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ORIGINAL ARTICLE

# *Streptococcus dysgalactiae subsp.-equisimilis* as an emerging secondary pathogen in leprosy foot ulcers

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## ABSTRACT

**Background and Objectives:** Leprosy foot ulcers (LFU) tend to become chronic due to secondary bacterial infections, leading to subsequent disfigurement and disability. Treatment modality for infected plantar ulcers thus so far is majorly based on conventional approach of empirical antibacterial therapy. However, this approach tends to overlook unconventional pathogens which are likely to be present in the LFU.

**Materials and Methods:** Twenty-six leprosy patients (17 males and 9 females) who had completed multidrug therapy (MDT) and those are suffering from foot ulcer were included. Using sterile cotton swabs, two wound swabs were collected, of these; one for bacterial culture and another for NGS (Next Generation Sequencing).

**Results:** Out of 26 samples tested on conventional bacterial culture, *Streptococcus* spp. (50%) was predominant organism. On NGS, 09/26 (34.61%) showed *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS 12* as the most abundant single organism, along with some unknown and unclassified organisms; 03/26 (11.5%) were *Arcanobacterium-haemolyticum-DSM-20595* alone and 02/26 (7.69%) were *Streptococcus-pyogenes alone*. A combination of *Arcanobacterium-haemolyticum-DSM-20595* and *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS 124* was found in nine (34.61%) specimens.

**Conclusion:** Polymicrobial infection with conventional and unconventional pathogenic bacteria is another notable finding suggesting appropriate interventions. The study findings also reiterate the need for understanding the polymicrobial infections and their role in the clinical progression of the LFU.

**Keywords:** Leprosy; Foot ulcer; 16S rRNA; Microbiome; Next generation sequencing

## INTRODUCTION

Leprosy, also known as Hansen's disease, is a chronic infectious disease of skin and peripheral nerves caused by *Mycobacterium leprae.* India has the highest leprosy burden in the world, with approximately 150,000 new cases detected each year (1). Chronic foot ulcers are one of the complications of leprosy secondary to peripheral nerve damage and subsequent sensory loss. Untreated secondary bacterial infections are one of the established causes of chronicity of ulcers, leading to subsequent disfigurement and disability (2). Current global leprosy control strategy by the WHO aims at achieving zero disability by the year 2030 (3). Secondary infections on chronic leprosy foot ulcers (LFUs) compel the affected persons

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to make multiple visits to the health facilities (even after many years of completing the multidrug therapy), which have cost implications in terms of the lost wages and recurrent health care expenditures (4). Hence proper identification of secondary pathogens and appropriate antimicrobial treatment forms one of the important components of managing chronic LFU (5). Previous studies from our laboratory and other settings demonstrated diversity and dynamicity of the secondary microbial profile across the geographies and time points, thus indicating the need for periodic surveillance for the secondary bacterial profile to formulate appropriate antimicrobial policies (6-8). Though, physicians in tertiary care settings largely rely on periodic profiling of microbial pathogens by conventional aerobic bacterial culture (CBC) for formulating customised antimicrobial policies, current management for infected LFU which happens mainly at primary care settings has been only through empirical antibiotic treatment without any specific bacterial profiling. Recent evidence on the occurrence of several newly emerging pathogen species such as *Streptococcus dysgalactiae* and *Arcanobacterium hemolyticus* on diabetes foot ulcers warrants the application of advanced molecular tools such as Next generation sequencing(NGS) for the microbial surveillance (9). NGS offers site specific characterization of microbial profiles that will help in understanding the pathogenesis of the infection and formulate targeted infection control strategies (10). With this background, the present study aimed at investigating the profile of secondary bacterial pathogens on infected LFUs using conventional and NGS-based bacterial 16s rRNA identification methods. The study findings are expected to inform the healthcare providers for formulating effective antimicrobial regimen covering the spectrum of the potential pathogens.

## MATERIALS AND METHODS

**Recruitment of subjects and specimen collection.** Leprosy patients with infected chronic foot ulcers (17 males and 9 females) who attended foot care clinic at LEPRA Blue Peter Public Health and Research Centre (LEPRA - BPHRC), Hyderabad, India, between April 2018 and October 2018 were enrolled into the study after obtaining an informed consent. All the 26 patients have completed multidrug therapy for leprosy at least 5 years before the study. The study protocol was approved by the institutional ethical committee of LEPRA Society- Blue Peter Public Health and Research Centre (LEPRA/IEC/2017/4.1).

**Specimen collection.** Two sterile swab (pure viscose swabs, Hi-media, Mumbai, India) specimens one each for bacterial culture and for NGS were collected from each of the LFUs. Briefly, the ulcer surface was superficially cleaned with a sterile moist swab which was subsequently discarded. The specimens for the microbial testing were then collected one after the other by rotating the swab on the entire ulcer surface and then transported immediately to the laboratory in two separate tubes with sterile 1X PBS (phosphate buffered saline). Specimens designated for the CBC were stored at 4°C and inoculated onto the bacterial culture media within 6 hours of collection and the specimen for NGS was frozen at -20°C until extraction of genomic DNA for the downstream processing.

**Aerobic bacterial culture.** The swab intended for culture was inoculated on sterile blood agar and McConkey agar and incubated overnight at 37°C. Bacterial isolates were identified by phenotypic characteristics such as colony morphology, Gram's stain, and biochemical tests as recommended by the standard guidelines (11).

**Next generation sequencing.** Genomic DNA was extracted from the swab using the QIAamp microbiome DNA Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany) according to the manufacturer's instructions. DNA was eluted with 20 µl of AVE buffer supplied by Qiagen. DNA quality was checked using Nanodrop1000 (Thermo Scientific, USA) and Qubit 2.0 Fluorometer (Life Technologies, India).

The V3-V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using primers 5' CCTACGGGNBGCASCAG 3' and 5'ACTACNVGGGTATCTAATCC 3'. PCR reactions were performed in 25 mL volume with 1.25 units of NEB DNA polymerase (M0273, New England Biolabs, USA), genomic DNA and 0.25µM forward and reverse primers with optimised thermal condition as described by previously by Takahashi et al. (12). Amplicon libraries were prepared according to the standard Illumina protocol described by Dehingia et al. (13). Libraries were sequenced (Illumina HiSeq 250bp × 2 paired-end protocol chemistry) at Agri Ge-

nome Labs Pvt Ltd, Kerala, India.

## RESULTS

**Bacterial profile by conventional aerobic bacterial culture.** Out of 26 specimens tested 13 (50%) were *Streptococcus* spp., 02 (7.69%) were *Staphylococcus aureus*, 02 (7.69%) mixed cultures of *Streptococcus* spp. and *Staphylococcus aurues* and another 02 (7.69%) were *Streptococcus* spp. and Gram-negative bacilli and Five (19.25%) yielded Gram positive bacillus which were originally considered as skin contaminants and two were culture negative (7.69%)

**Bacterial profile by16S RNA sequencing.** Out of 26 specimens on NGS, 09 (34.61%) were *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_12* as the most abundant single organism along with some unknown and unclassified organisms; 03/26 (11.5%) were *Arcanobacterium-haemolyticum-DSM-20595* alone and 02/26 (7.69%) were *Streptococcus-pyogenes*. Nine (34.61%) specimens demonstrated a combination of *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* and *Arcanobacterium-haemolyticum-DSM-20595.* Two specimens have demonstrated only unknown organisms which correlated with negative culture on CBC. 24/26 (92.3%) specimens had concordant results on both the methods. Sixteen specimens that yielded *Streptococcus* species on culture were also identified to species level as *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* on the NGS. Four specimens that yielded gram positive bacilli on the conventional culture were identified as *Arcanobacterium-haemolyticum-DSM-20595* and both the samples that were culture negative were also negative for any known pathogenic organisms on the NGS (Table 1). The operational taxonomic units (OUT) at species level were shown as Fig. 1.

There is no major discrepancy in the data generated by two methods. 92.3% (23/26) of specimens had concordant results on both the methods. One specimen yielded *Staphylococuus aureus* on culture while NGS yielded others (meaning no identifiable single species). Two specimens were culture negative; while on NGS, one of these two specimens yielded *Peptoniphilus* sp.BG3 (14.9%), uncultivanle bacterium (2.0%), *Helcococcus kunzii* (1.77%) and *Streptococcus dysgalactiae sub sp. Equisimilis* (1.7%) and *Corynebacterium* sp. (1.19%) with 77.8% mean abundance of other species on NGS. The second specimen yielded unknown species (30.9%) followed by uncultured bacterium (15.2%), Campylobacter ureolyticus (3.3%), *Peptoniphilus asaccharolyticus* (3.3%), *Streptococcus dysgalactiase sub sp. equisimilis, Corynebacterium sp.* (1.7%) and predominant abundance of anaerobic bacteria namely *Arcanobacterium haemolyticum* (41.1%) (Table 1).

## DISCUSSION

Secondary bacterial infections are one of the commonest causes for the chronicity and consequences of chronic ulcers in leprosy (6). Currently antimicrobial resistance (AMR) stewardship policies aim at preventing AMR through appropriate prescription practices basing on periodic microbial profiling (14). However, LFU management which is mostly being done at primary level health care does not follow any such standard for site specific or institution specific profiling. Current study findings formed a basis for undertaking such profiling exercise in a leprosy specific primary health care setting through comparing the usefulness of conventional and NGS based microbial characterization.

Present study reports for the first time the occurrence *Streptococcus dysgalactiae* as the most frequent bacterial pathogen on infected LFU followed by *Arcanobacterium haemolyticum* (Table 1). Our findings are different from those of Saha (India) and Gelatti (Brazil) who reported *Staphylococcus aureus,* Ramos (Ethiopia) who reported Proteus spp. and a previous study from this laboratory (India) which reported *Staphylococcus aureus* and *Pseudomonas* spp., (6-8, 15) could be due to the reason that these studies were reported based on CBC alone. *Streptococcus dysgalactiae*, which were earlier known to be part of the normal human microbial flora (16), have recently been implicated with severe soft tissue infections (17). Pathogenic molecules such as adhesins and fibronectin binding proteins that are secreted by the *Streptococcus dysgalactaiae* could explain their colonisation and invasion of skin tissues and are hence believed to be associated with the chronicity of the LFU (18). Malini et al. from India reported *Arcanobacterium haemolyticum* from cellulitis and wound infections in diabetic foot ulcers12 but this is the first report on such occurrence in leprosy foot ulcers. Polymicrobial infections as observed by both

**Table 1.** Summary of bacterial profile: Aerobic bacterial culture vs NGS

| S. No | Specimen Lab Id No. | Culture results | NGS based microbiome results* |
|---|---|---|---|
| 1. | 03 | Gram positive bacillus | *Arcanobacterium-haemolyticum-DSM-20595* |
| 2. | 05 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* |
| 3. | 06 | Gram positive bacillus | *Arcanobacterium-haemolyticum-DSM-20595, Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* |
| 4. | 07 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* |
| 5. | 08 | Gram positive bacillus | *Arcanobacterium-haemolyticum-DSM-20595* |
| 6. | 09 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* |
| 7. | 10 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* |
| 8. | 11 | *Streptococcus* spp., *Staphylococcus aureus* | *Streptococcus-pyogenes* |
| 9. | 12 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124, Arcanobacterium-haemolyticum-DSM-20595* |
| 10. | 13 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* |
| 11. | 14 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* |
| 12. | 15 | Culture negative | Others |
| 13. | 16 | Gram positive bacillus | *Arcanobacterium-haemolyticum-DSM-20595* |
| 14. | 17 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124, Arcanobacterium-haemolyticum-DSM-20595* |
| 15. | 21 | *Staphylococcus aureus* | *Streptococcus-pyogenes* |
| 16. | 23 | Culture negative | *Unknown and Others* |
| 17. | 24 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124, Arcanobacterium-haemolyticum-DSM-20595* |
| 18. | 29 | *Streptococcus* spp, Gram positive bacillus | *Arcanobacterium-haemolyticum-DSM-20595, Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* |
| 19. | 31 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* |
| 20. | 33 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* |
| 21. | 35 | *Streptococcus* spp., Gram positive bacillus | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124, Arcanobacterium-haemolyticum-DSM-20595* |
| 22. | 36 | Gram positive bacillus | *Arcanobacterium-haemolyticum-DSM-20595, Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* |
| 23. | 37 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124, Arcanobacterium-haemolyticum-DSM-20595* |
| 24. | 38 | *Streptococcus* spp., *Staphylococcus aureus,* | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124, Arcanobacterium-haemolyticum-DSM-20595* |
| 25. | 39 | *Staphylococcus aureus* | *Unknown and Others* |
| 26. | 40 | *Streptococcus* spp. | *Streptococcus-dysgalactiae-subsp.-equisimilis-GGS_124* |

*Most abundant organisms; Others: The taxa other than top 10 are categorized as others; unknown: The sequences that does not have any alignment against taxonomic database are categorized as Unknown

CBC and NGS is consistent with the common belief that chronic open wounds tend to be poly microbially colonised (19). However, what is alarming is the mixture of *Streptococcus dysgalactiae* and *Arcanobacterium hemolyticus*-DSM-20595 along with *Facklamia-ignava*-CCUG-37419 and *Staphylococcus aureus.* The presence of *Streptococcus dysgalactaiae* and its coexistence with opportunistic bloodstream pathogens such as *Facklamia-ignava*-CCUG-37419 sheds new light on the expanding aetiology of infected LFUs. Heat map analysis was performed to show the high abundance of microorganisms when distributed by species (Fig. 2). NGS can sequence DNA or RNA of all possible pathogens directly from clinical

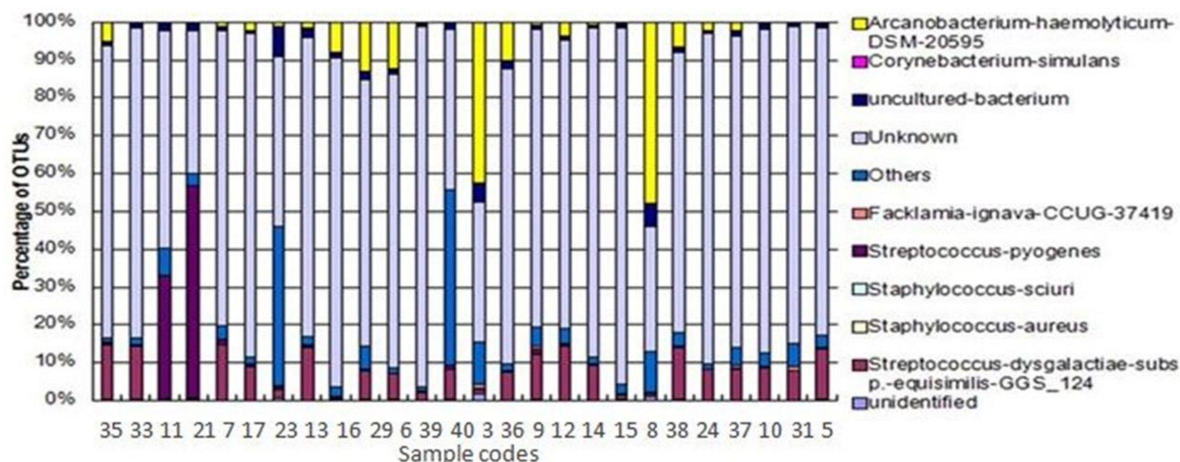


**Fig. 1.** The operational taxonomic units (OUT) at species level

**Fig. 2.** Heat map analysis showing high abundance of microorganisms when distributed by species

samples, without the need for colony isolation from culture medium or sequence-specific amplification (20). Sequencing can also help in identifying uncultivable pathogens and emerging pathogens if any. Hence, we used wound swabs as a test specimen for the source of microbial DNA, 16s rRNA amplification, and sequencing by NGS. We found some the rare and uncultured genus such as *Anaerovorax-sp, Filifactor, Finegoldia, Gemella Howardella, Howardella, Hydrogenophilus, Johnsonella, Macrococcus, Proteiniclasticum, Proteiniclasticum, Proteiniclasticum, Trueperella.*

LFU and DFU are different from one another, however they can both cause severe loss of sensation in the feet (21) which increases the risk of ulceration and painless injury (2). More Gram-positive bacterial infections, primarily with *S. aureus,* were seen in most leprosy cases with LFU (6, 7) whereas in DFU in diabetes, gram-negative bacterial infections were more common than gram-positive bacterial infections (22). Excess hyperglycemia causes infection in tissues, which in turn spreads tissue damage (23), in diabetic patients. All the leprosy patients with LFU enrolled in the study had infected ulcers with cellulitis i.e., swelling, pain, purulent discharge and involvement of regional lymph-nodes. Since this is a

preliminary cross-sectional study, only aerobic culture and NGS methods were performed to construct the microbial profile and did not include normal skin for its microbial flora for comparison. It is one of the limitations of the study at this point; longitudinal studies along with normal skin sampling at multiple time points may shed more light on this matter. The bacterial profile that emerged from the current study demonstrates the utility of a robust method such as NGS sequencing for identification of conventional as well as newly emerging pathogens. While the limited resources could still be a barrier to undertake the sequencing as a routine profiling method, it could certainly help in better understanding of the changing trends of secondary bacterial pathogens. This not only helps in formulating effective antimicrobial regimen but also in preventing antimicrobial resistance that could arise from an indiscriminate use of antimicrobials. The findings also help physicians in understanding the need for a high degree of clinical suspicion and asking for better tests to identify the emerging bacterial pathogens. Although NGS currently appears to be an advanced molecular assay, there is a likelihood of its improvisation and simplification to make it a field friendly and cost-effective tool that removes the access barrier.

**Study limitations.** The sample size is limited owing to the expensive nature of the NGS testing in its current format. Further studies on a larger number of samples are currently underway to validate the preliminary findings from this study. We tried to minimise the collection bias of open chronic wound, by cleaning the ulcer surface with sterile saline, just before the sample collection. Our results suggest the presence of polymicrobial infections in the majority of samples studied. However, the clinical significance of such a poly microbiome is yet to be studied. Longitudinal studies along with normal skin sampling at multiple time points may shed more light on this matter.

## CONCLUSION

The study reports for the first time the occurrence of *Streptococcus dysgalactiae* as the most frequent organism followed by *Arcanobacterium haemolyticum.* The study findings demonstrated for the first time the utility of the NGS for profiling the infected leprosy foot ulcers to identify the emerging and non-cultivable pathogens which will have far reaching implications in formulating effective antimicrobial regimen. We highlight the occurrence of polymicrobial infections with a mixture of conventional and unconventional pathogens, which indicate further longitudinal studies to understand their clinical implications. Infection prevention and control in foot ulcers is one of the essential components in preventing leprosy related disability. Hence NGS based profiling have a great potential in formulating suitable infection control and AMR prevention strategies. Attempts should also be made for development of field friendly NGS tools to make it more accessible for the leprosy endemic settings.

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