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Detection of T3SS, *oprI*, *aprA*, and *pvdA* Genes in Clinical Isolates of *Pseudomonas aeruginosa* obtained from Wound Samples

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Abstract

Background: *Pseudomonas aeruginosa* employs a varied number of virulence determinants, predominantly porins, type III secretion system (T3SS), alkaline protease and pigments to manipulate the host to establish infections. These factors contribute significantly to virulence in *P. aeruginosa* and are worrisome. This study is aimed at identifying the virulence genes in *P. aeruginosa* isolates from wound swabs of patients at two tertiary hospitals in Osun State, Nigeria.

Methods: Altogether, 237 participants consisting of 133 from State Hospital, Osogbo and 104 from General Hospital, Iwo with different types of wounds were enrolled. Swabs from the various wound types were collected, grown on cetrimide agar, and recovered isolates identified using conventional biochemical tests. Chromosomal DNA was extracted by thermal lysis and subjected to polymerase chain reaction using specific primers to affirm biochemical identification and detect the presence of *ExoT, ExoS, ExoU, ExoY, oprI, aprA, and pvdA* genes.

Results: Sixty-one (25.7%) *P. aeruginosa* isolates were recovered in the study. Based on the different wound types, the highest recovery was from surgical sites of caesarian sections (CS) (37.7%; 23/61) followed by trauma sustained from motorcycle and automobile accidents (36.1%; 22/61) and others wound types (26.2%; 16/71). Fifty-nine of the 61 recovered isolates were successfully amplified by PCR primers that targets *P. aeruginosa parugin* gene. Of these 59 PCR confirmed *P. aeruginosa,* the *oprI* gene was detected in 74.6% (n = 44/59) of isolates ; 18 from Osogbo and 26 from Iwo. No bands were detected for the other genes in all 59 isolates analysed.

Conclusion: The prevalence of *P. aeruginosa* was highest from surgical sites of caesarian sections, with the rates from Iwo higher than that from Osogbo. Detection of *oprL* gene in 74.6% of strains is significant as it's interaction with the peptidoglycan plays a part in the maintenance of the structural integrity of the cell, and may cause infections that impair wound healing.

Keywords: *Pseudomonas aeruginosa*, T3SS genes, *oprI*, virulence, porins, wounds

1.0 INTRODUCTION

Pseudomonas aeruginosa, is a well known nosocomial pathogen that is broadly distributed in nature especially in hospital environment, and it is an important cause of a variety of acute infections [1,2]. They occur predominantly in patients with cystic fibrosis, burns and other wound types [3,4,5,6], and in individuals that have debilitating disorders resulting in immunosuppressed systems [7,8]. *Pseudomonas aeruginosa* employ varied number of virulence determinants, including but not limited to, factors involved in host colonization, nutrient uptake and various other factors that modulate the host response to the infection. Predominant amongst these are the elaborations of porins, type III secretion system (T3SS), alkaline protease and pigment production. The Type III secretion system (T3SS) and its effectors are major virulence determinants of *P. aeruginosa* and other Gram-negative pathogens [9]. The T3SS forms a needle that directly injects proteinaceous virulence factors cytotoxins (*ExoS, ExoT, ExoU, ExoY*) [10,11] into the cytoplasm of eukaryotic host cell. These toxins are translocated directly from the bacterial cytoplasm into the host cytosol [12]. The T3SS similarly permits *P. aeruginosa* to elude killing by infiltrating host neutrophils [13,14].

The Gram-negative bacteria possesses linked outer membrane proteins (OPRs), which includes OprI, OprL, and OprF which attach it to the peptidoglycan and stabilizes the outer membrane [15]. *P. aeruginosa* has been reported to possess OprI, a small outer membrane peptidoglycan-binding lipoprotein about 8-kDa in size [16,17]. The OprI is involved in envelope integrity via interactions with peptidoglycan and other outer membrane proteins, majorly OprF and OprL [18], which has also been reported to influence membrane vesicle development in *P. aeruginosa* [19]. The *oprI* gene has also been tested as a phylogenetic marker for classifying rRNA group I Pseudomonads [20]. It is exceedingly abundant in the outer membrane of *P. aeruginosa* [16,21,22].

The alkaline protease gene, which codes for the production of alkaline protease (*aprA)* is one of the most widely studied virulence genes of *P. aeruginosa.* It is a zinc-dependent metalloprotease, and it breaks down several proteins such as laminin (a constituent of the basal lamina), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and human interferon-γ (IFN-γ). Both TNF-α and IL-6 are involved in the regulation of immune cells and host immune response during infections. The human interferon-γ (IFN-γ) plays a vital role in innate and adaptive immunity [23]. Consequently, aprA can degrade host defense and immunoregulatory proteins, damage epithelia [24,25] leading to impaired host immune response [26].

Iron, although not readily available as a result of binding by certain cellular proteins [27], is required by *P. aeruginosa* in considerable quantities to cause infection and invade host cells. *P. aeruginosa* has a remarkable ability to recover this iron through an array of complex processes including the production of two siderophores, pyoverdine and pyochelin [28,29]. Pyoverdine, a peptidic siderophore contains two hydroxamic groups and a fluorescent dihydroxyquinoline chromophore, which form a highly effective iron coordination unit [30,31,32].

These exotoxins are the most significant virulence determinants of *P. aeruginosa* and cause great concern in the clinical setting. It is essential to screen for virulence genes periodically in surveillance studies. This research therefore aimed at identifying certain virulence genes in *P. aeruginosa* isolates from wound swabs of out- and in-patients at two selected tertiary hospitals in Osun State, southwest Nigeria.

2.0 METHODOLOGY

2.1 Study Area and Sample Collection

The study was undertaken at two selected tertiary Hospitals (State Hospital, Osogbo, and General Hospital, Iwo) in Osun State, Southwest Nigeria. Altogether, 237 participants consisting of 133 patients from the State Hospital, Osogbo and 104 patients from the General Hospital, Iwo with different types of wound were enrolled. Ethical approval was obtained from the Ethical Review Board of the Osun State University, Osogbo, Nigeria. Wound swabs from automobile accidents, caesarian sections sites and other wound types were collected using sterile cotton-tipped applicators (Evepon, Nigeria) moistened with physiological saline, inoculated initially into Tryptone Soy Broth (TSB) for 24 hour growth at 37^{o}C. Subsequently, the cultures were streaked out onto cetrimide agar, and distinct colonies identified using Gram stain and conventional biochemical tests [33,34,35]. Pure cultures of the bacterial isolates were maintained in freshly prepared TSB with 15% glycerol and stored at -20^{o}C until further assay

2.2 Molecular Identification of Virulence Genes

2.2.1 DNA Extraction

The chromosomal DNA was extracted by thermal lysis as described by Adeleke *et al.,* [36]. The purity and concentration of the extracted DNA in the supernatant

were estimated using NanoDrop-One (ND-One) spectrophotometer (ThermoFisher Scientific).

2.2.2 Polymerase Chain Reaction

The recovered isolates were subjected to polymerase chain reaction (PCR) using specie specific primers to further confirm the biochemical identification of the *P. aeruginosa* isolates. Forward primer 5'-GGCGTGGGTG TGGAAGTC-3' and reverse primer 5'-GGTGGCGATCTT GAACTTCTT-3' were used to amplify the the segments of the bacterial gene. The PCR amplification was carried out in a total volume of 25µl in a solution containing 12.5µl of master mix (Biolabs, England), 0.5µl of 10µM each of the forward and reverse primers (Inqaba Biotec, South Africa), 5µl DNA template and 6.5µl of DNAse/RNAse free sterile water (Bio-Concept, New Hampshire). The presence of *oprl* for porins, *ExoT*, *ExoS, ExoU*, and *ExoY* for T3SS, *aprA*, and *pvdA* for alkaline protease and pyoverdine production respectively were chosen based on previous studies [37,38,39,40] and screened for in all the isolates that were confirmed genotypically as *P. aeruginosa*. Specific primers for the above genes were employed in the amplification of the virulence genes present in the chromosomal DNA of the isolates and details of oligonucleotide sequences the and expected size of amplicons are given in Table 1. Uniplex PCR was done for *oprl*, *aprA*, *pvdA*, and *ExoS* while multiplex PCR was done for *ExoT*, *ExoU*, and *ExoY* for T3SS. Uniplex PCR was done in 25µl volume as described previously. For the multiplex reactions, 0.5µl each of the forward and reverse primers (10µM) was added and made up to 25µl with DNAse/RNAse free sterile water. The PCR reactions were carried out with a negative control comprising of all the reagents but lacking the DNA template. Amplification was done in a Master Cycler Nexus Gradient 230 with the thermocycling parameters given in Table 2. PCR products (10µl) were run on 1.0% agarose gel electrophoresis and visualized using EZ-vision blue light DNA dye (VWR Life Sciences, Pennsylvania) staining under UV transilluminator (E-BOX-CX5.TS imaging system). GeneRuler 100bp and 1kb DNA ladders (Biolabs, England) were used as DNA molecular weight standards to evaluate the size of the amplified fragments as appropriate.

3.0 RESULTS

Altogether, 61 (25.7%) isolates of *P. aeruginosa* were recovered from 237 wound samples from the two hospitals selected for this study. The highest recovery rate was from surgical sites of caesarian sections (CS) (37.7%; 23/61) the trauma sustained from motorcycle and automobile accidents (36.1%; 22/61) and other wound types (26.2%; 16/71). Out of these, 42.6% were isolated from samples collected from Osogbo while 57.4% were from Iwo (Table 3). The rate of recovery of isolates from CS wounds was higher in Iwo than in Osogbo (60.9%); while a higher number of isolates were recovered from males involved in automobile accidents than from females from both locations (Table 3).

Table 1. The PCR Oligonucleotide sequences employed for amplification of virulence genes

	Primer/ oligo name	Target gene	Oligonucleotide sequence (5' to 3')	Amplicon size (bp)
Porins	*oprI*	*OprI-F*	ATGAACAACGTTCTGAAATTCTCTGCT	249 [37]
		OprI-R	CTGCGGCTGGCTTTTTCCAG	
T3SS	*ExoT*	*ExoT-F*	CAATCATCTCACAGAACCC	1,159 [38]
		exoT-R	TGTCGTAGATCTCCTG	
	ExoS	*exoS-F*	ATCCTCAGGCGTACATCC	328 [38]
		exoS-R	ACGAGTATCTCTCCAC	
	ExoU	*exoU-F*	GATCCATCACAGGCTCG	3,308 [38]
		exoU-R	CTACAATGCACTAATCG	
	ExoY	*exoY-F*	TATCGAGGTCATCGTCAGGT	1,035 [38]
		exoY-R	TTGATGCATCGACCAGCAAG	
Alkaline protease	*aprA*	*aprA-F*	5'GTCGACAGGCGGCGGAGCAGATA 3'	993 [39]
		aprA-R	5'GCCGAGGCCGCCGTAGAGGATGTC 3'	
Pyoverdine	*pvdA*	*pvdA-F*	GACTCAGGCAACTGCAAC	1,281 [40]
		pvdA-R	TTCAGGTGCTGTACAGG	

Table 2. PCR Protocol for the *P. aeruginosa* Identification and Virulence Genes

Target genes	Protocol for PCR Reactions					
	Initial Denaturation	**No. of Cycles**	**Denaturation**	**Annealing**	**Extension**	**Final Extension**
Parugin	96°C for 5mins	40	96°C for 60s	55°C for 60s	70°C for 60s	72°C for 10 mins
oprI	94°C for 5mins	30	94°C for 60s	55°C for 60s	72°C for 60s	72°C for 10 mins
ExoT ExoS ExoU ExoY	94°C for 10 mins	30	94°C for 40s	57°C for 50s	72°C for 55s	72°C for 10 mins
aprA	95°C for 2 mins	30	95°C for 40s	65°C for 60s	72°C for 2 mins	72°C for 10 mins
pvdA	94°C for 2 mins	30	94°C for 30s	60°C for 30s	68°C for 60s	68°C for 10 mins

Table 3. Distribution of *P. aeruginosa* isolates recovered from wound samples in Osogbo and Iwo

Locations	Gender	Wound Types (%)				Gross Total
		Caesarian sections	**Automobile accidents**	**Others**	**Total**	
Osogbo	Male	0 (0.0)	7 (31.8)	2 (12.5)	9 (14.7)	26 (42.6)
	Female	9 (39.1)	3 (13.6)	5 (31.3)	17 (27.9)	
Iwo	Male	0 (0.0)	7 (31.8)	6 (37.5)	13 (21.3)	35 (57.4)
	Female	14 (60.9)	5 (22.7)	3 (18.7)	22 (36.1)	
Total		23 (37.7)	22 (36.1)	16(26.)	61	61

3.1 Molecular Identification and Characterization of Recovered Isolates

Altogether, 59 (96.7%) out of the 61 recovered isolates revealed the presence *P. aeruginosa* using specie specific *Parugin* gene primer (Figure 1). One isolate each recovered from Osogbo and Iwo was negative. The frequency of *Parugin* gene in Osogbo and Iwo was 25/26 (96.2%) and 34/35 (97.1%), respectively.

3.2 Detection of Virulence Genes by PCR

The 59 PCR confirmed *P. aeruginosa* isolates were screened for the presence of virulence genes – *oprI, ExoT, ExoS, ExoU, ExoY, aprA,* and *pvdA*. Only *oprI* gene was detected in the isolates, occurring in 74.6% of the isolates (n = 44/59) (Figures 2 and 3). The type III secretion system genes (*ExoT, ExoS, ExoU,* and *ExoY*), alkaline protease gene *aprA*, and pyoverdine gene *pvdA* were not present in all the 59 *P. aeruginosa* isolates analyzed. Out of the 44 isolates that were positive for *oprl,* 18 were recovered from Osogbo while 26 were recovered from Iwo.

4.0 DISCUSSION

In the present study, a total of 61 isolates of *P. aeruginosa* isolates were screened for the presence of specie specific *Parugin* genes, and 59 of these for virulence genes – *oprl, ExoT, ExoS, ExoU, ExoY, aprA*, and *pvdA*. Out of the 61 isolates, the specie specific gene for *P. aeruginosa* was detected in 59 (96.7%) isolates. This result correlates well with the phenotypic identification of the isolates as only two isolates did not reveal bands using the specie specific primer for amplification. The difference in the genotypic and phenotypic identification could be as a result of the last two isolates belonging to other species of *Pseudomonas*. The *ExoT, ExoS, ExoU, ExoY, aprA* and *pvdA* genes were not detected in any of the 59 *P. aeruginosa* isolates. Feinbaum *et al*., [41] stated that some virulence factors including *ToxA, exoA, oprL*, and *oprI* genes are among the most frequent factors responsible for *P. aeruginosa* pathogenicity. The presence of virulence genes in *P. aeruginosa* depends largely on several causes which may include environmental factors, level of im-

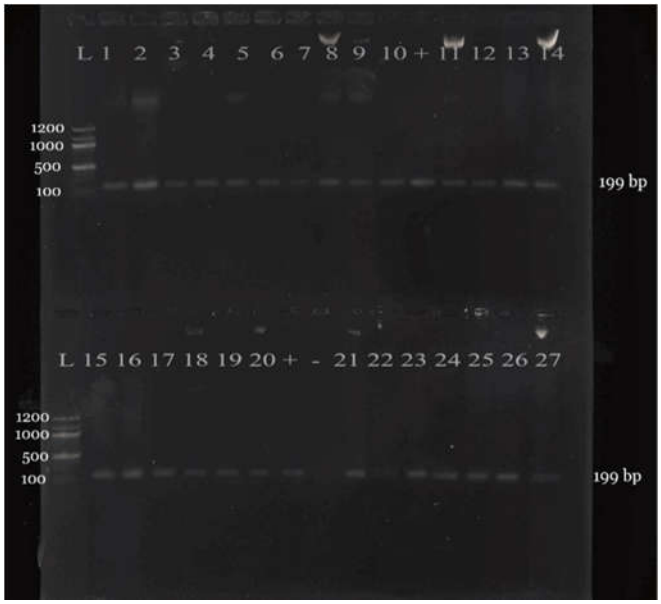

Figure 1. The gel electrophoresis of PCR products using specie specific primers for the identification of *Pseudomonas aeruginosa* isolated from wounds of hospital patients (199 bp).

Upper Row: Lane 1: (L) 100bp molecular weight marker; Lanes 2 - 11; 13 - 16 *Pseudomonas aeruginosa* isolates cultured from wounds of hospital patients; Lane 12 positive control

Lower Row: Lane 1: (L) 100bp molecular weight marker; Lanes 2 – 7; 10 – 16: *Pseudomonas aeruginosa* isolates; Lane 8: Positive control; Lane 9: Negative control.

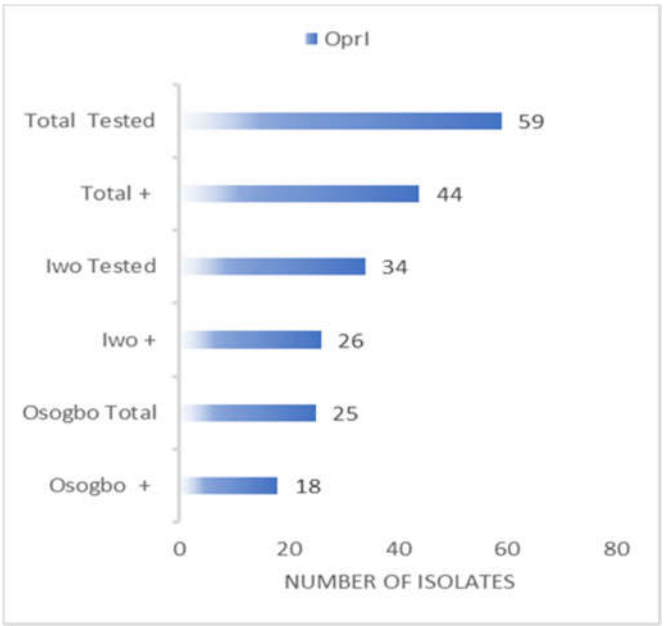

Figure 2. Distribution of *oprI* virulence gene in *P. aeruginosa* recovered from wound samples in Osogbo and Iwo

munity in patients, degree of contamination and nature and virulence of strain [42]. Of the seven virulence genes investigated, only the *oprI* gene was detected. It was observed that 74.6% (n = 44/59) of the strains screened

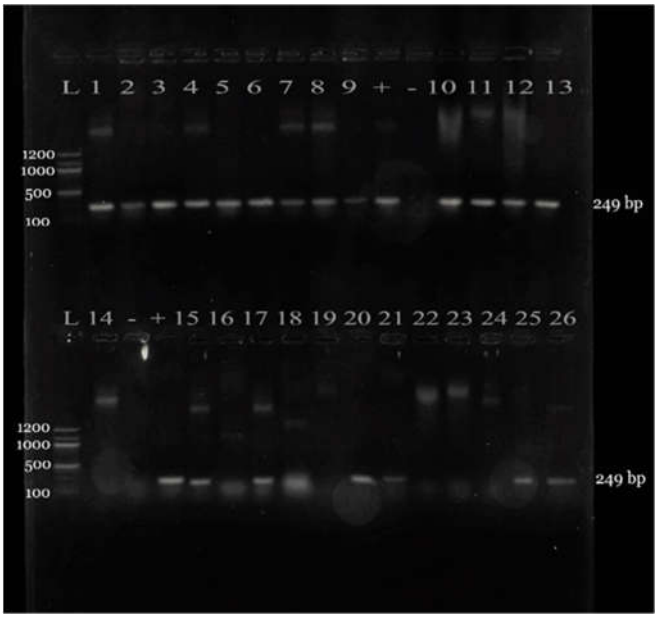

Figure 3. The gel electrophoresis of PCR products for the detection of *oprI* gene in *Pseudomonas aeruginosa* recovered from wounds of hospital patients (249 bp);

Upper Row: Lane 1: (L) 100bp molecular weight marker; Lanes 2 – 10; 13 – 16: *Pseudomonas aeruginosa* isolates cultured from wounds of hospital patients; Lane 11: Positive control; Lane 12: Negative control.

Lower Row: Lane 1: (L) 100bp molecular weight marker; Lanes 2; 5 – 16: *Pseudomonas aeruginosa* isolates; Lane 3: Negative control; Lane 4: Positive control

harbored the *oprI* gene.

oprI in *P. aeruginosa* associate non-covalently with peptidoglycan [43,44,45], and thus plays a part in maintaining the structural integrity of the bacterial cell. The interaction of *oprI* with the peptidoglycan has been reported to differ among *P. aeruginosa* strains [16,22,45]. The *oprI* loss directly impacts the production of outer membrane vesicles (OMVs) in *P. aeruginosa*. Wessel *et al.* [19] observed and reported that *oprI* mutants grew at comparable rates to wild-type *P. aeruginosa* but produced thrice as much OMVs; while the expression of *oprI* in trans in the *oprI* mutants reduced OMV production, demonstrating that increased OMV production was due to the loss of *oprI*. Loss of *oprI* decreases the tethering of the outer membrane to peptidoglycan, leading to the detachment of the outer membrane from the underlying peptidoglycan layer [19,46,47,48].

Another possibility suggested by Wessel *et al.* [19], however was that the inactivation of porin lipoproteins may modify the levels of *Pseudomonas* quinolone signal [PQS], a quorum-sensing signal which stimulates *P. aeruginosa* OMV production. This was proven to be true for *OprF* as its mutants produced four times more PQS than wild type *P. aeruginosa*; nevertheless, *oprI* mutants synthesized PQS levels equal to that of the wild type

strain. Alkaline protease is another important virulence determinant in *P. aeruginosa* which promotes the development of bacteria within the infected host and interferes with the host immune system. The aprA cuts several proteins such as laminin, human interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [23], degradation of which may well lead to weakened immunity in the host. Again, aprA can impede neutrophil activities by interfering with chemotaxis of neutrophils, permitting pathogens to get away from phagocytes of the host defense system [23]; and it has been reported that aprA restricted the classical and lectin pathway-mediated complement activation by cleaving C_2 [49]. Consequently, aprA contributes significantly to the pathogenicity of *P. aeruginosa*.

Various research has shown that analyses of *P. aeruginosa* clinical isolates from different sites suggested that both the infection site as well as the duration of infection influenced the virulence of the bacteria by altering the production of extracellular virulence factors [50]. As such, the expression of aprA, as well as other virulence determinants, may be related to infection in the site of injury. Study by Cotar *et al.* [50] reported a low prevalence of the *aprA* gene in *P. aeruginosa* isolates from the wound samples cultured. Results from this study, however, indicated that none of the isolates obtained from the samples was positive for *aprA* genes. The reasons for this are however not quite apparent. The failure to detect the genes probably was as a result of a failure in amplification or non production of the isolates to produce alkaline protease. Type III secretion system (T3SS), a highly sophisticated virulence factor is a major determinant of two pathogenic types (invasiveness or cytotoxicity). *ExoS* and *ExoT* both have GTPase-activating protein activities and ADP-ribosyl transferase activities. *ExoU* is a potent phospholipase, while *ExoY* acts as a secreted adenylyl cyclase [51]. The result for all these four types of T3SS genes showed that all the four virulence genes were not detected in all the isolates in this study.

Clinical isolates of *P. aeruginosa* commonly fall into one of three phenotypic categories: those that secrete *ExoU* and *ExoT*; those that secrete *ExoS* and *ExoT*; and those that do not secrete type III proteins [52]. Previous studies have reported that *ExoU* and *ExoS* seem to occur in a mutually exclusive association [52,53,54]. Strains that secrete *ExoU* and *ExoT* may be the most virulent, strains that secrete *ExoS* and ExoT may be intermediate in virulence, while strains that are incapable of type III secretion and therefore do not secrete *ExoU, ExoS* and *ExoT* may be the least virulent [52]. The non detection of the T3SS phenotype in this present study, implies that these isolates may be the strains of *P. aeruginosa* that is not capable of secreting the T3SS proteins. Also, *P. aeruginosa* isolates recovered from samples obtained from Osogbo and Iwo hospitals in this study may not be producing certain virulence factors as none of the wounds were infected at the time of sampling.

In conclusion, this study shows that the prevalence of clinical isolates of *P. aeruginosa* from the two selected hospitals studied was highest from surgical sites of caesarian sections, with the rates from Iwo higher than that of Osogbo. Furthermore, a high proportion of the isolates revealed the presence *oprI*, although none of the other genes were detected. This observation portends great hazard as potential infections arising from these strains may impair wound healing, or culminate in systemic infections arising from dissemination. Regular monitoring of virulence determinants in clinical isolates is therefore recommended as this may aid clinicians to devise efficient antimicrobial therapy for the effective treatment of wound infections.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Authors Contribution

FMA conceived and designed the study, contributed to data verification and interpretation, participated in bacteriological and molecular study, supervised the work and wrote the manuscript; **RRA** contributed to study design, sampling, bacteriological study, other laboratory procedures and manuscript writting; **AAA, N-AY and AAW** performed the bacteriological and molecular investigation. All authors approved the final version of the manuscript

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