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Correlation of Virulence Determinants of *Staphylococcus aureus* to the Severity of Diabetic Foot Ulcers in a Tertiary Care Centre, Egypt

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Authors' contributions

This work was carried out in collaboration between both authors. Author YN designed the idea of the research and was responsible for the processing of samples obtained from the included cases, writing of the manuscript, evaluating the results and submitting the manuscript for publishing. Author GB shared in processing of samples and writing of the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: This study aimed to estimate the correlation of the *Staphylococcus aureus* virulence determinants to the severity of the diabetic foot ulcers in patients admitted to diabetic foot Unit at Mansoura University Hospitals.
Study Design: A prospective study was performed.
Place of Study and Duration: This study was performed in diabetic foot Unit of Mansoura University hospital, Egypt.
Methodology: The study included 95 patients clinically diagnosed with diabetic foot ulcers from whom swabs were obtained from the foot lesions to be processed followed by detecting virulence determinants in isolated *S. aureus* by PCR.
Results: *Staphylococcus aureus* was isolated from 34 cases (35.8%). *icaA*, *icaD*, *pvl* and *tst* genes were detected in 61.8%, 67.6%, 53%, and 32.4% of *S. aureus* isolates respectively with higher

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prevalence in isolated strains from more severe infections and results were stastically significant. *coa* and *clfA* genes were positive 97.1% and 94.1% isolates respectively.
Conclusion: The results showed that the *S. aureus* strains causing infections in diabetic foot patients having genes: *icaA*, *icaD*, *pvl* and *tst* may be correlated to the severity of lesions whereas *coa* and *clfA* were not.

Keywords: Diabetic foot; icaA; icaD; pvl; tst; coa; clfA; Polymerase chain reaction.

ABBREVIATIONS

PCR: Polymerase Chain Reaction
Staphylococcus aureus: S. aureus

1. INTRODUCTION

Lots of studies consider infections in diabetic foot as a great clinical and financial problem to the patients suffering from diabetes [1,2,3] proved by being one of the disastrous complications that commonly proceed throughout the course of the disease up to septic gangrene which may end in amputation of the foot in up to 70% of diabetic patients especially if uncontrolled[2]. Studying the types of organisms causing diabetic foot infections showed a great diversity, from one patient to another, and also from one locality to another [4].

The polymicrobial nature of infected diabetic foot shown clearly through microbiological studies have demonstrated that, the most commonly detected aerobic isolates include coagulase-positive *Staphylococcus* spp, besides other coagulase-negative ones*, Enterococcus* spp., in addition to various types of Gram negative bacteria [3]. The most commonly reported anaerobic causative agents include *Clostridium* spp., *Peptostreptococcus* spp, and *Bacteroides fragilis* [5].

Staphylococcus aureus is one of commensal bacteria over skin and mucosa that may be a cause of lots of infections in human beings including diabetic patients, these infections may range from minor skin infections to severe infections that can end in septicaemia and osteomyelitis [6].

We can attribute the pathological effects of *S. aureus* to a variety of virulence factors including biofilm formation aiming to help the bacterium to evade the immune system and to resist to antibiotic therapies through the formation of adherent bacterial populations that grow inside a polymeric structure [7]. The biosynthesis of polysaccharide intercellular adhesion molecules in which bacteria is entrapped producing the biofilm, is attributed to several virulence genes like *icaA* and *icaD*.

Staphylococci, especially *S. aureus* produce other virulence factors like toxins, immune evasion factors and tissue degrading enzymes [8]. They produce coagulase enzyme that is encoded by *coa* gene and clumping factor which is encoded by *clfa* gene, it hinders phagocytosis and binds to fibrinogen changing it into fibrin leading to platelet activation [9].

Another virulence determinant is cytotoxin panton-valentine leukocidin (*pvl*), which is considered a major hazard causing severe necrosis in tissues. Its encoding gene locus is determined to be carried on a bacteriophage [10]. Its disastrous effect is produced through causing leukocyte destruction and tissue necrosis. Fewer than 5% of *S. aureus* strains can produce it [10]. Some isolates also secrete the toxic shock syndrome toxin 1 (*TSST-1*). It is a superantigen toxin encoded by the *tst* gene, scarlet fever and toxic shock syndrome caused by *S. aureus* strains are attributed to such type of toxin [11,12].

Main diagnostic methods of such wound infections are based mainly on clinical laboratory investigations supporting the isolation of the bacterial cause by culturing techniques [13]. This can be only fulfilled through obtaining the proper sample by swabbing techniques versus tissue sampling of infected diabetic foot ulcers [14].

To the best of our knowledge, finding the correlation between the presence or absence of various virulence factors and the grading of the diabetic foot ulcers was not yet studied in our locality.

In our study, we aimed to find a correlation between the *S. aureus* virulence determinants and the severity of the diabetic foot ulcers in patients admitted to diabetic foot Unit at Mansoura University Hospitals.

2. MATERIALS AND METHODS

2.1 Selection of Patients

This study was carried out in Medical Microbiology and Immunology department of Mansoura faculty of Medicine over a period beginning in October 2015 and terminating in December 2016. Ninety five patients clinically diagnosed with diabetic foot ulcers were enrolled in this study. The grading of foot ulcers was done following Wagner's classification [15] as follows (grade 0- showed hyperkeratosis; grade I – showed superficial ulcers; grade- II suffered from deep ulcers; grade- III showed tendonitis, osteomyelitis, cellulites, and or abscess; grade-IV presented by ulcers with gangrene of a toe or forefoot; and finally grade -V had massive gangrene of the whole foot. The study protocol was reviewed and accepted by our faculty's research board with a written informed consent from all included subjects.

2.2 Collection of Samples

A sterile cotton-tipped swab was used to obtain a superficial swab from the center of ulcer after cleaning the foot lesion with sterile saline. All obtained samples were transported to Medical Microbiology and Immunology Department for further processing. They were used for detecting bacterial causes of infection through different culturing techniques.

2.3 Isolation and Identification of Aerobic Bacterial Causes

Once samples were obtained, they were directly inoculated onto appropriate culturing media to be incubated aerobically at 37°C for 24 hs. Identification of different isolates was done by resulting colonies' morphological characters, Gram staining characters and suitable indicated biochemical reactions [16].

2.4 Identification of *Staphylococcus aureus*

Staphylococci were chronologically identified by their characteristic colony morphology including the produced pattern of haemolysis, Gram staining criteria, biochemical reactions such as catalase and coagulase activity testing, DNase test, and mannitol fermentation on mannitol salt agar. Results were confirmed by API Staph 32 (bioMe´ rieux, Marcy-l'E´ toile, France). Tests were carried out and interpreted according to the manufacturers' guidelines.

2.5 Screening for Virulence Factors in *Staphylococcus aureus* Isolates

Isolated *S. aureus* strains were subcultured on brain heart infusion agar to be subjected to a process of genomic DNA extraction using QIAamp DNA Extraction Kit (QIAGEN Hilden, Germany) then PCR amplification of studied genes.

- ***ica A* and *ica D* genes:** The set of primers used to amplify *icaA* gene was 5′-TCTCTTGCAGGAGCAATCAA-3′ & 5′-TCAGGCACTAACATCCAGCA-3′ to give a DNA fragment of 188-bps, and that for *icaD* was 5′-ATGGTCAAGCCCAGACAGAG-3′ & 5′-CGTGTTTTCAACATTTAATGCAA-3′ giving a DNA fragment of 198-bps [17].

  The reaction was carried out through a multiplex PCR in a 25-µl volume containing 1 µM of each primer with 150 ng of the extracted DNA, 100 µM of each neucleotide, 1 U of Taq DNA polymerase, and buffer. We applied the following conditions for the amplification process using (Norwall, CT, USA) thermal cycler, 5 mins of initial denaturation at a temperature of 94°C for, then 50 cycles at 94°C for 30 s for denaturation, annealing at 55.5°C for 30 s, extension at 72°C for 30 s, and finally elongation for 1 min at 72°C [17].

- ***coa* gene:** We used the following set of primers for amplification: coa-1 5′-ATA GAG ATG CTG GTA CAG G-3′ and coa-2 5′-GCT TCC GAT TGT TCG ATG C-3′ yielding a DNA fragment of 350-bps. The conditions used were: 94°C for 5 min for initial denaturation, then 30 successive cycles as follows 94°C, 1 min; 58°C, 1 min; 72°C, 1 min [18].
- ***clfA* gene:** The set of primers used was clfA-1 5′- GGC TTC AGT GCT TGT AGG -3′ and clfA-2 5′- TTT TCA GGG TCA ATA TAA GC -3′ for obtaining a DNA fragment of 540-bps. Initial denaturation was done for 5 min at 94°C and then 35 following cycles at 94°C for 1 min; 57°C for 1 min; 72°C for 1 min [18].
- ***pvl* gene:** We used the following set of primers for amplification: lukS-PV–5′-ATCATTAGGTAAAATGTCTGGACATGAT

CCA-3′ and lukF-PV 5′-GCATCAASTG-TATTGGATAGCAAAAGC-3′ to yield a DNA fragment of 433-bps. The amplification conditions used were 30 successive cycles: 30 s for denaturation at 94°C, 30 s for annealing at 55°C, and then 1 min for extension at 72°C [19].

- ***tst* gene:** The set of primers was TST-1 5′-TTCACTATTTGTAAAAGTGTCAGACCCA CT-3′ and TST-2 5′-TACTAATGAATTTTTTTATCGTAAGCCC TT-3′ to yield a DNA fragment of 740-bps. The conditions of the reaction were: denaturation, annealing, and extension for 1 min at 94°C, 1 min at 55°C and 1 min at 72°C respectively [20].

2.6 Statistical Analysis

We presented the data as numbers and percentage. We used SPSS software 17 to analyse data by the Pearson's Chi square and 1-sample K–S tests. Significance is considered when P value is less than 0.05.

3. RESULTS

We conducted this work in the department of Medical Microbiology and Immunology in Mansoura faculty of Medicine over a period beginning in October 2015 and terminating in December 2016. Ninety five patients clinically diagnosed with diabetes mellitus complicated by diabetic foot ulcers were included in the study. The mean of the studied cases age range was 43 ± 6.51 years and their body mass index showed a mean of 21.73 ± 3.96. According to Wagner's classification [15], 29 patients were classified as grade I with superficial ulcers, 34 patients were grade- II complaining of deep ulcers, 25 ones grade- III with abscess and only 7 patients were grade- IV having ulcers with gangrene of one toe or forefoot but none were diagnosed as grade zero or grade-V with severe gangrene of the foot as a whole.

Gram positive organisms were isolated from 52 cases (54.7%) whereas gram negative isolates were obtained from the remaining 43 ones (45.3%). *Staphylococcus aureus* was isolated from 34 cases (35.8%), *Staphylococcus epidermidis* from 10 cases (10.5%) whereas *Enterococci* were detected in 8 cases (8.4%). *Klebsiella spp*.were isolated from 4 cases (4.3%), *Pseudomonas spp*. from 15 patients (15.8%), *Escherichia coli* from 18 ones (18.9%) and finally *Proteus spp.* were found in 6 samples (6.3%), Table 1.

Table 1. Organisms isolated from infected diabetic ulcers

Isolated organism	№ (Total 95)	%
Gram positive isolates	52	54.7
Staphylococcus aureus	34	35.8
Staphylococcus epidermidis	10	10.5
Enterococci	8	8.4
Gram negative isolates	43	45.3
Klebsiella spp.	4	4.3
Pseudomonas spp.	15	15.8
Escherichia coli	18	18.9
Proteus spp.	6	6.3

icaA gene was positive in 18 *S. aureus* isolates (52.9%) with higher prevalence in grade III and IV diabetic foot cases than grade I and grade II ones, similarly *icaD* gene was positive in 23 isolates (67.6%) showing also higher association with increased severity of the disease, P value was 0.026*and 0.037* respectively, Table 2.

coa and *clfA* genes were positive in 33 (97.1%) and 32 (94.1%) isolates respectively with nearly similar prevalence in all studied grades of diabetic foot infections, P value was 0.59 and 0.71 respectively. *pvl* gene was demonstrated in 18 (53%) isolates, *tst* gene was found in 11 (32.4%) ones and both were mainly manifested in more severe infections in grades III and IV, P value was <0.0001* for both, Table 2.

4. DISCUSSION

The study carried out by Richard et al. [21] mentioned that *Staphylococcus aureus* was found to be the most prevalent pathogen that was isolated from diabetic foot ulcers. In spite of being a common inhabitant of humans' skin flora, that may also colonize the nasal cavities in addition to other human mucosa, *S. aureus* is considered as an opportunistic microorganism [22].

By studying different *S. aureus* strains isolated from diabetic foot ulcer: two different populations could be described which were: strains obtained from ulcers showing no evidence of severe infections and showing a low virulence potential in contrast to strains obtained from infected ulcers and having a higher virulence

Table 2. Results of polymerase chain reaction (PCR) in diabetic foot cases

	PCR								Total (34 cases)		Significance P value
	Grade I (9 cases)		Grade II (12 cases)		Grade III (6 cases)		Grade VI (7 cases)				
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
icaA gene	2	7	5	7	5	1	6	1	18	16	0.026*
icaD gene	3	6	9	3	4	2	7	0	23	11	0.037*
coa gene	9	0	11	1	6	0	7	0	33	1	0.59
clfA gene	8	1	11	1	6	0	7	0	32	2	0.71
pvl gene	0	9	5	7	6	0	7	0	18	16	<0.0001*
tst gene	0	9	1	11	4	2	6	1	11	23	<0.0001*

** Means stastically significant*

potential [23]. Wagner et al. [15] grading was used for studying the severity of the diabetic foot ulcers in correlation to the *S. aureus* virulence determinants.

Out of the 95 patients with diabetic foot ulcers, 29 patients were classified as grade I with superficial ulcers, 34 patients were grade- II with deep ulcers, 25 ones grade- III with abscess and only 7 patients were grade- IV having ulcers with gangrene of one toe or foot but none were diagnosed as grade zero or grade-V with severe gangrene.

Gram positive organisms were isolated from 52 cases (54.7%) whereas Gram negative isolates were obtained from the remaining 43 ones (45.3%). In a study done in Morocco over 3 years, the bacteria isolated from infected diabetic foot were Gram negative bacilli, Gram positive bacilli and Gram positive cocci in a percentage of 48.8%, 6.2% and 45% respectively [24].

The severity and outcomes of infected diabetic foot may be also influenced by the polymicrobial status of some cases besides the control measures applied for diabetes and infection.

In this study *S. aureus* was isolated from 34 cases (35.8%). Staphylococci, in addition to being the most frequently isolated organisms, are attributed to be the most virulent ones in diabetic foot infections [25,26,27]. Gardner et al. [28] showed that the culture results had higher relative abundance of Staphylococcus (46%). Previous studies done in France demonstrated a correlation between certain virulence genotypes in *S. aureus* isolates from diabetic foot ulcers and the ulcer prognosis [29,22,30]. Belefquih et al. [24] isolated *S. aureus* as the most predominent bacteria (12.6%) regardless of the sampling method.

According to the virulence genes, Sotto et al. [29] tried to establish a differentiation between colonized wounds and infected ones in foot ulcers that were culture-positive for only *S. aureus*. Virulence genes were not found in 92% clinically uninfected ulcers, but present in 98% infected ulcers.

Important adhesion virulence factors are *icaA* and *icaD* genes. *icaA* gene was positive in 21 *S. aureus* isolates (61.8%) with higher prevalence in grade III and IV diabetic foot cases than grade I and grade II ones. As regard *icaD* gene, it was positive in 23 isolates (67.6%) showing also higher association with increased grading of the disease and the results were stastically significant. In a previous study by Mottola et al. [31], all the *S. aureus* was positive for biofilm associated *icaA* and *icaD* genes. The pattern of biofilm growth may play an essential role in chronicity of wounds [32]; it can cause resistance even to higher concentrations of antibiotic than the ones usually needed to kill planktonic cells [33].

coa and *clfA* genes were positive in 33 (97.1%) and 32 (94.1%) isolates respectively with nearly similar prevalence in all studied grades of diabetic foot infections. Mottola et al. [34] found that all isolates were *coa* positive but only 70% were *clfA* positive.

pvl gene was found in 18 (53%) isolates, and *tst* gene in 11 (32.4%). Both were mainly manifested in more severe infections in grades III and IV and the results were stastically significant. *tst* gene was detected in only one isolate from diabetic foot ulcer (2.4%) in Mottola et al. study [34].

In a study done on samples from both major abscess and diabetic foot infection in order to determine the presence of *pvl* gene by realtime PCR, the majority of *S. aureus* real-time

PCR-positive major abscess samples were at the same instance *pvl* real-time PCR-positive (89%). In contrast, a minority of *S. aureus* real-time PCR-positive diabetic foot infection samples were giving *pv*l real-time PCR-positive results (14%). In both groups of major abscesses and diabetic foot infections, two samples were *pvl* real-time PCR positive but not *S. aureus* real-time PCR-positive [35]. Only fourteen percent of the diabetic foot infections were *pvl* positive.

PVL functions as a bicomponent pore-forming toxin. It is encoded by the *lukS-PV* and *lukF-PV* genes, and can induce damage of leukocytes [36]. A previous meta-analysis confirmed that there was an association between PVL and *S. aureus* skin infections, and that was not shown in invasive infections caused by *S. aureus* [37] All the *S. aureus* strains isolated by Mottola et al. [34] from diabetic foot ulcers were negative for *pvl* gene.

5. CONCLUSION

There is a positive correlation between the presence of certain virulence genes of the *S. aureus* especially *icaA*, *icaD*, *pvl* and *tst* and the grading or the severity of the diabetic foot ulcers when 95 diabetic patients with diabetic foot infections were studied. This conclusion needs to be supported by further studies over larger number of cases especially in our locality.

ETHICAL APPROVAL

Our study protocol was accepted by our research board and ethical committee in Mansoura faculty of Medicine under the code number of: R/16.12.42. A written informed consent was obtained from all included cases.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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