Variation in Clinical Presentation and Genotype of Causative *Leishmania major* Strain in Cutaneous Leishmaniasis in North and South Afghanistan

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Abstract. A different clinical picture and therapeutic response were observed when data from *Leishmania major*-infected Dutch military personnel stationed in southern ($N = 8$) and northern ($N = 169$) Afghanistan were analyzed. Clinical presentation of cutaneous leishmaniasis in personnel in the south was milder and seemed to respond better to antileishmanial treatment; molecular analyses of parasite isolates seem to indicate that these differences may be genetic.

INTRODUCTION

Dutch troops deployed to northern Afghanistan in 2005 suffered an attack rate of zoonotic cutaneous leishmaniasis (ZCL) of 18.3%.¹ Detailed information on ZCL (*Leishmania major* infection) from parts of Afghanistan other than the northern region is absent. In a review on cutaneous leishmaniasis (CL),² widespread occurrence of CL in the north was indicated, and less extensive occurrence was noted around Kandahar in the south. At the time of the mentioned outbreak in the north,¹ Dutch military were deployed in rural areas around Kandahar city, where eight acquired CL. The clinical picture of these patients differed from the picture in the north, and genetic differences in the parasites were found. We report on these differences.

PATIENTS AND METHODS

South Afghanistan. Eight Dutch military personnel deployed in Kandahar province were referred to the Academic Medical Center (AMC) in Amsterdam with a provisional diagnosis of CL.

Diagnostic methods and parasite characterization. CL was confirmed by demonstration of parasites in Giemsa-stained smears of biopsies and culture on Novy–McNeal–Nicolle (NNN)—medium and mini—exon repeat (MER) polymerase chain reaction (PCR)³ followed by sequence analysis of the non-transcribed spacer region. Additional molecular analyses were performed by PCR and sequence analysis of the GP63 and rDNA ITS1 loci⁴ of four isolates each from south and north Afghanistan and four isolates from unrelated civilian patients (two epidemiologically linked patients from Israel and two epidemiologically linked patients from Morocco); these isolates were compared with the sequences of the reference strains MHOM/TM/1973/5ASKH (zymodeme MON-4) and MHOM/IL/1980/Friedlin (zymodeme MON-103). Sequences were analyzed using the CodonCode Aligner (CodonCode Corporation, Dedham, MA) and MEGA4.⁵ One parasite isolate each from north and south Afghanistan were sent for multilocus enzyme electrophoresis (MLEE)⁶ to the

Center National de Référence des Leishmania, Laboratoire de Parasitologie, Université Montpellier I, France.

Treatment. Treatment was with intralesional injections of sodium stibogluconate (iSb^v; Pentostam; GlaxoSmithKline, Barnard Castle, United Kingdom) with cryotherapy as reported.¹ Follow-up was around 6 weeks and 6 months after treatment.

North Afghanistan. Details of an outbreak of CL in Dutch forces in 2005 have been reported.¹ In short, 172 of 938 deployed persons were infected. Most were diagnosed and treated in Afghanistan, some continued treatment in AMC, and a small group was diagnosed and treated in AMC. In Afghanistan, diagnosis was by microscopy of Giemsa-stained smears of biopsies, and treatment was with iSb^v. In AMC, diagnosis and treatment were as described above.

RESULTS

South Afghanistan. Clinical data showed a long period between noticing the lesion and visiting a physician as well as the presence of one lesion without satellite lesions in most cases (Table 1). The diagnosis was confirmed for all, and the parasites of six patients could be characterized. Six patients responded well to primary iSb^v treatment, and one patient received secondary iSb^v treatment because of recurrence after excision was performed at his insistence during deployment. One person refused treatment; when seen 9 months later, he was cured. At first follow-up 6–8 weeks after treatment, all seven treated patients were clinically cured. Five patients were seen for a second follow-up between 6 and 12 months after treatment and remained cured. Two patients were not seen at second follow-up (Table 2). Apart from pain at the injection site, the only adverse event was secondary bacterial infection with *Staphylococcus aureus* in one patient.

North Afghanistan. Parasitological investigations among 132 of 172 patients confirmed the diagnosis in 129 patients; three microscopy negative patients and 38 patients stationed in field sites where microscopy was not available were treated as cases of CL on clinical grounds. Parasitological investigations were not performed for two patients by mistake. Clinical data are presented in Table 1. One patient, knowing that lesions might cure spontaneously, refused treatment and was cured, one patient experienced spontaneous cure before treatment was available, and one patient was cured after excision at his request (note that this approach is not recommended by either the Dutch military or the authors). Three patients were treated

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TABLE 1
Clinical aspects of cutaneous leishmaniasis among Dutch military deployed in north and south Afghanistan in 2005

	South Afghanistan	North Afghanistan	P*
Time between noticing lesion and presentation	N = 8	N = 169†	
Days (IQR median; range)	54 (49–63; 21–63)	28 (14–35; 1–73)	< 0.01
Number of lesions	N = 8	N = 161	
1	5 (62.5%)	67 (41.6%)	ns
2	1 (12.5%)	28 (17.4%)	ns
3	0 (0.0%)	34 (21.1%)	ns
≥ 4	2 (25.0%)	32 (19.9%)	ns
Size (IQR median; range)	N = 8	N = 149	
Minimum diameter (mm)	10 (3.25–14.75; 2–20)	10 (5–13; 3–50)	ns
Maximum diameter (mm)	15.5 (8.25–19; 7–22)	20 (13–30; 4–80)	ns
Location of lesions	N = 8	N = 163	
Hands, neck, or head	4 (50.0%)	20 (12.3%)	< 0.05
Arms	4 (50.0%)	61 (37.4%)	ns
Trunk, legs, or feet	3 (37.5%)	132 (81.0%)	< 0.05
Aspect of lesions	N = 8	N = 149	
Ulcerated	5 (62.5%)	64 (43.0%)	ns
Nodular	2 (25.0%)	57 (38.3%)	ns
Mixed	1 (12.5%)	28 (18.8%)	ns
Lymphatic system involvement	N = 8	N = 153‡	
Lymphangitis	0 (0.0%)	7 (4.6%)	ns
Lymph node involvement	2 (25.0%)	32 (20.9%)	ns
Satellite lesions	N = 8	N = 121	
	0 (0.0%)	18 (14.9%)	ns

IQR = interquartile range.

*Differences in proportions were assessed using the Fisher's exact test, and differences in medians were assessed using the Mann-Whitney test; ns is defined as a *P* value ≥ 0.1.

†Of the 172 infected, three patients were seen elsewhere.

‡In one patient, both lymphangitis and lymph node involvement.

elsewhere, and three patients received primary miltefosine treatment (all six patients were cured). Of 163 patients who started iSb^v treatment, 65 (39.9%) received additional treatment with iSb^v or miltefosine.⁷ Ultimately, 125 patients (76.7%) were definitely cured, and 22 patients (13.5%) were probably cured, whereas 16 (9.8%) were lost to complete follow-up (Table 2). Details have been reported.¹

***L. major* genotypes.** For 6 of 8 patients from south Afghanistan and 79 patients from north Afghanistan, MER PCR products with *L. major* sequences were obtained. The MER PCR primers target the conserved mini-exon repeats, amplifying the non-transcribed spacer regions. Intragenomic variation in length of different non-transcribed spacer regions

is reflected in the electrophoretic mobility of the PCR products. Consistent differences between isolates from north and south Afghanistan were noted in the electrophoretic mobility of the PCR amplicon (Figure 1A), which was also reflected in the resulting sequences (Figure 1B). This showed 16 differences in 324 nucleotides between the Afghan isolates. Similar observations were made for two epidemiologically linked pairs of patients from other geographical areas.

Additional loci were amplified and sequenced for four isolates from each geographical area. In the rDNA ITS1 locus, northern isolates had an A at position 113 and were identical to the genotype LmB described.⁸ The southern isolates had a G at this position as well as an insertion at position 61,

TABLE 2
Treatment and outcome of cutaneous leishmaniasis in Dutch military deployed in south and north Afghanistan in 2005

	South Afghanistan	North Afghanistan	P*
Parasitological confirmation <i>n</i> per <i>n</i> examined	8/8 (100%)	129/132 (97.7%)	
Primary treatment	N = 7†	N = 166‡	
Primary miltefosine	0	3	
iSb ^v (number with cryotherapy)	7 (7)	163 (62)	
Number of iSb ^v sessions (IQR median; range)	3 (2–3; 2–3)	5 (4–8; 1–13)§	< 0.001
Number of cryotherapy sessions (IQR median; range)	1 (1–1; 1–1)	1 (1–2; 1–4)¶	ns
Treatment duration in days (IQR median; range)	6 (5–8; 3–12)	17 (10–34; 1–163)§	< 0.01
Outcome			
Unsatisfactory response and recurrence	0 (0)	65/163 (39.9%)	< 0.1
Additional iSb ^v		35	
Secondary miltefosine		30	
Definite cure at 6 months	5/7 (71.4%)	125/163 (76.7%)	ns
Clinical cure at 6 weeks; not seen at 6 months	2/7 (28.6%)	22/163 (13.5%)	ns
Unknown	0	16/163 (9.8%)	ns
Definite and probable cure	7/7 (100%)	147/163 (90.2%)	ns
Lymph system involvement during treatment	0	88/156 (56.4%)	< 0.01
Secondary bacterial infection	1/7 (14.3%)	40/154 (26.0%)	ns

IQR = interquartile range.

*Differences in proportions were assessed using the Fisher's exact test, and differences in medians were assessed using the Mann-Whitney test; ns is defined as a *P* value ≥ 0.1.

†Of the eight infected, one had spontaneous cure, and one had excision but recurrence and was treated with iSb^v/cryotherapy.

‡Of the 172 infected, 3 were treated elsewhere, 1 had spontaneous cure, 1 had cure after excision, and 1 refused treatment (and experienced cure).

§North Afghanistan patients receiving first-line treatment, excluding those with recurrence.

¶Only north Afghanistan patients who continued treatment at AMC received cryotherapy in combination with iSb^v.

||For definitions, see ref. 1.

FIGURE 1. (A) Difference in electrophoretic mobility of the PCR amplicon obtained from different patient samples in polyacrilamide gel electrophoresis (PAGE). Lane 1: 100-bp marker; lanes 2 and 3: isolates from north Afghanistan; lanes 4 and 5: isolates from south Afghanistan; lanes 6 and 7: isolates from Morocco; lanes 8 and 9: isolates from Israel. (B) Polymorphic sites in 324 nucleotides of the non-transcribed spacer regions of the MER locus for two reference strains, two epidemiologically linked strains from Israel, two epidemiologically linked strains from Morocco, four isolates from north Afghanistan, and four isolates from south Afghanistan. Note that the large number of ambiguous positions in the Friedlin strain is because of an indel in part of the MER copies in this strain. (C) Ten polymorphic sites in 574 nucleotides GP63 sequences of the same strains as in B. (D) Three polymorphic sites in 340 nucleotides of the rDNA ITS1 sequences of the same strains as in B.

resulting in seven TA repeats instead of six at positions 61–72. At the GP63 locus, there were differences at 6 of 425 positions. All of these comprised positions where multiple peaks were seen for a position in some electropherograms, indicating heterozygosity or a different distribution of alleles in this multicopy gene. In the GP63 sequences, one and two nucleotide differences were also observed between isolates from south Afghanistan in contrast with the epidemiologically linked isolates from other geographical areas. The southern isolate was typed by MLEE as MON 74, and the northern isolate was typed as MON 26.

DISCUSSION

The clinical picture of eight military personnel with *L. major* infection acquired in south Afghanistan suggested a milder dis-

ease pattern than the picture in the military personnel infected in the north of the country at the same time. Significant differences were the time between noticing the lesion and presentation, location of the lesions, number of treatment sessions, treatment duration, and response to treatment (Tables 1 and 2). Molecular characterization of the parasites showed consistent differences between 79 isolates from the north and 6 isolates from the south in the minixon repeat locus. Differences were also observed in two additional loci, ITS1 and GP63, for four representative isolates from the north and four isolated from the south. Genetic and biological variation among *L. major* strains have been shown, and correlations of genetic differences with geographical areas and reservoir hosts were proposed.^{4,9} A relationship to clinical manifestations in man was not described. Various molecular methods are available that serve different epidemiological purposes.^{10,11} There is a need to standardize molecular methods and relate

those to clinical findings,^{10–12} the MLEE system, and MON classification.⁶

MON-26, the northern isolate, has been reported from Senegal to Kazakhstan and Pakistan, and MON-74, the isolate from the south, has been reported from Africa (Sudan, Kenya, and Ethiopia) but not Asia. Both zymodemes are found in Egypt, Burkina Faso, Niger, Mauritania, Senegal, and Mali.¹³ Although MON-74 is a single enzyme variant (nicotinamide adenine dinucleotide [NADH] diaphorase) of MON-26,¹³ the molecular data for ITS and GP63 showed more similarity of the south Afghanistan isolates to the Moroccan isolates than to the north Afghanistan isolates. Additional research is needed to establish whether the isolates from south Afghanistan are related to the two Central Asian subpopulations observed by Al-Jawabreh and others⁹ or if they form a seventh, geographically separated *L. major* subpopulation.

Molecular methods have rapidly become available more widely. Multilocus molecular methods have higher discriminatory power for strain characterization than MLEE, and it is not unlikely that they will become the standard for strain typing.¹⁴ For example, Kuhls and others¹⁵ identified three subpopulations within the zymodeme MON-1 in *L. infantum*.

Host response and strain differences of the parasites influence the clinical manifestations of *L. major* infection, but the contribution of each component in a given region is unknown. We speculate that the differences in the reported groups of patients are related to differences between parasite strains, but we do realize that the comparison is between only 8 patients from the south and 169 patients from the north. The comparison may not be fully justified, but the eight patients from the south showed a rather uniform picture, whereas it was more varied in the northern population. The picture in the south was of an indolent skin lesion with quick and good response to local therapy, whereas in the north, the lesions relatively quickly progressed to multiple clinical manifestations, with slower and less effective response to local treatment that required additional treatment in a substantial proportion of patients.¹

In conclusion, we observed cutaneous leishmaniasis caused by *L. major* in south Afghanistan with clinical differences from the infection in the north and showed genotypical differences in the *L. major* strains isolated in the two geographical areas.

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