Visceral Leishmaniosis Caused by *Leishmania (L.) mexicana* in a Mexican Patient with Human Immunodeficiency Virus Infection

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A 36 year old male was admitted in December 1997 to hospital with afternoon fever, malaise and hepatosplenomegaly. He also had a dry cough, dyspnoea and anaemia. Pneumonia caused by Pneumocystis carinii *and human immunodeficiency virus (HIV) infection were documented. The HIV infection was confirmed in 1997 with 290,000 virus copies. The patient had been in the Mexican State of Chiapas which is known to be endemic for visceral leishmaniosis (VL) and localized cutaneous leishmaniosis (LCL). The visceral symptoms were diagnosed as VL and the causal agent was identified as* Leishmania (L.) mexicana. *Identification of* Leishmania *was carried out by the analysis of amplified DNA with specific primers belonging to the* Leishmania *subgenus and by dot blot positive hybridisation of these polymerase chain reaction derived products with kDNA from the* L. (L.) mexicana *MC strain used as probe. This is the first case in Mexico of VL caused by a species of* Leishmania *that typically produces a cutaneous disease form.*

Key words: visceral leishmaniosis - visceral leishmaniosis/human immunodeficiency virus co-infection - *Leishmania mexicana*

Leishmaniosis can present itself in man in four different forms, all with devastating consequences. The four forms of the disease are: localized cutaneous leishmaniosis (LCL), diffuse cutaneous leishmaniosis (DCL), mucocutaneous leishmaniosis (MCL) and visceral leishmaniosis (VL). The cutaneous forms are the most common, with 1 to 1.5 million cases per year. The visceral form, of which there are 500,000 cases per year, is the most serious form of the disease, and lethal if untreated, particularly in cases of co-infection with human immunodeficiency virus (HIV). VL is characterized by irregular fever, weight loss, swelling of the liver and spleen and anaemia. After recovery, patients sometimes develop chronic cutaneous leishmaniosis called post kala azar diffuse leishmaniosis (PKDL) and require long and expensive treatment (Desjeux & UNAIDS 1998). VL is caused by *Leishmania* species which are members of the *L. donovani* complex. This complex includes, *L. (L.) donovani*, *L. (L.) infantum* and *L. (L.) infantum/chagasi*. The later species is prevalent in the New World, where it is distributed from Mexico to the north of Argentina. In Mexico there have been few reports of VL, with about 17 cases reported in the period from 1985 to 1994 (OPS/OMS 1994).

Co-infection with *Leishmania* and HIV is emerging as a serious new disease and is becoming increasingly frequent with cases reported from 32 countries; however, Mexico has not been one of the countries reporting such leishmaniosis and HIV co-infection. The diagnosis of VL in *Leishmania*/HIV co-infected patients is difficult because the usual clinical features are not always present and may be masked by other opportunistic infections mimicking the same symptoms (Desjeux & UNAIDS 1998). Techniques for DNA analysis are useful for the diagnosis of VL and to identify the strain of parasite causing the disease.

MATERIALS AND METHODS

Case report - The patient studied was a 36 years old male, who was admitted in December 1997 to the hospital with afternoon fever, malaise and

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hepatosplenomegaly. He had symptoms of a dry cough, dyspnoea, anaemia and pneumonia, which was shown to be caused by *Pneumocystis cariniiin* (February 1998). He also had an HIV infection which had been confirmed since October 1997 with 290,000 virus copies. He had a $CD4^+$ cell count of $121/mm^3$; $CD8^+$ cell count of $1290/mm^3$ and a $CD4^+/CD8^+$ ratio of 0.93. Three years previously (1994) the patient had been to the State of Chiapas in Mexico, which is endemic for VL and LCL. He had also visited Guatemala for one day. For histological studies (April 1998) a liver biopsy was taken and bone marrow aspirate for DNA analysis was taken (May 1998).

Leishmania species and culture conditions - The reference strains of *Leishmania* used were: *L. (L.) infantum/chagasi* MHOM/BR/74/PP75 (code PP75); *L. (L.) donovani* MHOM/IN/80/DD8 (code DD8); Mexican strain *L. (L.) mexicana* MHOM/MX/88/HRC MC (code MC); *L. (L.) amazonensis* IFLA/BR/67/PH8 (code PH8); *L. (V.) braziliensis* MHOM/BR/84/LTB300 (code LTB300); and a Mexican isolate of *Trypanosoma cruzi*. These reference strains were cultured in RPMI medium supplemented with 10% foetal calf serum at 28°C.

Isolation of DNA - DNA from patient bone marrow aspirate sample was prepared by incubation at 65°C for 2 h in 200 µl of NET 100 (100 mM Tris HCl, pH 8.0; 100 mM EDTA; 100 mM NaCl), 1% SDS and 4 µl of 20 mg/ml proteinase K (Sigma) followed by 2 phenol-chloroform extraction and ethanol precipitation. The DNA precipitate was dissolved in 30 µl of TE (Tris-EDTA).

DNA from *Leishmania* reference strains was prepared by centrifuging 10^9 parasites of a exponentially growing culture at 1,900 g for 10 min at room temperature. The pellet was resuspended in 1 ml of NET 100 (100 mM Tris-HCl, pH 8.0; 100 mM EDTA; 100 mM NaCl), 1% SDS and 4 µl of 20 mg/ml proteinase K (Sigma) and incubated at 65°C for 2 h, followed by 2 phenol-chloroform extractions and ethanol precipitation. The DNA was dissolved in 300 µl of TE.

Polymerase chain reaction - Purified total DNA (100 ng) of the reference strains or 2 µl of DNA from the patient bone marrow sample, was amplified in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.01% gelatine in the presence of 0.2 mM of each deoxyribonucleotide (Pharmacia), 100 pmol of each primer and 2.5 units of Taq DNA polymerase (Perkin Elmer Cetus) in a final volume of 100 µl. Samples were overlaid with mineral oil (Sigma) and initially denatured at 96°C for 6 min. Cycles consisted of annealing at 60°C for 1 min for specific primers for the subgenus *Leishmania*; 63°C for 1 min for *L. braziliensis* complex specific primers, extension at 72°C for 1 min and denaturation at 93°C for 30 sec. When using primers specific for the *L. donovani* complex, the samples were denatured at 96°C for 1 min; then the temperature was lower to 55°C for 10 sec and increased to 94°C for 30 sec, in cycles with annealing temperature of 59°C for 30 sec and extension of 72°C for 1 min. With primers KNS1 and KNS2 specific for *T. cruzi* the cycles consisted of annealing temperature of 56°C and extension of 72°C for 1 min. All programmes were run for 35 cycles on a Perkin Elmer thermocycler (Perkin Elmer, USA), with a final extension at 72°C for 10 min. Products (10 µl) were fractionated by electrophoresis in 2% agarose or 8% acrylamide gels in TBE (90 mM Tris-HCl pH 8.3, 90 mM boric acid and 25 mM EDTA).

Dot blot of PCR products of biopsies and Leishmania reference strains - The PCR products of biopsies and reference strains amplified with the primers specific for subgenus *Leishmania* (AJS1 and DeB8) were denatured by boiling in a water bath during 10 min; 10 µl of each were blotted onto nylon membranes (Hybond N, Amersham) (Sambroock et al. 1989) and hybridized with probes which were labelled with the DIG Random Primer DNA labelling kit (Boehringer Mannheim). The hybridization and the subsequent washes were at medium stringency conditions as described previously (Hernandez-Montes et al. 1998). The filters were developed by luminescent detection with CSPD solution (Boehringer Mannheim). Probes used were cloned fragments of kDNA, B4Rsa which hybridises specifically to members of the *L. donovani* complex, probe B18 specific for members of the *L. braziliensis* complex and a probe made from kDNA from the Mexican isolate of *L. (L.) mexicana* MC.

RESULTS

Histological studies - The liver biopsy processed for histological studies, was negative for *Leishmania* infection.

PCR specificity for the Leishmania subgenus - A PCR specific for the *Leishmania* subgenus was carried out by using the DeB8 and AJS1 primers (Smyth et al. 1992). These primers, when used at an annealing temperature of 60°C and after 35 cycles, resulted in specific amplification of kDNA of *L. (L.) donovani* DD8, *L. (L.) infantum/chagasi* PP75 reference strains and the Mexican isolate of *L. (L.) mexicana* MC, with a band size of 700-800 bp. The DNA purified from the patient's biopsy also yielded an amplification product (Fig. 1, Table I).

PCR specificity for the L. donovani complex – A PCR specific for the *L. donovani* complex was carried out with the primers D1 and D2 (Piarroux et al. 1993). At an annealing temperature of 59°C

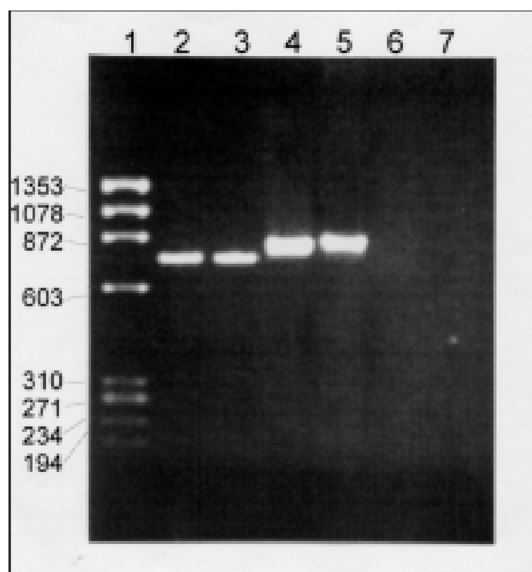

Fig. 1: specificity of polymerase chain reaction with primers specific for subgenus *Leishmania* AJS1 and DeB8

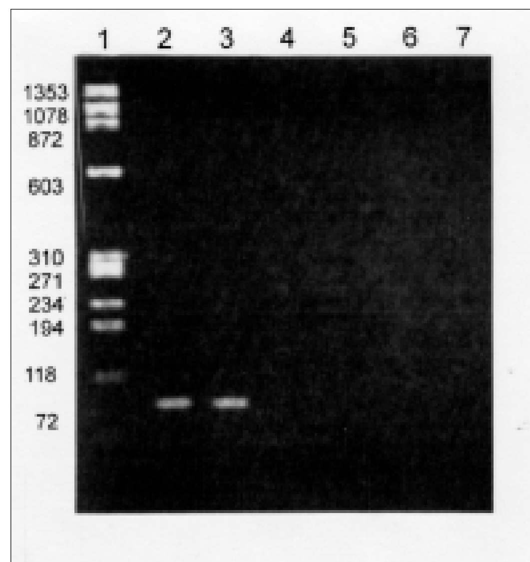

Fig. 2: specificity of polymerase chain reaction with primers D1 and D2 specific for *Leishmania donovani* complex

and after 35 cycles, these primers produced a kDNA amplification band of about 100 bp. With these primers the *L. (L.) donovani* DD8 and *L. (L.) infantum/chagasi* PP75 were amplified giving a band of 100 bp. Patient biopsy DNA was not amplified (Fig. 2, Table I).

PCR specificity for the L. braziliensis complex - A PCR specific for the *L. braziliensis* complex was carried out with B1 and B2 primers (De Bruijn & Barker 1992). At an annealing temperature of 63°C and after 35 cycles, these primers produced a kDNA amplification band of 750 bp. Only kDNA from the *L. (V.) braziliensis* LTB300 reference strain was amplified with these primers, giving a band of 750 bp. Biopsy DNA did not amplify (Fig. 3, Table I).

PCR specificity for Trypanosoma cruzi - A PCR specific for *T. cruzi* was carried out using the primers KNS1 and KNS2 (Monteon-Padilla et al. 1994). At an annealing temperature of 56°C and after 35 cycles, these primers only amplified *T. cruzi* kDNA giving a band of 270 bp. The kDNA from the

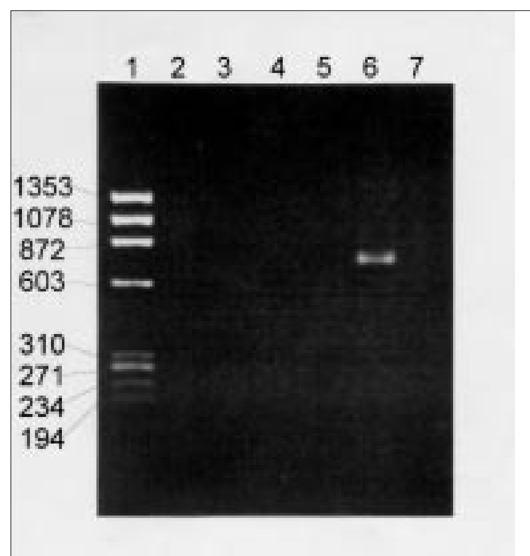

Fig. 3: specificity of polymerase chain reaction with primers B1 and B2 specific for *Leishmania braziliensis* complex

TABLE I

Results of polymerase chain reaction (PCR) and hybridization by dot blot. Specificity of patient DNA amplified by PCR with several primers

DeB8 and AJS1 Subgenus *Leishmania*	D1 and D2 *L. donovani* complex	B1 and B2 *L. braziliensis* complex	KNS1 and KNS2 *Trypanosoma cruzi*
+	-	-	-

Mexican isolate of *L. (L.) mexicana* MC and from the biopsy DNA, gave a smear but not the diagnostic amplification band (Fig. 4, Table I).

Dot blot analysis - PCR products derived from the amplification of *L. (L.) infantum/chagasi, L. (L.) mexicana* MC and biopsy material with primers specific for the *Leishmania* subgenus, were dot blotted onto nylon membranes. These were then tested by using the *L. donovani* complex specific probe, B4Rsa (Scrimgeour et al. 1998) and the kDNA of *L. (L.) mexicana* MC, used as a probe. The B4Rsa probe hybridized with kDNA from the *L. (L.) infantum/chagasi* PP75 reference strain only (Fig. 5a dot 3, Table II). The kDNA from *L. (L.) mexicana* MC, hybridized with high affinity to *L. (L.) mexicana* MC, with less affinity to the biopsy DNA but did not hybridize with *L. (L.) infantum/ chagasi* (Fig. 5b dot 3, Table II). The B18 probe, specific for members of the *L. braziliensis* complex (Alexander et al. 1998), hybridized only with kDNA from the *L. (V.) braziliensis* reference strain (data not shown).

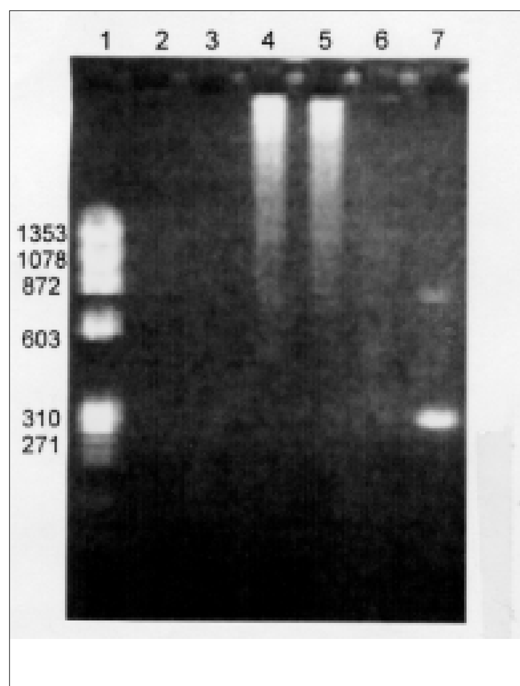

Fig. 4: specificity of polymerase chain reaction with primers KNS1 and KNS2 specific for *Trypanosoma cruzi;* lane 1, DNA size marker MWM fX174 Hae III; lane 2, *Leishmania (L.) donovani* DD8 reference strain; lane 3, *L. (L.) infantum/chagasi* PP75 reference strain; lane 4, *L. (L.) mexicana* MC; lane 5, DNA from patient; lane 6, *L. (V.) braziliensis* LTB300 reference strain; lane 7, *T. cruzi*

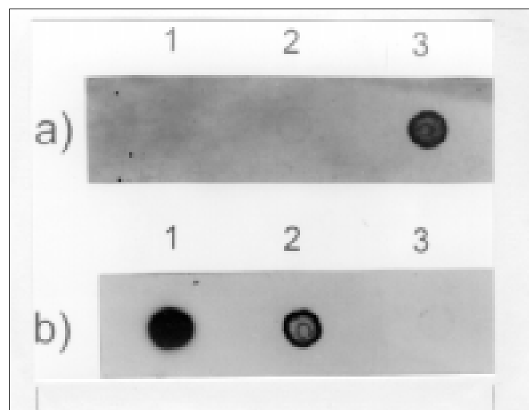

Fig. 5-a: specificity of *Leishmania donovani* complex specific probe B4Rsa; b: specificity for kDNA of *L. (L.) mexicana* MC used as probe. Hybridized with polymerase chain reaction products amplified with primers specific for subgenus *Leishmania*: dot 1, kDNA of *L. (L.) mexicana* MC; dot 2, DNA from patient; dot 3, kDNA from *L. (L.) donovani/chagasi* PP75

DISCUSSION

In Mexico there have been few reports of VL, and the causal agent of those reported has been shown to be *L. (L.) infantum/chagasi*. Furthermore, these cases were confined to central Mexican states such as Chiapas, Puebla and Guerrero. The statement of VL is now becoming more important due to an increase in the number of cases over the last ten years (since 1995 until 1999, 36 cases were recorded) and to its occurrence in six different Mexican states (the Mexican health authority, unpublished data). In the State of Chiapas most reported cases of VL have until now been caused by *L. (L.) infantum/chagasi* infection and the causal agent of most of the LCL are due to *L. (L.) mexicana* but some are due to *L. (V.) braziliensis.* The finding that patient DNA was amplified with primers specific for the *Leishmania* subgenus but

TABLE II

Hybridization of polymerase chain reaction products derived from the amplification of DNA patient amplified with primers DeB8 and AJS1

Probe B4Rsa *Leishmania donovani* complex	Probe B18 *L. braziliensis* complex	Probe kDNA of *L. (L.) mexicana* MC
-	-	+

was not amplified with primers specific for the *L. donovani* and *L. braziliensis* complexes (Figs 1, 2, 3) and the positive hybridization of these PCR products only with kDNA from the Mexican *L. (L.) mexicana* MC (Fig. 5b, dot 2), indicates that the patient was suffering from VL caused by *L. (L.) mexicana*. The less intense signal given by patient DNA compared to the MC strain is because the MC strain was hybridized with its own kDNA used as the probe (Fig. 5b, dots 1 and 2). We therefore conclude that this is the first case in Mexico of co-infection by *L. (L.) mexicana* and HIV, which is manifested as VL.

Patients with HIV infection have depressed $CD4^+$ cells. This contributes to the occurrence of visceral infection with *L. (L.) mexicana,* which is otherwise confined to the skin. In addition, immuno-suppressed patients could have VL caused by strains of *Leishmania,* which are different from *L. (L.) chagasi.* This is important because in Mexico there are endemic areas where MCL, LCL and VL co-exist. The results reported here are similar to those of Hernandez et al. (1994). These authors reported that in Venezuela a patient displaying the symptoms of VL had a co-infection with HIV and a *Leishmania* variant strain sharing kDNA sequences with *L. braziliensis* and *L. mexicana.* In order appropriate treatment to provide the patient, the disease must first be diagnosed and the *Leishmania* species identified. This is possible by using techniques for DNA analysis such as PCR and hybridization by dot and southern blot.

The Mexican isolate of *L. (L.) mexicana* MC and the patient DNA, amplified with primers specific for *T. cruzi,* gave a smear but not the diagnostic band for *T. cruzi* (Fig. 4, lanes 4 and 5). This could be because *Trypanosoma* and *Leishmania* share some DNA sequences in the kDNA or genomic DNA.

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