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ASEPTIC SEPARATION AND CHARACTERIZATION OF BACTERIA PRODUCING SECONDARY INFECTION IN CUTANEOUS LEISHMANIASIS LESIONS

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ABSTRACT
Leishmaniasis is a complex parasitic disease, endemic in 98 countries worldwide including Pakistan. Cutaneous leishmaniasis is a devastating illness and the most common kind of leishmaniasis that results in cuts, scrapes, and sores on the skin. Bacterial presence of skin lesions caused by leishmaniasis results in recurrent bacterial infection. This fatal infection impedes wound healing by causing serious localized and systemic skin problems. The data for this study was gathered from the Leishmania diagnostic and treatment unit located at Kuwait Teaching Hospital in Peshawar. Sterile cotton swabs were used to sample 37 ulcerative patients among 150 instances who were contaminated with secondary infections. Bacteria were identified and described using gram staining, morphology, and biochemical testing after samples were processed on a nutrient agar medium. *Staphylococcus aureus* was the most often identified bacterium in ulcerative lesions, accounting for 43% of cases. *Neisseria meningitides* was the second most prevalent, discovered in 29% of cases. *Staphylococcus epidermidis* accounted for 13% of cases, while *Escherichia coli* was present in 8%. *Micrococcus luteus* and *Propionibacterium acne* were both detected in one instance, representing 2.7% of cases each. Due to the serious complications caused by secondary bacterial infection in cutaneous leishmaniasis, it is necessary to recommend suitable antibiotics that may be used with antileishmanial drugs as a treatment approach for leishmaniasis.

INTRODUCTION
Leishmaniasis is a prevalent parasitic disease that affects regions with tropical, subtropical, and Mediterranean temperatures, including Pakistan (Ferreira-Sena *et al.*, 2023). The condition is widespread in 98 countries, affecting around 12 million persons worldwide (Madannejad, Rashidi, Sadeghhassani, Shemirani, & Ghasemy, 2018). From the curable cutaneous form of leishmaniasis to

the incurable visceral form, the disease manifests itself in a broad variety of ways (Silva *et al.*, 2009). The disease is caused by more than 20 types of Leishmania and is transmitted by 30 species of Phlebotomus sand-flies (Hammoudeh, Nguyen, & Sousa, 2014). Sandfly females feed their young by drawing blood from various animals, including humans. Rodents including gerbils, rats, and mice, as well as canines, wolves, and jackals, serve as reservoirs for zoonotic leishmaniasis (de Vries, Reedijk, & Schallig, 2015).

Leishmania has two distinguishable morphological stages. The initial phase involves the transmission of the infectious flagellated promastigote by the Phlebotomus sandfly. The second phase is characterized by the presence of non-flagellated amastigotes, which are found inside the monocyte-macrophage system of the vertebrate hosts. This information is depicted in Figure 1.1.(Feijó, Tibúrcio, Ampuero, Brodskyn, & Tavares, 2016).

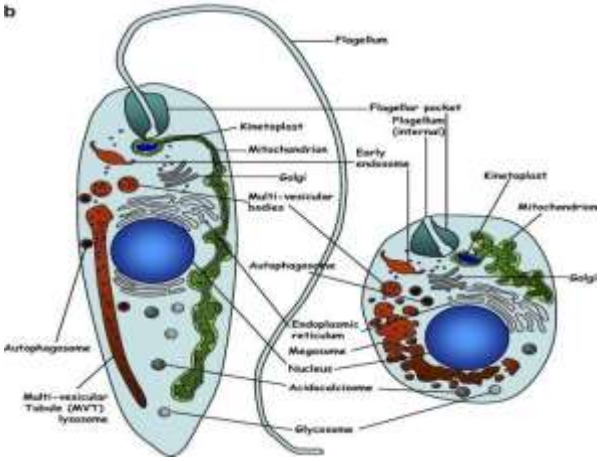


Figure 1: The Leishmania parasite has two distinct stages: promastigote and amastigote (Veras & Bezerra de Menezes, 2016).

There are three distinct clinical types of leishmaniasis: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and mucocutaneous leishmaniasis (MCL). The immune system can be affected in different ways by these kinds, which can lead to the host being sick or even dying (Sundar & Singh, 2018).

Cutaneous leishmaniasis (CL) is the predominant manifestation of leishmaniasis, characterized by the formation of skin lacerations and ulcers on exposed regions of the body, leading to lasting scars that can affect an individual's physical appearance (Willemze *et al.*, 2019). The most deadly type of leishmaniasis, cutaneous leishmaniasis (CL), usually goes away in three to eighteen months, however, it can occasionally leave lifelong scars (Sundar & Singh, 2018).

Leishmania species, including those found in the Old World (*L. major, L. infantum, and L. tropica*) and Central and South America (*L. mexicana, L. amazonensis, L. guyanensis, L. panamensis, and L. braziliensis*), can cause the deformity known as cutaneous leishmaniasis (CL) (de Vries & Schallig, 2022).

Pakistan is home to many instances of anthroponotic cutaneous leishmaniasis (ACL), which is mostly caused by Leishmania tropica. The areas of Baluchistan and Khyber Pakhtunkhwa (KP) have the greatest recorded incidence. Many areas in Khyber Pakhtunkhwa have been greatly affected by this problem. Kohat, Cherat, Bannu, Karak, Dir, Dargai, and Shangla are all a part of this. So are Nowshehra, Peshawar (Akhtar *et al.*, 2024), Cherat, D.I. Khan, and Khyber agency. Cutaneous leishmaniasis is mainly transmitted by the extremely common *Phlebotomus papatasi* and *Phlebotomus sergenti* (CL) in KP (Khalid, 2019).

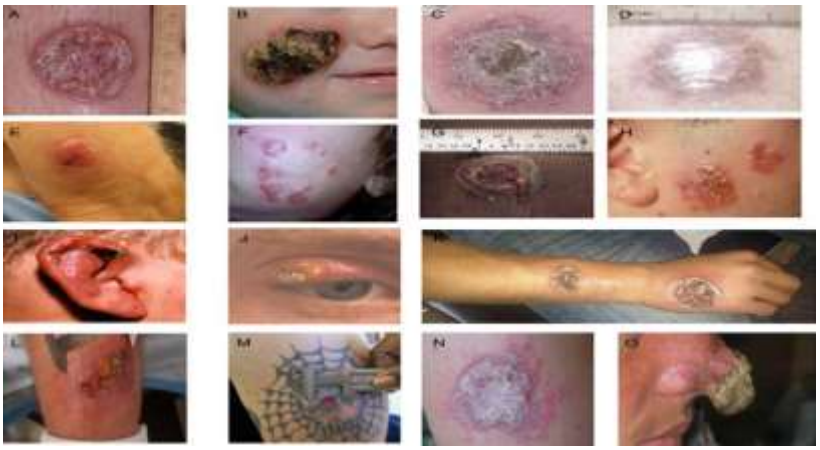


Figure 2: A variety of skin lesions caused by Leishmania can manifest clinically. (Aronson *et al.*, 2016).

Initial occurrences of cutaneous leishmaniasis in Pakistan were reported in the remote northwestern region of the country in 1960. However, the illness has now become widespread throughout the whole nation. Every year, this condition affects over 20,000 individuals. It has been shown that over 37 different species of Phlebotomus sand flies in Pakistan carry this illness (Hussain, Rigoni, & Orij, 2018).

Some believe that the influx of Afghan refugees into Pakistan's northwest areas is a major contributor to the growth of this illness. Itching leishmaniasis is very common in certain regions (Sophie *et al.*, 2017). The initial instance of cutaneous leishmaniasis in Pakistan was documented in 1997 at the Timargara refugee camp in Khyber Pakhtunkhwa (KP). The Leishmania tropica outbreak had a devastating effect on 38% of the refugee camp population. There have been occurrences of anthroponotic cutaneous leishmaniasis recorded in various sections of the Baluchistan and KP provinces. In Pakistan, Leishmania major is the most common causative agent of zoonotic cutaneous leishmaniasis (Meijer *et al.*, 2023).

Among Pakistani provinces, Khyber Pakhtunkhwa (KP) has the greatest number of confirmed cases of anthroponotic cutaneous leishmaniasis (ACL), a disease usually caused by *Leishmania tropica*. It is believed that residing near cowsheds raises the risk of ACL because sandflies prefer the organic waste of domestic animals. Due to the high incidence of *L. tropica* infection in dogs, these animals play a crucial role as vectors for the transmission of this sickness to humans. (Nawaz *et al.*, 2020). People who live near bodies of water, such as streams and ponds, where adult sandflies can thrive in the damp, humid environment. Phlebotomus sandfly eggs can also find a home in a mud building with gaps and crevices in the walls (M. Hussain *et al.*, 2018).

Figure 3: Both ulcerative and nonulcerative lesions were observed in the cutaneous lesions of cutaneous leishmaniasis victims at Kuwait Teaching Hospital in Peshawar.

Current research aims to determine the various species of bacteria that induce secondary infections in skin lesions of cutaneous leishmaniasis. The primary objectives of this study were to identify a variety of bacterial species, analyze the isolated bacterial species, and acquire uncontaminated bacterial cultures from cutaneous leishmaniasis ulcerative sores.

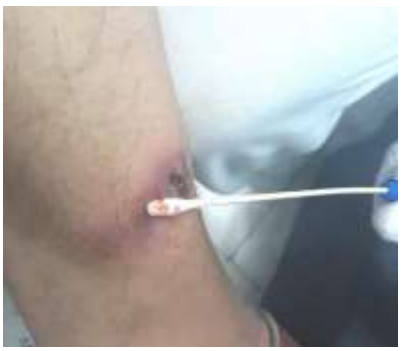
MATERIALS AND METHODS

The items included in Annexure-A are as follows: sterile cotton swabs, disposable Petri dishes, wire loops, a spirit lamp, a laminar airflow (class II), a 37ºC incubator, a compound microscope, 70% alcohol, distilled water, an autoclave (Dhaijan, Korea), test tubes, nutrient broth medium, agar technical No. 3, Simmons citrate agar, Christensen's urea agar, blood agar base (CM-BAB 109), Mannitol salt agar, hydrogen peroxide (H2O2), N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) oxidizing reagent, and Gram's stain.

Collection of Samples

Participants in the research were those who had cutaneous leishmaniasis (CL) and had a subsequent bacterial infection. From November 2020 to April 2021, these patients were seen at the dermatology department at Kuwait Teaching Hospital Peshawar, which is known for its Leishmania diagnostic and treatment capability.

A patient suffering from cutaneous leishmaniasis had ulcerative lesions from which only pus and exudate were taken. A total of 37 patients with ulcerative lesions were selected from a pool of 150 CL patients. Using sterile cotton swabs, pus was collected. Patients with CL had their ulcerative lesions gently cleaned with cotton soaked in 70% alcohol. As shown in figure 4. Lesions' edges were pricked and crushed with sterile, gloved fingers until pus discharged. After sterile cotton swabs were used to collect pus and exudate samples, they were sent to the Department of Zoology's microbiological laminar flow section for further investigation.



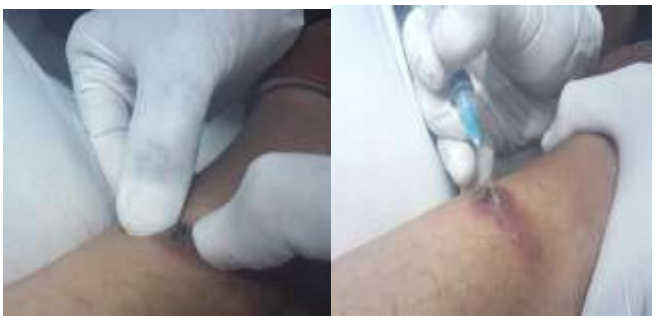


Figure 4: Patients with CL will have their pus or exudate collected from ulcerative lesions using sterile cotton swabs and a sterile syringe.

Results

Of 150 patients with cutaneous leishmaniasis who were investigated, only 37 developed ulcerative lesions that tested positive for subsequent infections caused by bacteria. By employing a technique that involved precise identification and characterization using Gram's staining and other biochemical assays, a total of six bacterial species were recovered from ulcerative lesions. Following isolation and classification, the following bacterial species were identified:

There was a prevalence percentage of 43% for *Staphylococcus aureus*, 13% for *Staphylococcus epidermidis*, 29% for *Neisseria meningitides*, 8% for *Escherichia coli*, and 2.7% for *Micrococcus luteus* and *Propionibacterium acne*, respectively, in the 16 subjects that tested positive.

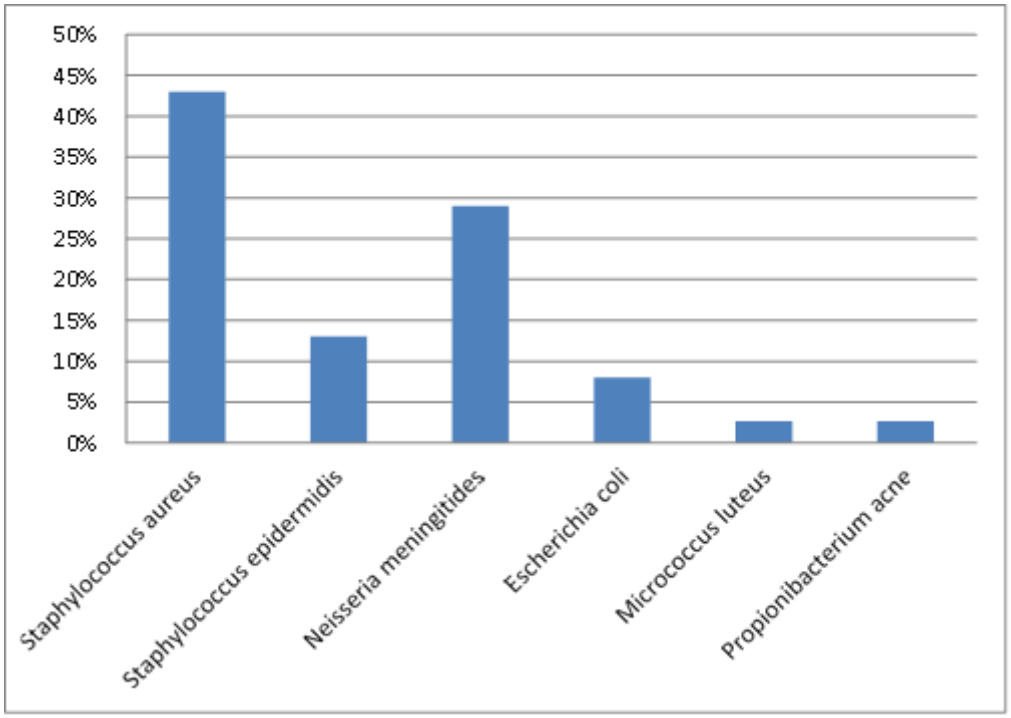


Figure 5: The frequency of a variety of bacterial species in ulcerative lesions of cutaneous leishmaniasis.

Gram Staining

The staining approach known as Gramme staining, or Gram's technique, is employed to classify and distinguish between two categories of bacterial species: Bacteria, both good and bad. The Danish bacteriologist Hans Christian Gramme, who developed the method, is named after it. Separating two big groups of bacteria based on their different cell wall components is the goal of Gramme staining, a collective technique. Due to the presence of a thick coating of peptidoglycan in their cell walls, Gramme-positive bacteria stain violet, whereas Gramme-negative bacteria have thin peptidoglycan

and stain pink.

Biochemical Examination of Isolated Bacteria

Additional verification of microorganisms was conducted using biochemical tests, as depicted in Table 1. To accomplish this purpose, we employed catalase, oxidase, mannitol, motility, citrate, urease, and hemolytic tests.

Staphylococcus aureus, Staphylococcus epidermidis, Neisseria meningitides, Escherichia coli, Micrococcus luteus, and Propionibacterium acne were the six bacterial species that were definitively identified by these biochemical assays.

Table 1: Results obtained from biochemical analysis on isolated cells from ulcerative lesions in individuals with cutaneous leishmaniasis.

Bacterial Species	Gram Stain/ Morphology	Fermentation Catalase	Oxidase	Mannitol	Motility	Urease	Citrate	Hemolysis
Staphylococcus aureus	Gram-positive, Cocci	+	+	+	Non-motile	+	+	Beta-hemolysis
Staphylococcus epidermidis	Gram-positive, Cocci	+	_	_	Non-motile	+	_	Gamma-hemolysis
Neisseria meningitides	Gram negative, Cocci	+	+	_	Non-motile	_	+	Gamma-hemolysis
Escherichia coli	Gram negative, Bacilli	+	_	_	Motile	_	_	Gamma-hemolysis
Micrococcus luteus	Gram Positive Cocci	+	+	+	Non-motile	+	_	Alpha-hemolysis
Propionibacterium acne	Gram positive, Bacilli	+	_	_	Non-motile	_	_	Gamma-hemolysis

Catalase Test

Culture and morphological examination are the mainstays of medical microbiology for documenting most microbiological samples, particularly bacterial isolates. The advanced microbiological laboratories employ precise microbial culture techniques to separate bacteria and classify them based on their biochemical properties. In public microbial laboratories when molecular testing is not available or feasible, documentation is done using conventional biochemical exams. We utilized a variety of biochemical assays to verify the morphological identification of bacteria from CL lesions. Some bacteria make an essential enzyme called catalase. The primary role of this chemical is to neutralize the effects of hydrogen peroxide (H2O2), which is produced in copious amounts by immune cells such as granulocytes, monocytes, and macrophages. To eliminate hydrogen peroxide and superoxide radicals, the enzymes catalase and superoxide dismutase are often present in facultative anaerobes and obligative aerobes, respectively. Due to the presence of catalases, all bacterial isolates showed good results for the test. Figure 3.14 shows the formation of bubbles as a result of the enzyme's action on hydrogen peroxide, which converted it into water and oxygen.

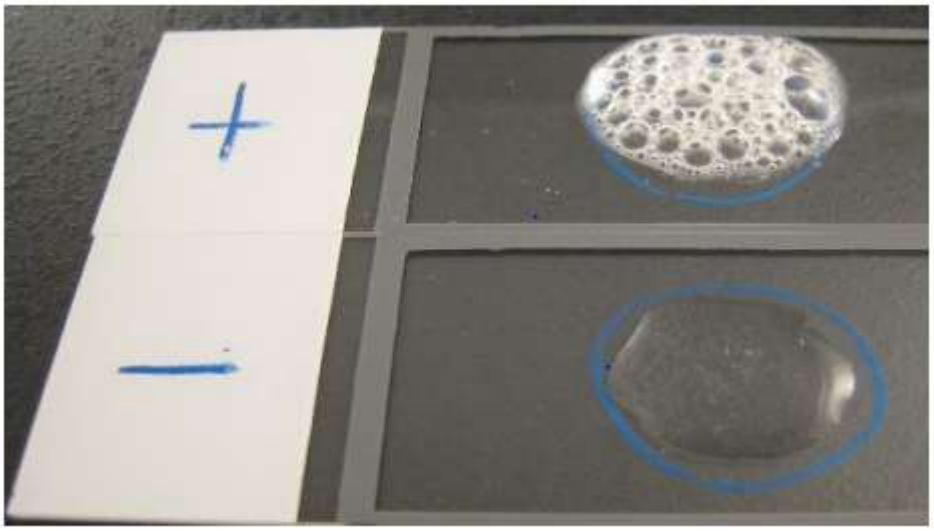


Figure 6: The catalase test indicates that (A). Positive for catalase (B). Catalase-negative colonies of isolated bacterial species.

Oxidase Test

This test can be used to identify bacterial species based on the presence of cytochrome c oxidase or indophenol oxidase. The testing method follows the principle of Redox (Reduction-oxidation) reaction, which entails the exchange of electrons. In oxidation, electrons are removed from an object, whereas in reduction, they are added. An electron-donating synthetic reagent called tetra-methyl-p-phenylenediamine dihydrochloride is used in the oxidase test. The colorless reagent becomes indophenol blue, a complex with a dark blue or purple hue when it is oxidized. Out of the six bacterial species that were isolated from CL lesions, the oxidase test was positive for *Staphylococcus aureus*, *Neisseria meningitides*, and *Micrococcus luteus*. The favorable outcome was attributed to the existence of the cytochrome-C oxidase enzyme, which led to the creation of a deep purple color, as seen in Figure 7.

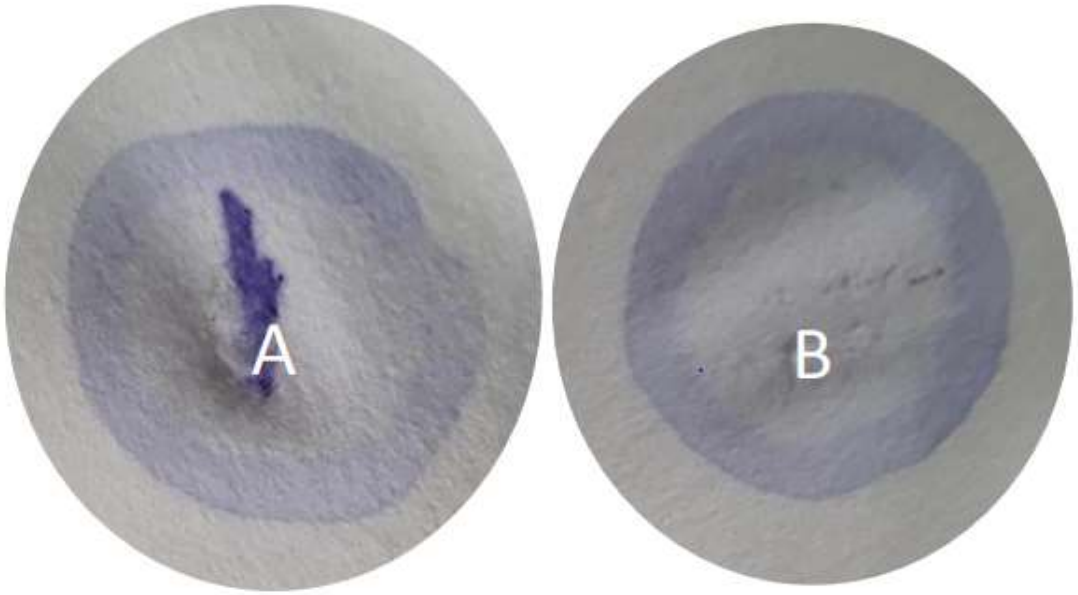


Figure 7:(A) Oxidase test results. Oxidase positive (B). Three bacterial species that were isolated from CL lesions yielded oxidase-negative results.

Test for Mannitol

Mannitol Salt Agar (MSA) contains mannitol sugar and phenol red, which is used to assess pH. A bacterial colony capable of metabolizing mannitol, when grown on mannitol salt agar, produces an acidic byproduct that causes phenol red to become yellow. *Staphylococcus aureus*, which is a kind of pathogenic *staphylococci*, undergoes mannitol fermentation. The inclusion of 7.5% sodium chloride in the MSA growth medium enables the selection of bacterial colonies that can withstand high salt concentrations, such as a 7.5% salt environment in MSA. *Staphylococci* species *Staphylococcus aureus* exhibits the ability to withstand a salt concentration of 7.5%. The MSA solution, which has a salt concentration of 7.5%, inhibits the growth of both gram-negative and gram-positive bacteria. Only *Staphylococcus aureus* and *Micrococcus luteus* showed promise when grown on mannitol salt agar among the bacterial species we recovered from cutaneous leishmaniasis lesions. Figure 8. displays the emergence of a yellow color zone surrounding the colonies. No color change was seen in any of the other detected bacterial species.

Figure 8: Mannitol fermentation test (A). showing Staphylococcus aureus positive mannitol Test (B). representing Negative Mannitol Test.

Motility Test

Motility is the ability of an organism to move by employing specialized flagella that resemble propellers. This type of motility is unique to bacteria that exhibit a sliding motion. Bacterial species exhibit motility when grown in a growth medium containing semi-solid agar. The medium employed for this objective is the Sulphide Indole Motility medium, which possesses a very pliable consistency that facilitates the vigorous movement of bacteria, resulting in the formation of opacity or cloudiness. *Escherichia coli* was the onlys one of six bacteria isolated from CL ulcers that showed signs of movement. This was observed as it migrated away from the stab line located in the center of the test tube after a 24-hour incubation period. In contrast, the growth of all the other bacteria was limited to the stab line, indicating their lack of motility. The Figure 9 clearly exhibits the presence of cloudiness and opacity.

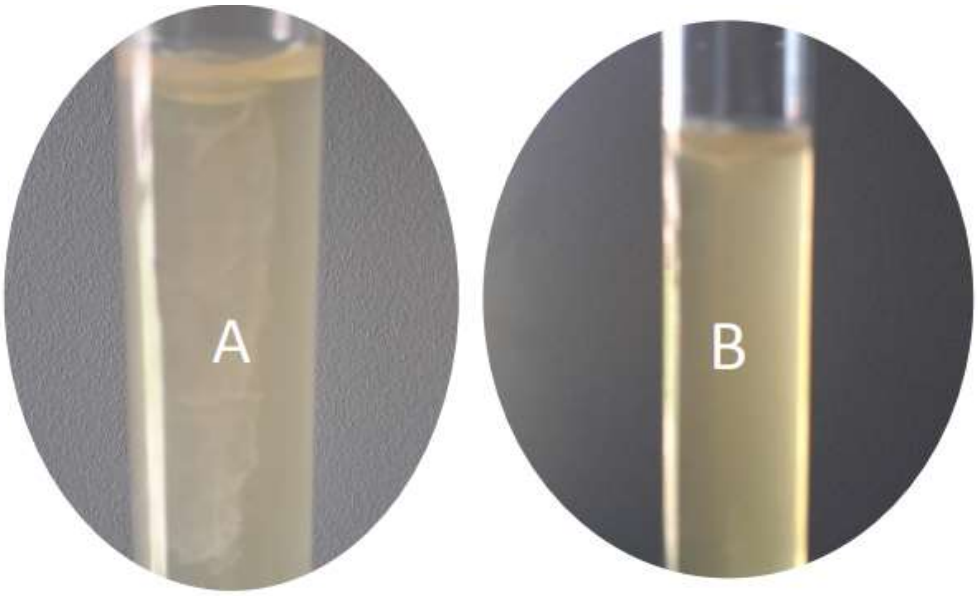


Figure 9: Semisolid medium motility testing (A). A positive result for Escherichia coli was observed (B). Negative Motility Test for the remaining bacterial isolates.

Citrate Test

The primary goal of this experiment is to determine whether or not a given organism can carry out its metabolic operations using citrate as its only carbon source, leading to the formation of alkaline waste products. Bacterial citrase catalyzes citrate hydrolysis, which yields oxaloacetic acid and acetic acid. There are two steps to this reaction:

Stage 1: Citrate → citrase enzyme → acetic acid + oxaloacetic acid
Stage 2: Pyruvic acid + CO2 → oxaloacetic acid

The bacteria that are to be examined are cultivated in a medium that contains sodium citrate and exhibits the indicator bromothymol blue. The pale green hue transitioning to blue is a consequence of an alkaline reaction, suggesting that the test bacteria have metabolized citrate. *Staphylococcus aureus* and *Neisseria meningitides* were the only bacterial species in our collection to yield positive results. The bacteria utilized citrate as a carbon source, which led to a noticeable shift in the media's color from green to blue, as illustrated in figure 10.

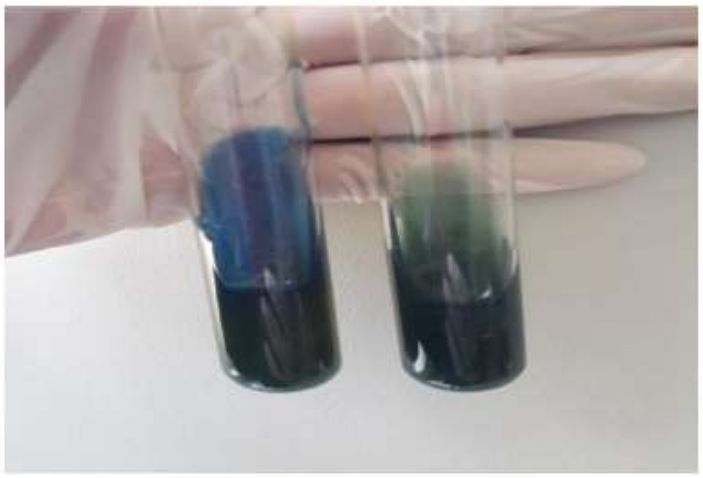


Figure 10: A positive citrate test is indicated by a change in the medium's colour from green to blue.

Urease Test

The test employed urease broth as the differential growth medium to assess an organism's capacity to produce the exoenzyme urease, which catalyzes the hydrolysis of urea into ammonia and CO2. The pH indicator phenol red and two pH buffers, urea, comprise the bouillon. Positive urease findings were observed in *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Micrococcus luteus* when urea was used, resulting in a change in the medium's colour ranges from orange to pink. Figure 11. illustrates this color transition.

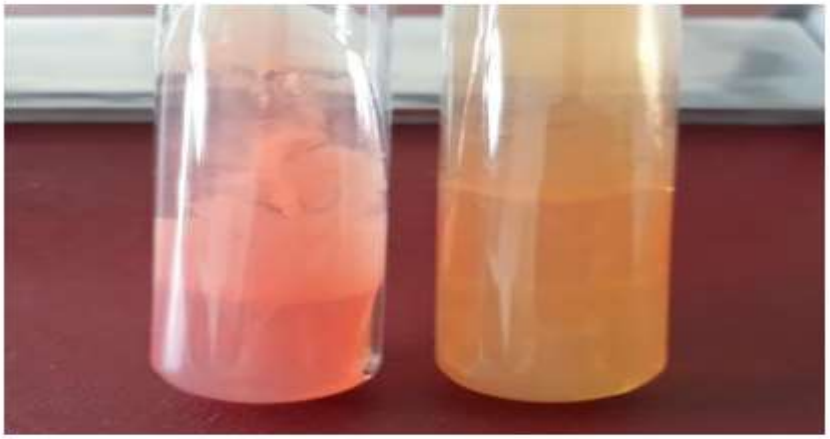


Figure 11: The pink colour of Staphylococcus aureus, Staphylococcus epidermidis, and Micrococcus luteus colonies is indicative of a positive urease test.

Hemolytic Test

Hemolytic response refers to the lysis or destruction of red blood cells derived from sheep or rabbits when exposed to a culture medium. Hemolysin is the term used to describe a substance that causes hemolysis. The words alpha, beta, and gamma represent three distinct forms of haemolytic processes. Our investigation found that *Staphylococcus aureus* exhibited beta hemolysis, which refers to the total lysis of red blood cells. This resulted in the formation of clear zones around the bacterial colonies. The colonies of *Micrococcus luteus* exhibited alpha hemolysis, characterized by the formation of greenish zones resulting from the partial degradation of hemoglobin. Every other bacterium displayed gamma hemolysis, since they did not show the breakdown of hemoglobin, as seen in figure 12.

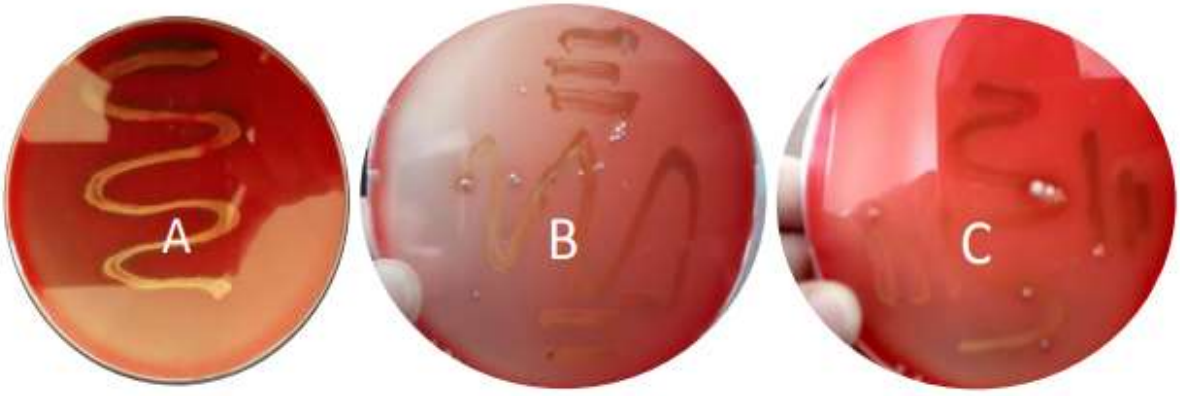


Figure 12: Staphylococcus aureus showing beta hemolysis (A), Micrococcus luteus showing alpha hemolysis (B) and other species showing Gamma Hemolysis (C).

Discussion

Bacteria thrive in the skin's diverse environment. The human skin is a perfect environment for the development of microorganisms. A wide variety of bacterial species, some of which are parasitic, symbiotic, or commensal, may be discovered on human skin. The quantity, genus, and population of bacteria determine the kind of illness. As a defense mechanism against microbial infections, the host skin's humidity, sebum volume, and sweat production work together. Serious problems might arise from untreated skin infections.

It could be difficult to cure the condition if these infections destroy soft tissues. The healing process of lesions, including those caused by cutaneous leishmaniasis, can be interrupted by secondary bacterial infections, which are among the most prevalent forms of skin diseases. In the absence of early and appropriate medical intervention, serious local and systemic consequences can develop (Manohar *et al.,* 2020). The mixed bacterial flora, which includes *Staphylococcus, Streptococcus, Proteus, Klebsiella,* and *Escherichia coli,* causes secondary bacterial infections in cutaneous leishmaniasis (Goonoo *et al.,* 2022).

Skin leishmaniasis (CL) ulcerative lesions are more likely to have secondary bacterial infection, which slows down the healing process. A slower pace of recovery is associated with secondary bacterial infection, which causes purulent development. Ulcerative lesions were rife with *Staphylococcus aureus*, a kind of bacteria known to produce an enzyme that promotes tissue death (Jafri, Ansari, & Ahmad, 2019).

Skin carbuncles, furuncles, and wound contagions are only a few of the many human skin illnesses that *Staphylococcus aureus* is involved with. Osteomyelitis, endocarditis, pneumonia, and bacteremia are other inflammatory conditions that can be caused by it. *Staphylococcus aureus* is a known provider of toxic shock syndrome (TSS) (Ongpipattanakul *et al.,* 2022).

Staphylococcus epidermidis was also detected in 5 cases (13% of the total) of ulcerative lesions in this investigation. In cutaneous leishmaniasis lesions, *Staphylococcus aureus* and *Staphylococcus epidermidis* were the most common bacteria (Conceição-Silva, Leite-Silva, & Morgado, 2018).

Staphylococcus epidermidis is a common and harmless skin bacterium. Epithelia, nasal passages, axillae, and mucous membranes are common places to find it in humans and other mammals (Severn & Horswill, 2023).

S. epidermidis is a frequent causative agent of nosocomial infections. The most common cause of these infections is the use of indwelling catheters or implanted devices, such as heart pacemakers, prosthetic hips, implantable cardioverter defibrillators (ICDs), or hardware for spinal fusion. The primary pathogen-causing component of *S. epidermidis* may also be its ability to create biofilms (Severn & Horswill, 2023).

Patients with ulcerative lesions of cutaneous leishmaniasis who visited the Kuwait Teaching Hospital in Peshawar were discovered to have 11 instances of *Neisseria meningitides*, which is rather surprising. *Nocardia meningitides* is a round, non-motile, Gram-negative bacterium. The findings for the catalase, oxidase, and citrate tests were positive, as can be shown in table 1.

The common human pathogen *Neisseria meningitides* is mostly found in the human nasal passages (nasopharynx) (Poole, Day, von Itzstein, Paton, & Jennings, 2018). Meningococcal sepsis and other complex systemic skin infections are caused by it. All throughout the body, patients with this condition will have hemorrhagic rashes and non-blanching petechiae (Zhu *et al.,* 2022). There is a significant risk of difficulties for laboratory technicians and workers when they get infections caused by *N. meningitides*, which is commonly encountered in these settings. Always use protective gear, such as a mask, gloves, and a lab coat, and be well-versed in all lab procedures, when working with potentially harmful germs. Regular checkups should be conducted by trained medical professionals (Borrow, Findlow, Gray, Taylor, & Kaczmarski, 2014).

Only a single instance of both *Micrococcus luteus* and *Propionibacterium acne* bacterium was identified in the present investigation. *Micrococcus luteus* exhibited a gram-positive staining pattern and was observed in the tetrad configuration when viewed under a microscope. The bacteria *Propionibacterium acne* was found to be gram-positive and rod-shaped. It tested positive for catalase,

but not for oxidase, mannitol, or urease. Eight species of the Micrococcus genus were abundantly obtained from the human skin, with *Micrococcus luteus* being the most discovered. *Propionibacterium acnes* is an anaerobic bacterium that mostly resides in the deeper layers of the skin, where oxygen is scarce (Hay & Morris-Jones, 2016). No publications were found that reported contamination of CL lesions with these two taxa.

Conclusion

Severe skin problems and the interruption of the healing process are caused by secondary bacterial infection in cutaneous leishmaniasis (CL) lesions. In CL ulcerative lesions, *Staphylococcus aureus* made up around 43% of the overall bacterial flora and was the most common bacterial species. *Propionibacterium acne* (2.7%), *Escherichia coli* (8%), *Neisseria meningitides* (29%) and *Micrococcus luteus* (2.7%) were the most common bacteria detected. Complications such as infective endocarditis, cellulitis, and lymphadenitis can arise from CL, hence it is crucial to use antibiotics and antileishmanial medications that are effective against both CL and bacterial infections. Finally, health care providers and members of the community must immediately begin to understand the seriousness of secondary bacterial infections in CL lesions.

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