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Multilocus sequence typing and molecular detection of phenol-soluble modulin in biofilm-positive *Staphylococcus Epidermidis* isolated from paediatric blood culture

John-Ugwuanya A Grace[1], Stephen K Obaro[1], Busayo O Olayinka[2], Josiah A Onaolapo[2], Fatimah Hassan-Hanga[3], Huda Munir[3] and Paul D Fey[4]

[1]*Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria*

[2]*Division of Pediatric Infectious Disease, University of Nebraska Medical Center, Omaha, Nebraska, USA*

[3]*Aminu Kano Teaching Hospital, Kano, Nigeria*

[4]*Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska, USA*

[*]**Corresponding author:** John-Ugwuanya A. Grace, Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria Tel: +2347061145614; E-mail: graceamy2k@yahoo.com

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Abstract

Aim: *Staphylococcus epidermidis* is a significant coagulase-negative staphylococci obtained from blood culture samples. However, there is limited information about phenol-soluble modulin (PSM), which is associated with virulence in *S. epidermidis* and its genetic relatedness in Nigeria. This study observed the presence of phenol-soluble modulin mec *(psm-mec)* gene and the multilocus sequence typing (MLST) of biofilm-positive *Staphylococcus epidermidis* (BPSE).

Method: Twenty-two biofilm-positive *S. epidermidis* isolates obtained from paediatric blood culture at three hospitals in north-west and north-central Nigeria were evaluated for the molecular detection of the *psm-mec* gene using conventional polymerase chain reaction (PCR). The biofilm formation was previously assessed by molecular detection of the intercellular adhesion *(icaA)* gene and the methicillin resistance using cefoxitin disk agar diffusion. Internal fragments of the respective seven housekeeping genes was sequenced for 21 BPSE strains and matched with the central MLST database.

Results: Out of 22 BPSE, only 4.5% had the *psm-mec* gene and it was methicillin resistant. About 91% methicillin resistance was observed among the *psm-mec* negative BPSE strains. Twenty-one BPSE strains were sequence type 1 (ST1) based on the MLST analysis of the seven housekeeping genes.

Conclusion: Considering the very low presence of *psm-mec* gene, the BPSE may be carrying the β-type PSMs related to biofilm formation and dissemination, and not the cytolytic α-type PSMs common in the aggressive form of *S. epidermidis*. Phenol-soluble modulin methicillin resistance island-encoded peptide toxin is involved in sepsis related methicillin-resistant *S. epidermidis*. The strains are genetically related to each other.

Keywords: Phenol-soluble modulin; Methicillin resistance; *Staphylococcus epidermidis*; Biofilm-positive; Multilocus sequence typing

Introduction

Almost all staphylococcal strains secrete multifunctional peptide toxins known as phenol-soluble modulins (PSMs) with multiple studies ascribing their importance to the pathogenesis of staphylococci [1]. The secreted virulence factor located precisely in the methicillin resistance mobile genetic elements of certain Staphylococcal cassette chromosome (SCCmec) subtypes is known as phenol-soluble modulins [2]. Generally, members of the PSM family contribute to biofilm structuring and detachment, promote inflammatory, receptor-mediated responses in different human cell types and also exhibit strong cytolytic activity to neutrophils [3]. Phenol-soluble modulins are the main cytolytic and inflammatory toxins of *S. aureus* and *S. epidermidis* [4].

The common types are the alpha (α-type) and beta (β-type) PSMs. Alpha-phenol-soluble modulins (PSMα) are a novel class of miniature peptides having amphipatic alpha-helical structure produced by most staphylococci species [4]. The α-type PSMs have the cytolytic capacity and a shorter size having between 20-25 amino acids which contribute to the evasion of the innate immune system. They have properties that mimic strong surfactants and are majorly produced by Staphylococcal strains, in particular *S. aureus* and *S. epidermidis* [5,6]. The β-type PSMs are non-cytolytic, active in biofilm formation and have a size of approximately 40-45 amino acids.

The first discovery of PSMs in *S. epidermidis* was on the phenol layer following the hot aqueous phenol extraction of culture supernatant in the course of investigating factors associated with pro-inflammatory activity in a macrophage cell line [7]. The role of PSMs in staphylococci includes the activation and lyses of neutrophils to evade immune damage, support the production of proinflammatory cytokine, enhance biofilm structuring and detachment, facilitate biofilm-associated Staphylococcal infection and effectively eradicate competing bacteria [5,8].

The PSMs belongs to the amphipathic phenol-soluble modulin family and it is the only staphylococcal toxin encoded by the *psm-mec* gene localized in the SCCmec element, which also contains the *mecA* genes, regulatory elements, recombinase genes, and some resistance genes [4,8].

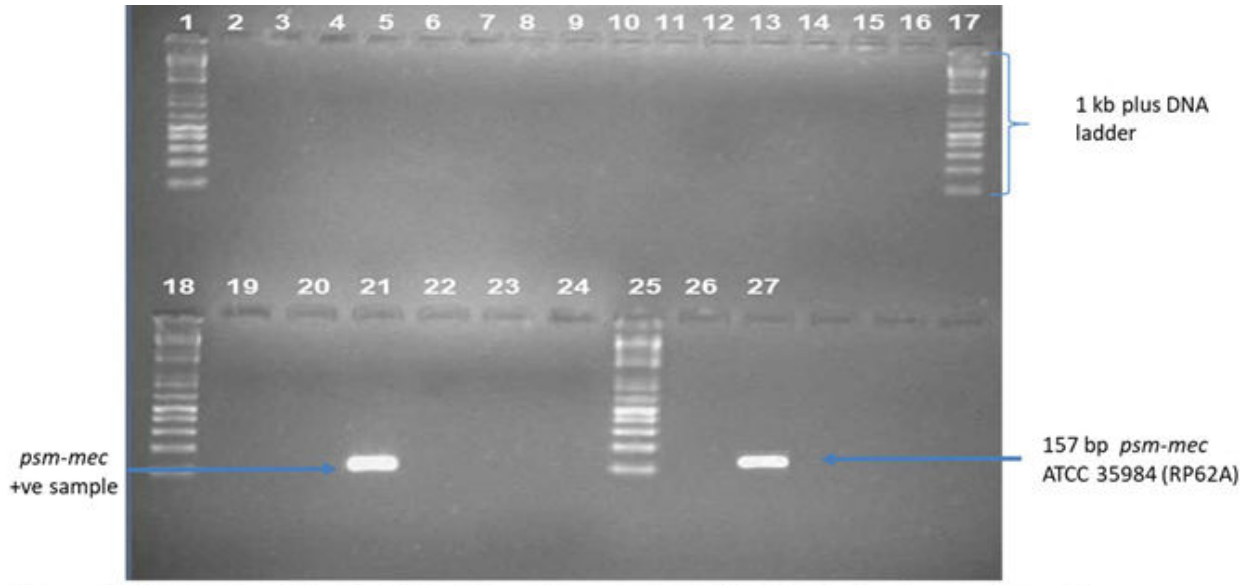
Multilocus sequence typing (MLST) is a reference genotyping method that is suitable for analysing the evolution and population genetics of organisms [9,10]. It is a vital and investigatory tool in bacterial typing, and studying of staphylococci evolution, population dynamics, mode of bacterial dissemination and epidemiology [11]. A major advantage of MLST as a reference method is due to the unambiguous nature of DNA sequences stored easily on internet-linked databases with the corresponding clinical information of the isolate [12,13].

Genetic nucleotide sequence variations are identified and correctly interpreted in understanding the molecular epidemiology of *S. epidermidis* [14]. Previously, three different schemes for MLST proposed for *S. epidermidis* comprising varying genes were analysed but they proved difficult in studying the clinically diverse *S. epidermidis* strains globally and did not yield adequate resolution for understanding its epidemiology [15]. However, an improved MLST scheme was developed based on the comparative study of the previous MLST schemes reported and this was recognized and adopted as the MLST scheme for *S. epidermidis* [13].

The MLST scheme focuses on seven housekeeping genes present in all isolates of each bacterial species, their varying location in the bacterial chromosome and functions. Every distinct nucleotide sequence at a locus is designated an allele. A sequence type (ST) refers to each unique classification of alleles across the loci [9,16].

Materials and Methods

This study was carried out on 22 biofilm-positive *Staphylococcus epidermidis* isolated from children under five with bacteraemia attending the neonatal and paediatric units of University of Abuja Teaching Hospital, Federal Medical Center, Keffi and Murtala Mohammed Specialist Hospital, Kano between 2009 and 2016. The initial study involved the detection of methicillin resistance using cefoxitin disk agar diffusion method, biofilm formation by Christensen biofilm assay and the molecular detection of the intercellular adhesion *(icaA)* gene in the *S. epidermidis* isolates obtained from positive paediatric blood cultures of seven hospitals in northern Nigeria [17].



a) Lane 1, 17, 18 & 25 - 1 kb plus DNA ladder; b) lane 2-16, 19-20, 22-24, 26 - absence of band for *psm-mec* gene; c) lane 21: *psm-mec* positive sample d) reference strain ATCC 35984 RP62A

Figure 1: PCR amplification for *psm-mec* gene in the biofilm-positive *Staphylococcus epidermidis*

Ethical approval was obtained from the Research Ethics Committee of the hospitals and consent was given by the International Foundation against Infectious disease in Nigeria (IFAIN) to gain access to the *S. epidermidis* isolates obtained from positive blood culture in the respective hospitals.

Polymerase chain reaction (PCR) amplification of the *psm-mec* gene was performed using primers designed for this study. The 25 µl PCR reaction consisted of 12.5 µl of Midas Mix with Taq DNA polymerase (Monserate Biotechnology Group), 1 µl diluted cell culture, 9.5 µl of DNase/RNase-free distilled water and 1 µl each of psm-mec forward primer (5'-TGCATATGGATTTCACTGGTGTTA-3') and reverse primer (5'-CGTTGAATATTTCCTCTGTTTTTTAGTTG-3'). The *psm-mec* gene used had a product size of 157 bp and the PCR amplification was run at 56°C annealing temperature for the 22 biofilm-positive S. epidermidis strains. One of the strains was lost [17].

Gel concentration was increased to 2% agarose gel by measuring 2 g of agarose powder into 100 ml of tris-borate-EDTA (TBE) due to the small product size of *psm-mec* gene used (Figure 1). The *S. epidermidis* RP62A (ATCC 35984) was used as a positive control. A second duplex PCR run for *psm-mec* gene was done with *icaA* primers [17] at 56°C to ensure that each of the PCR products had the respective *icaA*-positive *S. epidermidis* (Figure 2).

The internal fragments of the seven housekeeping genes (*arcC, aroE, gtr, mutS, pyrR, tpi, and yqiL*) were amplified by PCR, using the specific primers with varying amplicon size and annealing temperature (Table 1) with the purified PCR products as the DNA template [18].

A collection of 21 biofilm producing *S. epidermidis* isolates were analysed by MLST protocol. The PCR was performed with 25 µl reaction volume, composed of 1 µl each of the forward and reverse primer, 12.5 µl of Midas mix and 9.5 of RNAse/DNase free sterile water.

The conditions for running the PCR amplification involved an initial denaturation of 95°C for 3 min; 30 cycles of 95°C for 30 s, annealing of primers between 58-60°C for 1 min based on the seven housekeeping genes, extension at 72°C for 1 min; and a final extension of 72°C for 10 min.

The PCR products were analysed for DNA band separation by running 1% agarose gel electrophoresis in tris-borate-EDTA buffer (Figure 3). The amplified PCR products were analysed by 0.8% agarose gel electrophoresis with ethidium bromide. It was visualized and captured on UV transilluminator (Bio Rad Gel Doc). The size of the amplicon was determined by comparing with the GeneRuler^{TM} 1 kb plus DNA ladder [19,20].

Gene and function	Primer	Sequences (5'-3')	Amplicon Size (bp)	Annealing T °C
Carbamate Kinase *(arcC)*	arcC-F	TGTGATGAGCACGCTACCGTTAG	508	58°C
	arcC-R	TCCAAGTAAACCCATCGGTCTG		
Shikimate dehydrogenase *(aroE)*	aroE-F	CATTGGATTACCTCTTTGTTCAGC	459	60°C
	aroE-R	CAAGCGAAATCTGTTGGGG		
ABC transporter *(gtr)*	gtr-F	CAGCCAATTCTTTTATGACTTTT	508	60°C
	gtr-R	GTGATTAAAGGTATTGATTTGAAT		
DNA mismatch repair protein *(mutS)*	mutS-F3	GATATAAGAATAAGGGTTGTGAA	608	60°C
	muts-R3	GTAATCGTCTCAGTTATCATGTT		
Pyrimidine operon regulatory protein *(pyrR)*	pyrR-F2	GTTACTAATACTTTTGCTGTGTTT	851	60°C
	pyrR-R4	GTAGAATGTAAAGAGACTAAAATGAA		
Triosephosphate isomerase *(tpiA)*	tpi-F2	ATCAATTAGACGCTTTAGTAAC	592	58°C
	tpi-R2	TTAATGATGCGCCACCTACA		
Acetyl coenzyme A acetyltransferase *(yqiL)*	yqiL-F2	CACGCATAGTATTAGCTGAAG	658	60°C
	yqiL-R2	CTAATGCCTTCATCTTGAGAAATAA		

Table 1: Seven housekeeping genes for multi locus sequence typing of *S. epidermidis.*

The amplified fragments were purified using DNA Clean and ConcentratorTM-5 Kit (Zymo Reasearch). DNA quantification was done using the nanodrop spectrophotometer to measure the purified PCR products in nanogram/microliter. The DNA sequencing was performed using Illumina HiSeq 2500 sequence analyser at the Genomics Core Facility of University of Nebraska Medical Centre, Omaha, Nebraska. The sequence analysis was done using NCBI-BLAST tool to analyse the seven housekeeping genes and confirm their specificity to the corresponding GenBank sequence check for correct amplification of the primers [10].

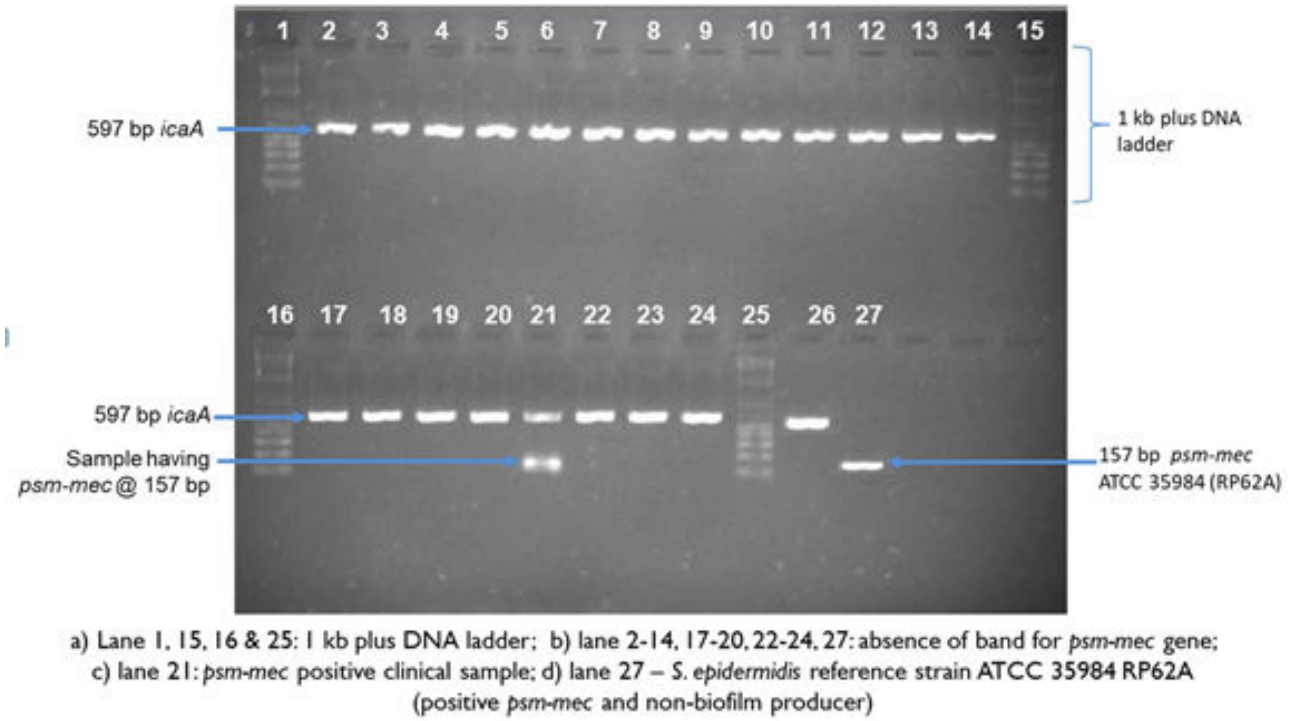
The nucleotide sequencing gives confirmation and precise identification of most of the bacteria, and it was run on the basic local alignment search tool (BLAST) against *S. epidermidis* genome to find the regions of sequence similarity. The sequence types (ST) of the 21 BPSE strains were assigned based on the MLST analysis of allelic profiles of the seven housekeeping genes by sequence comparison using the available *S. epidermidis* MLST database.

Results

Out of 22 biofilm-positives *S. epidermidis*, only 4.5% carried the virulence determinant *psm-mec* gene while 95.5% did not have the *psm-mec* gene (Table 2). The *S. epidermidis* strain having the *psm-mec* gene was also methicillin resistant (Figure 3). About 91% of the *psm-mec* negative *S. epidermidis* isolates were methicillin resistant (Figure 4).

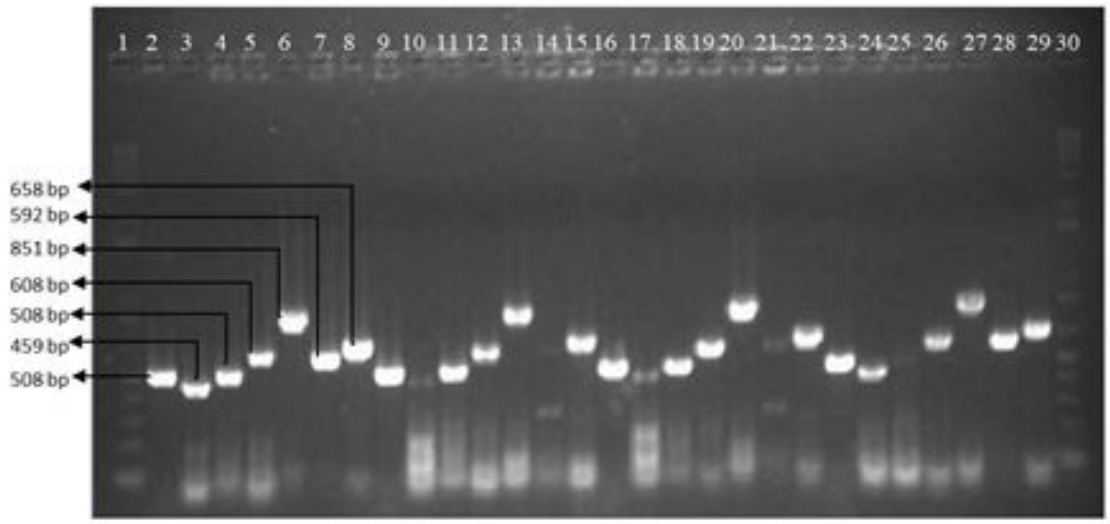
Parameter	Value
psm-mec +ve	4.5% (1)
psm-mec -ve	95.5%(21)

Table 2: Detection of *psm-mec* gene in biofilm-positive *Staphylococcus epidermidis.*



a) Lane 1, 15, 16 & 25: 1 kb plus DNA ladder; b) lane 2-14, 17-20, 22-24, 27: absence of band for *psm-mec* gene; c) lane 21: *psm-mec* positive clinical sample; d) lane 27 – *S. epidermidis* reference strain ATCC 35984 RP62A (positive *psm-mec* and non-biofilm producer)

Figure 2: PCR amplification of *icaA* gene and *psm-mec* gene in the biofilm-positive *Staphylococcus epidermidis*



PCR amplifications Lane 1 - 1kb DNA plus ladder, lane 2 – *arcC*, lane 3 – *aroE*, lane 4 –*gtr*, lane 5 – *mutS*, lane 6 – *pyrR*, lane 7 – *tpiA*, lane 8 - *yqiL*

Figure 3: PCR amplification using the MLST scheme for *S. epidermidis.*

The allelic profile match of the 21 biofilm-positive *S. epidermidis* isolates analysed using MLST were 1, 2, 2, 2, 1, 1, 10 resulting in sequence type 1 (ST1). The strain designation was DEN19 originating from Denmark. A detailed result of the biofilm-producing *S. epidermidis* strains in this study is indicated in (Table 3).

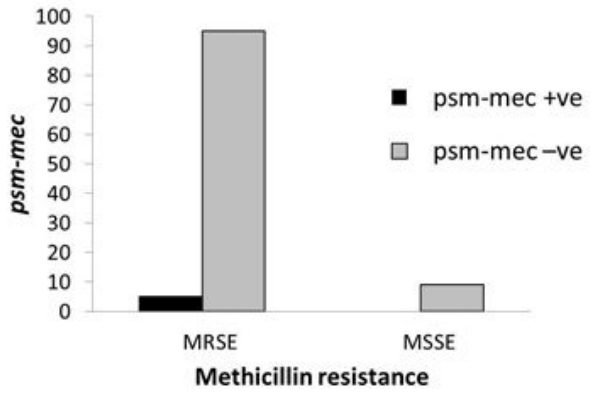


Figure 4: Correlation of *psm-mec* gene and methicillin resistance in the *S. epidermidis.*

Number	Isolate ID	(Grace et al., 2018)		(This study)	
		MRSE	*icaA*@597 bp	*psm-mec* @157 bp	Sequence Type (ST)
1	R1	+	+	-	ST1
2	R11	+	+	-	ST1
3	R14	+	+	-	ST1
4	R21	+	+	-	ST1
5	R28	+	+	-	ST1
6	R44	+	+	**	**
7	R46	+	+	-	ST1
8	R56	+	+	-	ST1
9	R75	+	+	-	ST1
10	R79	+	+	-	**
11	R87	+	+	-	ST1
12	R100	+	+	-	ST1
13	R120	-	+	-	ST1
14	R191	+	+	-	ST1
15	R201	+	+	-	ST1
16	R219	+	+	-	ST1
17	R231	+	+	-	ST1
18	R235	+	+	-	ST1
19	R240	+	+	-	ST1
20	R245	+	+	-	ST1
21	P13	+	+	-	ST1
22	P32	+	+	-	ST1
23	P71	+	+	-	ST1

Table 3: Detailed breakdown of MRSE, *icaA, psm-mec* and MLST of the *S. epidermidis* strains.

Discussion

Staphylococcus epidermidis is known to produce phenol soluble modulins (PSM) that exhibit selective antimicrobial activity against other organisms and are proinflammatory cytolysins. This selective activity enables them to dominate other microbes as a skin flora [18]. Only 4.5% of the twenty-two biofilm-positive *S. epidermidis* had the *psm-mec* gene in this study. It is believed that the β-type PSMs produced by *S. epidermidis* participate mainly in biofilm formation and facilitates in vivo dissemination of biofilm-associated infection, and in vitro biofilm detachment. However, this process can be blocked by anti-PSM β antibodies [1,21].

The production of cytolytic α-type PSMs is very low in *S. epidermidis* and it is a significant cause of its lower pathogenicity and aggressiveness when compared to *S. aureus* [22]. The PSM methicillin resistance island-encoded peptide toxin is involved in sepsis related methicillin-resistant S. *epidermidis* [23].

Presence of virulence determinants in *S. epidermidis* is very limited but the persistence of clinical infection is often associated with genes that code for methicillin resistance and biofilm formation [24]. This agrees with the study by Qin, et al., (2017) [23] indicating that *S. epidermidis* has a high resistance rate of methicillin resistance.

Using the multilocus sequence typing (MLST), a collection of 21 biofilm-producing S. *epidermidis* isolates analysed in the study were sequence type 1 (ST1). This suggests a low level of genetic diversity and the strains are genetically related to each other. ST1 has also been reported in Denmark, Iceland, Canada and the United States of America. However, in a study by Sharma, et al., (2014) [26], the most commonly identified sequence types (ST) among the *S. epidermidis* obtained from blood cultures were ST 2 (21%) and ST 5 (14%).

Although the *S. epidermidis* strains used in this entire study for MLST were biofilm producers, a study carried out in Finland on the MLST of methicillin-resistant *S. epidermidis* obtained from bacteraemic patients were all sequence type ST2, indicating a genetic relatedness [25]. The *S. epidermidis* sequence type 2 (ST2) is characterized by antibiotic resistance, biofilm formation and a very flexible genetic background. It seems to be the common nosocomial clone obtained from neonates [11].

The use of MLST is becoming necessary in understanding global epidemiology of most pathogenic bacteria [9]. The central MLST database for *S. epidermidis* is progressively developing and contains distinct catalogue of genetic variants, which aids the direct identification of nucleotide sequence variation. However, there is still limited knowledge in the population genetics in Nigeria.

Conclusion

Considering the very low presence of *psm-mec* gene, the biofilm-positive *S. epidermidis* strains may be carrying the β-type PSMs related to biofilm formation and dissemination, and not the cytolytic α-type PSMs common in the aggressive form of *S. epidermidis*. Additionally, *S. epidermidis* does not produce aggressive virulence factors or toxins; and the cytolytic PSMs when present are not secreted

at a significant toxic level. The strains analysed are genetically related to each other.

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