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ORIGINAL ARTICLE

Insights into global transcriptomic profile of biofilm producing *Staphylococcus aureus* clinical isolates from chronic foot ulcers

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ABSTRACT

Background and Objectives: *Staphylococcus aureus (S. aureus)* is one of the predominant biofilm producing pathogen in leprosy foot ulcer (LFU). The objective of this study was to identify the transcriptome profile through Next Generation Sequencing (NGS) approach in mature biofilm of leprosy foot ulcer isolate of *S. aureus.*

Materials and Methods: A cross-sectional study was conducted from July 2019 to May 2022 and a total of twenty-seven *S. aureus* isolates were collected from the foot ulcers of leprosy patients. All *S. aureus* isolates were screened for biofilm formation in vitro. Initially, two potential biofilm producing isolates and two planktonic cells were selected for transcriptome comparison.

Results: With reference to transcriptome profile, out of 2,842 genes, 2,688 genes in mature biofilm and 2,685 genes in planktonic cells were expressed. Among them, forty-five differentially expressed genes with 32 and 13 genes showing up and down regulation respectively were obtained.

Conclusion: The research emphasizes the need for continued exploration into the mechanisms of biofilm formation by *S. aureus*, particularly in the context of leprosy foot ulcers. Understanding these pathways not only aids in grasping the complexity of chronic infections but also paves the way for innovative therapeutic approaches aimed at mitigating biofilm-related complications in clinical settings.

Keywords: Leprosy foot ulcer; *Staphylococcus aureus*; Biofilm; Confocal microscopy; Transcriptome

INTRODUCTION

Plantar ulcers are one of the most common complications of leprosy and occur due to sensory loss and consequent chronic wound formation (1). Plantar ulcers lead to disability associated with stigma and discrimination and further impact the quality of life of the people affected by leprosy (2), which makes a significant economic burden not only to the patient but also to the health systems (3). Approximately one-third of leprosy patients with foot anesthesia are at risk of developing plantar ulcers during their lifetime (4). Interventions addressing chronic foot ulcers hence have a far-reaching effect on healthcare cost and life quality of the affected individuals (5).

S. aureus, Pseudomonas and members of *Enterobacteriaceae* are some of the common pathogenic bacteria that cause secondary infections on chronic foot ulcers in leprosy (6). Biofilm formation is known to be one of the key features of pathogenic bacteria

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not only for colonisation and multiplication but also for defending the host immune responses and antimicrobials (7). Chronic foot ulcers thus are often colonised by secondary bacterial pathogens which produce biofilms and eventually develop resistance to multiple antimicrobials (8). Previous studies from India and other countries have reported the profile of secondary bacterial infections in leprosy plantar ulcers and observed that most of these pathogens were multi drug resistant (9-14). Our earlier work demonstrated the presence of biofilm producing *S. aureus* infection on chronic leprosy foot ulcers and their association with antimicrobial resistance for the first time (15, 16). Biofilm formation by bacterial pathogens are known to be influenced by both internal factors such as their inherent biofilm producing ability and external environment including several host tissue factors (17). Understanding the causative mechanisms thus plays an important role in developing appropriate antibiofilm interventions. Studies conducted on these lines reported transcriptomic profiles of biofilm producing strains and identified some important differential genes and enrichment pathways such as MRSA clones with association with arginine pathways (18). However, most of these studies were conducted on type stains and lab controls which might not give a comprehensive picture of the mechanism, since the strains were not isolated from host tissue. There is a possibility that biofilm forming pathways in pathogenic bacterial type strains might not always closely correlate with that of a clinical isolate (19).

Present study hence attempted to address this gap through investigating the transcriptome profiles of clinical isolates of *S. aureus* collected from infected chronic foot ulcers in leprosy patients.

MATERIALS AND METHODS

Specimen collection, bacterial identification and biofilm screening. During July 2019 to May 2022, a total of 83 leprosy patients who were treated for chronic foot ulcers at the foot care clinic of LEPRA Blue Peter Public Health and Research Centre (LEPRA - BPHRC), Hyderabad, India enrolled the study after obtaining an informed consent. One swab was collected from each foot ulcer. Wound swabs were inoculated on MacConkey agar and Blood agar and incubated overnight at 37°C. Bacterial isolates were identified based on colony morphology, Gram's staining and biochemical tests as per the CLSI standards (CLSI). All the *S. aureus* were further subjected for biofilm detection by Congo red method and microtiter plate method, a semi qualitative method as described previously (16). The biofilm-producing isolates in this study were screened based on qualitative methods and classified as no biofilm producer, weak producer and strong producer. All the strong biofilm producing isolates were selected for this study. These isolates were also methicillin-resistant and exhibited multidrug resistance (MDR), as expected. Two strong biofilm positive isolates of *S. aureus* cells and two planktonic cells of *S. aureus* were selected to generate the transcriptome profile (C1: is *S. aureus* isolate-1-planktonic cells; C2 is *S. aureus* isolate- 2- planktonic cells; D1 is *S. aureus* isolate- 1 of matured biofilm cell and D2 is *S. aureus* isolate - 2 of matured biofilm cells.

Biofilm matrix through confocal microscopy. A 10 mL of bacterial TSB broth with 1% glucose containing McFarland equivalent (3.0) was transferred into a 50mL sterile falcon tube and a sterile cover slip (10 × 10 mm) was immersed into the broth and it was incubated at 37°C for 24 hours. Then, the adhering biofilm was washed, fixed with 4% formaldehyde for 60 minutes, washed twice with distilled water and stained with 200 µl of 1.67 µM Syto 9 fluorescent dye (Thermo Fisher Scientific, USA) for 30 min. The stained biofilms were washed again with autoclaved water and mounted on a glass slide (20). Confocal images were taken using Zeiss confocal laser scanning microscope (LSM 510) through argon laser excited at 450-490 nm.

RNA extraction. The bacterial cell pellets were washed with ice cold 1X PBS and then dissolved in 100ul of lysozyme in the TE buffer. This was incubated at 37°C for 30 minutes and followed by RNA extraction using HiPurA™ Bacterial RNA Purification kit. 350 µl of lysis buffer was added to the samples, mixed and passed through a shredder column. The flow through was collected and mixed with ethanol prior to loading onto the spin column. These columns were washed with the provided wash buffers and final elution was performed using 20 µl of RNAse free water. Initially the RNA presence was checked through 1% agarose gel. The RNA quality assessment was done using RNA ScreenTape System (Catalog: 5067-5576, Agilent) in a 4150 Tapestation System (Catalog:

G2992AA, Agilent) according to the manufacturer's instructions. RNA concentration was determined on Qubit® 3.0 Fluorometer (Catalog: Q33216, Thermo Fisher Scientific) using the Qubit™ RNA BR Assay Kit (Catalog: Q32853, Thermo Fisher Scientific). RNA sequences were performed on two potential biofilm cells of *S. aureus.* Planktonic cells were C1 & C2 and biofilm cells were D1 & D2. If the RNA integrity number (RIN) of the extracted RNA was more than 7, they were subjected to further downstream processing.

RNA depletion, library preparation, quantification. NEBNext Ultra II RNA Library Prep Kit (Catalog: Catalog: E7775S, New England Biolabs) for Illumina was used for the library preparation. The enriched transcriptome was chemically fragmented in a magnesium-based buffer at 94°C for 10 minutes. The fragmented samples were primed with random hexamers, and reverse transcribed to form cDNA and the first strand cDNA reactions were converted to dsDNA. The double stranded cDNA fragments obtained were cleaned up by using AMPure beads (Catalog: A63881, eBeckman Coulter). The cDNA fragments underwent end repair converting the overhangs resulting into blunt ends. The 3' to 5' exonuclease activity of the end repair mix removes the 3' overhangs and polymerase activity fills in the 5' overhangs. To the blunt ended fragments, adenylation was performed by adding single 'A' nucleotide to the 3' ends. To the adenylated fragments, loop adapters (Platform Specific) were ligated and cleaved with uracil-specific excision reagent (USER) enzyme. Size Selection was performed using AMPure beads (Catalog: A63881, Beckman Coulter) according to the manufacturer's protocol aiming the library size of 400-600bp. Furthermore, the DNA was amplified by 12 cycles of PCR by adding NEBNext Ultra II Q5 master mix, and "NEBNext® Multiplex Oligos for Illumina" to facilitate multiplexing while sequencing. The amplified products were then purified using 0.9X AMPure XP beads (Catalog: A63881, Beckman Coulter) and the final DNA library was eluted in 15µl of 0.1X TE buffer. The library concentration was determined in a Qubit.3 Fluorometer (Catalog: Q33216, Life technologies using The Qubit dsDNA HS (High Sensitivity) Assay Kit (Catalog: Q32854, Thermo Fisher Scientific).

Sequence data QC and alignment. The sequence data was generated using Illumina HiSeq. Data quality was checked using FastQC (21) and Multi QC software (22). Raw sequence reads were processed to remove adapter sequences and low-quality bases using fastp (23) and mapped to *S. aureus* genome using Bowtie v2 aligner (24). The cDNA libraries of four *S. aureus* [two each from planktonic (C1&C2) and biofilm cells (D1 & D2)] were sequenced and generated with a total of 41,415,910 to 57,126,002 reads which were mapped to the reference genome of *S. aureus* (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=1280).

Differential expression. Differential expression analysis was carried out using the DESeq2 package (25). Genes with less than total 5 reads were removed from further analysis. The read counts were normalized (variance stabilized normalized counts) using DESeq2. Expression profiles of biofilm samples were compared to those of planktonic cells. Gene level expression values were obtained as read counts using feature-counts software (26).

Pression gene ontology and pathway analysis. Enrichment analysis for biological process, molecular function, cellular component and KEGG pathway was performed using DAVID (27). Gene Ontology (GO) and pathway terms with multiple test q-value ≤ 0.05 were considered significant. The pathways were visualized using the Path view package (28) to check the differential expression level of the genes in the pathway.

RESULTS

Bacterial isolates and biofilm. A total of 83 leprosy patients with foot ulcers (Fig. 1A) were enrolled. The mean age was 54.30 + SD11.90 with male (n=44) and female (n=39) distribution. All ulcers were single except for five patients with two concomitant ulcers. Among 88 wound swabs tested, 93.18% (82/88) were found to be culture positive, of which 91.14% (75/82) were mono-bacterial and 8.53% (7/82) were poly microbial. Of the 89 bacterial isolates, 73.30% (65/89) were Gram positive and 26.96% (24/89) were Gram negative organisms. *Streptococcus* spp. (38/89) and *S. aureus* (27/89) were the frequent organisms among Gram positive bacteria. *Proteus* spp. (14/89) was the most common organism among Gram negative bacteria. The detailed antimicrobial susceptibility testing

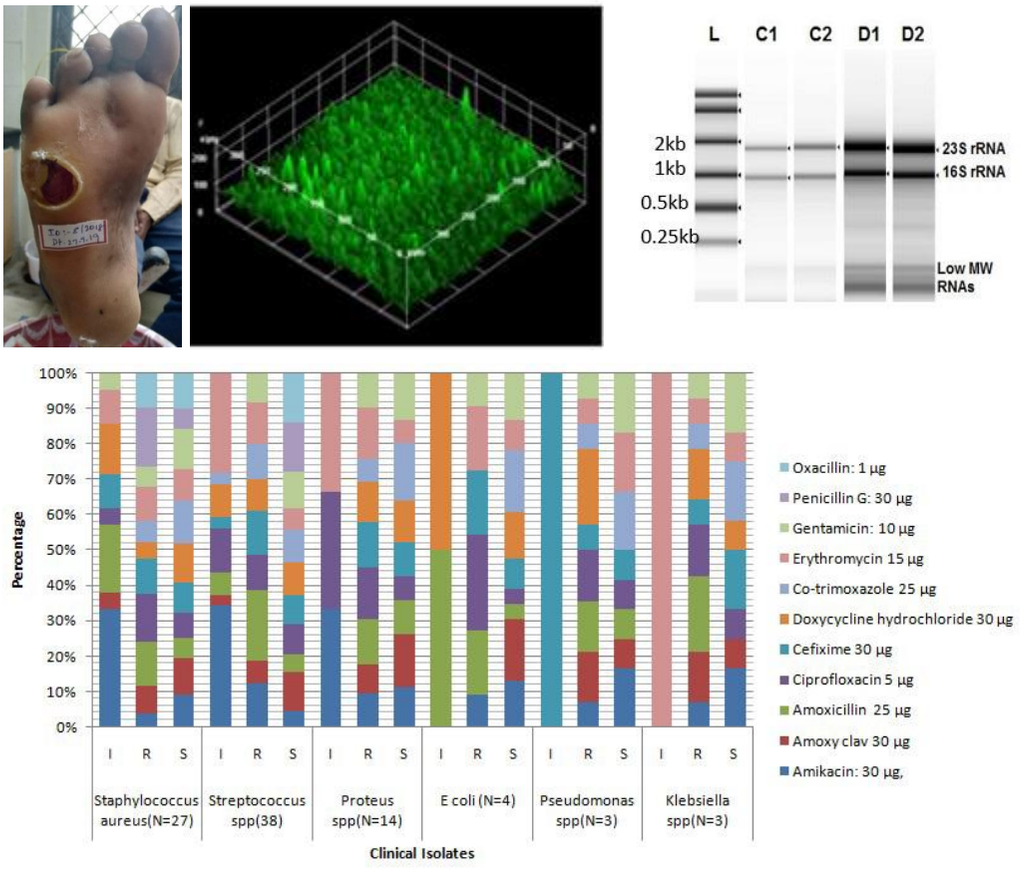

Fig. 1. A. Leprosy foot ulcer
B. 3D view of biofilm under confocal scanning microscope
C. Total bacterial RNA pattern
L: Ladder, C1& C2: planktonic RNA; C3 & C4 Biofilm RNA
D. Antibiotic susptibility pattern for all clinical isolates.

(AST) pattern is shown in Fig. 1D.

All isolates were tested for biofilm screening and we found that only *S. aureus* isolates were positive for biofilm formation (19/27). Biofilm was allowed to mature for 24 hours and the matrix was visualised under a confocal laser microscope (Fig. 1B). The high molecular (23 S rRNA and 16S rRNA) and low molecular weight RNA pattern was observed through agarose gel electrophoresis (Fig. 1C).

Differential gene expression by comparing biofilm associated cells with planktonic cells. Out of 2,842 genes, 2,688 and 2,685 genes were expressed in mature biofilm and in planktonic cells, respectively. Genes with absolute log2 fold change ≥ 1 and P value ≤ 0.05 were considered as significant (Fig. 2). Forty-five differentially expressed genes with 32 and 13 genes that were up and down regulated respectively were obtained. 22/32 upregulated genes encoded aminoacyl tRNA proteins, seven encoded hypothetical proteins and few others were related to multidrug resistance and cell wall peptidoglycan synthesis. Differential expressions for genes across the samples of planktonic cells and biofilm cells were represented in heat map analysis (Fig. 3).

DISCUSSION

Identifying the factors that contribute to recalcitrant foot ulcers is crucial for tailoring appropriate preventive and therapeutic interventions (29). Some of the key factors in leprosy-related foot ulcers are the peripheral neuropathy leading to sensory impairment and changes in bone architecture that result in elevated foot pressure leading to loss of skin and foot

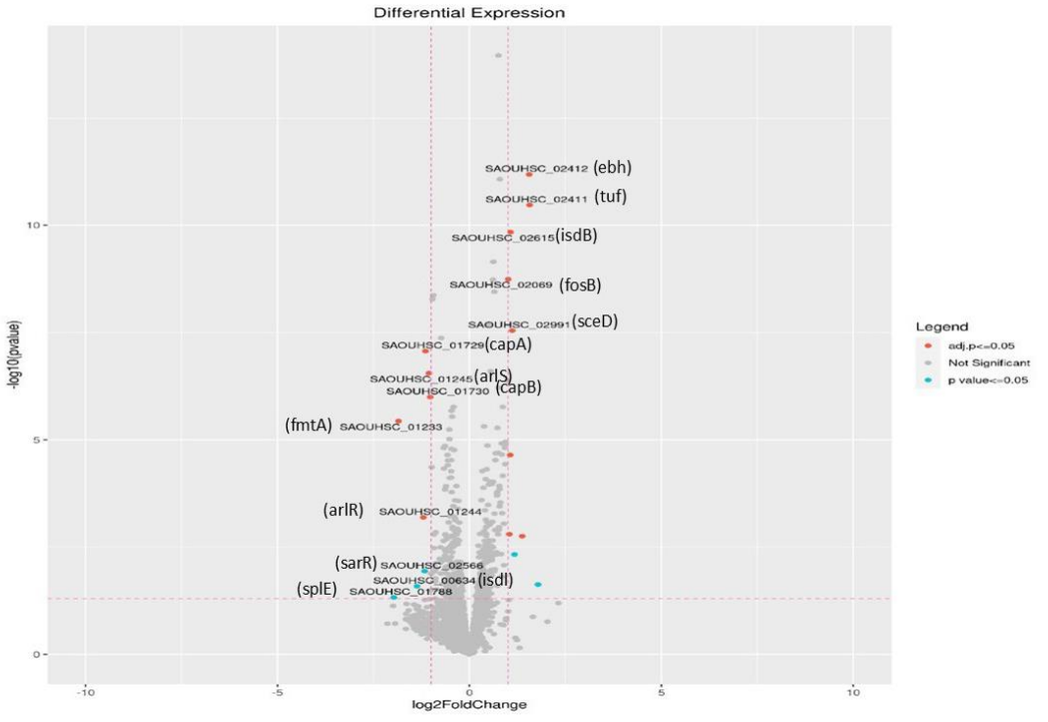

Fig. 2. Volcano-plot of the complete quantified genes in *S. aureus* biofilm

architecture and loss of sweating due to autonomic nerve impairment leading to chronic dry skin conditions. Unprotected dry soles thus lead to cracks, blisters which if unaddressed can attract microbial infections and lead to subsequent complications (30). It is well known that recalcitrant infections are one of the leading causes for delayed healing and chronicity of foot ulcers. Chronic colonisation through biofilm formation is one of the strategies used by bacterial pathogens for establishing their niche and defending the host immune responses (31). In an earlier study conducted by our group, we demonstrated that *S. aureus* (54%) is one of the predominant organisms followed by *Pseudomonas aeruginosa* (12%) (16). Current study confirms the same findings indicating the critical significance of *S. aureus* as a secondary bacterial pathogen on chronic foot ulcers with potential biofilm. Hence understanding the mechanism of biofilm production of *S. aureus*, could pave the way for tackling AMR on chronic foot ulcers. RNA-sequence is one of the modern approaches for transcriptomic studies (32), which was used for this study to profile the differential gene expression in biofilm producing of *S. aureus*, which were further analysed using GO and KEGG pathway analysis to predict the functions associated with these genes. To note, planktonic controls provide a standardized comparison, as they represent the same bacterial strain in a different physiological state rather than a genetically distinct non-biofilm-producing variant. This could potentially minimize variability and confounding. Previous studies have demonstrated that biofilm-associated *S. aureus* exhibits increased resistance to antibiotics and host immune responses compared to its planktonic counterpart (33, 34). To accurately assess the effects of biofilm formation on gene expression, it is essential to use the same *S. aureus* isolate for both biofilm and planktonic conditions. This approach ensures that the genetic background remains constant, allowing for a more precise determination of how biofilm formation influences gene expression. Therefore, using planktonic cells as a reference enables more accurate assessment of the biofilm's protective advantages to the bacterial pathogen and corresponding differential gene expression patterns. A wider gene expression profiling and the patterns if any identified will be further investigated for associated specific gene mutations.

In the current study with RNA sequence technology, a total of 2687 transcripts were obtained

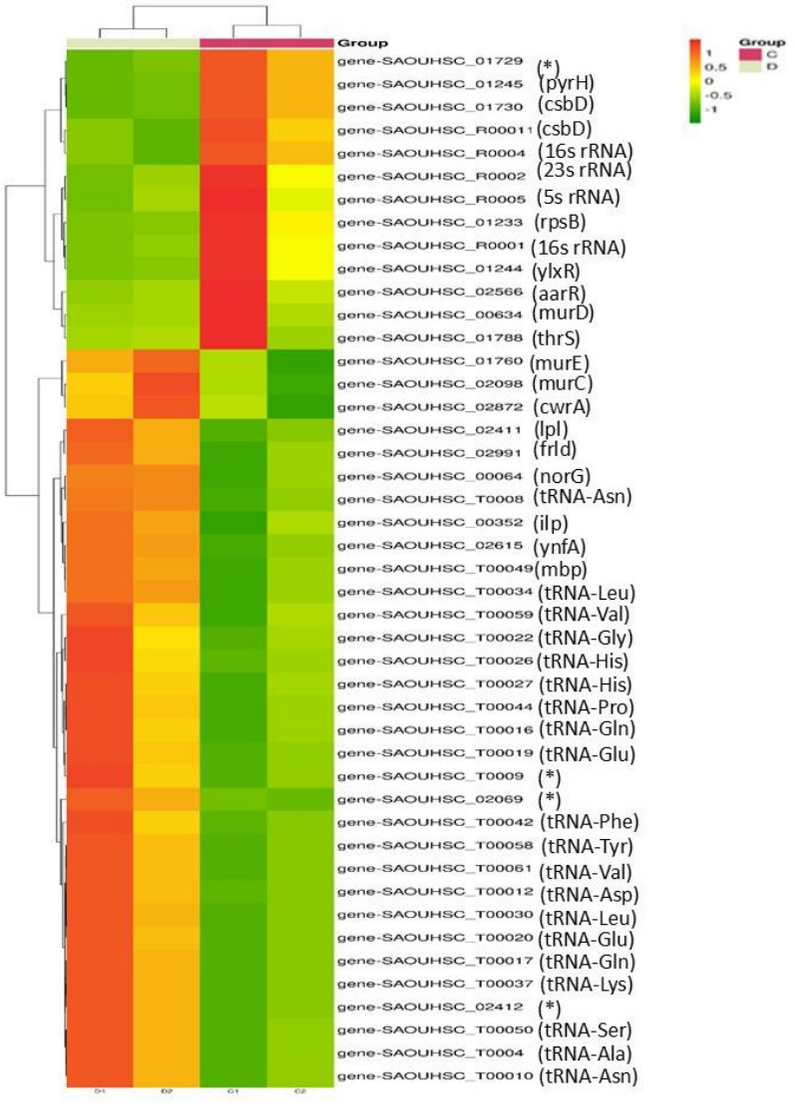

Fig. 3. Heat map analysis gene expression across the samples of planktonic (C) vs biofilm cells (D)
* hypnotical genes (uncharacterised)

representing the reference genome of *S. aureus* (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=1280). Forty-five differentially ex- pressed genes with 32 and 13 genes showed up and down regulation respectively. Out of the thirty-two up-regulated genes, 68.75% (22/32) of the genes [(SAOUHSC_100010 (aroE), SAOUHSC_T00012 (tRNA-Thr), SAOUHSC_T00016 (tRNA-Val), SAOUHSC_T00017 (tRNA-Gly), SAOUHSC_ T00019 (tRNA-Leu), SA UHSC_T00020 (tRNA-Pro), SAOUHSC_T00022 (tRNA-Met), SAOUHSC_T00026 (tRNA-Arg), SAOUHSC_T00027 (tRNA-Asp), SAOUHSC_T00030 (tRNA-Ser), SAOUHSC_T00034 (tRNA-Tyr), SAOUHSC_T00037 (tRNA-Arg), SAOUHSC_T0004, SAOUHSC_T00042 (tRNA-Gln), SAOUHSC_T00044 (tRNA-Leu), SAOUHSC_T00 49 (tRNA-Ile), SAOUHSC_T0050 (tRNA-Ala), SAOUHSC_T00058 (tRNA-Arg), SAOUHSC_T00059 (tRNA-His), SAOUHSC_ T00061(tRNA-Glu), SAOUHSC_T0008 (tRNA-Lys), SAOUHSC_T0009 (tRNA-Gly)] belonged to amino acyl t RNA associated genes. There is limited evidence that aminoacyl-tRNA synthesis pathways are involved in biofilm formation in microbial populations including *S. aureus.* Aminoacyl-tRNA synthetases (aaRSs) as a class of vital and universal 'house-keeping' enzymes catalyse the esterification of amino acids to their tRNA equivalents, which is

required for decoding genetic information during protein synthesis (35). Hence, targeting the aminoacyl tNRA biosynthesis pathway could be an effective antimicrobial intervention through its anti-biofilm function in chronic foot ulcers where *S. aureus* happens to be a major pathogen. Previous studies demonstrated the effectiveness of compounds such as hydroxycinnamic acid, norvaline and tribenuron methyl which target leucyl t-RNA synthase of oxacillin resistant *S. aureus* (36, 37). We also found similar kind of a t-RNA aminoacyl biosynthesis which facilitates the wide range of amino acids indicating similar design and additional therapeutic candidates that target acyl tRNA synthetases for tackling biofilm producing *S. aureus.*

Additionally, we found that about one fourth of the differentially expressed genes in mature biofilms were hypothetical proteins such as SAOUHSC_00064 (dnaA), SAOUHSC_01760 (sspB), SAOUHSC_02411 (tuf), SAOUHSC_02412 (ebh), SAOUHSC_02615 (isdB), SAOUHSC_02872 (sasG), SAOUHSC_02991 (sceD) indicating their likely involvement in biofilm formation. Further studies are warranted characterising the function and possible role of these hypothetical proteins in biofilm formation. This could further lead to developing robust antibiofilm formulations that target as many pathways as possible.

We also observed that the VraR (SAOUHSC_02098) gene was upregulated in the biofilm producing *S.aureus* isolates. VraR plays a role in cell wall peptidoglycan synthesis and hence is critical for populating the biofilm community in *S. aureus.* VraR has been already implicated in biofilm production as reported previously by Yan X.etal on *Staphylococcus aureus* subsp. *aureus* strain ATCC 6538 (38). Other genes like phi PV83 orf 20-like protein (SAOUHSC_02069), and integrase-like protein (SAOUHSC_00352) were also upregulated, however, the specific association of these genes to biofilm needs further investigation. We propose future studies that confirm or implicate the role of these upregulated genes in biofilm production through gene knockout studies using transposon-mediated targeted mutagenesis. Alternative strategies include inhibiting up-regulated genes with existing inhibitors or developing new ones.

Tomlinson BR et al., demonstrated six important ribosome assembly proteins (rplX, rplE, rpsN, rpsH, rplF and rplR,) and they were under expression up to 50% in 10 hours old biofilm in the *S. aureus* CDC-types (USA100-USA500) (38). Formation of biofilm architecture consists of several phases, including adsorption, adhesion, microcolony development, maturation, and dispersion. It has been observed that in *S. aureus*, a 24-hour gown biofilm is considered a mature biofilm (39). The current study subjected 24-hour-old mature biofilms to transcriptomic analysis, revealing a 38% decrease in the expression of two additional ribosome assembly proteins (rrs and rrf) in clinical isolates. The impairment of translational function, as indicated by reduced ribosomal protein expression, is a hallmark of physiological dormancy. Such dormancy promotes the persistence of bacterial populations by facilitating the continuous production of persister cells, which are more frequently present in biofilms than in planktonic populations (40). This altered physiological state further contributes to antimicrobial resistance, a feature commonly associated with biofilm-associated infections (41). Interestingly, other studies have also reported similar patterns of ribosome assembly protein downregulation and decreased transcriptional activity in 10-hour-old biofilm populations derived from reference lineage strains (42). The current study, however, is based on freshly isolated *S. aureus* from chronic foot ulcers, providing a real-time perspective on biofilm adaptation within the human host. While data from 10-hour-old biofilms remain limited, the observed downregulation of ribosome assembly proteins in mature (24-hour-old) biofilms suggests a continued transcriptional suppression mechanism that supports persister cell maintenance. This phenomenon could be a significant contributing factor to the persistence and colonization of *S. aureus* in chronic wound environments. To summarize, this is the first report that examines likely contributing factors for biofilms formation in *S. aureus* on fresh clinical isolates isolated from chronic leprosy foot ulcers, through comparing the global transcriptome profile of biofilm populations with that of the planktonic cells. This study was approved by the institutional review board of LEPRA Society – Blue Peter Public Health and Research Centre, Hyderabad, India.

Study limitations. The small sample size as one of the limitations of the study. Given the mechanistic and exploratory nature of the study, its leads could further be expanded to study large number of clinical isolates. The study specifically focused on the diversity observed among clinical isolates with respect

to the biofilm production. The study samples were selected based on strong biofilm positivity, a critical factor that is known to be associated with AMR and negative clinical outcomes. Authors also envisage incorporating standard strain in future comparative studies with large number of samples on various stages of wound healing in foot ulcers to further understand the biofilm formation pathways. Moreover, qPCR (real-time PCR) remains a widely accepted method for confirming RNA-Seq findings, especially when focusing on specific genes of interest. However, this was an exploratory study, which focused on global gene expression analysis rather than targeting specific gene expression patterns.

CONCLUSION

We concluded that biofilm producing *S. aureus* is the most common organism implicated in secondary infections of leprosy foot ulcers. We report the first global gene expression data of biofilm producing *S. aureus* freshly isolated from leprosy foot ulcers. The aminoacyl t- RNA biosynthesis pathway is one of the key mechanisms in biofilm forming clinical isolates. Simultaneously, cell wall peptidoglycan synthesis genes, integrase-like protein coding genes are up regulated. Study also confirms the downregulation of ribosomal assembly proteins and reduced translation within biofilms. Further research is suggested to identify appropriate molecular markers that target one of these critical pathways and thus prevent the formation or progression of biofilms.

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