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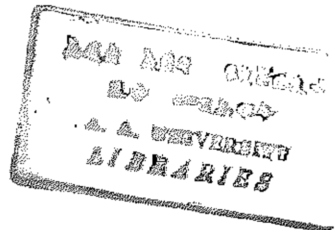
A Comparison of Surface Antigens of Four *Leishmania* Species (*L. aethiopica*, *L. donovani*, *L. major* and *L. tropica*)



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By

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INTRODUCTION

The genus *Leishmania* comprise different species all of which are obligate intracellular parasites of the mononuclear phagocytic cells of vertbrates. About 14 species and subspecies of the genus are known to infect man and cause a spectrum of disease states collectively known as leishmaniasis (Molyneux and Ashford, 1983). Transmission of the parasite is accomplished by sandfly vectors and almost always involve reservoir hosts. Man is generally considered as an accidental host (Marinkelle, 1980).

Leishmaniasis has been reported from more than 80 countries in the world with an estimated 400,000 new cases per year (Marinkelle, 1980; WHO, 1984). The disease stands as one of the major health problems of the world and in many countries it poses significant barriers to economic development (Lainson and Shaw, 1978; WHO, 1984). Clinically the disease is classified into three groups: viseral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL). Visceral leishmaniasis attacks the visceral organs - spleen, liver and bone marrow and is often fatal if untreated. Cutaneous leishmaniasis is characterized by ulcerative skin lesions and is rarely fatal, but can cause serious disfigurment: this is especially so for diffuse CL of both the Old World and the New World. South-American mucocutaneous leishmaniasis is highly multilating by destroy the nasal and pharyngeal tissues and causes sever mutilation (Chullay and Manson-Bahr, 1984).

Visceral leishmaniasis both, in the Old World and New World is ca
clients and control groups. 40

the complexes of *L. brazilensis* and *L. mexicana* (Lainson and Shaw 1978; WHO, 1984). Old World CL is caused by three different species: *L. major*, *L. tropica* and *L. aethiopica*. Whereas *L. major* and *L. tropica* often cause self healing localized lesions commonly known as oriental sore, *L. aethiopica* cause two clinically different types of CL: the localized self healing CL (LCL) and diffuse CL (DCL) (Marinkelle, 1980). At present whether this differences in clinical manifestation is a result of different strains of parasites or due to differences in host response to the same parasite is not clear (Mengistu and Fehniger, unpublished data; Bryceson, 1980).

The Leishmania parasites alternate between two developmental stages: the flagellated promastigote form in the insect vector gut, and the aflagelated amastigote form found intracellularly in vetebrate hosts (Chullay and Manson-Bahr, 1984). Infection is transmitted following inoculation of promastigotes by the sandfly vector during a blood meal. The promastigotes then enter macrophages and start to multiply inside the phago-lysosome (Mauel, 1984; Mauel and Behin, 1982).

Research on leishmaniasis has been greately facilitated by the ability to culture the parasite *in vitro*. Nevertheless, to date only the promastigote stage, the stage found in the insect vector gut, has been successfully grown in culture. Recently there have been some reports on the mass cultivation of amastigotes using different monocyte cell lines as hosts. (Monjour *et al.*, 1984; 1986; Chang; 1980; Looker *et al.*, 1986).

Identification and Classification of *Leishmania*

The identification and classification of *Leishmania* is controversial. To date there is no a single agreed upon criteria for the idenfitication and classification of the different species of *Leishmania* associated with the various clinical manifestations of the disease (WHO, 1984). Until now the identification of the different species of *Leishmania* is based largely on;

1. clinical manifestations of the disease and its geographical distribution;

2. direct microscopic identification of parasites from tissue aspirates ; and

3. culture of parasites from tissue aspirates and/or passage into experimental animals (Gardner, 1977; Chullay and MansonBahr, 1984). Immunodiagnostic tests has been applied to a limited extent (Zuckerman, 1975; Matossian-Rogers *et al.*, 1976).

Identification and classification based on clinical manifestations is not acceptable since host related factors may be involved in determining the outcome of the disease (Bryceson, 1980). The same parasite can cause different clinical manifestations in different patients. For example, *L. donovani* normally a viseralizing species in the Sudan was found to cause typical cutaneous leishmaniasis (Hoogstral and Heyneman, 1969; Abdella, 1982). Identification based on geographical distribution is also unreliable in areas where there is an overlap of the different speices of *Leishmania* (Gardner, 1977). Direct

microscopic identification of parasites from infected tissue samples is extremely difficult for the different species of Leishmania are morphologically indistinguishable even at the electronmicroscopic level (Bray, 1980). Moreover, this method is tedious and time consuming and requires the service of trained laboratory personnel (Lobel and Kagan, 1978). Culture of parasites from tissue aspirates or passage in experimental animals is an unreliable way of identifing since some isolates of Leishmania do not grow well in culture nor in experimental animals and in addition this method generally requires a long time to get results (Chullay and Manson-Bahr, 1980).

Immunodiagnosis of Leishmaniasis

Immunodiagnosis of leishmaniasis is based on measuring the antibody response (serodiagnosis) or cellular immune response (Leishmanin skin test). Infection with leishmania parasites elicit both humoral and cellular immune responses. Originally non-secific serologic tests like the formol test and complement fixation test have been used for diagnosis of leishmaniasis (Zuckerman, 1975). However these methods are unreliable because of the very high false positive results with other diseases, such as malaria, leprosy, syphilis etc. (Bray, 1980). The indirect immunoflorescence assay (IFA) and recently the enzyme linked immunosorbent assay (ELISA) have been developed for the diagnosis of cutaneous and viseral leishmaniasis using crude leishmania antigen (Zuckerman, 1975; Hommel, 1976; Hommel et al., 1978). These assays, too, although can give accurate results under optimal conditions, are still seriously affected by cross reaction with other co-endemic diseases (Mauel and Behin, 1982).

Another immunodiagnostic test involve the leishmanin skin test using crude leishmania antigen. Although this test is relatively fast and simple to perform for large scale studies, it is only genus specific and shows a high degree of cross reaction with other diseases (Carvalho et al., 1978). Perhaps the other major limitation of this technique is that it can not distinguish between past and present exposures to the parasite.

Recently new biochemical and molecular biology techniques have been used for accurate identification of Leishmania parasites to the species and subspecies level based on their intrinsic characterstic. These techniques are isoenzyme analysis (Kreutzer et al., 1980). DNA boyant density analysis (Chance et al., 1974;) DNA hybridization Barker et al., 1986; Lawrie et al., 1985) and Karyotype analysis (Giannini et al., 1986). Although these methods can give accurate and reliable identification of the different species of Leishmania, they all require the use of sophisticated and expensive equipments and reagents and some of these techniques involve complicated procedures making their routine use impractical (Wirth et al., 1986). This becomes even more important when we consider the fact that the disease is common in many developing countries. Thus other reliable, simple and cheap methods of diagnosis should be sought.

Perhaps the best method for reliable, fast, and cheap way of diagnosis both for routine clinical use and for large scale epidemiological studies of leishmaniasis would be to use an immunodiagnostic tests. The major limitation of these techniques, as outlined above, are the presence of cross reaction with other diseases and among the different species of Leishmania (Bray, 1980; Mauel and Behin, 1982). These

problems arise from lack of well characterized parasite antigens. Molecular, biochemical and immunological characterization of *Leishmania* antigens would, therefore, be important for the development of reliable and cheap immunodiagnostic tests (Mitchell *et al.*, 1982). Recent developments in hybridoma technology have led to the identification of different stage specific, strain specific, species specific and cross reactive *Leishmania* antigens (Handman and Hocking, 1982; Jaffe *et al.*, 1984; Jaffe and McMahon-Pratt, 1983; Williams *et al.*, 1986). Sixteen monoclonal antibodies produced by Jaffe *et al.*, (1984) against *L. donovani*, for example, were found to react only with *L. donovani* with no cross reaction with other parasites nor with other species of the genus. Some of these monoclonal antibodies were found to be useful for species identification. A number of investigators have suggested that monoclonal antibodies to defined *Leishmania* antigens may be useful in the development of a serologic assay for the clinical diagnosis and epidemiological studies of leishmaniasis.

Recently sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting have been developed and used in the study of parasite antigens (Zingales, 1984; Blackwell *et al.*, 1986). Using these techniques many investigators have demonstrated the antigen profile of different strains and species of *Leishmania* (Lepay, 1983; Sadik and Raff, 1985; Jammandas, *et al.*, 1983; Colomer-Gould *et al.*, 1985). Gardiner *et al.*, (1984) identified about 12 cross reactive antigens and many other species specific surface antigens from several *Leishmania* species by I labeling and immunoprecipitation followed by SDS-PAGE. Similar findings have been reported by other investigators for different *Leishmania* species (Lepay *et al.*, 1983;

Sadick and Rolff, 1985). One major protein reported by almost all investigators from all species investigated is a protein with molecular weight of 63 kilodanton (kD) called P63. It has been found both on promastigotes and amastigotes (Colomer-Gould *et al.*, 1986). The P63 is found to cover a large fraction of the promastigotes accounting about 1% of the total cellular protein (Bouvier *et al.*, 1985). Etages *et al.*, (1985) showed that P63 from 3 different Old World *Leishmania* species are structurally similar perhaps suggesting some functionsl similarity, too, and that it may be conserved in the course of evolution for an important function.

Reed *et al.*, (1987) identified *L. donovani* antigens that react with patient sera by Western blot technique. When a fraction of one of these molecules (in the molecular weight range of 55-62kD) was eluted and used in ELISA, it was found to be highly sensitive with a 100% specificity. Crude antigen preparation in the same test gave a high degree of cross reaction with other diseases. This strongly suggest the potential usefulness of specific, well characterized *Leishmania* antigens to develop sensitive and specific immunodiagnostic tests.

Immune Response to Leishmania Infection and the Potential for Vaccine Development

Besides their potential use for the development of immunodiagnostic tests, identification and characterization of *Leishmania* antigens would be important in the search for candidate vaccines to be used in the control of leishmaniasis (Anders *et al.*, 1982). Self cure in leishmaniasis is believed to be due to the immune response it evokes (Howard and Liew, 1984). In some parts of the world, notably in the

Middle East and some parts of USSR deliberate "Vaccination" of subjects with live strains of *L. tropica* at a cosmetically hidden site has been practiced to induce protective immunity (Zuckerman, 1975; Bryceson, 1980). Lesions developed from such leishmanization could last for months, sometimes even years.

Indirect evidence for the development of host protective acquired immunity has been obtained from experiments on model animals (Bryceson, 1980). Balb/c mice immunized with irradiated promastigotes of *L. major* were found to develop marked resistance to challenge infection with virulent strains of the same species decreasing mortality from 100% in control groups to 0-20% in immunized groups (Howard & Liew, 1984). Other investigators also showed development of protective immunity in different animal models immunized with different species of *Leishmania* with different antigen preparations (Holbrook *et al.*, 1981; Dennis *et al.*, 1986; Liew *et al.*, 1985; Alexander and Phillips, 1978).

In some species of *Leishmania* cross protection has been observed (Alexander and Phillips, 1978; Howard and Liew, 1984). These experiments on animal models strongly suggest that vaccines could be prepared from fragments or extracts of parasites if we could specifically identify which parasite molecules are responsible for inducing host protective immune response.

Surface antigens of leishmania have become the focus of attention in the search for host protective parasites antigens, for they are the first to come in contact with the immune response (Anders, 1982). Many investigators identified surface antigens on different *Leishmania*

strains using monoclonal antibodies and Western blot analysis (Handman and Curtis, 1982; Chang and Chang, 1986). The role of some of these molecules in inducing host protective immunity are being investigated. *Leishmania* being an intracellular parasite of macrophages requires a mechanism to enter to target host cells and once inside it must be able to protect itself from the harmful effect of enzymes and toxic metabolites of the host macrophage cells (Mauel, 1984; Moulder, 1986). The initial interaction between the parasite and the host macrophages is believed to involve a "receptor-ligand" interaction that involves molecules on the surface of parasites and macrophages (Chang and Dwyer, 1978; Blackwell *et al.*, 1985). This has been experimentally shown to be true (Klemper *et al.*, 1983). Macrophage plasma membrane vesicles specifically bind to promastigotes suggesting the presence of intrinsic determinants both on macrophages and promastigote surfaces. At a molecular level two promastigote surface molecules has been implicated in the initial attachment events: the major surface protein, P63 (Blackwell *et al.*, 1986) and a leishmania lipopolysacchride molecule (L-LPS) (Handman *et al.*, 1985). Afinity purified P63 of *L. major* has been shown to inhibit macrophage-promastigote binding up to 50% (Chang and Chang, 1986). Handman and Mitchel (1985) used purified L-LPS to immunize mice and found significant resistance to challenge infection with virulent *L. major*. This suggest the possibility of using such molecules as candidate vaccines. However, later work by Mitchel and Handman (1986) showed that whereas some portion of the L-LPS molecule has a protective role some portion of it induces suppression and promotes diseases. Further investigations might lead to the identification of some hitherto unidentified parasites surface molecules important for parasite entry.

Once the parasite is inside its target cell it must protect itself from harmful enzymes and metabolites released by target cells. Since macrophages are the very cells whose main function is to destroy any invading microorganisms, they produce hydrolytic enzymes and toxic metabolites (Mauel, 1984). The mechanisms of intracellular survival for leishmaniasis are not clearly known. Several possibilities have been proposed, all of which are not necessarily mutually exclusive (Moulder, 1986). The first possibility is that Leishmania spp. may secret some enzyme inhibitors or inactivators, like the carbohydrate rich excretory factors (El-On et. al., 1980) or produce ammonia in excess and interfere with the activity of enzymes by raising the pH (Coombs and Shanderson 1985). A second possibility is that Leishmania spp. may have evolved an enzyme resistant cell surface (Chang and Dwyer, 1978). Chang and Fong (Cited in Moulder, 1985) suggested that surface glycoproteins play crucial role in this type of adaptation. Some isolated surface molecules of leishmania were found to be resistant to proteases (Handman et al., 1981). However, the in vivo role of these molecules for leishmania intracellular survival is not yet determined. Recently Etges et al. (1986) found that the major surface protein, P63, of L. major is a protease. They postulated that the enzyme could function in protecting the parasite from microbicidal enzymes in the insect gut or during intracellular establishment in the macrophages. However, much is to be done to establish the role of this surface molecule for intracellular survival.

Other parasite antigens considered important in modulating host immune response are those expressed on the surface of infected macrophages (Berman and Dwyer, 1981; Handman and Hockong, 1982).

Because of an obligatory intracellular location of Leishmania, molecules expressed on the surface of infected macrophages may be important for the development of host protective immunity. Several mechanisms for the orgin of such molecules have been proposed: first incorporation of Leishmania antigen with the phago-lysosomes with subsequent translocation to macrophage plasma membranes or antigens excreted to the extracellular fluid may bind to the macrophages (Anders et al., 1982). Characterizaiton of such molecules, detailed studies on their origin, their role in the immune response of the host has to be investigated in a greater detail in order to establish a possible link between the development of host protective immunity to these molecules.

In Ethiopia both CL and VL have been reported in several regions of the country. While L. donovani is the causative agent of VL in the lowlands, L. aethiopica is considered to be the main causative agent of CL in the highlands. L. major is also suspected to cause CL in the lowlands. The disease affects the most productive sector of the population in the age range of 14-40 years (Ayele, 1982) and mostly occurs in fertile agricultural development areas. It can thus pose serious public health and economic problems. To date very little is known about the epidemiology of the disease, its host parasite relationship and the range of parasite species involved. Such information is highly required inorder to devise control strategies for the disease in areas of agricultural development and in new settlement areas.

The objective of this study was to identify species specific, subspecies specific and cross reacting antigens in four species of

Leishmania: *L. aethiopica*, *L. major*, *L. tropica* and *L. donovani* using leishmaniasis patient sera and rabbit immune sera. *Leishmania* antigens were separated using SDS-PAGE, transfered to nitrocellulose paper by the technique of Western blotting and reacted with the different patient sera and rabbit immune sera.

MATERIALS AND METHODS

Parasites:

Leishmania aethiopica (MHOM/ET/72/L100) originally isolated from a DCL patient and *L. tropica* (MHOM/SU/74/K27) were obtained from the WHO Leishmania Reference Strain Center, the London School of Tropical Medicine and Hygiene, U.K. *L. major* (LV-39) was obtained from Dr. David P. Humber, Department of Biology, originally isolated from animals and was a gift of the Bassel Institute, Swizerland. *L. donovani* originally isolated from Ethiopia, and *L. d. infantum* (LEM-235) were a gift of Dr. J. Blackwell, London School of Tropical Medicine and Hygiene. *L. aethiopica* (1467/85) was isolated from a localized cutaneous leishmaniasis patient at the Armauer Hansen Research Institute (AHRI) in 1985 and characterized as *L. aethiopica*.

Parasites were cultured at room temperature in a medium of RPMI 1640 (Flow Laboratories) containing 10% heat inactivated (56 C, 30 min) foetal calf serum, 100 u/ml penicilin and 100 ug/ml streptomycin. Stationary phase parasites were harvested by centrifugation and washed 5 times with Tris-Saline (TS) buffer (50mM Tris and 0.15M Nacl pH 7.4) containing 1.2 mM phenylmethyl sulfinylflouride (PMSF) and 200 U/ml trysol. Parasites were resuspended in TS-PMSF-Trysol buffer and stored at -70 until used.

Rabbit Immunization

Stationary phase parasites were harvested and washed 4 times with normal saline (pH 7.4) and fixed with 0.1% 1-Ethyl-3- (3-dimethylanmopropyl) carbodianide hydrochloride (Sigma) for 1 hour and

washed 2 times with 0.1 M glycine and three times with normal saline. Rabbits were immunized intramuscularly with the fixed and washed parasites (0.5 mg total protein) in complete Fruend's complete Adjuvant. Two weeks later booster injections were given intramuscularly with 0.5 mg protein in Incomplete Fruend's Adjuvant. Four weeks after the first booster injection, rabbits were re-boosted by 5 intravenous injection at 3 day intervals starting with 50 ug protein in the first injection and increasing the protein concentration to 100, 150, 250 and 500 ug protein respectively for the 2nd, 3rd, 4th and 5th injections. Rabbits were bleed 2,3 and 6 weeks after the last booster injection. Antibody titer was measured by the technique of dot blot (Pappas, 1983) and sera were stored at -20 C until used.

<u>Human Serum</u>

<u>L. aethiopica</u> (both LCL and DCL) and leprosy patient sera were obtained from the Serum Bank of AHRI. Visceral leishmaniasis patient sera were collected from patients clinically diagnosed as visceral leishmaniasis at the Army Hospital, Addis Ababa, and were kindly provided by Dr. Yohannes Negesse, Army Hospital. Visceral leishmaniasis endemic area normal human sera were collected from apparently healthy individuals at Aba Roba Kebele, Gamu Gofa Administrative Region and were a gift of Ato Tesfaye Getachew, Institute of Pathobiology, Addis Ababa University. Schistomiasis patient sera were collected from confirmed cases of <u>Schistosoma mansoni</u> infected patients at the Institute of Pathobiology and were a gift of Ato Astrat Hailu and Ato Hailu Birre, Institute of Pathobiology.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Washed promastigotes were mixed with an equal volume of final sample buffer (consisting of 0.2% glycerol, 0.1% mercaptoethanol, 0.04 SDS and 50 mM Tris buffer, pH 6.8 plus bromophenol blue) (Lammeli, 1970), boiled for 7 min. and centrifuged at 14000 RPM for 5 min. in an Eppendorf Centrifuge 5415. The supernatant was electrophoresed in 0.75mm thick slab gel. Throughout the experiment 11% separating gels and 4% stacking gels were used. Proteins of known molecular weight were run on a parallel lane to the sample to serve as molecular weight (mol. wt) markers. The relative mol. wt. of *Leishmania* antigens were extrapolated based on the migration of these mol. wt. markers.

Western Blotting

Following SDS-PAGE, the separated proteins were electrotransferred onto nitrocellulose papper using the method of (Towbin *et al.*, 1979) at 420V for 45 min. in Tris-glycine buffer (30mM Tris and 250mM glycine, pH8.3). Lanes containing molecular weight markers were cut and stained with Amido Schwartz Stain (0.001% w/v Amido Schwartz 0.3% v/v methanol and 0.1% v/v acetic acid).

Nitrocellulose sheets were cut into strips (0.6 cm wide) and blocked for 1 hr. with 0.05% Brij in TS buffer (pH 7.4) and incubated overnight with 1/40 dilutions of human sera (diluted in 0.05% Brij) or 1/20 dilution of rabbit immune sera (diluted with 10% foetal calf serum and 0.05% Brij in TS buffer). Strips were washed three times with 0.05% Brij each 10 min. and incubated with 1/100 dilution (in 0.05% brij) of peroxidase conjugated rabbit anti-human immunoglobulins for 2 hrs. (for strips initially incubated with human serum) or with

1/150 dilution of peroxidase conjugated swine anti-rabbit immunoglobulines for 3 hrs. (for strips initially incubated with rabbit serum). At the end of incubation strips were washed three times with 0.05% brij and incubated with 1/150 dilution of peroxidase conjugated swine anti-rabbit for 1 hour (for strips initially incubated with human sera). Strips were washed 3 times with brij and once with deionized water (5-10 min.). Color was developed by incubating strips with 0.01% 4-cholo-1- naphthol (prepared from a stock solution of 5 mg/ml 4-chloro-1-naphtal in DMSO) and 0.24% H O in TS buffer (pH 7.4) with vigrous shaking. The reaction was stopped by washing strips with tap water.

RESULTS

Leishmania donovani Antigens Recognized by Patient Sera

Whole cell lysates of L. donovani promastigotes were electrophoresed by SDS-PAGE and electrotransferred onto nitrocellulose paper. Nitrocellulose bound antigens were reacted with patient sera. Major antigens of mol. wt. 74, 54, 25, 18 and 15 kD and other weakly reactive antigens of 67, 64, 62, 46, 31 and 28 kD were recognized by human viseral leishmaniasis (VL) patient sera (Fig. 1 lane A).

Sera from L. aethiopica patients (LCL and DCL) recognized most of the antigens reactive with VL patient sera (Fig. 1, lanes B and C). One antigen of mol. wt. 18 kD is not reactive with LCL and DCL patient sera indicating that this molecule is species specific to L. donovani. The 31 and 28 kD antigens are reactive only with VL and DCL patients sera.

The same antigenic profile was demonstrated when L. donovani infantum blots react with VL, LCL and DCL patient sera (Fig. 2). The 18 kD antigen is not reactive with L. aethiopica patient sera.

Leishmania aethiopica antigens recognized by patient sera

When SDS-PAGE separated L. aethiopica (1467/85), an LCL parasite, reacted with an LCL patient sera, major antigens of 74, 67, 64, 62, 54 and 53 kD and weakly reactive antigens of mol. wt. 58, 45, 43, 36, 33, 26 and 15 kD are identified (Fig. 3 Lane A). DCL and VL patients sera recognized most of the antigens reactive with LCL serum (lane B and C). The 45 kD antigen is uniquely recognized by LCL and DCL patient sera while the 43 and 24 kD antigens are recognized only by an LCL

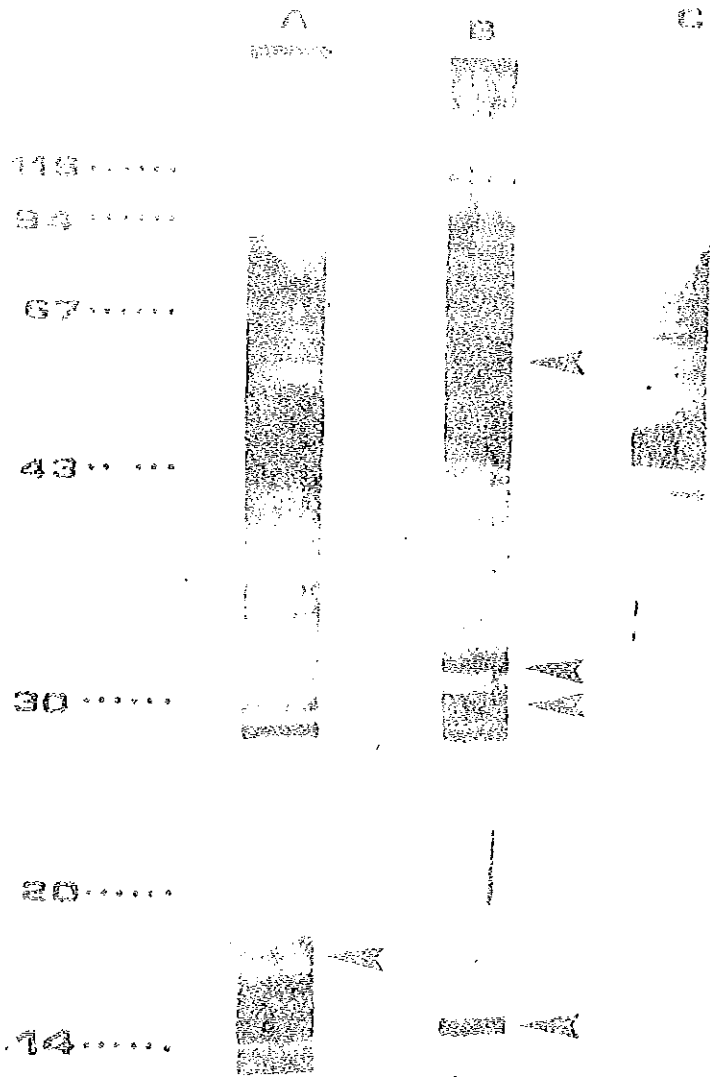

Fig. 1

Western blot of whole cell lysate of *L. donovani* reacted with leishmaniasis patient sera. (A) Viseral leishmaniasis (B) Diffuse cutaneous leishmaniasis (DCL). (C) Localized leishmaniasis (LCL). Arrows indicate antigens that are not recognized by all patient sera. Numbers at the left indicate molecular weight standards, in kilodalton (kD), run at the same time.

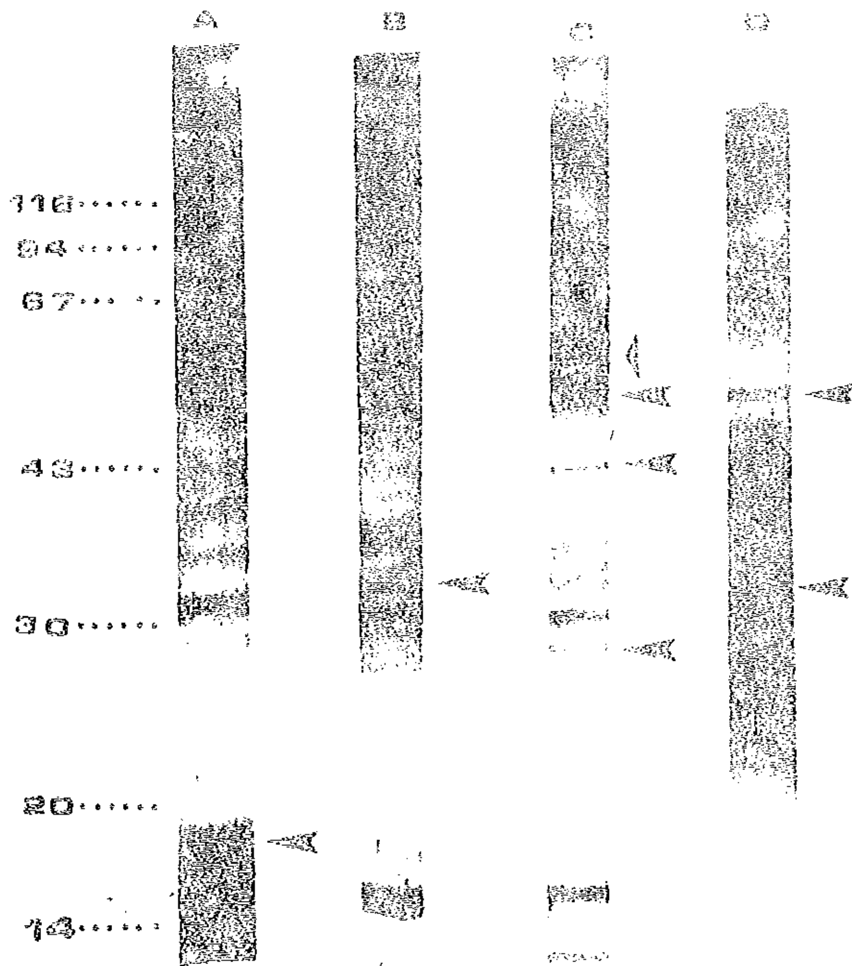

Fig. 2

Western blot of whole cell lysate of L. donovani infantum reacted with leishmaniasis patient sera and rabbit immune sera. (A) Visceral leishmaniasis. (B) DCL, (C) LCL, (D) anti-L. donovani rabbit sera. Arrows indicate antigens that are not recognized by all sera.

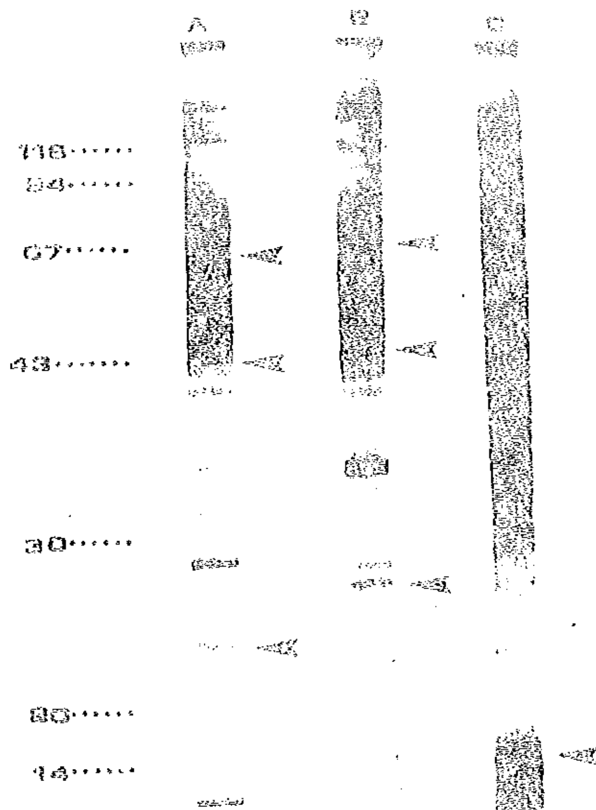

Fig. 3

Reactivity of *L. aethiopica* (1467/85), an LCl isolate antigens, with leishmaniasis patient sera on Western blots. Whole cell lysates were electrophoresed on SDS-PAGE and transferred onto nictocellulose paper. (A) LCl, (B) DCL, (C) Visceral leishmaniasis patient sera. Antigens that are not recognized by all sera are indicated with arrows.

sera. The 15 kD antigen of *L. aethiopica* (2467/85) is strongly reactive with VL sera but react very weakly with LCL and DCL patient sera.

A similar antigenic profile was observed when DCL parasite antigens were reacted the same way as above (Fig. 4). The 45 kD antigen is reactive only with LCL and DCL patient sera. One antigen of mol. wt. 27 kD is recognized only by DCL patient sera suggesting that it is specific for DCL patients.

Antigens of *L. major* and *L. tropica* recognized by *L. aethiopica* and *L. donovani* patient sera.

Antigens of *L. tropica* and *L. major* reactive with VL, LCL and DCL patient sera are displayed on Figures 5 and 6 respectively. It appears that most of the common antigens shared between *L. aethiopica* and *L. donovani* are also shared with *L. tropica* and *L. major*. The 33 kD antigens of *L. tropica* and *L. major* is reactive only with DCL patient sera.

The 54 kD antigen is recognized by all types of patient sera and it is the major antigen of all the species.

Leishmania Antigens Recognized by Rabbit Immune Sera

Blots of the different *Leishmania* species were reacted with rabbit immune sera raised against the promastigotes of the different species. Figure 7 shows antigens of *L. donovani* reactive with different rabbit immune sera (lane A-E). Sera from *L. donovani* promastigote immunized rabbits recognized major antigens of mol. wt. 93, 68, 47, 40, 35, 26

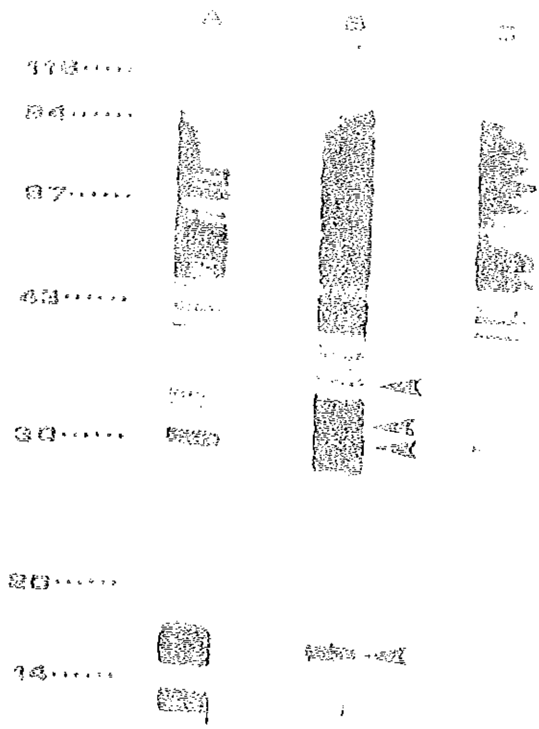

Fig. 4

The antigenic profile of whole cell lysates of *L. aethiopica* (MHOM/ET/72/L100), a DCL isolate, recognized by leishmaniasis patient sera on Western blots. (A) Visceral leishmaniasis patient sera. (B) DCL patient sera. (C) LCL patient sera.

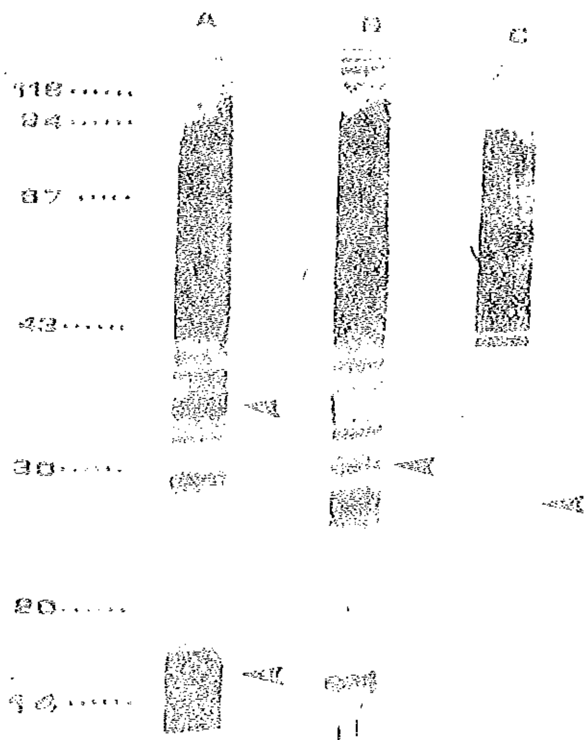

Fig. 5

Western blots of *L. tropica* whole cell lysate reacted with leishmaniasis patient sera. (A) Visceral leishmaniasis. (B) DCL. (C) LCL.

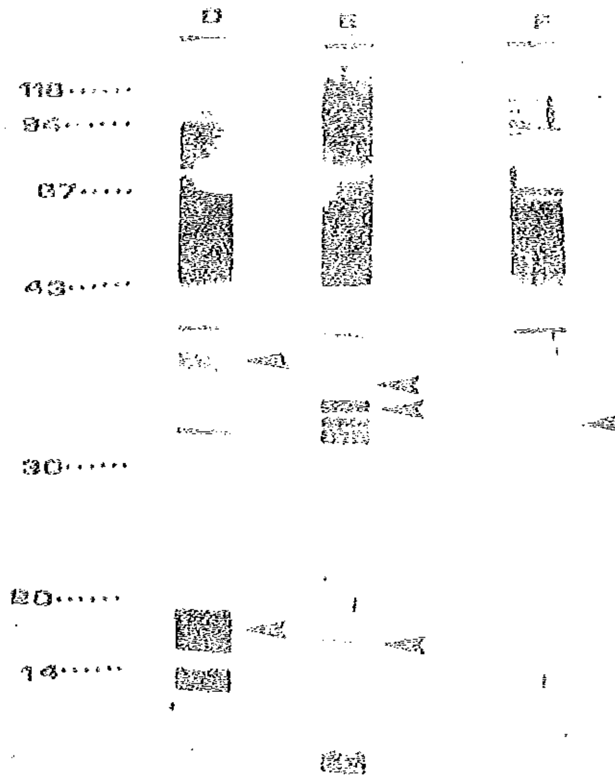

Fig. 6

Western blot of L. major whole cell lysates reacted with patient sera. (D) Visceral leishmaniasis. (E) DCL. (F) LCL.

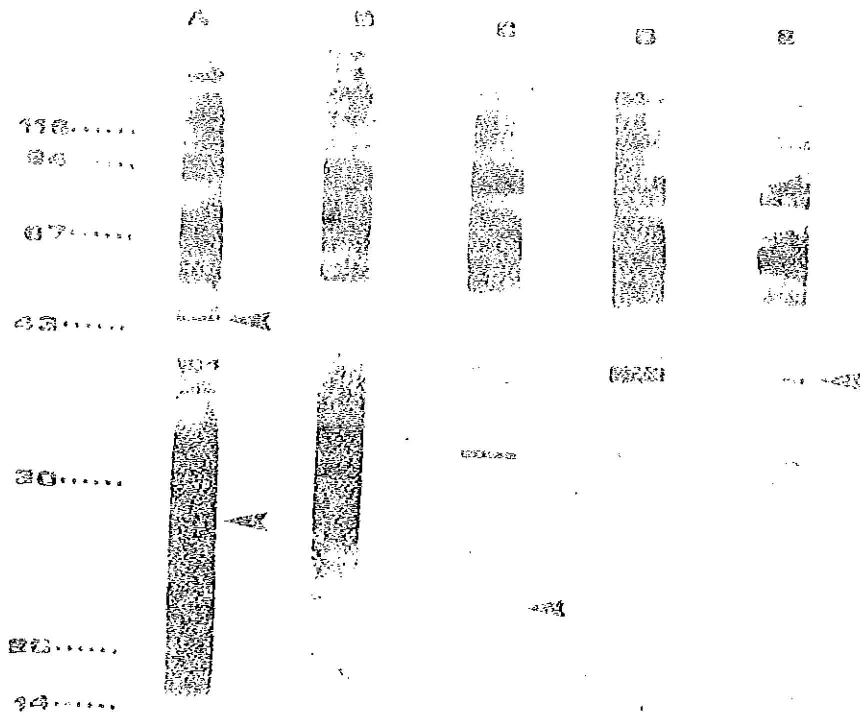

Fig. 7

Western blot of L. donovani whole cell lysates, reacted with rabbit immune sera. (A) anti-L. donovani, (b) anti-L. aethiopica (1467/85). (C) anti-L. aethiopica (MHOM/ET/72/L100). (D) anti-L. tropica. (E) anti-L. major. Arrows indicate antigens that are not shared among all the leishmaniasis species.

and 22 kD and other weakly reactive bands (Fig. 7, lane A). Four antigens of L. donovani recognized by immune rabbit serum, were not recognized by patient sera. Two of the Leishmania donovani antigens recognized by L. donovani rabbit immune sera (mol. wt. 47 and 27) apeared to be species specific antigens. The 40 kD antigen is reactive with L. major, L. tropica and L. donovani immunized rabbit sera but not with L. aethiopica (LCL and DCL) promastigote immunized rabbit sera.

L. aethiopica (1467/85) an LCL parasite, immunized rabbits sera recognized two antigens of molecular weight 70 and 60 that are not recognized by any other rabbit sera (Fig. 8 lane B). Three antigens reactive with rabbit immune sera are not reactive with patient sera. The 32kD antigen is strongly reactive with DCL parasite immunized rabbit sera and weakly reactive with LCL immunized rabbit sera. Sera from other rabbits do not react with this band.

L. aethipica (1467/85) and L. donovani immunized rabbit recognized a strongly reactive but diffuse band mol. wt. of around 15-33 kD.

Antigens of L. aethiopica (MHOM/ET/72/L100) - a DCL isolate - reactive with the different rabbit immune sera is shown in Fig. 9. A 32 kD antigen is strongly reactive with DCL immunized rabbits and weakly reactive with 1467/85 immunized rabbit sera. This antigen is not cross reactive with sera from other rabbits. Antother weakly reactive antigen (mol. wt. 27) appears to be unique to DCL parasites. The 70 kD antigen is specifically reactive with LCL and DCL rabbit immune sera, suggesting that the two antigens are specific for L. aethiopica.

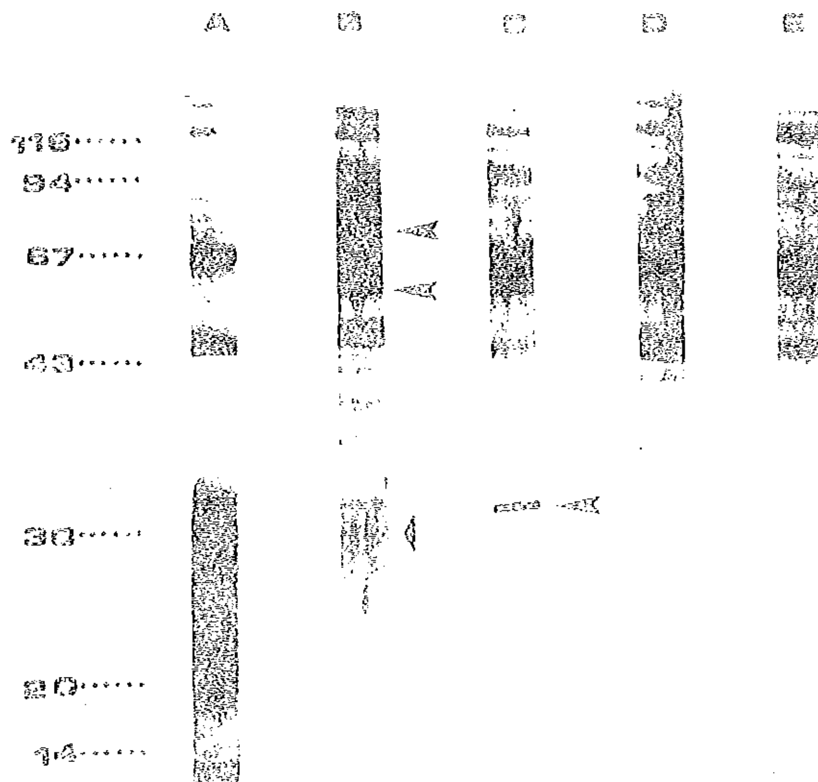

Fig. 8

Blots of *L. aethiopica* (1467/85) antigens reacted with rabbit immune sera. (A) anti-*L. donovani*. (B) anti-*L. aethiopica* (1467/85). (C) anti-*L. aethiopica* (MHOM/ET/72/L100). (D) anti-*L. tropica*. (E) anti-*L. major*.

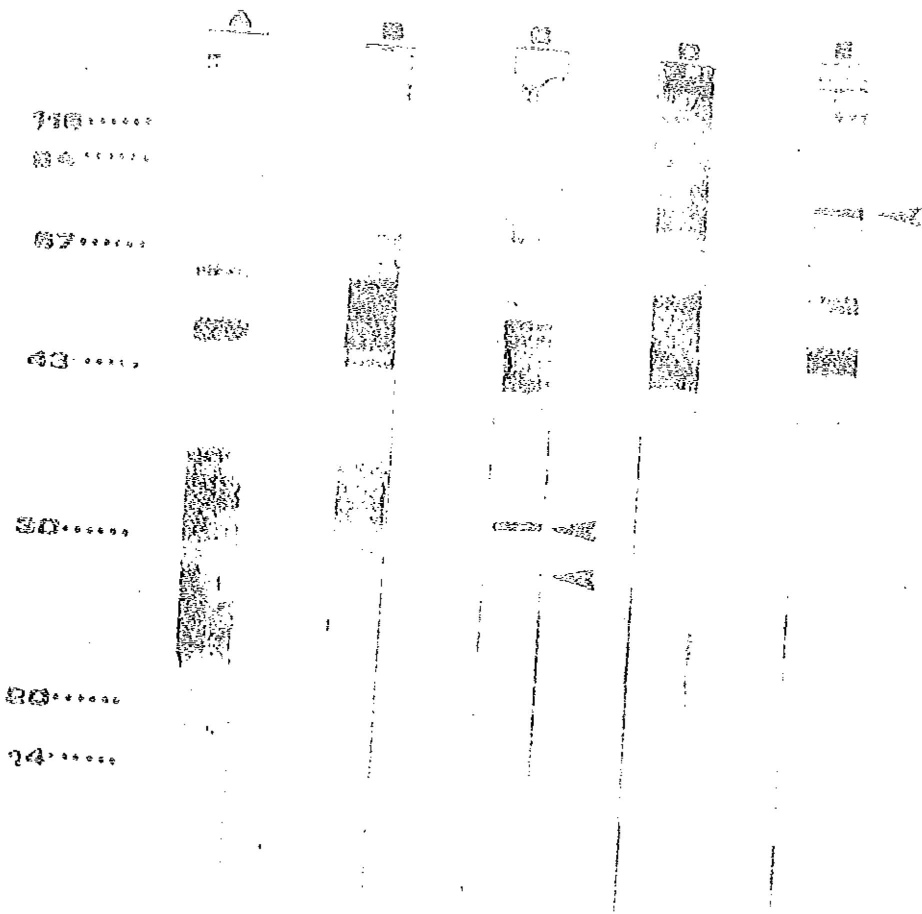

Fig. 9

Blots of *L. aethipoica* (MHOM/ET/72/L100) reacted with rabbit immune sera. (A) anti-*L. major*. (B) anti-*L. tropica*. (C) anti-*L. aethiopica* (MHOM/ET.72/L100). (D) anti-*L. aethiopica* (1467/85) (E) anti-*L. donovani*.

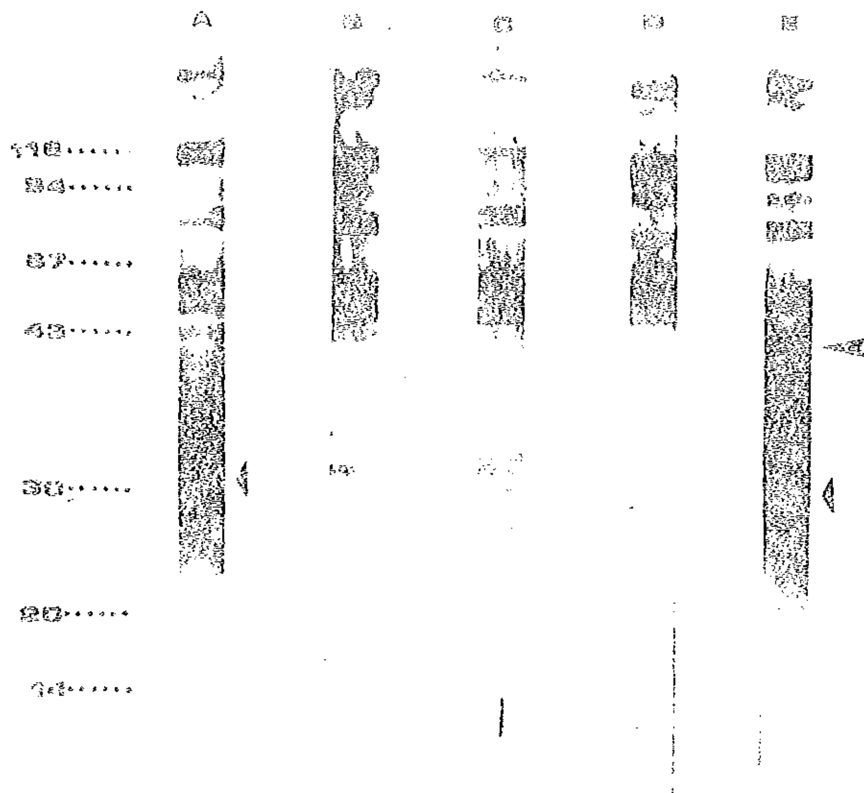

Fig. 10

Western blots of *L. major* whole cell lysates reacted with rabbit immune. (A) anti-*L. major*. (B) anti-*L. tropica*. (C) anti-*L. aethiopica* (1467/85). (D) anti-*L. aethipica* (MHOM/ET/72/L100). (E) anti-*L. donovani*.

Almost all antigens of L. major recognized by L. major rabbit immune sera (Fig. 10 lane E) are cross reacting other rabbit immune sera (Fig. 10, lanes A-D). The 40 kD antigen was consistently a common antigen among L. donovani, L. major and L. tropica but not shared by L. aethiopica (both LCL and DCL).

L. tropica immunized rabbits recognized one unique antigen mol. wt. 38 kD which is not shared with other leishmania species (Fig. 11, lane D). The rest of the antigens were common to all species. A consistant finding from all the leishmania species is that the 54 kD antigen is not immunogenic in rabbits.

Cross reaction of Leishmania antigens with leprosy and schistosomiasis patient sera

Sera from leprosy and schistosomiasis patients did not cross react with L. donovani (Fig. 12, lane B) or with leprosy patient sera although an antigen (mol. wt. 67) is recognized by all patients and normal indi-viduals. The same result was found for L. aethiopica (DCL isolate, data not shown).

Reactivity of Visceral Leishmaniasis Endemic Area Normal Human Sera to Leishmania donovani Antigens

To check for the possibility of subclinical infection by the presence of antibody production we have tested the reactivity of 10 sera collected from apparently normal individuals living in VL endemic area was tested on SDS-PAGE separated L. donovani antigens. None of the sera showed any specific reaction with L. donovani antigens (Fig. 12, lane D and E, the results of the other 8 sera not shown). The patterns were all similar to normal human serum from a non-visceral leishmania-sis endemic area (Lane B).

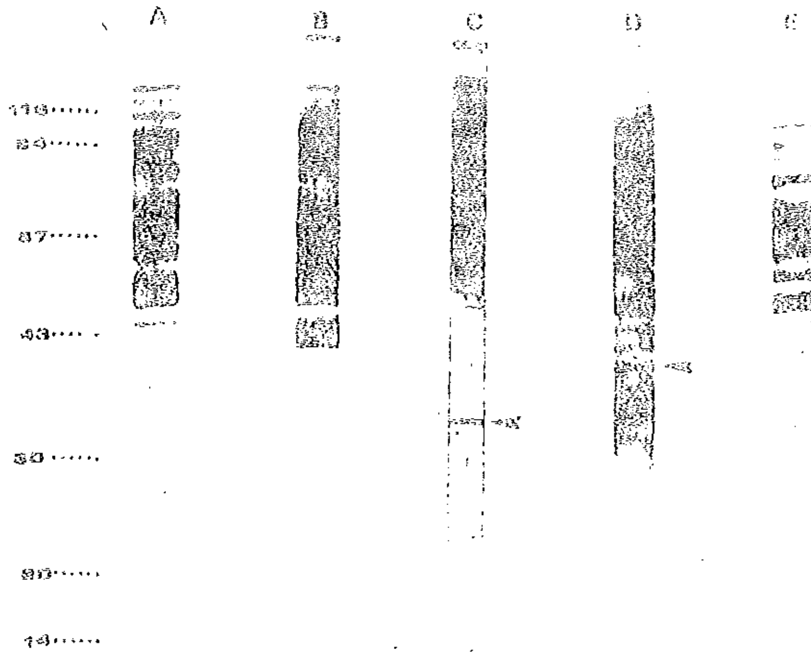

Fig. 11

Blots of L. tropica lysates reacted with rabbit immune sera. (A) anti-L. donovani. (B) anti-L. aethiopica (1467/85). (C) anti-L. aethiopica (MHOM/ET/72/L100). (D) anti-L. tropica. (E) anti-L. major.

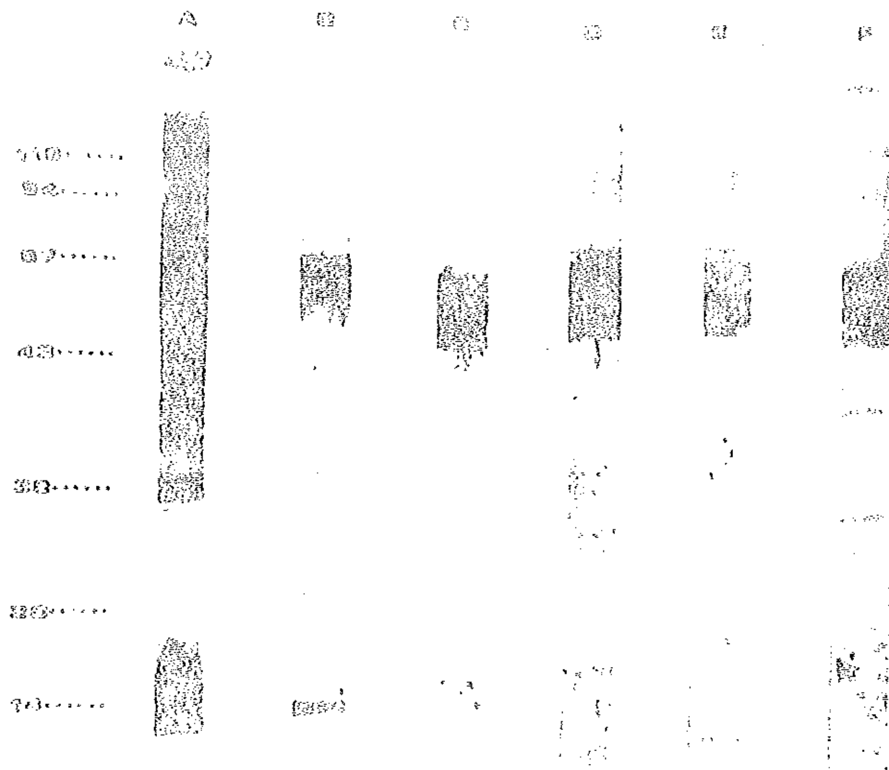

Fig. 12

Reactivity of Leishmania donovani antigens to different human sera. (A) Visceral leishmaniasis patient sera. (B) Normal human serum. (C) Schistosomiasis patient sera. (D) Leprosy patient sera. (E and F) Normal human sera from visceral leishmaniasis endemic area.

TABLE 1 Leishmania antigen recognized by different leishmaniasis patient sera and rabbit immune sera on Western blots

Antigen (kD)	Leishmania species expressing antigens				
	L. aethipica (LCL)	L. aethipica (DCL)	L. donovani	L. major	L. tropica
93	+	+	+	+	+
74	+	+	+	+	+
70	+	+	-	-	-
68	+	+	+	+	+
67	+	+	+	+	+
64	+	+	+	+	+
62	+	+	+	+	+
60	+	-	-	-	-
58	+	+	+	+	+
54	+	+	+	+	+
53	+	+	+	+	+
47	-	-	+	-	-
46	+	+	+	+	+
45	+	+	-	-	-
43	+	+	+	+	+
40	-	-	+	+	+
38	-	-	-	-	+
36	+	+	+	+	+
35	+	+	+	+	+
33	-	+	-	-	-
32	+	+	+	+	+
31	+	+	-	-	-
28	-	+	+	-	-
27	-	-	+	-	-
26	+	+	+	+	+
24	+				
22	+	+	+	+	+
18	-	-	+	-	-
15	+	+	+	+	+

TABLE 2

Cross reactive antigens found on _L. aethiopica_, _L. donovani_, _L. major_ and _L. tropica_

Reactive with human patient sera*	Reactive with rabbit antisera**
74	93
67	68
64	58
62	53
58	36
54	26
53	22
43	
31	
15	

* Reactive with serum from patients infected with either _L. donovani_ or _L. aethiopica_.

** Showed reactivity with all rabbit anti-sera against either _L. aethipica_, _L. donovani_, _L. major_ or _L. tropica_.

DISCUSSION

The lack of well characterized Leishmania antigens has greatly limited research on leishmaniasis and the potential to control the disease. One of the major problems is the lack of a reliable, simple and cheap method for identification of the different species and subspecies of Leishmania associated with the different clinical manifestations of the disease. The other limitation is the difficulty to investigate for parasite molecules that could induce host protective immune response.

The result of this study demonstrated that Old World Leishmania species express common and species specific antigens that are recognized by patient sera and rabbit immune sera (Table 1 & 2). L. donovani express a species specific antigen of 18 kD which is detected using patient sera. This same antigen was also detected in L. d. infantum, suggest-ing that the antigen is species specific.

The 45 kD antigen of L. aethiopica is recognized only by L. aethiopica (LCL & DCL) patient sera indicating that it is species specific. LCL parasites expressed two unique antigens, 43 and 24 kD while DCL parasites express one unique antigen of mol. wt. 27 kD. The question of whether these antigens are strain specific or represent possible inherent differences between DCL and LCL parasites will require further study.

These unique antigens of L. donovani and L. aethiopica were shown not to be shared with L. tropica and L. major as VL, DCL, and LCL patient sera did not recognize these antigens from the two species. Species specific molecules might play crucial roles in determining species

specific parasite behaviors, and the identification of such molecules could be important in developing an understanding of the molecular basis of disease pathogenesis.

A large part of *Leishmania* antigens identifiable by patient sera are common antigens shared among all the 4 species of *Leishmania*. A major antigen of interest, reported by many workers, is a glycoprotein of mol. wt. 63 kD (Etges; *et al.*, 1985, Lepay *et al.*, 1984; Bourdier *et al.*, 1986). This antigen was shown to be universally present in all leishmania species and was found to be surface located by surface iodination experiments. In this study a major antigen shared by all leishmania species and recognized by all patient sera was found around a mol. wt. of 54 kD. Preliminary results of AHRI (Mengistu, unpublished observations) have indicated that *L. aethiopica* does not express a 63 kD antigen, in contrast to all other species which have been exmained. The 54 kD common antigen has been identified as the alpha and beta tubulin proteins which are found in the plasma membrane of the organisms.

The 15 kD antigen was found in all the species studied. However, it is highly reactive with VL patient sera, less reactive with DCL patient sera and almost unreactive with LCL patient sera, both for the homologus and the heterologous antigen. This antigen may be expressed in greater amounts in *L. donovani* than in *L. aehtiopica* and may thus initiate the production of high titer antibodies. Another possibility is that the antigen is present in all the species but may be stearically inaccessible to the hosts immune response during *L. aethiopica* infection.

The common antigens of Leishmania might be involved in determing common parasite behaviours, such as entry into host target cells, intracellular survival etc. (Moulder, 1986; Mauel, 1984). We can thus assume that many of the common antigens to be conserved in the course of evolution. Etges et al., (1985) showed that gp 63, a common antigen of leishmania species, has a basic structural similarity in three Old World leiahmania species, indicating that the molecule is conserved in all the 3 species. Other investigators (Russel, et al., 1986; Chang and Chang, 1986; Blackwell, et al., 1986) showed that the gp63 is involved in the initial attachment of the parasite to macrophages. The use of such molecules as candidate vaccines has been suggested based on the observation that vaccination of mice with macrophage receptors does induce protective immunity (Handman et al., 1985).

Experiments using animal models (Howard & Liew, 1984; Alexander, 1982) and on human subjects (Convit et al., 1987; Green et al., 1983; Bryceson, 1980a) clearly showed that protective immune responses could be initiated using fragments, or extracts of Leishmania promastigotes. Identification and characterization of common and species specific antigens of leishmania parasites could, therefore, allow the determination of those antigens that stimulate protective immune responses. Some of the common antigens identified in this study might prove useful for immunoprophylaxis or for immunotherapy.

Rabbit sera raised against the promastigote stage of the different leishmania recognized common and species specific antigens some of which are not reactive with patient sera. Since rabbit immune sera are produced against the promastigote stage those antigens reactive only with rabbit sera might represent stage specific molecules, for

the patient sera are directed against the amastigote stage determinants which may or may not be shared with the promastigote stage.

L. donovani immunized rabbit sera recognized two species specific antigens 47 and 25 kD. One antigen of mol. wt. 40 kD is cross reactive among L. donovani, L. major and L. tropica but not reactive with L. aethiopica (both LCL and DCL) suggesting that there are probably common antigens that may not be present in some species.

Two antigens of L. aethiopica (mol. wt. 70 and 32kD) were found to be species specific and the 70 and 60 kD antigens of 1467/85 and the 27 kD antigen DCL parasites were shown to be strain specific antigens. Similarly L. triopica immunized rabbits recognized one species specific antigen of mol. wt. 38kD Other antigens recognized by immune rabbit sera are common antigens shared by all leishmania species. The 54 kD antigen recognized by patient sera was not immunogenic in rabbits using fixed organisms. To date there are no published data showing any correlation between immunogenicity in rabbits and in human subjects. However, the species secific leishmania antigens could be used as immunodiagnostic antigens.

Cross reaction between leishmania and other diseases both at the antibody and cellular immune response have been well documented (Zuckerman, 1985; Bray, 1980; Carvalaho et al., 1987). In this study, sera collected from schistosomiasis and leprosy patients did not react with SDS-PAGE separated leishmania antigens. This is highly encouraging since L. aethiopica DCL patients are often confused with leprosy (Sarojini, et. al. 1984). Perhaps the very high positivity of leprosy sera for leishmania antigens reported by other workers might be due to

misdiagnosis of DCL patients for leprosy or may be because of the presence of double infection by leprosy and leishmaniasis. Barneston and Bryceson (1978) reported about 8 cases of leprosy and leishmaniasis concomitant infections in Ethiopia.

There have been reports suggesting that in leishmaniasis endemic areas there is a very high subclinical infection rate which may be as high as 9 times the clinical rate (Sukker et al., 1986). If this is correct then it might be expected that individuals living in endemic areas would have significant immune response to leishmania antigens.

However, in this study 10 sera collected from aparently normal individuals living in VL endemic area and tested for reactivity on SDS-PAGE separated L. donovani antigens showed no evidence of specific reactivity to leishmania antigens.

CONCLUDING REMARKS

This study has identified a number of common and species specific antigens in 4 Old World *Leishmania* species using leishmaniasis patient sera and rabbit immune sera (Table 1 & 2). Analysis of common and species specific antigens of leishmania are required to identify antigens that would be of value for immunodiagnosis, vaccination, analysis of immunopathology and the analysis of various, immune responses in infected and vaccinated subjects (Anders, *et al.*, 1982; Handman *et al.*, 1981). Species specific antigens or antibodies produced against these antigens could be used as parasite typing reagents.

Most of the pathology of leishmaniasis is known to be a result of the interaction of parasite antigens with host immune system. Although it is impossible to assign specific functions to the antigens defined in this study, some of the antigens identified may be useful in immunodiagnosis and further understanding of the specific role of these molecules in the disease process might enable the identification of host protective antigens. Recent developments in recombinant DNA technology would then allow the large scale production of desired antigens.

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