Phenotypic, molecular and antimicrobial susceptibility assessment in isolates from chronic ulcers of cured leprosy patients: a case study in Southern Brazil*

Luciane Cristina Gelatti[1]
Ana Paula Becker[3]
Letícia Ganassini[5]
Renan Rangel Bonamigo[2]
Letícia Maria Eidt[4]
Pedro Alves d' Azevedo[2]

DOI: http://dx.doi.org/10.1590/abd1806-4841.20142688

Abstract: BACKGROUND: One of the most stigmatizing physical sequelaeof leprosy in cured patients is the development of chronic lower extremity ulcers. The bacterial diversity present in ulcers is considered one of the factors that can delay the healing process, as well as serve as a focus for severe secondary infections.
OBJECTIVE: To identify the microbiota and antimicrobial resistance profile of bacteria isolated from skin ulcers in patients cured of leprosy.
METHODS: After obtaining informed consent, material was collected from ulcers of 16 patients treated at the Outpatient Public Health Dermatology Clinic of Rio Grande do Sul and Hospital Colônia Itapuã. Sampleswere collected during dressing, and the material sent to the Microbiology Laboratory of the Federal University of Health Sciences of Porto Alegre for microbiological culture. Methicillin-resistant *Staphylococcus aureus* (MRSA) was characterized by two molecular methods, including detection of the mecA gene by PCR and SCCmecgene typing.
RESULTS: Cultures revealed microorganisms in all ulcers: Gram-negative bacilli in 80%, Gram-positive cocci in 63%, and mixed microflora in 36%. *Staphylococcus aureus* and Pseudomonas aeruginosa were the most prevalent bacteria. Assessment of the antimicrobial resistance profile was notable for the presence of MRSA. Molecular analysis of this isolate revealed presence of the mecA gene contained in a type IV staphylococcal cassette chromosome mec (SCCmec).
CONCLUSIONS: In patients with leprosy, laboratory culture of skin ulcers is essential for correct antibiotic selection and to control emerging pathogens, such as MRSA carrying SCCmec type IV.
Keywords: Bacteria; Leprosy; Methicillin-resistant *Staphylococcus aureus*; *Mycobacterium leprae*; *Staphylococcus aureus*; Ulcer

INTRODUCTION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*, an obligate intracellular bacterium. *M. leprae* is an acid-alcohol fast bacillus, with high infectivity and low pathogenicity, that mainly infects skin macrophages and Schwann cells in the nerves, and was first observed by Norwegian physician Amauer Hansen in 1871.[1,2] Transmission is believed to occur by direct person-to-person contact between a susceptible individual and a patient with multibacillary leprosy, particularly via the airborne route.[3] Unfavorable living conditions in the population influence the transmission of leprosy and hinder its control and elimination.[4,5]

The World Health Organization (WHO) regards leprosy as a public health issue, particularly in countries where its prevalence exceeds 1:10,000 population. India and Brazil, two countries where leprosy is considered endemic by WHO, are ranked first and second respectively worldwide in terms of absolute number of cases.[6]

Received on 11.4.2013.
Approved by the Advisory Board and accepted for publication on 20.06.2013.
* Work performed at the Graduate Program, Department of Microbiology and Parasitology, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Outpatient Public Health Dermatology Clinic (ADS) Rio Grande do Sul State Department of Health, and Hospital Colônia Itapuã - Viamão (RS), Brazil.
Financial Support: None
Conflict of Interest: None.

[1] Faculdade Serra da Mesa (FASEM) – Uruaçu (GO), Brasil.
[2] Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) - Porto Alegre (RS), Brazil.
[3] Centro Universitário Franciscano (UNIFRA) – Santa Maria (RS), Brazil.
[4] Ambulatório de Dermatologia Sanitária da Secretaria Estadual de Saúde do Rio Grande do Sul – Porto Alegre (RS), Brazil.
[5] Hospital Mãe de Deus – Porto Alegre (RS), Brazil.

One of the most stigmatizing sequelae occurring after treatment of leprosy is the development of chronic neuropathic ulcers in the lower extremities (plantar aspect of the feet, heels, and legs), or *mal perforant*. The plantar region is a site of particularly high risk of ulcer development, due to the biomechanical changes and loss of protective sensation that occur in patients with leprosy. Anhidrosis caused by sweat and sebaceous gland disfunction is another aggravating factor,as it dries the skin and facilitates rupture of its protective stratum corneum. Dry, inelastic skin is conducive to development of fissures on the lower extremities, which, in turn, act as a point of entry for infectious agents, slowing the healing process and occasionally causing muscle, bone, and joint involvement.[7,8]

In addition to clinical risk, which is essentially associated with secondary infections, affected patients are embarrassed by their lesions, which compounds the impairment in quality of life caused by the physical and motor consequences of leprosy.

During the wound treatment process, several factors may delay skin and tissue repair. Notable systemic factors include patient age, nutritional status, comorbid diseases, chronic medication use, smoking, stress, anxiety, and depression. Local factors that affect the healing process include the anatomical site of the wound and the presence of bacterial infection and devitalized tissues.[7,8]

Patients who have been discharged from care after cure of leprosy sometimes have several such factors in combination, includingadvanced age, comorbidities (such as diabetes, hypertension, and obesity), and presence of microorganisms with certain components (capsule, fimbriae, adhesins, toxins, protein A, biofilm production) that increase their virulence, hinder the healing process, and predispose to secondary infections, such as osteomyelitis. However, studies on the microbial flora that colonizes or infects skin ulcerations in patients cured of leprosy are scarce, thus justifying the present study.

Therefore, the objective of this study was to identify the bacterial microbiota of lower extremity skin ulcers in patients cured of leprosy and assess the antimicrobial susceptibility profile of these pathogens.

MATERIALS AND METHODS

This case series was conducted between September 2007 and February 2008 with patients treated at the Outpatient Public Health Dermatology Clinic of Rio Grande do Sul (ADS) and Hospital Colônia Itapuã, both of which are referral centers for the treatment and follow-up of patients with active and cured leprosy in the Brazilian state of Rio Grande do Sul. The sample included patients treated for leprosy who presented to the aforementioned centers for treatment of chronic lower extremity skin ulcers.

The study was approved by the Rio Grande do Sul School of Public Health Research Ethics Committeewith protocol no. 319/07. Before sample collection, all patients were informed of the risks and benefits of the study and provided written informed consent for participation.

The independent variables (factors of interest) were age, sex, time since diagnosis of leprosy, and time since discharge (cure of leprosy) for patients with trophic ulcers. The dependent variables (outcomes) were bacterial isolates from lower extremity ulcers (after phenotypic and molecular identification) and antimicrobial susceptibility.

Sample collection

Biological specimens were collected from skin lesions for bacterial culture during dressing changes, after decontamination of perilesional areas and of the ulcer bed with 0.9% saline solution and 70% rubbing alcohol. When devitalized tissue was present, it was mechanically debrided, and the wound prepared again. Material was collected from deep healthy tissues with a sterile swab and placed in Ames'medium for transport. The specimens were sent to the Microbiology Laboratoryof the Federal University of Health Sciences of Porto Alegre (UFCSPA), where conventional methods were used for isolation and identification of any microorganisms present.

Phenotypic identification

The following assays were used for phenotypic identification of *Staphylococcus aureus*: Gram staining, mannitol agar growth and fermentation, and coagulase and deoxyribonuclease (DNAse) detection. Gram-negative bacteria were identified by means of Gram staining, the oxidase test, and biochemical reactions in triple sugar iron (TSI) agar, lysine iron agar (LIA), Simmons' citrate agar, and urea agar slants, as well as motility-indole-ornithine (MIO) medium.

Antimicrobial susceptibility testing

Susceptibility to antimicrobials was determined by means of the disk diffusion method, in accordance with Clinical and Laboratory Standards Institute recommendations.[9] The disks used for testing contained the following antimicrobial agents: amikacin (30 µg), ampicillin (10 µg), aztreonam (30 µg), cefalotin (30 µg), cefepime (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), ciprofloxacin (5µg), clindamycin (2µg), chloramphenicol (30µg), erythromycin (15µg), gentamicin (10µg), imipenem (10 µg), meropenem (10 µg), piperacillin-tazobactam (100 µg/10 µg), and trimethoprim-sulfamethoxazole (25µg). The *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922 strains were used as quality control.

Molecular characterization

Molecular characterization for presence of the *mec*A gene and SCC*mec* typing was performed on methicillin-resistant *S. aureus* (MRSA) isolates by means of the PCR multiplex method, following the protocol developed by Zhang et al.[10]

Data analysis

Univariate descriptive analysis was conducted so as to obtain the absolute and relative frequencies of all dependent variables, as well as patient gender.

The chi-square test was used to compare the actual and expected frequencies of distribution of bacterial isolates, with statistical significance defined by a p-value< 0.05.

The database was compiled and stored in Microsoft Excel®, and all statistical analyses were carried out in SPSS 12.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The study sample comprised 16 patients. Mean age was 66 years (standard deviation, 10.5 years; range, 47-86 years). Ten patients were male and six were female. The mean time elapsed since diagnosis of leprosy was 34 years (standard deviation, ~8 years). Clinical discharge due to cure of leprosy had occurred a mean of 13 years before (standard deviation, ~2 years), with a coefficient of variation of approximately 15%.

Cultures from all ulcers grew microorganisms; 80% grew Gram-negative bacillin, 63% grew Gram-positive cocci, and 36% grew a mixed microbial flora. The most common combination was *S. aureus* and *P. aeruginosa*. A wide range of bacterial species were isolated; their frequencies are shown in table 1. *S. aureus* was the most common species (62.5%, p<0.05), followed by *P. aeruginosa* (43.75%) and the *Enterobacteriaceae* (68.75%).

All *S. aureus* isolates were sensitive to ciprofloxacin, gentamicin, and trimethoprim-sulfamethoxazole; 90% were sensitive to clindamycin, 80% to erythromycin, and 40% to chloramphenicol.

Among the *S. aureus* isolates, one was methicillin-resistant (MRSA). This isolate was resistant to cefoxitinand chloramphenicol, but susceptible to other, non-beta-lactam antimicrobials, including ciprofloxacin, clindamycin, erythromycin, gentamicin, and trimethoprim-sulfamethoxazole. Molecular analysis by polymerase chain reaction (PCR) included detection of the *mec*A gene and SCC*mec* typing. This *S. aureus* isolate was characterized as *mec*A-positive and carried a type IV SCC*mec*.

Klebsiella spp. and *Escherichia coli* isolates were sensitive to nearly all classes of antimicrobials tested. Decreased susceptibility was found only with trimethoprim-sulfamethoxazoledisks (33%). *Proteus* spp. isolates exhibited wide variation in sensitivity to the tested antimicrobials. Over 80% were sensitive to amikacin, cefepime, and ciprofloxacin. The non-fermenting Gram-negative bacillus *Pseudomonas aeruginosa* was 100% sensitive to imipenem and meropenem, followed by ceftazidime (86%) and amikacin, aztreonam, cefepime, ciprofloxacin, and piperacillin-tazobactam (57% each).

Table 1: Frequency of bacterial isolates from leprosy ulcers

Bacterial species	Frequency	
	N	%*
Staphylococcus aureus	10	62.5
Pseudomonas aeruginosa	7	43.75
Proteus spp.	5	31.25
Klebsiella spp.	3	18.75
Escherichia coli	2	12.5
Citrobacter freundii	1	6.25
Enterococcus spp.	1	6.25

DISCUSSION

Bacterial contamination of leprosy ulcers is a major issue, as the presence of these pathogens can contribute to slow healing and serve as a focus for secondary soft-tissue and skeletal infections. The bacterial microbiota observed in the present study was polymicrobial; the most common species was *S. aureus*, followed by *P. aeruginosa*. A similar microbiota was described by Quege et al. in their 2008 study of cured leprosy patients.[11]

S. aureus is recognized worldwide as a major pathogen implicated in the genesis of hospital- and community-acquired infections. Most pathogenic bacteria can infect bone, but *S. aureus* is the main etiological agent of osteomyelitis, accounting for 80-90% of cases.[12,13] A previous study demonstrated an association between staphylococcal infections and a higher rate of lower extremity amputation in patients with diabetes.[14] A series of virulence factors (capsule, peptidoglycans, teichoic acid, adhesins, protein A, leukocidin, biofilm production) contribute to the pathogenicity of *S. aureus*, as they facilitate its successfulestablishment, development, and persistence in host tissues.[15]

Analysis revealed the presence of a MRSA isolate – thus, one resistant to beta-lactam antibiotics. Methicillin resistance is related to modification in a penicillin-binding protein (PBP) encoded by the *mec*A chromosomal gene.[16,17] This gene is carried by a mobile genetic element known as the staphylococcal chromosomal cassettes (SCC). At least six types of

SCC*mec* have been well characterized: I, II, III, IV,V, and VI.[18,19,20,21] Types I–III are often reported in hospital-acquired clinical MRSA isolates, whereas types IV–VI are found in community-acquired MRSA.

Infections attributed to MRSA are a constant, well-known presence in the hospital setting. However, in recent years, community-acquired MRSA infection has been reported in several countries.[22,23,24] Community MRSA isolates are a frequent cause of skin and soft tissue infections, such as cellulitis and abscess.[25,26,27] However, they may also be implicated in severe infectious conditions, such as meningitis, pneumonia, bacteremia, and septic shock.[28,29,30] Molecular analysis was consistent with presence of the *mec*A gene , contained in a clonal type IV SCC*mec*. Molecular findings also confirmed an increased antimicrobial susceptibility profile; this is attributableto the smaller cassette, which, in most cases, does not harbor other resistance-determining factors, unlike classic hospital-acquired strains.

It is unclear whether the MRSA isolate found in this study was community- or hospital-acquired. Its phenotypic antibiotic resistance profile and the presence of *SCCmec* type IV suggest community origin. However, from an epidemiological standpoint, clinical MRSA isolates are defined as community-acquired if found in samples collected from outpatients or collected within 48 hours of hospital admission. Antimicrobial therapy, recent hospitalization, recent surgical or therapeutic intervention, severe underlying illness, indwelling medical device use, and nursing home admission must be ruled out.[31,32] Under these epidemiological criteria, the MRSA isolate reported herein could not be considered community-acquired, as it was obtained from a patient with severe chronic illness and subject to constant therapeutic interventions.

Pseudomonas aeruginosa, the second most commonly isolated pathogen, also has a broad armamentarium of virulence factors, including structural components, toxins, and enzymes that facilitate infection and tissue invasion and can potentiate tissue necrosis. The nutritional requirements of *P. aeruginosa* for survival are minimal,it tolerates a wide temperature range, and is resistantto many antibiotics and disinfectants.[33] Analysis of the antimicrobial susceptibility profile of these isolates revealed 100% sensitivity only to the carbapenem antibiotics (imipenem and meropenem). The presence of multidrug-resistant bacteria in these lesions could be a result of (particularly topical) antimicrobial use. This highlights the importance of performing culture and sensitivity testing to establish effective therapy, as antimicrobial susceptibility variessubstantially within the same species.

CONCLUSION

Bacteriological analysis of skin ulcerations is not routinely performed in patients with leprosy, as conventional wisdom holds that these wounds are infected or colonized by a range of microorganisms as a matter of course. However, the bacterial species isolated in this study highlight the importance of culture and sensitivity testing in determining the actual microbiota present and establishing proper therapeutic guidance. The information provided by microbial cultures makes it possible for appropriate measures to be implemented for the control of emerging pathogens, such as MRSA carrying SCC*mec* type IV. ❑

REFERENCES

1. Hansen GA, Looft C. Leprosy: in its clinical and pathological aspects. London Bristol: John Wright & Co; 1973. p.50-51.
2. Kaplan G, Cohn ZA. The immunobiology of leprosy. Int Rev Exp Pathol. 1986;28:45-78.
3. Shepard CC. Acid-fast bacilli in nasal excretions in leprosy, and results of inoculation of mice. Am J Hyg. 1960;71:147-57.
4. Andrade VLG, Sabroza PC, Araújo AJG. Fatores associados ao domicílio e à família na determinação da hanseníase, Rio de Janeiro, Brasil. Cad SaúdePúbl. 1994;10:281-92.
5. Rao PSS, Karat ABA, Karat S. Epidemiological studies in leprosy in Gudiyatham Taluk. II: Patterns of familial aggregation of leprosy in endemic area. Lepr Rev. 1969;40:93-8.
6. Who.int [Internet]. World Health Organization. Estratégia Global para aliviar a carga da hanseníase e manter as atividades de controle da hanseníase (Período de plano 2006-2010). 2005;2-27. [acesso 1 mar. 2013]. Disponível em: http://www.who.int/lep/Reports/GlobalStrategy-PDF-verison.pdf.
7. Brasil. Ministério da Saúde. Secretaria de Políticas de Saúde. Departamento de Vigilância Epidemiológica. Manual de Prevenção de Incapacidades. Mistério da Saúde: Brasília; 2008. 141p. Série A. Normas e Manuais Técnicos. (Cadernos de prevenção e reabilitação em hanseníase; n. 1).
8. Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. Manual de Condutas para Tratamento de Úlceras em Hanseníase e Diabetes. Ministério da Saúde; 2008. 94p. Normas e Manuais Técnicos (Cadernos de Prevenção e Reabilitação em Hanseníase, A, 2).
9. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk Susceptibility Tests. Approved Standard. M2-A8. 9th ed. Pennsylvania: Clinical and Laboratory Standards Institute; 2007.
10. Zhang K, McClure JA, Elsayed S, Louie T, Conly JM.. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome mec types I to V in methicillin-resistant Staphylococcus aureus. J Clin Microbiol. 2005;43:5026-33.
11. Quege GE, Bachion MM, Lino Junior RS, Lima ABM, Ferreira PS, Santos QR, et al. Comparação da atividade de ácidos graxos essenciais e biomembrana na microbiota de feridas crônicas infectadas. Rev Eletr Enferm. 2008;10:890-905.
12. Nair SP, Williams RJ, Henderson B. Advances in our understanding of the bone and joint infection pathology caused by Staphylococcus aureus infection. Rheumatology (Oxford). 2000;39:821-34.
13. Lew DP, Waldvogel FA. Osteomyelitis. Lancet. 2004;364:369-79.
14. Nather A, Bee CS, Huak CY, Chew JL, Lin CB, Neo S, et al. Epidemiology of diabetic foot problems and predictive factors for limb loss. J Diabetes Complications. 2008;22:77-82.
15. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WCJ. The Gam-positive cocci. Staphylococci and related organisms. In: Koneman EW, Allen SD, Janda WM, Schrekenberger PC, Winn WC Jr. Color atlas and Textbook of Diagnostic Microbiology. Philadelphia: Lippincott-Raven Publishers; 1997. p.539-76.
16. Chambers HF. Methicillin resistance in Staphylococci: molecular and biochemical basis and clinical implications. Clin Microbiol Rev. 1997;10:781-91.
17. Lowy FD. Staphylococcus aureus infections. N Engl J Med. 1998;339:520-32.
18. Ito T, Katayama Y, Asada K, Mori N, Tsutsumimoto K, Tiensasitorn C, et al. Structural comparison of three types of staphylococcal cassette chromosome mec integrated in the chromosome in methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2001;45:1323-36.
19. Ito T, Ma XX, Takeuchi F, Okuma K, Yuzawa H, Hiramatsu K.Novel type V staphylococcal cassette chromosome mec driven by a novel cassette chromosome recombinase, ccrC.Antimicrob Agents Chemother. 2004;48:2637-51.
20. Ma XX, Ito T, Tiensasitorn C, Jamklang M, Chongtrakool P, Boyle-Vavra S, et al. Novel type of staphylococcal cassette chromosome mec identified in community-acquired methicillin-resistant Staphylococcus aureus strains. Antimicrob Agents Chemother. 2002;46:1147-52.
21. Oliveira DC, Milheiriço C, de Lencastre H.Redefining a structural variant of staphylococcal cassete chromosome mec, SCCmec type VI. Antimicrob Agents Chemother. 2006;50:3457-9.
22. Coombs GW, Monecke S, Pearson JC, Tan HL, Chew YK, Wilson L, et al. Evolution and diversity of community-associated methicillin-resistant staphylococcus aureus in a geographical region. BMC Microbiol. 2011;11:215.
23. Wang X, Towers S, Panchanathan S, Chowell G. A population based study of seasonality of skin and soft tissue infections: Implications for the spread of CA-MRSA. PLoSOne. 2013;8(4):e60872.
24. Vardakas KZ, Kontopidis I, Gkegkes ID, Rafailidis PI, Falagas ME. Incidence, caracteristics, and outcomes of patients with bone and joint infections due to community-associated methicillin-resistant Staphylococcus aureus: a systematic review. Eur J Clin Microbiol Infect Dis. 2013;32:711-21.
25. Gelatti LC, Sukiennik T, Becker AP, Inoue FM, Carmo MS, Castrucci FMS, et al. Sepse por Staphylococcus aureus resistente à meticilina adquirida na comunidade no sul do Brasil. RevMed Trop. 2009;42:458-60.
26. Razera F, De Stefani S, Bonamigo RR, Olm GS, Dias CA, Narvaez GA.CA MRSA in furunculosis: case report of southern Brazil. An Bras Dermatol. 2009;84:515-8.
27. Caraciolo FB, Maciel MA, Santos JB, Rabelo MA, Magalhães V. Antimicrobial resistance profile of Staphylococcus aureus isolates obtained from skin and soft tissue infections of outpatients from a university hospital in Recife-PE, Brazil. An Bras Dermatol. 2012;87:857-61.
28. Naesens R, Ronsyn M, Druwé P, Denis O, Ieven M, Jeurissen A.Central nervous system invasion by community-acquired meticillin-resistant Staphylococcus aureus. J Med Microbiol. 2009;58:1247-51.
29. Otera H, Yamamoto G, Ohkusu K, Kozuki H, Hashimoto K, Tada K. Necrotizing pneumonia in the community. Intern Med. 2012;51:2463-7.
30. Gouveia C, Gavino A, Bouchami O, Miragaia M, Varandas L, de Lencastre H, et al. Community-Associated Methicillin-Resistant Staphylococcus aureus Lacking PVL, as a Cause of Severe Invasive Infection Treated with Linezolid. Case Rep Pediatr. 2013;2013:727824.
31. Salgado CD, Farr BM, Calfee DP. Community acquired methicillin-resistant Staphylococcus aureus: a meta-analysis of prevalence and risk factors. Clin Infect Dis. 2003;36:131-9.
32. DeLeo FR, Otto M, Kreiswirth BN, Chambers HF. Community-associated meticillin-resistant Staphylococcus aureus. Lancet. 2010;375:1557-68.
33. Kiska DL, Gilligan PH. Pseudomonas, In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, editors. Manual of Clinical Microbiology. 7th ed. Washington, D.C.: ASM Press;1999. p. 516-26.

MAILING ADDRESS:
Luciane Cristina Gelatti
Laboratório de Microbiologia / Universidade Federal de Ciências da Saúde de Porto Alegre
Rua Sarmento Leite, 245 - Sala 203
90050-170 - Porto Alegre - RS
Brazil
E-mail:lucianegelatti@hotmail.com

How to cite this article: Gelatti LC, Bonamigo RR, Becker AP, Eidt LM, Ganassini L, d'Azevedo PA.Phenotypic, molecular and antimicrobial susceptibility assessment in isolates from chronic ulcers of cured leprosy patients: a case study in Southern Brazil. An Bras Dermatol. 2014;89(3):404-8.