THERAPEUTIC ADVANCES in
Infectious Disease

Original Research

Spectrum of bacterial pathogens in inflammatory and noninflammatory cutaneous ulcers of American tegumentary leishmaniasis

Ther Adv Infect Dis

2024, Vol. 11: 1–14

DOI: 10.1177/20499361241274200

Ruwandi Kariyawasam, Bryan Gascon, Priyanka Challa, Jordan Mah, Rachel Lau, Braulio M. Valencia, Alejandro Llanos-Cuentas and Andrea K. Boggild

Abstract

Background: Cutaneous leishmaniasis (CL) ulcers exhibiting an inflammatory phenotype, characterized by purulent exudate, erythema, pain, and/or lymphatic involvement, are empirically treated with antibiotics.

Objective: The spectrum of bacteria present in localized versus inflammatory phenotypes of CL is elucidated herein.

Methods: Filter paper lesion impressions (FPLIs) from 39 patients with CL (19 inflammatory and 20 noninflammatory ulcers) were evaluated via real-time polymerase chain reaction (qPCR) and end-point PCR targeting: *Staphylococcus aureus*, *Enterobacter cloacae*, *Streptococcus pyogenes*, *Enterococcus* spp., *Citrobacter freundii*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and 16S rDNA. Whole genome sequencing (WGS) was performed on six specimens.

Results: In total, 30/39 (77%) patients' ulcers had ⩾1 bacterium detected, which included the following species: *S. aureus* ($n = 16$, 41%), *C. freundii* ($n = 13$, 33%), *P. aeruginosa* ($n = 12$, 31%), *E. cloacae* ($n = 12$, 31%), *K. pneumoniae* ($n = 11$, 28%), *Enterococcus* spp. ($n = 7$, 18%), *E. coli* ($n = 6$, 15%), and *S. pyogenes* ($n = 4$, 10). Prevalence of bacterial species did not differ by CL phenotype ($p = 0.63$). However, patients with inflammatory phenotypes were, on average, over a decade older than patients with noninflammatory phenotypes (42 years vs 27 years) ($p = 0.01$). The inflammatory phenotype was more prevalent among ulcers of *Leishmania Viannia braziliensis* (58%) and *L. V. panamensis* (83%) compared to those of *L. V. guyanensis* (20%) ($p = 0.0369$).

Conclusion: The distribution of flora did not differ between inflammatory and noninflammatory CL phenotypes. Further prospective analysis, including additional WGS studies of all CL ulcers for nonbacterial organisms, is necessary to determine the role of empiric antibiotic therapy in inflammatory and purulent CL.

Keywords: American tegumentary leishmaniasis; antibiotic stewardship, bacterial pathogens, inflammatory ulcers

Received: 20 May 2024; revised manuscript accepted: 25 July 2024.

Correspondence to:
Andrea K. Boggild
Temerty Faculty of Medicine, University of Toronto, ON, Canada

Tropical Disease Unit, Toronto General Hospital, 200 Elizabeth Street, 13EN-218, Toronto, ON M5G 2C4, Canada

Department of Medicine, University of Toronto, Toronto, ON, Canada
andrea.boggild@utoronto.ca

Ruwandi Kariyawasam
Public Health Laboratory, Alberta Precision Laboratories, Edmonton, AB, Canada

Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada

Bryan Gascon
Temerty Faculty of Medicine, University of Toronto, Toronto, ON, Canada

Priyanka Challa
Institute of Health Policy, Management and Evaluation, University of Toronto, Toronto, ON, Canada

Jordan Mah
School of Medicine, Duke University, Durham, NC, USA

Rachel Lau
Public Health Ontario Laboratories, Public Health Ontario, Toronto, ON, Canada

Introduction

Leishmaniasis is a vector-borne protozoan infection transmitted by female *Phlebotomus* spp. and *Lutzomyia* spp. sandflies.[1] While Leishmaniasis affects more than 12 million people worldwide, recent estimates show that approximately 350 million people in endemic countries across Northern Africa, the Middle East, Central and South America, and parts of Asia are at risk for contracting the disease.[2] Most causative species

Braulio M. Valencia
Instituto de Medicina Tropical "Alexander von Humboldt", Universidad Peruana Cayetano Heredia, Unidad de Leishmaniasis y Malaria, Lima, Peru

Kirby Institute, University of New South Wales, Sydney, NSW, Australia

Alejandro Llanos-Cuentas
Instituto de Medicina Tropical "Alexander von Humboldt", Universidad Peruana Cayetano Heredia, Unidad de Leishmaniasis y Malaria, Lima, Peru

of *Leishmania* are classified according to one of three major clinical manifestations with which they are associated: cutaneous leishmaniasis (CL), visceral leishmaniasis, and mucocutaneous/mucosal leishmaniasis (MCL/ML).[2] The more than 20 infecting species of *Leishmania* are also divided between New World and Old World.[3] Specific to the New World is the *Viannia* subgenus, including *Leishmania Viannia braziliensis*, *L. V. guyanensis*, *L. V. lainsoni*, *L. V. peruviana*, and *L. V. panamensis*, with a propensity to cause MCL/ML.[3] Pathogenesis relies primarily on the interplay between the infecting *Leishmania* species and host immune responses.[2] Disease manifestation is often the result of the prevailing Th1 or Th2-biased response, involving a number of cytokines and chemokines differentially expressed as a result of infecting species and underlying host immune status. CL is often characterized by a Th1-biased response, associated with healing; whereby a mixed Th1/Th2 profile is exhibited in MCL/ML.[4]

CL is the most common syndrome with an estimated 0.7–1.2 million cases worldwide.[1] CL is characterized by single or multiple ulcerating skin lesions typically localized at the site of the sandfly bite, but may also appear distally.[3] Ulcerative lesions are usually self-healing, but often persist for months or years if left untreated, with the risk of developing into ML approximately 1–5 years after initial healing of a CL lesion caused by primarily *Viannia* strains of *Leishmania* found in Latin America.[5] Moreover, specific strains of *L. V. braziliensis*, such as those containing genetic polymorphisms in parasite kinetoplast DNA minicircles and heat-shock protein 70, respectively, have been associated with treatment failure of CL and progression to MCL/ML.[6,7] Clinical manifestations of CL can range from small noninflammatory lesions < 4 that may self-heal—often referred to as "localized CL"—to more complicated inflammatory ulcers characterized by pain, erythema, and purulent exudate with or without lymphatic involvement suggestive of bacterial coinfection, requiring additional therapy.[8] In addition, ulcerations of the skin in CL are associated with strong local pro-inflammatory responses to *Leishmania*-infected host macrophages and necrosis of the dermis, thus producing an ulcer either heavily inflamed, erythematous, or painful.[3]

To date, causative species of *Leishmania* vary in their susceptibilities to different antimonial and other anti-leishmanial therapies.[9] As a result, there are CL treatment guidelines that recommend considerations for the size of the lesion, risk for developing ML, drug toxicities such as hepatotoxicity, cardiotoxicity, and nephrotoxicity, and prior treatment when prescribing CL therapy.[8–13] Currently, there is a paucity of literature devoted to the management of patients presenting with the inflammatory phenotype, despite the anecdotal clinical practice of universal treatment of these ulcers with antibiotics prior to anti-leishmanial therapy. Although such inflammatory ulcers may appear to mimic a secondary bacterial infection, there is a scant evidence base supporting these common antimicrobial treatment practices.[14–16]

Antimicrobial stewardship and evidence-based management guidelines for inflammatory CL ulcers would benefit from knowledge of the microbial differences between inflammatory and noninflammatory CL ulcers in order to elucidate potential bacterial contribution to CL phenotype. Fontes et al.[15] demonstrated the colonization of various bacterial contaminants and pathogens in ulcers of American tegumentary leishmaniasis, prompting clinicians to consider bacterial contaminants in the treatment of CL ulcers. Additionally, detection of *Enterobacterales* such as *Enterobacter cloacae*, *Klebsiella oxytoca Pseudomonas* spp., and *Staphylococcus* spp.,[14–16] and in particular, from the midgut of sandfly vectors,[17] has supported the notion that bacterial superinfection may be common. However, differences in clinical phenotype and how they might be influenced by the ulcer microbiome have been rarely considered.[15] Given the extensive knowledge surrounding common bacterial organisms as causative agents of skin and soft tissue infections (SSTIs),[18] we sought to document the spectrum of bacterial pathogens in inflammatory and noninflammatory ulcers of CL using polymerase chain reaction (PCR) and whole genome sequencing (WGS) to determine whether there were differences in bacterial spectra colonizing inflammatory compared to noninflammatory ulcers of CL.

Methodology

Specimen and clinical data collection

Surplus, discard filter paper lesion impressions (FPLIs) of CL ulcers submitted to the Public Health Ontario Laboratory (PHOL) between 2012 and 2018 were collected and stored at

−20°C following clinical diagnostic testing. Additional prospectively collected FPLIs for patients enrolled in clinical diagnostic studies at the *Leishmania* Clinic of the Instituto de Medicina Tropical "Alexander von Humboldt," Lima, Peru over the same time period were also enrolled following written informed consent. Research ethics board approval was obtained from Public Health Ontario (#2015-048.01 and 2017-052.01) and the Universidad Peruana Cayetano Heredia (#60401). De-identified clinical data for source patients collected from test requisitions and clinical charts were stratified into "inflammatory" and "noninflammatory" phenotypes as per the Infectious Diseases Society of America (IDSA) guidelines,[8] where an inflammatory phenotype was defined as an ulcer with associated erythema, purulent exudate, pain, and/or lymphatic involvement; whereas a noninflammatory phenotype was defined as localized CL (LCL) of <4 ulcers in number.[8]

DNA extraction

DNA extraction for end-point PCR and real-time PCR (qPCR) was performed using the Qiagen DNA Mini Kit (Qiagen, Germantown, MD, USA) by soaking FPLIs in 250 µL of 1 × TE (10 mM Tris-HCl containing 1 mM EDTA·Na2) for 5 min prior to extraction. DNA was eluted with 60 µL of Buffer AE (10 mM Tris-Cl; 0.5 mM EDTA) and stored at −20°C prior to use. DNA extraction for WGS was performed using the ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research, Tustin, CA, USA). Two sets of extractions were performed on each specimen where FPLIs were soaked in 500 µL of 1 × TE for 20 min prior to extraction. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were extracted using the DNA & RNA Parallel Purification protocol with a 20-minute bead beating step. DNA was eluted with 50 µL DNase/RNase-free water and combined to maximize yield.

Leishmania *species identification and confirmation*

Leishmania genus 18S real-time PCR was performed as previously described.[19] Species identification included analysis of the internal transcribed spacer 1 (ITS1), ITS2, cysteine proteinase B (CPB), heat-shock protein 70 (HSP70), and mannose phosphate isomerase (MPI) by PCR, restriction fragment length polymorphism (RFLP) analysis, and Sanger sequencing.[20–22] PCR–RFLP analysis of the ITS1 region can only differentiate *L. V. braziliensis* from the other species within the *Viannia* subgenus (*L. V. guyanensis*, *L. V. peruviana*, *L. V. panamensis*, *L. V. lainsoni*). Thus, PCR–RFLP and sequencing analysis of the CPB, HSP70, MPI, and ITS2 regions were required to differentiate species within the *Leishmania Viannia* subgenus complex and to provide a confirmation of the species identified in the initial ITS1 assay. Purified PCR product was used for Sanger sequencing as per Big Dye protocol (Life Technologies, Carlsbad, CA, USA). Sequenced products were purified and analyzed using the Applied Biosystems 3130xl Genetic Analyzer and Basic Local Alignment Search Tool (BLAST) searched for highest homology.

Detection of bacterial pathogens

Quantitative real-time PCR. 16S rRNA qPCR along with the following six qPCR assays targeting *Staphylococcus aureus*, *S. pyogenes*, *Escherichia*/*Shigella* spp., *Citrobacter freundii*, *Enterobacter*/*Klebsiella* spp., and *Enterococcus* spp. (Qiagen, Cat# BBID00314AR, BBID00332AR, BBID00146AR, BBID00108AR, BBID00139AR, BBID00141AR, respectively) (Table 1) was performed on the Applied Biosystems 7900 Fast RT-PCR System using 5 µL of DNA as per manufacturer protocol. A specimen with a cycle threshold (C_t) value less than or equal to 40 was considered positive for the microbial-specific target. A pan 16S rDNA qPCR assay was also performed on the Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using 12.5 µL Taqman Universal Master Mix and previously published primers and probes. The following conditions were used: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C (Table 1).

End-point PCR

The following bacterial targets were detected using end-point PCR assays performed on the Applied Biosystem Veriti® 96-well Thermal Cycler (Thermo Fisher Scientific): *S. aureus*, *E. cloacae*, *S. pyogenes*, *Enterococcus* spp., *C. freundii*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and pan-bacterial 16S rDNA (Table 1). The annealing temperatures including primer and probe sequences varied among different microbial

Table 1. Primer and probe* sequences of bacterial targets.

Primer and probe	Sequence (5′→3′)	Annealing temperature (°C)	Reference
16 S End-point PCR		55°	23
8FPL Primer	AGTTTGATCCTGGCTCAG		
806R Primer	GGACTACCAGGGTATCTAAT		
16S rRNA qPCR		60°C	24
16S rRNA fwd primer	TGGAGCATGTGGTTTAATTCGA		
16S rRNA rev primer	TGCGGGACTTAACCCAACA		
16S rRNA probe	CY5-CACGAGCTGACGACAR* CCATGCA-BHQ		
Klebsiella pneumoniae		52°C	25
ntrA fwd primer	CATCTCGATCTGCTGGCCAA		
ntrA rev primer	GCGCGGATCCAGCGATTGGA		
Pseudomonas aeruginosa (PA)		58°C	26 and 27
PA-SS fwd primer	GGGGGATCTTCGGACCTCA		
PA-SS rev primer	TCCTTAGAGTGCCCACCCG		
Enterococcus spp.		55°C	28
Ent1 fwd primer	TACTGACAAACCATTCATGATG		
Ent2 rev primer	AACTTCGTCACCAACGCGAAC		
Enterobacter spp.		54°C	In-house
Fwd primer	AGACCAAAGAGGGGGACCTT		
Rev primer	TCTGTGGATGTCAAGACCAGG		
Staphylococcus aureus		56°C	29
TStaG422 fwd primer	GGCCGTGTTGAACGTGGTCAAATCA		
TStaG765 rev primer	TACCATTTCAGTACCTTCTGGTAA		
Citrobacter freundii		56°C	In-house
Fwd primer	ATGCAAGTCGAACGGTAGCA		
Rev primer	GGAGTTAGCCGGTGCTTCTT		
Escherichia coli		56°C	30
UsPA fwd primer	CCGATACGCTGCCAATCACGT		
UsPA rev primer	ACGCAGACCGTAGGCCAGAT		
Streptococcus spp.		62°C	31
Fwd primer	GAAGAATTGCTTGAATTGGTTGAA		
Rev primer	GGACGGTAGTTGTTGAAGAATGG		

*Probe sequences for the species-specific qPCR assays are proprietary to Qiagen.
Fwd, Forward primer; qPCR, real-time polymerase chain reaction; Rev, Reverse primer, R*, A or G.

assays (Table 1). For each reaction, 3 µL of DNA was used in addition to 0.2 µL of Life Tech Native Taq, 2.5 µL of 10X Buffer, 0.5 µL of dNTP and 0.75 µL of $MgCl_2$. The following conditions were used: 5 min at 94°C, 40 cycles of 45 s at 94°C, 30 s at 56°C and 1 min at 72°C, followed by 10 min at 72°C. A total of 5 µL of product was loaded onto 1% agarose gel and visualized for bands under UV.

Sanger sequencing

All sequencing reactions were performed on Applied Biosystems 3130xl Genetic Analyzers using the Big Dye v3.1 Cycle Sequencing Kit. The following cycling conditions were used: 1 min at 96°C, 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. PCR product was cleaned using 45 µL of SAM™ Solution (Applied Biosystems, Carlsbad, CA) and 10 µL of XTerminator beads on a vortex for 30 min. The product was centrifuged for 2 min at 2000 g prior to being loaded. Data were standardized using the Sequencing Analyzer program, Vector NTI® software (ThermoFisher Scientific, Carlsbad, CA) was used to assemble the sequences and BLAST search engine on the National Centre for Biotechnology Information database was used to analyze the sequences with highest homology.

WGS and bioinformatics analysis

In a proof-of-concept sub-analysis, a selection of six samples were analyzed in parallel using WGS. Genomic DNA was submitted to The Hospital for Sick Children—The Centre for Applied Genomics facility in Toronto, Canada for genomic library preparation and sequencing as a fee-for-service. DNA samples were quantified using Qubit High Sensitivity Assay Qubit dsDNA HS Assay Kits (ThermoFisher Scientific, Carlsbad, CA, USA), and sample purity was checked using Nanodrop OD260/280 ratio. Two-hundred ng of DNA was used as input material for library preparation using the Illumina TruSeq Nano DNA Library Prep Kit following the manufacturer's recommended protocol. In brief, DNA was fragmented to 450 bp on average using sonication on a Covaris LE220 instrument; fragmented DNA was end-repaired, A-tailed and indexed TruSeq Illumina adapters with overhang-T were added to the DNA; adapter-ligated DNA was enriched by PCR under the following conditions: initial denaturation at 95°C for 3 min, followed by six cycles at 98°C for 20 s, 60°C for 15 s and 72°C for 30 s, and a final extension step at 72°C for 5 min and hold at 4°C. Libraries were validated on a Bioanalyzer DNA High Sensitivity chip to check for size and absence of primer dimers, and quantified by qPCR using KAPA Library Quantification Illumina/ABI Prism Kit protocol (KAPA Biosystems, Wilmington, MA). Validated libraries were pooled in equimolar quantities and paired-end sequenced on one lane on the Illumina HiSeq 2500 platform following Illumina's recommended protocol to generate paired-end reads of 125-bases in length using a high output flow cell with the V4 chemistry. Raw reads generated from WGS were analyzed by CosmosID (Rockville, MD).

Statistical analysis

Descriptive statistics—including mean with standard deviation, median with ranges, and calculated proportions—were performed on continuous (age) and categorical (e.g., sex and clinical phenotype) variables, respectively. Comparative statistics on categorical variables were analyzed using Fisher's exact test, chi-square, and chi-square test for trend (Cochran-Armitage test for trend); while continuous variables were compared by Mann–Whitney *U*-test. All statistical analyses were calculated via GraphPad Prism Version 6.01 (La Jolla, CA, USA) and MedCalc (MedCalc Software Ltd, Belgium). Significance was set at $p < 0.05$.

Results

Clinical and diagnostic data

In total, lesions from 39 patients were examined: 9 (23%) from the PHOL, 12 (31%) from Hospital Cayetano Heredia in Lima Peru, and 18 (46%) field isolate from Pichanaki, Peru—all of whom traveled from/currently reside in a CL endemic country. Nineteen (49%) ulcers fulfilled the criteria for an inflammatory phenotype, whereas the remaining 20 (51%) of ulcers had a noninflammatory phenotype (Table 2). Median age of all patients was 31 years (8–72 years), however, patients with the inflammatory phenotype were, on average, over a decade older than patients with the noninflammatory phenotype (42 years vs 26.5 years) ($p = 0.01$). No differences related to sex were observed, where 12 (63.2%) lesions in

Table 2. Clinical and demographic correlates of 39 patients with ulcers due to CL stratified by phenotype.

Clinical/ Demographic correlate	Total *N*	Inflammatory CL (*n* = 19)	Noninflammatory CL (*n* = 20)	*p* Value
Median age (range), years	31 (8–72)	42 (9–72)	27 (8–63)	0.01
Sex				0.32*
Male	19 (100%)	12 (63.2%)	7 (36.8%)	
Female	16 (100%)	7 (43.8%)	9 (56.3%)	
Unknown	4 (100%)	0 (0.0%)	4 (100%)	
Spectrum of causative *Leishmania* species				
L. V. braziliensis	12 (100%)	7 (58.3%)	5 (41.7%)	0.10 (compared to *L. V. guyanensis*)
L. V. panamensis	6 (100%)	5 (83.3%)	1 (16.7%)	0.04 (compared to *L. V. guyanensis*)
L. V. guyanensis	10 (100%)	2 (20.0%)	8 (80.0%)	
L. V. lainsoni	3 (100%)	2 (66.7%)	1 (33.3%)	
L. V. peruviana	2 (100%)	1 (50.0%)	1 (50.0%)	
Unknown	6 (100%)	2 (33.3%)	4 (66.7%)	

*Comparison of male and female.
CL, Cutaneous leishmaniasis.

the inflammatory group occurred in males versus 7 (35%) lesions in the noninflammatory group occurred in males ($p = 0.32$) (Table 2).

Causative species

Species identification revealed the following prevalence: 12 (31%) of 39 patients had lesions due to *L. V. braziliensis*, 10 (26%), 6 (15%), 2 (5%), 3 (8%), and 6 (15%) were due to *L. V. guyanensis*, *L. V. panamensis*, *L. V. peruviana*, *L. V. lainsoni* and unknown/unable to confirm species, respectively (Table 2). Species identification revealed no difference among clinical phenotypes as follows: 7 (37%) inflammatory ulcers and 5 (25%) noninflammatory ulcers were due to *L. V. braziliensis* compared to non-*L. V. braziliensis* ($p = 0.50$), 2 (10%) inflammatory ulcers and 8 (40%) noninflammatory ulcers were due to *L. V. guyanensis* ($p = 0.06$), 5 (26%) inflammatory and 1 (5%) noninflammatory ulcers were due to *L. V. panamensis* ($p = 0.09$), 1 (5%) inflammatory and 1 (5%) noninflammatory ulcers were due to *L. V. peruviana* ($p = 0.66$), 2 (11%) inflammatory and 1 (5%) noninflammatory ulcers were due to *L. V. lainsoni* ($p = 0.66$), 2 (11%) inflammatory ulcers and 4 (20%) noninflammatory ulcers were due to hybrid/unidentified species ($p = 0.66$) (Table 2 and Figure 1). However, compared to both *L. V. braziliensis* and *L. V. panamensis*, *L. V. guyanensis* ulcers were less likely to cause an inflammatory phenotype: 58% of ulcers due to *L. V. braziliensis*, 83% of ulcers due to *L. V. panamensis*, and 20% of ulcers due to *L. V. guyanensis* were inflammatory ($p = 0.0369$).

Bacterial organisms

Of the 39 ulcers, 30 (77%; 15 inflammatory and 15 noninflammatory) had at least one bacterial species detected while 9 (23%, four inflammatory and five noninflammatory) had no bacterial species detected ($p > 0.99$) (Table 3). The following organisms were identified in ulcers using non-WGS methods: *P. aeruginosa* was detected in 12 (30%) of 39 ulcers; while *S. aureus* was detected in 16 (41%), *C. freundii* was detected in 13 (33%), *E. cloacae* was detected in 12 (31%), *K. pneumoniae* was detected in 11 (28%), *Enterococcus* spp. was detected in 7 (18%), *E. coli* was detected in

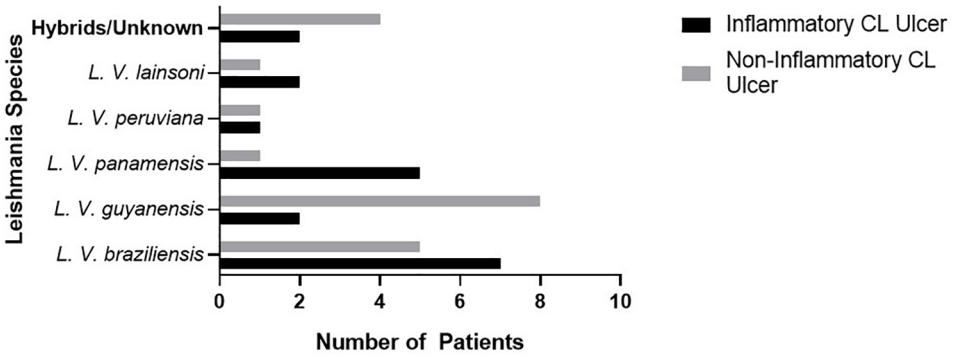

Figure 1. Distribution of causative *Leishmania* spp. across the inflammatory and noninflammatory phenotypes of CL.
CL, Cutaneous leishmaniasis.

Table 3. Spectrum of bacterial pathogens among inflammatory and noninflammatory CL.

Characteristics of bacterial organisms	Total *N*	Inflammatory CL (*n* = 19)	Noninflammatory CL (*n* = 20)	*p* Value
Number of bacterial organisms detected per patient				0.20*
0 detected	9 (100%)	4 (44.4%)	5 (55.5%)	
1 detected	7 (100%)	2 (28.6%)	5 (71.4%)	
2 detected	5 (100%)	2 (40.0%)	3 (60.0%)	
3 detected	7 (100%)	7 (100%)	0 (0.0%)	
4+ detected	11 (100%)	4 (36.4%)	7 (63.4%)	
Spectrum of bacteria detected				
P. aeruginosa	12 (100%)	7 (58.3%)	5 (41.7%)	0.54‡
S. aureus	16 (100%)	8 (50.0%)	8 (50.0%)	1.00‡
C. freundii	13 (100%)	6 (46.2%)	7 (53.8%)	1.00‡
E. cloacae	12 (100%)	4 (33.3%)	8 (66.7%)	0.36‡
K. pneumoniae	11 (100%)	5 (45.5%)	6 (54.5%)	1.00‡
Enterococcus spp.	7 (100%)	4 (57.1%)	3 (42.9%)	0.71‡
E. coli	6 (100%)	1 (16.7%)	5 (83.3%)	0.20‡
S. pyogenes	4 (100%)	3 (75.0%)	1 (25.0%)	0.35‡
S. oralis	2 (100%)	0 (0.0%)	2 (100%)	0.49‡
S. epidermidis	1 (100%)	1 (100%)	0 (0.0%)	0.48‡
S. thermophilus	1 (100%)	1 (100%)	0 (0.0%)	0.48‡
Solobacterium spp.	1 (100%)	1 (100%)	0 (0.0%)	0.48‡
P. puraquae	1 (100%)	1 (100%)	0 (0.0%)	0.48‡

*Grouped "0," "1," "2," and "3," "4+" for comparison.
‡Compared the respective bacteria to the other bacterial species.
CL, Cutaneous leishmaniasis.

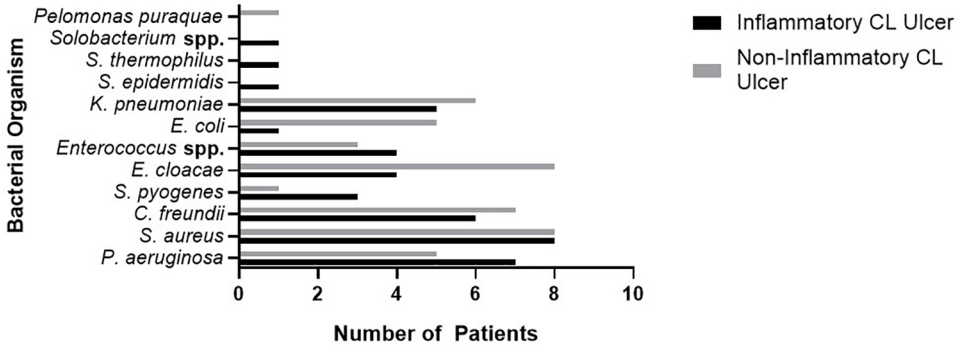

Figure 2. Distribution of bacterial organisms among patients with the inflammatory and noninflammatory phenotypes of CL.
CL, Cutaneous leishmaniasis.

6 (15%), *S. pyogenes* was detected in 4 (10%), *S. oralis* was detected in 2 (5%), and *S. epidermidis*, *S. thermophilus*, *Solobacterium* spp., and *Pelomonas puraquae* were detected in 1 (3%) each, respectively (Table 3). There was no difference observed in the presence of the following organisms across phenotypes: *P. aeruginosa* was detected in 7 (37%) inflammatory versus 5 (25%) noninflammatory ulcers ($p = 0.50$); *S. aureus* was detected in 8 (42%) inflammatory versus 8 (40%) noninflammatory ulcers ($p > 0.99$); *C. freundii* was detected in 6 (32%) inflammatory versus 7 (35%) noninflammatory ulcers ($p > 0.99$); *S. pyogenes* was detected in 3 (16%) inflammatory versus 1 (5%) noninflammatory ulcers ($p = 0.34$); *E. cloacae* was detected in 4 (21%) inflammatory versus 8 (40%) noninflammatory ulcers ($p = 0.30$); *K. pneumoniae* was detected in 5 (26%) inflammatory versus 6 (30%) noninflammatory ulcers ($p > 0.99$); *E. coli* was detected in 1 (5%) inflammatory versus 5 (25%) noninflammatory ulcers ($p = 0.18$); and *Enterococcus* spp. was detected in 4 (21%) inflammatory versus 3 (15%) noninflammatory ulcers ($p = 0.69$) (Table 3 and Figure 2). There was no difference in the number of ulcers containing pathogenic (*S. aureus*, *S. pyogenes*, *P. aeruginosa*, *C. freundii*, *Enterobacter*/*Klebsiella* spp., *E. coli*) versus opportunistic (*S. epidermidis*, *S. thermophilus*, *Enterococcus* spp., *S. oralis*, *Solobacterium* spp., *P. puruqae*) bacterial organisms observed across inflammatory and noninflammatory phenotypes of CL ($p > 0.99$) (Figure 3).

WGS analysis

Of six ulcer FPLIs sent for WGS, 4 (68%) lesions were caused by *L. V. braziliensis*, 1 (16%) was due to *L. V. peruviana*, and 1 (16%) was below detection limits for *Leishmania* species identification; only four had sufficient sequence data for further WGS analysis. Among the four FPLIs with sufficient sequence data, WGS yielded identification of many environmental bacterial contaminants, with *Brevundimonas nasdae*, a gram-negative opportunistic pathogen, identified in all four samples. Other viral, parasitic, and fungal organisms were identified, including *Melampsora pinitorqua*, *Staphylococcus* phage, and *Toxoplasma gondii*. WGS and conventional PCR identified *S. aureus* and *C. freundii* concordantly. WGS remained more sensitive for *P. aeruginosa*, *S. pyogenes*, *E. coli*, and *Enterococcus* spp., however, remained less sensitive for *Enterobacter* spp. and *K. pneumoniae* (Table 4).

Discussion

The present study demonstrates that detection of potentially pathogenic and nonpathogenic bacteria is common in ulcers of CL, regardless of phenotype, with bacterial species detected in approximately 77% of CL ulcers in this study. Although the frequency of CL ulcers harboring no detectable bacteria by targeted molecular assays was only 23%, this may be a result of our limited set of microbial assays, few samples with available WGS data, as well as low relative abundance and possibly restricted bacterial diversity that has been previously documented in CL ulcers.[32] Overall, we reported equal distributions of detectable organisms, pathogens, and nonpathogens among inflammatory and noninflammatory ulcers of CL. Furthermore, prevalence of bacterial species did not differ by CL phenotype.

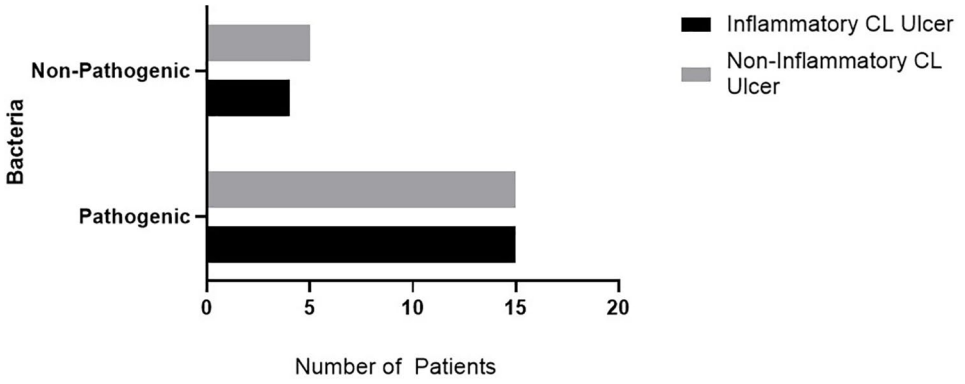

Figure 3. Distribution of pathogenic (*S. aureus*, *S. pyogenes*, *P. aeruginosa*, *C. freundii*, *Enterobacter/Klebsiella* spp.) versus nonpathogenic (*S. epidermidis*, *S. thermophilus*, *Enterococcus* spp.) bacteria among ulcers of inflammatory and noninflammatory CL.
CL, Cutaneous leishmaniasis.

Table 4. Comparison of WGS to conventional PCR methods for pathogen detection in subset of six samples.

Clinical sample	*S. aureus*	*C. freundii*	*S. pyogenes*	*Enterococcus* spp.	*Enterobacter* spp./*Klebsiella* spp.	*P. aeruginosa*	*E. coli*
1	WGS/PCR	—	PCR	PCR	PCR	WGS	PCR
2	WGS/PCR	—	PCR	WGS	—	—	WGS
3	WGS/PCR	—	WGS	WGS	WGS/PCR	WGS/PCR	WGS/PCR
4	WGS/PCR	—	WGS	PCR	—	WGS	—
5	NA	NA	NA	NA	NA	NA	NA
6	NA	NA	NA	NA	NA	NA	NA

NA, not applicable; PCR, polymerase chain reaction; WGS, whole genome sequencing.

This finding suggests a minimal contribution of detected bacterial organisms to the pathogenesis of the severe inflammatory CL phenotype. Patients with the inflammatory phenotype were, on average, 15 years older than patients with the noninflammatory phenotype, which may have influenced ulcer bacterial microbiome due to age-related immune dysfunction (immunosenescence), however, this study was not designed to test such a hypothesis.

Case reports and studies have emerged examining the impact of secondary bacterial infections and healing times of patients with CL ulcers,[14,33] which have shown conflicting results. High proportions of *S. aureus* present in the CL ulcers in our study were not particularly surprising, as this species is known to frequently inhabit many regions of the skin—exerting pathogenesis when it infects deeper tissues, the blood, or expresses pathogenic endotoxins or exotoxins.[34] The proportions of *P. aeruginosa* were descriptively different among the two groups, with *P. aeruginosa* detected in 44% of inflammatory CL ulcers versus 18% of all noninflammatory CL ulcers, however, this was not statistically significant. This relatively higher frequency of *P. aeruginosa* in inflammatory ulcers may suggest its potential association with the severe inflammatory phenotype given its highly pathogenic nature and propensity for multi-drug resistance, though the sample size in this study was too small to detect this difference. Based on the prevalence of *P. aeruginosa*, a larger sample size of 50 (power of 0.8 and significance of 0.05) in each of the respective inflammatory and noninflammatory ulcer arms would be required to detect a significant difference in pseudomonal prevalence across

phenotypes. Previous studies have also reported *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Pseudomonas* spp., and other opportunistic bacteria in LCL lesions that are free of signs of secondary infection, again supporting a lack of bacterial causality to the inflammatory phenotype.[15]

Previous clinical reports have suggested earlier and prompt antibiotic therapy in cases where *P. aeruginosa* is present in cutaneous lesions in order to prevent severe mutilation, such as those seen in inflammatory CL ulcers.[35] It is known that *P. aeruginosa* has the intrinsic potential to develop antibiotic resistance through a variety of different mechanisms, causing pathogenesis in immunocompromised or highly comorbid individuals who are immunocompromised.[36,37] We did note an association between age and disease severity, whereby older patients were more likely to manifest the inflammatory phenotype, yet we did not find a difference in bacterial spectra that may explain this observation.

Strengths of the current study include the use of both bacterial-specific qPCR and pan-bacterial end-point PCR assays, as well as WGS on a subset of samples, which allowed for proof-of-concept of improved sensitivity and specificity for detecting bacterial organisms relative to culture-based methodology carried out by other studies evaluating the microbiome of CL ulcers.[15,38] Both WGS and PCR produced concordant results for the detection of *S. aureus* and *C. freundii*, however, depending on the organism, WGS or PCR would outperform the other. Culture-based methodology can largely exclude bacteria of low relative abundance and fastidious microorganisms, which may result in the under-representation of species present in the CL microbiome.

Limitations of this study include the small number of ulcers in each group, with varying causative *Leishmania* spp. We found no association between causative *Leishmania* species and CL phenotype, but whether or not this represents a true absence of association or was a result of a small sample size cannot be determined. Consequently, beyond WGS, we had no molecular corroboration of nonbacterial members of the ulcer microbiome, nor could we infer a colonization role versus infection for any detected organisms. Another limitation is our inability to detect the production of virulence factors or specific resistance genes in any of the organisms detected as such an objective was beyond the scope of this study. However, understanding which detected organisms are elaborating virulence factors within the ulcer microbiome is another dimension potentially underpinning causality that should be investigated in a prospective fashion in a larger clinical study.

Finally, ulcer chronicity and previous use of topical therapeutics are potential modifiers of representative organisms, such as use of antimicrobial ointments or oral cephalexin reducing burden of *S. aureus* and *S. epidemmidis*.[39] Comorbidities including diabetes and vascular disease would also theoretically contribute to ulcer microbiome, as would smoking and micronutrient status due to the likely synergistic effects of reduced vascular perfusion of tissue, impaired wound healing, dysfunction of adipose tissue leading to insulin resistance and consequent immunological changes, although we have no access to such data in this cohort.[40] Moreover, genetic polymorphisms of human leukocyte antigen (HLA) genes have been found to confer protection against or impart susceptibility to leishmaniasis.[41,42] Again, these potential modifiers could be investigated prospectively in a larger clinical study and a thorough examination of both host- and parasite-specific factors.

Beyond the role of targeted antimicrobial therapies for secondarily infected CL ulcers, phytochemicals—that is, biologically active compounds found in plants—such as capsaicin, may also have a concomitant role in anti-leishmanial and antibacterial action.[43] Despite the very low quality of existing observational studies, ethnopharmaceutical compounds used in traditional medicine as well as novel compounds such as garlic, garad, neem, *Mat Lippia*, *Physalis minima*, and *Morinda citrifolia* were evaluated on humans with some level of cure achieved in participants of these studies, with comparable efficacy to current anti-leishmanial therapeutics.[44–47] Future evaluation of such plant-based compounds in the context of secondarily infected ulcers may prove to be an all-encompassing therapeutic modality to combat both bacterial and leishmanial organisms and improve wound healing.

The overall representation of pathogenic and nonpathogen bacterial organisms was not associated with the inflammatory phenotype, however, *P. aeruginosa* may be present in higher frequencies

in inflammatory compared to noninflammatory ulcers. Future studies with a larger sample size to study this association are warranted. As well, further examination of additional ulcers using WGS will clarify the bacterial composition between the two ulcer phenotypes, which may have implications for our understanding of antimicrobial stewardship in ulcerative leishmaniasis. The identification of other viral, fungal, and parasitic organisms raises the question of nonbacterial agents contributing not just to the ulcer microbiome but to the inflammatory phenotype, in particular. Such organisms could include the endosymbiotic *Leishmania* RNA Virus-1,[48] and may enhance parasitic virulence and drive host immune responses, a tantalizing explanation of older patients in this cohort developing the more inflammatory phenotype. Future work should focus on disentangling the interrelationships between host, parasite, and ulcer microbiome in order to improve clinical response, direct wound care, and better target antimicrobial therapy according to principles of stewardship.

Conclusion

This study demonstrated that the bacterial microbiology of the severe inflammatory phenotype of CL ulcers was not significantly different compared to that of the noninflammatory phenotype. Considering the alarming spread of antimicrobial resistance, our findings may inform clinical practice as antimicrobial therapy may not truly impact resolution of inflammatory CL ulcers. Rather, the diminution of the inflammatory appearance may simply be predicated upon adequate wound care and timely anti-leishmanial therapy. Future studies should include substantiating the current findings with larger sample sizes and focusing on other contributions to the pathogenesis of CL ulcer phenotypes to further inform management guidelines. The use of WGS may be warranted to assess bacterial and nonbacterial contributions to the CL phenotype as observed in this study, where other viral, fungal and parasitic organisms not captured from the outputs of singleplex assays can be identified.[49]

Declarations

Ethics approval and consent to participate

Research ethics board (REB) approval was obtained from Public Health Ontario (#2015-048.01 and 2017-052.01) and Institutional Review Board (IRB) approval was obtained from the Universidad Peruana Cayetano Heredia (#60401). For the Peruvian cohort, written informed consent for participation was provided by patients prospectively enrolled via the Instituto de Medicina Tropical "Alexander von Humboldt." For the Ontario cohort, requirement for informed consent to participate was waived by the research ethics board of Public Health Ontario due to use of biobank anonymized, surplus, discarded clinical specimens submitted for *Leishmania* testing.

Consent for publication

Not applicable.

Author contributions

Ruwandi Kariyawasam: Data curation; Formal analysis; Investigation; Methodology; Validation; Writing – original draft; Writing – review & editing.

Bryan Gascon: Data curation; Formal analysis; Investigation; Methodology; Writing – original draft; Writing – review & editing.

Priyanka Challa: Data curation; Formal analysis; Investigation; Writing – review & editing.

Jordan Mah: Data curation; Formal analysis; Investigation; Writing – review & editing.

Rachel Lau: Data curation; Formal analysis; Investigation; Methodology; Software; Supervision; Validation; Writing – original draft; Writing – review & editing.

Braulio M. Valencia: Data curation; Formal analysis; Investigation; Methodology; Writing – review & editing.

Alejandro Llanos-Cuentas: Funding acquisition; Data curation; Investigation; Methodology; Project administration; Resources; Supervision; Writing – review & editing.

Andrea K. Boggild: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing – original draft; Writing – review & editing.

Acknowledgements

The authors would like to thank Dominac Gomez-Baldeon and Ana Luz Quispe for their contributions to field operations and logistical support.

Funding
The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This body of work was supported by the Leishmaniasis Working Group of the Instituto de Medicina Tropical "Alexander von Humboldt," Lima, Peru, the University of Toronto, and the Public Health Ontario Laboratory, Toronto, Canada. The study was supported by the Projehct Initiative Fund (PIF; #2015-048.01 and 2017-052.01) from Public Health Ontario Laboratories Toronto Public Health Ontario, Toronto, Ontario, Canada. Dr. Boggild is supported as a Clinician Scientist by the Departments of Medicine of the University of Toronto and the University Health Network.

Competing interests
Dr. Boggild oversees the Tropical Disease Fund for Excellence of the University Health Network Foundation, which has received an unrestricted educational grant from Seegene Canada. All other authors do not report a conflict of interest.

Availability of data and materials
All relevant data has been made available through this publication.

ORCID iD
Andrea K. Boggild https://orcid.org/0000-0002-2720-6944

References

1. Alvar J, Vélez ID, Bern C, et al. Leishmaniasis worldwide and global estimates of its incidence. *PloS One* 2012; 7: e35671.
2. Castellucci LC, Almeida LFd, Jamieson SE, et al. Host genetic factors in American cutaneous leishmaniasis: a critical appraisal of studies conducted in an endemic area of Brazil. *Memorias do Instituto Oswaldo Cruz* 2014; 109: 279–288.
3. Reithinger R, Dujardin J-C, Louzir H, et al. Cutaneous leishmaniasis. *Lancet Infect Dis* 2007; 7: 581–596.
4. Hartley MA, Kohl K, Ronet C, et al. The therapeutic potential of immune cross-talk in leishmaniasis. *Clin Microbiol Infection* 2013; 19(2): 119–130.
5. Rhajaoui M, Nasereddin A, Fellah H, et al. New clinicoepidemiologic profile of cutaneous leishmaniasis, Morocco. *Emerging Infect Dis* 2007; 13: 1358.
6. Rego FD, Lima AC, Pereira AS, et al. Genetic variant strains of Leishmania (Viannia) braziliensis exhibit distinct biological behaviours. *Parasitology Res* 2018; 117(10): 3157–3168.
7. Gagini T, Schubach AO, Madeira MF, et al. Genotypic profiles of Leishmania (Viannia) braziliensis strains from cutaneous leishmaniasis patients and their relationship with the response to meglumine antimoniate: a pilot study. *Parasite* 2017; 24: 34.
8. Aronson N, Herwaldt BL, Libman M, et al. Diagnosis and treatment of leishmaniasis: clinical practice guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). *Clin Infect Dis* 2016; 63: e202–e264.
9. Hodiamont CJ, Kager PA, Bart A, et al. Species-directed therapy for leishmaniasis in returning travellers: a comprehensive guide. *PLoS Negl Trop Dis* 2014; 8: e2832.
10. Shmueli M and Ben-Shimol S. Review of leishmaniasis treatment: can we see the forest through the trees? *Pharmacy* 2024; 12(1): 30.
11. Periferakis A, Caruntu A, Periferakis AT, et al. Availability, toxicology and medical significance of antimony. *Int J Environ Res Public Health* 2022; 19(8): 4669.
12. Blum J, Buffet P, Visser L, et al. LeishMan recommendations for treatment of cutaneous and mucosal leishmaniasis in travelers, 2014. *J Travel Med* 2014; 21: 116–129.
13. Eiras DP, Kirkman LA and Murray HW. Cutaneous leishmaniasis: current treatment practices in the USA for returning travelers. *Current Treat Options Infect Dis* 2015; 7: 52–62.
14. Vera LA, Macêdo VdO, Magalhães AVd, et al. Cutaneous leishmaniotic ulcers with Corynebacterium diphtheriae. *Revista da Sociedade Brasileira de Medicina Tropical* 2002; 35: 311–313.
15. Fontes CO, Carvalho MAR, Nicoli JR, et al. Identification and antimicrobial susceptibility of micro-organisms recovered from cutaneous lesions of human American tegumentary leishmaniasis in Minas Gerais, Brazil. *J Med Microbiol* 2005; 54: 1071–1076.
16. do Gonçalves EdG, dos Reis FSA, de Oliveira EG, et al. Bacterial infection in cutaneous leishmaniasis: bacterial pattern and sensitivity to antibiotics. *Revista da Sociedade Brasileira de Medicina Tropical* 2009; 42: 219–221.

17. Gunathilaka N, Perera H, Wijerathna T, et al. The diversity of midgut bacteria among wild-caught *Phlebotomus argntipes* (Psychodidae: Phlebotominae), the vector of leishmaniasis in Sri Lanka. *BioMed Res Int* 2020; 2020: 5458063.

18. Ki V and Rotstein C. Bacterial skin and soft tissue infections in adults: a review of their epidemiology, pathogenesis, diagnosis, treatment and site of care. *Can J Infect Dis Medical Microbiol* 2008; 19: 173–184.

19. Wortmann G, Sweeney C, Houng H-S, et al. Rapid diagnosis of leishmaniasis by fluorogenic polymerase chain reaction. *Am J Tropical Med Hygiene* 2001; 65: 583–587.

20. Boggild AK, Valencia BM, Espinosa D, et al. Detection and species identification of Leishmania DNA from filter paper lesion impressions for patients with American cutaneous leishmaniasis. *Clin Infect Dis* 2010; 50: e1–e6.

21. Schönian G, Nasereddin A, Dinse N, et al. PCR diagnosis and characterization of Leishmania in local and imported clinical samples. *Diagnostic Microbiol Infect Dis* 2003; 47: 349–358.

22. de Almeida ME, Steurer FJ, Koru O, et al. Identification of Leishmania spp. by molecular amplification and DNA sequencing analysis of a fragment of rRNA internal transcribed spacer 2. *J Clin Microbiol* 2011; 49: 3143–3149.

23. Persing DH, Tenover FC, Hayden RT, et al. *Molecular microbiology: diagnostic principles and practice*. 3rd ed. Hoboken, NJ: John Wiley & Sons, 2020.

24. Hardick J, Won H, Jeng K, et al. Identification of bacterial pathogens in ascitic fluids from patients with suspected spontaneous bacterial peritonitis by use of broad-range PCR (16S PCR) coupled with high-resolution melt analysis. *J Clin Microbiol* 2012; 50: 2428–2432.

25. CDC. Multiplex real-time PCR detection of Klebsiella pneumoniae carbapenemase (KPC) and New Delhi metallo-β-lactamase (NDM-1) genes, https://www.cdc.gov/hai/settings/lab/kpc-ndm1-lab-protocol.html (2011, accessed 31 April 2021).

26. De Vos D, Lim A Jr, Pirnay J-P, et al. Direct detection and identification of Pseudomonas aeruginosa in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane lipoprotein genes, oprI and oprL. *J Clin Microbiol* 1997; 35: 1295–1299.

27. Spilker T, Coenye T, Vandamme P, et al. PCR-based assay for differentiation of Pseudomonas aeruginosa from other Pseudomonas species recovered from cystic fibrosis patients. *J Clin Microbiol* 2004; 42: 2074–2079.

28. Ke D, Picard FJ, Martineau F, et al. Development of a PCR assay for rapid detection of enterococci. *J Clin Microbiol* 1999; 37: 3497–3503.

29. Martineau F, Picard FJ, Ke D, et al. Development of a PCR assay for identification of staphylococci at genus and species levels. *J Clin Microbiol* 2001; 39: 2541–2547.

30. Nyström T and Neidhardt FC. Cloning, mapping and nucleotide sequencing of a gene encoding a universal stress protein in *Eschericha coli*. *Molecular Microbiol* 1992; 6: 3187–3198.

31. Collado M, Delgado S, Maldonado A, et al. Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR. *Lett Appl Microbiol* 2009; 48: 523–528.

32. Salgado VR, de Queiroz AT, Sanabani SS, et al. The microbiological signature of human cutaneous leishmaniasis lesions exhibits restricted bacterial diversity compared to healthy skin. *Memorias do Instituto Oswaldo Cruz* 2016; 111: 241–251.

33. Sadeghian G, Ziaei H, Bidabadi LS, et al. Decreased effect of glucantime in cutaneous leishmaniasis complicated with secondary bacterial infection. *Indian J Dermatol* 2011; 56: 37.

34. Davis C. *Normal flora in medical microbiology*. In: Baron S (ed.) *Medical Microbiology*. 4th ed., Chapter 6, Galveston, TX: University of Texas Medical Branch at Galveston, 1996.

35. Van Der Vliet D, Le Guern A-S, Freitag S, et al. Pseudomonas aeruginosa otochondritis complicating localized cutaneous leishmaniasis: prevention of mutilation by early antibiotic therapy. *Am J Tropical Med Hygiene* 2006; 75: 270–272.

36. Li X-Z, Livermore DM and Nikaido H. Role of efflux pump (s) in intrinsic resistance of Pseudomonas aeruginosa: resistance to tetracycline, chloramphenicol, and norfloxacin. *Antimicrob Agents Chemother* 1994; 38: 1732–1741.

37. Strateva T and Yordanov D. Pseudomonas aeruginosa–a phenomenon of bacterial resistance. *J Medical Microbiol* 2009; 58: 1133–1148.

38. Rhoads DD, Cox SB, Rees EJ, et al. Clinical identification of bacteria in human chronic wound infections: culturing vs. 16S ribosomal DNA sequencing. *BMC Infect Dis* 2012; 12: 1–8.

39. Layegh P, Ghazvini K, Moghiman T, et al. Bacterial contamination in cutaneous leishmaniasis: its effect on lesions' healing course. *Indian J Dermatol* 2015; 60(2): 211.

40. Oliveira FMS, Cruz FE, Pinheiro, et al. Comorbidities involving parasitic diseases: a look at the benefits and complications. *Experimental Biol Med* 2022; 247(2): 1819–1826.

41. Ribas-Silva RC, Ribas AD, dos Santos MCG, et al. Association between HLA genes and American cutaneous leishmaniasis in endemic regions of Southern Brazil. *BMC Infect Dis* 2013; 13: 198.

42. Eimanzadeh M, Mohebali M, Zarrabi M, et al. The association of human leukocyte antigen (HLA) Class I and II Genes with cutaneous and visceral leishmaniasis in iranian patients: a Preliminary Case-Control Study. *Iranian J Parasitology* 2023; 18(2): 155–164.

43. Periferakis AT, Periferakis A, Periferakis K, et al. Antimicrobial properties of capsaicin: available data and future research perspectives. *Nutrients* 2023; 15(19): 4097.

44. Sattar FA, Ahmed F, Ahmed N, et al. A double-blind, randomized, clinical trial on the antileishmanial activity of a Morinda citrifolia (Noni) stem extract and its major constituents. *Nat Prod Commun* 2012; 7(2): 195–196.

45. Khalid FA, Abdalla NM, Mohomed HEO, et al. Treatment of cutaneous leishmaniasis with some local sudanese plants (Neem, Garad & Garlic). *Turkiye Parazitoloji Dergisi* 2004; 28(3): 129–132.

46. Rahman A, Choudhary MI, Yousuf S, et al. United states patent application publication-formulations against cutaneous leishmaniasis. Pub No. US2827921B1, 2011.

47. Soomro FR, Pathan GM, Abbasi P, et al. Clinical trial of 20% Mat lippia (bucan) topical ointment for cutaneous leishmaniasis; a preliminary trial. *J Pakistan Assoc Dermatol* 2005; 15(2): 108–112.

48. Kariyawasam R, Grewal J, Lau R, et al. Influence of Leishmania RNA virus 1 on proinflammatory biomarker expression in a human macrophage model of American tegumentary leishmaniasis. *J Infect Dis* 2017; 216: 877–886.

49. Didelot X, Bowden R, Wilson DJ, et al. Transforming clinical microbiology with bacterial genome sequencing. *Nat Rev Genetics* 2012; 13: 601–612.