Current Trends in Biotechnology and Pharmacy
Vol. 20(2) 2978-2991 April 2026, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2026.2.21

Screening of Biofilm forming Bacterial Isolates from Chronic Leprosy Foot Ulcers

Dr. Sreedevi Sarsan[1]*, Adithi Pavan Sai[1], K. Ruthvik[1], Dr. Kumar Ebineshan[2]

1: Department of Microbiology, School of Allied and Healthcare Sciences, Malla Reddy University, Hyderabad, Telangana, India
2: Scientist, Department of Microbiology, LEPRA Society - Blue Peter Public Health and Research Centre, Hyderabad, Telangana, India

***Corresponding Author:** dr.sreedevi@mallareddyuniversity.ac.in

Abstract

Leprosy, a chronic infectious disease caused by *Mycobacterium leprae*, often leads to severe complications such as chronic foot ulcers due to nerve damage and loss of protective sensation. These ulcers are frequently colonized by secondary bacterial pathogens, further delaying wound healing. One of the major challenges in treating these ulcers is the presence of biofilm-forming bacteria, which protect microbes from antibiotics and the host immune System, making infections persistent and difficult to eradicate. This study was conducted to isolate bacterial species from chronic leprosy foot ulcers and evaluate their biofilm-forming abilities. Swab samples were collected from ulcer sites of leprosy patients and cultured on selective media. The bacterial isolates were identified through standard cultural, morphological, and biochemical methods. Biofilm production was assessed using Congo Red Agar (CRA) and the Microtiter Plate (MTP) assay. Among the isolates, *Staphylococcus aureus* was the most predominant species, followed by *Pseudomonas* spp., *Klebsiella* spp., and *Escherichia coli*. A significant proportion of these isolates exhibited strong biofilm-forming ability, particularly *Staphylococcus aureus*. The findings emphasize the importance of early detection of biofilm-producing bacteria in chronic leprosy ulcers to guide appropriate antimicrobial therapy, reduce complications, and improve patient outcomes.

Keywords

Leprosy, Foot ulcers, Biofilms, secondary bacterial pathogens, Congo Red Agar, Microtiter Plate assay

Introduction

Leprosy, also known as Hansen's disease, is a chronic infection mainly caused by *Mycobacterium leprae*. These bacteria primarily affect the skin and peripheral nerves. Despite being curable with multidrug therapy, leprosy remains a significant public health concern due to its ability to cause permanent nerve damage, disability, and social stigma. Leprosy is classified into various types based on clinical presentation, bacteriological load, and immune response of the patient (1). Environmental factors like malnutrition, poverty, and poor sanitation have been linked to an increased risk of developing leprosy. The clinical manifestations of leprosy vary depending on the immune response. Common symptoms include hypopigmented or reddish skin lesions, often with loss of sensation due to nerve damage. Patients may also experience thickened peripheral nerves, muscle weakness, numbness, dry or ulcerated skin, and in advanced cases, deformities of the hands, feet, and face. Nerve damage can cause trophic ulcers and progressive disabilities if treatment is delayed (2).

Leprosy foot ulcers

Leprosy foot ulcers primarily result from the peripheral neuropathy associated with *Mycobacterium leprae* infection, which damages the sensory, motor, and autonomic nerves supplying the feet. Leprosy foot ulcers are chronic, non-healing wounds that typically develop in areas of the foot subjected to repeated pressure or trauma, especially in patients with sensory loss due to peripheral neuropathy. Motor nerve damage leads to muscle weakness and deformities such as claw toes or foot drop, which cause abnormal weight distribution during walking, creating pressure points that further predispose to ulcer formation. These ulcers often begin as small blisters or superficial wounds that progressively deepen due to continuous unnoticed trauma, particularly in weight-bearing areas like the metatarsal heads, heels, and lateral borders of foot (3, 4, 5)**.** The hallmark characteristic is their painless nature, resulting from damage to sensory nerves by *Mycobacterium leprae* (6). Over time, these ulcers may lead to deformities, contractures, and even amputation if not properly treated. Socioeconomic factors, such as poverty and stigma associated with leprosy, further delay timely medical intervention, contributing to ulcer chronicity and severity (4, 7). Leprosy foot ulcers can be broadly classified into neuropathic ulcers, neurotrophic ulcers, and trophic ulcers, all of which primarily result from peripheral neuropathy associated with *Mycobacterium leprae* infection (Table 1).

Table 1: Types and Characteristics of Leprosy-associated Foot ulcers

S.No	Types of Leprosy Foot Ulcers	Characteristics
1	Neuropathic Ulcers	Painless wound, Pressure points, Callus formation, Well-defined edges.
2	Neurotrophic Ulcers	Specifically related to sensory nerve damage, Deep, chronic, and slow-healing.
3	Trophic Ulcers	Pressure and poor skin nutrition, muscle weakness, deformities, and dry skin.
4	Plantar Ulcers	Located on the sole of the foot, Usually occur at metatarsal heads, heels.
5	Secondary Infected Ulcers	Pus formation, Swollen surrounding tissue, Foul odor.

Pathogenic bacteria in leprosy foot ulcers

Secondary bacterial infections are common, especially when ulcers remain open for prolonged periods frequently leading to foul-smelling discharge, cellulitis, or even osteomyelitis (8, 9)**.** Leprosy foot ulcers are frequently complicated by secondary bacterial infections, as the loss of protective sensation in leprosy patients often leads to chronic, open wounds that are susceptible to microbial invasion. These open wounds serve as an ideal environment for bacterial colonization, with *S. aureus* being the most commonly isolated organism. The bacterium adheres to damaged tissue, forming biofilms that protect it from both host immune responses and antibiotics, making infections persistent and recurrent (10,11)**.** *Pseudomonas aeruginosa* is particularly associated with ulcers that have a foul-smelling discharge and a greenish exudate, especially in cases with prolonged moisture exposure (6)**.** This gram-negative bacillus thrives in moist, warm environments, making infected leprosy ulcers, especially those with inadequate wound care, an ideal site for colonization. Due to peripheral neuropathy in leprosy patients, foot ulcers often go unnoticed and untreated, creating an ideal environment for colonization by opportunistic pathogens like *K. pneumoniae.*

Significant bacterial contamination of ulcers was observed comprising of Gram-negative bacteria in 80% of cases, Gram-positive cocci in 63% of cases and mixed microflora in 36% of cases suggesting a complex microbial environment where different bacteria interact, potentially making infections more difficult to treat (12)**.** Several pathogenic bacteria have

been commonly isolated from infected leprosy foot ulcers. The most frequently identified organisms include *Staphylococcus aureus*, particularly methicillin-resistant Staphylococcus aureus (MRSA) strains, *Pseudomonas aeruginosa*, *Proteus* species, *Escherichia coli*, and *Klebsiella species* (13,14,15). These bacteria often colonize the ulcer surface and can penetrate deeper tissues, leading to complications such as cellulitis, abscess formation, and even osteomyelitis in advanced cases. Additionally, anaerobic bacteria such as *Bacteroides* and *Clostridium* species may be involved, particularly in deep or necrotic ulcers with poor oxygenation, leading to foul odour and potentially gas gangrene in severe cases (16). Fungal infections caused by *Candida albicans* or *Aspergillus* species may co-infect ulcers, especially in immunocompromised leprosy patients or those on prolonged antibiotic treatments (14). The polymicrobial nature of many leprosy foot ulcers complicates treatment, often requiring broad-spectrum antibiotics guided by culture and sensitivity testing. Inadequate hygiene, delayed medical attention, and compromised immunity in affected individuals further contribute to bacterial colonization and infection.

Biofilm forming bacteria in leprosy foot ulcers

Biofilm formation by pathogenic bacteria plays a critical role in the chronicity and poor healing of leprosy foot ulcers. A biofilm is a structured community of microbial cells encased in a self-produced extracellular polymeric substance (EPS) that adheres to wound surfaces. This protective layer shields the bacteria from antibiotics and the host immune system, leading to persistent infections (11, 17, 18). Several bacteria commonly isolated from leprosy foot ulcers are notorious biofilm formers, including *Staphylococcus aureus*, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli*, and *Proteus* species (10, 19, 20). Biofilms play a critical role in the chronic nature of leprosy ulcers by creating a protective barrier around bacteria, making infections persistent and difficult to treat. These microbial communities allow bacteria to evade immune responses and resist antibiotics, leading to prolonged inflammation and delayed healing. The presence of biofilms in leprosy foot ulcers makes conventional antibiotic treatments largely ineffective, often necessitating mechanical debridement, long-term antimicrobial therapy, and in some cases, the use of anti-biofilm agents like silver dressings or enzymatic debrides. Recognition of biofilm formation is therefore essential in developing effective management strategies for chronic, non-healing leprosy ulcers.

The study on biofilm formation in leprosy foot ulcers primarily aims to understand the role of bacterial biofilms in the chronicity and treatment resistance of these ulcers. The presence of biofilms in leprosy foot ulcers necessitates additional research to understand their impact on clinical outcomes and to develop targeted treatments. This knowledge is essential for creating combination treatments that integrate biofilm-disrupting agents with traditional antimicrobial therapies, offering new hope for patients suffering from chronic leprosy ulcers. Thus, the aim of the present study was to screen for biofilm forming bacteria from leprosy foot ulcers.

Although several studies have reported the bacteriological profile and biofilm- forming ability of pathogens isolated from leprosy foot ulcers, region-specific data remain limited, particularly from South India. The microbial spectrum, biofilm prevalence, and clinical behavior of chronic ulcers are influenced by local factors such as climate, wound-care practices, antibiotic usage patterns, and access to healthcare services. This study represents one of the few systematic investigations from the Telangana–South India region assessing both the bacteriological profile and biofilm-forming capacity of isolates from chronic leprosy foot ulcers. In addition, the present study employs both Congo Red Agar (CRA) and Microtiter Plate (MTP) methods simultaneously, allowing a compar-

ative evaluation of qualitative and quantitative biofilm detection techniques in a leprosy- specific clinical context. This dual method approach provides practical methodological insight for routine diagnostic laboratories in resource-limited settings. Therefore, this study aims not only to confirm the presence of biofilm-forming bacteria in leprosy ulcers but also to generate region-specific evidence and clinically relevant insights that may aid in improving wound management strategies in Indian leprosy care programs.

Methods and Materials

Sample collection

Swab samples were collected from chronic foot ulcers of leprosy patients using sterile gloves, ensuring aseptic technique throughout the procedure. A total of 24 samples were obtained from both male and female patients (Fig 1). Prior to sample collection, the ulcer surface was gently cleansed with sterile saline or water to remove any debris, slough, or surface contaminants, taking care to avoid the use of antiseptics that could interfere with the recovery of microorganisms. A sterile swab was then carefully inserted into the deeper portion of the ulcer and gently rotated to collect exudate and microbial flora from the wound bed. Care was taken to avoid contact with the surrounding skin or ulcer margins to prevent external contamination of the sample.

Inclusion and exclusion criteria

Adult patients aged 18 years or older with leprosy-related foot ulcers who are willing to participate in research studies were included, while patients with non-leprosy-related ulcers, those under 18 years of age, and those unwilling to participate were excluded in the study.

Isolation of bacteria

Clinical samples collected were inoculated onto nutrient agar and selective media such as Mannitol Salt Agar, Mac Conkey Agar, and Blood Agar, and incubated at 37 °C for 24 to 48 hours (Fig 2). Following incubation, distinct bacterial colonies were observed for their colony morphology characteristics, including size, shape, margin, elevation, and pigmentation. To obtain a pure culture, a well-isolated single colony was carefully picked and subcultured onto a fresh agar plate. The subculture was incubated under the same conditions to ensure the development of isolated bacterial growth. Once pure colonies were obtained, they were subjected to Gram staining and a series of biochemical tests to facilitate accurate bacterial identification.

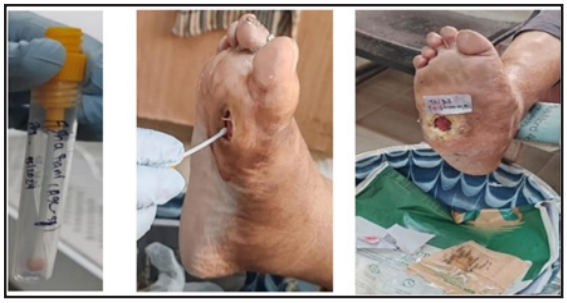

Figure 1: Collection of Samples from Patients with Leprosy Ulcers

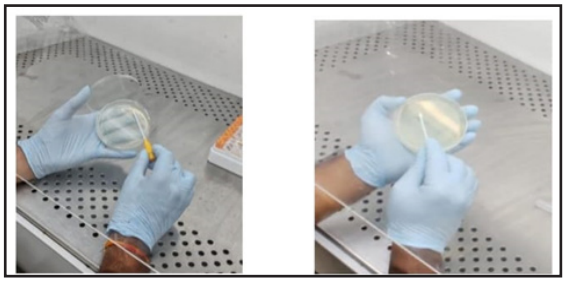

Figure 2: Inoculation of Ulcer Swabs on Media Plates

Identification of bacterial isolates

Bacteria identification involves microscopic observations, cultural characteristics, and biochemical tests. Microscopic observation includes Gram staining to differentiate between Gram-positive bacteria and Gram-negative bacteria. Biochemical tests, such as Indole test, Methyl red test, Voges-Proskauer test, citrate test (IMViC tests), catalase, coagulase, and urease, were performed for preliminary identification of the bacteria (21).

Biofilm detection

The biofilm forming ability of the bacterial isolates was determined by Congo Red Agar (CRA) Method and Microtiter Plate Method (MPT). The Congo Red Agar (CRA) method is a widely used, qualitative technique for detecting biofilm formation by bacterial isolates, especially from clinical samples such as leprosy foot ulcers. The method relies on the ability of biofilm-producing bacteria to synthesize exopolysaccharide (slime), which binds to Congo red dye, leading to characteristic color changes in bacterial colonies (22, 23)**.** To prepare CRA, a medium of Brain Heart Infusion (BHI) agar supplemented with 5% sucrose and Congo red dye (0.8 g/L) is used. The presence of sucrose promotes slime production, which is a key indicator of biofilm synthesis. After inoculation of bacterial isolates onto the CRA plate, the plates are incubated aerobically at 37°C for 24–48 hours. Biofilm-producing strains typically form black or darkly pigmented, dry, crystalline colonies, while non-biofilm producers yield red to pink, smooth colonies. Intermediate or weak biofilm formers may show colonies with a dark red or Bordeaux coloration (24)**.**

The MTP method is a quantitative assay that measures biofilm formation using crystal violet staining. The MTP method provides a more precise measurement of biofilm formation. This method is simple, inexpensive, and suitable for screening multiple bacterial isolates under various conditions. Bacterial suspensions were inoculated into 96-well microtiter plates containing Brain Heart Infusion (BHI) broth growth medium. Plates were incubated at 37°C for 24–48 hours to allow biofilm formation. Wells were gently washed with phosphate-buffered saline (PBS) to remove non-adherent cells. Biofilms were stained with 0.1% crystal violet for 15 minutes, followed by washing to remove excess dye. The bound crystal violet was solubilized using ethanol or acetic acid, and absorbance is measured at OD 570 nm using a spectrophotometer. Based on the optical density (OD) values obtained, bacterial isolates can be categorized as Non Biofilm, weak, moderate, or strong biofilm producer (Fig 3).

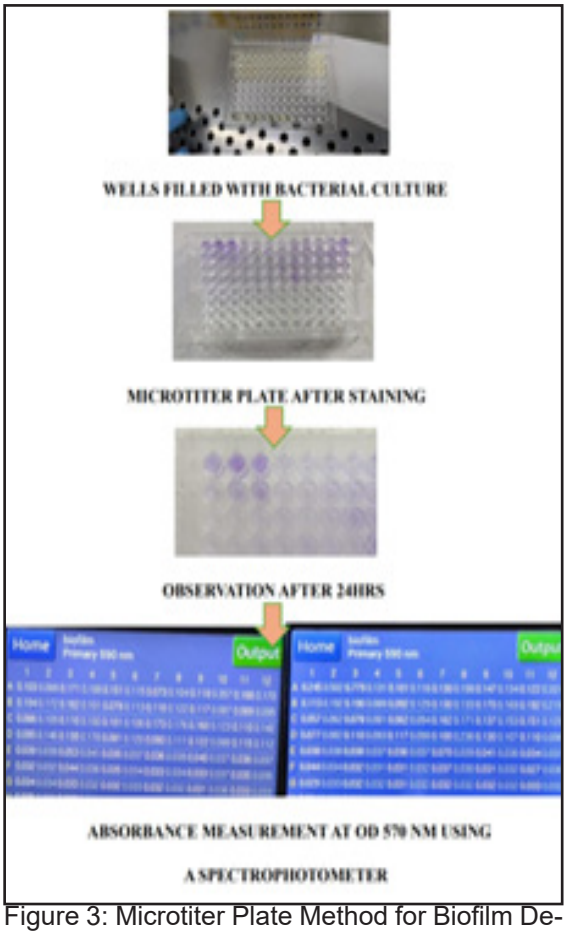

Figure 3: Microtiter Plate Method for Biofilm Detection

Results and Discussion

Isolation of bacteria

Leprosy foot ulcer swabs collected from the patients were inoculated onto the culture media like Nutrient agar, Mac Conkey agar, Blood agar, Mannitol salt agar and incubated at 37°C for 24-48 hours and cultural characteristics of the colonies obtained were observed. A detailed overview of the results is provided in (Table 2).

Table 2: Cultural Characteristics of Bacterial Isolates from Leprosy Foot ulcer Swabs

Sample	Patient Details		Bacterial Isolates	Media			
Leprosy foot ulcer swabs	Age	Gender		Nutrient Agar	Mac Conkey Agar	Blood Agar	MSA
S1	52	F	B1	Greyish-white smooth colonies	Pink colonies	Non-hemolytic	No growth
S2	22	M	B2	Creamy, Sticky , mucoid colonies	Pink mucoid colonies	Non-hemolytic	No growth
S3	41	F	B3	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies
S4	47	M	B4	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies
S5	21	M	B5	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies
S6	61	F	B6	Green pigmented flat colonies	Non lactose fermenting colonies	β-hemolytic, metallic sheen	No growth
S7	60	M	B7	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies
S8	27	M	B8	Greyish-white smooth colonies	Pink colonies	Non-hemolytic	No growth
S9	61	M	B9	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies
S10	40	M	B10	Green pigmented flat colonies	Non lactose fermenting colonies	β-hemolytic, metallic sheen	No growth
S11	50	M	B11	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies
S12	53	F	B12	Creamy, Sticky , mucoid colonies	Pink mucoid colonies	Non-hemolytic	No growth
S13	46	M	B13	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies
S14	66	F	B14	Creamy, Sticky , mucoid colonies	Pink mucoid colonies	Non-hemolytic	No growth

S15	65	F	B15	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies
S16	69	M	B16	Greyish-white smooth colonies	Pink colonies	Non-hemolytic	No growth
S17	27	M	B17	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies
S18	24	M	B18	Green pigmented flat colonies	Non lactose fermenting colonies	α-hemolytic	No growth
S19	32	F	B19	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies
S20	47	M	B20	Creamy, Sticky , mucoid colonies	Pink mucoid colonies	Non-hemolytic	No growth
S21	32	M	B21	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies
S22	52	M	B22	Green pigmented flat colonies	Non lactose fermenting colonies	α-hemolytic	No growth
S23	58	F	B23	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies
S24	59	M	B24	Golden yellow colour colonies	No growth	Non-hemolytic	Yellow coloured colonies

Preliminary identification of bacterial isolates

The bacterial isolates were subjected to preliminary identification tests like Gram staining and Biochemical tests such as IMVIC, Catalase, Coagulase, Oxidase, Triple Sugar Iron (TSI) and Urease tests. Based on cultural, morphological, and biochemical characteristics, the clinical samples were found to be positive for gram positive and gram negative bacteria. The corresponding results are presented in the (Table 3). The results indicated that the isolates identified from clinical samples comprised *Staphylococcus aureus* (54.1%), *Escherichia coli* (12.5%), *Klebsiella* (16.7%), and *Pseudomonas aeruginosa* (16.7%). The distribution patterns are depicted in the Fig 4.

Table 3: Preliminary Identification of Bacterial Isolates from Leprosy Foot ulcer Swabs

Bacterial Isolates	Gram staining	I	MR	VP	C	TSI	U	Catalase	Coagulase	Oxidase	Identified organism
B1	Gram-negative bacilli (short rods)	+	+	–	–	A/A, G	–	+	–	–	E. Coli

B2	Gram-negative bacilli	–	–	+	+	A/A, G	+	+	–	–	Klebsiella spp.
B3	Gram-positive cocci (clusters)	–	–	–	–	–	–	+	+	–	Staphylococcus spp.
B4	Gram-positive cocci (clusters)	–	–	–	–	–	–	+	+	–	Staphylococcus spp.
B5	Gram-positive cocci (clusters)	–	–	–	–	–	–	+	+	–	Staphylococcus spp.
B6	Gram-negative bacilli	+	–	–	+	K/K	–	+	–	+	Pseudomonas spp.
B7	Gram-positive cocci (clusters)	–	–	–	–	–	–	+	+	–	Staphylococcus spp.
B8	Gram-negative bacilli (short rods)	+	+	–	–	A/A, G	–	+	–	-	E. Coli
B9	Gram-positive cocci (clusters)	–	–	–	–	K/A	–	+	+	–	Staphylococcus spp.
B10	Gram-negative bacilli,	+	–	–	+	K/K	–	+	–	+	Pseudomonas spp.
B11	Gram-positive cocci (clusters)	–	–	–	–	K/A	–	+	+	–	Staphylococcus spp.
B12	Gram-negative bacilli,	–	–	+	+	A/A, G	+	+	–	–	Klebsiella spp.
B13	Gram-positive cocci (clusters)	–	–	–	–	K/A	–	+	+	–	Staphylococcus spp.
B14	Gram-negative bacilli	–	–	+	+	A/A, G	+	+	–	–	Klebsiella spp.
B15	Gram-positive cocci (clusters)	–	–	–	–	K/A	–	+	+	–	Staphylococcus spp.
B16	Gram-negative bacilli (short rods)	+	+	–	–	A/A, G	–	+	–	-	E. Coli
B17	Gram-positive cocci (clusters)	–	–	–	–	K/A	–	+	+	–	Staphylococcus spp.
B18	Gram-negative bacilli,	+	–	–	+	K/K	–	+	–	+	Pseudomonas spp.
B19	Gram-positive cocci (clusters)	–	–	–	–	K/A	–	+	+	–	Staphylococcus spp.

B20	Gram-negative bacilli,	–	–	+	+	A/A, G	+	+	–	–	Klebsiella spp.
B21	Gram-positive cocci (clusters)	–	–	–	–	K/K	–	+	+	–	Staphylococcus spp.
B22	Gram-negative bacilli,	+	–	–	+	K/K	–	+	–	+	Pseudomonas spp.
B23	Gram-positive cocci (clusters)	–	–	–	–	K/A	–	+	+	–	Staphylococcus spp.
B24	Gram-positive cocci (clusters)	–	–	–	–	K/A	–	+	+	–	Staphylococcus spp.

I – Indole; MR – Methyl red; VP – Voges Proskauer; C – Citrate; U – Urease; TSI – Triple sugar iron

+ve – positive; -ve – negative

A/A – Acid / Acid; K/A – Alkaline / Acid , G- Gas

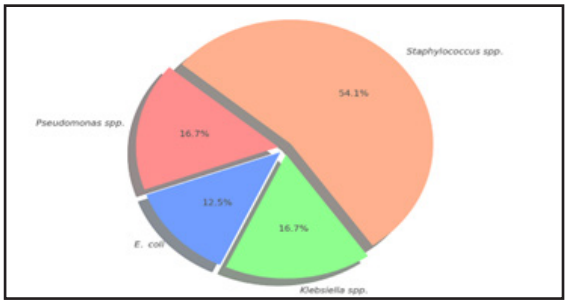

Figure 4: Percentage of bacterial pathogens isolated from leprosy foot ulcer samples

Screening of bacterial isolates for biofilm formation

Bacterial isolates were screened for biofilm formation by Congo Red Agar method and Microtiter Plate method. These isolates were subjected to biofilm screening using Congo Red Agar (CRA) and also 96-well Microtiter plate method (MTP) to determine their ability to form biofilms. The detailed results of biofilm forming ability of bacterial isolates by CRA and MTP are presented in Table 4 & 5. After incubation, the appearance of dark black colonies with a dry, crystalline texture on Congo Red Agar (CRA) indicates Strong Biofilm formation (Positive), while non-biofilm-producing microorganisms form smooth, red colonies (Negative) and Weak Biofilm Producers show some intermittent darkening and appear as Mild Black Coloured Colonies (Fig 5). The results revealed variations in biofilm production among the bacterial isolates. The CRA method predominantly classified the isolates as strong biofilm producers (45.8%), followed by non-biofilm producers (37.5%), with a smaller proportion identified as weak producers (16.7%) (Fig 6). In contrast, the MTP method showed a more balanced distribution, with weak biofilm producers (33.3%) being the most common, followed by non-biofilm producers (25%), moderate (20.8%), and strong biofilm producers (20.8%) (Fig 7).

Table 4: Biofilm Formation Ability of Bacterial Isolates by Congo Red Agar Method

Bacterial Isolates	Biofilm Forming Ability - Categories
B1	Non - Biofilm Producer
B2	Strong Biofilm Producer
B3	Non - Biofilm Producer
B4	Strong Biofilm Producer
B5	Strong Biofilm Producer
B6	Strong Biofilm Producer
B7	Weak Biofilm Producer
B8	Weak Biofilm Producer
B9	Non - Biofilm Producer
B10	Non - Biofilm Producer
B11	Non - Biofilm Producer

B12	Non - Biofilm Producer
B13	Weak Biofilm Producer
B14	Strong Biofilm Producer
B15	Strong Biofilm Producer
B16	Strong Biofilm Producer
B17	Non - Biofilm Producer
B18	Non - Biofilm Producer
B19	Weak Biofilm Producer
B20	Strong Biofilm Producer
B21	Strong Biofilm Producer
B22	Strong Biofilm Producer
B23	Non - Biofilm Producer
B24	Strong Biofilm Producer

Table 5: Biofilm Formation Ability of Bacterial Isolates by Microtiter Plate Method

Bacterial Isolates	Biofilm Formation - Categories
B1	Strong Biofilm
B2	Strong Biofilm
B3	Strong Biofilm
B4	Moderate Biofilm
B5	Moderate Biofilm
B6	Weak Biofilm
B7	Weak Biofilm
B8	Weak Biofilm
B9	Weak Biofilm
B10	No Biofilm
B11	No Biofilm
B12	No Biofilm
B13	Moderate Biofilm
B14	Moderate Biofilm
B15	Moderate Biofilm
B16	Strong Biofilm
B17	Weak Biofilm
B18	Weak Biofilm
B19	Strong Biofilm
B20	Weak Biofilm
B21	Weak Biofilm
B22	No Biofilm
B23	No Biofilm
B24	No Biofilm

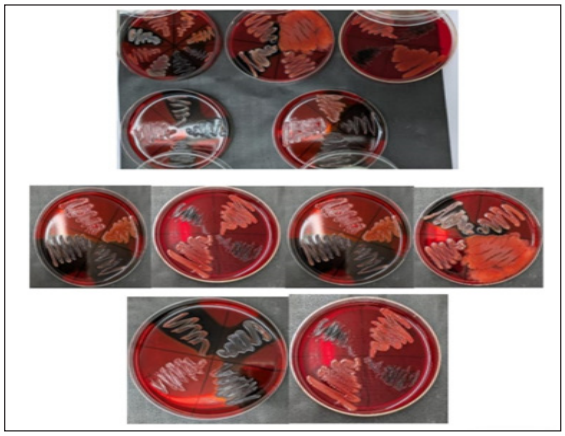

Figure 5: Biofilm formation by bacterial isolates on Congo-red agar

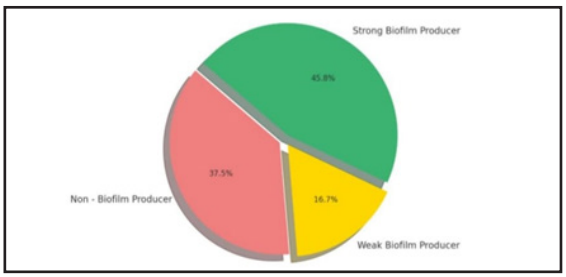

Figure 6: Categorization of biofilm forming bacterial isolates by CRA method

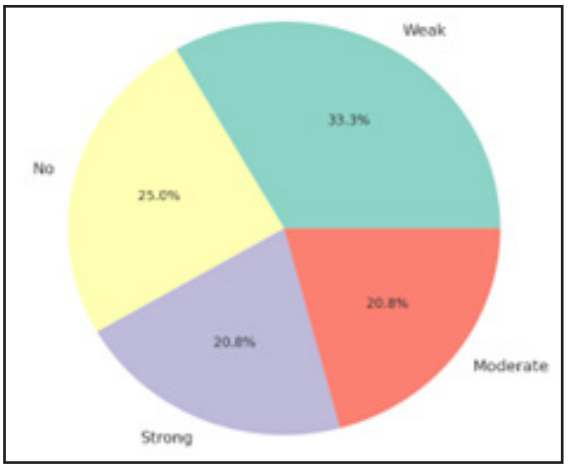

Figure 7: Biofilm formation among isolates by Microtiter plate method

Discussion

Chronic leprosy foot ulcers provide a favorable niche for persistent bacterial colonization due to prolonged tissue exposure, impaired local immunity, and repeated mechanical trauma. In the present study, *Staphylococcus aureus* was the predominant isolate (54.1%), followed by *Pseudomonas aeruginosa, Klebsiella spp.,* and *Escherichia coli,* highlighting a polymicrobial environment commonly associated with chronic non-healing ulcers. Similar organismal patterns have been reported earlier, confirming that secondary bacterial infection remains a major contributor to delayed wound healing in leprosy-associated ulcers (9, 15, 18).

A key finding of this study was the high prevalence of biofilm-forming isolates, particularly among *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The findings align with previous literature emphasizing the clinical implications of biofilm formation in chronic wounds significantly contributing to chronicity and therapeutic resistance in leprosy-related ulcers (11,18). The ability of these organisms to form strong biofilms can be attributed to multiple virulence mechanisms. *S. aureus* possesses surface adhesins, extracellular polysaccharide production, and regulatory systems that promote attachment and maturation of biofilms on damaged tissue. Similarly, P. aeruginosa is known for robust biofilm architecture mediated by alginate production, quorum sensing, and environmental adaptability, allowing it to persist in moist, necrotic ulcer environments. In contrast, *Escherichia coli* and *Klebsiella* spp. showed comparatively variable or weaker biofilm formation, suggesting that while these organisms contribute to infection, they may play a secondary role in ulcer chronicity unless supported by synergistic interactions within polymicrobial biofilms. This organism-specific variability explains why certain ulcers remain refractory to treatment despite antibiotic sensitivity seen in planktonic cultures.

Bacterial isolates from chronic leprosy ulcers were successfully screened for their biofilm-forming capacity using both qualitative (Congo Red Agar method) and quantitative (Microtiter Plate Assay) techniques. The Congo Red Agar method provided rapid visual identification of biofilm-producing colonies, while the Microtiter Plate Method allowed for a more precise and measurable assessment of biofilm biomass.The MTP method showed a more even distribution of biofilm formation among the isolates: strong biofilm formation was seen in 5 samples (21%), moderate in 6 (25%), weak in 7 (29%), and no biofilm in 6 samples (25%). In comparison, the CRA method identified strong biofilm producers in 12 samples (50%), weak biofilm formation in 4 (17%) and 8 (33%) were non-biofilm producers. The Congo Red Agar method identified a higher proportion of strong biofilm producers compared to the Microtiter Plate assay. This difference reflects the qualitative nature of CRA, which detects slime production, versus the quantitative sensitivity of the MTP method, which measures adherent biomass. The use of both methods strengthened the reliability of the findings by minimizing false-negative detection and allowing cross-validation of biofilm-forming capacity.

While the bacteriological profile of leprosy foot ulcers has been described previously, the scientific contribution of the present study lies in its regional focus, methodological comparison, and clinical relevance. This study provides localized evidence from a South Indian leprosy care setting, where environmental conditions, patient behavior, and healthcare access differ from previously reported regions.

A notable strength of this work is the parallel application of Congo Red Agar and Microtiter Plate assays, which enabled comparative assessment of biofilm detection methods under routine laboratory conditions. The findings demonstrate that CRA may overestimate strong biofilm producers, whereas the MTP assay offers finer discrimination between weak, moderate, and strong biofilm formation. This comparison highlights the importance of combining qualitative and quantitative methods for

accurate biofilm screening in chronic wound infections. Importantly, the high prevalence of biofilm-producing *Staphylococcus aureus* and *Pseudomonas aeruginosa* observed in this study has direct clinical implications for Indian leprosy care settings, where delayed presentation, limited microbiological testing, and empirical antibiotic therapy are common.

The findings of the study support the need for early biofilm detection, aggressive wound debridement, and incorporation of anti-biofilm strategies to prevent chronicity, recurrence, and limb-threatening complications in leprosy patients. Biofilms act as physical and metabolic barriers, reducing antibiotic penetration and allowing bacteria to survive hostile conditions, leading to recurrent infections, delayed epithelialization, and chronic inflammation. In leprosy patients, where sensory loss delays early ulcer detection, biofilm-associated infections further increase the risk of deep tissue involvement, osteomyelitis, and eventual amputation. Importantly, the high proportion of strong biofilm producers observed in this study underscores the limitations of conventional antibiotic therapy alone. Standard culture-based antibiotic selection may fail to eradicate biofilm-embedded bacteria, explaining frequent treatment failures in chronic leprosy ulcers. These findings reinforce the need for integrated management strategies, including mechanical debridement, prolonged or combination antimicrobial therapy, and the incorporation of anti-biofilm approaches.

Conclusion

This study highlights the significant role of biofilm-forming bacteria in the chronicity of leprosy foot ulcers. The formation of biofilms by bacterial pathogens not only protects the microorganisms from the host immune system but also dramatically increases their resistance to antimicrobial treatments. This contributes to the persistence of infection, delayed healing, and recurrent ulceration in leprosy patients. Early identification of biofilm-forming pathogens can guide clinicians toward more effective wound-care strategies, including biofilm-disrupting agents, advanced dressings, and targeted antimicrobial regimens. Screening for biofilm production should therefore be considered an essential component of microbiological evaluation in chronic leprosy foot ulcers.

The study underscores the urgent need for integrated treatment strategies that combine traditional antimicrobial agents with anti-biofilm therapies, including nanoparticles, quorum sensing inhibitors, bacteriophage therapy, and photodynamic therapy. Implementing such combined approaches could significantly improve treatment outcomes, reduce recurrence, and promote faster healing of chronic leprosy foot ulcers. In conclusion, early detection of biofilm-producing bacteria in chronic wounds, combined with targeted, multidisciplinary treatment protocols, will be essential in improving the quality of care for patients with leprosy-associated ulcers.

Acknowledgements

We would like to express our special thanks to Dr. S. Aparna, Dean, School of Allied and Healthcare Sciences, Malla Reddy University, for providing the support and encouragement

We would like to express sincere gratitude to Mr. Prasant Kumar Naik, Chief Executive & Dr. Michael Sukumar Pallapati, Research Medical Officer cum In-charge, LEPRA Society Blue Peter Public Health and Research Centre, India (Co- funded by LEPRA, UK), for providing the necessary facilities and resources.

Funding source

No funding was received.

Ethical approval and informed consent

Ethical Approval for the study was sought from the Institutional Ethics Committee (IEC) of Malla Reddy Hospital, Suraram, Hyderabad, Telangana. The certificate of approval number is MRIMS/DHR-IEC-MSC-CMB/2025/51

Documented consent was obtained from all participants before their involvement. All patient information is anonymized, and strict confidentiality is upheld throughout the study (Informed consent form attached as Annexure).

References

1. Ridéey, D.S. and Jopling, W.H. Classification of leprosy according to immunity. A five-group system. *International Journal of Leprosy and Other Mycobacterial Diseases,* 1966, 34 (3):255-73.
2. World Health Organization. Leprosy [Internet]. Geneva: WHO; 2023 [cited 2025 Sep 22].
3. Brandsma, J.W. and Van Brakel, W.H. WHO disability grading: operational definitions. *Leprosy review*, 2003, *74*(4): 366-373.
4. Rafferty,J. Curing the stigma of leprosy. *Leprosy review*, 2005, *76*(2): 119-126.
5. Govindasamy, K., Darlong, J., Watson, S.I. and Gill, P. Prevalence of plantar ulcer and its risk factors in leprosy: a systematic review and meta-analysis. *Journal of Foot and Ankle Research*, 2023, *16*(1): 77.
6. Ganapati, R. & Pai, V.V., Neuropathic ulcers in leprosy. *International Journal of Dermatology,* 2002, 41(9): 599–606
7. Khan, A., Ali, Y.H., Saeed, I.K., Mostafa, H.M., Ahmed Alshahrani, R.M., Abdullah Alhunaydi, et al., Emerging Therapeutic Strategies for Leprosy: Trends, Challenges, and Innovations. *Journal of Pure & Applied Microbiology*, 2025, *19*(3): 1569.
8. Sarin, R. & Kumar, S. Management of plantar ulcers in leprosy: role of surgery. *Indian Journal of Leprosy*, 2016, 88(2), pp.73–81.
9. Ebineshan, K. Bacteriological profile of chronic leprosy ulcers. *Indian Journal of Leprosy,* 2024, 90(1):23–31.
10. Otto, M. Staphylococcal biofilms. *Bacterial biofilms*, 2008, 207-228.
11. Ebineshan, K., Pallapati, M.S. and Srikantam, A., Occurrence of bacterial biofilm in leprosy plantar ulcers. *Leprosy Review*, 2020, *91*(2): 130-138.
12. Bandy, A., Wani, F.A., Mohammed, A.H., Dar, U.F., Dar, M.R. and Tantry, B.A. Bacteriological profile of wound infections and antimicrobial resistance in selected gram-negative bacteria. *African health sciences*, 2022, *22*(4):576-586.
13. Majumdar, M., Chakraborty, U., Das, J., Barbhuiya, J.N., Mazumdar, G. and Pal, N.K. Bacteriological study of aerobic isolates from plantar ulcers of paucibacillary leprosy patients. *Indian Journal of Dermatology*, 2010, *55*(1): 42-43.
14. Anju, V.T., Busi, S., Imchen, M., Kumavath, R., Mohan, M.S., Salim, S.A., et al. Polymicrobial infections and biofilms: clinical significance and eradication strategies. *Antibiotics*, 2022, *11*(12): 1731.
15. Saha, R., Sarkar, S., Majumder, M. and Banerjee, G. Bacteriological profile of aerobic and anaerobic isolates of trophic ulcer in leprosy: A study from Eastern India. *Indian Journal of Dermatology*, 2019, *64*(5): 372-376.
16. Ganapati, R., Pai, V.V. and Kingsley, S. Disability prevention and management in leprosy: A field experience. *Indian Journal of Dermatology, Venereology and Leprology*, 2003, *69*: 369.
17. Donlan, R.M. and Costerton, J.W. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical microbiology reviews*, 2002, *15*(2): 167-193.
18. Gelatti, L.C., Bonamigo, R.R., Becker, A.P., Eidt, L.M., Ganassini, L. and d'Azevedo, P.A. Phenotypic, molecular and antimicrobial susceptibility assessment in isolates from chronic ulcers of cured leprosy patients: a case study in Southern Brazil. *Anais Brasileiros de Dermatologia*, 2014, *89*(3): 404-408.

19. Gellatly, S.L. and Hancock, R.E. Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. *Pathogens and disease*, 2013, *67*(3):159-173.
20. Peng, Q., Tang, X., Dong, W., Sun, N. and Yuan, W. A review of biofilm formation of Staphylococcus aureus and its regulation mechanism. *Antibiotics*, 2022,*12*(1):12.
21. Mac Faddin, J.F. Biochemical tests for identification of medical bacteria, williams and wilkins. *Philadelphia, PA*, 2000, *113*(7).
22. Freeman, D.J., Falkiner, F.R. and Keane, C.T.New method for detecting slime production by coagulase negative staphylococci. *Journal of clinical pathology*, 1989, *42*(8): 872-874.
23. Mathur, T., Singhal, S., Khan, S., Upadhyay, D.J., Fatma, T. and Rattan, A.Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. *Indian journal of medical microbiology*, 2006, *24*(1): 25-29.
24. Hassan, A., Usman, J., Kaleem, F., Omair, M., Khalid, A. and Iqbal, M. Evaluation of different detection methods of biofilm formation in the clinical isolates. *Brazilian journal of infectious diseases*, 2011:*15*: 305-311.