Development of a PCR based diagnostic system for kala azar

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF DHAKA IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF PHILOSOPHY IN MICROBIOLOGY

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Dedicated

To

My Beloved Parents

Certification

It is hereby certified that the thesis entitled **"Development of a PCR based diagnostic system for kala azar"** submitted by Mahbuba Khatun, Reg. No. – 225, Session: 2009 – 2010, carried out her research under our supervision. It is further certifying that is an original work and suitable for the partial fulfillment of her Master of Philosophy Degree in Microbiology from the University Of Dhaka, Bangladesh.

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Declaration

I hereby declare that the whole work submitted as a thesis entitled "**Development of a PCR based diagnostic system for kala azar**." in the Department of the microbiology, University of Dhaka, for partial fulfillment of the requirements for the degree of Master of Philosophy in Microbiology under the joint supervision of Dr. Md. Manjurul Karim, Professor, Department of Microbiology, University of Dhaka and Dr. Md. Shariful Alam Jilani, Professor & Head, Department of Microbiology, Ibrahim Medical College & BIRDEM Hospital, Dhaka.

I, further declare this thesis or part thereof has not been concurrently submitted for the award of any Degree or Diploma anywhere.

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Abstract

Kala-azar or black fever is a severe form of Leishmaniasis, a disease caused by protozoan parasite, *Leishmania* spp. one of the largest parasites-borne illnesses, this disease could be fatal, if untreated, as a consequence of infection to different internal organs such as liver, spleen, and bone marrow resulting in death. The conventional approach for the diagnosis of the disease relies on collecting samples from bone marrow and splenic tissues from suspected patients, a method which is not only painful, stressful and risky for patients, but also cumbersome. This prompted us to develop a simple, rapid PCR-based diagnostic assay for the detection of *Leishmania* using patient's venous blood sample. A total of thirty five clinically suspected febrile patients who had sign and symptoms for the disease, and produced positive reaction for rk39 immunochromatographic test were taken for the diagnosis. In addition to the blood samples, specimens from bone marrow/ splenic aspirations were collected from those individuals. While presence of *Leishmania* spp. was confirmed in twenty six patients either by microscopy and by culture on Nicolle Novy McNeal (NNN) culture media from the samples collected from bone marrow/spleen aspirations of patients with Kala-Azar, however their buffy coat preparations of blood samples could not produce the same, indicating the unsuitability of the blood samples to be used as a specimen for diagnosis by conventional methods. In an attempt to address whether those blood samples could be used for diagnosis, a pair of oligonucleotides was designed using conserved sequences of kinetoplast DNA minicircles of *Leishmania* spp and was used as primers to detect the presence of *Leishmania* genome in a polymerase chain reaction. In addition, blood samples collected from sixteen and twenty five healthy individuals from the endemic and non-endemic regions respectively, and twenty five patients with other similar diseases (TB, malaria and dengue) were used as controls. All the twenty six blood samples from patients with Kala-azar yielded specific amplifications in PCR, while blood samples taken as controls produced no amplicon, producing the sensitivity and specificity records as 98% and 100% respectively. Such an analysis was found superior when compared with two other sets of primers reported in the literature. The identity of the amplicon was subsequently confirmed as *Leishmania* by DNA sequencing and BLAST search. Therefore, the primers

designed for this study could be used to score the presence of Leishmania in a PCR-based detection system.

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Abbreviations

Symbols	
%	Percentage
&	and
≤	Less than equal
≥	Greater than equal
°C	Degree Centigrade
cm	Centimeter
µg	Microgram
µl	Microliter
g	Gram
M	Molar
ml	Milliliter
mm	Millimeter
mM	Millimolar
General Terms	
ACP	Acid phosphatase
AIDS	Acquired immune deficiency syndrome
AMP	Adenosine monophosphate
APC	Antigen presenting cell
AT	Aldehyde test
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CDC	Centers for Disease Control
CFT	Complement fixation test
CL	Cutaneous Leishmaniasis
DAT	Direct agglutination

DDT	Dichloro Diphenyl Trichloroethane
DGHS	Director General for Health Services
DNA	Deoxy ribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTH	Delayed type of hypersensitivity
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme linked immunosorbant assay
e.g.	Examplia gratia
et al	at alia (all others)
FCS	Foetal calf serum
GBP	Gene B protein
gp	Glycoprotein
Hsp	Heat shock protein
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
ICDDRB	International Center for Diarrhoeal Diseases and Research, Bangladesh
ICT	Immunochromatography test
IFAT	Indirect fluorescent antibody test
IgG	Immunoglobulin G
IL	Interleukin
INF	Interferon
IU	International unit
KA	Kala-azar
KCl	Potassium chloride
kDa	Kilo-dalton
kDNA	Kinetoplast DNA
kb	Kilo base
kbp	Kilo base pair

L	*Leishmania*
LAAMB	Lipid associated amphotericin B
LACK	Leishmania homologue of receptor for Activared C Kinase
LAT	Latex agglutination test
LD bodies	*Leishmania donovani* bodies
LdMT	*L. donovani* miltefosine transporter
LdRos	*L. donovani* Ros protein
LMDR	*Leishmania* multi-drug resistant
Ln-PCR	Leishmania nested Polymerase chain reaction
LPG	Lipophosphoglycin
LST	Leishmanin skin test
M-CSF	Macrophage colony stimulating factor
MHC	Major Histocompetibility complex
MIF	Macrophage inhibitory factor
MgCl2	Magnesium chloride
medRNA	Mini exon derived RNA
MDR	Multi-drug resistant
n	Number
NCBI	National Center for Biotechnology Information
NNN	Novy McNeal Nicolle
NO	Nitric oxide
NK	Natural killer
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PKDL	Post kala-azar dermal leishmaniasis
PCR-SHEL	PCR solution hybridisation enzyme linked assay
PCR-SSCP	PCR single stranded conformation polymorphisms
pH	Negative logarithm of hydrogen ion concentration

RT PCR	Reverse Transcriptase PCR / Real time PCR
RNA	Ribonucleic acid
RPM	Rotation Per Minute
Sec	Second
TB	Tuberculosis
UV	Ultraviolet
WHO	World health Organization

Chapter 1

INTRODUCTION AND OBJECTIVES

1.0 Introduction

1.1. General Introduction

The protozoan parasite *Leishmania donovani* is the causative agent for visceral leishmaniasis (VL) or kala-azar (KA), which is a vector born disease. It affects people in many tropical and sub tropical region and is transmitted by the bite of female phlebotomine sand fly (*Sacks, 2001; Sacks and Kamhawi, 2001*). Clinical forms of leishmaniasis are numerous, depending on the species of *Leishmania* and the genetic background of the patient, different form includes kala-azar (KA) or visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (MCL), zoonotic cutaneous leishmaniasis (ZCL) and post-kala-azar dermal leishmaniasis (PKDL). Kala-azar is typically caused by the *Leishmania donovani* complex, which includes three species: *L. donovani*, *Leishmania infantum*, and *Leishmania chagasi*. *L. donovani* is predominantly found in Indian subcontinent (*Pearson et al, 1996; Begum et al., 2002,*).

Leishmania causes a large spectrum of diseases, ranging from spontaneously healing skin lesions to fatal visceral symptoms. The visceral form, also known as black sickness or kala-azar in Asia, is characterized by prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, pancytopenia, and hypergammaglobulinemia and is complicated by serious infections (*WHO, 1984*). It is the most severe form of the disease and if left untreated, is usually fatal (*Ashford, 2000*). Leishmaniasis development depends on several risk factors such as malnutrition, immunosuppression and genetic factors (*Assimina et al., 2008).*

Kala azar afflicting primarily rural and periurban populations exposed to the infected sand fly vector. These sand flies are breed in damp soil, cracks and crevices of mud house and for that reason it mainly affect people of lower socioeconomic group *(Ahluwalia et al, 2003*) . The parasite exists in two morphological stages: Amastigote stage which

occurs in mammalian and Promastigote stage which occurs in sand fly. The amastigote form residing in the phagolysosomal system of mononuclear phagocytic cells in human reticulo-endothelial (RE) system, like macrophage, neutrophil and endothelial cells and multiplies by binary fission till the RE cell becomes enlarged (*Antonie et al, 1998; Rittig and Bogdan, 2000*). The cell is ruptured and the parasites are liberated in the circulation and invade fresh cells; the cycle is repeated until all cells of RE system are affected. During blood sucking, sand flies draw these amastigote. In the insect gut amastigote transform into promastigote, multiply by binary fission, from which they are introduced into a new host when the sand fly again feeds (*Molyneux et al, 1987; Killick- Kendrick, 1990*). Proliferation of RE cells leads to massive splenomegaly and hepatomegaly. The bone marrow is also involved, compression of bone marrow resulting in pancytopenia.

The World Health Organization considers leishmaniasis as one of the most important neglected parasitic diseases (*WHO, 1990; Herwaldt B L, 1999).* Leishmaniasis is endemic in 88 countries. World Health Organization (WHO) has estimated that more than 12 million people are currently infected with *Leishmania and* around 2 million infections occur each year *(Rai et al., 2008)* and 350 million people at risk of contracting the disease (*Desjeux P, 2004; Murray et al., 2005*). Recently the WHO estimated that worldwide about 200,000 to 400,000 new cases of VL and 50,000 to 60,000 deaths occur annually with the majority of VL cases (>90%) concentrated in only four countries: Bangladesh, India, Brazil and Sudan *(WHO 2010; Alvar et al. 2012)*. The official estimate of 430,000 VL cases in Bihar State of India over the past 11 years may represent only a fraction of the real numbers. The actual number is believed to be at least five times as great *(Lira R et al, 1999).*

In Bangladesh kala-azar cases were reported from 43 of 64 districts, including 105 upazillas *(Bern C et al, 2007)*. More than 90 percent of cases were reported from just 10 districts. Mymensingh district representing over 50% of kala azar cases followed by Pabna, Tangail, Jamalpur, Sirajganj, Gazipur, Natore, Naogaon, Manikganj, Rajshahi and Nawabganj, although there is substantial under reporting *(Rahman et al,2008).* The estimated numbers of active cases were 1, 36,500 in the year 2007 however; only less

than 5,000 cases were reported in that year *(Joshi et al, 2008)*. A survey in 2008, showed that the prevalence of PKDL is 6, 2/10,000 population – years *(Mondal et al, 2010).*

One of the major threats to control of visceral leishmaniasis (VL) is its interaction with HIV infection. Visceral leishmaniasis (VL) has emerged as an opportunistic infection associated with HIV *(Alvar et al, 2008; WHO,2013)*. The risk of developing active VL is between 100 to 2320 times higher in case of concomitant HIV infection *(Ababa A, 2007)*. In Southern Europe, up to 70% of cases of Visceral Leishmaniasis in adults are associated with HIV infection. HIV infected people are particularly vulnerable to VL, while VL accelerated HIV replication and progression to AIDS thus the two diseases are mutually reinforcing. Till date as many as 35 countries throughout the world have reported the cases of VL/ HIV co infection *(Desjeux P 1998, Desjeux P et al 2000, 2003)*. Till now no VL/ HIV co infection has been reported from Bangladesh.

Kala azar is a progressive disease and the mortality rate in untreated cases ranges from 75% to 95%. Death usually occurs within 2 years *(Salam et al, 2009)*. In a relatively mild form, the disease may persist for many years, but can be cured by successful treatment after accurate diagnosis. So, early diagnosis of leishmaniasis is important in order to avoid severe clinical manifestations to the patient including mortality for VL patients.

Laboratory diagnosis of leishmaniasis rests upon: (i) Identification of parasite in bone marrow or splenic aspiration by light microscopic examination of the stained specimen (ii) in vitro culture in specific media, (iii) detection of parasite DNA in different samples; or (iii) immune diagnosis by detection of parasite antigen in different samples, or by detection of nonspecific or specific anti Leishmanial antibodies (immunoglobulin) in serum *(Sundar and Rai, 2002).*

Spleen, bone marrow, or lymph node aspirated stained smears are used for identification of parasites in light microscopy *(WHO, 1984; Zijlstra et al, 1992).* Microscopy is invasive, as spleen or bone marrow aspiration is required as a sample. Although the splenic smears are highly sensitive (95%), but the recovery of splenic tissue carries the risk of fatal hemorrhage and may leads to death *(Kager et al., 1983)*. This procedure also

requires expert personnel *(Boelaert et al 2007)*. The sensitivity of bone marrow smears is unsatisfactory (56%) *(Salam et al, 2010).* Bone Marrow aspiration is cumbersome and very much painful. It is not also helpful in detecting early infection *(Chowdhury et al., 1993; Boelaert et al., 1999).*

The Aspirates (bone marrow, spleen or lymph node) can also be used to culture in NNN (Nicolle, Novy, and McNeal) media. Culture might improve the sensitivity; but it is expensive, time consuming, needs expertise and sophisticated equipments, so not suitable for field diagnosis *(Lightner et al 1983, Sundar and Rai, 2002).*

Currently used serological tests includes Aldehyde test [AT], Complement fixation test [CFT], Indirect fluorescent antibody test [IFAT], direct agglutination test [DAT], rK39-based rapid immunochromatographic test [ICT], enzyme-linked immunosorbent assay [ELISA] etc.

Aldehyde test [AT] is simple and highly sensitive (74.6%) *(Parvin R et al, 2007)*. But it becomes positive after 3 months of infection and it is a non specific test, it becomes false positive in other diseases associated with hypergummaglobinaemia *(Chandra J, 1994; Koirala S 2004).* Complement fixation test [CFT] is useful for diagnosing early cases (in 3 weeks) of Kala azar *(Chih et al, 1953)*. CFT is a non specific test and become positive in tuberculosis, leprosy and Chagas disease *(Chandra J et al 1994).* It gives false positive results even in normal population of an endemic area *(Monsur et al 1957, Rahman et al, 1983).*

A promising ready-to-use immunochromatographic [ICT] strip test based on rK39 antigen has been developed as a rapid test for use in difficult field conditions. Several studies from the Indian subcontinent reported the test is 100% sensitive and 98% specific. But antibodies against rK39 may be present in serum for an extended period after treatment for VL; thus, patients with suspected relapse of VL with a past history of infection would not be candidates for diagnosis by strip testing. Another drawback of this test is that an individual with a positive rK39 strip test result may suffer from another illness (es) like malaria, typhoid fever or tuberculosis with clinical features similar to those of VL yet is misdiagnosed as suffering from VL. Notwithstanding these limitations,

the rK39 immunochromatographic strip test has proved to be versatile in predicting acute infection, and it has an acceptable sensitivity and specificity levels in diagnosis of VL. It is also inexpensive, simple and can be performed even by paramedics in prevailing difficult field conditions *(Rahman et al, 1983).*

Enzyme Linked Immunosorbent Assay [ELISA] has a sensitivity of 90% and specificity of 100% and can be a tool for epidemiological studies *(Srivastva et al, 1988).* Direct agglutination test [DA T] has a sensitivity and specificity of 100% if a titer of 1:1600 is considered suggestive of Kala azar. Latex agglutination test [LAT] has a relatively lower sensitivity (80%) and specificity (96%). Indirect fluorescent antibody test [IFAT] detects antibodies against Leishmania that appears early in the course of the disease and persists as long as six months after cure. This test, therefore, can also be used for monitoring treatment *(Manson Bahr et al, 1991).*

However the sensitivity and specificity of such diagnostic methods depends on the type, source and purity of antigen employed, as some of *Leishmania* antigens have common cross-reactive epitopes shared with other microorganisms *(Sarkari et al., 2005).* Many of them have low sensitivity and specificity. Immunological methods fail to distinguish between past and present infections thus limiting their use for the diagnosis of relapses or reinfections and detecting asymptomatic infections in areas of endemicity. Moreover, they are not reliable in case of immunocompromised patients *(Lachaud et al, 2000; Salotra et al 2001).* Serological methods usually failed to detect VL in HIV/ VL co infected patients. Furthermore, these methods cannot address the problem of species identification, which is important for determining appropriate treatment regimens and designing control measures. For this reason, development of a specific and sensitive method for early and rapid detection of kala-azar has become very much essential.

Amongst the molecular methods used for clinical diagnosis, PCR has been proved to be most sensitive and specific technique. In recent years, PCR-based diagnostic methods have been evaluated for the diagnosis of visceral leishmaniasis which has shown excellent sensitivities and specificities *(Adhya et al 1995, Pizzuto et al 2001; Wortman et*

al 2001; Motazedian et al 2002; Gangneux J Pet al 2003; Schonioan et al 2003; da Silva, 2004). The PCR assay can detect parasite DNA or RNA a few weeks ahead of appearance of any clinical signs or symptoms. The specificity of the PCR can be adapted to specific needs by targeting conserved region of the gene. Gene amplification through the PCR has several advantages compared to traditional techniques, because of its extremely high sensitivity, rapidity and the ability to be performed with a broad range of clinical specimens. Also the detection or identification of the causative agent is possible directly from the clinical specimens. The important gene targets are 18S-rRNA, small subunit rRNA (SSU rRNA), a repetitive genomic sequence of DNA, the miniexon (spliced ladder) gene repeat, the β-tubulin gene region, gp63 gene locus, internal transcribed spacer (ITS) regions; micro-satellite DNAs such as maxi- and minicircles of kinetoplast DNA *(Santos-Gomes et al., 2000; El Tai et al., 2001; Wortman et al., 2001; Ostria et al, 2002).* Different researchers use different target for kala azar identification and their sensitivity and specificity varies.

We have designed a new primer which is *Leishmania donovani* complex specific. A major target of this primer is the kinetoplast mini-circle DNA, which is present at thousands of copies per cell and will be used as targets for selective amplification of parasite DNA *(Jha et al 1999).* The identification of conserved sequence elements represented within the kinetoplast DNA (kDNA) of a given species of *Leishmania* would allow to use for species-specific identification of parasites in clinical samples. PCR was done with our designed primer, and its amplification pattern is compared with the amplification pattern of other existing primers which is previously used in others work. Designed new primer's efficacy is observed by comparing the PCR result with the gold standard (culture and microscopy) tests. PCR is found to be sensitive enough to detect parasite DNA from buffy coat of peripheral blood of patients with KA, which is less invasive and allow us to avoid invasive procedures like, spleen or bone marrow aspiration. As PCR require only a few hours to complete, so rapid identification is possible by this method. Thus, the result of the study will help the clinicians to minimize the mortality and morbidity from kala azar by fast accurate diagnosis and early management of the disease.

1.2. Aims and Objectives:

Aims:

The aim of this study is to develop a PCR-based less invasive diagnostic system for rapid detection of kala azar by using peripheral blood.

Specific objective:

1. To design new primers for a PCR-based detection system for the presence of *Leishmania donovani* in buffy coat of peripheral blood.
2. To develop a less invasive technique for kala azar detection from peripheral blood.
3. To compare the efficacy of the newly designed primer with that of the primers currently used in overseas.
4. To compare the PCR-based detection system with conventional culture and microscopy.
5. To sequence the PCR amplified region to see the primers identity.

Chapter 2

LITERATURE REVIEW

2. Review of Literature:

Leishmaniasis caused by several species of Leishmania, is transmitted by blood feeding sand flies and causes infection in viscera e.g. liver, spleen, and bone marrow, skin and mucous membrane. The natural transmission is carried out by a certain species of sand fly of the genera *phelobotomus* (Old World Leishmaniasis) and *Lutzomya* (New World leishmaniasis) *(Joshi et al, 2008).*

The clinical manifestations of the leishmaniasis depend on complex interactions between the virulence characteristics of the infecting *Leishmania* species and the immune responses of its host. The result is a spectrum of disease ranging from localized skin lesions to diffuse involvement of the reticuloendothelial system. Human disease has traditionally been divided into three major clinical syndromes visceral, cutaneous and mucocuteneous leishmaniasis; however, a number of variants exist. Furthermore, a single *Leishmania* species can produce more than one clinical syndrome and each syndrome is caused by multiple species *(Pearson and Sousa, 1996).*

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2.1. Historical Review

Sir William Leishman, a Glasgwegian doctor serving with the British Army in India, developed one of the earliest stains of Leishmania in 1901. In Dum Dum, a town near Calcutta, Leishman discovered ovoid bodies in the spleen of a British soldier who was experiencing bouts of fever, anemia, muscular atrophy and swelling of the spleen. Leishman described this illness as "dum dum fever" and published his findings in 1903. Lnt. Col. Charles Donovan also recognized these symptoms in other kala-azar patients and published his discovery a few weeks after Leishman. After examining the parasite using Leishman's stain, these amastigotes were known as Leishman-Donovan bodies and officially, this species became known as, *L. Donovani*. By linking this protozoan with kal-azar, Leishman and Donovan discovered the genus, *Leishmania (Encyclopedia Americana 1969).* The genus Leishmania was created by Ross in 1903 to include *Leishmania donovani.* In India, the disease is known as kala-azar, meaning "black sickness or fever" as the disease turns the color (pigmentation) of the skin black, the word '*kala*' means '*black'* and '*azar'* means '*deadly'*, thereby, signifying a fatal illness *(Chatterjee, 1982)*. In 1904,

Roger cultivated the organism in sodium citrate solution and observed the conversion of amastigotes to promastigotes in culture *(Chulay, 1991)*.

Similar parasites were observed in a disease of children in the mediterrenean countries by Cathorie in 1904, Pianese in 1905 and Nicolle in 1908. Nicolle proposed the name of infantile kala-azar for this disease and designated the parasite as *L. infantum (Chatterjee, 1982).* Further investigation revealed that it was a strain of *L. donovani.* The parasite of South American VL originally named as *L. chagasi* in 1937, was also found to be identical to *L. donovani.*

Alder and Theodor found Promastigotes in sand flies in 1925 *(Chulay, 1991)***.** In 1940, it as was demonstrated the *Plebotomus argentipes* was the vector of India kala-azar. Nicolle in 1908 reported that mammals including dogs could act as reservoir hosts for the *leishmania* parasite. *Swaminathan et al* in 1942, proved using human volunteers that the *leishmania* parasite could be transmitted by the phlebotomus sandflies.This is not to say that leishmaniasis did not exist before 1903, on the contrary. *Archibadi in 1922,* described an epidemic of Kala-azar which occurred in the Garo hills of Assam as far back as 1870.

Irregular epidemic waves have swept through Assam, Bengal and Bihar since the 1800s with a frequency of 15-20 years *(Birley, 1993).* In 1890-1900 an epidemic swept Assam, which depopulated whole villages and reduced populations over large areas. In 1917 another epidemic started in Assam and Bengal and reached its height about 1925 and mysteriously subsided, unit by 1931, it was almost disappeared. A new outbreak began in Bihar In 1937. Before the Second World War, kala-azar was endemic in Assam, Bengal (a part of which is now Bangladesh), Bihar and some other parts of India subcontinent. Within 1958-1964 kala-azar almost disappeared from eastern states of India and Bangladesh due to insecticide spraying as a part of malaria eradication campaign. But in 1977 with the cessation of insecticide spraying there has been a resurgence of VL in these regions *(Rahman et al., 1983).*

2.2. Geographical Distribution, Prevalence and Incidence

Visceral leishmaniasis (VL) has a worldwide distribution in more than 88 countries in four continents. Visceral leishmaniasis is endemic in Europe in Mediterranean and neighboring countries; in Asia, north and west China, Middle East, Central Asia, India and Bangladesh; in Africa on the Mediterranean region, East and part of West Africa; and Central and South America *(Desjuex, 1993)*.

Although it is thought to be under reported, rough estimate suggests that 350 million people are at risk of the infection and approximately 12 million are infected with Leishmania species (Ashford *et al.,* 1992). Annual incidence of VL is around 5,00,000 cases, ninety percent of all VL cases occur in Bangladesh, India, Nepal and Sudan *(Desjuex, 1993)*. Post kala-azar dermal leishmaniasis (PKDL) is sequelae of VL. It occurs in about 20% cases of VL in India and Bangladesh while only in 2% cases in Africa *(Manson–Bahr et al, 1991).*

In neighboring India Kala-azar is endemic in the states of Bihar, Uttar Pradesh and West Bengal. One of the largest epidemics occurred in 1978 in North Bihar were over half a million people fell victim to Kala-azar. In the first eight months of 1982, 7500 cases were reported in India and in one year alone between 1987 and 1988, 22 000 cases of Kala-azar were registered *(WHO, 1991)*. Recently the foci of Kala-azar has shifted from rural villages to large cities probably as a result of migration of settlers from villages into these cities creating dense population and living in sub-standard house with improper sanitation and keeping farm animals in their gardens. In most countries, males are almost twice more likely to be Kala-azar than females, with young children being at the highest risk. Kala-azar is also endemic with higher prevalence among children below the age of 15 years. Males are three times more susceptible than females *(Shiddo et al., 1995)*.

Table2.1: Reported and estimated incidence of visceral leishmaniasis in the Indian subcontinent and Southeast Asia in 2012 (Alvar J et al, 2012)

Country Name	Reported VL cases / year	Years of report	Estimated annual VL incidence		
Bangladesh	6224	2004–2008	12,400	to	24,900
Bhutan	2	2005–2009	10	to	20
India	34,918	2004–2008	146,700	to	282,800
Nepal	1477	2004–2008	3000	to	5900
Thailand	2	2006–2010	25	to	105
Sri Lanka	no data		6	to	10
Region	42,623		162,100	to	313,600

2.3. Leishmaniasis in Bangladesh:

The parasitic disease Kala-azar was first described in 1824, in Jessore district of Bengal which is now in Bangladesh *(Bern et al, 2006).* Available reports show that the current prevalence is estimated to be 45,000 in Bangladesh. And it is endemic in 34 districts out of 64 districts but 90% of them are from 10 districts *(Rahman et al, 2008)*. The cumulative reported incidence of kala-azar by district from 1994 - 2004 shows that the worst hit area in Bangladesh is Mymensingh followed by Pabna, Tangail, Jamalpur, Sirajganj Gazipur, Natore, Naogaon, Manikganj, Rajshahi and Naawabgaj *(Ahasan et al.,2008)*. In the past decade, there has been a resurgence of VL in Bangladesh. An incidence rate of one per thousand (1/1000) population in affected areas is considered at present a conservative estimate as the disease is under reported and continuously spreading to areas previously free from the disease. It also revealed that about 90% of

cases of Kala-azar came from low-income group who were living in the houses plastered with mud and cow-dung *(Sarker et al, 2003).*

2.4. Etiology

2.4.1. Causative Agents

Leishmaniasis is caused by different species of *Leishmania.* Seven complexes consisting of 17 Leishmania species i.e. *Leishmania donovani complex, Leishmania tropica complex, Leishmania major complex, Leishmania aethiopica complex, Leishmania mexicana complex, Leishmania braziliensis complex and Leishmania guyanensis complex* have been identified as causative agents of leishmaniasis all over the world *(WHO, 1990)*.

Visceral leishmaniasis is caused by parasite species of the Leishmania donovani complex that include *Leishmania donovani, Leishmania donovani infantum* and *Leishmania donovani chagasi* (in this thesis the three species has been referred to as *L. donovani, L. infantum* and *L. chagasi* respectively). Some undefined or unspecified species belonging to the *L. donovani* complex has been isolated from VL patients in Kenya, Ethiopia and Somalia *(Pearson and Sousa, 1990).*

Old World, anthroponotic visceral leishmaniasis is caused by *L. donovani i*n India, Bangladesh, Nepal, parts of China and East Africa. VL is believed to be caused by *L. donovani* other species has been identified as mentioned above. Zoonotic visceral leishmaniasis in Mediterrenean region, China, Middle East and parts of Sub-Shara Africa is caused by *L. infantum*. New World zoonotic visceral leishmaniasis is caused by *L. chagasi* and *L. infantum*.

Old world cutaneous Leishmaniasis is caused by *L. tropica*, *L. major* and *L aethiopica;* the latter is also the etiological agents of diffuse Leishmaniasis. In the New world, cutaneous Leishmaniasis is mainly caused by *L. braziliensis, L. Mexicana, L. amazonesis, L. venezuelensis, L. panamensis and L. guyanensis, L. braziliensis and L. panamensis* are mainly responsible for mucocuteneous leishmaniasis, a condition in which cutaneous lesion spreads to the mucosa of the mouth and nose.

Table 2.2: Summary of the main features of old world Leishmaniasis (Arnold H et al, 2005)

Leishmania spp.	Diseases in human	Geographical distribution	Important mammalian hosts	Impotant sandfly hosts
Leishmania donovani	Visceral leishmaniasis(VL), Kala – azar(KA), Post Kala Azar Dermal Leishmaniasis(PKDL).	Indian subcontinent, East Africa	Humans	Phlebotomous argentipes, P. orientalis, P. martini
Leishmania major	Rural, zoonotic, cutaneous leishmaniasis, oriental sore.	North Africa, Sahel of Africa, Central and west Asia	Great gerbil, *Rhombomys opimus*, fat sand rat, *Psammomys obesus*	Phlebotomous papatasi, P. dubosqi, P. salehi.
Leishmania tropica	Urban, anthroponotic cutaneous leishmaniasis, oriental sore.	Central and West Asia.	Humans.	Phlebotomous sergenti.
Leishmania aethiopica	Cutaneous leishmaniasis, diffuse cutaneous leishmaniasis.	Ethiopia, Kenya	Rock hyraxes, Heterohyrax brucei and Procavia spp	Phlebotomous longipes, P. pedifer
Leishmania infantum	Infantile visceral leishmaniasis	Mediterranean basin, Central and West Asia.	Domestic dog	Phlebotomous arisi, P. pernicious.

2.4.2. Reservoirs

Broadly There are two types of VL, namely zoonotic VL and anthroponotic VL. In the anthroponotic form, humans act as reservoir and in the zoonotic form animals are the reservoir *(WHO, 1990)*. In the anthroponotic form, humans are directly involved as reservoir which are predominantly found in India, Bangladesh and some countries of East Africa, *(WHO, 1990)*. Dogs are the principal reservoir in the zoonotic form, predominantly found in Europe and Africa around the Mediterrenean regions. Dogs are also important reservoirs in China and south and Central America. Foxes with in apparent infection are the reservoir in southern France and central Italy. The common peri-domestic rat *Rattus rattus*, has been found to be infected with various Leishmania species in both Old World and New World. While its role in the maintenance of parasite populations has not been fully established, it is strongly suspected as a secondary reservoir host of *L. infantum* in Italy. *L. donovani* has been isolated from *Arvicanthis nilotica* and other rodents in Sudan and rodents are probably important in maintaining enzoonotic foci in interepidemic period *(WHO, 1990).*

2.4.3. Vectors

The only proven vectors of Leishmania is Sand fly which is a invertebrate hosts are small insects of the order Diptera, belonging to the *Phlebotominae* subfamily and only two of the six genera described are of medical importance: *Phlebotomus* of the "Old World" (Africa, Asia, and Europe) and *Lutzomyia* of the "New World" (the Americas) *(Killick-Kendrick, 1990).*

In the Central and South America, *Lutzomyia longipalpis* is the main vector for transmission of VL. In the Mediterrean region, *Phlebotomus pernicious chinensis* and *Phlebotomus alexandri* are the proven vectors. In East Africa including Sudan, *Phlebotomus martini* and *Phlebotomus orientalis* and considered as vectors for the disease. In India, for the anthroponodic form of VL the proven vector is *Phlebotomus*

argentipes. Besides, many other species have been implicated as vectors of leishmaniasis *(Swaminathan et al., 1942).*

In Bangladesh, during an outbreak of kala-azar at Kalihati thana of Tangail districts sandfly species of *Phlebotomus argentipes, sergentomyia babu babu, Sergentomyia barrudi* and *Sergentomyia shortii* were identified *(Masum et al., 1990a).* The sand flies collected from Thakurgaon district of Bangladesh during an outbreak of VL were identified as *Phlebotomus argfentipes, Phlebotomus papatasi, Sergentomyia babu babu and Sergentomyia barrudi (Masum et al., 1990b).*

An entomological study on vector of kala-azar in Shahjadpur thana of Sirajgonj district showed that the species were *Phlebotomus argentipes, Phlebotomus malabaricus* and *Phlebotomus minutus.* Among these *Phlebotomus argentipes* was 70.6% *(Ahmed et al., 1983). Phlebotomus argentipes* is a recognized vector of kala - azar in India and in Bangladesh and this species occurs in areas where cases of kala azar have been found *(Elias et al., 1989).*

2.5. Taxonomy of the genus Leishmania:

Kingdom: Protista
Subkingdom: Protozoa
Phylum: Sarcomastigophora
Subphylum: Mastigophora
Class: Zoomastigophora
Order: Kinetplastida
Family: Trypanosomatidae
Section: Salivaria
Genus: *Leishmania*
Species: *donovani, tropica, mexicana, braziliensis*

2.6. Mode of Transmission

Worldwide, vector-borne transmission is the most common mode of transmission (*Herwaldt B L 1999).* Other modes of transmission such as parenteral, congenital, sexual,

occupational (needle stick) exposures, and person to- person transmission could also occur *(Magill A J, 1995*). Because in endemic areas it is generally presumed that leishmaniasis is transmitted through sandfly bites, transmission via other modes might be underestimated.

2.7. Morphological forms

The Parasite *Leishmania* are existing in two forms so it is a digenetic protozoa which exists as:

i. Amastigote form and
ii. Promastigote form.

2.7.1. Staining characteristics:

Giemsa (one type of Romanowsky dyes) stains chromatin of the nucleus and nucleic acid containing kinetoplast a brilliant red or violet, whereas the cytoplasm is stained pale blue *(Neva et al., 1990).* With Leishman's stain, the cytoplasm appears blue, the nucleus pink or violet and the kinetoplast, bright red *(Chatterjee, 1982).*

2.7.2. Amastigote form:

Amastigotes are ovoid and nonflagellated obligate intracellular form of *Leishmania*, which reside within mononuclear phagocytes of their vertebrate hosts including man. measuring 3-5 μm in length *(Ross R, 1903)*. The size of amastigote from different species is known to vary. On simple light microscopy, a central round or oval nucleus and adjacent but smaller round or rod shaped kinetoplast can be discovered. An infolding of the surface membrane creates an internal space, termed as 'flagellar pocket' *(Webster et al, 1993).* The flagellum is not functional in amastigotes and does not extend beyond the cell body. In addition to anchoring the flagellum the main function of the pocket is to function as a site of endocytosis and exocytosis *(Webster P et al 1993).* Immediately below the origin of the flagellum lies a dense mass of mitochondrial DNA known as

kinetoplast. The kinetoplast DNA is composed of several thousand circular DNA molecules linked together in a catenated network *(Lukes et al 2002)*. These DNA networks are of two types: each kinetoplast contains 25-250 maxicircles of approximately 30kb, and 5000 - 10,000 minicircles of about 2kb size each. Together these constitute the mitochondrial genome. The cytoplasm contains both rough and smooth endoplasmic reticulum. The Golgi complex is typically found in the vicinity of the flagellar pocket, which probably reflects the role of this organelle in the endocytic and exocytic pathways. Lysosomes are also found in the cytoplasm together with an organelle unique to kinetoplastids, the glysosome *(Parsons et al, 2001)*. The cytoplasm of the amastigotes often stains the same as the host cell cytoplasm and only the nucleus and kinetoplast can be distinguished. The kinetoplast stains more densely than the nucleus *(Neva et al., 1990).*

2.7.3. Promastigote form:

Promastigotes are flagellated extracellular form, which are found in the gut of sandflies and in *in-vitro* culture. The flagellar promastigote form measures 10-20 μm in length not including the length of the flagellum, which may equal the body length. The pale blue staining cytoplasm contains a centrally placed nucleus. The kinetoplast lies about 2 μm from the anterior end and the flagellum emerges anteriorly. The overall shape is that of a spindle with the posterior end gradually tapering to a point *(Neva et al., 1990)*. The transformation of amastigotes to promastigotes starts within hours of ingestion of the amastigotes (either free or intracellular) and occurs exclusively in the gut. The amastigotes are completely transformed into motile promastigotes within 24-48 h and keep on dividing by binary division. The mature metacyclic promastigotes are accumulated in the midgut and foregut *(Lainson R et al, 1987).* The main difference from amastigotes is that the cell body is elongated, in the range of 8-15 μm, the flagellum emerges from the cell body, and is functional, making these cells motile. The promastigote flagellum has a paraxial rod, a paracrystalline structure running parallel to the microtubules of the axoneme. There is a variety of different promastigote forms that can be separated on morphological grounds but functional distinction is less complete (*e.g.*, procyclic promastigotes, paramastigotes, nectomonad promastigotes, haptomonad

promastigotes, paramastigotes and metacyclic promastigotes). The first developmental event in the sandfly is probably the transformation of amastigotes to procyclic promastigotes. These events occur in the posterior midgut of the sandfly *(Bates P A, 1994b).* Multiplication of procyclic promastigotes occurs, they elongate and transform to nectomonad forms of 15-20 μm body length. Approximately 3 days after blood feeding the peritrophic membrane (a secretory sheath) which contains these parasites usually begins to breakdown and promastigotes begin to set free and they forward to the anterior midgut *(McConville et al, 2002).*

2.8. Molecular determinants of virulence of Leishmania

These determinants include most, not all Leishmania molecules have been studied as "virulence factors". Virulence factor includes: Surface Lipophosphoglycan (LPG), Glycoprotein (gp) 63, Acid Phosphatases (ACP), Nucleotidases, Transporters, Cysteine Proteinases and Megasomes, Heat-Shock Proteins. All these molecules appear to play certain roles in *Leishmania* infection of macrophages *(Chang et al, 1990).* They are referred to as invasive/evasive determinants because they help *Leishmania* successfully establish intracellular parasitism in the following sequential events: (A) evasion of humoral lytic factors; (B) attachment of parasites to macrophages followed by their intracellular entry into these phagocytes; (C) the intracellular survival of the endocytized parasites; (D) promastigote-to-amastigote differentiation; and (E) replication of the amastigotes.

2.8.1. Surface Lipophosphoglycan (LPG):

Lipophosphoglycan (LPG) is the major cell surface glycolipid macromolecule and plays a key role in determining parasite virulence and survival in the mammalian macrophage *(McConville et al., 1987).* LPG has approximately 5 million copies per cell; covers the entire surface of the promastigote to form a dense glycocalyx and appears to be involved in facilitating the initial attachment of promastigotes to macrophages and their subsequent uptake into the phagolysosome. LPG molecules are also expressed on the cell surface of the amastigotes and may be necessary for parasite survival in the macrophage

phagolysosome compartment *(Handman et al, 1984)*. LPGs from all species of Leishmania have an identical lipid anchor and glycan core. The tissue tropism of different Leishmania species may be related to the variations in their surface glycolipids *(Handman et al., 1987)*.

LPG and related glycolipids plays important biological role in Leishmania macrophage interactions. Lipophosphoglycan (LPG) has multifunctional virulence factors that includes: attachment to the sand fly vector midgut, attachment and entry into macrophages, induction of neutrophil extracellular traps (NETs), inhibition of protein kinase C (PKC), retardation of phagosome maturation, disruption of NADPH oxidase, assembly at the phagosome membrane, modulation of NO production and induction of protein kinase R and heme oxigenase-1 *(Winberg M E, 2009, Luz N F et al, 2012).* Although the lipid anchor is conserved, different studies shows that the changes in the carbohydrate structure of procyclic LPG and GIPLs can account for variations in macrophage modulation *(Assis R R et al, 2012)*. The Leishmania LPG and related glycolipids are highly immunogenic owing to their unique structural features *(Tolson et al., 1989)*. They have long been exploited as excretory factors for serotyping of Leishmania species *(Turco, 1988)*.

2.8.2. Glycoprotein (gp) 63:

Surface glycoprotein is present 500,000 copies per cell and constitutes about 1% of the total cellular proteins of Leishmania *(Bordier, 1987).* Surface glycoprotein is also called gp63 and it was initially recognized as a major surface antigen of promastigotes by using monoclonal antibodies and surface radio iodination of living cells *(Chang et al 1990).* Glycoprotein63 has been found in all major pathogenic Leishmania species by peptide mapping and/ or immunological cross reactivity and in both stages endowed with proteolytic activity *(Bourvier et al., 1987)*. It is the major antigenic protein of most promastigotes *(Colomer-Gould et al., 1985).* Gp63 has been shown to cleave C3 into C3b and other C3 products and to protect lipid-protein substrates from intralysosomal degradation within macrophages *(Chaudhuri G et al, 1989)*.

By virtue of its proteolytic activity, universal presence, abundance and surface localization, gp63 is thought to be Leishmania virulence factor. The abundance of gp63 is often correlated with Leishmania infectivity *and* host-parasite interactions *(Kweider et al 1987)*. Because of gp63's proteolytic activity, it may function in the CR-mediated endocytosis of promastigote and protection of Leishmania from intralysosomal microbicidal factors *(Chang et al., 1990)*. Leishmania gp63 is immunogenic; anti-gp63 antibodies have been reported in sera from patients with leishmaniasis *(Murray P J et al, 1989)*.

2.8.3. Acid Phosphatases (ACP):

Two forms of Acid phosphatases (ACP) are present in Leishmania namely membrane-bound ACP and secretory ACP *(Glew R H et al, 1988)*. The two forms are antigenically distinct and each probably exists as multiple isoenzymes present in both stages of most Leishmania species *(Bates P A et al, 1989)*. The membrane-bound ACP enzymes purified from promastigotes reduces the respiratory burst of neutrophils and is itself resistant to oxidative metabolites *(Remaley et al., 1985; Saha et al., 1985)*. In addition, it has been shown to dephosphorylate certain phospholipids and phosphoproteins *(Remaley et al., 1985)*. Thus, the ectoenzyme is thought to protect Leishmania species by interfering with the regulatory mechanism of the macrophages that produces microbial free radicals *(Glew et al., 1988)*. Secretory ACP production in large quantity elicits humoral immune response of the host and may conceivably contribute to the pathobiology in leishmaniasis *(Chang et al., 1990)*.

2.8.4. Nucleotidases:

5' nucleotidase and 3' nucleotidase/nuclease have been reported to exist on the surface of some trypanosomatid protozoa including Leishmania species *(Gottlieb M, 1989)*. The latter enzyme is most active with 3' AMP and prefers RNA to DNA as substrate. Both enzymes are glycosylated and non stage specific but alkaline pH optimum of both enzymes suggest that they probably serve such functions for promastigotes in the sandfly

gut better than for amastigotes in the phagolysosomes of the macrophages *(Chang et al., 1990).*

2.8.5. Transporters:

The plasma membrane of Leishmania has been shown biochemically and genetically to possess the transport systems for folate, glucose, nucleosides, proline and ribose. It also possess a cation or proton-transporting ATPase, which is apparently crucial for the pH homeostasis of Leishmania species and the transport of nutrients necessary for their adaptation to the changing environment in their life cycle *(Chang et al., 1990).* ATPase Ib is much more abundant in amastigotes than in promastigotes. ATP Ib expression is crucial for the transition of Leishmania from the sand fly gut into the acidic environment of phagolysosomes (*Meade J C et al, 1989).*

2.8.6. Cysteine Proteinases and Megasomes:

Megasome-a modified lysosome, constitute as much as 15% of total cell volume, which is noticeable in the promastigotes grown to stationary phase and becomes fully developed as they differentiate into amastigotes *(Lockwood B C et al ,1987).* It has a optional proteolytic activity at acidic pH against peptide substrate *(Pupkis M F et al, 1984).*This enzyme is proposed to serve a degenerative role, possibly for the nutritional benefits of the amastigotes and for releasing ammonia or other amines to modulate the host lysosomal activity for the parasites' intracellular survival and is functionally important to amastigotes *(Chang et al., 1990).*

2.8.7. Heat-Shock Proteins:

Exposure of promastigotes to elevated temperatures results in an over-expression of the classic heat-shock genes and other genes. Multiple protein bands emerge shortly after heat-shock and their number varies with different species. Only one of these proteins has been positively identified immunologically as equivalent to Hsp 70 protein *(Shapira M et al, 1988).* Most intriguing is the increase in the virulence seen with briefly heat-shocked

promastigotes *(Smejkal R M, 1988).* Which type of molecular changes in these promastigotes account for virulence remains uncertain (Chang *et al.,* 1990).If the over expressed heat shock proteins are involved in this phenomenon, their precise role remain equivocal.

2.9. Life Cycle

2.9.1. In sand fly host:

The amastigotes are ingested in the female sand fly with the first blood meal either within infected macrophage or free amastigotes forms. The macrophages disintegrate over a matter of hours, so the first event is transformation. In case of promastigote form, it takes 24 - 48 hours *(Bates and Rogers 2004).* Promastigotes of various kinds grow and divide in the sandfly gut. The ultimate products of the developmental cycle are the ‘ metacyclic promastigotes’ which is the mammalian infective form. Then migrate forwards to the anterior part of the thoracic mid gut or ‘*cardia’*. From here they move forward to contaminate the mouthparts to be regurgitated into the wound caused by the bite at the second blood meal. In all cases, the infections are transmitted by bite. Development in the sand fly from amastigote to infective promastigote stage (metacyclic promastigotes) varies from 5-10 days *(Manson-Bahr et al., 1991).*

2.9.2. In mammalian host:

After inoculation by the sand fly, either into a capillary or the dermal tissue, the promastigotes encounters macrophages, which actively search them out and phagocytose them by receptor-mediated enocytosis *(Chatterjee, 1982).* The promastigote changes into amastigote in a phagolysosome where it multiplies by binary fission. Multiplication goes on continuously till the cell becomes packed with parasites. The host cell is thereby enlarged and eventually ruptures or the amastigotes leave the macrophages by penetrating the cell membrane. The amastigotes thus released into the circulation are again either taken up by, or invade fresh macrophages and the cycle is repeated. In this way to entire reticuloendothelial system becomes progressively infected. In the blood stream, some of the free amastigote are phagocytosed by the neutrophilic granulocytes and monocytes

(macrophages). A blood-sucking sand fly draws these free amastigote forms as well as those within the monocytes during its blood-meal *(Manson–Bahr et al., 1991).*

2.10. Pathogenesis

Although all clinical and geographical varieties of the Leishmania infection share a common histological feature - namely, the early accumulation of mononuclear phagocytic cells in the invaded tissues leading to hyperplasia of reticuloendothelial cell (REC) of the organs involved but wide range of variation of clinical manifestations seen in Leishmania infection. The REC hyperplasia that follows infection with *L. donovani* affects spleen, liver, mucosa of small intestine, bone marrow and lymph nodes. The cardinal histopathological feature of hepatic resistance to visceralizing species of *Leishmania* is the development of granulomas *(WHO, 1990).*

The metacyclic (infective) forms of parasite are inoculated into the skin by the bites of infected female sand fly. Here the promastigotes are exposed to IgG and IgM, which opsonize them and are killed by activation of the membrane attack complex of complement through the classical pathway (Pearson *et al.*, 1980). Those escaping the lethal effect of serum bind to macrophages. Lipophosphoglycan (LGP) and gp63 has been considered parasite ligands with complement receptor CR1 and CR3 and mannose-fructose receptor as the corresponding macrophage receptors *(Da Silva et al, 1989; Rizvi et al., 1988; Russell et al., 1988).* After receptor-mediated endocytosis the parasite resides within the phagolysosomes, thus are sheltered from the body's immune system. Side by side, they overcome the microbicidal conditions of the macrophage phagolysosomes. Amastigotes and metacyclic promastigotes appear to evade oxygen-dependent destruction by triggering a minimal respiratory burst during infection due to the use of C3 receptors for internalization *(Da Silva et al., 1989)*. LPG scavenges oxygen free radicals *(Chan et al., 1989)* and inhibits relevant enzymes e.g. lysosomal glycosidases and protein kinase C that may enhance the survival of promastigotes in macrophages *(Chang et al., 1990)*. Glycoprotein63, a parasite ectoenzyme, has been shown to protect lipid-protein substrates from intralysosomal degradation within

macrophages. Protection against low pH that exists within the phagolysosmes, is accomplished by the action of a membrane proton translocating ATPase which is located

The parasites multiply within the phagolysosomes by binary fission. The infected macrophages secrete colony-stimulating factors, which stimulate precursor cells of macrophages thereby providing new target cells for the parasites, and form a granuloma with epitheliod cells and giant's cells in the dermatotropic species and hyperplasia of the reticuloendothelial cells in the viscerotropic species *(WHO, 1990).* The next stage depends on the toxicity and immunogenicity of the parasites and on host resistance. In visceral leishmaniasis, the major host response is cellular and the amastigotes in macrophages are killed due to increased production of oxygen and nitric oxide metabolites in the macrophages *(Liew et al., 1990)* stimulated by lymphokines particularly interferon-y (IFN-y), from activated T-helper 1 cells generated during the immune response. The released amastigotes are destroyed extracellularly with the appearance of delayed type hypersensitivity. So, in majority cases, the infections are mild and self-limiting (WHO, 1990). A small fraction of individuals who develop specific suppression of cell-mediated immunity permits the dissemination and uncontrolled multiplication of parasites leading to disease and complications.

2.11. Immunological responses in Kala azar:

There is a wide range of variation in immune response in Kala azar. Some are completely resistant to infection but diseases in man are variable. All form of Leishmaniasis shows natural immunity against reinfection with homologous strain. Once recovered from Kala azar infection acquire lifelong immunity and there is no reliable record of second attack of Kala azar *(Manson-Bahr and Bell, 1991)*. The fundamental principle of the immunoregulation of leishmaniasis is that the parasite, which replicates in quiescent macrophages, is killed by activated macrophages. Murine models of L. major disease exemplify the th1/Th2 paradigm, in which the outcome of disease is determined by the nature and magnitude of the T-cell and cytokine responses early in infection. In infected inbred mice, production of interferon gamma by Th1 and natural killer cells mediates

resistance, whereas expansion of interleukin-4-producing Th2 cells confers susceptibility *(Reed et al., 1993).*

2.12. Visceral Leishmaniasis

Visceral leishmaniasis (VL) or kala-azar (KA) is usually an insidious in origin, chronic disease among the inhabitants of endemic areas. Symptoms of Visceral leishmaniasis (VL) include prolonged undulant fever, weight loss, decreased appetite, signs of anemia, and abdominal distention with splenomegaly and hepatomegaly, pancytopenia and hypergammaglobulinaemia *(Manson and Bhar et al, 1991)*. Untreated VL is frequently fatal, often from secondary infection especially in patients co-infected with HIV *(Mathur et al, 2006; Shah et al 2010).*

Clinically, according to National guideline for Kala – azar case management in Bangladesh (2013), an individual in an endemic area who has fever for more than 2 weeks, splenomegaly and 'rK39' test is positive should be diagnosed as a case of Kala – azar.

Post – kala azar dermal Leishmaniasis (PKDL) occurs after recovery in some cases of visceral Leishmaniasis. This syndrome includes maculopapular, macular or nodular rash around the mouth, which spreads.

2.13. Clinical features

Kala-azar is also called Dum-dum fever, Sikari disease, Burdwan fever and Shahib.s disease. But due to its characteristic symptoms, blackening or darkening of the skin of the hands, feet face and the abdomen most commonly known as Kala-azar, which in Hindi means black sickness or black fever *(Lainson and Shaw, 1987).* Its incubation period ranging from 2-6 months depending on the patient's age, immune status and the species of *Leishmania.* In endemic cases of Kala-azar, the disease is chronic and onset is gradual. People of all ages are susceptible in the old world *(Rab and Evans, 1995).* The symptoms of Kala-azar vary person to person according to geographical foci. Common symptoms included high undulating fever often with 2-3 peaks in 24 hours and drenching sweats

which can easily be misdiagnosed as malaria. Chills, rigors, weight loss, fatigue, cough, burning feet, insomnia, abdominal pain, joint pain, epistaxis and diarrhoea might be associated *(Hashim et al., 1995).* Most commonly observed clinical signs include massive splenomegaly, hepatomegaly and anemia. Splenic enlargement along with hepatomegaly causes an abdominal protuberance in these patients *(Sundar et al., 2001).*

2.14. Complications of Kala-azar

Kala-azar is commonly complicated by secondary infections, such as pneumonia, bronchial infections, tuberculosis, malaria, diarrhoea or dysentery, viral infections, bacterial skin infections, otitis media and Cancarum oris. Thrombocytopenia may cause epistaxis or bleeding from other sites and this may precede death. *Leishmania* enteritis may be a cause of diarrhoea and malabsorption and pulmonary involvement may mimic pneumonia, Mortality is related to immunosuppression causing secondary infections and hemorrhage and in untreated cases mortality ranges from 75-95% *(WHO, 1996; Peter, 2000).*

2.15. Consequences of Kala-azar and post Kala-azar dermal leishmaniasis (PKDL)

PKDL has been reported from India *(Prasad, 1999)* occur in approximately 10% of cases after the treatment of Kala-azar. Lesions can develop at late as 1-2 years after treatment for the original disease and manifest on the face, trunk or extremities and may persist for as long as 20 years. In Africa, it has reported that dermal lesions occur in only 2% of cases and tend to appear during or shortly after the treatment and persist only for a few months *(Zijlstra et al., 2000; Prasad, 1999).* PKDL is characterized by a patchy and raised maculopapular rash and changes in skin color. Late manifestations are papules, papules or nodules. Pentavalent antimony at a dose of 20mg/kg/ day for 4 months or longer is used to treat Indian PKDL *(WHO, 1996).*

2.16. Diagnosis of Visceral Leishmaniasis:

Conventionally Kala azar was diagnosed by clinical evaluation. The disease present with fever, hepatosplenomegaly, weight loss, anemia etc. But it is impossible to differentiate kala azar from other causes of fever with splenomegaly. At present diagnosis is based on (i) Identificatiom of parasite in tissues of relevance by light microscopic examination of the stained specimen or in vitro culture, or animal inoculation; (ii) detection of parasite DNA in tissue samples; or (iii) immunodiagnosis by detection of parasite antigen in tissue, blood, or urine samples, by detection of nonspecific or specific antileishmanial antibodies (immunoglobulin), or by assay for Leishmania-specific cell-mediated immunity *(Sundar and Rai, 2002).*

2.16.1. Microscopic examinations

The confirmatory used method for diagnosing Kala-azar has been the demonstration of parasites in splenic or bone marrow aspirate. The presence of the parasite in lymph nodes, liver biopsy, or aspirate specimens or the buffy coat of peripheral blood can also be demonstrated. Under Microscope amastigotes appear as round or oval bodies measuring 2 to 3 µm in length and are found intracellularly in monocytes and macrophages. In preparations stained with Leishman's stain, the cytoplasm appears pale blue, with a relatively large nucleus that stains red. In the same plane as the nucleus, but at a right angle to it, is a deep red or violet rod-like body called a kinetoplast *(Chatterjee, 1982).* The amastigote stage seen in clinical samples is commonly known as Leishman-Donovan (LD) bodies. The sensitivity of the bone marrow smear is about 70% or lowers *(Chowdhury et al., 1993, Boelaert et al., 1999).* Splenic aspirate, though associated with rise of fatal hemorrhage in inexperienced hands, is one of the most valuable methods for diagnosis of kala azar, with a sensitivity exceeding 90% *(Kager et al., 1983; Zijlstra et al., 1992).* For patients suspected of have Kala-azar, splenic aspirate can be performed even when spleen is not palpable, after demarcating the area of splenic dullness by percussion. Occasionally amastigotes have also been demonstrated in liver biopsy (50-80% sensitivity) and lymph node aspirate (56% sensitivity) *(Zijlstra et al., 1992).*

Splenic aspiration microscopy is most reliable but a high risk procedure due to chance of hemorrhage and shock. It is a slow, painful and cumbersome procedure. Beside this bone marrow aspiration is comparatively safer procedure but it is much painful. And its sensitivity is much lower (54 – 86%). The aspiration procedures required expert personnel and are not useful for screening for epidemiological purpose and for detecting early infection.

2.16.2. Culture of Leishmanial parasites:

Culture of parasite might improve the sensitivity of parasite isolation but Leishmania culture is rarely used in routine clinical practice. Culture is done in Nicolle, Novy, Mcneal (NNN) medium with 100 IU of penicillin and 100 µg of streptomycin or in Tobies medium *(Sundar et al., 2001).* Kala azar culture is simple to use and satisfactory for diagnosis but it is expensive. However, culture tubes are inoculated with 1 to 2 drops of bone marrow or splenic aspirate and incubated at a temperature between 22°C and 28°C. The tubes are examined weekly for the presence of promastigotes by phase contrast microscopy or by wet mount of culture fluid for 4 weeks before being discarded as negative. Blood can also be used to isolate the parasite but the method is slow and takes longer. Contamination of the culture media by bacteria of yeast species or other fungi usually complicates the culture but can be avoided by use of good sterile techniques and be the addition of penicillin (200 IU/ml), streptomycin (200µg/ml) and 5-flucytosine (500µg/ml) to the medium *(Schur and Jacobson, 2001).*Culture are usually required for (i) obtaining a sufficient number of organisms to use an antigen for immunologic diagnosis and speciation (ii) obtaining parasites to be used in inoculating susceptible experimental animals (iii) in vitro screening of drugs (iv) accurate diagnosis of the infection with the organism. *Leishmania* strains can be maintained as promastigotes in artificial culture medium *(Sundar et al., 2001).*

2.16.3. Aldehyde Test (AT):

When a drop of formalin is added to 1 - 2 ml of serum from a case of kala azar, immediately an opaque – white gel is formed this phenomenon is called aldehyde test

(AT). Visceral Leishmaniasis is associated with a great rise in gammaglobulin, which can give a positive aldehyde test *(Chatterjee, 1982).* AT is simple and highly sensitive. But it's usually becomes positive after 3 months of infection so early diagnosis can't be made. At is also a non specific test because it gives false positive results in other diseases associated with hypergummaglobinaemia such as multiple myeloma, cirrhosis of liver, African trypanpsomiasis, leprosy, tuberculosis, chronic mamaria, sarcoidosis etc. *(Ho et al, 1983).*The test is negative n cases of cutaneous Leishmaniasis. In Indian subcontinent AT is commonly used as a diagnostic test particularly in rural areas where other facilities for diagnosis of VL is not available. Although it is a simple, sensitive and economical test but it has a little value in diagnosis recent cases which is required in seroepidemiological studies *(Chowdhury , 1991; Cheesebrough, 1999).*

2.16.4. Complement Fixation Test (CFT):

An antibody when combines with an antigen can also activate the complement system. In Complement fixation test, the test serum (thought to contain the antibody), antigen and complement are mixed and after a period of incubation antibody coated sheep blood is added *(Chih et al, 1953).* A positive CFT test indicates absence of lysis of red blood cells. Complement fixation test was introduced more than 60 years ago in the diagnosis of Kala-azar (Smith *et al.,* 1984). Since then it has been used as a serodiagnostic tool in different countries. Although, CFT becomes positive in the early stage of infection (in 3 weeks) it is a non-specific test. Sensitivity of this test varies with the source and method of extraction of antigen and also with different batches of antigen *(Aikat et al., 1979).*But the test gave a few false positive results with tuberculosis and leprosy cases. In addition, the test has shown to be of limited value in routine laboratory diagnosis *(Duxbury and Sadun, 1964)* and large-scale epidemiological studies *(Hommel et al., 1978).* Due to its complicated procedure that test can only be performed in reference laboratories *(Rahman and Islam, 1979).*

2.16.5. Direct agglutination test (DAT):

Human cells and different types of microbial species can be directly agglutinated by addition of antibody. Test to detect specific antibody are carried out by serially titrating

antisera in 2 fold dilutions in the presence of a constant amount of antigen. After incubation agglutination is complete and particles are examined directly or microscopically for evidence of clumping *(Gari- Toussaint et al, 1994)*. The direct agglutination test is a highly specific and sensitive test. It is inexpensive and simple to perform making it ideal for both field and laboratory use. The method uses whole, stained promastigotes either as a suspension or in a freeze-dried form. The freeze-dried form is heat stable and facilitates the use of DAT in the field. However, the major disadvantage of DAT is the relative long incubation time of 18 hours and the need for serial dilutions of serum (*Schallig et al., 2001)*. DAT has no prognostic value too. The test may remain positive for several years after cure.

2.16.6. Immuno-chromatography test (ICT):

A recombinant antigen, rK39 has been shown to be specific for antibodies in patients with Kala-azar caused by members of the *L. donovani* complex (*Badaro et al., 1996*). This antigen, which is conserved in the kinesin region, is highly sensitive and predictive of the onset of acute disease. The antigen is derived from *L. chagasi*. This antigen has been reported to be 100% sensitive and 100% specific in the diagnosis of Kala-azar and PKDL by ELISA *(Singh et al., 1995, Kumar et al., 2001)*. Another important feature of anti-rK39 antibody is that the titer correlates directly with the disease activity, indicating its potentiality for use in predicting response to chemotherapy. A promising ready to use immunochromatographic strip test based on rK39 antigen has been developed as a rapid test for use in difficult field conditions. The recombinant antigen is immobilized on a small rectangular piece of nitrocellulose membrane in a band form and goat-anti-protein A is attached to the membrane above the antigen band. After the finger is pricked, half a drop of blood is smeared at the tip of the strip, and the lower and end of the strip is allowed to soak in 4 to 5 drops of phosphate buffered saline, placed on a clean glass slide or tube. If the antibody is present, it will react with the conjugate (protein A colloidal gold) that is pre dried on the assay strip. The mixture moves along the strip by capillary action and reacts with rK39 antigen on the strip, yielding a pink band. In the strip of patients who are infected, two pinkish lines, appear in the middle of the nitrocellulose membrane (the upper pinkish band serves as a procedural control. Several studies from

the Indian subcontinent reported the test to be 100% sensitive *(Bern et al., 2000; Sundar and Rai, 2002)*. However, when evaluated in Sudan, the sensitivity of the test was only 67%. In the Sudan study, all the parasitologically confirmed Kala-azar patients who tested negative by the rK39 strip test showed IgG against rK39 by micro ELISA at lower titers *(Zijlstra et al., 2001).* In a study done in southern Europe, the rK39 strip test results were positive in only 71.4% of the cases of Kala-azar *(Jehinek et al., 1999)*. These differences in sensitivity may be due to differences in the antibody responses observed in different ethnic groups *(Singh et al., 1995).*While such reactions might be considered to be false positive, these probably represent subclinical infections: PCR assay for *L. donovani* was positive in a few of these cases *(Salotra et al., 2001; Sundar and Rai, 2002).* Anti-rK39 IgG may be present in serum for an extended period after successful treatment for Kala-azar; thus, patients with suspected relapse of Kala-azar with a past history of infection would not be candidates for diagnosis by strip testing. Another drawback of this method is that an individual with a positive rK39 strip test result may suffer from an illness (malaria, typhoid fever or tuberculosis) with clinical features similar to those of Kala-azar yet be misdiagnosed as suffering from Kala-azar. Besides these limitations, the rK39 ICT strip test has proved to be versatile in predicting acute infection. It is the only available format for diagnosing Kala-azar with acceptable sensitivity and specificity. It is also inexpensive and simple *(Sundar and Rai, 2002).*

2.16.7. Enzyme Linked Immunosorbent Assay (ELISA) and Indirect fluorescent antibody test (IFAT):

The Enzyme Linked Immunosorbent Assay (ELISA) is a valuable tool in the serodiagnosis of leishmaniasis. The test is useful for laboratory analysis as well as for field applications *(Rajasekariah et al, 2001).* The Indirect fluorescent test is one of the most sensitive tests available. The basic principle of IFAT and ELISA are same. The only difference is in the antigen antibody detection system. The antigen fixed to a solid surface either wells of microtitre plates (ELISA) or glass slides (IFAT) are allowed to react with specific antibodies in patient's sera. In ELISA, antigen antibody detection is detected by using enzyme conjugated antihuman IgG antibody with specific dye

substrate, the result is read either visually or spectrophotometrically *(Davidson, 1998)*. In IFAT, antigen antibody reaction is detected by the use of flourochrome conjugated antihuman IgG antibody and observed under fluorescence microscope. The sensitivity and specificity of ELISA is greatly influenced by the antigen used. Both ELISA and IFAT is highly sensitive and specific test. IFAT was found to be 100% sensitive and 94 – 100% specific. ELISA was 96.5 – 100% sensitive and 87.3 – 98.6% specific *(Harith et al, 1987; Srivasta, 1989)*. But when African trypanosomiasis and Chaga's diseases is taken in consideration specificity of both test fall significantly *(Harith et al, 1987).* There is a possibility of a cross reaction with trypanosomal sera. Although IFAT is more sensitive and specific than soluble antigen of ELISA, it is cumbersome and not suitable for field conditions *(Sassi et al., 1999).*

2.16.8. Limitations of serological tests:

Serodiagnostic assay based on whole cells or crude antigens may be suitable for diagnosis of acute visceral Leishmaniasis. Early detection of infection using assays based on crude antigen has not been practical because of false negative reactions *(Evans et al, 1990)*. The antibody persists in high titer at human body for a long period after chemotherapy. So serological methods can't differentiate between present and past infection that's why it can't detect relapse or reinfection. It has a limited use for monitoring drug therapy. Presence of cross reactive antibodies may cause difficulties in diagnosis of VL where Leishmaniasis and trypanosomiasis are co endemic. The commonly used serodiagnostic assays either uses whole promastigotes, therefore these tests are unable to detect antibody response to specific antigen of the organism *(Harith et al, 1987, Hailu, 1990).*

2.16.9. Immunoblotting:

Most of the work concerning the use of immunoblotting in the diagnosis of leishmaniasis has been done on Kala-azar. In CL, anti-leishmanial antibodies though detectable, are present in low titres, Hence Immunoblotting is not widely adopted for diagnosing CL. The biggest advantage of immunoblotting, as a general rule, is that various antigens

expressed and antibodies recognized during the course of infection can be documented. It also has an advantage of permanent documentation. However, the technique is not user-friendly and limited only to research laboratories *(Herwaldt, 1999; Maalej et al., 2003).*

2.16.10. Molecular methods:

Microscopy and culture several limitations like low sensitivity and are time consuming. Sample used for culture or microscopy is bone marrow or splenic aspiration which is very painful and there is a risk of hemorrhage and may cause death. The immunological methods fail to distinguish between past and present infections and are not very reliable in immunocompromised patients. While the molecular approach is capable of detecting nucleic acids unique of the parasite, it would address these limitations. A variety of nucleic and detection methods targeting both DNA and RNA have been developed. Recently molecular probes, using kinetoplast DNA (kDNA), ribosomal RNA (rRNA), miniexon derived RNA (med RNA) and genomic repeats have been evaluated and used to a much higher sensitivity and specificity. The distinct advantages of DNA hybridization are that large numbers of samples can analyzed quickly without compromising efficiency and many different types of samples can be processed including blood spots, tissue samples, splenic and bone marrow aspirates *(Wilson, 1995)*. The probes are more specific since they are made to target regions on the kDNA or rRNA, which are unique to a particular Leishmania species, complex or isolate *(Williams et al., 1995).* However, these were for laboratory use only and were not suitable as diagnostic kits, since ^{32}p has a short half-life of about 14 days and being radioactive they presented safety problems. Recently, a chemiluminescent hapten digoxigenin labeled kDNA probes have been developed. These probes are as sensitive as the ^{32}p labeled probes (can detect as low as 100 parasites) and are stable enough to use in diagnostic kits, which are more practical in the field. However, high background signals make result interpretation difficult *(Wilson, 1995; Williams et al., 1995).* The latest developments in molecular diagnostic technology have come about as a direct result of the advent of the polymerase chain reaction (PCR).

The PCR is able to amplify small amounts of DNA or RNA to larger usable quantities (*Nuzum et al., 1995; Wilson, 1995*). Although the PCR is able to detect a single copy of target DNA, repeat sequences are used to improve sensitivity. The PCR assay can detect parasite DNA or RNA a few weeks ahead of appearance of any clinical signs or symptoms. Different DNA sequence in the genome of *Leishmania* like its region, gp63 locus, telomeric sequences, sequence targets in rRNA genes such as 18s rRNA and SSU-rRNA and both conserved and variable regions in kinetoplast DNA (kDNA) minicircles are being used by various workers *(Santos-Gomes et al., 2000; El Tai et al., 2001; Pizzuto et al., 2001; Wortman et al., 2001; Ostria and Tezeda, 2002).*

In recent years, PCR-based diagnostic methods have been described for leishmaniasis, with a wide range of sensitivity and specificity. An excellent target for a sensitive and rapid detection method is the kinetoplast mini-circle DNA, which is present at thousands of copies per cell. The mini-circles have been used as targets for selective amplification of parasite DNA in various studies *(Nuzum et al., 1995; Smyth et al., 1992)*. The identification of conserved sequence elements represented within the kinetoplast DNA (kDNA) of a given species of Leishmania would allow the design of oligonucleotide primers to be used for species-specific identification of parasites in clinical samples. We have analyzed kDNA sequences from Old World leishmaniasis and designed primers specific for L. donovani complex to detect kDNA from a single parasite in the presence of huge excesses of human DNA. The utility of the primers designed for L. donovani has been examined in clinical samples from patients with KA and healthy endemic and nonendemic individuals and with those individuals who have diseases with similar sign and symptom such as Malaria, Tuberculosis, and Dengue etc in Bangladesh. The PCR test was found to be sensitive enough to detect parasite DNA from peripheral blood of patients with KA. Furthermore, the test was specific for L. donovani complex of the parasite, leading to simultaneous identification of the parasite that causes kala azar in Bangladesh.

The identification and characterization of Leishmania from parasites, as well as reservoir hosts and vectors, is important for understanding the epidemiology and transmission of leishmaniasis. In addition, the characterization of parasites isolated from patients may

affect decisions regarding drug treatment, since disease pathology is generally associated with specific Leishmania species. In the Old World, cautaneous leishmaniasis (CL) is usually caused by *leishmania major*, *L. tropica*, or *L. aethiopica*; mucocutaneous leishmaniasis (MCL) by *L. aethiopica*; and visceral leishmaniasis (VL) by *L. donovani donovani* or *L. d. infantum*. Diagnosis and parasite identification is still largely based on the microscopic observation of parasites in stained tissue or growth in culture, with isoenzyme analysis the "gold standard" for strain characterization. This latter technique, though relatively simple, is time consuming, since each isolate must be examined by multiple enzyme reactions. Other techniques for Leishmania characterization, such as monoclonal antibodies or excreted factor (EF) typing, may be affected by antigenic changes during growth in culture or differences between species isolated from geographically distant regions *(Lainson et al., 1987; Eisenberger et al., 1997).*

Recently, DNA-based technologies have been adapted for the detection and characterization of Leishmania. However, many of these techniques are still expensive and/or time consuming and even the most promising systems appear to have some drawbacks. PCR and Southern blotting with species-specific primers or probes have been used for direct diagnosis, although the latter technique appears to be less sensitive than PCR and employs radioisotopes which may be difficult to obtain in some developing regions or Old World species and subspecies of Leishmania has been used to characterize both promastigotes grown in culture and amastigotes directly in tissue samples. This system is extremely sensitive and can detect even 1 of parasite DNA. Primers derived from miniexon and intergenic sequences have also been used with equal success for leishmanial characterization by PCR.

Random amplified polymorphic DNA (RAPD) and arbitrary primer (AP)-PCR, based on fragment-length polymorphisms, has the advantage that they do not require prior knowledge of specific DNA sequences for primer design. Instead, these two methods utilize nonspecific primers, which give rise to a variety of amplification products and produce unique fingerprint patterns of genomic DNA. The diverse products are sensitive enough to show Leishmania species-specificity and can often identify individual isolates

derived from geographically diverse regions. The disadvantage is that the nonspecific primers used for RAPD and AP-PCR are able to amplify non target DNA from host and /or other contaminating organisms present in the sample.

Researchers showed that PCR could distinguish between the different Old World Leishmania complexes: *L major, L. tropica. L. donovani and L. aethiopica*. The PCR products can be analyzed directly by agarose gel electrophoresis and give simple patterns, which are unique for each complex. DNA from non kinetoplastid sources is not amplified, suggesting that it may be possible to use PCR for the direct diagnosis of Leishmania parasites in host tissue *(Eisenberger et al., 1997).*

Leishmaniasis is widely distributed and endemic in 88 countries around the world. Visceral leishmaniasis (VL), or kala-azar, if untreated, is usually fatal. Even in treated patients, the fatality rate may be as high as 30% because of late or missed diagnoses. Early diagnosis and efficient treatment could reduce mortality if a simple and accurate diagnostic technique was available.

Antibodies specific for Leishmania may remain in the serum long after patients are cured. Therefore, current serologic tests cannot be used to differentiate between active and cured cases, although many efforts have been attempted for this purpose and for evaluation of the efficacy of chemotherapy. Polymerase chain reaction (PCR) has several advantages over other diagnostic techniques. In addition to diagnosing VL accurately, PCR can differentiate the species or strain of the pathogen. Definitive diagnosis of VL now relies on the identification of the parasites in stained smears or culture of bone marrow, spleen, or lymph node, all of which require invasive procedures.

In the present study, we detect *L. donovani* complex specific kinoplastic conserved region from gene bank. Sequence analysis of the conserved region of kDNA minicircles allowed us to develop a pair of oligonucleotide primers (I and II) to amplify a minicircle kDNA fragment of 125bp by PCR. The reaction was used to detect L. donovani complex *(Leishmania donovani, Leishmania donovani infantum, Leishmania chagasi*) which mainly occurs in Bangladesh. Primers efficacy has been tested with the bone marrow or splenic aspiration and blood of 35 patients and also with blood of 25 healthy non endemic

control, 16 endemic healthy control and 25 sick controls that have similar sign and symptom like Malaria, Tuberculosis and Dengue.

2.17. Primer designing:

Primers: A primer is a short, single-stranded piece of DNA that anneals (attaches) to its complementary sequence on the template. A pair of primers will bind to either side of the target DNA segment providing initiation sites for DNA synthesis. The two primers used in a PCR are often designated by the terms forward and reverse *(Dieffenbach et al, 1993).*

PCR primers are oligonucleotides, typically 15-30 bases long, hybridizing to opposite strands and flanking the region of interest in the target DNA. When choosing two PCR primers, it is important that they not contain bases complementary to themselves or with each other. Complementarity at the 3' ends should especially be avoided to minimize the formation of an artifactual product, often called "primer-dimer" or "primer-oligomer." A 40% - 60% G+C content is recommended for each primer, avoiding internal secondary structure and long stretches of any one base. Also, primers should not sit on regions of secondary structure (within the target) having a higher melting point than the primer. Non-template, complementary 5' extensions may be added to primers to allow a variety of useful post-amplification manipulations of the PCR product without significant effect on the amplification itself. These 5' extensions can be restriction sites, promotor sequences, etc *(Dieffenbach et al, 1993, Burpo F J, 2001).*

Optimal annealing temperatures and primer concentrations must be determined empirically. *Taq* DNA polymerase has activity in the 25 °C-72 °C range. Therefore, primer extension will occur during the annealing step and the hybrid will be stabilized. Primers are always present at an excess and equal concentration in conventional (symmetric) PCR amplification and, typically, are within the range of 0.1 μM to 1 μM. It is generally advisable to use purified oligomers of the highest chemical integrity.

Chapter 3

MATERIALS AND METHODS

3. Materials and Methods:

This investigation aims at evaluation of a newly designed primer for diagnosis of kala azar. The primers efficacy is tested with serologically and microbiologically confirmed kala azar positive cases, clinically suspected cases and with healthy endemic and non endemic individuals and also with other persons who are suffering from other similar diseases like malaria, dengue, tuberculosis etc. The sensitivity and specificity of the primer was calculated. And its amplification pattern is compared with other primer's amplification patterns which were previously used by other investigators in overseas. The PCR amplified product is sequenced and aligned with gene bank to see the amplified products authenticity. PCR amplification pattern was also compared with traditional culture and microscopy test. The overall study design is outlined in Fig. 3.1. All media compositions, chemicals & reagents, apparatus used in this study are given in the Appendix I, II and III respectively.

3.1. Study Place and period

The samples were collected in the months of January, 2011 to June, 2013. All culture, microscopy and rK39 tests were done in department of Microbiology of Ibrahim Medical College and BIRDEM hospital. Molecular procedures including Primer designing, PCR and sequencing were done in Department of Microbiology, University of Dhaka.

3.2. Type of the study

The study was designed as an experimental study.

3.3. Study population

Two group of population were included in the study. Suspected kala azar (KA) patients were included from patients who were attending Mymensing SK Hospital and were referred to BIRDEM and BSMMU hospital. Because kala azar is an endemic disease and its prevalence is highest in Mymensing district. Healthy endemic controls were collected from Mymensing district and healthy non endemic controls and sick controls were collected from Dhaka city. A total of 101 individuals were included in this study. 136

samples were collected from 101 individuals because 25 kala azar suspected patient's bone marrow and blood were collected and 10 kala azar suspected patient's splenic aspiration and blood were collected. Among them 16 patient's bone marrow were previously collected by Prof. Dr. Zafar Ullah Chowdhury and were preserved at -70°C in BIRDEM hospital. 41 healthy control individuals (both endemic and non endemic) and 25 sick control individuals only blood were included in this study. Bone marrow or splenic aspirations from control individuals were not sampled because it is not ethically justified to aspirate these specimens from a healthy person.

Table 3.1.: Criteria for sample collection

Type of Subjects	Inclusion Criteria	Exclusion criteria
1.suspected Kala-azar cases	1. Patients had history of prolonged low grade irregular fever. 2.Weight loss, Pallor, Bleeding, Anemia 3. Organomegaly 4. Blackening of skin 5. ICT for kala azar positive	1. Febrile persons showing negative results in ICT.
2. Healthy non endemic controls:	1. Individuals from non endemic region. 2. Have no sign, symptom, apparently healthy declared by a physician. 3. ICT for kala azar Negative.	1. Healthy persons showing positive results in ICT.
3. Healthy endemic controls	1. Individuals from endemic region. 2. Have no sign, symptom, apparently healthy declared by a physician. 2. ICT for kala azar Negative.	1. Healthy persons showing positive results in ICT.
4. Sick control	1. Fever, Hepatosplenomegaly, Anemia, Pancytopenia, Weight loss, Pallor, Bleeding. 2. ICT for kala azar Negative	1. Sick persons showing positive results in ICT.

*ICT = Immunochromatographic test.

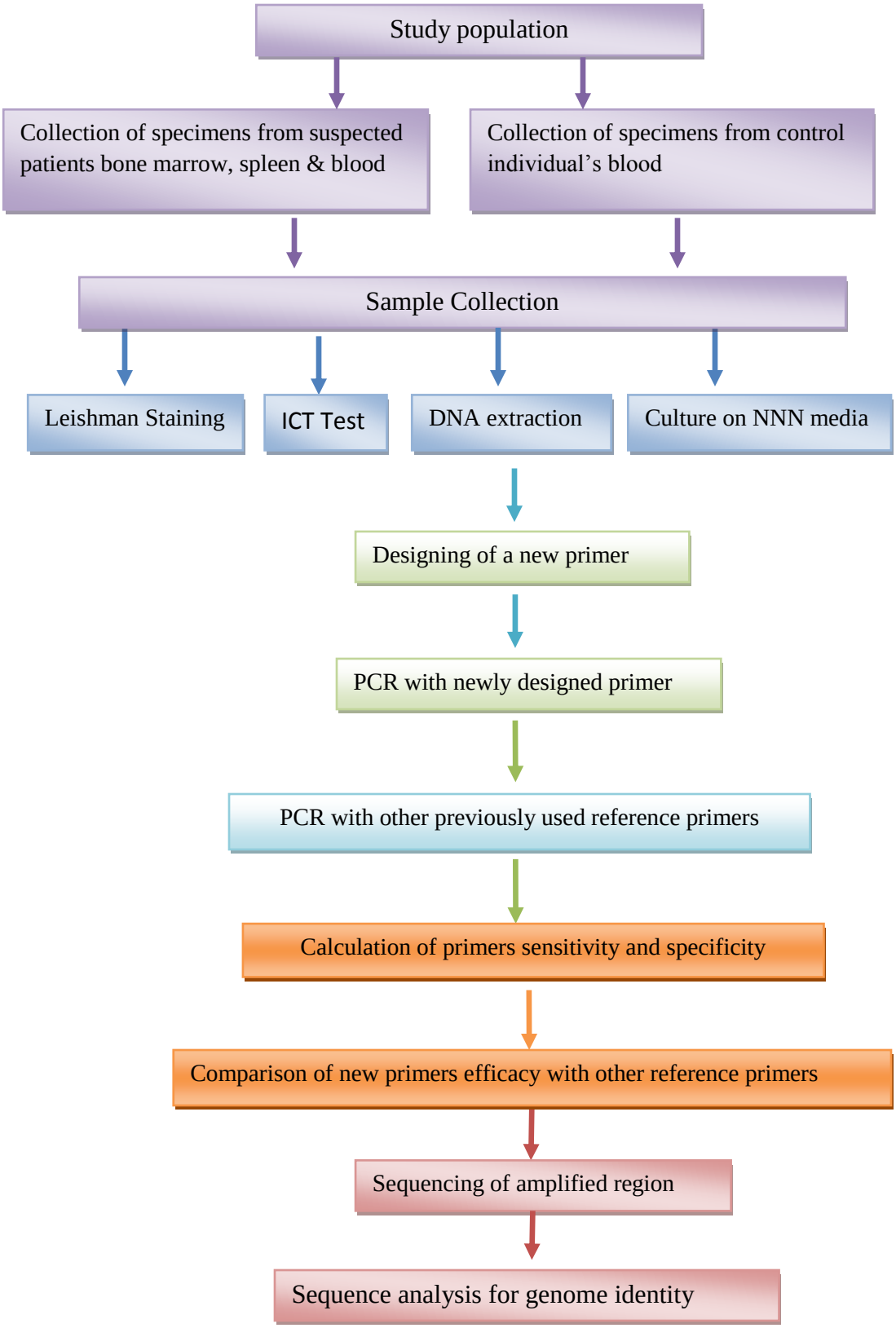


Figure 3.1.: Sequential analysis of development of a PCR based diagnostic system for kala azar.

3.4. Collection of specimens

3.4.1. Collection of bone marrow aspirates:

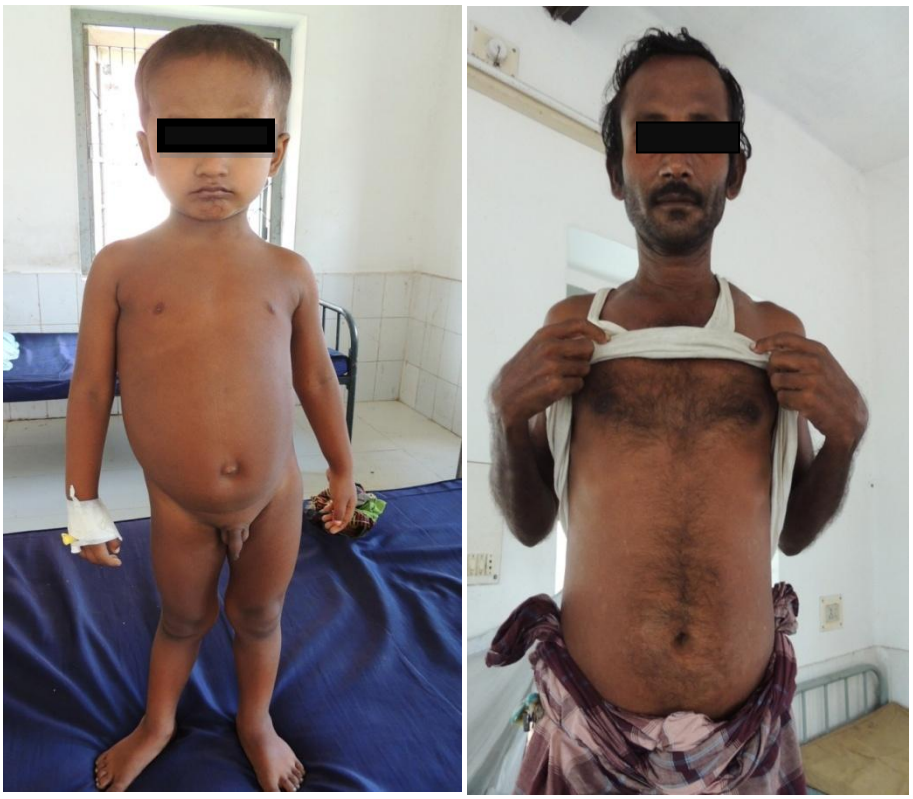
The preferred site for bone marrow aspiration include sternum preferably manubrium of the sternum, iliac crest and sometimes tibia. Bone marrow was collected from the patients who were admitted in Kala azar Unit of S K Hospital, Mymensingh. The purpose of bone marrow collection was explained clearly to the patient and the attendant. After adequate motivation, written or verbal consent was taken from the legal guardian or from the patient. Keeping the patient lying on the side, site was palpated and fixed. The area was cleaned by tincture iodine and rectified spirit. Injection Jasociane (1%) 1.5-2.0 ml was pushed into the site as local anesthetic. Allowing 5-10 minutes time, appropriate bone marrow needle was inserted up to the marrow cavity. Then up to 1 ml of marrow material was aspirated by gentle suction through a 10 ml disposable syringe. After completing aspiration, the syringe was removed from the needle and the needle was drawn out. The local wound was sealed by sterile gauge and microspore adhesive tape. After aspiration of bone marrow, thin films of the aspirate were made on at least three microscopic slides and were allowed to dry in air. After air drying slides were dipped in Absolute alcohol. With proper labeling bone marrow smear was carried to the laboratory for staining and microscopic examination. One drop was inoculated in NNN medium and one aliquot was preserved at - 20°C for DNA extraction for PCR. 25 suspected kala azar patient's bone marrow were collected.

3.4.2. Collection of splenic aspirate:

If patient had a prolonged coagulation time prior to splenic aspiration in order to promote the coagulability of the blood, the patient was given intravenous or intra muscular injections of 10% calcium gluconate 5 ml for two or three days.If the coagulation time (CT) of blood of the patients was normal no preparation is required prior to splenic aspiration.

The splenic area over which the puncture was to be made was chosen at a spot half to one inch below the costal margin, sterilized by painting with tincture of iodine. The sterilized needle of the syringe was first introduced into the skin and then plunged directly into the

spleen by the doctor, while the assistant help in fixing the enlarged spleen by pressing it upwards. Forcible aspiration is now made and some of the splenic pulp or blood is invariably drawn although it may not appear in the barrel of the syringe, the needle is quickly withdrawn. After the splenic puncture, the area is sealed with benzoin and an abdominal bandage with pressure pad is applied over the splenic area. The patient should be kept lying down for an hour or two and the pulse rate should be recorded for the detection of any evidence of internal hemorrhage. The patient may be allowed to take food one hour after the puncture. Immediately after aspiration, thin film of the aspirate was made on at least three microscopic slides by ejecting one or two drops of the fluid on the glass slide. A little sterile citrated saline was aspirated into the syringe to dilute the splenic juice that is left behind then one drop was inoculated in NNN medium and one aliquot was preserved at -20°C for DNA extraction for PCR. 10 suspected kala azar patient's splenic aspiration were collected.



(a) (b)

Figure3.2: Sample collection procedure from child (a) and adult individual (b).

3.4.3. Collection of blood:

Under all aseptic precautions (cleaning the area with povidone iodine followed by adequate rubbing by alcohol pad containing 60% isopropyl) 5 ml of venous blood was collected from median ante-cubital veins by gentle suction. The collected blood sample was immediately introduced into EDTA containing container and one drop in culture tube. Rest of the blood was used to prepare peripheral blood film, ICT strip test. Immediately after collection with proper labeling, blood was carried to the laboratory for buffy coat preparation which was used for DNA extraction for PCR. A total of 101 individuals (both case and controls) blood was collected.

3.5. Primer designing:

The genomes of three Leishmania species (*L donovani, L infantum, and L. braziliensis*) have already been sequenced revealing more than 8300 protein- coading and 900 RNA genes including tRNA, rRNA and/or snRNA genes *(Ryan el al, 2004).* Leishmania also contained a kinetoplast which is a mitochondrial genome. The Kinetoplastic DNA (kDNA) consists of a catenated network, which is composed of two types of DNA rings, consisting of 5,000–10,000 minicircles and 25–50 maxicircles. Depending on the species, the minicircles range from 0.5 to 10 kilobases (kb) in size, whereas maxicircles are approximately 20–40 kb. Kinetoplast DNA represents approximately 30 percent of the total cell DNA. It differs from nuclear DNA in several properties, including its buoyant density. Using DNA sequencing to analyze the kDNA, scientists know that maxicircles encode typical mitochondrial proteins, such as cytochrome *b*, cytochrome oxidase subunits, NADH dehydrogenase subunits, ATPase subunit 6, and ribosomal RNA. And "guide RNA" (gRNA) molecules are mainly encoded by minicircles which presumably directed the RNA editing process *(Vargas-Parada L, 2010).* This is one of the most unusual DNA structure in nature. Kinetoplasts are only found in Protozoa of the class kinetoplastida. So kinetoplast is a highly specific structure for Leishmania and also minicircles of kinetoplast presents thousands copy per cells so can be identified easily

through PCR. That's why; kinetoplast minicircle genes were used as a target for primer designing.

Leishmania sequences were downloaded from databases like National Center for Biotechnology Information (NCBI) *(http://www.ncbi.nlm.nih.gov/GenBank)* database. This sequences were aligned and doing a multiple sequence alignment by using Ebi-ClustalW program to find the region that is mostly consensus. This consensus sequence is then used to design primer for that region. Primer 3 plus program was used for primer designing. Using several parameters some primers of 18~22 bases were designed and one of the pair which showed our expected values in terms of melting temperature (Tm), Guanine cytosine percentage (GC%), amplicon size, Dimerization and hairpin loop formation was selected. For this IDT oligo analyzer 3.0 program was used. NCBI was used to check primer specificity, on which sequences this primer sequence is present. After finishing evaluation designed primer was ordered through "bioserve (Andhra Pradesh, India)" and checked in wet lab for diagnosis.

3.6. Staining of bone marrow, splenic aspirate and peripheral blood film:

At first the films (bone marrow, splenic aspiration and blood) were placed on a staining rack and was covered with Leishman stain (Appendix II) and kept for one minute. Then double quantity of distilled water was added and kept for 12 minutes. Finally more distilled water was flowed over the marrow film for washing and dried in the air and the smear was examined under oil immersion objectives (*DGHS, 2009; Duguid, 1996).*

3.7. Microscopic examination of bone marrow, splenic aspirates and peripheral blood film:

At least 1000 fields per slide preferably around the edges of the preparation were examined under oil immersion lens to detect amastigote form of *Leishmania.* Amastigotes appeared as small round or oval bodies measuring about 3µm x 5 µm size inside or outside of the monocytes and macrophages. In preparations stained with

Leishman stain, the cytoplasm appears pale blue, with a relatively large nucleus that stains red. In the same plane as the nucleus, but at a right angle to it, is a deep red or violet rod-like body called kinetoplast. Presence of amastigotes in the slide was reported as positive.

3.8. Preparation of buffy coat (by gradient centrifugation):

Buffy coat was separated according to *Sengar et al;* after thorough mixing, 8 ml. of blood were transferred in 13 x 100 mm. tubes for the preliminary leukocyte separation. The tubes were centrifuged at 80g for 6 - 7 minutes. This centrifugation gives rise to a buffy coat layer which is quite thick in EDTA treated blood but not so in the heparinized blood. About 1 ml. of plasma from the top was first removed in a tube and the buffy coat was then aspirated with a Pasteur pipette and kept separately. The buffy coat was removed with a thin superficial layer of the red blood cells in order to obtain a greater yield of leukocytes. The final volume of this suspension was later made up to 2 ml with the plasma that was removed earlier. Such leukocyte-rich suspensions were well mixed to disperse the cells and loaded in a second tube. The tube were balanced and centrifuged at 80g for five minutes. After centrifugation the plasma was removed by means of another Pasteur pipette. The tube containing sedimented leukocytes were then taken and the buffy coat was washed into a tube with plasma. In this procedure buffy coat may contain some RBC's but these will not hampered DNA extraction procedure as the extraction kit contains RBC lysis buffer. Buffy coat was stored at -20°C for DNA extraction and further analysis.

3.9. Culture of Leishmania parasite

With strict aseptic precautions one drop of bone marrow from 25 suspected kala azar patients and one drop of splenic aspiration from 10 suspected kala azar patients were inoculated into the liquid phase of Novy McNeal Nicolle (NNN) medium (1908) and incubated at 22^0C. One drop of blood from all 35 suspected patients and all control (healthy endemic, non endemic and sick) individuals were also inoculated in NNN media. After 7 days, using sterile pasture pipette, a drop of fluid was transferred from the culture

tube on a slide and examined under microscope for the presence of promastigotes. If no parasites were seen, the cultures were re-incubated and reexamined weekly for up to 4-6 weeks. If positive, cultures were maintained by sub culturing in NNN media and also Promastigotes were separated and washed with Phosphate buffer saline and stored at -20°C for DNA extraction for PCR.

3.10. Identification of anti-leishmanial antibody by Immuno-Chromatography Test (ICT):

Antibody was detected by Immunochromatographic test (ICT) by using the CTK Biotech Leishmania IgG/ IgM Combo Rapid test kit (CTK Biotech, SanDiego, USA), Catalog No.R0121C, as per manufacturer instructions.

3.10.1. Principle of the test:

ICT for Kala-azar is a qualitative, membrane based immunoassay for the detection of antibodies in Kala-azar in human serum. The membrane is pre-coated with a novel recombinant Kala-azar (rk39) antigen on the test line region and goat anti rabbit IgG on the control region. During testing, serum sample reacts with the dye conjugate (protein A colloidal gold conjugate), which has been pre-coated in the test device. The mixture then migrates upward on the membrane chromatographically by capillary action to react with recombinant Kala-azar antigen on the membrane and generates a red line. Presence of this red line indicates a positive result, while its absence indicates a negative result. Regardless of the presence of antibody to Kala-azar antigen, as the mixture continues to migrate across the membrane to the immobilized goat anti rabbit IgG region, a red line at the control line region will always appear. The presence of this red line serves as verification for sufficient sample volume and proper flow and as a control for the reagents.

3.10.2. Test Procedure:

Serum and Supplied Buffer were allowed to reach room temperature prior to testing. The ICT test strip for Kala-azar was removed from the foil pouch and 1 drop (20 µl) of serum was added to the test strip in the area beneath the arrow. Then 2 drop (70 – 100 µl) of the supplied Buffer solution (provided with the test kit) was added. The result was taken within 10 minutes. Background was clear before the result of the test was taken. Results interpreted after 10 minutes could be misleading.

3.10.3. Interpretation of ICT test

A) Positive result: The test was positive when a colored control line and colored test line appeared in the test area. A positive result indicated that the ICT detected antibodies to members of *L. donovani complex (Leishmania donovani, Leishmania infantum, Leishmania Chagasi)*

B) Negative result: The test was negative when only the colored control line was appeared. A negative result indicated that the ICT did not detect antibodies to members of *L. donovani* complex.

C) Invalid result: When no colored lines appeared either at the control line or at the line areas, the test was taken as invalid. The test was also invalid if no control line was appeared but a test line was seen, retest using a new test strip and fresh serum was recommended.

3.11. Molecular study (Polymerase chain reaction)

Molecular analysis of samples includes five major steps:

A. DNA extraction from samples,

B. Measurement of DNA concentration

C. Optimization of PCR

D. DNA amplification in thermal cycler,

E. Gel electrophoresis and visualization / documentation under UV light

3.11.1. Extraction of DNA

The blood samples were collected with anticoagulant and buffy coats were separated after centrifugation. Buffy coat DNA was extracted by the following steps. Bone marrow and splenic aspiration and culture positive promastigotes were also extracted by following the same steps by using EZ-10 Spin column genomic DNA minipreps kit (Biobasic, Canada), Catalog No. BS 483, as per manufacturer instructions.

Genomic DNA extraction protocol

1. 500µl whole blood was taken in a centrifuge tube. 800 µl TBP buffer (supplied with kit) was added to the tube. Gently vortexes and let the tube stand for 1 min at room temp.
2. The tube was spin at 4,000 rpm for 3 minutes and the supernatant was discarded.
3. If supernatant and blood pellet remain red in color then again add 800 µl TBP buffer to the tube. Gently vortexes and let the tube stand for 1 min at room temperature. The tube was spin at 4,000 rpm for 3 minutes supernatant was discarded.
4. If the blood pellet looks mauve or colorless, the pellet was dissolved in 200µl TE buffer.
5. 500 µl of TBM buffer was added to the centrifuge tube. The tube was vigorously vortexes and then 3µl proteinase K was added. Incubated at 55°C for 30 minutes.
6. The tube was centrifuged for 2 minutes at 5000 rpm. Supernatant was transferred to another 2.0 micro centrifuge tube & 260µl absolute ethanol was added.
7. The mixture was poured to EZ – 10 columns that were placed in a2.0 ml collection tube. The tube was spin at 10,000 rpm for 2 minutes. The flow through in the collection tube was discarded.
8. 500 µl of wash solution was added & the tube was spin at 10,000 rpm for 1 minute.
9. Again 500 µl of wash solution was added & the tube was spin at 10,000 rpm for 1 minute.

10. The flow- through was discarded. The tube was Spin at 10,000 rpm for an additional minute to remove any residual amount of wash solution.
11. The column was placed into a clean 1.5 ml Eppendrof tube. 30 – 50 µl Elution Buffer was added into the center part of membrane in the column. The tube was incubated at 37°C or 50°C for 2 minutes.
12. The tube was spin at 10,000 rpm for 1 min to elute DNA from the column.
13 Eluted DNA was stored at - 20°C for PCR analysis.

3.11.2. Measurement of DNA concentration:

Extracted DNA was measured as ng/ µl using Nanodrops (Thermo Scientific, USA). The ratio between the readings at 260 nm and 280 nm (OD 260 /0D 280) provides an estimate of the purity of the DNA. Pure DNA preparations have OD 260/OD 280 values of 1.8 *(Maniatis et al., 1989)*

3.11.3. Primer selection:

Two oligonucleotides primers were selected by choosing the sequences from published paper. One new primer was designed for this study. Primers were obtained from "bioserve (Andhra Pradesh, India)". The Primers used are included in the following table.

Table 3.2: Primer sequences used in this study:

Primers Name	**Sequences**	**Amplicon Size (bp)**	**References**
MK1F MK1R	5′ – CCC AAA CTT TTC TGG TCC TC – 3 5′ – GAG CCG ATT TTT GGC ATT T – 3′	104	*This study*
LD1F LD1R	5′ – AAA TCG GCT CCG AGG CGG GAA AC – 3′ 5′– GGT ACA CTC TAT CAG TAG CAC -3′	600	*Srivastava et al,2011*
BHUL 18SF BHUL 18SR	5′– CGT AAC GCC TTT TCA ACT CAC – 3′ 5′ – GCC GAA TAG AAA AGA TAC GTA AG – 3′	311	*Maurya et al, 2005*

3.11.4. Optimization of PCR:

PCR was first optimized using parasitic DNA and then on clinical samples. The annealing temperature of the primers was determined by setting up a temperature gradient PCR (Veriti, ABI,Foster City, USA) in increments of 1°C starting from 43°C. The reaction mixture using primer MK1F/R was amplified in ABI Prism 3130 Genetic Analyzer PCR system (ABI, Foster City, USA) with heated lid option at 95°C for 10 min followed by 40 cycles, each consisting of 40 secs at 95°C, 30 secs at 45°C, and 30 secs at 72°C, and a final extension step of 7 min at 72°C. For each experiment, negative controls were included to check for possible contamination.

3.11.5. PCR Reactions

The reaction mixture (25 mL) contained 1X Taq Polymerase Buffer (Promega), 1.5 mM MgCl2,200 mM concentration of each deoxynucleotide triphosphate (dNTP), 10 pmole of each primer, 1.5 U of Taq DNA Polymerase (Promega) and 50 ng of template. After mixing the reaction mixture with the template DNA, the PCR tube containing reaction mixture was capped and centrifuged briefly to spin down the contents. The PCR tubes were then placed in a thermal cycler (Biometra, Germany). For each experiment, one negative controls and one positive control were included to compare the obtained result.

Table 3.3: Programmed steps of PCR in the thermal cycler

Steps	**For MK1F,1R**	**For LD1F,1R**	**For BHUL18SF, SR**
1.Initial denaturation	95°C for 10 min	94°C for 2 min	95°C for 4 min
2. Denaturation	95°C for 40 sec	94°C for 1 min	94°C for 45 sec
3. Annealing	45°C for 30 sec	45°C for 1 min	62°C for 45 sec
4. Elongation	72°C for 40 sec	72°C for 2 min	72°C for 1 min
5.Looping (step 2 - 4)	40 times	40 times	40 times
6. Final elongation	72°C for 7 min	72°C for 3 min	72°C for 5 min
7. Hold	4°C for 24 hrs	4°C for 24 hrs	4°C for 24 hrs

3.12. Post - PCR detection of amplified DNA by electrophoretic analysis

The successful amplifications of newly targeted kinetoplastic gene and and other previously used different target genes were examined by resolving the PCR products in 2% agarose gel. Agarose (Sigma, USA) was dissolved in 1X Tris-acetate EDTA (TAE) buffer to give a final concentration of 2% agarose and was heated to dissolve in a microwave oven for about 2.5 - 3 minutes.When the temperature came down to 50^{O}C, 5% ethidiumbromide was mixed with the gel and was poured onto the gel tray (Labnet Gel XL Ultra V-2 Mini-Gel System, USA) already fixed with appropriate combs. Following solidification of the gel, it was submerged in 1X TAE buffer in a gel running tank. 5 µl PCR product was mixed with 1 µl of 6X gel loading dye and loaded into the slots of the gel with the aid of a micropipette. Electrophoresis was continued with 100 volts until DNA fragments were separated. The ethidiumbromide stained DNA bands were observed on a UV transilluminator (UVstar, USA). Photographs were taken using Gel Doc (AlphaImager, USA) machine attached to a computer and bands were analyzed. The PCR products were further purified using a system such as Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and was stored at –20°C.

3.13. Sequencing and analysis

3.13.1. Purification of amplified PCR product and sequencing

The newly designed kinetoplastic gene PCR products of representative strains Leishmania donovani were purified with the Wizard PCR SV Gel and PCR Clean-Up System kit (Promega, USA) according to the manual instruction. Briefly, the PCR products were separated through electrophoresis. Following electrophoresis, if only single band in the target region was seen each PCR product was taken. PCR product was dissolved in equal volume of Membrane Binding Solution (Appendix-II). Dissolved gel mixtures were then transferred to the mini column assembly and were centrifuge to bind the PCR amplicon. After discarding flow through, the column was reinserted into a collection tube. Amplified DNA bound to the spin column was then washed twice with 750 µl of Membrane Wash Solution (Appendix-II) and was eluted with 30 µl of nuclease-

free water, added to the spin column. Finally, the column was centrifuged for 1 minute at room temperature and the pure amplified DNA was stored at –20°C for further analysis. Representative purified kinetoplastic DNA PCR products were sequenced from "1st BASE, Laboratories, Malaysia" by using forwards MK1F and reverse MK1R primers (designed primer) (Table 3.2).

3.13.2. Sequence alignment and identification

Partial sequences, obtained using forward and reverse primers (Table 3.2), were combined to full length sequences (104 bp) via the SeqMan Genome Assembler (DNAstar, USA) and were compared to the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) by means of the basic local alignment search tool (BLAST) to find its iedntity.

a. Reference sequences were download from, NCBI: http://www.ncbi.nlm.nih.gov
b. Acquired sequences were aligned, checked and trimmed by using
    - ClustalW and GeneDoc
    - MEGA 5

Briefly, the multiple sequence alignment of the retrieved reference sequences from NCBI, EMBL or DDBJ and representative isolates' sequences were performed with the ClustalW *(Larkin, Blackshields et al. 2007)* software (Fig. 2.4). Aligned sequences were exported to the GeneDoc software for sequence trimming and conserved region identification.

Chapter 4

RESULTS

4. Results

Kala azar is an endemic disease in Bangladesh. It is a progressive disease and the mortality rate in untreated cases ranges from 75% to 95%. Death usually occurs within 2 years (*Salam et al, 2009).* But it can be completely cured by successful treatment after accurate diagnosis. So, early diagnosis of leishmaniasis is important in order to avoid severe clinical manifestations to the patients. The traditional diagnosis employs isolation of parasite in bone marrow or splenic aspiration under microscope or in vitro culture. But the invasiveness and potentially fatal complications associated with collecting samples from bone marrow or splenic aspiration had been a cause of concern that demanded to develop a noninvasive diagnostic approach without compromising the reliability and efficacy of the detection system. Detection of *Leishmania donovani* in peripheral blood buffy coat by Polymerase chain reaction (PCR) is a minimally invasive procedure for the diagnosis of visceral leishmaniasis (VL) which has been tried as an alternative (*Maurya et al, 2005; Srivastava et al,2011*), however its success is yet to be ascertained with great sensitivity and specificity. This study attempts to address this issue along with traditional diagnosis system, for a comparative analysis.

4.1. Samples for the study

One hundred and thirty six samples were included in this study. Blood samples from 35 patients were appeared positive in an immunochromatographic test, rK39 strip as this method scores the presence of circulating antibodies, if any, in blood, as a result of immune response, past or present. This result was presumptively considered as "positive" with VL for the study and therefore, collection of bone marrow and splenic aspirations from 25 and 10 patients were conducted respectively. It may be mentioned that bone marrow or splenic aspiration are used as specimen for the conduction of traditional diagnosis, like culture and microscopy.

As far as the control reactions are concerned, this study included two types of individuals. I) Healthy individuals: A total of sixteen blood samples were collected each from sixteen volunteers. They were from endemic areas of kala azar, i.e. Mymenshing, Gazipur,

Natore etc. In addition, twenty five blood samples were drawn each from twenty five volunteers, hailed from non endemic zone, i.e., Dhaka city. II) Diseased individuals: Twenty five blood samples were taken from twenty five patients infected with other similar diseases or having similar sign and symptom like kala azar, for example, Tuberculosis, Dengue, Malaria etc. Both types of Healthy and diseased individuals produced rK39 test negative, hence were considered as negative controls.

Individuals contributing their valuable specimen were asked to fill up a questionnaire after taking their oral consent. Questionnaire form and ethical clearance statements are attached in Appendix IV.

Table 4.1: Distribution of collected specimens:

Specimen type	Sample type	Number	Percent
Tests	Samples from suspected VL patient's bone marrow	25	18
	Samples from suspected VL patient's splenic aspiration	10	08
	Samples from suspected VL patient's Blood	35	26
Controls	Blood samples from healthy individuals from non endemic zone	25	18
	Blood samples from healthy individuals from endemic zone	16	12
	Diseased control (Patient have similar diseases like MTB, Malaria, Dengue etc)	25	18
	Total	136	100

4.2. Patient's Sample:

A total of seventy specimens were collected from thirty five patients suspected with Visceral Leishmaniasis (VL). Thirty five blood samples were drawn from 35 patients*.Out of these 35 rK39 positive cases, bone marrow and splenic aspirations were collected from 25 and 10 patients respectively. Among these 35 patients 22 patients were male and the rest 13 were female (Fig. 4.1)

* These samples were all appeared rK39 positive.

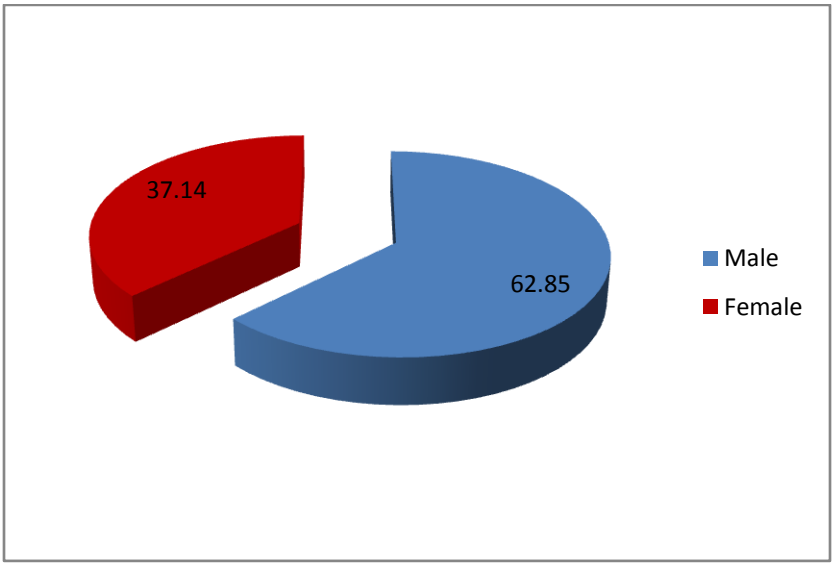


Fig.: 4.1. Sex – wise percentile distribution of kala azar patients.

4.3. Control samples:

Blood samples from 25 healthy individuals* from areas considered endemic (e.g. Mymensing, Gazipur, Natore etc) and blood samples from 16 healthy individuals* from areas considered non endemic (e.g. Dhaka city) for kala azar were collected. Blood samples were also collected from 25 patients* diagnosed with various types of diseases other than Leishmaniasis. Among this 66 control samples 35 (53%) were male and the rest 31 (47 %) were female. Their sex distribution is mentioned in a pie graph (Fig 4.2)

* These samples were all appeared rK39 negative

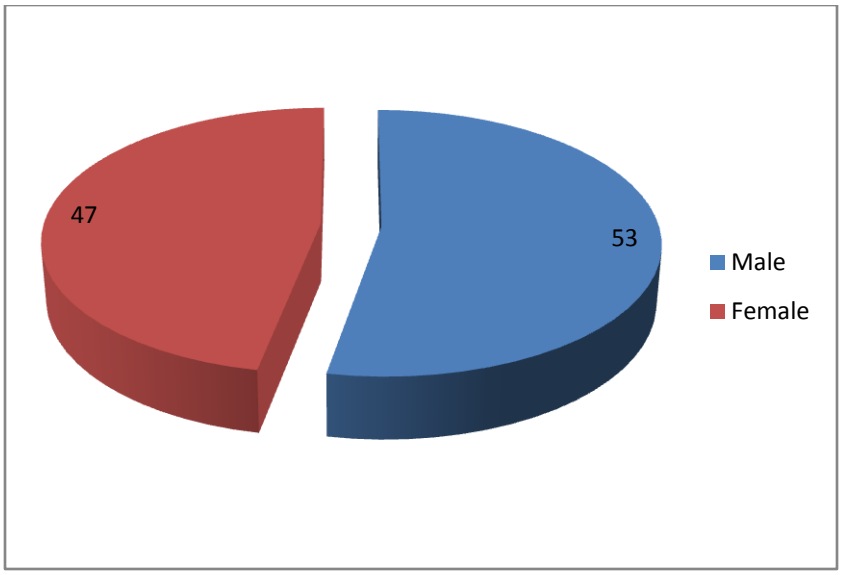


Fig.: 4.2. Sex – wise Percentile distribution of control samples.

4.3.1. Distribution of Diseased Control:

Twenty five blood samples were collected each from 25 patients diagnosed with various similar types of diseases other than Leishmaniasis. The distribution of these types of patients is illustrated in (Fig. 4.3)

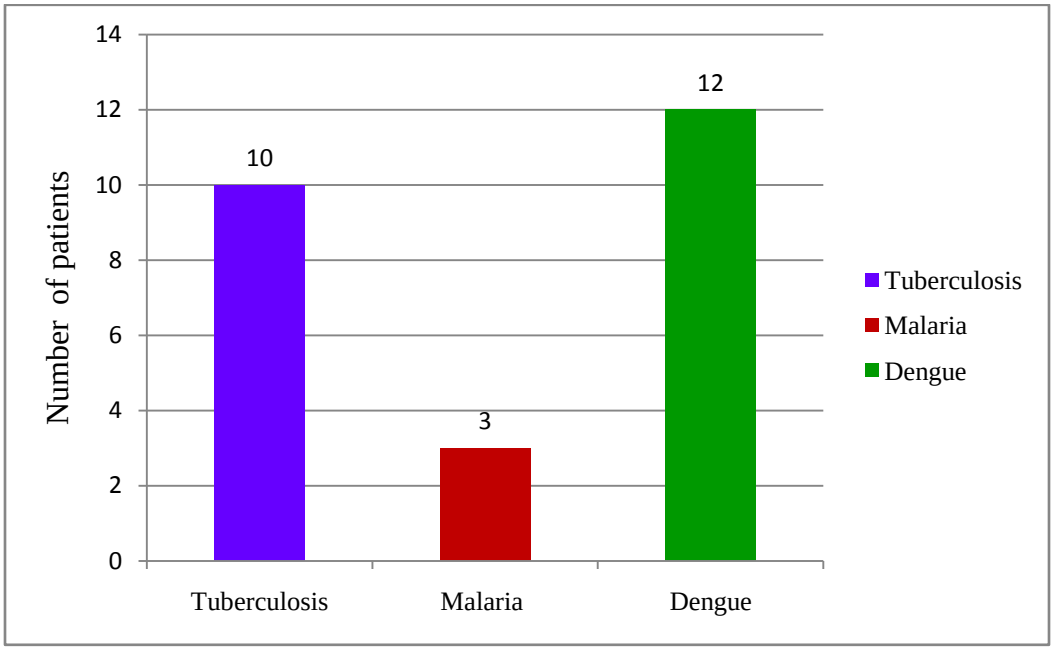


Fig.: 4.3. Distribution of Diseased control according to disease type.

4.4. Clinically suspected VL patient's characteristics (According to History):

The clinical histories of patients suspected with kala azar were taken and tabulated accordingly (Table 4.2). It appears that all the patients had the typical characteristics such as Splenomegaly, anemia, weight loss etc. Hepatomegaly or enlargement of liver was prominent in about 69% of patients.

Table 4.2: Distribution of patient's characteristics

Analyzed parameters	Percentages (%)
Male	62.9
Female	37.1
Fever	100
Duration of fever in Months (Mean)	1.6
Maximum patient's age group (3-13 yrs)	57.1
Anemia	100
Anorexia	100
Weight loss	100
Darkening of Skin	68.6
Splenomegaly	100
Hepatomegaly	68.5
Lymphadenopathy	48.6
Past history of VL	14.3
Family history of VL	11.5
Housing Material (Mud made house)	88.6

After the successful collection and storage of samples, each of them was then subjected to investigation as per the following order:

a. rK39 Immunochromatographic (ICT) based dip stick test
b. Culture on Nicolle Novy McNeal(NNN) medium
c. Microscopy
d. Molecular assay

4.5.: rk39 Immunochromatographic (ICT) dipstick test:

Plasma samples from all one hundred and one subjects were taken and tested for rK39 immunochromatographic (ICT) dipstick test in order to detect the presence of antibody in patient's plasma against visceral leishmaniasis (Fig.4.4). All the suspected kala azar patients show a positive rK39 test. None of the control (Including healthy endemic, non endemic and diseased control) gave rK39 positive test (Table 4.3). As rK39 was used as a screening test, individuals with rK39 positive were included as patients or test samples in the study and individuals having rK39 negative were considered as controls.

Table 4.3: rk39 Immunochromatographic (ICT) dipstick test result:

	rK39 Test		
Individual Types	Positive	Negative	Total
Samples from suspected VL patient's (case)	35	0	35
Samples from healthy Non endemic control	0	25	25
Samples from healthy endemic control	0	16	16
Samples from diseased control	0	25	25
Total	35	66	101

Note: Bar diagram indicates percentages.

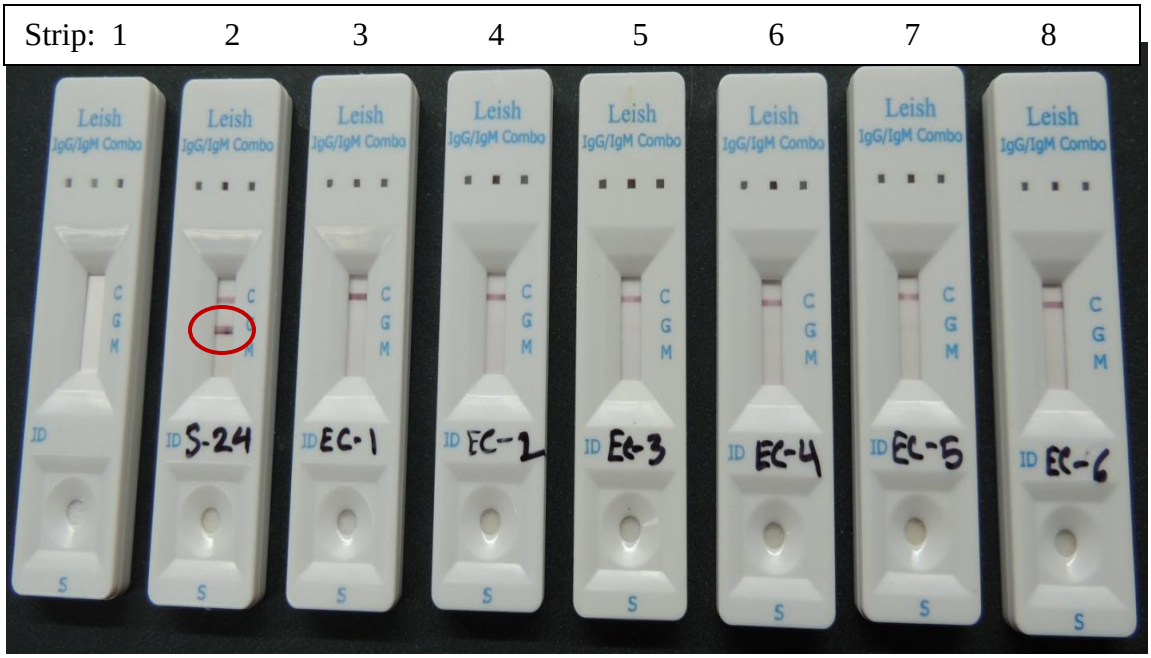


Fig.4.4: Result of rK39 immunochromatographic test. Strip 1: Blank strip, strip2: Positive for Leishmania indicated as circle around the signal band, Strip 3 - 8: Negative for Leishmania.

4.6.: Culture of Bone marrow or splenic aspiration in Nicolle McNeal Novy (NNN) medium:

Fourteen out of 25 bone marrow aspirations from suspected kala azar patients showed growth of promastigotes in Nicolle Novy McNeal (NNN) medium and 11 patients show no growth in culture (Fig 4.6 and Fig 4.7). Likewise, 6 splenic aspirations out of 10 suspected kala azar patients' exhibited positive in culture while four produced no growth in culture (Table 4.4). Bone marrow or splenic aspirations from control individuals were not sampled because it is not ethically justified to aspirate these specimens from a healthy person.

Table 4.4: Result of culture growth of bone marrow and splenic aspiration specimens

Individual Types	Culture Positive	Culture Negative	Total
Samples from suspected VL patient's bone marrow	14	11	25
Samples from suspected VL patient's splenic aspiration	6	4	10
Total	20	15	35

4.7.: Culture of Peripheral Blood in Nicolle Novy McNeal (NNN) medium:

The peripheral blood from control and patients were subjected to centrifugation and Buffy coat layer was separated (Fig. 4.5) and cultured in Nicolle Novy McNeal (NNN) medium. None of the preparation, be it test or control samples produced growth of promastigotes in NNN medium (Table 4.5)

Table 4.5: Result of Peripheral Blood culture

Individual Types	Culture Positive	Culture negative	Total
Samples from suspected VL patient's blood	0	35	35
Samples from Healthy Non endemic control's blood	0	25	25
Samples from healthy endemic control's blood	0	16	16
Samples from Diseased control's blood	0	25	25
Total	0	101	101

4.8.: Bone marrow or splenic aspiration microscopy for LD body:

Seventeen out of 25 bone marrow aspirations and 6 out of 10 splenic aspirations had *Leishmania donovani* parasite detected under microscope (Table 4.6) (Fig 4.8). As described earlier, bone marrow or splenic aspirations were not sampled from control subjects. The parasite burdens in the smear positive cases were graded as described by Chulay and Bryceson (1983). Around thirty three percent (8/24) parasite positive patients had grade 1+ parasite load, and about sixty two percent (15/24) had equal or greater than grade 2+ parasite load.

Table 4.6: Bone marrow or splenic aspiration microscopy

Individual Types	Microscopy Positive	Microscopy Negative	Total
Samples from suspected VL patient's bone marrow	17	8	25
Samples from suspected VL patient's splenic aspiration	6	4	10
Total	23	12	35

Note: Bar diagram indicates percentages

4.9.: Peripheral blood microscopy for LD body:

The peripheral blood from control and patients were observed under microscope. None of the specimen, be it test or control showed *Leishmania* parasite under microscope (Table 4.7) (Fig 4.9).

Table 4.7: Result of Peripheral blood microscopy

Individual Types	Microscopy Positive	Microscopy Negative	Total
Samples from suspected VL patient's blood	0	35	35
Samples from healthy Non endemic control's blood	0	25	25
Samples from healthy endemic control's blood	0	16	16
Samples from diseased control's blood	0	25	25
Total	0	101	101

Note: Bar diagram indicates percentages

Fig. 4.5: Separation of buffy coat from peripheral blood.

Fig 4.6: Culture of Bone marrow, splenic aspiration and blood in NNN medium

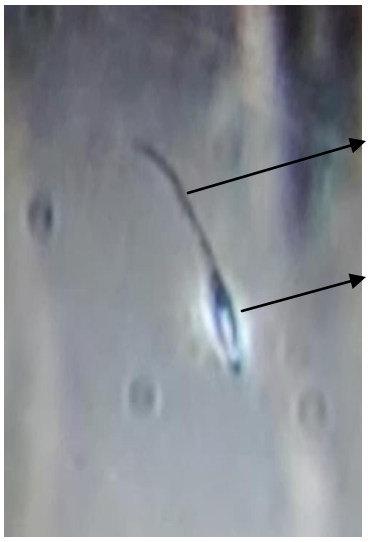


Fig.4.7: Promastigote body of *Leishmania* spp under 40X microscope obtained from liquid portion of culture medium.

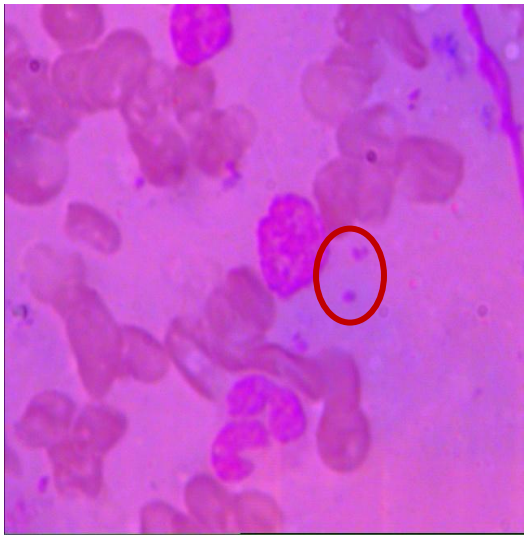


Fig.4.8: *Leishmaniain* in splenic aspiration smear at 100X (oil immersion) lens under microscope, as circle around the amastigotes.

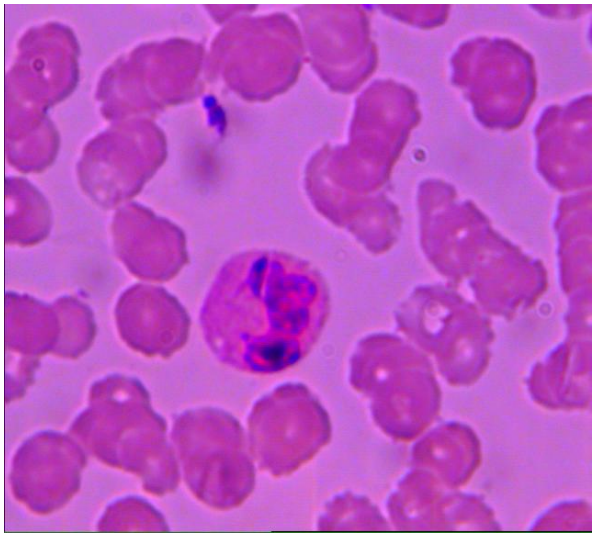


Fig.4.9: Negative for Leishmania in peripheral blood film at 100X (oil immersion) lens under microscope.

4.10. Result of microscopy and culture of different samples from suspected kala azar cases:

Out of 35 different samples collected from bone marrow and splenic aspiration a total 26 (74.3%) were positive in either culture or microscopy. But only 23 (65.7%) samples were positive in microscopy and 20 (57.1%) samples were positive in culture. Among 25 bone marrow samples, 17 were positive for microscopy but 14 were positive for culture. Out of 10 splenic aspiration samples, 6 were positive for both microscopy and culture.

Table 4.8: Result of microscopy & culture of different samples from suspected cases

Samples of Suspected kala azar cases	Total number	Result positive by		
		Microscopy n (%)	Culture n (%)	Either culture or microscopy n (%)
Bone marrow	25	17 (68)	14 (56)	19 (76)
Splenic aspiration	10	6 (60)	6 (60)	7 (70)
Total	35	23 (65.7)	20 (57.1)	26 (74.3)

4.11.: Microscopy and culture positivity among the kala azar cases:

Out of 35 bone marrow or splenic aspiration samples 17 samples were found positive by both culture and microscopy. Three (8.6%) were positive only by culture and 6 were positive only by microscopy that were negative by culture. In total 26 (74.3%) samples were positive either by culture or microscopy and 9 (25.7%) were negative by both.

Table 4.9: Microscopy and culture positivity among the suspected kala azar cases

Tests	Positive	%
Both culture and microscopy	17	48.5
Only culture	3	8.6
Only Microscopy	6	17.1
Either culture or microscopy	26	74.3

Analyzing the above mentioned findings it can be concluded that bone marrow and splenic aspiration produced 57.2% culture positive and 65.7 % microscopy positive results. Although, on the other hand the buffy coat preparations from peripheral blood samples produced rK39 tests positive, they all failed to produce even a single positive reaction either in culture or in microscopy, indicating the parasite load in circulating blood was too low to be detected during the period of investigation. This observation prompted us to use molecular investigation in order to use blood samples for detecting kala azar.

4.12. Molecular investigations:

4.13. Design of a new primer:

A pair of primers was designed by using the Primer 3 plus programm (code available at http://www-genome.wi.mit. edu/genome_software/other/primer3.html) from conserved sequences of the kinetoplastic DNA of Leishmania gene (GenBank accession numbers: AF169137, AF103739.1, AF190476, Y11401.1, X84844.1, Z35273.1). Forward primer designated as MK1F which reads 5'- CCC AAA CTT TTC TGG TCC TC -3' (from 39 to 58 bases) and reverse designated as MK1R was 5'– GAG CCG ATT TTT GGC ATT T -3' (from 123 to 141 bases). This primer pair can amplify a (141 – 39) = 102 bp DNA fragment specific for Leishmania (GenBank accession number: AF169137, AF103739.1, AF190476, Y11401.1, X84844.1, Z35273.1) as illustrated in Fig. 4.10. Specificity of the primer pair was examined by using the BLAST program and aligning their sequences with all DNA sequences entered in the databases and checking for similarities.

Figure 4.10: Multiple sequence alignment of full mitochondrial kinetoplast sequence of three different *Leishmania* spp (L donovani, L chagasi and L infantum) and position of both forward (MK1F) and reverse (MK1R) primer.

4.14. Optimization of PCR:

The reaction was carried out as explained in section 3.11.4 of Chapter: materials and method and was finally optimized as illustrated in Fig. 4.11.

The leishmanial kinetoplastic minicircle DNA was purified from promastigotes obtained from bone marrow culture using EZ-10 Spin column genomic DNA minipreps kit; blood (Biobasic, Canada). These were used as template in a PCR reaction with the newly designed primer MK1F and MK1R (Fig 4.12). These promastigote were used as positive control in next steps.

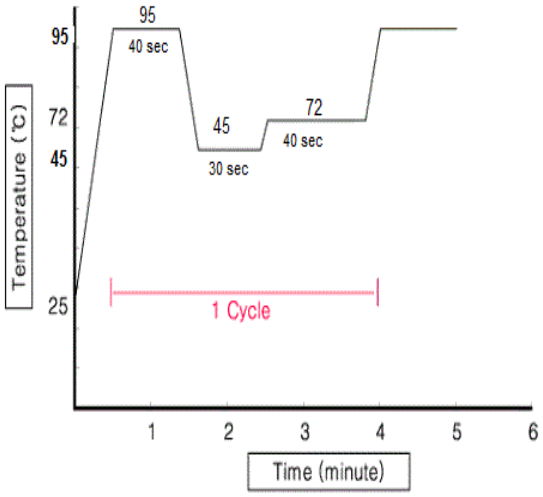


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Fig. 4.11: Optimization of PCR

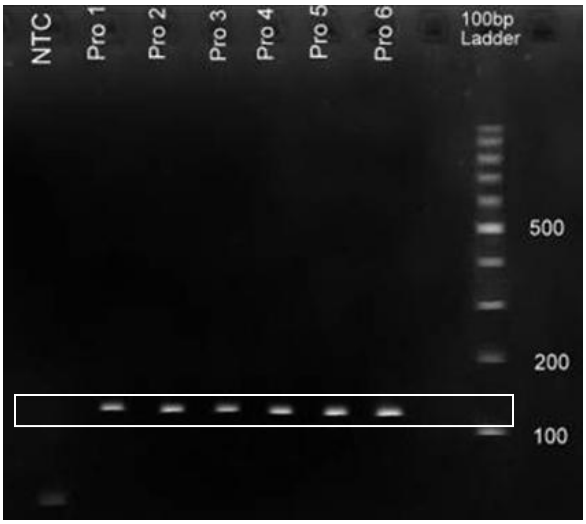


Fig 4.12: PCR amplification of the 102 bp product of Leishmanial kinetoplastic minicircle gene with newly designed primer MK1F and MK1R. Lane 1: NTC or negative template control with no added primer, Lane 2 - 7: Positive Leishmania (promastigotes) samples, Lane 7: 100bp DNA ladder

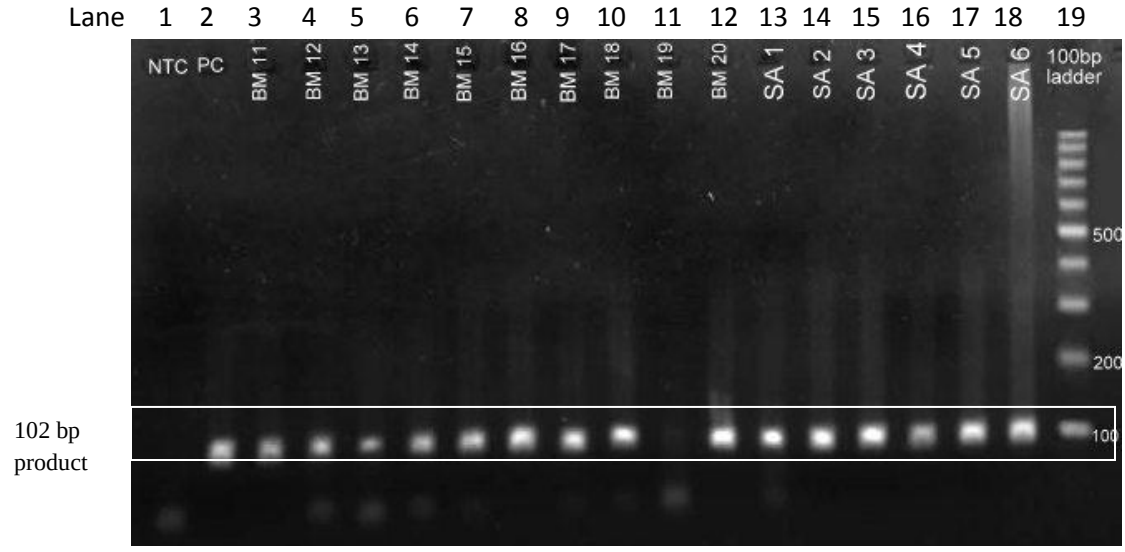
4.15.: PCR amplification of Visceral Leishmaniasis (VL) specimens with newly designed primers (MK1F & MK1R):

PCR has been done with samples from VL positive patient's bone marrow, splenic aspiration and buffy coat preparations with newly designed primer. The PCR products, as revealed on a 2% agarose gel, were appeared positive in 95% specimens of bone marrow and 100% specimen of splenic aspiration. Interestingly all the buffy coat's DNA yield positive result after PCR. PCR was done with only those patient's samples which were positive for VL either by culture or microscopy.

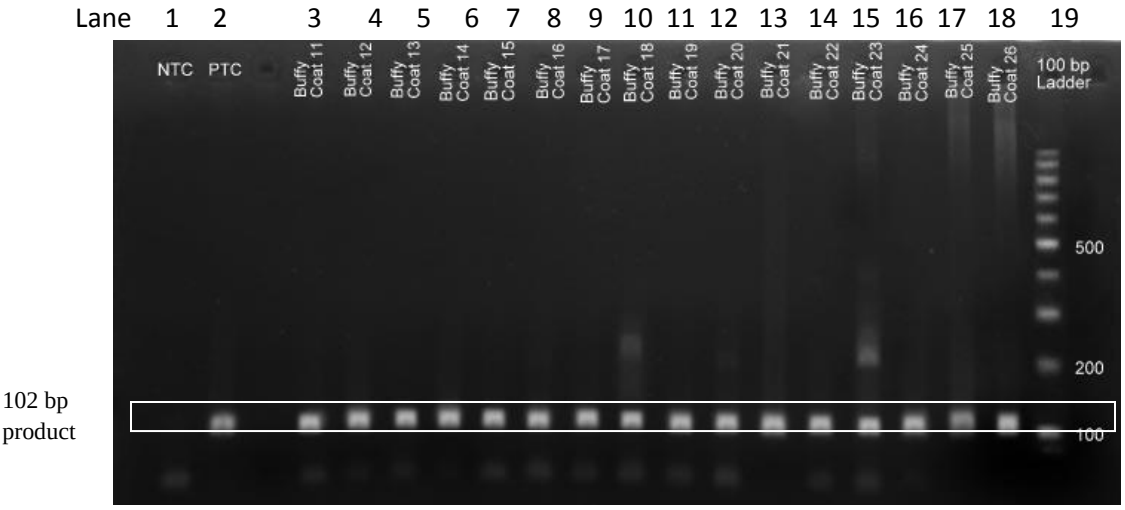
Table 4.10: PCR amplification of VL patients samples with newly designed primers:

Individual Types	Positive (%)	Negative (%)	Total
Samples from VL positive patient's bone marrow	18 (94.74%)	1 (5.26 %)	19
Samples from VL positive patient's splenic aspiration	7(100%)	0 (0 %)	07
Samples from VL positive patient's Buffy coat (i. e. Blood)	26 (100%)	0 (0 %)	26
Total	51	01	52

Note: Parenthesis indicates percentage.



4.13 (a)



4.13(b)

Fig.4.13: Amplification of the 102 bp product of *L. donovani* by PCR from 4.13.(a) bone marrow (BM) and splenic aspiration(SA) , 4.13.(b) blood of suspected VL patients samples with MK1F & MK1R primer. 4.13.(a) Lane 1: Negative template control, Lane2: Positive template control, Lane 3 - 10,12 - 18: Positive Leishmania sample, Lane11: Negative Leishmania sample, Lane19: 100bp DNA ladder. 4.13.(b) Lane 1: Negative template control, Lane2: Positive template control, Lane 4 - 18: DNA from buffy coat preparation of positive Leishmania sample, Lane19: 100bp DNA ladder

4.16.: PCR amplification of Healthy controls (both endemic and non endemic) individuals with newly designed primers (MK1F & MK1R):

No PCR amplification was seen in both cases of healthy endemic and non endemic controls with designed primer.

Table 4.11: PCR amplification result of healthy endemic and non endemic controls:

Individual Types	Positive (%)	Negative (%)	Total
Blood samples from healthy Non endemic control	0 (0 %)	25 (100%)	25
Blood samples from healthy endemic control	0 (0 %)	16 (100%)	16
Total	00	41	41

Note: Parenthesis indicates percentage.

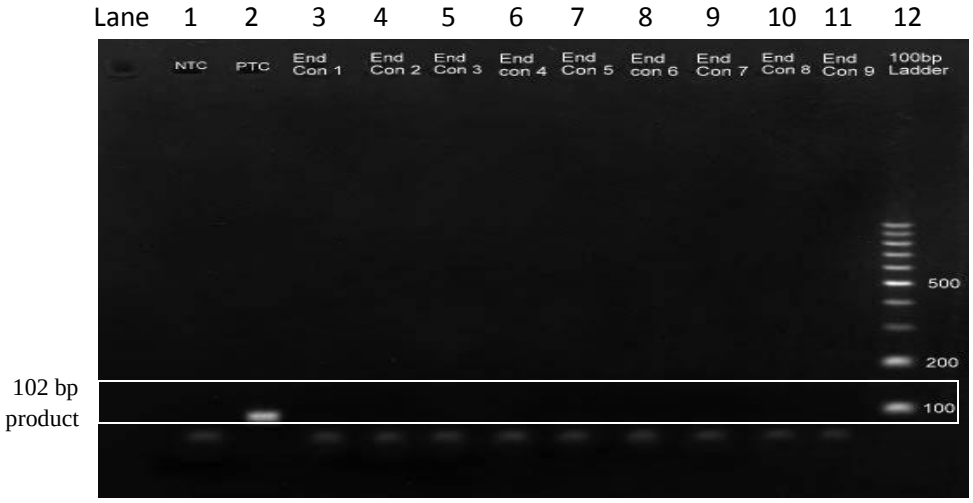
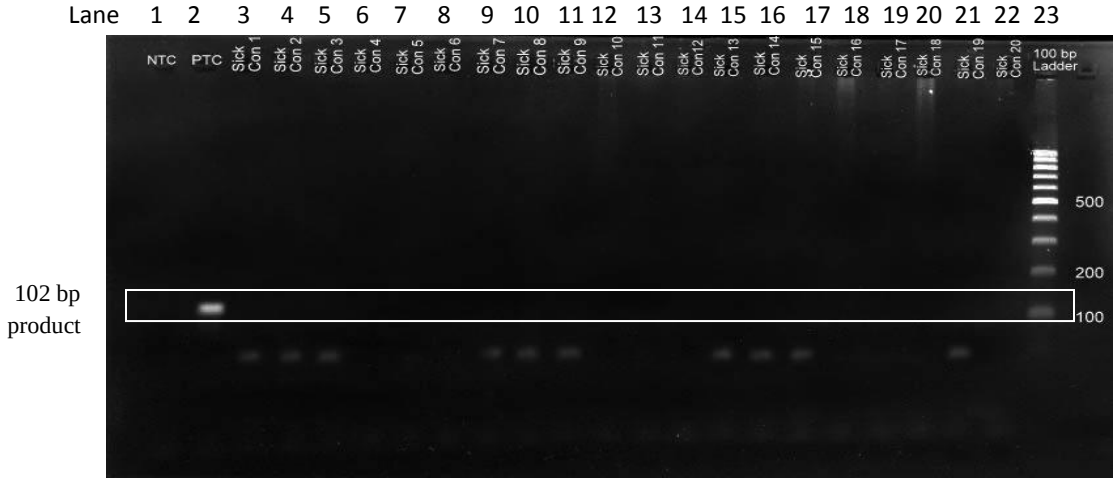
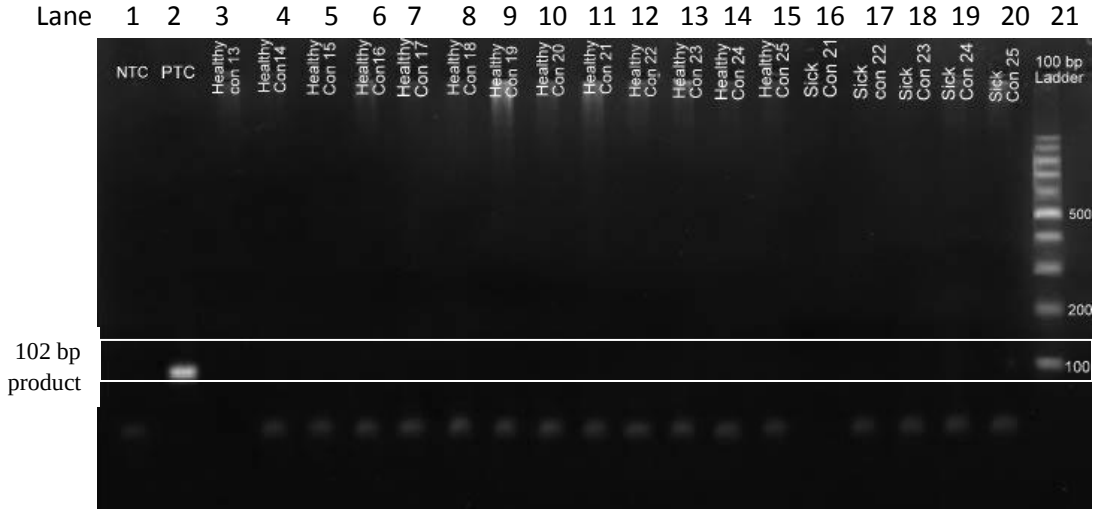


Fig.4.14: Amplification of the 125bp product of *L. donovani* by PCR from blood of endemic control individuals with MK1F & MK1R primer. Lane 1: Negative template control, Lane2: Positive template control, Lane 4 - 11: Negative Leishmania sample, Lane 12: 100bp DNA ladder.



4.15.(a)



4.15.(b)

Fig.4.15: Amplification of the 125bp product of *L. donovani* by PCR from blood of healthy and diseased control individuals with MK1F & MK1R primer. 4.15. (a) Lane 1: Negative template control, Lane2: Positive template control, Lane 3 - 22: Negative Leishmania sample, Lane 23: 100bp DNA ladder. 4.15. (b) Lane 1: Negative template control, Lane2: Positive template control, Lane 3 - 20: Negative Leishmania sample, Lane 21: 100bp DNA ladder..

4.17.: PCR amplification of diseased control individuals with newly designed primers (MK1F & MK1R):

No PCR amplification was seen in any cases of disease controls (Malaria, Tuberculosis, Dengue patients) with our designed primer (Fig: 4.15).

Table 4.12: Newly designed primers PCR amplification result with diseased controls:

Individual Types (Diseased control)	Positive (%)	Negative (%)	Total
Blood samples from Tuberculosis patients	0 (0 %)	10 (100%)	10
Blood samples from Dengue patients	0 (0 %)	12 (100%)	12
Blood samples from Malaria patients	0 (0 %)	3 (100%)	3
Total	00	25	25

Note: Parenthesis indicates percentage.

4.18. Sensitivity and specificity of Designed primer:

Sensitivity and Specificity of our primer for PCR reactions has been calculated. While more than 98% sensitivity was recorded for primer Pair MK1F & MK1R, their specificity was flawless i. e. 100%.

Table 4.13: Sensitivity and specificity of designed primer:

Test	Culture &/or Microscopy positive	Culture & Microscopy negative	Total	Sensitivity	Specificity
PCR positive	(a)True positive 51 (98.08%)	(b) False positive 0 (0.00%)	(a + b) = 51	98.08%	100%
PCR negative	(c)False negative 01 (1.92%)	(d)True negative 66 (100%)	(c + d) = 67		
Total	(a + c) = 52	(b + d) = 66	(a + b + c + d) = 118		

Sensitivity and specificity of the designed primer for PCR reactions was calculated by the following formula:

Sensitivity= a / (a + c) ×100

Specificity=d / (b + d) × 100

4.19.: Sequencing of designed primer amplified region:

Two DNA samples, one obtained from promastigote, collected from splenic aspiration culture (Fig.4.16) and the other obtained from amastigote collected from human blood (Fig.4.17) were used as templates in a PCR system to have PCR product that were sent for sequencing. The 102 bp long PCR product was purified by Wizard PCR SV Gel and PCR Clean-Up System kit (Promega, USA) according to the manufacturer's instruction. After analyzing the obtained sequences with NCBI the data shows maximum identity with Leishmania donovani complex (*Leishmania donovani, Leishmania Chagasi, Leishmania infantum*) (Fig: 4.18).

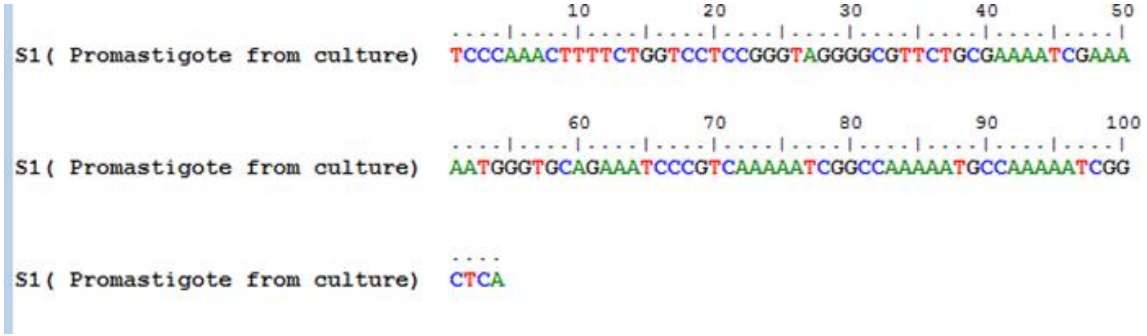


Fig 4.16: Sequences of designed primer amplified region obtained from Promastigote from culture.

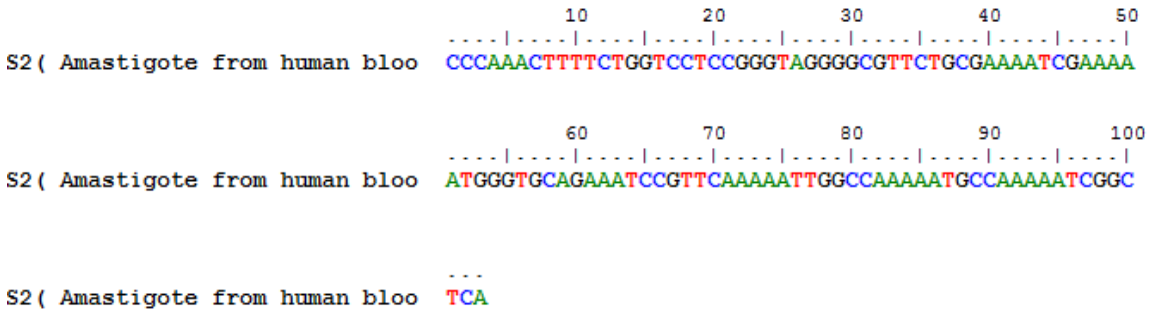


Fig 4.17: Sequences of designed primer amplified region obtained from Amastigote from human blood.

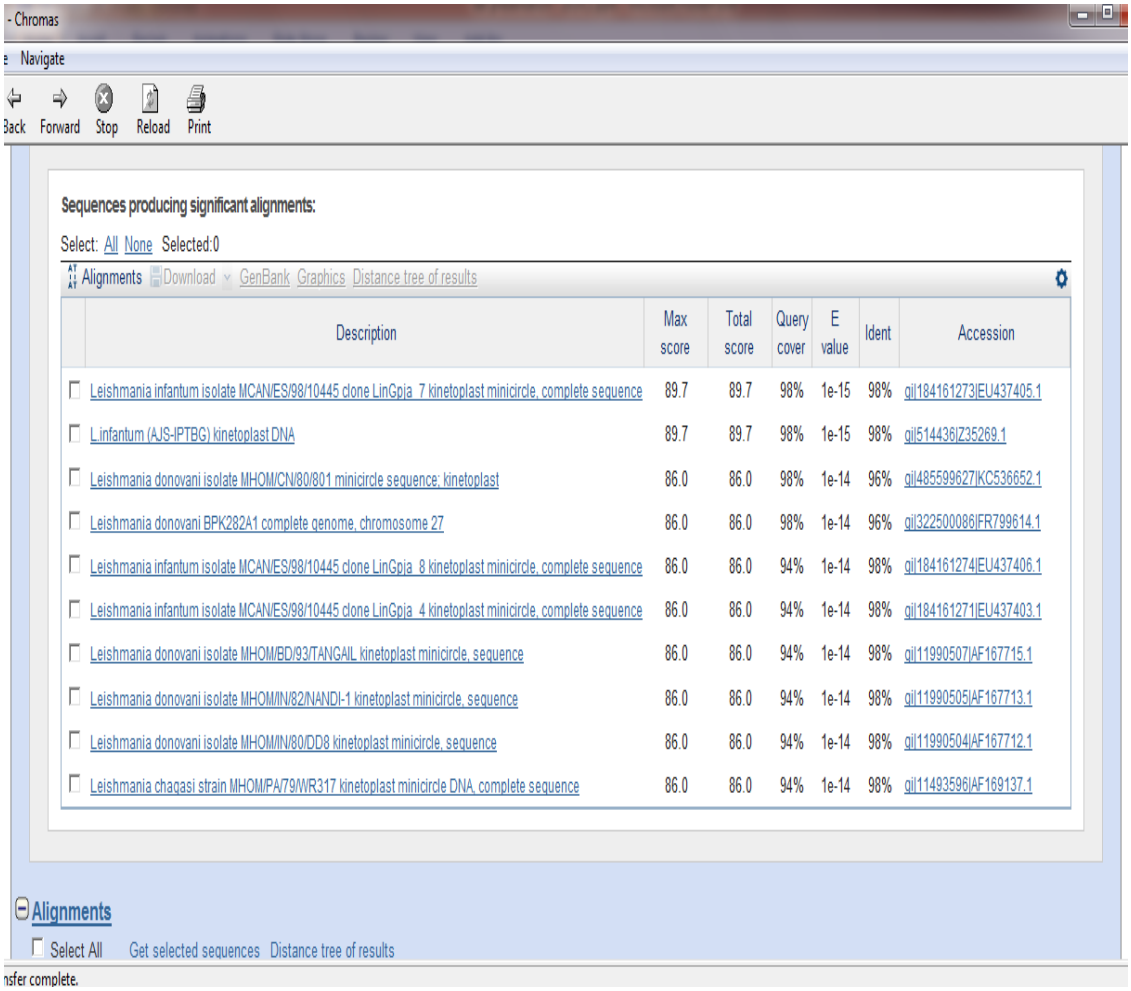


Fig. 4.18: BLAST searching showing maximum identity with Leishmania donovani complex.

The identity of the PCR products with Leishmania donovani complex (*Leishmania donovani*, *Leishmania Chagasi*, *Leishmania infantum*) further confirms the reliability of the detection procedure.

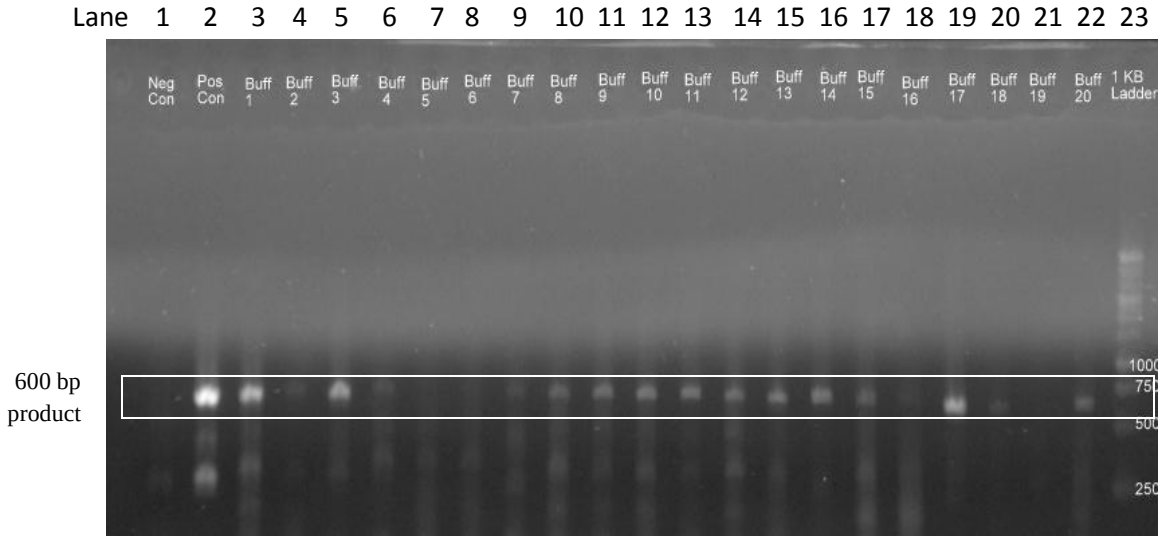
4.20.: PCR amplification with Ref 1 (LD1F, LD1R) primer:

In order to compare the primer designed for this study with other primer sequences already in practice, two primer pair sequences were ordered and thereby were served as reference primer. The first primer, used in this study was referred by *Maurya et al, 2005*. PCR has been done with Ref1 (LD1F, LD1R) primer which target is kinetoplastic minicircle DNA. Among 26 VL patient's Buffy coat 21(80.08%) was amplified with Ref 1 primer and 5(19.92 %) was not amplified with ref1 primer. Among 10 controls (including Healthy endemic, non endemic & diseased) individuals blood all remains negative with Ref 1 primer (Fig: 4.19).

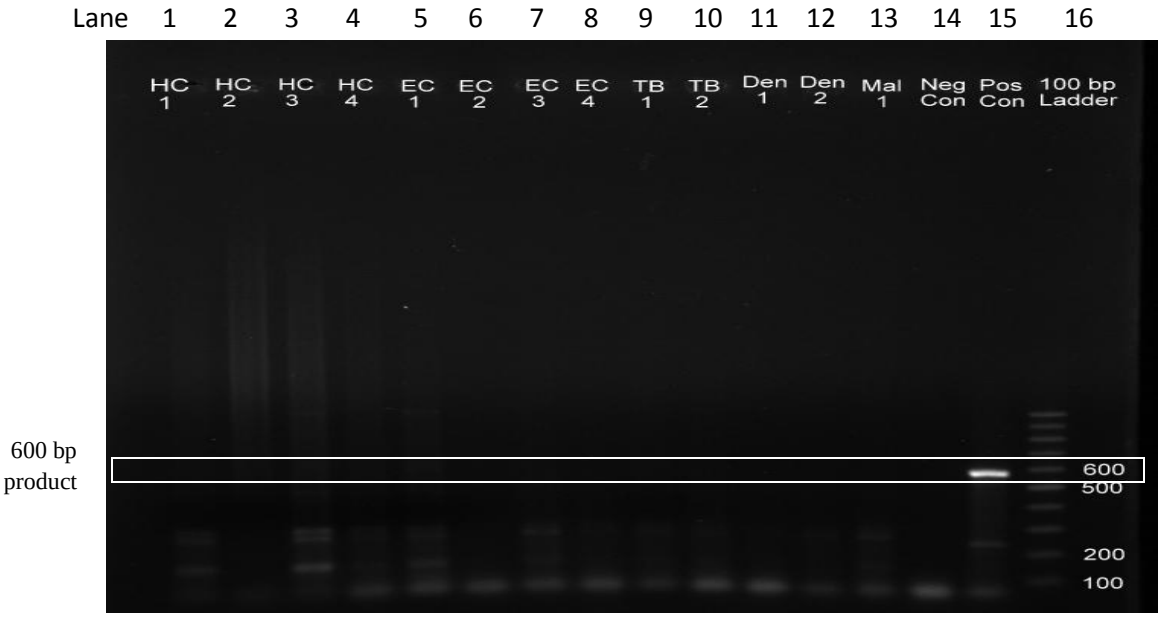
Table 4.14: Ref 1 (LD1F, LD1R) primer amplification result with VL patient's and control individuals:

Individual Types	Positive (%)	Negative (%)	Total
VL positive patient's Buffy coat (i. e. Blood)	21(80.08%)	5 (19.92 %)	26
Control (Healthy, endemic & diseased) individuals blood	0 (00%)	10 (100%)	10
Total	21	15	36

Note: Parenthesis indicates percentage.



4.19.(a)



4.19.(b)

Fig.4.19: Amplification of the 600bp product of *L. donovani* by PCR with Ref 1 (LD1F, LD1R) primer. 4.19.(a)Lane 1: Negative template control, Lane2: Positive template control, Lane 3,5,6,9,10,11,12,13,14,15,16,17,19,20, 22: Positive Leishmania sample, Lane 4,7,8,18:Negative Leishmania sample, Lane 23: 100bp DNA ladder. 4.19.(b) Lane 1 - 13: Negative Leishmania sample, Lane 14: Negative template control, Lane15: Positive template control, Lane 16: 100bp DNA ladder.

4.21. Sensitivity and specificity of Ref 1 (LD1F, LD1R) primer:

Sensitivity of Ref 1 (LD1F, LD1R) primer has been calculated as per the formula mentioned in Section 4.17 and it was 80.76%. Specificity was also calculated and it was 100% (Table 4.15).

Table 4.15: Sensitivity and specificity of Ref 1 (LD1F, LD1R) primer:

Tests	Culture &/or Microscopy positive	Culture & Microscopy negative	Total	Sensitivity	Specificity
PCR positive	(a)True positive 21 (80.76%)	(b)False positive 0 (0.00%)	(a + b) = 21	80.76%	100%
PCR negative	(c)False negative 05 (19.24%)	(d)True negative 10 (100%)	(c + d) = 05		
Total	(a + c) = 26	(b + d) = 10	(a + b + c + d) = 36		

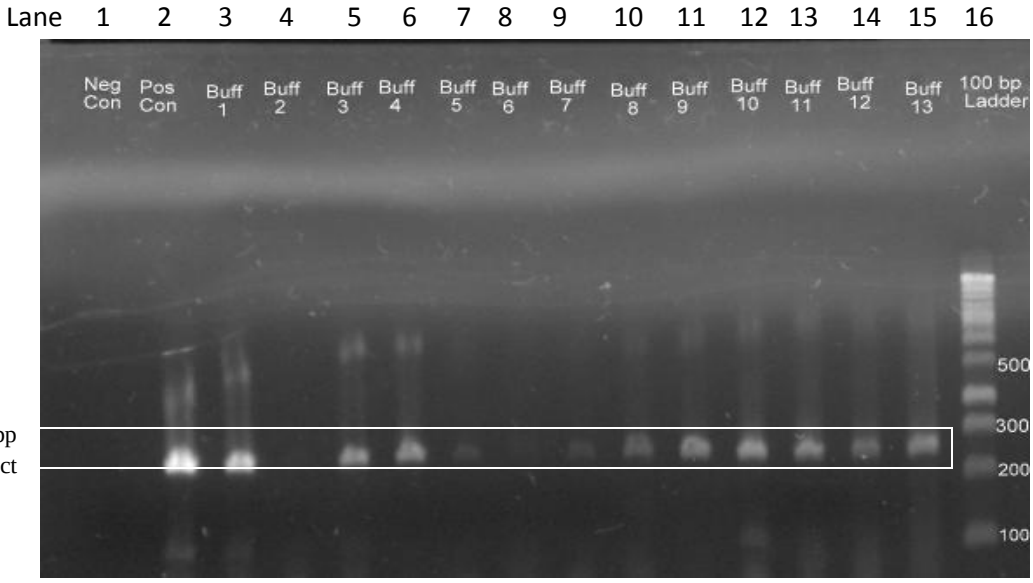
4.22.: PCR amplification with Ref 2 (BHUL18SF, BHUL18SR) primer:

PCR has been done with Ref 2 (BHUL18SF, BHUL18SR) primer which target is Leishmania nuclear rRNA gene. Among 26 VL patient's Buffy coat 22 (84.62%) was amplified with Ref2 primer and 4 (15.38 %) was not amplified with Ref2 primer. Among 10 controls (including Healthy endemic, non endemic & diseased) individuals blood all remains negative with Ref 2 primer (Fig 4.20).

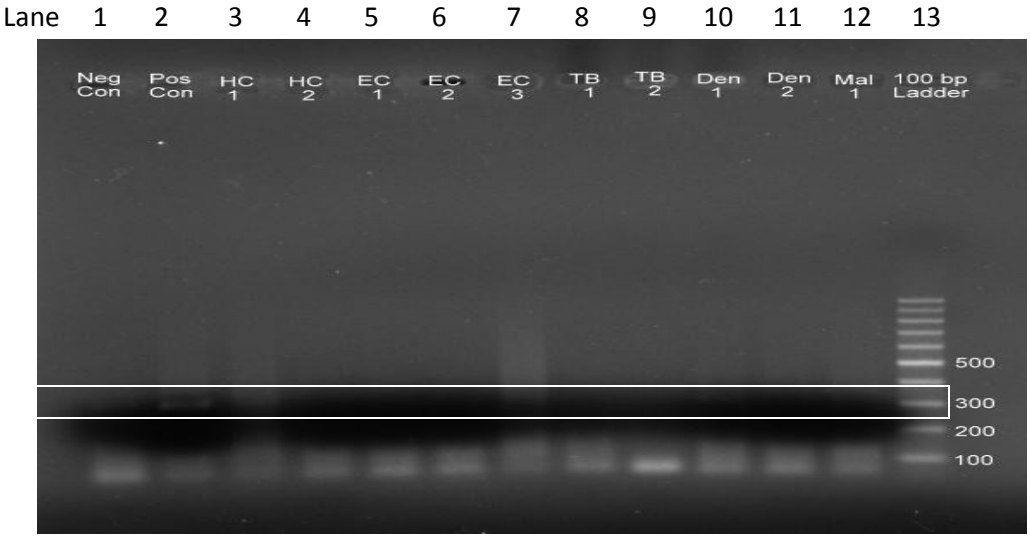
Table 4.16: Ref 2 (BHUL18SF, BHUL18SR) primer amplification result with VL patient's and control individuals:

Individual Types	Positive (%)	Negative (%)	Total
VL positive patient's Buffy coat (i. e. Blood)	22(84.62%)	4 (15.38 %)	26
Control (Healthy, endemic & diseased) individuals blood	0 (00%)	10 (100%)	10
Total	22	14	36

Note: Parenthesis indicates percentage.



4.20.(a)



4.20.(b)

Fig.4.20: Amplification of the 311bp product of *L. donovani* by PCR with Ref 2 (BHUL18SF, BHUL18SR) primer. Lane 4.20.(a)1: Negative template control, Lane2: Positive template control, Lane 3,5,6,9,10,11,12,13,14,15: Positive Leishmania sample,Lane4,7,8: Negative Leishmania sample, Lane 16: 100bp DNA ladder. Lane 4.20.(b) Lane 1: Negative template control, Lane2: Positive template control, Lane 3 - 12: Negative Leishmania sample, Lane 14: 100bp DNA ladder.

4.23. Sensitivity and specificity of Ref 2 (BHUL18SF, BHUL18SR) primer:

Sensitivity of Ref 2 (BHUL18SF, BHUL18SR) primer has been calculated as per the formula mentioned in Section 4.17.and it was 84.6%. Specificity was also calculated and it was 100%.

Table 4.17.: Sensitivity and specificity of Ref 2 (BHUL18SF, BHUL18SR) primer

Tests	Culture &/or Microscopy positive	Culture & Microscopy negative	Total	Sensitivity	Specificity
PCR positive	(a)True positive 22 (98.08%)	(b)False positive 0 (0.00%)	(a + b) = 22	84.62%	100%
PCR negative	(c)False Negative 4 (1.92%)	(d)True negative 10 (100%)	(c + d) = 04		
Total	(a + c) = 26	(b + d) = 10	(a + b + c + d) =36		

4.24. Comparison of primers sensitivity and specificity:

Comparison between our designed primer and two reference primers were done. Among these three primers our primer shows superior sensitivity and it was 98.08% and specificity was 100%.

Tebale4.18: Comparison of primers sensitivity and specificity:

Serial No	Primers Name	Amplicon Size	Sensitivity (%)	Specificity (%)
Ref 1	LD1F, LD1R	600 bp	80.76	100
Ref 2	BHUL18SF, BHUL 18SR	311 bp	84.62	100
Designed primer	Desigened Primer, Named: 1F, 1R	125 bp	98.08	100

Chapter 5

DISCUSSION

5. Discussion:

Kala-azar is a socio economic status related disease. Since the poorest segment of the community mostly suffer from this disease, due attention is usually not paid on behalf of the health authority *(Assimina et al, 2008).* Moreover, developments of researches are also difficult to perform because of remote and backward approach to reach the endemic locality. Thereby, it has appeared as a neglected disease, though it has constituted a major health problem at regional level in the Indian sub-continent *(Salotra et al, 2001)*. It is the essential demand of the time to control Kala-azar. For this purpose, timely and adequate treatment may contribute significantly to reduce the human reservoir of this disease. Antimonials therapies are the first-line drugs for all clinical forms and chemotherapy is also critically important in reducing the burden of disease. Treatment is long, expensive and not devoid of adverse side effects *(Sundar S et al, 2001).* In many states of India, treatment failure is already well documented due to low responsiveness of parasites to sodium antimony gluconate *(Sundar S et al, 2001; Bora D, 1999).* In Bangladesh, resurgence of Kala-azar has been noticed in the decade of 1970 that has gradually increased to an epidemic form in many areas. Currently Kala-azar appears at a rate in excess of half a million per year *(Bari et al, 2010).*

A large number of studies were carried out mostly on epidemiological, clinical and comparative diagnostic and therapeutic aspects *(Berman J D, 1997).* The conventional approach for definitive diagnosis of kala-azar is technically difficult as collection of splenic or bone marrow aspirate is painful, cumbersome and risky *(Salotra et al, 2001).* These samples are not collected or examined at community and rural health care center as routine practice. In order to address this problem this study has described the development of an easy definitive PCR based diagnostic method for diagnosis of kala azar from buffy coat of peripheral blood and usefulness of this method for differentiation of kala azar from other similar diseases. This study has also compared the newly designed PCR method with other previously used PCR methods as well as traditional diagnostic methods such as culture and microscopy.

In this study a total of 35 clinically suspected kala azar patients and 66 controls (Endemic, non endemic and diseased controls) were included. Different clinical samples collected from both the suspected kala azar cases and controls (endemic, non endemic, diseased) were subjected to microscopy, culture, serological tests and PCR with three pairs of primers.

In the present study, regarding the sex distribution of the seropositive cases, it was found that among the male members seropositive status is little bit higher than the female members (Table 4.2). Out of 35 suspected kala azar cases, 22 (61.54%) were male and 13 (38.46%) were female giving a male to female ratio of 1.7:1 (Table 4.2.). The result was consistent with the study findings of ICDDR, B (*2002 and 2003*), which was male to female ratio of 1.2:1 and 1.04:1 respectively. Several local studies were also found consistent demonstrating male to female ratio as 1.38:1 *(Sarker et al., 2003),* 1.8:1 (*Musa et al., 1996*), 2:1 (Talukder *et al.,* 2003) and 2.4:1 (Shamsuzzaman *et al.,* 2003). Similar finding was reported from abroad where ratio between male to female was 1.3:1 (Antonio *et al.,* 2002). In a study, from Sudan, it was reported that like many countries males are almost twice (1.8:1) more likely to be affected by Kala-azar than females (Zijlstra *et al.,* 1994). Males are infected more often than females; most likely because of their increased exposure to sandflies due to professional activities as was also suggested by *Berman, 1997*.

The study findings showed that, majority of cases (57.1 %) were in the 3-13 years age group (Table 4.2). Almost similar findings were also found in many studies. In a study by *Nuttal et al.,* (2002) observed that majority patients of Kala-azar were in the age group of 5-15 years. In one study performed on 65 patients, over 60% cases of KA were under 20 years of age (Talukder *et al.,* 2003). Therefore it is reasonable to believe that young people are more vulnerable to kala azar infection, because of children's outdoor movement which may correlate.

In the present study, living houses of most of the kala-azar cases (88.6. %) were made of earthen walls (mud made) and secondly tin houses (Table 4.2). People made their houses

with mud due to low socio-economic condition. One study showed that, increased incidence of the diseases was among those who lived in the houses with walls made of mud and cow – dung (*Chowdhury et al, 1993*). Tiny cracks and crevices are easily developed in the earthen houses which are favorable places for sandflies (*Desjeux, 2004*). So, from the above findings, it may be concluded that, people residing in earthen houses must bear more risk for being infected by kala azar agents.

The remarkable presentation of kala azar patients is prolonged low-grade irregular fever associated with organomegaly of spleen and liver. Anemia and darkening of skin are also found in majority of cases (Gradoni *et al.,* 1995). In our study, prolonged low-grade irregular fever was found in 100% cases and mean duration of fever was 1.6 months (Table 4.2). Similar findings were also reported by other authors both in home and in abroad describing association of fever in 95-100% cases of kala-azar (*ICDDR, B, 2003; WHO, 1996; Maltezou et al., 2000; Sarker et al., 2003*).

Hepatomegaly and splenomegaly are common presentation for kala azar patients, and these were evident in 68.5% and 100% cases respectively in the present study (Table 4.2). *ICDDRB (2003) and Sarker et al., (2003)* reported splenomegaly in 100% cases and hepatomegaly in 79% cases. WHO (1996) reported 98% hepatosplenomegaly from India. Hepatomegaly was found in 85% and splenomegaly in 99% cases by *Maltezou et al., (2000)*.

In this study, rk39 Immunochromatographic (ICT) dipstick test was used as screening test for differentiating between case and control group. Those individuals, who have sign, symptom and rK39 positive were included as suspected case group and those individuals who have no sign and symptom and rK39 negative were included as control group. That's why; rk39 Immunochromatographic (ICT) dipstick test was found 100% positive in suspected kala azar cases and 100% negative among control individuals (including healthy endemic, non endemic and diseased control) (Table 4.3). Likewise, *Cunningham J et al, (2012)* reported rK39 ICT test using serum samples become 93 – 100% sensitive and 96 – 100 % specific among Indian subcontinent. *Khan et al (2010)* reported the

sensitivity and specificity of the rK-39 strip test using urine samples was 95% and 93.3%, respectively. The high sensitivity and specificity rate of our rK39 ICT test could be due to careful case selection. We have included only those cases which have most florid clinical features of kala azar.

In this study it was evident that, peripheral blood samples produced rK39 tests positive in every suspected kala azar samples. This test identifies anti- Leishmanial antibodies in the circulation by specific interaction with recombinant 39 amino acid antigens immobilized in the paper strip. Such an observation of detecting antibodies however has some limitations. These are as follows: (1) detectable antibodies may not be found in early stage of illness, (2) antibodies tends to persist in the circulation several years after cure of VL and hence could be misleading for diagnosis, (3) anti - Leishmanial antibodies found among residents of endemic areas who had no history of leishmaniasis.

Leishmania donovani bodies were demonstrated in seventeen (68%) bone marrow samples and six (60%) spleen samples of suspected kala azar patients in microscopy (Table 4.6). The high positivity rate by microscopy could be due to case selection. We have included only those cases, which have most symptomatic clinical features of kala azar. Though we have detected LD bodies in around 65% of cases, others have reported the detection rate being 61.1% previously *(Alam et al, 1996).* It is noteworthy that we could not detect LD bodies in blood by microscopy (Table 4.7).

Leishmania donovani bodies were grown in fourteen (56%) suspected kala azar patients bone marrow and six (60%) patients spleen in NNN media (Table 4.4). It was interesting to note that, though LD bodies were present in 68 % of cases in microscopy, the culture positivity rate was 57%. Culture was negative in eleven bone marrow and four splenic aspirations of patients suspected with kala azar. The isolation rate of parasite by culture could have been further increased if we could culture the specimens immediately after collection. We suspect the time spent in between the collection of some samples from S K hospital, Mymensing and the laboratory in Dhaka could be the factor of this under performance. Other researchers reported the isolation rate around 76.2% in Italy *(Antinori*

S et al, 2007). Like microscopy we could not detect LD bodies in blood by culture in NNN medium either (Table 4.5).

The specimens of bone marrow and splenic aspiration produced around 57% culture positive and 68% microscopy positive results respectively. Although, culture and microscopy have low sensitivity, it was evident that, diagnostic yield was increased when both tests considered together. Positivity of combined culture and microscopy was found 74% (26 out of 35), which were higher than the individual test results. If diagnosis was made only on the basis of culture result 6 (17%) cases could have been missed. Similarly, 3 (8.6%) cases could be missed if only microscopy result was considered. The reason of microscopy negativity might be due to the low concentration of *Leishmania* in sample i.e. below the detection limit of 10,000 organisms / ml.

Peripheral blood samples failed to produce a positive reaction either in culture or in microscopy, indicating the parasite load in circulating blood was too low to be detected during the period of investigation. On the other hand, the conventional methods of diagnosing VL by detecting antibodies have some disadvantages. The sensitivity of these methods is low. Hence the choice of sample rests on collection of bone marrow and splenic aspiration, collection of these specimens is a painful, cumbersome and risky procedure and their culture on laboratory media is complex, time consuming and laborious. They are usually interfered by bacterial and/ or fungal contamination.

In order to use “blood” as a specimen for diagnosis of kala azar that would reduce shear pain of the individuals coming to the clinic, we took a PCR based molecular approach, for what we aligned the DNA sequences of *Leishmania* spp, available in NCBI database in order to construct oligo sequences that would help to amplify the *Leishmania* genome in a Polymerase chain reaction. Hence the main objective of this work was to design, optimize and evaluate the PCR based detection system to sore kala azar infection.

So, we targeted kinetoplastic minicircle DNA and designed a pair of primer (MK1F & MK1R). This primer pair was able to amplify the DNA of promastigotes from culture.

This promastigotes were used as positive control in the next steps. It was reported that, PCR assay with buffy coat preparations to detect *Leishmania* was 10 times more sensitive than that with whole blood preparation (*Sundor and Rai, 2002*). *Leishmania* are obligate intracellular parasites (in the vertebrate host) and therefore are supposedly more concentrated in the buffy coat, hence the buffy coat preparation of peripheral blood was used for PCR amplification.

The designed primer pair was able to amplify *Leishmania* kinetoplastic DNA from suspected patient's bone marrow and from splenic aspiration and even from buffy coats to a size what it should be. PCR described in this work yielded a unique product of approximately 102 bp and no non specific side product or artifacts appeared on the gel. It had the advantages that results were easily and unequivocally interpreted upon analysis of agarose gel. On the other hand no PCR amplification was seen in cases of healthy endemic and non endemic and diseased controls with our designed primer. The Sensitivity of PCR reactions was recorded 98% for primer pair MK1F & MK1R as only one out of 26 kala azar positive samples become non responsive which was microscopically positive. The high level of sensitivity was reflected by the ability of the assay to detect parasite DNA in peripheral blood of patients with kala azar.

Out of 26 kala azar positive samples 25 were positive by PCR (Table: 4.10) but unexpectedly only one case was found negative by PCR with designed primer though it was microscopically positive. This false negativity can be explained either by sampling errors (not uniform distribution of microorganisms), low parasitic load, inefficient extraction of DNA, insufficient sample for extraction or to the presence of PCR inhibitors. Monitoring and accurate evaluation of PCR inhibitors is possible only if an internal control is included during DNA amplification which was absent in this study.

The sensitivity of the designed primer pairs was compared with two other previously used primers, Ref 1 (LD1F, LD1R) (*Maurya R et al, 2007*) and Ref 2 (BHUL18SF, BHUL18SR) *(Srivastava et al, 2011*) in polymerase chain reaction to detect *Leishmania donovani* genome, and the designed primer produced superior sensitivity and specificity

over the other tested primer pairs (designed primer pair was 98% sensitive whereas Ref 1 and Ref 2 primer pair were 88.76% and 84.6% sensitive respectively).

The authenticity of PCR amplified product by the primer designed for this study was checked by DNA sequencing followed by BLAST search and the identity of amplicon was found to be 98 % identical with *Leishmania Donovani* complex (*Leishmania donovani, Leishmania infantum* and *Leishmania chagasi*). The identity of the PCR product with *Leishmania Donovani* complex further confirms the reliability of the detection procedure.

The identification of *Leishmania* specific genome from peripheral blood specimens of patients with kala azar with such a high degree of sensitivity and specificity as proved in this study could shed a new light for its diagnosis in quick time, thus reducing the burden of risks of the patients to hemorrhage and saving valuable time, so the patient management could be initiated without delay.

Chapter 6

CONCLUDING REMARKS

6. Concluding remarks:

This study was carried out to develop a PCR based technique, to assess the sensitivity and specificity of the newly designed primer pair and compare its efficacy with other previously used PCR primers as well as conventional culture, microscopy and serodiagnosis. Analyzing the findings of the study, it can be said that male individuals are more susceptible to kala-azar than females, majority of the victims were children of under 12 years of age and mud made houses are related with kala azar infection. In addition to these observations this study attempted to develop a PCR based detection system and its findings concluded as follows:

- PCR with the primer pair designed in this study is a rapid and more sensitive and specific alternative approach compared to culture and microscopy.
- Invasive specimens like bone marrow or splenic aspiration could be avoided for PCR reaction with the primer pair designed in this study, as it can be done with peripheral blood as specimen.
- The primers designed for the study shows superior sensitivity and specificity compared to the primers reported in the literatures.
- These primers can be used for commercial kala azar detection kit preparation.

Chapter 7

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Appendix-I

Media used

Unless otherwise mentioned, all media were sterilized by autoclaving at 121° C for 15 minutes at 15 lbs pressure. Distilled water was used for preparation of all media. The media used in this thesis have been given below:

NNN (Nicolle, McNeal, Novy) media:

Ingredients	**Amount**
Blood agar base (Difco)	1.4 gm
Sodium Chloride (NaCl)	0.6 gm
Double distilled water	90.0 ml
Defibrinated rabbit blood	10 ml
Storage temprature	4° C

Overlay solutions (to be used with NNN medium)

Ingredients	**Amount**
Sodium Chloride (NaCl)	4.5 gm
Double distilled water	500.0 ml
pH	7.4
Storage temperature	4° C
pH	7.3

Antibiotics solutions (to be used with NNN medium)

Ingredients	**Amount**
Sodium penicillin G	20,000 U
Streptomycin sulfate	20,000 μg
Amphotericin B	40μg
Sterile double distilled water	1 ml
Storage temperature	- 20° c

Appendix-II

Solutions and Reagents used

Preparations of the stock solutions used in this work are given below: (all the working solutions used in this work were prepared from the stock solutions).

Leishman stain

This stain was prepared by 0.2 gm of powdered dye & was transferred to a conical flask of 200-250 ml capacity. 100 ml of methanol was added and warmed the mixtures to 50°C for 15 mins, occasionally shaking it. The flask was allowed to cool & filtered. It was then ready for use.

Normal saline

Normal saline was prepared by dissolving 0.85 g NaCl in 100ml of distilled water and sterilized by autoclaving, pH was adjusted to 7.8.

1 M Tris-Cl

121.1 g tris-base was dissolved in 800 ml of distilled water. The pH was adjusted to the desired value by adding concentrated HCl and the final volume was made up to 1 L by distilled water. The solution was sterilized by autoclaving and stored at RT.

3M NaCl

175.3 g of NaCl was dissolved in distilled water to a final volume of 1L. The solution was autoclaved and stored at RT.

10 M NaOH

40 g of NaOH pellet was dissolved in 100 ml distilled water. The solution was stored in an airtight bottle at RT.

0.5 M EDTA

186.1 g of $Na_2EDTA.2H_2O$ and 20.0 g of NaOH pellets were added and dissolved by stirring to 800 ml distilled water on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10 M NaOH and final volume was made up to 1L with distilled water. The solution was sterilized by autoclavingand stored at RT.

3 M sodium acetate

40.81 g of $Na_2 (CH_3COOH).H_2O$ was dissolved in 80 ml of distilled water. The pH was adjusted to 5.2 with glacial acetic acid. The final volume was adjusted to 100 ml with distilled water and the solution was sterilized by autoclaving. It was stored at 4°C.

TAE buffer

242 g of tris-base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA (pH 8.0) was talen and distilled water was added to the mixture to make 1L. 1X concentrated TAE buffer was made by adding 10 ml 50X TAE buffer with 490 ml idtilled water and stored at room temperature.

Ethidium bromide solution

10 µl of ethidium bromide was dissolved in 100 ml TAE buffer to make a final concentration of 20 mg/ml and stored at 4°C in the dark.

Gel loading buffer

Ingredients	**Amount (g/L)**
Sucrose	6.7
Bromophenol blue	0.04
Distilled water	Up to 1 L

*Stored at 4°C

EZ-10 Spin column genomic DNA minipreps kit, Blood (Biobasic, Canada), Catalog No. BS 483

Reagents	**Purpose**
TBP Buffer	For the lysis of red blood cells
TBM Buffer	For lysis of cells
EZ-10spin Column	For Binding of the DNA molecules
Collection Tube	For collection of flothrow
TE Buffer	For washing purposes
Wash solution (ethanol added)	For washing purposes
Elution Buffer	For elution of DNA from the EZ column

Wizard® SV Gel and PCR Clean-Up System. Catalog No. A9280

Reagents	**Purpose**
Membrane Binding Solution	Help in binding of PCR product
SV Minicolumn	For Binding of PCR product
Collection Tube	For collection of flothrow
Membrane Wash Solution	For washing purposes
Nuclease-Free Water	For elution of the purified DNA from the GD column

Appendix III

Instruments & Apparatus

The important instrument and apparatus used through the study are listed below:

ABI Prism 3130 Genetic Analyzer	USA
AlphaImager HP System Versatile Gel Imaging	USA
Autoclave, Model no: HL-42AE	Hirayama corp, Japan
Centrifuge, Mode1:5804	Eppendorf, Germany
Class II Microbiological safety cabinet	Labcaire, USA
Electric balance, Scout, SC4010	USA
Freezer (-30°C)	Liebherr, Germany
Horizontal gel elctrophoresis apparatus Hl-SET	UK
Incubator	Japan
Microcentrifuge, Mikro20	Germany
Microcentrifuge tube	Eppendorf, Germany
Micropipettes	Eppendorf, Germany
Microwave oven, Model: D90N30 ATP	Butterfly, China
NanoDrop 2000	Thermo Scientific, USA
PCR Thermocycler	Biometra , Germany
pH meter, Model no: MP220	Eppendorf, Germany
Power pack	Toledo, Germany
Refrigerator (4°C)	Vest frost
Room temperature horizontal shaker	Gerhardt, Germany
Sterilizer, Model no: NDS-600D	Japan
Water bath, Model:SUM	England

Appendix-IV

Questionnaire form and Ethical clearance certificate

Leishmania Project

Questionnaire form

Patient ID: Date of collection:

Name of the sample:

Patient Name:

Age: Sex:

Address:

Village…….. Union……

Upazilla…….. District……..

Housing Material (Mud made house): Yes [ ] No [ ]

History of illness:

1. Fever: Yes [ ] No [ ]
2. Duration of fever:
3. Anemia: Yes [ ] No [ ]
4. Anorexia: Yes [ ] No [ ]
5. Weight loss: Yes [ ] No [ ]
6. Darken skin: Yes [ ] No [ ]
7. Splenomegaly: Yes [ ] No [ ]
8. Hepatomegaly: Yes [ ] No [ ]
9. Lymphadenopathy: Yes [ ] No [ ]
10. Past history of VL: Yes [ ] No [ ]
11. History of VL in the family: Yes [ ] No [ ]
12. Result of rk39
13. History of drug intake:

Findings:
Microscopy:
Culture:

বাংলাদেশ চিকিৎসা গবেষণা পরিষদ
Bangladesh Medical Research Council
BMRC

Ref: BMRC/NREC/2013-2016/1816 Date: 08-05-2014

National Research Ethics Committee

Dr. Md. Shariful Alam jilani
Professor of Microbiology
Ibrahim Medical College and
BIRDEM Hospital
Shahbag, Dhaka.

Subject: Ethical Clearance

With reference to your application on the above subject, this is to inform you that your Proposal entitled **"Development of PCR based non invasive diagnostic system for Kala azar from peripheral blood"** has been reviewed and approved by the National Research Ethics Committee (NREC).

You are requested to please note the following ethical guidelines as mentioned at page 2 (overleaf) of this memo-

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Director

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