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Bacterial superinfection in Zoonotic Cutaneous Leishmaniasis

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Zoonotic Cutaneous Leishmaniasis (ZCL) is a polymorphic disease. It is generally accepted that bacterial superinfection may play a role in the clinical appearance of the lesions and may delay or prevent the healing process. However, the pattern of bacterial pathogens involved has rarely been investigated.

Material/Methods: The aim of this study was to identify the bacterial species contaminating the suspected ZCL and their susceptibility to commonly used antibiotics. Microscopic examination of stained smears and cultures were used to differentiate ZCL from non-ZCL lesions in a rural area north of Isfahan, Iran from July to December 2009. Bacteria were isolated from the lesions and identified and antibiotic susceptibility was determined by standard microbiological techniques.

Results: The results show that 602 (68%) of 855 patients were positive for ZCL, of which 83.4% with volcano-shape, 8.8% psoriasiform, 6.6% popular form and 1.2% with other atypical forms of ZCL. The bacteria were isolated from 66.8% of ZCL (70% of volcano-shape, 60% of psoriasiform and 25% of popular form) and 64.7% of non-ZCL lesions. The most common species were *Staphylococcus aureus* (41.7%) and *S. epidermidis* (28%) followed by *Bacillus sp. Streptococcus pyogenes, Escherichia coli, Klebsiella sp., Proteus sp., Enterobacter sp.* and *Pseudomonas aeroginosa.* Ciprofloxacin, Erythromycin, Cefazolin and Clindamycin were the most effective antibiotics.

Conclusions: Bacterial superinfection appears to be very common in ZCL, but its prevalence is not different from that of non-ZCL lesions and it has little effect on the clinical appearance of anthroponotic cutaneous Leishmaniasis (ACL). Local lesion care and management of bacterial superinfection must be considered in the treatment of ZCL.

key words: **Cutaneous Leishmaniasis • bacterial superinfection • *Leishmania major***

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Background

Zoonotic Cutaneous Leishmaniasis, a vector-borne protozoal infection, is among the most important causes of chronic ulcerating skin lesions in the tropics. It is a major public health problem in many parts of the world, including Iran [1,2]. The diseases are hyperendemic in rural areas, where it may cause considerable morbidity. ZCL shows various clinical manifestations, ranging from asymptomatic infection without apparent lesions, to extensive lesions that may cause severe disfiguring [3–5]. It is generally accepted that secondary bacterial infection of the ZCL lesions may a factors that can influence the size, shape and severity of these skin lesions and also may delay or prevent the healing process [6,7]. Furthermore, it has been suggested that Leishmania parasites that are present in the skin lesions may induce a local suppressive effect, providing a better condition for the growth and survival of contaminating bacteria [8]. However, the scant evidence regarding the relationship between the secondary bacterial infection and cutaneous leishmaniasis is currently restricted to the work of El-on et al. and Edrissian et al. [9,10]. The first report is based on an investigation in experimentally infected mice and the later is the only field study on the bacterial superinfection in ZCL [9]. Therefore, because of the lack of a systematic bacteriological investigation, in the present study the secondary bacterial infection associated with the 3 clinically different forms of ZCL lesions and their susceptibility to common antibiotics were studied in naturally infected human hosts and the results were compared to non-ZCL skin lesions in patients living under similar environmental conditions in the same geographical area.

Material and Methods

Study area and population

The subjects of the study were all patients aged between 1 to 50 years (average, 9 years) with clinically suspected ZCL lesions and a disease history of 2 to 4 weeks, living in the Borkhar area, a rural district of northern Isfahan, Iran, where ZCL is highly endemic [2,11,12]. They were referred to the research center for skin diseases, Isfahan University of Medical Sciences, Isfahan, Iran by primary health care centers and private clinics, for microscopic diagnosis during a 6-month period from July to December 2009, the season during which ZCL is most common in the area [5].

Sampling and culturing methods

The lesions and the surrounding skin areas were disinfected with a single piece of cotton wool moistened with 70% alcohol. For parasitological investigation, 3 samples were collected from the edge of the lesions using a flame-sterilized lancet. Two samples were used to prepare methanol-fixed and Giemsa-stained smears for direct microscopy and observation of amastigotes. The third sample was inoculated into sterile screw top tubes containing 10 ml of Novey-Nicol-Mac Neal (NNN) medium [13] and incubated at 20–25°C. They were examined microscopically for the development of promastigotes at 2-day intervals for 2 weeks [14].

The skin lesions were regarded as non-ZCL skin lesion when no amastigotes was found in the smears after careful examination and promastigotes were not observed in the culture media after 2 weeks. No attempt was made to determine the cause of non-ZCL skin lesions. Clinically different forms of ZCL were named according to [6] with respect to their size, shape and severity of the lesions. For bacterial examination, 3 sterile cotton swabs wetted with sterile saline were moved from the center to the edge of lesions immediately after collection of samples for parasitological studies. The samples were transferred to nutrient broth (NB; Merck, Germany), blood agar (BA; Merck, Germany) and MacConkey agar (MC; Merck, Germany) separately and incubated at 37°C for 24 to 72 hours under aerobic conditions. Identification of colonies was done by Gram staining and standard biochemical tests as described by [15]. The susceptibility of bacteria to 16 common antibiotics was assessed using the Kirby-Bauer method [15] and Muller Hinton agar medium (MHA; Merck, Germany).

Statistical analysis

The Fisher's exact test was used to evaluate and analyze the data.

Result

Amastigotes were observed in Giemsa-stained smears taken from 602 (68%) of 885 patients with clinically suspected cases of ZCL who attended Isfahan research center for skin diseases during a 6 month-period from July to December 2009. The clinical feature of the ZCL-positive skin lesions is summarized in Table 1. The median age of 9 years of the patients reflects the age distribution of ZCL in the area. Table 2 shows the prevalence of 3 clinically distinguished different forms of ZCL and the distribution of secondary bacterial infection in these lesions.

Among 602 patients with ZCL and 283 patients with non-ZCL skin lesions, bacteria were isolated from the skin lesions of 402 (66.8%) and 183 (64.7%), respectively. The secondary bacterial infections were observed in about 70% of volcano-shape, 60% of psoriasiform and 25% of popular forms of ZCL. Table 3 shows the 9 bacterial species isolated from the lesions. *Staphylococcus aureus* and *S. epidermidis* were the most common bacteria isolated from both ZCL and non-ZCL lesions, followed by *Bacillus sp.* and *Streptococcus pyogenes.*

The bacterial species isolated from clinically different forms of ZCL is summarized in Table 4. *Staphylococcus aureus* and *S. epidermidis* were isolated from all 3 types of ZCL and they were the only species isolated from the popular form. The bacterial species isolated from volcano-shape and psoriasiforms were similar, except for *Bacillus sp.* and *Proteus sp.*, which were isolated from volcano shape and S. *pyogenes*, which was isolated from psoriasiform (Table 4).

Only 1 bacterial species was isolated from the skin lesions of 337 (83.8%) patients with bacterial contaminated ZCL and 153 (83.6%) with non-ZCL lesions. Mixed infection with 2 or 3 bacterial species were observed in 59 (14.7%) and 6 (1.5%) of the ZCL lesions and 28 (15.3%) and 2 (1.1%) of the non-ZCL lesions, respectively. The most common combination was *S. aureus* and *S. epidermidis.*

The results of *in vitro* testing of bacterial resistance to 16 common antibiotics are summarized in Table 5.

Table 1. The clinical feature of 3 different forms of ZCL present in Borkhar area, Isfahan, Iran.

Type of ZCL*	Main characteristics	Size**	Duration***	Site****
Volcano-shape	Raised turgid margins. Central serous crust Resembles to a flattened Volcano. Painless	2–3	2–4	Face, Foot Leg, Hand
Psoriasiform	Superficial with raised patches of skin covered with silvery scales Resembles psoriasis, Painless Firm-levated erythematous	6–8	2–4	Face Hand Foot Face
Papular form	Papule with exudative Surface, Plainless	<0.5	3–4	Upper arm

***** The shape was named according to Griffithis (1987); ****** size of ulcerated area expressed in cm; ******* number of weeks after first appearance of the lesions according to the patients; ******** the most common site of the lesions.

Table 2. Prevalence of clinically different forms of ZCL and the distribution of bacterial superinfection in Borkhar area Isfahan, Iran.

Type of ZCL	Number and% of patients		
	Total	Infected	Non infected
Volcano-shape	502 (83.4)*	355 (88.3)	147 (73.5)
Psoriasiform	53 (8.8)	32 (8)	21 (10.3)
Popular form	40 (6.6)	10 (2.5)	30 (15)
Other atypical form	7 (1.2)	5 (1.2)	2 (1)
Total	602 (100)	402 (100)	200 (100)

***** Figures in brackets are percentages.

Table 3. Bacterial species isolated from ZCL and non-ZCL lesions in Borkhar area Isfahan, Iran.

Bacterial	ZCL lesions %n=453	Non-ZCL Lesions %n=415
Staphylococcus aureus	189 (41.7)*	170 (41)
Staphylococcus epidermidis	127 (28)	110 (26.5)
Bacillus sp.	36 (7.9)	10 (2.4)
Streptococcus pyogenes	30 (6.6)	27 (6.5)
Escherichia coli	24 (5.3)	20 (4.8)
Klebsiella sp.	19 (4.2)	38 (9.2)
Proteus sp.	11 (2.4)	10 (2.4)
Enterobacter sp.	10 (2.2)	20 (4.8)
Pseudomonas aeroginosa	7 (1.5)	10 (2.4)

Table 4. Bacterial species isolated from 3 clinically different forms of ZCL.

Volcano-shape	Psoriasiform	Popular form
S. aureus	*S. aureus*	*S. aureus*
S. epidermidis	*S. epidermidis*	*S. epidermidis*
Bacillus sp.	*E. coli*	
E. coli	*Klebsiella sp.*	
Klebsiella sp.	*Enterobacter sp.*	
Proteus sp.	*P. aeroginosa*	
Enterobacter sp.	*S. pyogenes*	
P. aeroginosa		

No obvious differences were observed in the pattern of resistance to antibiotics between the bacteria isolated from ZCL and non-ZCL skin lesions.

The results of the present study show that secondary bacterial infections were not present in about 30%, 40% and 70% of volcano- shape, psoriasiform and popular forms of ZCL, respectively. Furthermore, the bacterial species isolated

Table 5. Percentage resistance of bacteria isolated form ZCL lesions to common antibiotics.

Antibiotics	Gram Positive Bacteria				Gram Negative Bacteria				
	Sa	Se	Sp	Bs	Ec	Ks	Es	Pa	Ps
Amoxcillin	87	89	17	41	99	60	94	91	94
Ampicillin	92	85	14	49	95	86	10	91	92
Cefazolin	12	20	10	27	20	18	16	25	11
Ceftizoxime	25	18	11	19	30	38	50	85	71
Cephalothin	13	11	20	28	65	62	51	75	38
Ciprofloxacin	8	10	12	14	20	17	13	13	15
Clindomycin	40	45	36	26	20	11	15	13	14
Cloxacillin	45	24	18	11	38	29	25	14	12
Chloramphenicol	60	50	35	43	50	70	81	93	85
Cotrimaxozole	83	90	35	35	80	78	70	91	93
Erythromycin	12	19	22	10	60	58	27	53	51
Gentamycin	36	30	20	12	55	60	29	71	59
Kanamycin	30	25	10	13	55	38	31	53	15
Neomycin	23	22	12	10	30	32	20	30	49
Penicillin	96	90	8	21	93	94	92	97	98
Tetracycline	71	63	35	43	89	60	81	62	69

Sa – *Staphylococcus aureus*; Se – *Staphylococcus epidermidis*; Sp – *Streptococcus pyogenes*; Bs – *Bacillus* sp.; Ec – *Escherichia coli*; Ks – *Klebsiella* sp.; Es – *Entrobacter* sp.; Pa – *Pseudomonas aeroginosa*; Ps – *Proteus* sp.

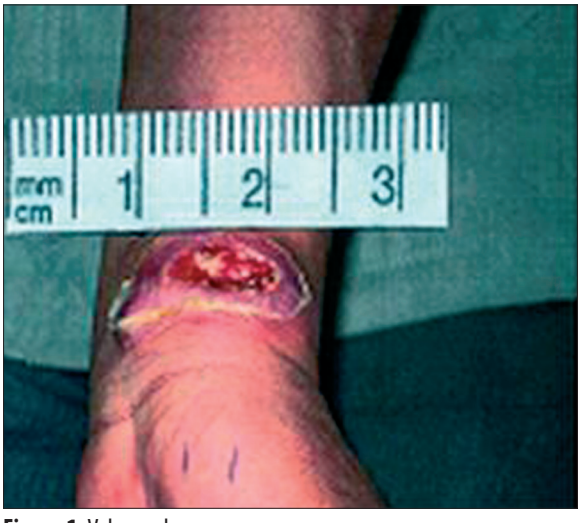

Figure 1. Volcano shape.

Figure 2. Psoriasiform.

from volcano-shape and psoriasiform of ZCL were similar, and no obvious differences were observed between the clinical appearance of those with and without bacterial infection, except a more severe inflammatory reaction in the surrounding skin of those with bacterial infections. These observations suggest that the secondary bacterial infection does not have an important effect on the shape, size and severity of the skin lesions in ZCL (Figures 1–3).

Discussion

Many authors believe that secondary bacterial infection of skin lesions in ZCL is common, particularly in areas where the disease is endemic. However, very little empirical evidence has been provided by systematic bacteriological studies. In fact, the only field study is that reported by Edrissian et al. [10], who reported secondary bacterial infection in

Figure 3. Popular form.

26.5% of ZCL skin lesions in military personnel deployed in Khuzestan province, an endemic area for ZCL in southwest Iran. Therefore, the present report is the first study in which native patients with clinically different forms of ZCL were examined bacteriologically and compared with non-ZCL skin lesions in the patients living in the same geographical area under similar condition.

In the present study, the results show that ZCL skin lesions are infected with bacterial pathogens in 66.8% of cases, and the differences in the prevalence of bacterial infection in ZCL lesions and non-ZCL lesions (64.7%) is not statistically significant (P<O.OO1). This finding confirms that in most cases the ZCL lesions are contaminated with potentially pathogenic bacteria, but does not support the idea that ZCL lesions are more susceptible to secondary bacterial infection as a result of a local immunosuppression induced by the parasite.

Nine different species of bacteria were isolated from both the ZCL and non-ZCL lesions and *S. aureus* and *S. epidermidis* were predominant, followed by *Bacillus sp.*, *S. pyogenes*, *E. coli*, *Klebsiella sp.*, *Enterobacter sp.* and *P. aeroginosa*, respectively, in both cases. The high frequency of *S. aureus and S. epidermidis* in both ZCL and non-ZCL lesions were not unexpected, as they are common on the skin and mucous surface of healthy people. They live harmlessly on the host, but when the skin is broken for any reason, they can enter the wound and cause an infection. These bacteria produce enzymes such as fibrinolysins, hyaluronidase and lipases, which break down components of tissue and facilitate invasion [16,17]. Most of these infections are minor, causing a mild to sever inflammation, as was observed in the present study; however, they can cause serious infections and under particular circumstances these bacteria should indeed be considered as true pathogens [18].

The rather high isolation rates of Enterobacteriaceae could be due to the use of night-soil as fertilizer on farms, resulting in an environment contaminated with fecal material. Furthermore, lack of hygiene, sewage disposal, walking barefoot and playing on the farms, particularly by children, provide favorable conditions for colonization of the skin, especially of the feet and hands, by these bacteria. This also may explain the total absence of these bacteria in popular form of ZCL, which are mostly located on the face and upper arms, with the consequence of less exposure to infection.

Although anaerobic cultures were not used in this study, microscopic examinations of Gram-stained smears did not suggest presence of any important anaerobic bacteria. This is not surprising in view of the erythema frequently surrounding the lesions, resulting in increased blood flow with a high oxygen concentration that inhibits the growth of anaerobic bacteria. Moreover, in the vast majority of cases the wound infections are caused by aerobic bacteria due to the lower number of anaerobic bacteria on the skin or in the environment [17].

The present study indicates that both Gram-positive and Gram-negative bacteria (except *S. pyogenes*) are highly resistant to penicillin, ampicillin and amoxicillin. Ciprofloxacin, cefazolin, and erythromycin are the most effective antibiotics against Gram-positive bacteria, and ciprofloxacin and clindamycin are the most effective antibiotics against Gram-negative bacteria.

Bacterial resistance to antibiotics has been widely investigated all over the world, but the present study is the first in this area and there is no previous information for comparison. However, in many cases, studies are difficult to compare due to the differences in patient demographics, etiologies and infections status of ulcers.

Conclusions

In conclusion, the present findings indicate that bacterial superinfection is very common in ZCL, but not more so than non-ZCL skin lesions. The bacterial superinfection has little if any effect on clinical appearance of ZCL which is a polymorphic disease. Local lesion care and management of secondary bacterial infection are essential and anti-Leishmanial therapy in ZCL may be more effective when combined with antibiotics.

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