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Serodiagnosis of Leishmaniasis

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ABSTRACT: Leishmaniasis is a spectrum of diseases ranging in severity from cutaneous (CL), post-kala-azar dermal (PKDL), and diffuse cutaneous (DCL) to mucocutaneous (MCL) and visceral (VL) infections that are endemic in 86 tropical and subtropical countries around the world, accounting for 75,000 deaths per year. Different forms of leishmaniases are generally caused by different distinct species of *Leishmania* having a digenetic life cycle alternating between an aflagellated amastigote form replicative within the macrophages of the host and a flagellated promastigote form that multiplies within the gut of the sandfly. VL, MCL, PKDL, DCL, and CL forms of the disease can be arranged on a priority basis in accordance with the humoral immune responses of host. Generally, the cell-mediated immunity, particularly the delayed-type hypersensitivity to leishmanial antigens, is associated with CL, MCL, PKDL, and cured VL cases. The serodiagnosis of leishmaniasis appears to be an alternative to parasite detection in biopsy samples either by the staining of amastigotes or by culturing the amastigotes, which transform to a promastigote form and replicate. A battery of immunological procedures have been developed or adapted to demonstrate either humoral or cell-mediated immune responses against *Leishmania* for diagnosis and epidemiological survey. The sensitivity and specificity of such diagnostic methods depend on the type, source, and purity of antigen employed, as some of the leishmanial antigens have common cross-reactive epitopes shared with other microorganisms, particularly *Trypanosoma, Mycobacteria, Plasmodia,* and *Schistosoma.* Serodiagnostic techniques for the detection of antileishmanial antibodies have been employed with about 72 to 100, 23 to 90, 83, and 33 to 100% success in VL, CL, MCL, and PKDL patients, respectively. The Leishmanin skin test (LST) is useful to detect MCL and CL, with about 100 and 84% success, respectively. In PKDL, the gradual fall of antileishmanial antibody titer to some extent and the rise of delayed hypersensitivity to the parasite antigen are the characteristic features associated with the chronicity of the disease. The use of whole promastigote as the source of antigens in the direct agglutination test (DAT) and immunofluorescent test (IFAT) gave cross-reactions with the sera of leprosy, tuberculosis, and African trypanosomiasis patients. Again, the use of cell-free extracts of promastigotes generally gave false positive results with the sera of normal human and Chagas' disease, leprosy, tuberculosis, and malaria patients in enzyme-linked immunosorbent assay (ELISA), dot ELISA, immunodiffusion, immunoelectrophoresis, and counter-current immunoelectrophoresis tests. Leishmanial proteinase, gp63, is not species specific, but appears to be *Leishmania* specific, so it can be used to detect a case of leishmaniasis. Acid phosphatase and the dp72-kDa protein of *L. donovani* cross-reacts with the sera of leprosy patients. Active VL cases are confirmed by the positive results obtained for antileishmanial antibody detection and negative for LST. The titers of antibodies against excreted factor (EF) or lipophosphoglycan (LPG) or lipophosphopolysaccharide (LPPS) of *Leishmania* were found to be insignificant for serodiagnostic purposes. Use of ConA-positive glycoproteins released or secreted by promastigotes of *L. donovani* showed 100% positive reactions with VL in immunoelectrophoresis. An LPS-like antigen of *L. major* promastigotes could detect 90% of confirmed CL cases with radioimmunoassay. Detection of leishmanial antigens circulating in immune complexes is considered an indication of active leishmanial infection.

KEY WORDS: leishmaniasis, serodiagnosis of leishmaniasis, visceral leishmaniasis, kala-azar, cutaneous leishmaniasis, mucocutaneous leishmaniasis, post-kala-azar dermal leishmaniasis, diffuse cutaneous leishmaniasis, direct agglutination test, indirect fluorescence antibody test, gel diffusion test, counter-current immunoelectrophoresis, immunoelectrophoresis, enzyme-linked immunosorbent assay, dot ELISA, radioimmunoassay, monoclonal antibodies, circulating antigen, leishmanin skin test, *Leishmania*.

I. INTRODUCTION

This review on the serodiagnosis of leishmaniasis is not intended to be an exhaustive one. A historical perspective is given to more recent developments and the employment of new, sensitive, economic, more reliable methodologies and different antigens of *Leishmania*, and their results

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TABLE 1
Leishmaniasis: Causative Agents, Disease Types, Distribution, Reservoir, and Some Immunological Parameters

Clinical syndrome	Causative *Leishmania* sp.	Common name of the leishmaniasis	Distribution	Animal reservoir	Antibody (enough for sero-diagnosis)[a]	Delayed-type hypersensitivity reaction (DTH) to leishmanin test[b]	Tendency to self-healing
Visceral leishmaniasis: generalized involvement of the reticuloendothelial systems (spleen, bone marrow, liver, etc.)	*L. donovani donovani*	Kala-azar	India, Bangladesh, Pakistan, Eastern China, East Africa	Man	++++	–	No
	L. d. chagasi	Kala-azar	South and Central America, Argentina, Brazil, Columbia, Ecuador, French Guiana, Honduras, Surinam, Paraguay, Venezuela, Island of Guadeloupe	Dog, Savanath fox	++++	–	No
		Kala-azar	Southern Russia, northern Iran China	Jackel Raccoon dog	++++	–	No
	L. d. archibaldi	Kala-azar	Sudan, Kenya, Ethiopia	Rodent	++++	–	No
	L. d. spp.	Kala-azar	Kenya, Somalia	Dog	++++	–	No
	L. d. donovani	Kala-azar	West Africa (Hoggar, Upper Volta, Republic of Congo, Zaire, Togo, Zambia)	Dog	++++	–	No
	L. d. infantum	Infantile kala-azar	Mediterranean area, Gambia, Kenya	Dog	++++	–	No
			Southern France, Italy	European fox			
			Italy, Yugoslavia, Iraq, Brazil	Rodent *(Rattus rattus only)*			
			Kzyl-Orda in Kazakahastan, Dzhalidabad in Azerbaizan	Dog, fox, jackel			
			East Asia (Himalayan valleys in northern Pakistan)	Canine			
			Northeast China	—			
	L. mexicana amazonensis		Brazil (Bahia State)	Rodent	++++	–	No
	L. tropica		Rare cases reported from Israel and India	—	+++	–	No
	L. major		Africa, Europe	Rodent	+++	–	No
Post-kala-azar dermal leishmaniasis (PKDL)	*L. d. donovani*		India, Bangladesh	Man	++	+/–	No
	L. d. spp.		Kenya, possibly Ethiopia, Somalia	—	++	+/–	No

Old World cutaneous leishmaniasis (single or limited number of skin lesions)	*L. major*	Oriental sore (west, rural)	Northwest India (Rajasthan) Libya, North Africa	Jird	+	–	Yes (rapid)
			Soviet Asia, northern Iran, northern Afghanistan	Great gerbil			
			North Africa (Algeria, Morocco) Libya, Dead Sea area of Israel and Jordan, southern Iran, Iraq Saudi Arabia, Kuwait, Yemen	Sand rat			
			Senegal, Sudan, Khartoum	Gerbil, Nile grass rat, multimammate rat			
			Rift valley of Kenya	Rodent			
	L. tropica	Oriental sore (dry, urban)	Northwest India, Afghanistan (Kabul)				
	L. aethiopica	Oriental sore	Ethiopian highlands, Kenya	Hyrax, rat	+	+++	Yes
	L. d. infantum		Occasional cases in Mediterranean basin	Fox, dog, rat, wolf, jackel	++	+	No
	L. d. archibaldi		Sudan, East Africa	Rodent	++	+	No
	L. tropica	Oriental sore (dry, urban) (ACL)	Mediterranean littoral, Middle East, West Asiatic area, Pakistan	Dog, cat	+	+++	Yes (slow)
	L. tropica	Ricidivan (nonhealing)	Northwest India, Afghanistan	Dog	++	+++	No
	L. donovani spp.		Kenya	Dog	++	+	No
Diffuse cutaneous leishmaniasis (DCL)	*L. aethiopica*		Ethiopian highlands, Mount Elganin, Kenya, Yemen, Namibia	Hyrax, rat	+++	–	No
	L. m. amazonensis		Amazon basin and neighboring areas	Rodent, fox	+++	–	No
	L. m. pifanoi		Venezuela	Spiny pocket mouse, spiny rat, cane mouse	++	–	No
	L. m. mexicana		Mexico, Central America (rare)	Rodent	++	–	No
	L. m. spp.		Dominican Republic	Hyrax	++	–	No
New World cutaneous leishmaniasis (single or limited number of skin lesions)	*L. m. mexicana*	Chiclero's ulcer	Mexico, Guatemala, Belize, Central America, Texas	Cricetid rodent, big-eared climbing rat, vesper rat, cotton rat, spiny pocket mouse	+	–	No
	L. m. amazonensis		Amazon basin and neighboring	Rodent,	+	+++	Yes

TABLE 1 (continued)
Leishmaniasis: Causative Agents, Disease Types, Distribution, Reservoir, and Some Immunological Parameters

Clinical syndrome	Causative *Leishmania* sp.	Common name of the leishmaniasis	Distribution	Animal reservoir	Antibody (enough for sero-diagnosis)[a]	Delayed-type hypersensitivity reaction (DTH) to leishmanin test[b]	Tendency to self-healing
			areas, Brazil, Panama, Venezuela	marsupial, fox spiny rat (in Panama)			
	L. m. pifanoi		Venezuela, Trinidad	Rodent, spiny pocket mouse, spiny rat, cane mouse	+	+++	Yes
	L. m. garhami		Venezuela	Rodent, opossum	+	++	
	L. m. venezuelensis		Venezuela		+	++	
	L. m. spp. (undetermined)		Dominican Republic		+	++	
	L. b. guyanesis	Pian bois (involvement of lymphatics), bush yaws	Amazonian Brazil, Guayana, Surinam, French Guayana	Sloth, edentates, anteater, opossum	+	+++	Yes
	L. b. peruviana	Uta	Dry scrubland valleys of Western Andes in Peru and Argentina, Santa Eulalia Valley	Dog	+	+++	Yes
	L. b. panamensis	Panamanian leishmaniasis	Central America, Panama, northwestern South America, Costa Rica	Sloth, night monkey, dog, Geoffroy's tamarin, olingo, coati, kinkajou	+ +	+++	Yes
	L. d. chagasi		Rare cases in Latin America	Dog, fox	++	+++	No
Mucocutaneous involvement (MCL) or Espundia	*L. b. braziliensis*	Espandia	Brazil, Peru, Ecuador, Bolivia, Paraguay, Argentina	Rodent, opossum	++	+++	No
	L. b. panamensis	Espundia	Panama and adjacent areas	Sloth, dog	++	+++	No
	L. b. guyanensis	Espundia	Panama, Brazil		++	+++	No
	L. t. aethiopica		Sudan		++	+++	No

Note: The taxonomy of *Leishmania* species is still in a state of flux. Three "species complexes" have been traditionally identified (*L. donovani, L. mexicana,* and *L. braziliensis,* each having multiple subspecies, as indicated above, but the various subspecies have been accepted recently as distinct species (e.g., *L. donovani chagasi* as *L. chagasi*).

[a] Antileishmanial antibody titers raised in patients are arbitrarily ranked: +, low; ++, low to moderate; +++, moderate; ++++, high.

[b] –, negative; +/–, development of positivity, in most cases with the chronicity of the disease. Lower (+), moderate (++), and higher (+++) degrees of reactivity.

are interpreted as a whole in the context of their significance as an alternative to parasite detection in the conclusive diagnosis of the disease. Some older literature is referenced where logical links seem necessary. Less emphasis is given to describing the details of individual assay methods. This review may assist researchers as well as medical personnel to assess their data and to improve their experimental design for better results in many ways.

Leishmaniasis comprises a spectrum of diseases that are widely distributed in tropical and subtropical countries, ranging in severity from self-healing skin lesions to severely mutilating mucocutaneous involvement or visceral infections (kala-azar) caused by protozoan hemoflagellate *Leishmania,* consisting of a group of species (Table 1). The parasite has a digenetic life cycle: the obligate, intracellular, rudimentary flagellated, nonmotile, small, and ovoid-shaped amastigotes multiply within the acidic environment of the endocytic phagolysosomal vacuole of some vertebrate endothelial cells, mainly macrophages and extracellular flagellated, motile, slender, and spindle-shaped promastigotes, which replicate in the hydrolytic environment of the digestive system of the invertebrate vector Phlebotomine insect (sandfly). About 20 million people worldwide are reported to be the victims of various forms of leishmaniasis, and about 400,000 new cases appear every year.[1,2]

The leishmanial diseases, except for cutaneous leishmaniasis (CL), have a lengthy incubation period, an insidious onset, and a chronic course. Kala-azar (KA) or visceral leishmaniasis (VL) is characterized by irregular fever, progressive enlargement of the spleen and liver, leukopenia with marked neutropenia, anemia, emaciation, and blackish discoloration of the skin. Post-kala-azar dermal leishmaniasis (PKDL) is generally found in 10 to 15% of Indian KA patients after a few years of cure. Mucocutaneous leishmaniasis (MCL) is characterized by metastasis to the oronasal or pharyngeal mucosa after a few years of primary skin lesion. These degenerative mucocutatenous lesions can be mistaken for leprosy. Uncomplicated CL is associated with high morbidity, a disfiguring lesion, and life-long scarring, posing a great need for early diagnosis and treatment. Chemotherapeutic treatment is unsatisfactory with the use of toxic drugs such as sodium stibogluconate, pentamidine, and amphotericin B. The situation is becoming still more alarming, with an increasing proportion of cases reported to be unresponsive to antimonials, the drug of choice.[3]

II. IMMUNOLOGICAL RESPONSES OF HOST

It was noted very early that leishmanial infections are accompanied by a dramatic humoral response against some forms of leishmaniasis (Tables 1 and 2). Based on this observation, methods of detection were devised within 2 decades of the discovery of the parasite. The immunology of leishmaniasis passed through a period of stagnation for a long time. The recent explosive growth of immunological knowledge and its practice in the diagnosis of leishmaniasis and related problems are the subject of this review. The most relevant immunological characteristics of leishmaniasis for serodiagnosis are as follows (Table 2).

Nonprotective hypergammaglobulinemia is associated with reversal of the albumin/globulin ratio, mostly in VL. This is not confined to leishmaniasis alone.[4–8] A marked increase in serum IgG and, to a lesser extent IgM, is the common feature in the majority of KA patient sera.[9] In the case of gammopathies such as multiple myeloma and macroglobulinemia, the serum IgG levels were found to be very high. The IgG does not rise to a very high level in tropical splenomegaly, where the IgM is elevated.[5] The presence of high levels of galactosyl α(1–3) galactose antibodies in VL patient sera as well as in American trypanosomiasis[8] and galactose α(1–3)-mannose antibodies in human sera infected with *L. mexicana* and *L. braziliensis* has been demonstrated.[6]

Except for some forms of the cutaneous type, leishmaniasis generally are not self-healing (Table 1). This is probably due to the lack of specific $CD8^+$ T helper cells (Type 1) responsible for the activation of macrophages mediated by (IFN-γ), the expansion of specific type-2 T helper

TABLE 2
Serodiagnostic Potentiality of Active and Cured Cases of Different Forms of Leishmaniasis

Significant antibody level	Significant cell-mediated delayed hypersensitivity reaction (DTH)	Insignificant in both antibody and DTH
VL	VL after cure	Diffuse CL
Multiple sore (by metastasis)	PKDL (not in all cases)	Early single-sore CL
CL during middle and late infection with *L. braziliensis* spp.	Old World CL at and after the commencement of resolution	Many cases of Old World uncomplicated CL, even when resolving
Any single-sore CL that involves the lymphatic draining the lesion and possibly the draining lymph nodes themselves	Leishmaniasis recidivans during infection	PKDL (in some cases)
PKDL < VL	Apparently quite early in the New World CL as caused by *L. braziliensis* or *L. mexicana* MCL	

cells that exacerbates the disease by deactivating macrophages with IL-4, IL-5, and IL-10,[10] and the lack of protective antibodies, particularly the IgG-2 subclass.[11]

Patients gain protective cell-mediated immunity against second-time infection in VL. However, there are exceptions in some KA cases in India, Sudan, and Bangladesh, where PKDL develops in patients who have relapsed from KA. It has been suggested that a certain population of VL-causing parasites escapes from the drug and undergoes change in its tropism to dermal macrophages. However, the biochemical nature of these changes is largely unknown. The simultaneous occurrence of KA and disseminated pustular and macronodular skin lesions were found in Iran.[12]

The parasite antigens consist of a repertoire of at least 30 somatic antigens and an unknown number of surface components, and the existence of both heterospecific antigens and specific parasite antigens have been established.[13,14] As a result, to date, the immunodiagnostic methods have been hampered by the problem of cross-reactivities of species within the family as well as within phylogenetically distant microorganisms such as *Mycobacteria*.[13,15–17] The problem is further complicated in geographical areas where different forms of leishmaniasis and trypanosomal infections occur simultaneously.[14]

III. TECHNIQUES OF DIAGNOSIS

Different techniques are used for the diagnosis of leishmaniasis (Table 3). The signs and symptoms of different forms of leishmaniasis are very confusing and often seem to be similar to other diseases (Table 4). Differential diagnosis of the disease is hampered by many factors that must be considered before treatment. Although conclusive diagnosis of leishmaniasis depends on detection of the parasite either by the staining of bone marrow or splenic aspirates from visceral cases and other forms of biopsy samples taken from the nodules of PKDL cases or infected tissues from cutaneous lesions or mucocutaneous involvement, the detection of amastigotes in stained samples or the detection of promastigotes by culturing the biopsy samples in a suitable medium (Table 5) also sometimes give negative results. The use of S-α-MEN with 5% heat-inactivated fetal bovine serum was found to be better for VL and PKDL cases in India than any other commonly used media[27] (Table 5) (author's unpublished observation). The culturing of biopsy samples is considered where the parasite is rare. These procedures require laboratory facilities and expertise, and are cumbersome, risky, and unsuitable for a large-scale epidemiological survey. Moreover, in developing countries where hospital facilities are limited, patients often do not have access to such

TABLE 3
Schematic of Diagnosis of Leishmaniasis in Man

Leishmaniasis

(1)	(2)	(3)	(4)	(5)	(6)
Detection of the parasite in biopsy materials by histological observation of amastigote form or by *in vitro* culture of the sample for detection of promastigotes	Immunodiagnosis: (1) detection of antibodies against *Leishmania*, (2) detection of parasite antigens, and (3) detection of cell-mediated immunity	Detection of parasite DNA, particularly Kinetoplast DNA (KDNA) (Use of PCR techniques)	Differential diagnosis: necessary in in conjucation with other tests for the exclusion of other diseases (Table 4)	Clinical sign and symptoms for the primary assessment of the disease (i.e., irregular fever indicates VL; (see Table 4)	Geographical origin of the patient (previous exposure to endemic area)

TABLE 4
Differential Diagnosis: Considering the Diseases Commonly Appearing as Different Forms of Leishmaniasis on the Basis of Their Apparent Sign and Symptoms

Sign and symptoms	Disease type	Resemblance	Diagnostic technique for exclusion of suspected disease from leishmaniasis
Irregular fever and splenomegaly	Malaria	VL	Examination of peripheral blood smear for *Plasmodium*
	Typhoid fever	VL	Blood culture of *Salmonella*, Widal test, low pulse-temperature ratio, characteristic toxemia, and rose spots
	Schistosomiasis with associated prolonged *Salmonella* sepsis	VL	Stool examination for schistosome eggs
	Leukemia	VL	Bone marrow examination
	Lymphoma	VL	Lymph node biopsy
	Bacterial endocarditis	VL	Blood culture
	Tuberculosis	VL	X-ray, culture of sputum, or biopsy samples
	Histoplasmosis	VL	Histological studies of appropriate biopsies, culture
	Sarcoidosis	VL	
	Brucellosis	VL	
	Cirrhosis of liver	VL	
Skin lesions	Deep mycoses	CL[a]/DCL	Histological examination, culture, detection of paracoccidioidomycosis, histoplasmosis, sporotrichosis (lymphatic spread)
	Leprosy	DCL	Palpable thickened nerves, demonstration of anesthesia, demonstration of *Mycobacterium* Laprae in skin, nasal mucous membrane, or nerve biopsy
	Skin tuberculosis *(lupus vulgaris)*	DCL (mimics tuberculoid relapsing leishmaniasis)	Culture
Varicose ulcer on anterior tibial surface	Insect bite	CL/DCL	History
Tropical ulcer	*Borelia vincent, Bacillus fusiformis*	CL	
Chronic extending ulcer with rolled edge	Basal cell carcinoma	CL	Histological examination

Mucosal involvement	Mucosal cases of paracoccioidomycosis	MCL	Nodular shadows on chest X-ray, histological detection of fungus
	Squamous cell carcinoma	MCL	Histological diagnosis
Mucosal involvement: saddle nose	Congenital syphillis and leprosy	MCL	Rarely seen in MCL
Mucosal involvement in nose	Rinoskleroma	MCL	Histological stain by pappenheim's method for detection of foam cells
Tropical ulcer skin lesions	Cutaneous diphtheria	CL/MCL	Histological examination, gram stain for *C. diphtheriae,* culture in doffler's or tellurite medium
	Furuncular myiasis	CL/PKDL (early stage in some cases)	Histological examination, Appearance of the spiracles in lesions

[a] CL characteristics: indolent skin lesion of more than a month's duration with many nodular lesions, especially with crusted tops or ulcers with raised margins.

TABLE 5
Media for Isolation of *Leishmania* Promastigotes

Medium			
Name	**Type**	**Composition**	**Ref.**
NNN	Biphasic	Difco blood agar base, 15% defibrinated rabbit blood, 0.5 ml Locke's sol used as overlay	18–20
Senekje's medium	Biphasic	Bacto-beef (Difco), 5.0%; Neopeptone (Difco), 2.0%; Bacto-agar (Difco), 2.0%; NaCl, 0.5%; defibrinated rabbit blood, 10% (v/v); Locke's solution used as overlay	21
USAMRU medium	Biphasic	Bacto blood agar (Difco), 4.0%; defibrinated rabbit blood, 15% (v/v)	22
Evans modified Tobie's medium	Biphasic	Beef extract, 0.3%; bacteriological peptone, 0.5%; NaCl, 0.8%; agar, 2.0%; defibrinated horse blood or rabbit blood, 15%; proline-containing balanced salt solution (PBSS) used as overlay	23
MS medium McConnell's modification of Senekje medium)	Biphasic	Oxoid nutrient agar, 15% defibrinated rabbit blood, 0.9% saline as overlay	24
Blood-free medium	Biphasic	Beef extract (Oxoid Lab hemco L29), 0.3%; bacteriological peptone (Oxoid L37), 0.5%; NaCl, 0.8%; agar (Oxid L28), 3.0%; fetal calf serum (1:8 v/v) used as overlay	25
	Semi-solid medium	Brain heart infusion, 3.1%; bactoagar, 0.5%; rabbit blood, 5% (v/v)	26
Schneider *Drosophila* medium	Liquid medium	Schneider *Drosophila* medium and 30% (v/v) heat-inactivated fetal bovine serum	20, 24
S-α-MEM	Liquid media	MEM (α-modification) supplemented with hemin, HEPES, folic acid, D-biotin, and D-glucose	27

facilities. In many VL patients there is an increased risk of bleeding, which further complicates the severity of the clinical symptoms. Repeated culturing of stained tissue smears of biopsy samples may be clinically unacceptable.[28,29] Splenic aspiration is more efficient (98% positivity) than bone-marrow (54 to 86%) or enlarged lymph node (64%) aspirates for the detection of amastigotes in stained tissue smears or promastigotes in culture.[1,30] The rate of success is limited for CL, DCL, and MCL,[31] and has not yet been determined for PKDL. Examination of peripheral blood for parasites is not a viable diagnostic procedure, although amastigotes have been reported in direct blood smears in India and East Africa.[32,33] *Leishmania* circulate in the bloodstream inside large mononuclear cells and polymorphonuclear leukocytes, which can be detected in the earlier stages before any clinical signs appear.[32] However, parasitemia was demonstrated by various methods in 15 of 20 VL patients in Kenya.[33] Alternative, serodiagnostic tests have been developed to screen VL since 1922, when Napier[34] developed the very simple and convenient formal gel test, which is still carried out as a preliminary test in many hospitals. The test is based on the presence of a high amount of immunoglobulins, and so diagnostic techniques are largely employed for antibody detection in patients. The techniques used for the immunodiagnosis of leishmaniasis can be grouped into three categories: (1) antibody detection, (2) antigen detection in circulating immune complexes, and (3) detection of cellular immune responses (Tables 2 and 3). Use of the polymerase chain reaction (PCR) for the detection of kinetoplast DNA (kDNA) of *Leishmania* in active leishmaniasis cases is in progress.[35,36] Details of this technique are beyond the scope of this review.

IV. NONIMMUNOLOGICAL TEST

The earliest serological tests — the aldehyde (formol gel) test of Napier,[34] and Brahmachari,[37] or Sia's[38] precipitation ring test with distilled water, and the antimony test of Chopra[39] — were nonspecific and nonimmunological, but based on chemical techniques, including opalescene or gel formation in positive sera.

A. Formol Gel or Aldehyde Test

This is a nonspecific test but very simple to perform and convenient for field work in leishmaniasis case detection. This test is particularly useful in VL. One drop of 30% formaldehyde is added to serum collected from about 5 ml of venous blood and shaken. A positive result is indicated when the mixture changes to a solid, opaque white, like that of a boiled egg, within 20 min. A lesser degree of opacity indicates the earlier stages of the disease. A positive test does not develop during the first 3 months of infection and after 6 months of cure. It is related to the level of IgG, and positive results can be obtained in other cases where the immunoglobulin levels are raised, whether IgG or IgM, as in African trypanosomiasis, malaria,[40] tropical splenomegaly, hepatosplenic schistosomiasis, macroglobulinemia, multiple myeloma, and leprosy.[16,41–45] The test helps in the primary assessment of VL cases but is not conclusive.

B. Chopra's Antimony Test

This test was developed by Chopra et al. in 1927[39] and is used in India for the diagnosis of VL cases. The principle is the same as the previous test, but an antimony compound such as stiburea is used. Two drops of blood are drawn into a Dreyer's tube containing 0.25 ml of a 2% solution of potassium acetate. A mixed solution of antimony compound (4.0% w/v) is added to the mixture by a capillary pipette and allowed to percolate just below the blood mixture. The formation of a flocculant precipitate at the junction after 10 to 15 min reflects a positive result. The character of the precipitate is very important; it is not easily broken up and disappears with 24 h. Alcohol-free test tubes must be used.

V. DETECTION OF ANTIBODIES

A. Complement Fixation Test (CFT)

The Complement fixation test is the first true immunodiagnostic test. In earlier works, an acetone extract of washed promastigotes was used as an antigen, which gave a positive reaction in VL and MCL as well as Chagas' disease.[46–49] However, similar reactions were obtained with antigens prepared from other microorganisms, including *Mycobacterium pheli,* Kedrowsky's bacillus, and BCG.[50,51] *Leishmania* has the common cross-reacting antigens shared by *Mycobacteria.*[52] Sonicated amastigotes of leishmaniae (usually *L. donovani*), when used as antigens, have proved satisfactory, but a polysaccharide fraction of promastigotes gave negative results.[53] South American workers pioneered the use of this test for the detection of KA. Gravel et al.[54] used CFT for the diagnosis of KA with Witebsky, Klingenstein, and Kuhn (WKK) antigen, which is an extract of the human tubercle bacillus in benzol and other solvents. Ghosh et al.[55] detected an antibody to KA in 192 out of 194 confirmed cases using promastigote antigens. Titers of 1:10 or higher are considered as significant, and titers of 1:40 develop in early infection, falling rapidly after cure and disappearing within 6 months, but persistent titers have been considered in seroepidemiological surveys to detect cryptic or past infection.[56] The test gave a cross-reaction with other parasitic *(Trypanosoma cruzi)* and mycotic infections and certain sera with anticomplementary activity.[14] Complement-fixing antibodies were used in a seroepidemiological survey for KA.[57,58] CFT has not been studied in PKDL cases and could not be found in *L. tropica* or *L. major* infection, but complement-fixing antibodies are found in a mucocutaneous infection caused by *L. braziliensis.*[5] The test is considered very useful in early diagnosis and in monitoring the effect of treatment.

B. Indirect Hemagglutination Test (IHA)

The IHA test is a specific serologic test that was introduced by Cascio and co-workers[57] and well studied by Bray and Lainson,[59] who detected

a titer up to 1:10240 in four out of four KA sera. Mukherjee et al.[60] detected antibodies in all six patients studied. Using the same technique, Manson-Bahr[61] found antibodies in the sera of 8 out of 11 patients with active KA. The famous Noguchi-Adler test, which has been used for differentiating *Leishmania* species,[62] is based on agglutination of the parasite in homologous hyperimmune rabbit serum.[63] IHA tests have been used for PKDL cases.[64] However, of 12 chronic PKDL cases, only four were found to be serologically positive and all eight fresh cases tested were seropositive, indicating that the antibody titer reduced during the chronicity of the disease.[64] The antigen is usually prepared from promastigotes grown in culture. Cross-reaction can occur with sera from malaria patients. A titer of 1:200 or higher is considered significant.[61,65] Antibodies against excreted factors (EF) of *Leishmania* were detected in patient sera by using the test.[66] Serum from patients with VL may cross-react with *T. cruzi* and *Mycobacteria* in this test.[67–69]

C. Direct Agglutination Test (DAT)

Allian and Kagan[70] first described the DAT for the diagnosis of leishmaniasis. The test has been modified by Harith et al.[44] for the serodiagnosis of VL, and is simple, economical, and at least as sensitive and specific as the enzyme-linked immunosorbent assay (ELISA)[71] and indirect fluorescent antibody test (IFAT).[72,73] The DAT can effectively distinguish between active VL and African trypansomiasis, something which neither the ELISA nor IFAT can do when an antigen complex is used.[44] The DAT and ELISA gave positivities of 67 and 60%, respectively, with sera of CL using *L. donovani* as an antigen.[71] A wide range of titers have been reported in cured patients up to a period of 2 to 3 years.[44] The test is unable to distinguish between early infection and after cure. In one study of 41 parasitologically confirmed Indian VL cases, the DAT gave a titer of 1:3200; the control groups, including 13 normal human sera, 6 lepromatous leprosy, 6 pulmonary tuberculosis, 5 cirrhosis of liver and thalassemia, 4 malaria, and 4 typhoid patients sera, had a titer of 1:1600.[74] Four PKDL cases of the macular type (i.e., with white patches), which may be confused with leprosy, were DAT negative. However, ELISA could discriminate PKDL from leprosy cases.[74] In Bangladesh, PKDL sera gave a high DAT titer (62%, 1:5200), which is indistinguishable from confirmed VL and PKDL.[75] The strains used in the DAT may differ in the make-up of surface membrane antigens, as the differential expression of gp 63, a major surface glycoprotein,[76] acid phosphatase,[77,78] lipophosphoglycan, and glycolipid[79] for virulent and avirulent cases are well documented. Thus, standardization of DAT is essential for differentiating among PKDL, VL, and other cross-reacting sera. Endemic controls with the use of different strains of *Leishmania* provide better results, and the differential surface membrane biochemistry of the parasite is needed for wider use of the test. The use of a cloned strain of MHOM/IN/78/UR6 that lacks the detectable lipophosphoglysaccharide gave a significantly lower titer for tuberculosis than the parent stock of UR6 (author's unpublished observation). The DAT was used successfully in the treatment of susceptible VL where the parasite could not be detected. A positive agglutination test can distinguish PKDL from DCL caused by *L. aethiopica*.[80] About 50% of 20 patients with localized CL had titers above those of normal control subjects in the Ethiopean highlands, whereas VL and DCL had a significant antibody titer against *L. major, L. donovani,* and *L. aethiopica* but not *L. tropica* antigen preparations.[81] DAT showed a significant titer in VL (100 to 67%) patients after treatment, even up to 8 years of recovery, vs. on the ELISA of 50 to 11%.[82] Hence, ELISA is recommended for clinical prognosis and patient follow-up.[82] In a seroepidemiological survey of KA in Sudan, Zijlstra et al.[83] found 8% negative results out of 67 tissue smear-positive cases.

D. Indirect Fluorescent Antibody Test (IFAT)

The IFAT is the most sensitive test and is group specific.[43] In this method, washed formalin or acetone-fixed promastigotes are used as antigen[41,84] for detecting circulating antibodies in

KA.[41,85] Hedge et al.[86] used *Crithidia* sp. as antigen in the serodiagnosis of KA by the immunofluorescence method. Similarly, Lopez-Brea,[87] using the same species as antigen, diagnosed KA in a child by IFAT a month before *L. donovani* was detected in the direct smear from the spleen. The production of humoral antibodies was demonstrated in humans infected with *L. braziliensis* (New World leishmaniasis) by IFAT using amastigotes liberated from monkey kidney cell culture as the substrate smear.[88] Frozen sections from infected tissues also can serve as the substrate.[85,89,90] When promastigotes are used as the antigen, serologic cross-reactivity between related and unrelated species occurs, which is generally noted with *T. cruzi,* which causes African trypanosomiasis and some cases of pulmonary tuberculosis.[67–69,91–93] The cross-reactivity can be eliminated with the use of amastigotes as the antigenic substrate,[88,94] indicating that promastigote surface antigens are more nonspecifically cross-reactive than amastigotes in the surface membrane. The IFAT satisfied the required specificity and sensitivity, but it is not readily adaptable to large-scale seroepidemiological studies due to limitations in the availability of amastigotes and fluorescent microscopes. Recently, axenic amastigote-like forms of the parasite have been cultivated[94–97] that can be evaluated for their efficacy as potential antigen sources for the serodiagnosis of leishmaniasis. The antigenic pattern of the amastigote differs from that of the promastigote.[97] The major promastigote antigens of *L. m. pifanoi* are 23-, 52-, and 68-kDa proteins, whereas 38-, 70-, and 74-kDa proteins are prominent in the amastigotes culture form or isolated amastigotes from macrophage culture.[95]

The efficiency of ELISA and IFAT was studied for the seroepidemiology of KA.[35,98,99] Certain malarial sera reacted positively in IFAT with *L. braziliensis,*[100] but the cross-reactivity was not found in ELISA.[101,102] An IFAT titer of 1:20 or greater is significant and 1:128 is diagnostic of active infection. Titers of 1:128 or higher were found in 90% of children with KA in Iran.[103] In the diagnosis of mucocutaneous cases, *L. major*-like and *L. b. braziliensis* promastigotes were used as antigens; both strains were equally responsive (77.2%), and cross-reactivity was restricted to Chagas' disease sera, VL, antinuclear factor, and paracoccidioidomycosis.[104] *L. major*-like antigens were better than *L. b. braziliensis* in disclosing cross-reacting antibodies in Chagas' disease.[104] IgA-IFAT was found to be highly specific (100%), but its sensitivity was very low.[99]

In VL there is a high titer (1:640 or 1:1280) of specific antileishmanial IgG against promastigotes. In cases that develop PKDL, the titers were much lower (in 40), whereas in recovered VL without PKDL, they remained comparatively high.[105]

IFAT suffers from certain inherent disadvantages with the use of fluorescent isothiocyanate (FITC), which can be removed by the use of aminomethyl coumarin acetic acid, a new fluorescent label for protein, yielding better results regarding nonspecific background staining and stability, with slower photodecomposition under the ultraviolet light used for the detection of leishmanial antibodies.[106] The new labeling agent emits a yellow/green color with an excitation filter of 450 to 490 nm and a barrier filter of 520 nm.

IFAT has generally been unproductive in CL due to the low level of circulating antibodies or the weak antigenic potential of the parasite.[107,108] Significant antibody levels were present in patients with large or multiple lesions caused by *L. major* in Saudi Arabia.[109] Varying antibody levels up to 1:1000 were obtained by using freeze-dried antigen prepared from *L. donovani* and *L. major* in a proportion of active CL cases in Beirut[92] and in children in Iraq.[110] Although a control group of Lebanese gave negative results, 25% of Saudi Arabians showed significant titers (1:10 to 1:500), but 60% had a strongly positive leishmanin tests from previous exposure to leishmaniasis. These results should be considered with caution, as 5 of 15 cases of tuberculosis gave a significant titer. Circulating immunofluorescent antibodies were not detectable in cases of leishmania recidiva, where the parasite can seldom be found on direct examination of biopsies or in cultures of biopsied materials. However, the leishmanin reaction is always present.[92] Fluorescent antibodies were absent in all four cases of DCL tested by Shaw and Lainson.[111] IFAT is useful in MCL. Titers range from 1:16 to 1:1024 in 89% of cases, and the test is especially useful

in determining the presence of latent infection in the quiescent period and as a test of cure.[88]

E. Gel Diffusion Test (GDT)

Immunodiagnosis of leishmaniasis has been widely made by using GDT, and the test is simple. It was successfully used for studying VL in human, canine, and hamster sera[112] and employed in seeking canine reservoirs of VL in North Africa.[113] Freeze-dried antigen was used to detect antibodies by this test.[114] Fraction 5 of the soluble supernatant of *L. donovani* promastigote homogenate obtained by centrifugation at 105,000 × g gave 100% positive reactions for seven confirmed KA cases.[115] Homologous and heterologous antigens were used in GDT in the diagnosis of human and canine leishmaniasis.[103,112,116] The antigen complex and crude parasite extract can only be used in this test for areas where no cross-reacting infections occur. A cell-free extract of freshly isolated *Leishmania* from the bone marrow of VL patients, cultured in NNN medium for 5 to 6 d, gave 28% negative results for 50 parasitologically confirmed KA cases in Sudan, but also gave 6% false positive results with normal human sera from an endemic focus.[117] Such a large number of negative results may be due to the use of diluted antigens or the presence of a low titer of antileishmanial antibodies in such patients. However, some antigen antibody reactions were reported to be specific to VL.[118] Exometabolites of *L. donovani* can be used for the detection of VL in patients.[119]

F. Counter-Current Immunoelectrophoresis (CCIE)

CCIE is more sensitive than GDT, and the time required to perform the test is less (15 to 60 min). An antigen concentration 100 to 300 μg/ml is sufficient, and the test gives more detail about the antigens.[103,120] Mansueto et al.[121] used CCIE for epidemiological studies on leishmaniasis. They found positive CCIE results in 15 cases having positive ELISA results. The test is used in India, with a result of 81.8% positive in KA of less than 30 d duration and 100% positive in 30 to 90 d duration of infection.[5] It compares favorably with the CFT, which is significantly less sensitive. A few precipitation bands were found in other febrile conditions, and a relatively high positive rate was observed in children in endemic areas who had no sign of KA, suggesting a previous subclinical infection.[122] The test appears to be more suitable than other serological tests for the detection of cryptic infection in field surveys in association with the leishmanin skin test to measure previous exposure to the parasite. In CCIE, the number of precipitation bands observed varied from one to four in 36 VL cases[117] and from two to nine and two to seven in seven Indian KA cases against crude homogenate and F_5 (as described previously) of *L. donovani* as antigens, respectively.[115] However, CCIE gave 28% negative results in parasitologically confirmed VL in Sudan and 4.6 and 1.3% false positive results with 150 normal sera from an endemic zone and 300 normal blood donors, respectively.[117] The test gave 100% positive results with parasitologically confirmed KA patients, while aldehyde, complement fixation, and IHA tests were 80, 80, and 73% positive, respectively.[122] This discrepancy may be attributed to the variation of different strains of *Leishmania* or the same strain of a different subpassage and different phase (i.e., log phase or stationary phase, including metacyclic promastigotes of a different antigenic make up). CCIE showed positive results in 50% of the population of an endemic zone, indicating exposure to infection without any apparent clinical manifestation.[122] Exometabolites of *L. donovani* (UR6 strain) gave two precipitation bands against KA patient sera that could not be recognized by leprosy, tuberculosis, and malaria patient sera.[119]

G. Immunoelectrophoresis (IEP)

A ConA-positive glycoprotein fraction of exometabolites of *L. donovani* (UR6 strain) gave two precipitation bands with Indian KA sera and did not cross-react with a control group containing tuberculosis, leprosy, and malaria patients sera and normal human sera by IEP.[119] Antibody against the major excreted glycoconjugate lipopho-

sphopolysaccharide (LPPS) of *L. donovani* could not be detected by IEP.[119] Proteins and glycoproteins present in the organic phase of the phenol extraction of exometabolites can be used for the serodiagnosis of VL.

H. Enzyme-Linked Immunosorbent Assay (ELISA)

This test is a precise, sensitive as radioimmunoassay and convenient for the screening of large number of leishmaniasis. Hommel[123] introduced ELISA for the serodiagnosis of leishmaniasis. The sensitivity of ELISA approaches that of IFAT.[64,101] At low serum dilution, positive reactions with sera from co-endemic diseases such as Chagas' disease, malaria, leprosy, and mucocutaneous and cutaneous leishmaniasis have been observed with *L. donovani* membrane antigens. Two purified parasite proteins (i.e., 70 kDa and dp72 kDa) of *L. donovani* promastigotes were found to be specific against VL sera, with 7% misdiagnosed cases belonging to New World CL and Chagas' diseases, and leprosy only with the dp72 antigen.[45,124] When sonicated promastigotes of *L. braziliensis panamensis* were used as antigens in ELISA, antibody was detected in 16 of 21 confirmed cases of New World leishmaniasis and in 39 of 49 cases of Chagas' disease.[93] Using the soluble fraction of sonicated *L. donovani* promastigotes, antibodies were detected in CL and cross-reactions were found with other diseases such as malaria, typhoid, larva migrans, dysentery, and liver diseases.[125] In ELISA, *L. t. major* antigen could recognize CL as well as VL and MCL and to some extent cross-reacted with sera from patients with lepromatous leprosy, tuberculosis, or African trypanosomiasis.[102] In a comparative study, Rassam and Al-Mudhaffar[126] demonstrated the superiority of the micro ELISA when compared with GDT, IEP, and CCIE for the serodiagnosis of VL. Specific antibodies were detected by micro ELISA in PKDL cases, but the titers were definitely lower than those found in VL.[64] Chronic PKDL cases gave 83% positive results.[64] Parasitological and ELISA results correlated in 72% of CL patients who were diagnosed clinically or parasitologically.[127]

Use of the ELISA with intact promastigote (Sudan 1-S strain of *L. donovani*) as antigen to coat the microtiter plates was more sensitive and more specific than either IFAT or DAT for VL and MCL.[128] However, the use of intact promastigotes as antigen for ELISA gave cross-reactions with schistosomiasis and African trypanosomiasis patient sera and to some extent with leprosy and tuberculosis patient sera at a serum dilution of 1:3200, for which about 25% of 25 VL cases were found to be borderline.[129] Thus, any ELISA with serial dilution of tested serum should be retested for confirmation.

Wang et al.[130] developed an ELISA kit consisting of PVC film prepared by coating antigens from cultured promastigotes in RPMI-1640, an enzyme conjugate of anti-human γ-chain monoclonal antibody, and found a 100% positive reaction rate in 37 cases of VL and no false positives from normal, schistosomiasis, malaria, cysticercosis, trichinosis, and donorchiasis patients, but a 10% positive reaction with leprosy. However, the authors did not test tuberculosis, one of the major cross-reacting diseases.

Use of protein A or protein G is more sensitive (fourfold), versatile, and specific than anti-Ig in ELISA.[131] Recombinant gp63 (a metaloprotease, rgp63), the major surface antigen of *Leishmania*, was used successfully for the diagnosis of VL.[131]

The ELISA for IgG and IgM yielded indices of diagnostic utility for CL with *L. major*-like parasite antigen instead of *L. b. braziliensis* or *L. b. guyanensis*, which are prevalent in the study area of northern and northeastern Brazil.[99]

I. Dot ELISA

This is a modified and simplified form of ELISA, where antigen-coated nitrocellulose paper is used in place of antigen immobilization to polystyrene or a polyvinylmicrotiter plate,[132] and the insoluble color development on the nitrocellulose paper is easily read visually. The test has been gradually adapted to the diagnosis of leishmaniasis[124,133,134] and can be successfully used in the field for epidemiological surveys of leishmaniasis.[134] Dot ELISA and ELISA have a good correlation, and dot ELISA does not require

expensive, sophisticated equipment and materials such as an ELISA reader, ELISA plates, etc. Pappas et al.[133] observed prozone phenomena at lower dilutions of sera, which they attributed to hypergammaglobulinemia or the presence of immune complexes. Dot ELISA and ELISA could not diagnose 2% of parasitically positive VL cases with the use of 1.5% formalin-sensitized whole *L. donovani* promastigotes.[134] However, there are no studies for other cross-reacting diseases such as tuberculosis, leprosy, etc. The test has been simplified and modified further by the use of protein A colloidal gold conjugate instead of antiimmunoglobulin-enzyme conjugate and the substrate for the enzyme, and hence can be used successfully in the field survey.[135]

J. Direct ELISA

This test, developed for the diagnosis of KA, is simple, specific, and quantitative.[136] The methodology is as follows. An intact formalinized (1% v/v) *L. donovani* promastigote suspension in saline is combined with the antibodies of the patient sera in small test tubes or microfuge tubes instead of microtiter wells. Unreacted antibodies are removed by centrifugation. Then, anti-human IgG-alkaline phosphatase conjugate (0.1 ml of 1:1000 dilution per tube) is added, vortexed, incubated at 37°C for 1 h, and washed as described above. To each tube, 0.1 ml of the substrate is added, vortexed, and kept at 37°C for 30 min. Then the colored supernatant is transferred to separate tube(s) after centrifugation for the measurement of optical density (OD), read at 405 nm in a spectrophotometer. Considering the mean plus three standard deviations as the lowest limit of positivity, 14 of 16 parasitologically confirmed KA cases were positive (87.5%). The results of PKDL cases were not significantly different from KA cases, although the results of the PKDL cases are lower than KA. No significant differences were observed between PKDL and VL. A higher dilution of test serum (1:3,200) is preferred. The use of intact promastigote to detect antileishmanial antibodies is better than the use of soluble antigens because soluble antigen preparations become negative at lower dilutions of sera, which is attributed to the presence of fewer antigens eliciting antibody responses.[129] The detection of antibodies in PKDL cases was contradicted by direct ELISA and DAT, using the same strain of *L. donovani*. The difference is that trypsinized promastigotes were used in the DAT.[136] In the latter case, loss of some surface antigens is possible, which seems to be more specific for the detection of PKDL cases.

K. Detection of Antiparasite Enzyme Antibodies[137]

Leishmania parasites are equipped with acid phosphatase in their surface membrane. Antibody against this acid phosphatase raised in humans during leishmanial infection can be isolated by binding along with other IgG to Protein G Sepharose 4B, followed by incubation with *Leishmania* membrane extract, and then acid-phosphatase-enzyme activity is assessed by the addition of the substrate 4-nitrophenyl phosphate and the color product 4-nitrophenol quantitated by measuring the OD at 405 nm. Seventeen of 18 KA sera gave positive results. A cross-reaction was found in leprosy patient sera, and hence its use is limited, or it can be used in conjunction with differential diagnosis for the exclusion of leprosy cases with the aid of other tests. Antibody against leishmanial acid phosphatase could not be detected in amoebiasis, toxoplasmosis, and malaria patient sera. In this study, leishmanial acid phosphatase appeared as a common cross-reacting antigen between *Leishmania* and *M. leprae*.

L. Radioimmunoassay (RIA)

In recent years, solid-phase RIA has been used to detect antibodies in leishmaniasis.[138–141] The test gave results similar to those from ELISA. RIA is sensitive at the level of 2 µl of serum and 1 to 2 µg of antigen.[140] El-On et al.[138] used a poly-L-lysine-coated microtiter plate for better binding of the excreted factor (EF) of *L. donovani*. They found that 1 to 2 µl of antiserum is sufficient for the test and 0.06 to 0.12 µg/ml of anti IgG or 2.5 µg/ml of EF could be detected. However, the authors found an insignificant titer in leishmaniasis patients. A curious finding was that KA patient

sera recognized the EFs of both *L. tropica* and *L. donovani*, but antibody against EF could not be detected in the sera of patients with cutaneous infection. Lipid antigens extracted from promastigotes with hexane:isopropanol (3:2) were found to be considerably species specific in RIA. Lipids from *L. donovani* were practically recognized by sera raised against *L. t. major* in rabbits and humans.[139] Slutzky et al.[139] also isolated an LPS-like antigen of *L. t. major* other than EF and found 18 of 20 confirmed cases of CL positive. However, they did not extend their test for common cross-reacting diseases. Using freeze-thawed sonicated (FTS) *L. major* (LRC-L 137) promastigotes and a carbohydrate-lipid-containing fraction (CLF) obtained by extraction with hexane-isopropanol, Rosen et al.[140] developed solid-phase RIA, and found that FTS cross-reacted with the sera of CL and VL patients, and that CLF showed strain specificity. Sera from leprosy patients did not cross-react with FTS, but four of eight from leprosy patients were positive for CLF, which limits its use for the serodiagnosis of leishmaniasis patients. About 12% of CL patients could not be recognized by CLF. Glycolipid antigens of CLF of *L. major* are different from excretory factor (EF). These antigens (100 µl of 30 to 60 mg/ml) were dissolved in ethanol, mixed with phosphatidyl choline (PC) (200 µg/mg lipid), and absorbed onto 96-well microtiter plates for RIA.

M. Western Blotting/Immunoblotting

This test is highly sensitive and specific and gives greater detail about the antigen profiles of the parasite. Generally, radioiodinated protein A or antihuman IgG is used for visualization of the antigens in the autoradiogram. The use of protein A-enzyme conjugate or anti-human IgG-enzyme conjugate largely substitutes the radioactive compounds and simplifies the procedure. *T. cruzi*-infected patient sera recognized a band of 38 kDa in *Leishmania* lysates, and sera from patients with *Leishmania* infections recognized a band of 66 kDa in *Leishmania* lysates that did not cross-react with *T. cruzi* antigens.[142] A 32- to 35-kDa antigen of *L. d. chagasi* was found to be specific for sera from persons with *L. d. chagasi* infections and did not cross-react with sera from persons with *L. m. amazonensis* infection.[16] Antigens of 62 to 68 kDa were recognized by all individuals with *L. d. chagasi* and *L. m. amazonensis*, indicating that it is not species specific, but did not cross-react with sera from *T. cruzi*-infected persons. Sera of VL recognized a major antigen of 65 to 66 kDa of *L. donovani* amastigotes.[143]

Serum antibodies from localized cutaneous leishmaniasis (LCL) patients recognized a limited number of 50-kDa somatic antigens of *L. aethiopica*, which were recognized by antibodies present in DCL patients.[144] The lack of antibody in LCL patients to LCL and DCL patient-derived promastigote antigens was evidenced by an immunoblot assay.[144] The American CL patient sera reacted with a 60-kDa protein of *L. b. braziliensis* promastigotes, whereas American VL, schistosomiasis, malaria, and Chagas' disease sera were negative.[145] Anti-ribonucleoprotein antibodies were present in many VL patients, and binding of these to ribonucleoproteins was inhibited by prior incubation of VL serum with either leishmanial membrane antigens from four different species of *Leishmania* or intact cells of *L. donovani*, implying molecular resemblance between common leishmanial antigens and ribonuclear antigens.[146] Greater reactivity of serum antibodies from patients with active *Leishmania* infections was found against the 38-kDa antigenic determinants expressed by promastigotes cultured at 37°C than was obtained for the 25°C-cultured organism.[147] Several components, particularly at 5 and 50 kDa, were recognized by most of the CL patients in Western blotting.[148]

N. Application of Monoclonal Antibodies

Single specific monoclonal antibodies make it possible to choose which antigen or antigens are promising for the detection of antibody for serodiagnosis. Similarly, monoclonals can be used for the detection of antigen present in the sera of active leishmaniasis cases. Western blot, competitive ELISA, and competitive RIA are generally used to detect anti-leishmanial circulating antibodies with the use of monoclonal antibody. Western blot, RIA, and ELISA can be used to detect the reaction of monoclonal antibodies against leishmanial antigens. Monoclonal anti-

bodies have been shown to differentiate between species and subspecies of leishmanias from South America[149,150] among *L. donovani* strains[151] and between life-cycle stages of *L. tropica*[152] by immunoprecipitation of radiolabeled antigens. Some monoclonal antibodies, raised against one *Leishmania* species, also bind to species showing a geographically distinct distribution.[151,153] Moreover, many cross-reactive antigens are cell-surface structures.[149,153] A monoclonal antibody has been used to detect *L. tropica* from Sudanese mucosal leishmaniasis.[154]

Jaffe et al.[155] developed 16 monoclonal antibodies (D-1 to D-16) against a *L. donovani* membrane affected by preincubation with KA sera but unaffected by preincubation with normal human sera. None of the species-specific proteins identified by these monoclonal antibodies is recognized by the X-1 or X-3 monoclonal antibodies specific for common cross-reactive determinants.[156–158] A 65-kDa glycoprotein is the major cross-reactive antigen immunoprecipitated from *L. donovani* and *L. b. braziliensis* promastigotes by KA sera as well as by the sera of patients with CL,[157] and this glycoprotein is the major promastigote surface antigen of different stocks of *Leishmania* spp.[157–160] Presently, this glycoprotein is termed gp63, a protease that may be present as monomeric and oligomeric forms in *L. infantum* as 65-kDa glycoproteins.[161] A number of reports indicated that the antigens in the 65-kDa range reacted with sera from patients with VL.[16,157,159,162]

Using D-13 monoclonal antibody, White and McMahon-Pratt[163] isolated an 80-kDa membrane protein from *L. donovani* that was unaffected by periodic acid and tunicamycin treatment. D-13 and two other (D-2 and D-11) monoclonal antibodies have been noted for their positive serodiagnostic importance, using a sensitive and highly specific competitive ELISA.[145] White and McMahon-Pratt[163] purified p80 from solubilized *L. donovani* membrane by acid elution of a D-13 affinity column and ion-exchange chromatography, while using a D-13 affinity resin and the same *L. donovani* strain. Jaffe and Zalis[164] purified another protein, dp72, by alkaline elution. However, both groups of workers indicated that the epitope identified by the D-13 monoclonal antibody is species specific and has a serodiagnostic value. White and Hanham[165] found that D-2 is more species specific than D-13. Monoclonal antibody V1, specific for *L. (L). venezuelensis*, did not cross-react with *L. (L). major*, which recognized a high molecular mass component of 130 to 198 kDa.[166] Monoclonal T1 was found to be *L. major* specific.[156]

O. Genetic Engineering

Expression cloning has been used to isolate complementary DNA (cDNA) or genomic DNA clones corresponding to antigens of *Leishmania*.[167–169] Sheppard and Dwyer[167] produced 90 clones that express *L. donovani* antigens, some of which were found to be specific for antibodies present in the sera of VL patients. Blaxter et al.[168] isolated a cDNA clone from a gtl1 expression library constructed using mRNA from promastigotes of an African *L. donovani* (MHOM/ET/67/HU3) that expressed a β-galactosidase/parasite antigen fusion protein corresponding to a 60-kDa membrane-associated antigen of *L. donovani*. The antigen specifically recognized antibody present in the sera of VL patients independent of geographical origin, but did not cross-react with the sera of CL, MCL, and Chagas' diseases. The antigen is not gp63, the surface protease of *Leishmania*.

Osland et al.[169] employed genetic engineering for the development of recombinant antigens from *L. aethiopica* that react with human antibodies. In the case of DCL, high-molecular-weight antigens (30 and 90 kDa) were found to be specific, whereas low-molecular-weight antigens (25 kDa) were specifically reactive in LCL.

VI. DETECTION OF CIRCULATING ANTIGENS

Circulating immune complexes (CIC) in leishmaniasis can be estimated by the $C1_q$ binding assay, polyethylene glycol (PEG) precipitation with radiolabeled staphylococcal protein A, or by measuring cryoglobulins. The identification of parasite antigens in CIC could have potential diagnostic and prognostic implications.[170,171] Evans and Pearson[172] demonstrated a prominent antigen of 70 kDa of *L. d. chagasi* in the sera of patients

with American VL by analyzing CIC obtained by 2.5% PEG precipitation and SDS-PAGE. There are reports that although many individuals are infected with *L. donovani* in endemic areas giving antibody response, they experience an asymptomatic, self-resolving infection,[173] and in such cases identification of the circulating parasite antigen might be indicative of active infection. The level of CIC in VL is much higher than that in control human sera. The CIC of KA cases contains at least six protein antigens, of which the 32-, 55-, and 68-kDa antigens were prominent.[174] Desjeux et al.[175] have shown that an IgG-anti-IgG complex is present in MCL patients with several lesions.

The monoclonal enzyme-linked immunotransfer blot technique (McAB-EITB) was used for the detection of circulating antigens in patients with VL.[176] Four reaction bands of 130, 100, 50 and 25 kDa were found in each of the KA patient sera tested. No reaction band of 130, 100, and 25 kDa was present in the sera from normal controls, cured KA patients, or patients with schistosomiasis, malaria, or tuberculosis, and very few cross-reactions were detected in the 50-kDa band. Monoclonal antibody $L_{12}F_7$ targeted against *L. donovani* promastigote was labeled with peroxidase and used in dot ELISA for detecting circulating antigen in the sera of VL. The test gave 90.6% positive results out of 159 serum samples, and no positive result was obtained from 50 patients who had been recovering from the disease for 3 months to 16 years.[177] A positive correlation was exhibited between the titer of circulating antigen in the sera and the parasite load in the patients. Antigen-specific circulating immune complexes may be useful in monitoring therapeutic effects and recovery.

VII. TEST FOR THE CELLULAR IMMUNE RESPONSE

A. Leishmanin Skin Test

The delayed skin reaction is universally known as the Montenegro test, originally introduced by Wagner[178] using an extract of *Leishmania* promastigotes, and as an important feature of leishmaniasis. The antigen was then modified using Coca's fluid by Montenegro[179] at a strength of 10^6 cells per milliliter and a dose of 0.1 ml. Its diagnostic value was confirmed for *L. b. braziliensis*[180] and *L. tropica*.[181] The antigen usually used is a suspension of promastigotes in 0.5% phenol saline, which is known as "leishmanin" and which is injected intradermally, generally on the inner forearm. Bray[182] suggested that the antigen should be prepared at 500 μg/ml saline and sterilized by membrane filtration, the dose being 0.1 ml. The reaction is an indurated nodule associated with erythema, developed within 48 to 72 h. In active DCL and Indian KA, the test is negative, but positive after 6 to 8 weeks of recovery when some cellular immunity has developed. Indian KA patients failed to show a delayed-type hypersensitivity reaction to leishmanin, while 72% of 16 cases reacted to a purified protein derivative of tuberculin.[183] Six out of nine PKDL cases showed a DTH reaction to the leishmanin skin test.[183] In CL, the leishmanin reaction becomes positive within 2 to 3 months of the appearance of the lesion and remains positive for life after recovery. The leishmanin test is an important tool in the diagnosis of lesions where parasites are very scanty and in the diagnosis of visitors to endemic areas, as they have a background of leishmanin sensitivity. Shaw and Lainson[111] reported 84 and 100% positivity for simple cutaneous and mucocutaneous lesions, respectively, and negative results for DCL were obtained by the test. The leishmanin skin test fails to distinguish current from prior leishmanial infection (Table 2). Ten PKDL patients with a past history of VL were leishmanin negative before treatment, whereas all cured VL patients were skin-test positive.[184]

Promastigotes of any species of *Leishmania* can be used as an antigen source for the test.[185] Other flagellates *(Strigomonas oncolpelti, T. cruzi, T. equiperdum, Leptomonas ctenocephali, L. pessoai, L. adleri,* and *Crithidia fasciculata)* also can serve this purpose.[5]

Side effects such as vesiculation, lymphoadenopathy, and ulceration at the site of leishmanin skin test increased with the antigen dose.[186] Storage of leishmanin skin test antigen at 2 to 8°C for more than 15 months did not affect its potency.[186]

It has been suggested that the leishmanin skin test antigens should be prepared from homologous species that are prevalent in the survey area.

This would reduce the dose of antigen without loss of potency and sensitivity and successively reduce the side effects.[186]

Exoantigens or EF of *Leishmania* that are liberated by the parasite to the surrounding medium have also been used as an alternative to leishmanin (whole cell) in the determination of hypersensitivity responses in CL.[111]

In CL due to *L. m. amazonensis* in Amazonian Brazil, the Montenegro skin test is negative.[187] Reed et al.[188] demonstrated that 25 to 50 μg of protein (soluble extract of *L. d. chagasi*) was more sensitive (95 to 100%) than whole cells and gave a positive skin test reaction in past American VL, but produced no positive responses in normal controls, tuberculosis patients, or schistosomiasis patients, and 5% positive responses in Chagas' disease. The same amount of soluble extract of *L. m. amazonensis* produced 82% positive results in past American VL.

Host factors affect the sensitivity and potency of a given leishmanin antigen. For example, lesion type (mucocutaneous vs. cutaneous),[111,189,190] duration of the active lesion,[189,190] lesion stage (active vs. healed),[189] and time interval after healing.[189,190] Development of a skin-test response to leishmanial antigens,[173,191] proliferative responses to specific antigens in T cell assays *in vitro*,[191] and hepatic granulomata[56] without ever presenting clinical disease were found in many people of endemic areas.

In a leishmanin test survey of school children aged 6 to 12 years in an area of the Yemen Arab Republic where mixed infestation with *L. tropica, L. donovani,* and *L. infantum* occur, Dereure et al.[192] reported that the positivity decreased from 45.6% (of 92) to 11.2% (of 102) with increasing altitude from 950 to 1430 m. At the lowest altitude, all three species of parasite occur; at the highest, *L. tropica* is rarer and VL is negative. This distribution is paralleled by sandlfy members.

Proteins of approximately 66, 55, 45, 28, and 26 kDa of *L. panamensis* were related to a specific delayed-type hypersensitivity of CL of the New World.[193] Furuya et al.[31] reported that the skin test and ELISA positive rates among 72 subjects with active dermal lesion were 81.8% (36/44) and 81.3% (52/64), respectively, with the use of ruptured promastigotes of *L. t. braziliensis* antigens, while parasites were observed in 31 (44.9%) of 69 patients presenting active lesions. Simple cutaneous lesions and mucocutaneous lesions caused by *L. b. braziliensis* gave a positive leishmanin test in 45 of 53 (85%) and 19 of 19 (100%); simple cutaneous lesions and multiple cutaneous lesions caused by *L. b. guyanensis* gave positive results in 15 of 18 (83%) and 5 of 8 (62%), respectively, and simple cutaneous lesions and DCL caused by *L. m. amazonensis* showed positive in 5 of 6 (83%) and 0 of 4 cases, respectively.[111]

B. Lymphocyte Proliferation Assay

No response to promastigote surface protease was found; however, weak proliferation of lymphocytes to lipophosphoglycan was consistently present in CL patients, and a *L. major* promastigote antigen of 72 to 82 kDa was found to be specific for lymphocyte proliferation, as revealed by T cell blots.[148]

A total blood lymphocyte proliferation assay was negative in CL patients with lesions less than 3 months old and afterward showed positive.[194]

Peripheral blood lymphocyte of Indian KA patients failed to be stimulated by *Leishmania* antigens but could be stimulated by phytohemagglutinin.[183] A gradual fade up of the cell-mediated immune response to *Leishmania* antigen was found with the chronicity of PKDL.[183]

VIII. CONCLUSIONS

In the detection of leishmaniasis cases and epidemiological survey of the disease, serodiagnostic methods are of immense importance prior to attempts at parasite detection. The major findings in the use of different serodiagnostic techniques using whole parasite, its cell-free extracts, and its different antigenic fraction or purified antigens are summarized in Table 6. The performance of the tests is dependent on the antigen specificity to reduce the cross-reaction with cross-reacting diseases such as African trypanosomiasis, Chagas' disease, leprosy, tuberculosis, malaria, etc. and to reduce the false positive reaction with a normal human, particularly endemic origin. More work remains to be done on the

TABLE 6
Leishmaniasis: Summary of Specificity of Different Serodiagnostic Tests with More Commonly Used Antigen(s)

Test	Source of antigen	Reactivity of leishmaniasis						Other cross-reactive cases
		VL	CL	MCL	DCL	PKDL	Cured VL cases	
CFT	Cell-free extract of *Leishmania* promastigotes	+[a]	–[b]	+	?c	?	+	*T. cruzi*, mycotic infection
	Amastigotes	+	?	?	?	?	+	
IHA	Cell-free extract of promastigotes	+ (73–100)[d]	?	?	?	+ (100 in fresh cases; 33 in chronic cases)	?	Malaria
DAT	Whole promastigotes	+ (92–100)	+ (50–67)	–	+	+/–[e] – (macular)	+	Leprosy
IFAT	Whole promastigotes	+ (100)	+ (25–82)	+ (77–89)	–	+/–	?	*T. cruzi*, tuberculosis
	Whole amastigotes	+ (100)	?	?	?	?	?	—
GDT	Cell-free extract of promastigotes (soluble antigens)	+ (72)	?	?	?	?	?	Normal human
CCIE	Cell-free extract of promastigotes (soluble antigens)	+ (72–100)	?	?	?	+/–	?	Normal human
IEP	Exometabolites	+ (100)	?	?	?	?	?	—
	ConA-positive glycoproteins	+ (100)	?	?	?	?	?	—
	Lipophosphopolysaccharide	–	?	?	?	?	?	—
ELISA	Cell-free extract of soluble antigens	+ (94–100)	+ (72)	+	?	+ (83)	+	Chagas' disease, malaria, leprosy, tuberculosis
Dot ELISA	Whole promastigotes	+ (98)	?	?	?	?	?	?
	Soluble antigens of promastigote	+	?	?	?	?	?	?
Direct ELISA	Whole promastigotes	+ (87–100)	?	?	?	+	?	—
Competitive ELISA or RIA with monoclonal antibody (D-13)	Soluble antigens of promastigotes	+ (100)	+ (23)	?	?	?	+	Leprosy, Chagas' disease, normal human
RIA	EF	+/–	–	?	?	?	?	?
	LPS-like antigen	–	+ (90)	?	?	?	?	?
	FTS (freeze thaw sonicated *L. major* promastigotes)	–	+	?	?	?	?	Leprosy

TABLE 6 (continued)
Leishmaniasis: Summary of Specificity of Different Serodiagnostic Tests with More Commonly Used Antigen(s)

Test	Source of antigen	Reactivity of leishmaniasis						
		VL	CL	MCL	DCL	PKDL	Cured VL cases	Other cross-reactive cases
Western blotting	Soluble antigens							
	qp63 (M_r, 62–68 kDa)	+	+	+		?	?	—
	Antigens (M_r, 60 kDa) of *L. b. braziliensis*	–	+	?	?	?	?	—
Leishmanin skin test	Whole promastigotes	–	+ (84) (62 in multiple CL lesions)	+ (100)	–	+ (66)	+	?
	Soluble antigen	–	+	?	–	+/–	+	?
	EF	?	+	?	?	?	?	?

[a] +, positive in the test.
[b] –, negative in the test.
[c] ?, test not performed and adequate data not available.
[d] Numbers in parentheses are the percent of positive results reported to date by different workers.
[e] +/–, both positive and negative results were reported in a group of patients and weekly positive in the test.

identification and characterization of *Leishmania*-specific and species-specific antigen(s) and evaluation of these antigens by different serodiagnostic methods for different leishmaniasis and other cross-reacting diseases. The leishmanial surface membrane protease gp63 (62 to 68 kDa, the best characterized antigen that is *Leishmania* specific) may be used for antibody detection. Two ConA-positive glycoproteins of exometabolites of *L. donovani* are of serodiagnostic importance. The detection of antibody and DTH reaction should be evaluated simultaneously for conclusive diagnosis of leishmaniasis. In active VL, tests for antibody are always positive and the leishmanin skin test is negative, and the latter test is positive in cured VL. In PKDL, the antibody level in the serum gradually reduces to some extent with the chronicity of disease, and the DTH reaction becomes positive in many cases. In MCL, the DTH reaction is always positive, whereas in CL it is negative. In CL, both antibody detection and the DTH reaction could diagnose about 90% of the cases. There are very few data for MCL, DCL, and PKDL cases. DAT, ELISA, and dot-ELISA techniques are convenient to use in field conditions and epidemiological surveys of the disease.

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