the Molecular and Clinical Characteristics of Methicillin-resistant *Staphylococcus aureus*. Osong Public Health Res Perspect 2013; 4(5): 225-232.
42. Eleftheriadou I, Tentolouris N, Argiana V, *et al.* Methicillin-resistant *Staphylococcus aureus* in diabetic foot infections. Drugs 2010;70(14):1785-97.
43. Murugan S, Mani KR, Uma Devi P. Prevalence of Methicillin Resistant *Staphylococcus Aureus* among Diabetes Patients with Foot Ulcers and their Antimicrobial Susceptibility Pattern. Journal of Clinical and Diagnostic Research 2008; 14(2): 979-984.
44. Alizargar J, Sharif M, Sharif A.Risk Factors of Methicillin-Resistant *Staphylococcus aureus* Colonization in Diabetic Outpatients, A Prospective Cohort Study. International Journal of Microbiological Research 2013;4 (2): 147-151.
45. Hall-Stoodley L, Stoodley P. Evolving concepts in biofilm infections. Cell Microbiol 2009;11(7):1034-43.

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