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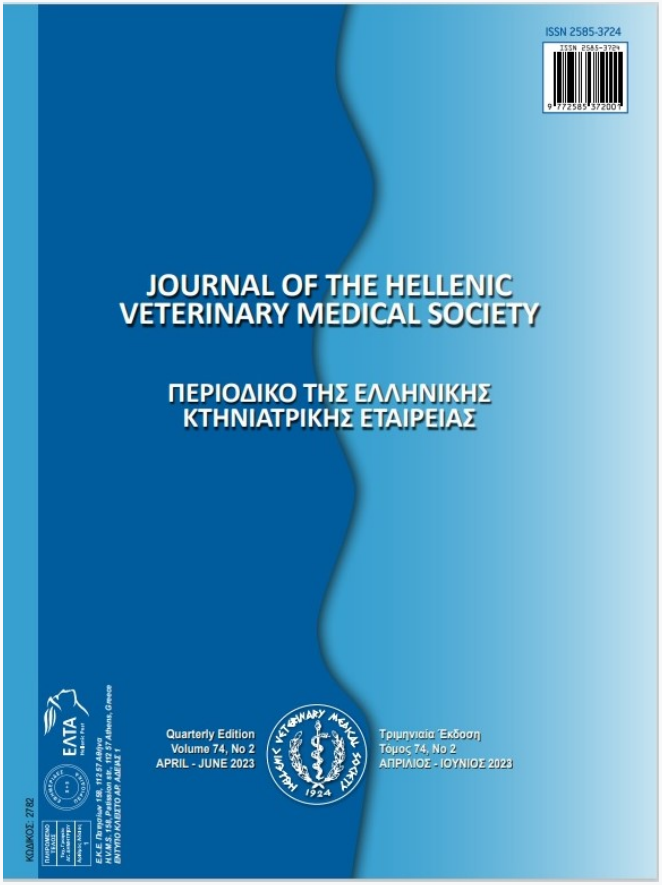

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Research article
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A Review on Recent Advancement in the Molecular Diagnostics of Leishmania

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ABSTRACT: Leishmaniasis is a protozoan infection with chronic manifestation, having high morbidity and mortality rates, and adversely affecting almost every species of animals. It is a globally prevalent vector-borne disease throughout all tropical and subtropical regions. Twenty intracellular species belonging to the genus *Leishmania* cause all types of leishmaniasis infection, including cutaneous, mucocutaneous, and visceral leishmaniasis in animals and human. Sandfly, as a vector, is responsible for its transmission between different hosts. Animals and humans affected with

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leishmaniasis may be prone to re-infection of another disease, especially the human immunodeficiency virus (HIV) syndrome, through trans-activation modification of the immune system. Several diagnostic procedures have been developed and are being used for its detection and confirmation. Hence, there is a need for standard as well as advanced diagnostic tools that are immediately required to identify the species for further treatment and to adopt precautionary and safety measures against leishmaniasis. The current review constitutes a brief picture flowing from microscopic evaluation to all possible immunological techniques that can detect the species and can differentiate between different types of leishmaniasis, along with the morphology and various routes of transmission of the parasite. These methods include serological antigenic screening like direct and indirect agglutination tests, indirect fluorescent antibody test, enzyme-linked immunosorbent assay, western blotting, and immuno-chromatographic test to advanced molecular techniques like nucleic acid sequence-based amplification, polymerase chain reaction, loop-mediated isothermal amplification assay and some modern techniques like proteomics, transcriptomics and protein biomarkers. The aim of this brief overview is to explain morphology, its ways of transmission and explain the current development in the types of diagnostic techniques for better detection and control of the Infection.

Keywords: Diagnostic techniques, Leishmania, morphology, sandfly, transmission,

INTRODUCTION

Leishmaniasis is a vector-borne parasitic disease caused by obligate intracellular protozoan parasites of the genus *Leishmania* that belongs to the family trypanosomatidae and order kinetoplastida. Twenty species are included in this genus that causes Infection in humans CDC (2019). These parasites can develop different forms of the disease, including cutaneous, mucosal, and visceral leishmaniasis (Ashford, 2000). The cutaneous form is caused by *L. tropica, L. major,* and *L. aethiopica;* those are found in Asia, the Middle East, the Mediterranean region, India, and Africa and belong to Old World cutaneous leishmaniasis. While *L.amazonensis, L. mexicana, L. braziliensis, L. panamesis, L. pruvians,* and *L. guayanensis* found in Central America and South America belong to New World cutaneous leishmaniasis(CL) (Inceboz, 2019). There is a sub-form of CL that is diffuse cutaneous leishmaniasis (DCL) (Singh, 2006). Mucosal (ML) form of the disease is due to *L. brazilensis, L. panamensis* and *L. guyanensis* in the New World region, while the Old-World Infection occurs by *L. infantum and L. donovani*. The third type of visceral leishmaniasis infection is caused by *L. donovani* complex comprising of *L. infantum, L. donovani and L. chagais,* commonly famous with the name kala-azar, Dumdum fever, or black fever. Globally, 0.2-0.4 and 0.7-1.2 million cases were seen per year for visceral (VL) and cutaneous leishmaniasis (CL), respectively (Fig.1) (Alvar et al., 2012). A large number of infected people were diagnosed worldwide, including America, Europe, Asia, and Africa, but still, it could be assumed that a large number of cases had not been reported (Desjeux, 1996). Transmission of the understudy parasite is usually carried out by the biting of an infected female sandfly that belongs to the genus *Phlebotomus* in the case of Old World and *Lutozomia* in the case of New World species (Cruz et al., 2002). The Infection could also be transmitted by re-use of infected syringes (Cruz et al., 2002), blood transfusion, and venereal route (Owens et al., 2001). For the last two decades, leishmaniasis has been included in the list of emergent and re-emergent parasitic diseases in many parts of the world. Even in this era of advanced scientific research, diagnosis and control of leishmaniasis is still a significant public health concern (Rani et al., 2022). Since the principal obstacle in the treatment of the disease is false-negative results as the diagnosis is also a little bit time taking. Hence, there is a need for standard as well as advanced diagnostic tools that are immediately required to identify the species for further treatment and to adopt the precautionary and safety measures against various parasitic diseases (Herwaldt, 1999; Mohsin et al., 2021; Aleem et al., 2022). The best diagnosis of leishmaniasis could be based on the correlation of many aspects of the study, including epidemiological surveys, clinical findings, and data from various laboratory analyses. The current piece of writing provides a comprehensive note on all the types of *Leishmania* diseases, including parasite morphology, mode of transmission, and pathophysiology. Moreover, it also provides the latest information about the advancement in immunological and molecular diagnostic techniques of the disease along with their sensitivity, specificity, efficacy, advantages, and disadvantages.

2.MORPHOLOGICAL FEATURES OF *LEISHMANIA*

There are two primary forms of *Leishmania* parasites:(1) Promastigote (2) Amastigote,

Promastigote type:

Promastigote type is only seen in the vector sand-fly, and its conversion starts within hours from amastigote to promastigote form and completes the process within 24-48 hrs. inside the gut of the fly. The mature promastigote form is mostly seen in the fore and mid-gut (Herwaldt, 1999; Desjeux, 2001). The length of the amastigote is about 8-15µm, and the functional flagellum is attached to its cell body to provide motility, while the flagellum of the promastigote form is a paraxial rod-shaped structure. So, there is a lot of difference between promastigote and amastigote forms based on their morphology (Fig. 2a). The new adaptation that occurs within the sand-fly is the conversion of amastigote to procyclic promastigoteand then multiplication occurs in the posterior part of the mid-gut and change in the form of nectomonad (Maia et al., 2018). After three days, peritrophic membranes around the parasite are broken down, and promastigotes move freely to the anterior mid-gut (Bates,

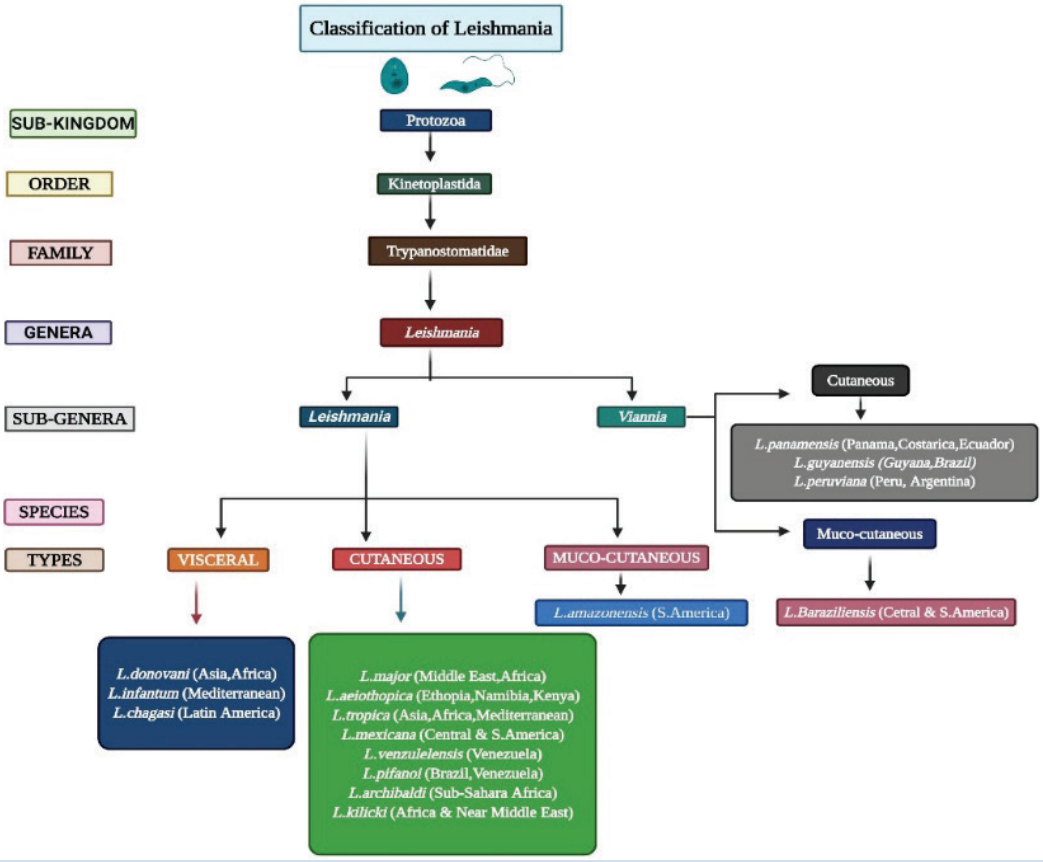

Figure 1. Worldwide classification of Leishmania

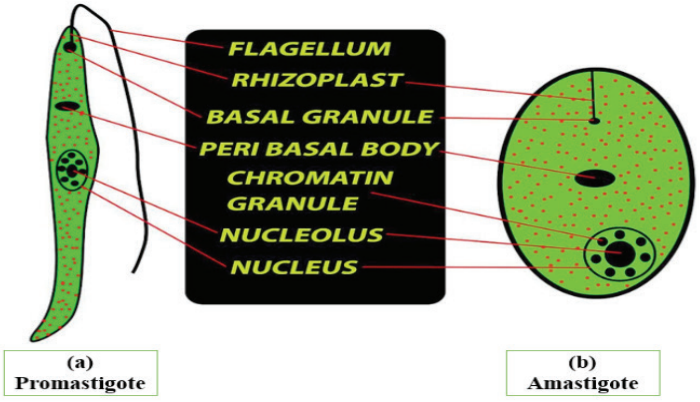

Figure 2. Parasite biology of Leishmania; (a): Promastigote type (b): Amastigote type

2007). A phospholipid lipophosphoglycan (LPG) showed an important role in boosting up the Infection in two ways; First, directly attached to the gut, and second by the maturation of the disease (Turco et al., 1994; Bates, 1994; Forestier et al., 2014). The Infection reaches the fore-gut within five days, and haptomonad promastigote is linked with the stomodeal surface valve. After the fifth day, metacyclic promastigote is found in the mid-gut, foregut, and sometimes at both sites. Metacyclogenesis occurs when the gut pH drops; hence it's important to use acidified media during the in-vitro growth culture (Bates and Tetley, 1993; Skalický et al., 2017). Hence a suitable medium is required for the growth of different stages of *Leishmania* (Serafim et al., 2012).

Amastigote type:

The shape of *amastigote* is oval; they are non-flagellated the size is about 3-5µm. At the center of the organism, there is around to oval nucleus, and a rod shape kinetoplast can be seen by a light microscope. An internal gap between the surface membranes is called a "flagellar pocket." The flagellum is not active in the *amastigote* form, and its size is remarkably reduced as compared to the cell body. The primary function of the flagellar pocket is endocytosis and exocytosis (McKean and Gull, 2010). Kinetoplast is a mitochondrial DNA present at the originating point of the flagellum and consists of several thousand round DNA molecules that form a mesh-work. There are two types of DNA networks in every kinetoplast that consists of 25-250 maxicircles of 30kb size and another type of 5000-10000 maxicircles of 2kb size; both unite to form the mitochondrial genome (Fig. 2b). Golgi complex is present in the surrounding of the flagellar pouch that plays a significant function in the pathway of endocytosis and exocytosis. Lysosomes are also present in the cytoplasm that resembles that of the kinetoplast (Shlomai, 1994). The cycle is started by the communication of metacyclic promastigote in the presence of body coat macrophages that are uptaken by phagosome, and the parasite inactivates the secondary lysosomes and then converts into amastigote form within the short period of 12-24h. This process continues until the breaking up of the phagosome (Bates, 1993; Zilberstein and Shapira, 1994).

MODE OF TRANSMISSION OF THE PARASITE

Globally the transmission of the disease occurs by a vector sandfly (Herwaldt, 1999; Kumar et al., 2001). At the same time, other ways are also present for its transmissions like parental route, transplacental, coitus, and direct contact among the people. Since in the endemic areas, the transmission of leishmaniasis mostly occurs by bite of the sand fly (Fig. 3), but other

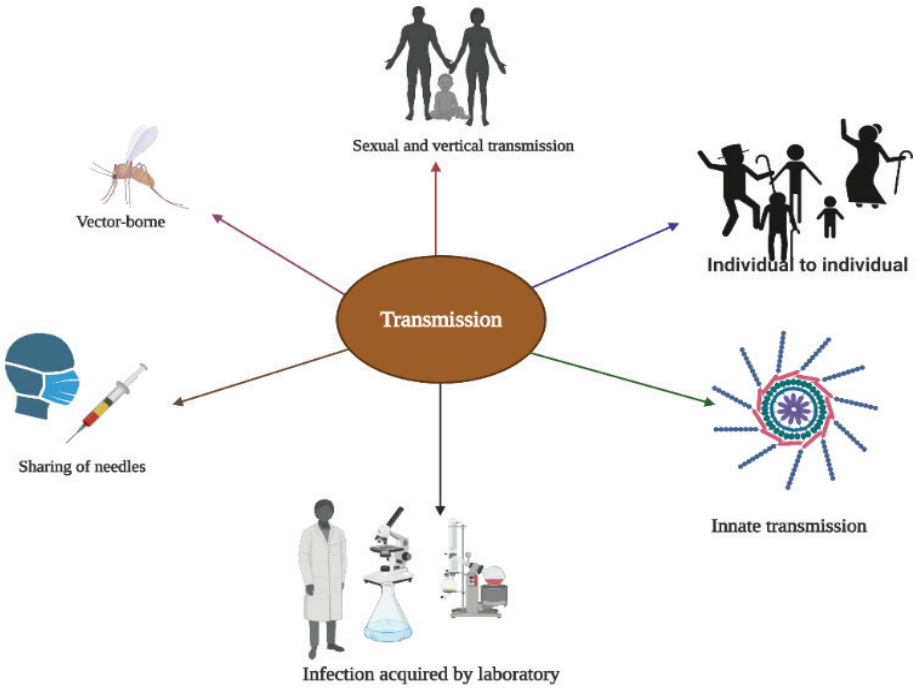

Figure 3. Transmission of leishmanial parasite by different ways

routes are still under consideration (Herwaldt, 1999; Singh and Sivakumar, 2003).

Vector-borne transmission

A vector plays a vital role in the spread of the disease. Leishmaniasis is also a vector-borne disease whose vector is sandfly. Once sand fly bites the relative host, it ingests the *Leishmania* amastigotes from the host blood. After developing metacyclic amastigote, sand fly transfers it to the second host along with active vasodilators i.e.maxadalin which causes long-term erythema (Añez et al., 2003). The function of macrophages is to phagocytose the promastigote, capable of staying alive within the phagolysosome, that reconverts to amastigote and is ready for multiplication by simple binary fission for the breakdown of host cells and then again ready for attacking the surrounding macrophages (Toepp et al., 2019). For transporting the disease to another host, a new sand fly nourishes the previous host, hence the ecological cycle is continuous (Fig. 4).

Sexual and vertical transmission

Some evidence has been collected that gives a clear indication of its sexual transmission. Urine and secretion of the prostate gland were collected from the patients suffering from visceral leishmaniasis, and a large number of promastigotes were observed both in urine and prostate secretions (Paredes et al., 2003; Wegmann et al., 1993). Moreover, the transmission occurs through sexual contact through the transfer of spermatic fluid, notably observed in the cases in Spain (Paredes et al., 2003). Moreover, leishmaniasis has also been observed in new individuals that carried the Infection from their dams (Turchetti et al., 2014). *Leishmania* species had been found in the lymph node, liver, and spleen of the newborn pup of an infected bitch. Wherein, 8 out of 31(26%) puppies from symptomatic or asymptomatic naturally infected bitches were found positive for *Leishmania* species as detected through polymerase chain reactions PCR (Masucci et al., 2003).

Sharing of needle

The transmission of the parasite was also evident by the re-use of an infected needle. About 85% of cases were seen in those people that had AIDS and HIV-related Leishmaniasis in Spain. About 17% of infections were found among 111 cases of bone marrow aspirates were observed in the patients infected with HIV. Fever was seen due to the presence of an amastigote form of *L. infantum* zymodemes species that was present in the people involved in the processing of intravenous drug application (Alvar et al., 1997; Safar, 2017). Indirect xeno-diagnosis was conducted for the detection of visceral leishmaniasis

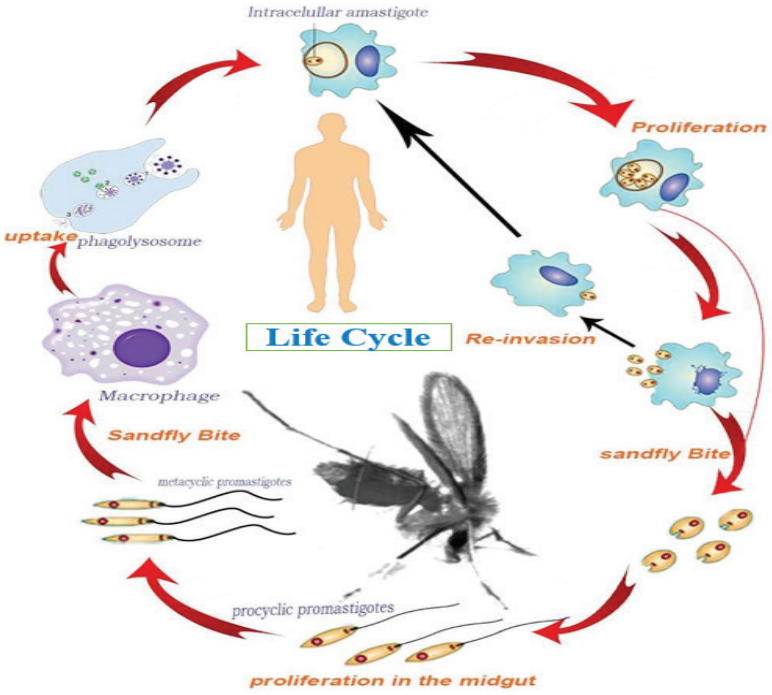

Figure 4: General Life Cycle of Leishmania

in the patients infected with HIV and observed that 0.3-0.5ul blood showed the Infection of *Phlebotomus perniciosus* (Molina et al., 1994).

Infection acquired by laboratory

Three dominant species of *Leishmania,* i.e., *L. tropica*, *L. donovani,* and *L. braziliensis* were observed that are involved in the Infection acquired by humans from a clinical laboratory (Herwaldt and Juranek, 1993; Paredes et al., 2003; Safar, 2017). Various factors responsible for the Infection transmitted in the laboratory are needle injury that caused ulcers at the inoculation site, some were involved in the management of infected specimens, and a few were exposed by oral contact. A fruitful attempt for this parasitic diagnosis must be based on the analysis of those persons that have not shown signs of complete sickness at the time of disease (Alvar et al., 1997). Hence laboratory-acquired Infection is another mode of disease transmission.

Innate transmission

Some evidence was also reported for the transplacental transmission of disease from the parent to the young, and about ten cases of innate disease transmission have already been reported (Elamin and Omer, 1992; Loke, 1982; Nyakundi et al., 1988; Yadav et al., 1989; Meinecke et al., 1999). An aborted case of a Syrian hamster was found in which although no parasite was found in the fetal organ, a number of amastigotes were observed in the placenta (Loke, 1982). This shows that the disease is transmitted when blood exchanges between the mother and the fetus by birth tube.

Individual to individual transmission

The current scenario is that the individual to the individual transmission of the disease is quite exceptional. A little information has been collected from the individuals who got the disease by contact with different body secretions, including blood, bone marrow, urine, and lymphatic fluid of diseased persons (World Health Organization, 2012; Safar, 2017). The disease also transferred from a contaminated female hamster to her offspring by direct contact with the infected area (Rosenthal et al., 1991).

PATHOPHYSIOLOGY OF LEISHMANIASIS

Leishmaniasis in spite of having a large number of clinical and geographical verities with a wide range of manifestations, shares some common histological features, including hyperplasia of the reticuloendothelial cells in the organs due to the accumulation of mononuclear cells in the damaged tissues (Omachi et al., 2017). The spleen produces the pre-active T-lymphocytes that are highly specific to the leishmania and are migrated to the liver, which is the site of parasite multiplication, where this cell becomes an effector T-lymphocyte in association with $CD8^+$ T cells (Gomes-Pereira et al., 2004). Ca^{2+} has an important role in giving the signals when leishmanial promastigote form transforms into amastigote, which then enters into the macrophages and increases the virulence (Prasad et al., 2001). The suppression of cell-mediated immunity and an increase in the parasite number due to its adverse effects on the hematopoietic system (usually termed as bloody dyscrasias) was also reported in the case of VL. Moreover, imbalance in predominance behavior between type 2 helper T-cells (Th2) over (Th1) and regulatory cells (Treg) over type 17 helper cells (Th17) carried out leishmanial Infection and disrupted the normal cytokines pattern that leads to failure of pregnancy both in animals as well as humans (Berger et al., 2017). The hypothesis is that Leishmania targets the Hoffbauer cells and the macrophages of the placenta that acts as the mainline during pregnancy as occurring in the case of the zika virus (Quicke et al., 2016). Sex hormones like estrogen also play an essential role in regulating the immune system via nitric oxide (NO) killing leishmania in hamsters (Osorio et al., 2008). Progesterone suppresses the components of the innate immune system and promotes the Th2 milieu involved in parasite killing, but the actual effects are still unknown (Robinson and Klein, 2012). The disease and multiplication in the monocyte/macrophage are the common processes that are carried out by both leishmanial and HIV diseases. The immunopathogenic mechanisms of these infections support each other to express by interacting with the immune system of the body. Moreover, leishmania disease favors the trans-activation of HIV in the monocytes and $CD4^+$ T cells that are latently infected (Cunha et al., 2017).

DIAGNOSIS OF VISCERAL LEISHMANIASIS (VL)

VL or Kala-azar is an infection of deprived and deserted people in 79 countries, and about 58,000 new patients are reported every year (Alvar et al., 2012). Amongst these, 90% of the infected cases belong to the sub-continent region, especially from the Bihar state of India shows a higher number of diseased pa-

tients (Sundar et al., 2010). In advanced countries, Infection is not so widespread, and laboratory services provide an efficient diagnosis of the disease. However, due to an increase in the number of infected people in rural areas, diagnostic approaches are essentially required in the field area (Elmahallawy et al., 2014). Hence for the detection of visceral leishmaniasis, different diagnostic techniques are used based on microscopic observation, detection of antigen, and serological analysis, including indirect fluorescent antibody test, ELISA, immunoblotting test, direct agglutination, immune-chromatic test (both in human and canine) (Herrera et al., 2019), latex agglutination, PCR, Loop-mediated isothermal amplification (LAMP) test and Nucleic acid sequence-based amplification (NASBA) test (Table.1).

Microscopic analysis

The microscopic examination was used for the demonstration of parasites in the specimens and (Artan et al., 2006; Del Olmo Martínez et al., 2009). Different stages of the parasite were isolated and diagnosed under the microscope like an amastigote form was collected from the tissues, and the promastigote form was collected from the culture. For microscopic detection of amastigote, samples were collected mostly from spleen, bone marrow, liver biopsy, buffy coat of blood, and liver tissue samples. In bone marrow biopsy, the sensitivity of Giemsa stain was 60% to 85%, while the sensitivity of spleen was 93% for the detection of this parasite (Boelaert et al., 2007; Galaï et al., 2011; Srividya et al., 2012; Elmahallawy et al., 2014). For boosting the diagnostic activity of a microscope, a fluorescent antibody coated with conjugated dye could also be used (Zijlstra et al., 1992). However, the diagnostic results were deficient at the margin of the blood smear, particularly of those people that suffered from parasitic fever. A recent technique has also been used named as a micro-culture method (MCM) by using single marginal nucleus blood cells and Buffy coat (Allahverdiyev et al., 2005; Maurya et al., 2010). In this method, experimental samples are cultured by two ways; in the first step, sample is cultured in a single phasic medium in Schneider's insect medium. Other media that could also be used are Novy-Mc-Neal-Nicolle medium and Evans modified Tobie's medium for the transformation of parasitic forms especially from amastigote to promastigote. Moreover, other types of medium including M199, Grace's insect medium for increasing the number of parasites could was also used (Zijlstra et al., 1992;Sundar and Rai, 2002). So, microscopic examination is the simplest tool for diagnosis of parasites that can be easily performed in the field conditions.

Detection of antigen

Antigen detection is a more reliable method used for the detection of VL (Srividya et al. 2012). This test was primarily used for patients which are immunocompromised, especially those having a correlated infection like HIV-*Leishmania* and devoid of antibodies (Cruz et al., 2006). Theoretically, this technique could be more critical than the detection of antibodies because they avoid cross infection and provide the current status of an older disease (Chappuis et al., 2006). Additionally, the serum is not the sole appropriate method for the detection of antigen, but many other methods are also specific for the determination of antigen based on immunological factors (Cruz et al., 2006). Many studies showed that parasitic antigen, especially *Leishmania*, was found to be present in the urine of diseased persons and detected by urine analysis, especially in the case of VL and by another test named latex agglutination test (KAtex); an easy, fast and dependable method. The specificity of this test was ranged 82-100%, while sensitivity is 47-95% (Riera et al., 2004; Diro et al., 2007; Salam et al., 2011; Akhoundi et al., 2013). Two reports were published, one in 1995 about the presence of polypeptide antigens ranging from 72-75KDa in the urine, and the second was reported in 2002 about the existence of carbohydrate antigen varied from 5-20KDa, especially seen in VL (De Colmenares et al., 1995; Sarkari et al., 2002). In short, KAtex is the best test for screening visceral leishmaniasis in developed countries.

Direct agglutination test (DAT)

DAT depends directly on the mechanism of agglutination, especially the promastigote form of *Leishmania* that mainly reacts with serum antibodies of leishmania. For staining of promastigotes, two kinds of stains, trypsinized and coomassie-stain, could be used in the form of suspension or dried ice (Akhoundi et al., 2010; Mahdi Fakhar, 2012; Elmahallawy et al., 2014). It is a partially quantitative test that is cheap to use in common areas of developing countries. The antigen used in this test is a promastigote form, and the test is performed by complete blood (Srividya et al., 2012; Elmahallawy et al., 2014). Microtiter plates are used for the dilution of samples, and agglutination of promastigote has occurred within 18h if particular antibodies are present. At 1:3200 was the highest

titer was found where agglutination was observed (Boelaertet al., 2007; Abass et al., 2015). However, many other positive results regarding the titer were reported that ranged from 1:800 to 1:6400 (Bern et al., 2000). The benefit of this test is to calculate the lowest level of antibodies by use of different antigens (Maia et al., 2012). This test is highly sensitive and specific, but the final value doesn't depend only on its sensitivity and specificity. The prognostic results are also based on epidemiological surveys and the occurrence of the disease (Akhoundi et al., 2010). A study data were collected from thirty different locations with probably 94.8% sensitivity and 97.1% specificity (Chappuis et al., 2006). The comparison of the Indian subcontinent and East Africa for DAT performance and result showed immuno-suppression was more in those patients that belong to East Africa (Diro et al., 2014). But DAT has some boundaries like an extensive incubation period ranging from 12h to 18h is required, and the second thing is the sequential dilution of the samples (Akhoundi et al., 2010). Moreover, a particular antibody might show a positive sign that covers above 50VL for five years; that's why this test has a limitation in endemic areas (Elmahallawy et al., 2014). To avoid the disadvantages of DAT, there is another screening test named as Fast Agglutination Screening Test (FAST) that was used for quick and early detection of particular antibodies. For that purpose, one serial dilution and an incubation period of only three hours are required. The sensitivity of this test was 95.4% and specificity 88.5% that is relatively better than DAT (Akhoundi et al., 2010; Elmahallawy et al., 2014). In a study, 9.4% (n = 13) of the cattle were found to be positive by ELISA. Of the 13 ELISA-positive cattle, only four (30.8%) were positive in direct agglutination tests (DAT). Parasite DNA was not detected in either of the molecular assays Nested PCR and Loop-mediated isothermal amplification (Ln PCR and LAMP) (Alam et al., 2011).

Indirect Fluorescent Antibody test (IFAT)

This test depends upon the detection of antibodies that are at the starting stage of the disease and are not measurable before a treatment of 6 to 9 months that determines the least level of titer. The test sensitivity is 87% to 100% and specificity 77% to 100%, and the source of antigen is promastigote or amastigote (Elmahallawy et al., 2014). For a positive response, the titer is more than 1:160 (Iqbal et al., 2002). It is a regular serological test of endemic areas that requires fluorescence microscopy with highly equipped laboratories (Cota et al., 2013). The promastigote type of *Leishmania* is more critical for the diagnosis of visceral leishmaniasis since they reduced the cross-reaction with sera of *Trypanosoma* (Badaro et al., 1983). Due to the limitations of the direct method, the best results have been reported by the control program of Brazilian leishmaniasis with 88-92% sensitivity and 81-92% specificity, respectively (Machado de Assis et al., 2012). Four major human diseases show cross-reaction with visceral leishmaniasis-included malaria, trypanosomiasis, brucellosis, and typhoid fever (Elmahallawy et al., 2014). Moreover, IFAT can also be considered as a good standard test in non-endemic areas for canine leishmaniasis, but its accuracy declines in endemic areas due to the complexity of the disease (Adel et al., 2016).

Indirect hemagglutination test (IH)

The principle of this test is to mix the serum of a patient with human erythrocytes that are sensitive to the antigen of *L. donovani*. Its positive titer is more than 1:64, while sensitivity was reported as 90-100% and specificity up to 86% (Iqbal et al., 2002; Dakic et al., 2009), but the graph of titer remains at a peak level after upturn (Artan et al., 2006). This test was used for diagnostic purposes in non-endemic areas where a smaller number of regular cases of visceral leishmaniasis were observed (Iqbal et al., 2002), and also for canine leishmaniasis (da Silva et al., 2006).

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a serological technique used for all types of infectious diseases, including visceral leishmaniasis. The method is more sensitive than specific because of the dependence of specificity on the variety of antigens used. Different antigenic molecules are present at the outer surface of antigens, including ribosomal, histones, and kinesin type proteins (Srividya et al., 2012; de Paiva-Cavalcanti et al., 2015; Sarkari et al., 2018). Crude soluble promastigote antigen (CSA) was used in the ELISA kit and was collected by freezing and thawing of alive promastigote. The sensitivity of this test was reported to be 80%-100%, while specificity was ranged from 84-95% (Ryan et al., 2002). Its cross-reaction was observed mostly in three diseases, including toxoplasmosis, tuberculosis, and trypanosomiasis, when serum was collected from the infected patients (Kumar et al., 2001; Sundar and Rai, 2002; Elmahallawy et al., 2014). Recently, a specific promastigote antigen (BHUP3) of *L. donovani* has been found to have 12.6kDa size that is in the soluble form

and used for the diagnosis of visceral leishmaniasis with a specificity of 95% and low sensitivity up to 88%, while infected patients of malaria and tuberculosis showed only 3% cross-reaction (Kumar et al., 2011). From the previous two decades, a specific antigen named rK39 was used to detect visceral leishmaniasis (Sundar and Rai, 2002; Maia et al., 2012; Savoia, 2015). The results that were collected from different endemic countries like Nepal and India ranged from 93%-100% sensitivity and 97%-98% specificity of the antigen, although lower in East Africa but lower titer was observed against rK39 (Chappuis et al., 2006). A study was conducted about two specific hydrophilic antigens named rK9 and rK26 (*L. chagasi*) used for the diagnosis of visceral leishmaniasis (Mohapatra et al., 2010). The rKE16 was a different recombinant antigen that was available for the diagnosis of visceral leishmaniasis, but in China, Turkey, and Pakistan, the most sensitive antigen used was named rK39 (Sivakumar et al., 2006). In Bangladesh and Sudan, a new antigenic fusion protein was detected named K28 with a sensitivity and specificity of 96% and 98%, respectively (Pattabhi et al. 2010). Furthermore, for the serodiagnosis of visceral leishmaniasis, four types of heat shock proteins were also used named H2A, H2B, H3, and H4 (Pérez-Alvarez et al., 2001). Moreover, another type of antigen was also used called lipid-binding protein (LBPs) that has a peak value of sensitivity and has no cross-reactivity (Brito et al., 2000). Another study, 9.4% cattle (n = 13) were found positive the ELISA results. Out of 13 ELISA-positive cattle, only four (30.8%) were positive in direct agglutination tests (DAT) (Alam et al. 2011).

Western blotting (Immunoblotting) test

It is an advanced type of testing to diagnose the presence of visceral leishmaniasis. The log phase was used for the culturing of promastigotes, and SDS-PAGE was used for the separation of proteins. Those proteins were probed for the detection of antibodies in the patient's sera by the process of electro-transfer on a nitrocellulose film. This test provides a complete antibodies picture for different leishmanial antigens (Brito et al., 2000; Ravindran et al., 2004). The sensitivity of this test is better than ELISA and IFA (immuno-fluorescent assay) specifically for the detection of the disease in the patients suffering from HIV-VL co-infection (Cota et al., 2012).

Immuno-chromatographic test (ICT)

This is a secure, fast, and accurate method relying on the antigen named rK39 for the diagnosis of visceral leishmaniasis (Sundar et al., 2010). It is a highly sensitive and highly specific test that shows 100% accuracy even in low endemic areas and is also used for the diagnosis of HIV-VL co-infection (Monno et al., 2009). Different types of recombinant antigens of VL are accessible, but the rK39 type is the most efficient in Brazil and India but does not show more effectiveness in East Africa (Abass et al., 2013). The samples of urine and saliva could also be collected but have low sensitivity of parasite detection Phumee et al. (2013); Mohapatra et al., 2016). Another type of antigen named rKE16 collected from *L. donovani* showed the same result as rk39 sensitivity of 92.8-100%, especially in Brazil and Africa (Abass et al., 2013). Newly, a fast-significant test (TRALd) has used two relevant antigens named rK26 and rK39 for the diagnosis of visceral leishmaniasis with high sensitivity and specificity of 100% and 98%, respectively (de Paiva-Cavalcanti et al., 2015). However, diagnostic research on both human and canine sera samples showed that the sensitivity (S) of both ICTs for human samples (Ad-bio *Leishmania* IgG/IgM Combo Rapid Test and Kalazar Detect™) was 91.5% and specificity (E) were 93.2 and 89.2% respectively, while for the ICTs tested on canine samples (Kalazar Detect™ Rapid Test, Canine and DPP® CVL rapid test) we found S values between 82.9 and 85.7% and E values between 79.6 and 92.6%. They found *L. infantum* by PCR and sequencing in 2 human samples, and *L. braziliensis* and *L. amazonensis* in canine serum samples that were negative by both ICTs (Herrera et al. 2019).

Nucleic acid sequence-based amplification (NASBA) test

Due to advancements in the world, especially in the diagnostic field, this new test has been introduced that showed better results for the diagnosis of visceral leishmaniasis by using RNA sequence amplification (Srivastava et al., 2011; Basiye et al., 2010). This test is more applicable, PCR dependent, and has self-containing kits both for animals and human (Alam et al. 2011). Along with this, another test is introduced named the Oligo-test that has the same sensitivity and specificity, especially in the area of Sudan, Peru, and Kenya (Mugasa et al., 2010; Saad et al., 2010).

Polymerase Chain Reaction (PCR) and qPCR

Many types of molecular methods are used for *Leishmania* diagnosis, among which one is PCR which is an essential diagnostic tool used for the de-

termination of leishmaniasis to check out the immuno-suppressant level of the patient (Antinori et al., 2007). Samples are usually collected from different parts of the body, including splenic tissues, blood, aspirate of bone marrow, buffy coat to identify the DNA of visceral leishmaniasis (Martín-Sánchez et al., 2004). This method has outstanding specificity and sensitivity, but due to certain boundaries, this technique is only used for serology (Srividya et al., 2012; de Paiva-Cavalcanti et al., 2015). The asymptomatic type of leishmaniasis is easily be detected by PCR, and it could also diagnose re-infection in the patient (Cota et al., 2013; Fukutani et al., 2014). Different types of PCR are used as diagnostic tools like nested PCR, partial-nested PCR, and real-time PCR (de Paiva-Cavalcanti et al., 2015). Nowadays, different types of tests are also PCR based like urine analysis with 96.6% sensitivity and 100% specificity (Motazedian et al., 2008) and oral fluid dependent real-time PCR that has 94.6% sensitivity and 90% specificity (Galaï et al., 2011). It's quite important to mention that PCR is a quantitative technique used for the diagnosis of visceral leishmaniasis that requires very little time for a perfect diagnosis (Cruz et al., 2013). Real-time quantitative PCR (qPCR) is a quick molecular qualitative and quantitative technique used for the identification of *Leishmania spp* both in canine and human (Herrera et al., 2019) by using species-specific primers and has been used in many diagnostic research methods for the detection of coding and non-coding regions in the genome of *Leishmania* (Galluzzi et al., 2018).

Loop-mediated isothermal amplification (LAMP) test

It is a molecular test that does not have a complicated technology and maintains the cold chain to preserve the reagent and judge the turbidity of the mixture; thus, this test could easily be applied in the field. In Bangladesh, experiments were conducted by LAMP to check its sensitivity and specificity that ranged from 90.7% and 100% (Khan et al., 2012; Nzelu et al., 2019; Mukhtar et al., 2018). This technique also used to diagnose visceral leishmaniasis in cattle (Alam et al., 2011).

Detection of Species

Identification of species is essential for the diagnosis of a disease. With the advancement of new techniques and technologies, species identification is not so much problematic. To check the sequencing of the genotype of *Leishmania*, PCR amplification is used based on restriction fragment length polymorphism (PCR-RFLP), other tests like RAPD (random amplified polymorphic DNA) and SSCP (single standard conformation polymorphism) for genotypic examination (Almeida and Araujo, 2013). Multi-locus enzyme electrophoresis is also a new technique used for the last few years and is best for the detection of strain on enzymatic sequence (Schnian et al., 2011; Aluru et al., 2015). This method is the alternative of another technique called high-quality multi-locus sequence typing (MLST) that depends on the analysis of many genes (Van Der Auwera et al., 2014).

DIAGNOSTICS OF CUTANEOUS & MUCOCUTANEOUS LEISHMANIASIS

In cutaneous leishmaniasis, the parasite multiplies in the phagocyte present in the skin. The complex of *L. mexicana* and *L. braziliensis*is is the main causative agent that causes CL (Grimaldi et al.,1989; Kato et al., 2010; Zerpa et al., 2018). In mucocutaneous leishmaniasis, the disease only affects the mucosa of the oral cavity, nasal mucosa, pharynx, larynx, and the Infection is not cured so easily (Goto and Lindoso, 2010). The direct way to detect cutaneous leishmaniasis depends on the microscopic and histological examinations and culturing of parasites and indirect identification by molecular and serological tests (Masmoudi et al., 2013). The choice of the test depends on the accuracy required and the availability of the instruments.

Direct microscopy for cutaneous Leishmaniasis

Amastigote form was diagnosed in the biopsy lesions, stained by Giemsa, and elliptical or round shape parasitic bodies were seen under the microscope with typical nucleus and kinetoplast. Mostly the material was collected at the margins of the wound on the skin by scraping. The best result was shown by aspiration of the needle that has more chances to find out the amastigote type, micro-granuloma, comfortability of patients, and lesser background of the slide than skin scraping (Hosseinzadeh et al., 2012). There is another quick method, press imprint smear (PSI), that is simple and showed 85.3% results in assumed cases and 44% in the case of histo-pathological examination of CL (Sousa et al., 2014). Novy-MacNeal-Nicolle is a culture medium used for parasite culturing and showed less sensitivity due to the development of small culturing techniques but has some benefits like simple to use and showed extra sensitivity even parasite load was less (Boggild et al., 2008; Pagheh et

al., 2014). The major disadvantage of this technique is that it could not differentiate between different species (Pagheh et al., 2014).

Immuno-diagnostic methods for CL

The recent serological tests are mostly dependent on ELISA, immunofluorescence antibody test (IFAT), western blot, flow assay, and DAT (direct agglutination test) also used for canine leishmaniasis (Adel et al., 2016). But sometimes, these tests are not generally used for the detection of CL due to less humoral response by the Infection and thus showed less sensitivity (Soto et al., 1996; Zeyrek et al., 2007; Goto and Lindoso, 2010). A specific recombinant type heat shock protein (HSPs; HSP83) is used for the diagnosis of CL that has more sensitivity and specificity when evaluating ELISA-dependent *L. major* antigen. Another type of chemiluminescent ELISA used to check the sera of human antibody is named as anti-a-galactosyl, mostly found in the Infection of *L. tropica* or *L. major*. This technique has more sensitivity than a microscopic test. After two years of study, the level of titer of anti-a-Gal was found 28 folds more than normal healthy persons. Hence, it shows dual properties; as a diagnostic tool and as a biomarker (Al-Salem et al., 2014).

Intradermal *Leishmania* skin test (LST)

It is also named the Montenegro skin test (MST) which acts as an indicator of the cellular immune response, mostly used in the diagnosis of CL, and has 86.4% sensitivity that could also lead to 100%. The LST is considered positive if its size ranges between 6-14mm and is considered negative if its value is between 0-5mm. Moreover, if the size is above 15mm, then the test is considered strongly positive. If a patient illustrates negative effects, then there are more chances to be non-responsive to the treatment Antonio et al. (n.d.). The sole drawback of the test is that it needs more production of MST antigen and could not differentiate between the old and recent antigens (Schnorr et al., 2012). LST depends on the production of IFN-c (antigen-specific interferon-c) to check the *Leishmania* infection, and it showed more Infection when compared with IFN-c (Alimohammadian et al., 2012). This test also used in dogs and cats (Trevisan et al., 2015).

Amplification of nucleic acid for CL and MCL

Different types of tests are used for the amplification of nucleic acid of the *Leishmania* parasite, among which are PCR, isothermal platform type, and molecular identification of parasites are included *Leishmania spp* both in canine and human (Herrera et al., 2019). PCR is used for the amplification of many gene sequences, especially the spacer 1 sequence that is transcribed ribosomal DNA (Ogado Ceasar Odiwuor et al., 2011; Toz et al., 2013); Monroy-Ostria et al., 2014). The main objective is to diagnose the kinetoplast DNA of genus *Leishmania*, but other types of essays like high-Tec fluorescence resonance and HPS70 are also used for its identification (Reithinger and Dujardin, 2007; Khosravi et al., 2012; Satow et al., 2013; Jara et al., 2013; Tsukayama et al., 2013; Van Der Auwera et al., 2014). Recently an innovative test was also introduced named an isothermal platform that mostly targets the RNA sequence of the parasite for amplification (Mugasa et al., 2010). For the post-amplification study, we used oligo-chromatographic analysis to check the suitable diagnostic performance (Mugasa et al., 2010; Saad et al., 2010; Schnorr et al., 2012). Further progress in the diagnosis of CL is the loop-mediated isothermal reaction (LAMP) by using the Bst DNA polymerase enzyme for amplification of DNA within half to one hour. This reaction has high specificity since six primers are used at a time. At the start, the function of the LAMP test was to transcribe 18S units of rRNA, but nowadays, by use of reverse transcriptase enzyme, we could detect about 10 to 100 infected parasites per mL at a time, and its efficiency reaches up to 98% (Adams et al., 2010). Many research groups use this technology for the diagnosis of CL and have the advantage of being employed in the endemic areas where therapeutic resources are imperfect (Caliendo et al., 2013; Nzelu et al., 2014); Mikita et al., 2014).

Enzymes

Adenosine deaminase (ADA) present in the macrophages causes the deamination of adenosine to inosine. It is observed that level of ADA has been found to be elevated in the sera and in the lymphocytes of the patients affected with CL (Erel et al., 1998). The concentration of the enzyme decreases as the treatment progresses; this shows that level of ADA could be considered as a parameter for the diagnosis of CL (Vijayamahantesh et al., 2016). Arginase and nitric oxide synthase use a common substrate of L-Arginine. Arginine performs both functions; nitric oxide (NO)-mediated killing of the parasite and also promoted polyamine-mediated parasite replication. However, in the case of acute CL duration (duration ≤

1 year), the concentration of arginase activity is more than in chronic CL (duration ≥2 years) (Mortazavi et al., 2016). Adenosine deaminase activity (ADA) and its isoenzymes activity were also identified in dog sea (Erel et al., 1998).

PROTEOMIC AND TRANSCRIPTOMIC APPROACHES FOR THE DIAGNOSIS OF *LEISHMANIA*

Leishmania regulates gene expressions at the post-transcriptional and post-translational levels. Therefore, a weak correlation is experienced between mRNA contents and translated proteins. The completion of the genomic sequencing of different leishmanial species has enormous importance in the study of leishmaniasis. The combination of the accessible genomic resources of these parasites with proteomic as well as transcriptomics analysis has shed light on different characteristics of leishmania biology as well as on the mechanisms underlying the disease (de Jesus et al., 2014; Santos, 2014). Different proteomic and transcriptomic approaches have been used to designate and catalog global proteins and RNA profiles of *Leishmania* species, expose alteration in their expression during development, determine the subcellular localization of gene products, evaluate host-parasite interactions, and explain the drug resistance mechanisms. The characterization of these proteins and RNAs has advanced, although several fundamental questions remain unanswered. The recurrent identification of a group of proteins and RNAs isolates that relate to the most abundant ones (in *Leishmania* and different biological systems) precludes the identification of the less abundant proteins and RNAs and obscures the studied phenomena (Veras and De Menezes 2016; Rastrojo et al. 2018). So, modern transcriptomic and proteomic technologies with bioinformatics tools and databases may be appreciated for better utilization and interpretation of Leishmania biology and for revealing inter and intra-specific discriminations (Sundar and Singh, 2018).

BIOMARKER APPROACH FOR THE DIAGNOSIS OF *LEISHMANIA*

In this advanced technique, used for the identification of parasites, the parasitic proteins are used as a biomarker to distinguish between healthy and diseased persons. Biomarkers of various forms are derived at different larval stages (Ndao, 2009) and used as an indicator of the normal biological process as well as an efficient method for diagnosis, prognosis, and monitoring of the disease progression (Mayeux, 2004). The use of biomarkers is considered to be safe, sensitive, and has the potential to diagnose the disease in different genders and ethnic groups (Theppeang et al., 2008; Strimbu and Tavel, 2010). Due to the formation of different lesions, the diagnosis of CL is considered a little bit difficult task, so modified and more sensitive diagnostic biomarkers are required (Akhoundi et al., 2017). In conditions like bacterial ulcers, leprosy, sarcoidosis, and lupus vulgaris, similar symptoms are observed as that of CL. Previously, the visual method by direct smear followed by culturing and animal inoculation were used by (Maia and Campino, 2018). With the passage of time, diagnostic methods at the molecular level got attention as they could differentiate between different species of leishmania by using specific probes. Some non-invasive techniques, like DNA isolation, followed by parasitic detection by using PCR, are also used (Taslimi et al., 2017).

T-Cell sub-populations and cytokines as putative biomarkers

Researches show that in the case of *L. major,* Th1 and Th2 are parts of regulatory CD^{4+}. T cells are supposed to be responsible for controlling the Infection (Alexander and Brombacher, 2012); however, in humans, these facts are not clear. Due to excessive secretion of pro-inflammatory IFN-γ and cytokine, Th1 cell-mediated immune response cause problems resulting in the formation of lesions (Maspi et al., 2016). Two other T cell populations Treg and Th17, are considered to regulate immunity to CL. Treg controls excessive damage during inflammatory responses (Suffia et al., 2005). Functional Treg moved towards the lesion site in the case of CL caused by *L. braziliensis* and *L. guyanensis* (Campanelli et al., 2006). Th17 is supposed to maintain a balance between pro and anti-inflammatory cytokines in CL and attract the neutrophils towards the site of Infection (Gonçalves-de-Albuquerque et al., 2017). IL-10 is considered a biomarker indicator if treatment is failed. In CL lesion, the mRNA of regulatory cytokine is directly linked with treatment failure. CL lesion size is directly associated with the production of TNF-α (Bourreau et al., 2001). Cytokine and phenotypic cell profiles of *leishmania infantum* infection in the dog were also measured (Maia and Campino, 2012).

CONCLUSION AND FUTURE ASPECTS

While the transmission modes, characterization,

and pathophysiology related to all *Leishmania* species are well recognized, understanding and detection of this intracellular protozoan at an early stage has lagged. Many reports of severe clinical outcomes are present due to lacking viability. However, the groundwork of understanding the interactive mechanism of the parasite with an immune system from a diagnostic perspective is progressing. This review briefly focuses on the morphology, its transmission and immunological as well as molecular diagnostic techniques that are considered more specific and sensitive. However, it requires a highly equipped laboratory with expert personnel to perform these tests. There are still factors that cause the paucity in serological and molecular detection mechanisms and limit the generalization of the recent knowledge of leishmaniasis; that needs to be studied and resolved. In the modern era, omics studies **(genomics, transcriptomics, proteomics, and metabolomics)** are useful approaches in the diagnosis and treatment of leishmaniasis. The use of mathematical models to study, cost-effective early detection of *Leishmania* species, and to explore valuable and implementable control approaches remains an active and study-worthy area in future research.

ETHICAL STANDARD

Not applicable

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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