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Research Article

Prevalence of methicillin resistance and virulence determinants of *Staphylococcus aureus* in diabetic foot ulcers

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ABSTRACT

Background: Diabetic foot ulceration (DFU) is a multifactorial process and is responsible for considerable morbidity and contributes to the increasing cost of health care worldwide. The diagnosis and identification of these ulcers remains a complex problem. Bacterial infection is promoted in the diabetic foot wound by decreased vascular supply and impaired host immune response. As conventional clinical microbiological methods are time-consuming and only identifies about 1% of the wound microbiota, detection of bacteria present in DFUs using molecular methods is highly advantageous and efficient. The aim of this study was to assess the virulence and methicillin resistance profiles of *Staphylococcus aureus* detected in DFUs using DNA-based methods.

Methods: A total of 223 swab samples were collected from 30 patients from March to October 2012. Bacterial DNA was extracted from the swab samples using standard procedures and was used to perform polymerase chain reaction (PCR) using specific oligonucleotide primers. The products were visualized using agarose gel electrophoresis.

Results: *S. aureus* was detected in 44.8% of samples. 25% of the *S. aureus* was methicillin-resistant *S. aureus* harboring the *mecA* gene. The alpha-toxin gene was present in 85% of the *S. aureus* positive samples. 61% of the *S. aureus* present in DFU samples harbored the exfoliatin factor A gene. Both the fibronectin factor A and fibronectin factor B gene were detected in 71% and 74% of the *S. aureus* positive samples.

Conclusions: DNA-based detection and characterization of bacteria in DFUs are rapid and efficient and can assist in accurate, targeted antibiotic therapy of DFU infections. The majority of *S. aureus* detected in this study were highly virulent and also resistant to methicillin. Further studies are required to understand the role of *S. aureus* in DFU trajectory.

Keywords: Diabetic foot ulcer, Polymerase chain reaction, *Staphylococcus aureus*, Methicillin resistant *Staphylococcus aureus*, Virulence

INTRODUCTION

Diabetes mellitus is the most commonly encountered health problem worldwide.[1] Studies have shown that 6-8% of the adult population worldwide is diagnosed with diabetes.[2] According to World Health Organization reports, the prevalence of this condition will double by the year 2025. It is the metabolic disorder, which damages target organs and is characterized by chronic hyperglycemia.[3] One of the most common complications associated with diabetes is diabetic foot ulcer (DFU), which accounts for around 20% of hospital admissions around the world.[4,5] Furthermore, DFUs are one of the most common causes of lower limb amputations, prolonged outpatient care and hospitalization. It is estimated that about 15% of all the diabetic patients develop foot ulcers during their lifetime.[4,6]

Infection is one of the complications associated with DFUs, and an infected DFU is also the second major cause of amputation.[3] Bacterial colonization occurs in deep tissues once the protective layer of the skin is broken.[1] Therefore, DFUs are highly prone to infections. Once the bacterial infection occurs, it rapidly spreads and leads to severe destruction of tissues that requires subsequent amputation.[1,6-9] Despite the presence of various mixed (Gram-positive and Gram-negative) bacterial species in DFUs, the most frequently isolated species is *Staphylococcus aureus*.[1] *S. aureus* is considered to be the major pathogen causing

DFU infections and it is also one of the most adaptable human pathogens responsible for causing a wide range of illnesses.[10]

A major threat to public health is the increasing prevalence of antimicrobial resistance which occurs due to excess usage of antibiotics.[11] In 15-30% of DFUs, methicillin-resistant *S. aureus* (MRSA) are isolated.[12] Different factors are responsible for this resistance but mainly it is due to inappropriate antibiotic treatment and poor hygiene.[6] Infrequent patient contact and follow-up has also contributed to resistance development as they may encourage the clinician to overprescribe.[13] The virulence potential of *S. aureus* present in DFUs is also a factor, which can contribute to wound severity as these factors enables this pathogen to access and destroy the host tissue.

The aim of the present study was to detect the presence of *S. aureus* in infected DFUs along with its virulence and antibiotic resistance profiles. An understanding of the pathogenic and antibiotic resistance profiles of *S. aureus* in DFUs will assist health professionals to manage this ailment more efficiently by using targeted therapy.

Aims and objectives

- To detect the presence of *S. aureus* in DFU samples using molecular methods
- To determine the prevalence of MRSA in DFUs
- To determine the virulence profile of *S. aureus* in DFUs.

METHODS

DFU swab samples were collected from 30 patients at different time points (from March-October 2012) using the Z-swab technique. All swabs were kept at 4°C until transported to the laboratory, whereupon they were stored at −80°C until further analysis.

DNA extraction

DNA was extracted from *S. aureus* ATCC control strains (ATCC 29213 and USA 300) and patient swabs using an optimised Qiagen protocol as per manufacturer's guidelines.

Detection of S. aureus and its methicillin resistance and virulence determinants in DFUs

The gene for *S. aureus* speciation was *nucA* and *mecA* for methicillin resistance. Virulence gene targets included: Panton–Valentine leukocidin (*PVL*), alpha toxin, exfoliatin factor A (*exfA*), fibronectin factor A (*fnbA*) and fibronectin factor B (*fnbB*). Primers were selected from previously published studies (Table 1). After primer optimization, DNA extracted from wound swabs was subjected to individual polymerase chain reaction (PCR) reaction conditions depending on the targeted gene.

PCR reaction for S. aureus (nucA, mecA, PVL, alpha toxin, exfA, fnbA and fnbB)

Each 25 µl PCR reaction contained 2 µl of DNA which was added to 23 µl of master mix containing 0.1 µl of Taq polymerase (Roche, Australia), 0.5 µl of dNTPs (Roche, Australia), 0.625 µl each of reverse and forward primers (Table 2) (Sigma Aldrich, Australia), 1.5 µl of $MgCl_2$ (Roche, Australia), 2.5 µl of 10X PCR buffer (Roche, Australia) and 17.15 µl of water (DNAse and RNAse free - Life Technologies, Australia). PCR was performed using the program, including initial denaturation at 95°C for 10 mins, followed by 30 cycles of annealing and extension (72°C for 30 sec). An annealing temperature of 55°C was used for *nucA* and *mecA* gene amplification. 60°C was used as an annealing temperature for *alpha toxin*, *fnbA* and *fnbB* genes. *exfA* gene was amplified using an annealing temperature of 49°C. Final extension was carried out for 5 mins at 72°C. The PCR products were visualized using agarose gel (2%) electrophoresis. Gel images were captured using the GeneSnap program of G: BOX gel documentation system, Syngene Australia.

RESULTS

This study included a total of 30 diabetic patients with DFUs. The concentration of DNA extracted from 223 swab samples ranged from 2 to 80 ng/µl. PCR was used to detect and characterize the commonly detected microbial species *S. aureus* from DFUs. It was found that 44.8% of DFU swab samples were positive for the *nucA* gene confirming the presence of *S. aureus* in these samples. All the *S. aureus* positive samples were examined further for the presence of MRSA by targeting the *mecA* gene. 25% of *S. aureus* positive samples were found to be MRSA. None of the *S. aureus* positive samples were positive for PVL. The *alpha toxin* gene was detected in 85% of the *S. aureus* positive samples, and the *exfA* gene was detected in 61% of the *S. aureus* positive samples. For the *fnbA* and *fnbB* genes, 71% and 74% of the *S. aureus* positive samples were positive for these two genes (Figure 1).

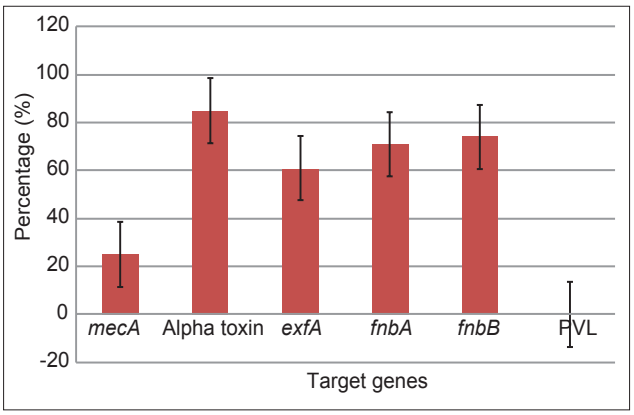

Figure 1: Methicillin resistance and virulence profile of *Staphylococcus aureus* present in diabetic foot ulcerations.

Table 1: Prevalence of *S. aureus* in DFUs.

Country	*S. aureus* prevalence (%) in infected DFUs	MRSA prevalence (%) in infected DFUs	References
USA	35.5	12.0	Ge et al., 2002[25]
Santa Monica	76.0	20.0	Goldstein et al., 1996[22]
UAE	28.0	8.1	El-Tahawy., 2000[26]
UK	48.3	30.2	Tentolouris et al., 1999[27]
Kuwait	38.4	5.9	Abdulrazak et al., 2005[28]
UK	42.0	19.0	Stanaway et al., 2007[29]
Spain	47.5	19.9	Aragón-Sánchez., 2008[30]
France	36	19.7	Richard et al., 2008[15]
India	75	60	Mehta et al., 2014[31]

S. aureus: Staphylococcus aureus, DFU: Diabetic foot ulceration, MRSA: Methicillin resistant *S. aureus*

Table 2: Oligonucleotide primers used in this study.

Target species	Gene	Forward primer	Reverse primer	Reference
S. aureus	*nucA*	5'GCGATTGATGG TGATACGGTT3'	5'AGCCAAGCCTTGA CGAACTAAAGC3'	Huygens et al., 2006[24]
	mecA	5'GATCGCAACG TTCAATTTAATTTTG3'	5'GCTTTGGTCTTTCT GCATTCCT3'	Huygens et al., 2006[24]
	PVL	5'TATCTCTAACGG CTTGTCAGGT3'	5'TGCTTCAACA TCCCAACC3'	Huygens et al., 2006[24]
	alpha-toxin	5'GACCAGCAATGG TACCTTTC3'	5'GCTAATGCCGC AGATTCTG3'	Unpublished (Huygens et al.)
	exfA	5'GGCTAATAACAC TTCGATAA3'	5'CAGGACTAGTC TTAGGATTA3'	Unpublished (Huygens et al.)
	fnbA	5'CCACCTGGGTTT GTATCTTCTTC3'	5'GATTACCACACAGC TATAGATGGTG3'	Unpublished (Huygens et al.)
	fnbB	5'CGTGACCATTTTCA GTTCCTAAACC3'	5'GATACAAACCC AGGTGGTGG3'	Unpublished (Huygens et al.)

PVL: Panton-Valentine leukocidin, *exfA*: Exfoliatin factor A, *fnbA*: Fibronectin factor A, *fnbB*: Fibronectin factor B, *S. aureus: Staphylococcus aureus*

DISCUSSION

From the 30 patients recruited, 223 DFU swab samples were analyzed for the presence of *S. aureus*. The majority of previous studies have documented that *S. aureus* is the most commonly isolated Gram-positive bacteria from infected DFU.[1,2,12-16] An Indian study showed that *S. aureus* was present in 21% of a total of 112 samples.[3] Our results show that 66.6% of diabetic patients had *S. aureus* in their foot ulcers, and 44.8% of all the DFU swab samples were positive for *S. aureus*. This data are similar to what has been reported previously in a study performed at the Royal Melbourne Hospital in 2009 by Yates et al., however, their study used traditional culture-based methods.[17]

S. aureus has a number of virulence factors that facilitates the infection of soft tissues and bones. PVL is one of these virulence traits, and it seems to be associated with severely infected DFUs.[18] In our study, none of the DFU patient samples were positive for PVL. Alpha-toxin is considered to be the most renowned *S. aureus* toxin and it has cytolytic activity.[19] Its role in DFU infection is not well documented in the literature. We found that this toxin was present in 85% of *S. aureus* positive DFU swab samples. One of the previous studies have reported that the *exfA* gene was absent in their DFU samples.[20] In our study, *exfA* was detected in 61% of the *S. aureus* positive DFU swab samples. *fnbA* and *fnbB* are members of microbial surface components recognizing adhesive matrix molecules family and mediate *S. aureus* adhesion to the host cell. In our study, *fnbA* gene was present in 71% of *S. aureus* positive DFU swab samples and the *fnbB* gene was present in 74% of *S. aureus* positive DFU swab samples.

DFUs are found to be increasingly inhabited with MRSA.[21] A major health threat to the hospital and community setting is the increasing frequency of MRSA isolation from DFU. The majority of findings have shown that the prevalence of MRSA among diabetic patients with infected foot ulcers is 15-30%.[2,22,23] In the United Kingdom, a study was conducted in which 30% of all cultured DFUs had MRSA. This MRSA colonization was linked to prior antibiotic usage, and it

adversely affected the wound healing process.[3] In our study, we have found that 25% of all the *S. aureus* positive samples were MRSA. This trend is similar to that found by Yates et al. (2009), who isolated MRSA from 23% of DFUs. The factors predisposing to MRSA infection include chronic duration of DFU and prolonged use of antibiotics.[17]

The rationale for targeting only *S. aureus* in our study is based on its increasing prevalence in DFUs, as described by previously published studies (Table 2). Our study reports the prevalence of *S. aureus*, MRSA and associated *S. aureus* virulence factors using molecular methods. To the best of our knowledge, this is the first Queensland study which has used molecular methods to characterize the virulence and methicillin resistance traits of *S. aureus* associated with DFUs.

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Ethical approval: Approval was obtained from the QUT's Ethics Committee prior to the commencement of this study

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