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Identification and antimicrobial susceptibility of micro-organisms recovered from cutaneous lesions of human American tegumentary leishmaniasis in Minas Gerais, Brazil

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An evaluation of the microbiota present in cutaneous ulcers from 31 patients with a clinical and parasitological diagnosis of American tegumentary leishmaniasis (ATL) was carried out by the standard filter paper disc technique, including antimicrobial susceptibility of the bacterial isolates. Microbial examination indicated that 21 patients (67·7 %) were contaminated with one to four bacteria and some of them also with yeast. A total of 142 micro-organisms were isolated. *Staphylococcus aureus* was the most frequently recovered bacterium (95·2 % of positive patients) and was found to produce type B (70 % of the staphylococcal isolates) and type C (50 %) enterotoxins as well as toxic shock syndrome toxin (60 %). *Proteus mirabilis* (33·3 % of the positive patients), *Streptococcus pyogenes* (19·0 %), H_2S-negative *Proteus* species (19·0 %), *Klebsiella oxytoca* (14·3 %), *Enterobacter* species (9·5 %), *Peptostreptococcus* species (9·5 %), *Pseudomonas* species (4·8 %), *Prevotella bivia* (4·8 %), *Escherichia coli* (4·8 %), *Streptococcus agalactiae* (4·8 %), *Bacteroides fragilis* (4·8 %), *Candida albicans* (9·5 %) and *Candida tropicalis* (4·8 %) were also isolated. Surprisingly, *Staph. aureus* isolates were susceptible to almost all tested drugs, although some of them were resistant to penicillin (69 %) and ampicillin + sulbactam (68 %). Concerning obligate anaerobes, all the Gram-negative isolates (25 % of the total) were resistant to metronidazole. The results of the present study show that microbial secondary contaminants, particularly *Staph. aureus*, should be considered in the diagnosis and treatment of ATL lesions.

INTRODUCTION

Leishmaniasis is a disease caused by parasites of the genus *Leishmania*, which produce diverse clinical manifestations depending upon the characteristics of the parasite and on the immune response of the host. *Leishmania braziliensis* is the species responsible for the majority of cases of American tegumentary leishmaniasis (ATL) in Brazil, where the disease represents a public health problem (Passos *et al.*, 2001).

A typical lesion is a painless ulcer at the site of parasite delivery, with a raised, indurated margin and a necrotic base that is often covered with an adherent crust of dried exudate. Most patients have one or two lesions, usually on exposed sites, varying in size from 0·5 to 3 cm in diameter. When the crust that covers the nodule of the ATL lesion falls off, the lesion becomes susceptible to colonization/infection with a number of micro-organisms, such as pathogenic or opportunistic fungi and bacteria that could cause secondary infections. Such secondary infections may play an important role in the size and shape of the lesion, as well as in scar development (Potter *et al.*, 1983). Most of these contaminating micro-organisms are known to be derived from the transient or indigenous skin microbiota, but data are scarce.

The skin is an intricate habitat for many bacteria, and their type and density are determined by anatomy, humidity, sebum production and host hormonal status. Bacterial skin microbiota are commensal, symbiotic or parasitic relative to the host; the type of relationship established is often inherent to the bacteria but also depends on alterations in the host

†Deceased.

Abbreviation: ATL, American tegumentary leishmaniasis.

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immune status. In virtually all studies of skin and soft-tissue infections, *Staphylococcus aureus* is the most common pathogen. In most studies, *Streptococcus pyogenes* ranks second in frequency, although some investigators do not list the individual species of streptococci, and in one study '*Streptococcus milleri*' was the second most frequently isolated pathogen (Summanen *et al.*, 1995). Because the skin is a fairly dry habitat, Gram-negative bacteria are, with one exception, rarely found on the skin in comparison with the Gram-positive bacteria. The exception is the genus *Acinetobacter*, which is found colonizing the moister areas of skin such axillae, groin and antecubital fossa. A number of *Enterobacter* species can be found on the hands, which become temporarily colonized and constitute a source for cross-infection. However, *Pseudomonas aeruginosa* and *Proteus mirabilis* can be found in the toewebs of normal individuals and can be found as skin invaders in persons with very moist feet (Tannock, 1999). A recent study of the SENTRY Antimicrobial Surveillance Programme on the major pathogens isolated from skin and soft-tissue infections showed that, in Latin America, the most common pathogens, in decreasing order of prevalence, were *Staph. aureus* (32·8 %), *Escherichia coli* (13·1 %), *Pseud. aeruginosa* (11·9 %), *Enterococcus* species (7·7 %), *Klebsiella pneumoniae* (5·8 %), *Enterobacter* species (5·6 %) and *Acinetobacter* species (4·1 %) (Sader *et al.*, 2002). Anaerobes play an important role in complicating skin infections, and peptostreptococci typically are the most common of the anaerobic isolates (Bowler *et al.*, 2001).

Few studies were found in the literature on the prevalence of bacteria and/or fungi in lesions of ATL. Edrissian *et al.* (1990) described the predominant presence, in Iran, of coagulase-positive staphylococci in ulcers of ATL as well as of *Streptococcus* species, *Pseudomonas* species, *Klebsiella* species and *E. coli*. Pereira *et al.* (1999) and Vera *et al.* (2001) also observed that *Staph. aureus* was prevalent in cutaneous leishmaniasis lesions in, respectively, Maranhão and Minas states, Brazil. However, in these few reports, the presence of obligate anaerobic bacteria and fungi was not determined.

The purpose of the present study was to determine the prevalence of bacteria and yeasts in secondary infections in ATL lesions as well as the antibacterial susceptibility of the isolates.

METHODS

Patients. From November 1999 to August 2000, 31 patients with clinical and parasitological diagnoses of ATL were investigated at a medical centre of Caratinga, Minas Gerais, Brazil. Chronic ATL lesions were not included in the study. The ages of the patients ranged from 11 to 84 years, with a mean ± SD of 34·2 ± 20·7 years. Twenty (64·5 %) of the patients were men and 11 (35·5 %) were women. The lesions observed in 21 patients were large and purulent, and 10 patients showed smaller lesions with few (three) or without (seven) secretion. Procedures were performed according to the legal rules of the Federal University of Minas Gerais.

Sampling procedures. The skin areas surrounding the lesions were thoroughly cleaned using cotton wool moistened with alcoholic iodine. Then a sterile filter paper disc, 5 mm in diameter, was applied with sterile tweezers onto the centre of the lesion for a period of 1 min. After application, the disc was transferred to 2 ml pre-reduced anaerobically sterilized (PRAS) Ringer solution under CO_2 flow. The time between specimen collection and receipt by the laboratory was 8–12 h.

Isolation and identification of the microbial strains. The clinical specimen was introduced into an anaerobic chamber (Forma Scientific Company) containing an atmosphere of 85 % N_2, 10 % H_2 and 5 % CO_2. After homogenization by hand, serial decimal dilutions in PRAS Ringer solution from 10^{-1} to 10^{-6} were performed. For each dilution, 0·1 ml quantities were spread onto the following media: brain heart infusion agar (Difco) supplemented with 0·5 % yeast extract, 0·1 % haemin, 0·1 % menadione and 5 % horse blood (BHI-S); *Bacteroides* bile aesculin (BBE) agar supplemented with 100 μg gentamicin (Livingstone *et al.*, 1978); selective agar medium for *Fusobacterium* (Omata & Disraely, 1956) and tryptic soy agar serum-bacitracin-vancomycin (TSBV) (Slots, 1982). Then portions of the same dilutions were transferred from the anaerobic chamber for plating under aerobic conditions onto MacConkey agar (Difco), BHI-S agar, phenyl ethanol agar (Difco) and Sabouraud dextrose agar (Difco). The plates were incubated at 37 °C for 24–48 h and 7 days under aerobic and anaerobic conditions, respectively.

After incubation, about two colonies representative of each morphotype were cultivated onto BHI-S agar in order to obtain pure culture. The identification of the bacterial isolates was based on the following procedures: determination of the obligate or facultative anaerobic character, Gram staining, physiological and biochemical characterization using the API 20A and API 20STREP identification kits (BioMérieux) and complemented, when necessary, with tests for catalase, coagulase, thermonuclease, indole production, H_2S production, urease production, lysine decarboxylase, tryptophan deaminase and carbohydrate fermentation.

For yeast identification, the following tests were done: presence of germ tube, micromorphological features, carbohydrate assimilation (glucose, sucrose, maltose, lactose, galactose, raffinose, cellobiose and xylose) and carbohydrate fermentation (glucose, sucrose, maltose, lactose, galactose and trehalose).

Determination of staphylococcal toxin production. Enterotoxigenicity of *Staph. aureus* isolates was evaluated by the dialysis-membrane-over-agar method as described by Casman *et al.* (1969). Plates were prepared with 25 ml BHI agar. The agar layer was covered with a membrane disc made from Spectra/Por Membrane Dialysis Tubing 6000-8000 (Thomas Scientific) with a flat width of 100 mm. Using aseptic technique the membrane was inoculated with 0·5 ml of a suspension containing the isolated *Staph. aureus*, and the plates were incubated at 37 °C for 24 h. The cultures were then removed from the membrane by washing with 2·5 ml 0·02 M $NaHPO_4$ (pH 7·4) in two steps. The washings were centrifuged at 10 000 ***g*** for 15 min, and the supernatant was collected for enterotoxin testing using the optimum sensitivity plate method as described by Bergdoll & Bennet (1984). The toxins and specific antiserum were provided by the Laboratório de Enterotoxinas, Fundação Ezequiel Dias, Belo Horizonte, Minas Gerais, Brazil.

Antimicrobial susceptibility. MICs for all the bacterial isolates were determined by agar dilution method according to guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, 2000). The obligate anaerobic bacteria were tested against penicillin G (Sigma), clindamycin (Sigma), metronidazole (Rhodia Farma), meropenem (Sigma), chloramphenicol (Schering Plough), tetracycline (Sigma) and ampicillin+sulbactam (Sigma). For Gram-negative facultative anaerobic bacteria the following antibiotics were used: penicillin G

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(Sigma), clindamycin (Sigma), chloramphenicol (Schering Plough), tetracycline (Sigma), ampicillin + sulbactam (Sigma) and gentamicin (Schering Plough). The same antibiotics plus vancomycin were used for Gram-positive anaerobic facultative bacteria. For each drug, appropriate amounts of stock solutions were added to melted agar media to obtain the following final concentrations: the break point concentration, two concentrations twofold higher than the break point and two concentrations twofold lower than the break point. Isolates were grown for 24 h in BHI (Difco) and inoculated by the use of a Steers' replicator (Steers *et al.*, 1959), with a final inoculum of approximately 10^5 c.f.u. per spot. All the tests were done in duplicate.

Quality control measures were utilized by testing *Bacteroides fragilis* ATCC 25285, *Staph. aureus* ATCC 33591, *Staph. aureus* ATCC 29213 and *E. coli* ATCC 11775.

RESULTS

In total 142 micro-organisms were isolated from 21 of the 31 patients (67·7 %). All the lesions that were positive for bacteria or yeast culture were large and purulent, whereas lesions with a negative result for the microbial culture showed a smaller size with few (three) or without (seven) secretions. The number of species recovered per site examined ranged from one to four. Most of the positive cultures were monocontamination (10/21), followed in decreasing order by bi- (6/21), tetra- (3/21) and tricontamination (2/21). As expected, *Staph. aureus* was isolated from most of the monocontaminated lesions (6/10). Four of the six bicontaminations were combinations of *Staph. aureus* with *Strep. pyogenes*.

Table 1 shows that the 142 specimens recovered pertained to 14 microbial species, with a prevalence of *Staph. aureus* (95·2 % of positive patients). In decreasing order of prevalence, the other bacteria recovered from the ATL lesions were: *Prot. mirabilis* (33·3 % of the positive patients), *Strep. pyogenes* (19·0 %), H_2S-negative *Proteus* species (19·0 %), *Klebsiella oxytoca* (14·3 %), *Enterobacter* species (9·5 %), *Peptostreptococcus* species (9·5 %), *Pseudomonas* species (4·8 %), *Prevotella bivia* (4·8 %), *E. coli* (4·8 %), *Streptococcus agalactiae* (4·8 %) and *B. fragilis* (4·8 %). The yeasts *Candida albicans* (9·5 % of the positive patients) and *Candida tropicalis* (4·8 %) were also isolated. Of the 68 *Staph. aureus* strains recovered from the leishmaniasis lesions, most produced type B (70 %) and type C (50 %) enterotoxins, as well as toxic shock syndrome toxin (60 %).

Table 2 shows the distribution of bacterial isolates according to the infection site. These micro-organisms were most frequently isolated from the legs (47·6 %), arms (19·0 %), face (14·3 %), abdominal site (9·5 %), cervical site (4·8 %) and feet (4·8 %), and *Staph. aureus* was found in all the sites. Most of the obligate anaerobic bacteria (*Peptostreptococcus* and *Prevotella* species) were recovered from the abdominal site. Lesions negative for culture were most frequently on the face (50 %), legs (40 %) and chest (10 %).

Table 3 shows the antimicrobial susceptibilities of all the bacterial isolates. Surprisingly, the *Staph. aureus* strains were

Table 1. Identification and frequency of microbial isolates recovered from cutaneous leishmaniasis lesions

Micro-organism	No. (%) of isolates recovered from lesions*	No. (%) of contaminated lesions positive for the microbial species†
Facultative anaerobic bacteria		
Staphylococcus aureus	68 (47·9)	20 (95·2)
Proteus mirabilis	15 (10·6)	7 (33·3)
Streptococcus pyogenes	11 (7·8)	4 (19·0)
Klebsiella oxytoca	10 (7·0)	3 (14·3)
Enterobacter species	10 (7·0)	2 (9·5)
Proteus H_2S negative	8 (5·7)	4 (19·0)
Pseudomonas species	3 (2·1)	1 (4·8)
Escherichia coli	1 (0·7)	1 (4·8)
Streptococcus agalactiae	1 (0·7)	1 (4·8)
Obligate anaerobic bacteria		
Peptostreptococcus species	9 (6·3)	2 (9·5)
Prevotella bivia	2 (1·4)	1 (4·8)
Bacteroides fragilis	1 (0·7)	1 (4·8)
Yeasts		
Candida albicans	2 (1·4)	2 (9·5)
Candida tropicalis	1 (0·7)	1 (4·8)

*Total number of isolates = 142.

†Of 31 patients studied, 21 had lesions contaminated with at least one microbial species.

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Table 2. Anatomical sites with positive culture and respective isolated bacteria

Isolation site	No. (%) of positive lesions*	Bacteria and no. of positive sites
Leg	10 (47·6)	*Staphylococcus*, 9; *Proteus*, 4; *Klebsiella*, 2; *Streptococcus*, 2; *Pseudomonas*, 1; *Bacteroides*, 1; *Enterobacter*, 1; *E. coli*, 1
Arm	4 (19·0)	*Staphylococcus*, 3; *Proteus*, 2
Face	3 (14·3)	*Staphylococcus*, 2; *Streptococcus*, 2; *Enterobacter*, 1
Abdominal site	2 (9·5)	*Peptostreptococcus*, 2; *Staphylococcus*, 1; *Streptococcus*, 1; *Prevotella*, 1
Cervical site	1 (4·8)	*Staphylococcus*, 1; *Peptostreptococcus*, 1
Foot	1 (4·8)	*Staphylococcus*, 1

*Total number of positive lesions = 21.

Table 3. Antimicrobial susceptibilities of bacteria recovered from cutaneous leishmaniasis lesions

VAN, vancomycin; PEN, penicillin; AMP, ampicillin + sulbactam; CHL, chloramphenicol; CLI, clindamycin; GEN, gentamicin; TET, tetracycline; MZOL, metronidazole; MNEN, meropenem.

Bacteria	Antibiotic	No. (%) of isolates in each susceptibility category		
		Resistant	Intermediate	Sensitive
Gram-positive facultative anaerobes ($n = 80$)				
Staphylococcus aureus ($n = 68$)	VAN	0	0	68 (100)
	PEN	47 (69·1)	0	21 (30·9)
	AMP	46 (67·6)	0	22 (32·3)
	CHL	0	0	68 (100)
	CLI	3 (4·4)	0	65 (95·6)
	GEN	0	0	68 (100)
	TET	12 (17·6)	0	56 (82·3)
Streptococcus species ($n = 12$)	VAN	0	0	12 (100)
	PEN	0	0	12 (100)
	AMP	0	0	12 (100)
	CHL	0	0	12 (100)
	CLI	0	0	12 (100)
	TET	6 (50·0)	0	6 (50)
Gram-negative facultative anaerobes ($n = 45$)*	PEN	45 (100)	0	0
	AMP	27 (60·0)	12 (26·7)	6 (13·3)
	CHL	4 (8·9)	0	41 (91·1)
	CLI	44 (97·8)	0	1 (2·2)
	GEN	0	1 (2·2)	44 (97·8)
	TET	15 (33·3)	9 (20·0)	21 (46·7)
Obligate anaerobes ($n = 12$)	PEN	2 (16·7)	0	10 (83·3)
	AMP	0	0	12 (100)
	CHL	0	0	12 (100)
	CLI	2 (16·7)	0	10 (83·3)
	TET	0	0	12 (100)
	MZOL	3 (25·0)	0	9 (75·0)
	MNEN	0	0	12 (100)

*Two *Prot. mirabilis* isolates were lost before MIC determination.

susceptible to almost all tested drugs, although some were resistant to penicillin (69 %) and ampicillin+sulbactam (68 %). Concerning obligate anaerobes, all the Gram-negative bacteria were resistant to metronidazole.

DISCUSSION

In Iran, the bacteriological examination of ATL lesions from 2202 individuals indicated that 35·7 % were also infected

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with one or more pathogenic bacteria, including coagulase-positive staphylococci, β-haemolytic streptococci and other opportunist pathogenic bacteria (Edrissian *et al.*, 1990). In Bahia state, Brazil, a similar study revealed that secondary bacterial infections were found in 54·2 % (45/83 patients) of ATL lesions, with a prevalence of *Staph. aureus* (89 %) (Vera *et al.*, 2001). In Mexico, Chiclero's ulcers, a form of cutaneous leishmaniasis due to *Leishmania mexicana*, have a high prevalence of bacterial contamination (90·9 %), but *Staph. aureus* is found at similar levels (20 %) to other micro-organisms such as *Strep. pyogenes*, *Pseud. aeruginosa*, *Morganella morganii* and *Enterococcus* species (Sader *et al.*, 2002).

The frequency of bacterial contamination found in the present study (67·7 %) can be considered high. Although we expected *Staph. aureus* to be the predominant species, the frequency of other pathogens, particularly enterobacteria, was quite surprising (Table 1). These high frequencies may reflect the low level of personal hygiene and poor sanitary conditions found in the region where the study was done.

Obligate anaerobes were recovered with a low frequency (19·1 % of the lesions) and the isolates pertained to the indigenous human microbiota. Similarly to the results described by Brook (1995) and Bowler *et al.* (2001), peptostreptococci were the most common of the anaerobic isolates. In a microbiological study of infected pustular psoriasis lesions, bacterial growth was noted in 52 % of the patients, and anaerobic bacteria (*Peptostreptococcus* species and *B. fragilis*) were recovered from 17 % (Brook *et al.*, 1999). In some studies, especially those involving diabetic foot infections, anaerobes have accounted for a higher percentage of the total isolates when compared to the present study (Gerding, 1995). Although methodological factors associated with detection of anaerobes could have been involved, such as collection of an adequate specimen and rapid transport to the laboratory, clinical conditions are also determinant to explain this finding. Thus, diabetic foot ulcers reflect the general and local 'status' of the patient that favours anaerobic polymicrobial infections, while the erythema frequently surrounding ATL lesions indicates increased flow of blood with a high oxygen concentration in the neighbouring tissue, which, in turn, inhibits the growth of anaerobic pathogens (Gerding, 1995).

We have found no report in the literature on the isolation of yeast from ATL lesions. In the present study, the frequency of yeast isolation was low and the prevalence of *C. albicans*, a component of the indigenous human microbiota, reflects its opportunistic character.

Spira & Rabinowitz (1975) showed that the capacity of normal hamster macrophages harbouring *Leishmania major* to ingest and digest *Staphylococcus epidermidis* was severely impaired. Moreover, El-On *et al.* (1992) showed that *Klebsiella* species and *E. coli* resistant to paromomycin treatment were eliminated from cutaneous leishmanial lesions only following total elimination of the parasites. Additionally, bacteria that were inoculated together with live *Leishmania* into normal mice showed better resistance to the host's killing than did bacteria inoculated alone. Finally, bacteria inoculated into either ATL nodules or ATL lesions were almost totally protected against the host's killing mechanisms. These data suggest that the *Leishmania* parasites that are present in the ATL lesion induce a local suppressive effect, allowing better survival of both the parasite and the contaminating bacteria. However, interrelationships between leishmanial and bacterial infections are not always synergistic. In the study of Edrissian *et al.* (1990), the prevalence of bacterial infections in lesions in which *Leishmania* parasites were detected was significantly lower (26·5 %) than for lesions in which no parasite was found with the bacteria (45 %). This apparently suggests some antagonistic mechanism of the bacteria against the *Leishmania* parasites. In the present study, bacterial contaminants and *Leishmania* parasites were always present simultaneously.

Although few data are available on the topic, some reports show no influence of secondary infection on complete healing of the epithelium of ATL lesions after conventional therapy (Vera *et al.*, 2001). Similar results were observed for venous leg ulcers (Skene *et al.*, 1992) and decubitus ulcers, particularly those involving *Staph. aureus*. However, when present, other bacteria, such as *Proteus* species, can retard the healing process (Daltrey *et al.*, 1981).

Various studies have suggested that toxins such as SEA and SEB, which are produced by *Staph. aureus*, act as superantigens. These staphylococcal enterotoxins have been implicated in the exacerbation of inflammatory skin lesions in patients. Superantigens can bind to major histocompatibility complex (MHC) class II molecules on epidermal Langerhans cells or macrophages, and cause the release of pro-inflammatory mediators such as interleukin (IL)-1α and tumour necrosis factor (TNF)-α (Marrack & Kapler, 1990). In addition, through cross-linking of MHC class II molecules on antigen-presenting cells and the β element of the T-cell receptor, T cells can be stimulated to proliferate and secrete a range of inflammatory cytokines (Tokura *et al.*, 1994). Exposure to staphylococcal enterotoxin B (SEB) skews the immune response toward Th2 cells, leading to allergic skin inflammation and increased IgE synthesis (Laouini *et al.*, 2003). Additionally, histamine and leukotriene generation from basophiles stimulated with staphylococcal enterotoxins A, B, D and E and toxic shock syndrome toxin-1 also indicates a role for these toxins as possible allergens (Wehner & Neuber, 2001). In the present study, the high prevalence of staphylococcal isolates producing type B (70 %) and type C (50 %) enterotoxins as well as toxic shock syndrome toxin (60 %) could be involved in the severity of the ATL lesions.

In regard to facultative anaerobes, all of the tested bacteria were sensitive to gentamicin and the Gram-negative bacteria showed a higher frequency of resistance to penicillin (100 %) and clindamycin (97·8 %) than the Gram-positive ones (Table 3). Among the Gram-positive facultative anaerobic bacteria, *Staph. aureus* showed some resistance to penicillin G (69·1 %) and ampicillin + sulbactam (67·6 %). This fact

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suggests a resistance mechanism other than enzymic inhibition of the β-lactam ring. The finding that three of the obligate anaerobe strains (*B. fragilis* and *Prev. bivia*) were resistant to metronidazole confirms the increasing resistance in this bacterial group (Freeman *et al.*, 1997).

In conclusion, *Staph. aureus* is an important secondary pathogenic bacterium in ATL ulcers. This should therefore be borne in mind in the diagnosis and treatment of such lesions.

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