TRANSMISSION DYNAMICS OF VISCERAL LEISHMANIASIS IN NEPAL

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF MICROBIOLOGY INSTITUTE OF SCIENCE AND TECHNOLOGY TRIBHUVAN UNIVERSITY NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN MICROBIOLOGY

BY

SHAILA BASNYAT

September, 2022

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DECLARATION

Thesis entitled **"Transmission Dynamics of Visceral Leishmaniasis in Nepal"** which is being submitted to the Central Department of Microbiology, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of Prof. Dr. Anjana Singh, Central Department of Microbiology, Tribhuvan University and co-supervised by Prof. Dr. Prakash Ghimire and Assoc. Prof. Dr. Megha Raj Banjara of Central Department of Microbiology, Tribhuvan University.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

……………………..

Shaila Basnyat

RECOMMENDATION

This is to recommend that **Shaila Banyat** has carried out research entitled **"Transmission Dynamics of Visceral Leishmaniasis in Nepal"** for the award of Doctor of Philosophy (Ph.D.) in **Microbiology** under our supervision. To our knowledge, this work has not been submitted for any other degree.

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph.D. degree.

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On the recommendation of Prof. Dr. Anjana Singh, Prof. Dr. Prakash Ghimire and Assoc. Prof. Dr Megha Raj Banjara this Ph.D. thesis submitted by Shaila Banyat entitled **"Transmission Dynamics of Visceral Leishmaniasis in Nepal"** is forwarded by Central Department Research Committee (CDRC) to the Dean, IOST, T.U.

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……..………………

Shaila Basnyat

September, 2022

ABSTRACT

Visceral leishmaniasis (VL) is also well-known as kala-zar is one of the neglected tropical disease, caused by a protozoan parasite, *Leishmania* speices and transmitted to humans by the bite of infected female *P. argentipes* sandflies. Humans are assumed to be the only established reservoir of VL including Nepal, India and Bangladesh.In human, VL may be either symptomatic or remain asymptomatic. So, it becomes necessary to know the role of symptomatic and subclinical infections in transmission of VL. Therefore, the main objective of this study was to elucidate the transmission of VL in Morang, Saptari, Sarlahi (confirmed endemic districts with VL control program) and Palpa (recently confirmed as endemic to VL) districts of Nepal.

For this study, 331human blood samples, 3976 sandflies samples from 142 HHs and 100 domestic animals (cow, goats, dog, buffaloes and ox) blood samples were collected from 2016 to 2019 from the villages of the four districts. Using the rK39 rapid immunochromatographic strip test, the human blood samples, were tested for anti-*Leishmania* antibodies. And from genomic DNA extracted from human blood, female *P. argentipes* sandfly and animal blood samples, amplification of kDNA of *L. donovani* was done PCR amplification.

32 and 16 out of 331 people screened were rK39 test positives and by PCR respectively. Among the 16 PCR test positives in human blood sample, 10 were from Sarlahi, 2 from Saptari, 1 from Palpa and 3 were from Morang districts. Among these 16 PCR positives, 10 were VL symptomatic and 6 were asymptomatic cases of VL.

This study also showed that the sandfly species *P. argentipes* is the most predominat species. Most of the sandfly collected harbor *Leishmania* parasites. We did not found *Leishmania* infection in domestic animals blood samples. The existence of VL cases, detection of *Leishmania* DNA and anti-rK39 antibodies in human blood samples showed that there is continuing transmission of VL in Morang, Palpa, Sarlahi and Saptari districts. However the maximum transmission was detected in Ishworpur village, Sarlahi district. This study reveals domestic animals were not the reservoir for *L. donovani* in tested villages.

Thus, humans can be consider as reservoir host for *L. donovani* in Nepal. Thus, continuous investigation is essential to identify transmission in the new foci and previous endemic districts.

LIST OF ACRONYMS AND ABBREVIATIONS

bp : Base pair

BPKIHS : B P Koirala Institute of Healthy Science

CDC : Centers for Disease Control and Prevention

CL : Cutanous Leishmaniasis

DAT : Direct Agglutination Test

DHO : District Health office

DNA : Deoxyribonucleic acid

EDCD : Epidemiology and Disease Control Division

ELISA : Enzyme Linked Immunosorbent Assay

HH / HHs : Household / Households

ISC : Indian sub-continent

KA : Kala-azar

kDNA : Kinetoplast DNA

LST : Leishmanin Skin Test

NTDs : Neglected tropical diseases

MCL : Mucosal Leishmaniasis

Na_2 EDTA : Disodium ethylenediaminetetraacetate

PCR : Polymerase Chain Reaction

PKDL : Post kalazar dermal leishmaniasis

pVL : past VL treated

RDT : Rapid Diagnostic Test

VL : Visceral Leishmaniasis

WHO : World Health Organization

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Visceral leishmaniasis (VL), is a fatal vector-borne disease caused by a protozoan parasitic *L. donovani* and are transmitted by female *P. argentipes* sandflies. An assessed prevalence of VL cases which occurs worldwide annually is 50, 000 to 90, 000 (WHO, 2019). Over 90% cases of VL are found in India, Bangladesh, Nepal, Sudan and Brazil (Sundar et al., 2008).

In Nepal, 23 districts are confirmed as VL endemic districts and above 8.6 million people are at risk of infection (EDCD, 2019). In 2003, the maximum number of VL cases were reported, in Nepal and ever since the cases are in a declining trend. In 2005, Nepal targeted to eliminate VL by 2015, defined as below 1 case in 10,000 population at the district level. However, Nepal reached this goal, in 2013, sporadic cases are constantly reported from previously non-endemic districts and as a result the elimination threshold has been surpassed (Rijal et al., 2019).

In the Indian subcontinent, including Nepal, India and Bangaladesh, the major parasite is *L.donovani* and the vector of VL is female *P. argentipes* (Burza et al., 2018; Sharma et al., 2008). VL may be either symptomatic or asymptomatic.VL has a signs and symptoms of persistent fever, enlargement of spleen and liver, weight loss, and anemia. A positive serum/blood test or PCR or Leishmanin skin test in people who are healthy is generally considered as asymptomatic leishmanial infection (Hasker et al., 2013; Srivastava et al., 2013).

In Nepal VL is considered to be anthroponotic (human to human transmission) where female *P. argentipes* transmits the *L. donovani* (Desjeux, 1996; Desjeux, 2004). Subclinical cases of VL are considered to be possible reservoirs of VL and may be major source for transmission of the disease in endemic areas. The rK39 rapid diagnostic test (RDT) can detect asymptomatic infections with high levels of circulating anti-*Leishmania* antibodies (Burza et al., 2018).

However, asymptomatic infections with low titers of antibodies generally cannot be detected with rK39 RDT, although some asymptomatic infections with high titer

antibodies can also be detected with the rK39 RDT (Hire et al., 2010). Therefore, there will be more asymptomatic infections in an endemic population than can be detected with the rK39 RDT.

In human *Leishmania* parasites are transferred by the bites of *L. donovani* infested female *P. argentipes*. For surveillance of vector borne diseases, it is important to monitor infection in the vectors. Since, *L. donovani* infection rate in sandfly varies in different endemic settings and the cases of VL in Nepal are decreasing sharply, it is the indicator to show whether VL has been actually decreased or can re-emerge in the future. So, we also detected the presence of infected sandflies in villages of four districts from where human blood samples were collected.

It has become crucial to detect the main role of domestic animals in the VL transmission in Nepal, because of their close association with the sandfly vector and to reassess transmission patterns of VL. So, we performed PCR to detect kDNA of *L. donovani* in all the collected domestic animals blood fom households of known VL cases and their neighbors.

1.2 Rationale

VL still remains a significant public health problem in Nepal with 23 districts endemic and over 8.6 million people are at risk. We, therefore examined the current *L. donovani* transmission in Morang, Palpa, Sarlahi and Saptari districts known to be endemic where VL cases has been detected. Morang, Saptari and Sarlahi are VL endemic districts for many years with ongoing control activities from the national program where as Palpa is recently confirmed VL endemic district without control activities in place.

Most of *L. donovani* infections remain asymptomatic and are considered as potential reservoirs of VL in an endemic area. So, it is essential to monitor asymptomatic cases for early detection of symptoms and for the improvement of more operational VL control strategies. Humans are the only proven reservoir of VL, in Nepal, so it is crucial to know the role of acute VL and subclinical cases in transmission of VL. So, we investigated seroprevalence of VL among the family members and neighbors of known VL patients in these four districts of Nepal.

An increasing numbers of VL cases have been reported from the hilly districts of Nepal, but none of these cases have been confirmed to be the local transmission. If local

transmission is confirmed in these hilly distrcts, this will challenge the current VL elimination program. So, Palpa as a hilly area, selected for this study.

Identification of vector and detection of infection in the vector population is an important tool for study of vector borne diseases. To achieve this, collection of female *P. argentipes* sandflies were done from the different villages of four districts.Then densities of sandfly were calculated and the parasite infection rate in the collected sandflies were detected by using PCR amplification of kDNA.

An extensive study in location of active VL transmission can be done by mapping Leishmania infections among asymptomatic persons and domestic animals, to explore the role of domestic animals in maintenance and transmission of VL in Nepal, as it is not clear.

The risk for VL may increase by keeping domestic animals in the same room at night as it attracts more sandflies into the houses. Having animals around the house may be protective, as animals acting as preferred bloodmeal source for sandflies. To understand the role of domestic animals in the maintence and transmission of VL, further research is necessary in this field.

Thus, to understand the present status of the transmission dynamics of VL in Nepal more precise epidemiological pattern can be obtained by collecting, entomological and human data in the same geographical locations.

1.3 Objectives

General Objective

To investigate the transmission dynamics of visceral leishmaniasis in Nepal.

Specific Objectives

- To determine the seroprevalence of VL in endemic areas of Nepal.
- To determine the seroprevalence of VL in hilly district of Nepal.
- To determine whether the sandflies contained *L. donovani*.
- To assess asymptomatic cases of VL as a reservoir of VL in Nepal.
- To detect domestic animals as a source of reservoir of VL in Nepal.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

VL is also known as Kala-zar is a fatal vector borne disease caused by the bite of *L. donovani* infected *P. argentipes* sandflies (Burza et al., 2018). Nepal is among the five countries, endemic for VL globally (WHO, 2020). The first confirmed case of VL was first recorded officially in Nepal in 1980 (Bista, 1998). In Nepal, the first cases of VLwere reported from 12 districts of central and eastern Terai region. Bhojpur, Okhaldhunga, Makwanpur, Palpa, Surkhet and Kailali were confirmed as new districts endemic to VL in 2016. The most of the VL elimination efforts are being focused on VL endemic areas of Nepal (EDCD, 2019). Now, incidence of VL has been expanded to new districts which was formerly known as non endemic to VL. This has prompted to verify the existence of vector and reservoir hosts, to know the expansion of VL incidence in non program districts (EDCD, 2019). In Nepal, maximum number of cases VL was reported in 2003, and ever since the cases of VL are in declining trend (EDCD, 2019).

To eliminate kala-azar defined as incidence < 1 case per 10,000 population at district level, Nepal formulated a national plan in 2005, with aim of accomplishing elimination by 2015. Though Nepal reached this target, in 2013, periodic cases of VL from non-endemic districts were being regularly reported which provide challenges to inverse the maintenance of the elimination target (Rijal et al., 2010).

2.2 Discovery

In 1900, William Boog Leishman, discovered the protozoan parasites, as ovoid bodies from the tissue sample of the enlarged spleen of a soldier by using microscopy. But he mistakenly considered the protozoan parasites, to be degenerate trypanosomes. In April 1903, Charles Donovan, found the parasites at the Government Hospital in Madras. Donovan after reading Leishman paper confirmed, the parasites which was known as "Leishman bodies" were the aetiological agents of kala-azar. Laveran and Felix Mesnil identified the protozoan as member of Piroplasmida and named as *Piroplasma donovanii*. Ross identified the species as member of the genus *Leishmania*, and gave the

name "Leishman-Donovan bodies."In1904 the valid binomial term *Leishmania donovani* was generally adopted.

2.3 Causative agent

The aetiological agent of VL in Nepal is *L. donovani*. Atleast 20 species of *Leishmania* can cause VL and is transferred by the female sandfly of the genus *Phlebotomus*. Human Leishmaniasis is caused by 21 species that also infect mammals. These include the *L. donovani* complex with 2 species *L. donovani* and *L. infant*um (also known as *L. chagasi*).

2.3.1 Structure

L. donovani is a unicellular eukaryote with a nucleus, kinetoplast and flagellum. It exists in two structural forms depending on its host: amastigote form and promastigote form.

1. Amastigote forms are found in human and in mammalian host's mononuclear phagocyte and circulatory systems. Amsastigote is round or oval in shape, 3–6 µm in length and 1–3 µm in breadth. It is non-motile form, lacking external flagellum. The kinetoplast and basal body are found at the anterior end.
2. Promastigote forms are found in the mid gut of sandfly and in culture. It is spindle-shaped, tapering at both ends, 15–30 µm in length and 5 µm in width. It is an extracellular and motile form with a long flagellum projecting externally at the anterior end. At the centre is the nucleus and kinetoplast and basal body are in front of it.

2.3.2 Life cycle

Leishmania donovani parasite requires two different hosts to complete its life cycle; definitive host (human and mammals) and intermediate host (female sandflies).

Life cycle in human (definitive host):

The infected female sandflies inject the promastigotes forms of *L.donovani* in human, through their proboscis when biting human. The promastigotes are phagocytized by macrophages. They are also engulfed by other types of mononuclear phagocytic cells in liver, spleen and bone marrow. Inside these cells promastigotes are transformed into amastigote forms which then multiplies by binary fission.

There are as many as 50 to 200 amastigotes in these fully congested cells. Now each individual amastigote can invade fresh cells.

Life cycle in sandfly (intermediate host):

Female sandflies becomes infected when sucking the infected blood of human containing free and phagocytosed amastigotes. The amastigotes change to procyclic promastigotes and then to metacyclic promastigotes in the midgut of the sandfly. Then metacyclic promastigotes multiply by longitudinal binary fission and produce large numbers of promastigotes. In 6 to 9 days, after initial blood meal, the number of parasites becomes massive and profoundly spread into the pharynx and buccal cavity. If the infected sandfly bites the susceptible human host, transmission of infection occurs into a new host.

2.3.3 Transmission of VL

In Nepal VL is considered to be anthroponotic, and female *P. argentipes* is the vector. Anthroponotic VL transmission is found in East Africa, Bangladesh, India and Nepal (Desjeux, 2004). In Nepal, female *P. argentpies* sandfly is the only vector responsible for transmission of VL in human and *L. donovani* is the main parasite. Thus, in Nepal, VL is transmitted to humans by the bite of *L. donovani* infected female *P. argentipes* sandfly. Other modes of transmission of VL is rare.

2.3.4 Pathogenicity

Visceral leishmaniasis is highly lethal, if left untreated. The incubation period of VL among symptomatic person, generally ranges from 3 to 6 months, and more than a year in some cases. Symptoms of VL includes high fever for more than 2 weeks, enlargement of spleen and liver. The patient shows emaciation and anemia as the disease progress. Fatality, usually occurs within 2 years in untreated VL cases and is mainly due to anemia or organ failure, or secondary infections.

There are 3 main forms of the Leishmaniasis:

1.Visceral leishmaniasis (VL), 2. Cutaneous leishmaniasis (CL) and 3. Mucosal or mucocutaneous leishmaniasis (MCL).

1.Visceral leishmaniasis

V L is the most severe form of leishmaniasis. VL develops within months and sometimes as long as year after the bite of *L. donovani* infected sandfly. Symptoms of VL includes high fever for more than 2 weeks, enlargement of spleen and liver, and heavy skin pigmentation, weight loss, and low blood counts.

Post-kala-azar dermal leishmaniasis (PKDL) is a sequale that often appears after treatment in VL patients. PKDL has also been reported in people without history of VL. Clinical manifestations of PKDL are macular, maculopapular, and nodular skin rash. However, the symptoms of PKDL varies especially in those without a history of VL.

2. Cutaneous leishmaniasis

Cutaneous leishmaniasis is the most common form of skin manifestation of leishmaniasis. In CL, skin lesions develop within a few weeks or months after the bite of sandfly. The lesions are painless but can be painful in some cases. The sores may erupt as papules or nodules and later develops into ulcers. Skin ulcers might be covered by scab or crust. In some people, swollen glands develops near the sore.

3. Mucosal leishmaniasis

Mucosal leishmaniasis is less common form of leishmaniasis. Mucosal leishmanisis can be a sequela of infection with some of the species of the parasite that cause CL. MCL causes partial or total destruction of mucous membranes of the nose, mouth and throat.

2.4 Epidemiology of leishmaniasis

Epidemiology of leishmaniasis depends on interactions between human, parasite and sandfly (Lukes et al., 2007; Patz et al., 2000; Ready, 2008; Ready, 2014). VL is usually more common in rural than in urban areas. About, 98 countries and territories, are endemic for VL but eco-epidemiological hot spots for VL are East Africa, Brazil and the Indian sub-continent, including Nepal, Bangaladesh and India. More than 50% of the global incidence of VL has been reported in India, Nepal and Bangladesh (Burza et al., 2018; WHO, 2020). 90% of global VL cases occur in India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil. About 30% of cases of CL are from the Americas, the Mediterranean basin, and western Asia from the Middle East to Central Asia. The highest cases of CL were found in Algeria, Afghanistan, Brazil, Colombia, Iran,

Ethiopia, North Sudan, Syria, Costarica and Peru. An estimate of 20,000 to 40,000 deaths occurs due to leishmaniasis annually (Alvar et al., 2011).

If VL is left untreated death occurs and result in case-fatality rates of 10–20% (Bern et al., 2005; Collin et al., 2004; Desjeux,1996; Rey et al.,2005; Zijlstra et al., 1994). The case-fatality rates reported, from the Indian subcontinent, ranged from 6.2% in Nepal, 2.4% in India and 1.5% in Bangladesh.

In India, VL is predominant in West Bengal, Bihar, Eastern Uttar Pradesh, Assam and Sikkim (Mahajan, 1996). Athough not clearly confirmed as local transmission, VL transmission has been reported from hills in India (Mahajan et al., 2004) and in Bhutan (Yangzom et al., 2012).

2.5 Visceral leishmaniasis in Nepal

The first confirmed case of VL was officially recorded in Nepal in 1980 (Bista, 1998). Since 1993, a national kala-azar control program has been running in Nepal. The main strategies are early diagnosis and treatment along with vector control using indoor residual spraying. No noticeable impact on the VL incidence has been seen inspite of this VL control efforts conducted between 1993 and 2005. VL was highly prevalent before 1958 in Nepal. But in the 1960s, DDT spraying was considered to eliminate the disease during the Malaria Eradication Program. (Zijlstra et al., 2003).

In Nepal, VL was officially reported again in 1980. It may have been reintroduced in Nepal from Bihar, where a large outbreak took place in 1977 (Bern et al., 2008). From 1980 to 1989, the incidence rate of VL remained below10per 100,000 person-years. Between 1980 and July 2006, more than 25,000 cases and around 600 deaths were reported from 16 affected districts of Bihar, and the majority of patients were above 15 years (Zijlstra et al., 1995).

Since 2005, Nepal, participated in a regional VL elimination program jointly with Bangladesh and India, targeting to reduce the incidence rate of VL in endemic districts by less than 1/10,000 population. A sharp decline in the number of VL cases has been observed in 2015. But, between 2007 and 2010, VL cases was notified from an increasing number from 14 districts in 2007 to 26 districts in 2010. In 9 districts, of Nepal the target of VL elimination has been completed (Rijal et al., 2010).

The risk factors for VL infection are poor nutrition of the people, housing conditions, with cracked mud walls and damp floors, and outdoor sleeping behavior (Desjeux & Ramesh, 2011). In a sero-survey conducted from 1996 to 1997, in two villages of Nepal a *Leishmania* infection rate was found 6.1%, with a male to female ratio of 1.44:1 (Koirala et al., 2004). In another survey done, VL infection was found 7.5% in Nepal (Schenkel et al., 2006).

The number of VL patients is much lower in Nepal than in Bangladesh or India. In 1990s there was an increase in VL infection but since 2006 the case of VL has decreased in Nepal. Based on a screening of cases treated by the BPKIHS, Nepal, the prevalence of PKDL, has been estimated 2.3% in patients treated for VL (Rijal *et al*., 2006).

Since 2000, VL cases from the hilly regions of eastern Nepal have been reported in increasing numbers. Although, sporadic cases of VL from the hills at altitudes of 1,000 meters above sea level, has been reported none of these cases were confirmed from local transmission (Joshi et al., 2006; Pandey et al., 2011). However, Ostyn et al. (2015) demonstrated that the infection of VL at hilly districts of Nepal were locally transmitted, and also found circulating *L. donovani* in hilly districts. Thus Ostyn et al reported the expansion of VL in new endemic hilly areas of Nepal from the study of epidemiology, serology, clinical, entomology and molecular test.

2.6 Transmission dynamics of VL

Visceral Leishmaniasis due to *L. donovani* infection has been documented as an anthroponotic disease with transmission in human and sandflies vector. (Malaviya et al., 2011; Stauch et al., 2011).

This transmission cycle of VL is initialized by a bite of sandfly sucking blood from *L. donovani* infected human. The parasites develop inside the female sandfly. The infected sandfly transmits the parasites to the susceptible human through bite. The transmission cycle of VL completes when the susceptible human after a period of incubation time becomes infectious and is ready to transfers on the infection to another female sandfly.

Even after becoming infectious, if the infected human is not treated, will most probably die within two years. But will recover after a period of one month if gets VL treatment. Only, a few fraction of the recovered human can develop PKDL. PKDL also constitute

a source of VL infection that spreads through the sandfly vector to susceptible human. Thus, the transmission cycle of VL occurs.

2.7 Vector and vector surveillance

Identification of vector and observing infection in vector population, is an essential tool for survelliance of vector-borne diseases. Detection of evidence for the presence of Leishmania DNA within the collected sandflies is more relevant to confirm Leishmania vector. Vector study is the indicator to show whether VL has been actually decreased or can reemergence in future.

Female *P. argentipes* sandfly is the vector for the transmission of VL in human in Nepal (Desjeux, 1996). *P. argentipes* sandfly is a small insect dark brown in colour, about 1.5 to 2.5mm in length with their body and densely clothed wings with hairs. *P. argentipes* survives best in areas with high humidity, relatively controlled temperatures, and in muddy soil. The lifecycle of sandfly is complete metamorphosis, comprising of egg, larvae, pupal and adult stages. Breeding places for sandfly, is usually a habitation in dark, humid soil protected from the sunlight. The favourite breeding place in the cattle sheds, is underneath cattle troughs and cracks and crevices within walls in households. Sandflies are nocturnal pests and they invade in night and take shelter in holes and crevices in walls, holes in trees, and store rooms in the day. The female sandflies bite human every third or fourth day, as they need a blood meal for ovipositioning. June to October is the peak months for risk of transmission of VL in Nepal, due to high humidity and high sandfly density.

Sandfly collection methods

The common collection methods sandfly are by using CDC Light trap, by mouth aspiration method and by using sticky traps. Sandfly collection by miniature CDC light traps is an attractive sampling method commonly used for collection of adult sandflies in the field. It consists of battery operated light suction traps that can be left overnight to collect active sandflies. These traps can attract sandflies at short distance (2- 3 m) and so in any one given sampling site, many light traps may be required.

Sandflies are collected on one whole night and the traps are replaced early morning.

Sandfly collection by using adhesive traps is a cheap and easy method for collecting adult specimen and especially useful in dry areas.The sampling methodology include

the uniform use of 20cmx20cm sticky traps impregnated with castor oil set once a month for several days. Sandfly collection by aspiration method is one of the most convenient method to collect sandflies resting in holes and crevices in walls of the room. It is the easiest method of collection of sandfly but requires a team of trained insect collector.

2.8 Domestic animal as a risk factor or a reservoir of VL

In Nepal, VL is considered to be anthroponotic and humans are assumed to be the only reservoir of VL. Because of their close association with the sandfly vector, domestic animals might play a potential role in the transmission of VL. The risk for VL may increase by keeping domestic animals in the same room at night as it attracts more sandflies into the houses. Having animals around the house may be protective because of animals as preferred bloodmeal source for sandflies. Keeping animals inside the house can attract more sandflies indoor, which might be a risk factor for VL. To understand the potential role of domestic animals in maintenance and transmission of VL, further research is necessary in this field.

In a study done in India, Palit et al., (2005) showed no significant associations between VL and ownership of domestic animals. However, in Nepal, livestock ownership was found protective against VL (Bern et al., 2000); but in India it was found a risk factor for VL (Barnett et al., 2005) and *L. donovani* infection (Saha et al., 2008). Having cows around the house was found protective against VL, in a study done in Bangladesh, Bern et al., (2005), but they did not proved the protective effect of livestock ownership on VL. Keeping small animals around the house was found protective against *L. donovani* infection by Schenkel et al. (2006). In a study done in Nepal, *Leishmania* infections was found positive by PCR in 6.1% people, 5% cows, 4% buffaloes and 16% goats (Bhattarai et al., 2010). But their findings do not clearify domestic animals as a reservoir of *L. donovani.*

In a survey done, for detection of anti-Leishmania antibodies and *Leishmania* DNA in domestic cattle in VL endemic area of Bangladesh, 9.4% (n = 13) of the cattle were found positive by ELISA and among these, only 4 (30.8%) were DAT positive but in PCR analysis, *Leishmania* DNA in domestic cattle was not detected (Alam et al., 2011). *Leishmania* DNA was detected in several domestic animals and cattle in one study done in Nepal (Bhattarai et al., 2010).

Many studies have suggested that *Leishmania* parasites can infect, wild, domestic and synanthropic mammals. These animals have been identified also as reservoir host (Akhoundi et al., 2016).

2.9 Laboratory diagnosis

Case detection is done on basis of clinical symptoms, a prolonged fever, enlargement of spleen and liver, and weight loss, for VL. Clinical diagnosis can be confirmed using a wide range of serological, parasitological and PCR tests.

Leishmaniasis can be diagnosed in the laboratory by: by microscopic examination of parasite in tissues; by PCR detection of parasite DNA in samples; or by detection of parasite antigen in blood or urine samples, by detection of anti leishmanial antibodies

2.9.1 Microscopic examination of parasite in tissues

The definitive diagnosis of VL is the microscopic confirmation of the amastigotes in tissue aspirates (spleen, bone marrow or lymph nodes).

On microscopic examination of Giemsa stained slides, amastiogotes can be seen round or oval 2-4 µm in diameter intracellularly in monocytes or macrophages. The nucleus stains red and cytoplasm stains pale blue. A kinetoplast stains a deep red or violet rod-like in the same plane but at a right angle to it.

2.9.2 Immunological methods for diagnosis of leishmaniasis

In immunological methods, detection of antigens or anti-Leishmania antibodies in serum or urine samples from patients are performed. The immunological methods are: indirect fluorescent antibody test; enzyme linked immunosorbent assay; Western blot; Direct agglutination test (DAT); and immunochromatographic rapid strip test.

Direct agglutination test (DAT)

DAT test can be performed using serum or urine samples of patients for detection of VL due to *L. infantum* and *L. donovani*. DAT is done to detect antibodies produced against antigen in VL infection caused by *L. donovani* and *L. infantum.*

This test shows high sensitivity (74.6–96.6%) and specificity (77.8–100%). The only limitations of this test are, increases cross-reactivity with other infectious diseases due to use of whole parasite antigen, its requires serial dilutions, and a long incubation time.

2.9.5 rK39 rapid diagnostic test (InBiosKalazar Detect rapid test Seattle, WA)

The rK39 rapid diagnostic test is done to detect antibodies against *L. donovani* in human serum (Srivastava et al., 2011; Sundar et al., 2002; WHO, 2010). The kit contains recombinant rK39 *Leishmania* species antigen. It can be used to detect the subclinical cases of VL, and case incidence in VL endemic countries (Moamly et al., 2012; Sundar et al., 2002; WHO, 2010).

Principle: The InBios Kalazar Detect rapid test contains the recombinant *L. chagasi* antigen rK39. This is a 39-amino-acid repeat section in the 230-kDa Lc Kinprotein (C terminus of the kinesin protein). The gene encoding the LcKin protein is found in *L. chagasi, L. donovani*, and *L. infantum*. A membrane strip is coated with this protein which also contains a conjugate dye region. The patient serum will react with the dye and antigen through capillary action and quickly indicates the presence of anti-rK39 immunoglobulin G (IgG) in a patient sample.

Interpretation:

A positive result: The test is positive when both a control line and test line appear. The positive result indicates that the rK39 strip detected antibodies to *L. donovani* in samples. A faint red line in the test line is interpretated as weakly positive result.

A negative result: The test is negative when only the control line appears. A negative result indicates that the dipstick did not detected antibodies to *L. donovani.*

An invalid result: If no lines appear at the control or test line areas, then the test is invalid. The test is also invalid if no control line appears, even though a test line appears.

The rk39 RDT is easy to perform, is rapid (10–20 min), and high diagnostic performance. Thus, rk39 RDT can be used to detect VL in VL endemic areas or in blood sample collected by finger prick method at collection sites. The rK39 test has high diagnostic accuracy of 100% sensitivity and 98% specificity.

To compare the sensitivity of the rK39 RDT on serum versus blood from the same infected person from India, Matlashewski, et al. (2013) showed that the concordance between serum and blood was excellent. However it is possible to mistake some rK39 positive individuals when using blood. If the symptoms of VL persist in an individual from an endemic area even if the blood rK39 RDT is negative, then it is recommended that the test should be redone 2–3 weeks later.

2.9.6 PCR-based methods

The sensitive and specific methods for diagnosis of VL is PCR, amplfications. Most commonly used amplification targets used for VL diagnosis are nuclear DNA such as small subunit ribosomal rRNA gene (Srivastava et al., 2013; Salotra et al., 2001); extra-chomosomal DNA such as kinetoplastid DNA (Maurya et al., 2005; Salotra et al., 2001); mini exon genes (Katakura et al., 1998); and ribosomal internal transcribed spacer (ITS) region (Schonian et al., 2003).

Amplification of mini-circle kinetoplast DNA is an excellent target for detection of VL, which is present at thousands of copies per cell (Schonian et al., 2003). In many studies, the mini-circles kinetoplast DNA have been used for selective amplification of parasite DNA (Aviles et al., 1999; Bhattacharyya et al., 1996; Nuzum et al., 1995; Rodgers et al., 1990). Different DNA-PCR assays have been designated to detect *Leishmania* in clinical samples and to detect species of *Leishmania* (Kuhls et al., 2005). In detecting *Leishmania* DNA in clinical samples, PCR amplification of kDNA has shown >90% sensitivity and 100% specificity (Nasreen et al., 2012; Salotra et al., 2001). Several researchers have used kDNA PCR amplification to detect VL cases and obtained better results (Disch et al., 2003; Ozerdem et al., 2009; Salotra et al., 2001). By PCR amplifications, *Leishmania* DNA has been detected, in peripheral blood of persons with asymptomatic infections in Brazil, India and Nepal (Bhattarai et al., 2009; Costa et al., 2002; Topno et al., 2010).

Now, PCR tests has been confirmed as one of the most sensitive method to detect infection of asymptomatic *L.donovani* infection in VL endemic areas (Srivastava et al., 2013; Topno et al., 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Ethical approval

Ethical approval of this study was obtained from Nepal Health Research Council (NHRC Red.No.16 /2017). Written consent from each individuals and from guardian in case of children below 1 year or more, was taken from before collecting human blood samples. Before collection of domestic animal blood samples by a veterinarian, consent from the owners of the domestic animals was taken. Household consent was taken from the owners of household for installing CDC light traps to collect sandflies.

3.2 Human study

3.2.1 Study sites for human blood collection

The study sites selection were done by tracing past VL cases obtained from patient's record from District Health office (DHO) of the districts and from VL patient's record from EDCD, Teku, Kathmandu. The human blood samples were collected during 2016 to 2019. Jahada-7, Morang, Pipaldanda and Rampur villages, Palpa, Belhichapena village, Saptari and Ishworpur village, Sarlahi districts were selected for sample collection respectively.

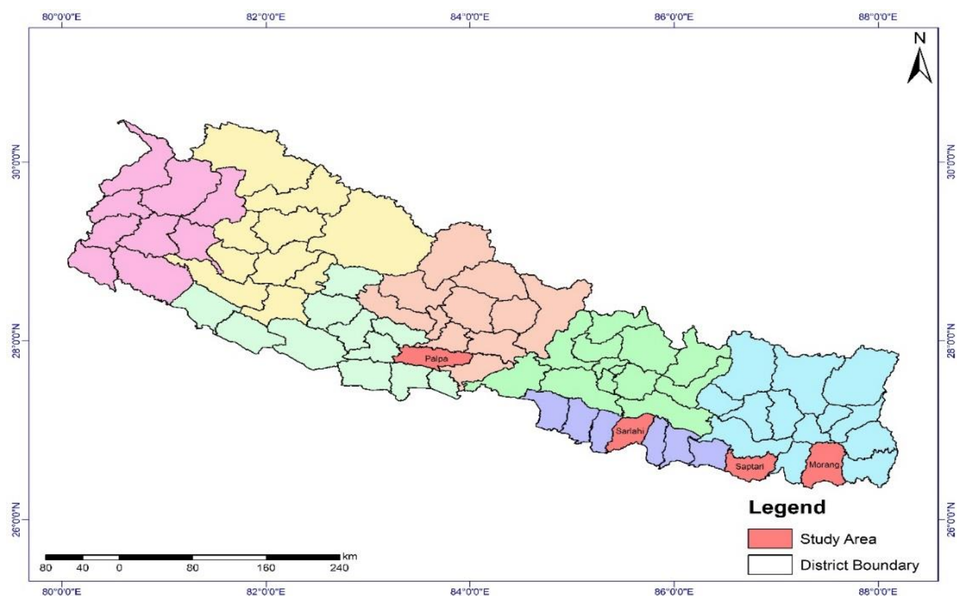


Figure 1: Location of study districts

3.2.2 Data collection for human study

For human study, data were collected by developing a structured questionnaire. Information on demographics, clinical signs & symptoms, history of VL treatment, occupation, owing domestic animals, sleeping behavior and types of houses were collected from all the villages of four districts as assessed by health workers.

3. 2.3 Case definition for human study population selection

Case definitions:

VL case was defined as a person from an endemic area, having signs and symptoms of prolonged fever, enlargement of spleen and liver, weight loss, and anemia, and a rK39 test positive.

Past case of VL was defined as person or individuals having record of previous treatment, confirmed by prescriptions and or case records from the health facility.

Asymptomatic case of VL

A positive serum/blood test or PCR or Leishmanin skin test in people who are healthy is generally considered as asymptomatic leishmanial infection.

Relapse case of VL is a patient who successfully completed a course of standard VL treatment, who later returns with symptoms of VL. And Leishmania parasites are found in the parasitological tests.

For the human study, the study population were selected targeting household members and neighbors of VL patients. All symptomatic and asymptomatic cases of VL from all the four districts were included in this study.

3. 2.4 Human blood sample collection

About 2ml of human blood sample from each individuals were collected by a vein puncture by a trained laboratory technician in a 3.2% sodium citrate vials. All collected blood sample were labelled properly and transported to the laboratory of Central Department of Microbiology within 24 hours in an ice box for further processing.

3. 2.5 Screening by rK39 test

From the collected human blood samples serum was obtained by centrifuging it at 3000 rpm for 15 minutes and anti-*Leishmania* antibodies in sera samples were tested by using the rK39 test (InBios, International, Seattle WA) kit, following the manufacturer instructions.

3. 2.6 Follow-up for human population

All the asymptomatic cases of VL in Sarlahi, Saptari and Palpa districts were followed up serologically within 1 year from the initial blood sample collection. Then the rK39 RDT test were performed. But in Morang district, we were not able to do follow up due to Covid-19 pandemic.

3. 2.7 Genomic DNA extraction from human blood specimens

From the collected human blood specimens genomic DNA extractions were done by following the manufacturer's instructions (Bio Basic, EZ-10 Column Blood Genomic DNA Miniprep Kit).Then the extracted genomic DNA was quantified using a Nano Drop (Thermo Scientific). The purity of the DNA was found satisfactory as the ratio of OD at A260/A280 was within 1.7–1.9 for all DNA samples. To avoid DNA damage during repeated freeze thawing, all the extracted genomic DNA samples were aliquoted in duplicate and stored at -80°C.

3. 2.8 PCR amplification of kinetoplastid minicircle DNA from human blood

PCR amplification were done from the extracted genomic DNA from the human blood samples by using the primers LIN4 (forward) (5′-GGG GTT GGT GTA AAA TAG GG-3′) and LIN19 (reverse) (5′-CAG AAC GCC CCT ACC CG-3′) which amplifies a *L. donovani* species- specific KDNA fragments of 720bp size in the infected human blood samples.

The PCR amplification reaction was carried out in a volume of 25 µL using, 3µl of these DNA, the pair of primers and the QIAGEN Multiplex PCR Master Mix Kit. *L. donovani* 1S2D strain was used as a positive control (kindly provided by Dr. Greg Matlashewski, Professor, Department of Microbiology and Immunology, McGill University, Canada), nuclease free water, as the negative control and a 1Kb DNA ladder, RTU (50 µg /500 µL) was used as a marker.

The amplified PCR products were separated on 1.5% agarose gel stained with ethidium bromide by electrophoresis.The amplified 720 bp fragments of *L. donovani* species-specific KDNA in the infected human blood samples were visualized under UV transillumination (Azure Biosystem).

Animal study

3.3.1 Study area for domestic animal blood collection

The study was conducted on domestic animals of known VL patients and the neighbors of VL patients in Pipaldanda and Rampur villages, Palpa and Belichapena village, Saptari districts during 2016 to 2019. Most of these villages had at least one animal (buffalo, cow, goat, ox or dog) as a domestic animals in their HHs. At night, the most of the goats, cows, and buffaloes, were kept atleast 10 meter away from the HHs, but in some HHs a few goats were kept inside the house at night.

3.3.2 Data collection for animal study

For animal study, by using pre-tested questionnaires data were collected from the HHs of the villages to evaluate the potential risk factors, such as whether treated for the VL or not, a history of VL in the owners, and keeping the domestic animals outside or inside the house at night.

3.3.3 Blood sample collection from domestic animals

About 2ml of blood sample from domestic animals were collected by a jugular vein puncture by a trained veterinary technician in a 3.2% sodium citrate vials. All collected blood sample were labelled properly and transported to the laboratory of Central Department of Microbiology within 24 hours in an ice box for further processing.

3.3.4 DNA extraction from domestic animals blood samples

From the collected domestic animals blood samples genomic DNA extractions were done by following the manufacturer's instructions of Bio Basic, EZ-10 Column Blood Genomic DNA Miniprep Kit, Animal (Canada). Then purity of all the extracted genomic DNA samples were checked by using a Nano Drop (Thermo Scientific). To avoid DNA damage during repeated freeze thawing, all the extracted genomic DNAs were aliquoted in duplicate and stored at -80°C.

3.3.5 PCR amplification of kinetoplastid minicircle DNA from animal

PCR amplification were done from the extracted genomic DNA from the animal blood samples by using the primers LIN4 (forward) (5′-GGG GTT GGT GTA AAA TAG GG-3′) and LIN19 (reverse) (5′-CAG AAC GCC CCT ACC CG-3′) which amplifies a *L. donovani* species- specific KDNA fragments of 720bp size in the infected domestic animal blood samples.

The amplification reaction was done in 25 μL volume, with 3μl of these DNA, the pair of primers and the QIAGEN Multiplex PCR Master Mix Kit. *L. donovani* 1S2D strain was used as a positive control (kindly provided by Dr. Greg Matlashewski, Professor, Department of Microbiology and Immunology, McGill University, Canada), nuclease free water, as the negative control and a 1Kb DNA ladder, RTU (50 μg /500 μL) was used as a marker.

The amplified PCR products were separated on 1.5% agarose gel stained with ethidium bromide by electrophoresis.The amplified 720 bp fragments of *L. donovani* species-specific KDNA in the domestic animal blood samples were visualized under UV transillumination (Azure Biosystem).

3.4 Sandfly study

3.4.1Study area for sandfly collection

The sandfly collections sites were 10 villages in Palpa:- Phoksingkot, Siluwa, Pipaldanda, Darmaldanda, Gothadi, Kachal, Argali, Khanichap, Bhuwanpokhari and Mashyam; 9 villages in Morang:- Sundarharaicha, Sundarharaicha-9, Rangeli, Katahari, Biratnagar, Sunbarshi, Jahada-2, .Jahada-5 and Jahada-7; 6 villages in Saptari :-West pipra, Kamalpur, Daulatpur, Malhaniya, Belhichapena and Tikuliya, and 1 village, Ishworpur in Sarlahi districts of Nepal.

3.4.2 Sandflies collection

Sandfly collection were done from overall 142 HHs from the villages of four districts. Collection of sandflies were done for 2 consecutive nights by trained insect collectors supervised by the entomologist. The sandfly collection in the villages of Palpa, Morang and Saptari was done by using CDC miniature light traps method and by mouth aspiration method in Ishworpur village of Sarlahi district. Before sunset, two CDC light

traps were installed per night per HH; 1 light trap was installed inside the HHs and the other in the door, by trained insect collectors on the observation of the entomologist. Then in the next day, in early morning, all the trapped sandflies were transferred in a petridish with chloroform-containing cotton balls. For identification the sandflies were placed in to 1.5ml collection vials. In this study, identification of female *P. argentipes* was done by the entomologist using the standard taxonomic key as described by Lewis [1982]. Only morphologically proven female *P. argentipes* sandfly samples were placed in the vials with 80% ethanol and were transported to the laboratory for the further investigations.

Sandfly density calculation:

The sandfly density was calculated as:

i). No. of sandflies /HHs/night/CDC light trap for CDC light trap collection method.

ii). No. of sandflies/ man/HHS/night for mouth aspiration method.

3.4.3 DNA extraction from sandflies

For the extraction of DNA from sandflies, sandfly pooled samples were homogenised using a disposable pestle. DNA from sandflies were extracted using QIAmp the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), following the manufacturer's instructions. Then purity of all the extracted DNA were checked by using a Nano Drop (Thermo Scientific). To avoid DNA damage during repeated freeze thawing, all the extracted genomic DNAs were aliquoted in duplicate and stored at -80°C.

3.4.4 PCR Amplification of kinetoplastid minicircle DNA from sandflies

PCR amplification were done from the extracted DNA from the sandfly samples by using the primers LIN4 (forward) (5′-GGG GTT GGT GTA AAA TAG GG-3′) and LIN19 (reverse) (5′-CAG AAC GCC CCT ACC CG-3′) which amplifies a *L. donovani* species- specific KDNA fragments of 720bp size in the infected female sandflies samples.

PCR was carried out in a volume of 25 µL using 5µl of these DNA, the pair of primers and the QIAGEN Multiplex PCR Master Mix. *L. donovani* 1S2D strain was used as a

positive control (kindly provided by Dr. Greg Matlashewski, Professor, Department of Microbiology and Immunology, McGill University, Canada), nuclease free water was used as negative control. 1Kb DNA ladder, RTU (50 µg /500 µL) was considered as a marker.

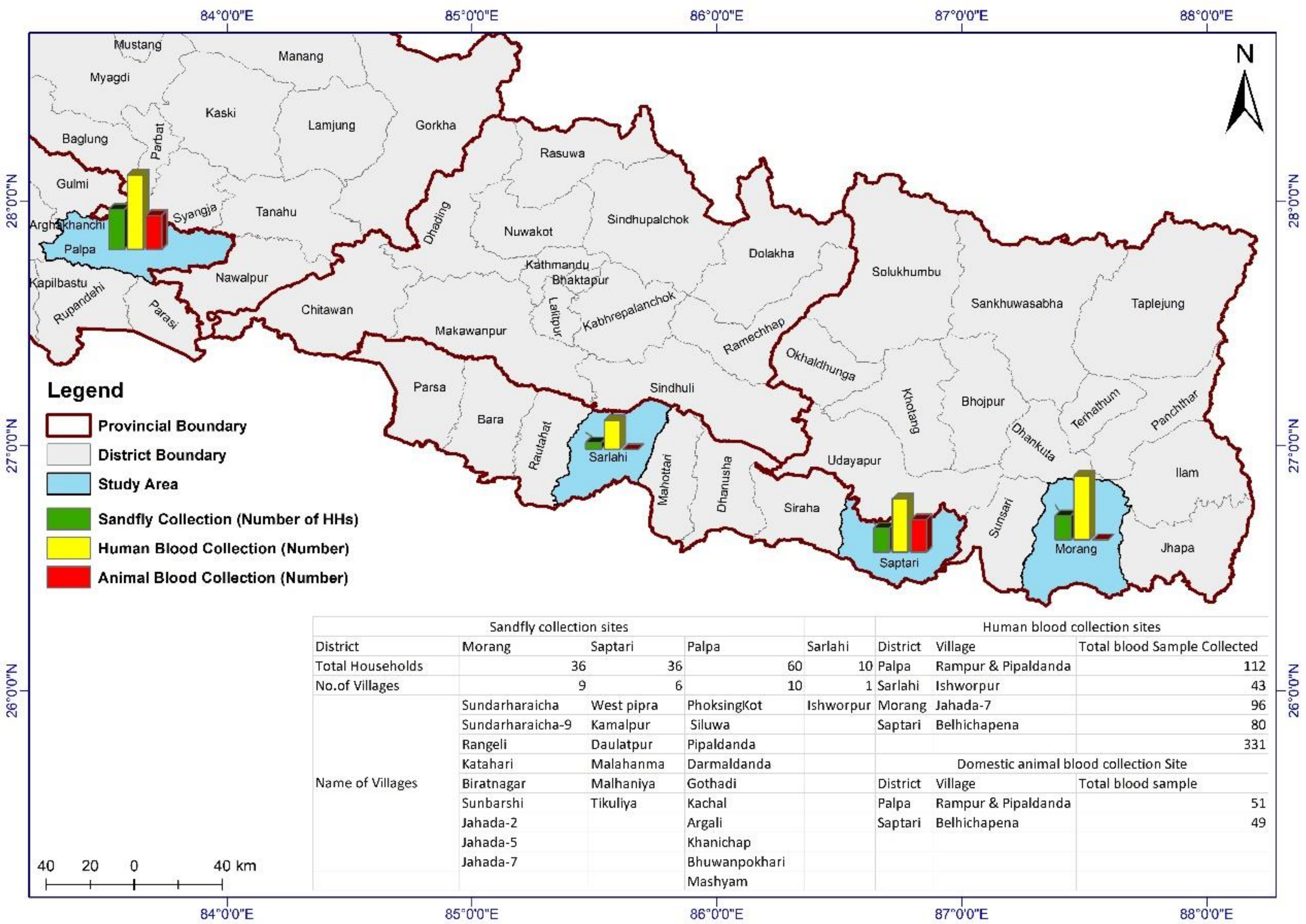
The amplified PCR products were separated on 1.5% agarose gel stained with ethidium bromide by electrophoresis.The amplified 720 bp fragments of *L. donovani* species-specific KDNA in the infected female *P. argentipes* sandflies were visualized under UV transillumination (Azure Biosystem).

Table 1: Polymerase chain reaction condition

PCR	Primers	Amplification condition
KDNA *L.donovani* species-specific PCR (Nasreen et al., 2012; Salotra et al., 2001)	LIN4 (forward) and LIN19 (reverse)	Initial denaturation at 95°C for 10 min]1 cycle Denaturation at 94°C for 30 sec Annealing at 63°C for 90 sec 40 cycles Extension at 72°C for 90 sec Final Extension at 72°C for 10 min

3.5 Data analysis

The data were entered into using SPSS version 21software and analyzed. Descriptive analysis was performed. Frequencies and percentages were calculated. Data on variables like risk factors of VL were compared using the chi-square test. A p-value of <0.05 were considered statistically significant.



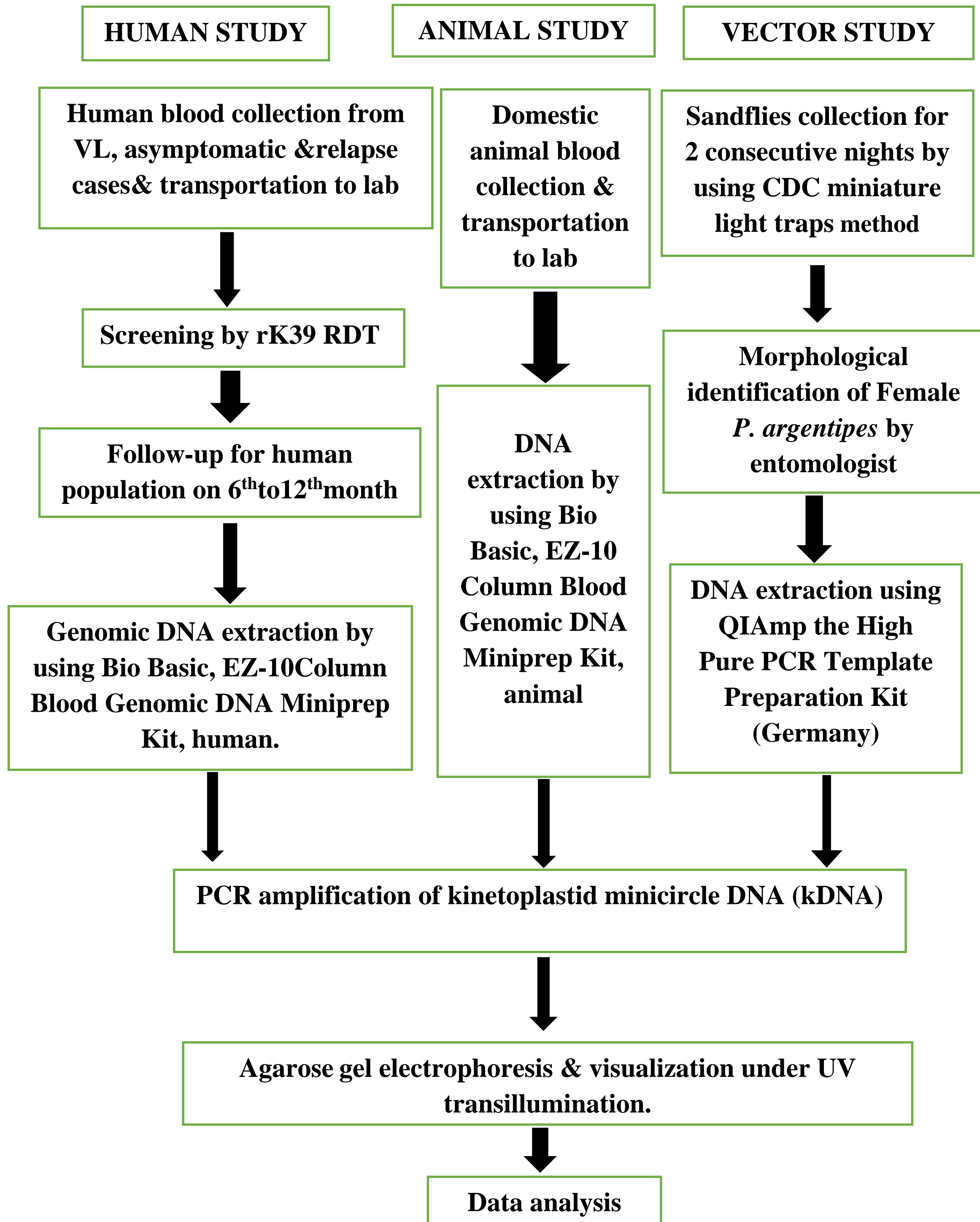
Sandfly collection sites				
District	Morang	Saptari	Palpa	Sarlahi
Total Households	36	36	60	10
No.of Villages	9	6	10	1
Name of Villages	Sundarharaicha	West pipra	PhoksingKot	Ishworpur
	Sundarharaicha-9	Kamalpur	Siluwa	
	Rangeli	Daulatpur	Pipaldanda	
	Katahari	Malahanma	Darmaldanda	
	Biratnagar	Malhaniya	Gothadi	
	Sunbarshi	Tikuliya	Kachal	
	Jahada-2		Argali	
	Jahada-5		Khanichap	
	Jahada-7		Bhuwanpokhari	
			Mashyam	

Human blood collection sites		
District	Village	Total blood Sample Collected
Palpa	Rampur & Pipaldanda	112
Sarlahi	Ishworpur	43
Morang	Jahada-7	96
Saptari	Belhichapena	80
		331

Domestic animal blood collection Site		
District	Village	Total blood sample
Palpa	Rampur & Pipaldanda	51
Saptari	Belhichapena	49

Figure 2: Location of study sites.

FLOW CHART OF METHODOLOGY



CHAPTER 4

RESULTS AND DISCUSSION

4.1 Seroprevalence of VL

The seroprevalence of VL was investigated among 331 people of different villages of four VL endemic districts of Nepal during 2016 to 2019 using the rK39 RDT.. In this study seroprevalence of VL was found 46.5% in Sarlahi district which is high than in other three districts. In hilly district Palpa, the seroprevalence of VL was found 1.8%. With 9.7% positivity, serological analysis reveals there is still ongoing transmission of VL in these districts.

Table 2: Seroprevalence (%) of VL in four study districts

Districts (Villages)	**Individuals tested No.**	**rK39 test positive No.**	**Seroprevalence (%)**
Palpa (Rampur & Pipaldanda)	112	2	1.8
Morang (Jahada-7)	96	4	4.2
Sarlahi (Ishworpur)	43	20	46.5
Saptari (Belhichapena)	80	6	7.5
Total	331	32	9.7

Age and gender specific distribution of rK-39 result

In our study districts, the study population composed of 133 (40.2%) males and 198 (59.8%) females.

The positivity of rK39 test was found high in 1-15 years and 31-45 years of age group. in Sarlahi district More proportion of males were rK39 positive than females. In Saptari district, rK39 test positivity was found high in females and rK39 positive cases were found up to 61 years of age. In Palpa and Morang districts, there was no differences in

rK39 positivity of VL according to gender. In all 4 districts, there was no rK39 positive cases above 75 years age group.

Table 3: Age and gender specific distribution of seroprevalence of VL

Age (years)	Sarlahi		Saptari		Palpa		Morang	
	Individuals tested No.	rK39 Positive No. (%)	Individuals tested No.	rK39 Positive No. (%)	Individuals tested No.	rK39 Positive No. (%)	Individuals tested No.	rK39 Positive No. (%)
1-15	16	8 (50.0)	26	2 (7.7)	14	0 (0.0)	18	0 (0.0)
16-30	9	4 (44.4)	22	0 (0.0)	45	1(2.2)	41	3 (7.3)
31-45	12	6 (50.0)	20	2 (10.0)	16	0 (0.0)	19	0 (0.0)
46-60	6	2 (33.3)	9	2 (22.2)	28	1(3.6)	15	1 (6.7)
61-75	0	0 (0.0)	0	0 (0.0)	5	0 (0.0)	3	0 (0.0)
76-90	0	0 (0.0)	3	0 (0.0)	4	0 (0.0)	0	0 (0.0)
Total	43	20(46.5)	80	6 (7.5)	112	2 (1.8)	96	4 (4.2)
Gender								
Male	21	14 (66.7)	43	2 (4.7)	37	1(2.7)	32	2(6.3)
Female	22	6 (27.3)	37	4 (10.9)	75	1(1.3)	64	2 (3.1)

4.2 Follow up results of rK39 tests

In Rampur and Pipaldanda villages of Palpa, 1/60 tested was positive on the rK39 RDT in the initial blood collection time. In Belichapena, village of Saptari, 3/50 tested were positive on the rK39 RDT in the initial blood collection time. In Ishworpur village,of Sarlahi, 10/28 tested were positive on the rK39 in the initial blood collection time.In Jahada-7 village of Morang, 4/96 tested were positive on the rK39 RDT (Table 4).

Asymptomatic cases of VL were followed up within 1year from initial blood collection, to observe disease progression. In this study, we observed 1/62 tested was rK39 positive from Palpa, 3/30 tested were rK39 positive from Saptari and 10/15 tested were rK39 positves in the blood samples collected in the follow up after 6th to 12thmonth.

Table 4: Result of rK39 test done initially and follow-up after 6th to 12thmonth

District (Villages)	First collection		Follow up collection	
	Individuals Tested No.	rK39 test positive No. (%)	Individuals tested No.	rK39 test positive No. (%)
Palpa (Rampur & Pipaldanda)	60	1 (1.7)	62	1 (1.6)
Saptari (Belhichapena)	50	3 (6.0)	30	3 (10.0)
Sarlahi (Ishworpur)	28	10 (35.7)	15	10 (66.7)
Morang (Jahada-7)	96	4 (4.2) 96	-	- 3 (3.1)

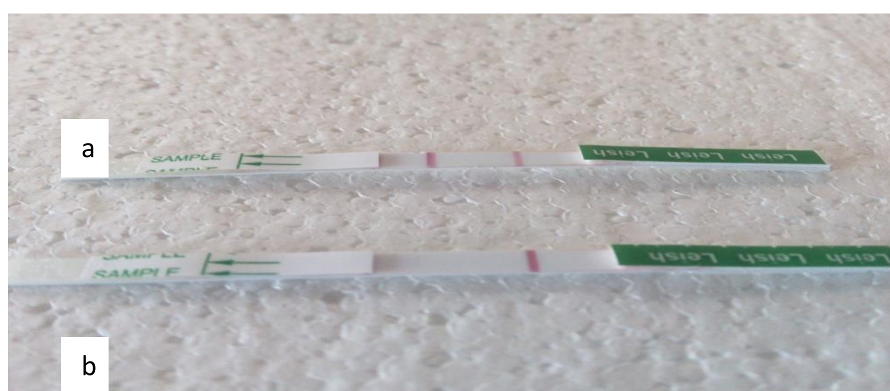


Figure 3: rK39RDT results (InBio International, WA).

(a) rK39 test positive result with both a control line and and test line.

(b) rK39 test negative result with only the control line.

4.3 Distribution of rK39 positive persons among symptomatic, asymptomatic and relapse cases of VL

Out of 32 rK39 positive cases observed in this study, 23 cases showed symptoms of VL, and 7 cases were without the symptoms of VL and 2 cases were relapses of VL. These 2 people were from Jahada -7 village of Morang, who completed the standard VL

treatment 6 years earlier but showed clinical signs of VL during screening of VLcases and were found rK39 test positive on RDT.

Table 5: Distribution of rK39 positive persons among symptomatic, asymptomatic and relapse cases of VL

Districts (Village)	**Individuals tested No.**	**rK39 test positive No.**	**Symptomatic cases No.**	**Asymptomatic cases No.**	**Relapse cases No.**
Palpa (Rampur & Pipaldanda)	112	2	2	0	0
Morang (Jahada-7)	96	4	1	1	2
Sarlahi (Ishworpur)	43	20	18	2	0
Saptari (Belichapena)	80	62	2	4	0
Total	331	32	23	7	2

4.4 Molecular analysis of human blood samples

In PCR amplification, 35.7 % (10/28) in the Ishworpur village of Sarlahi, 4% (2/50) in Belichapena village of Saptari district, 1.6 % (1/60) in the Pipaldanda villages of Palpa district and 3.1% (3/96) in the Jahada-7 village of Morang district were found PCR positive in the first collected blood samples (Table 6).

In comparison to Sarlahi district, there were much fewer positive samples in Saptari, Palpa and Morang districts but each of these districts did have positive PCR tests. All the blood samples collected after 6 to 12 months from initial sample collection in Palpa, Sarlahi and Saptari districts, showed PCR negative results.

Table 6: PCR results of human blood samples

Districts (Village)	First collection		Follow up collection	
	Individuals tested No.	PCR positives No. (%)	Individuals tested No.	PCR positives No. (%)
Sarlahi (Ishworpur)	28	10 (35.7)	15	0(0)
Saptari (Belichapena)	50	2 (4.0)	30	0(0)
Palpa (Rampur & Pipaldanda)	60	1(1.6)	62	0(0)
Morang (Jahada-7)	96	3 (3.1)	-	-

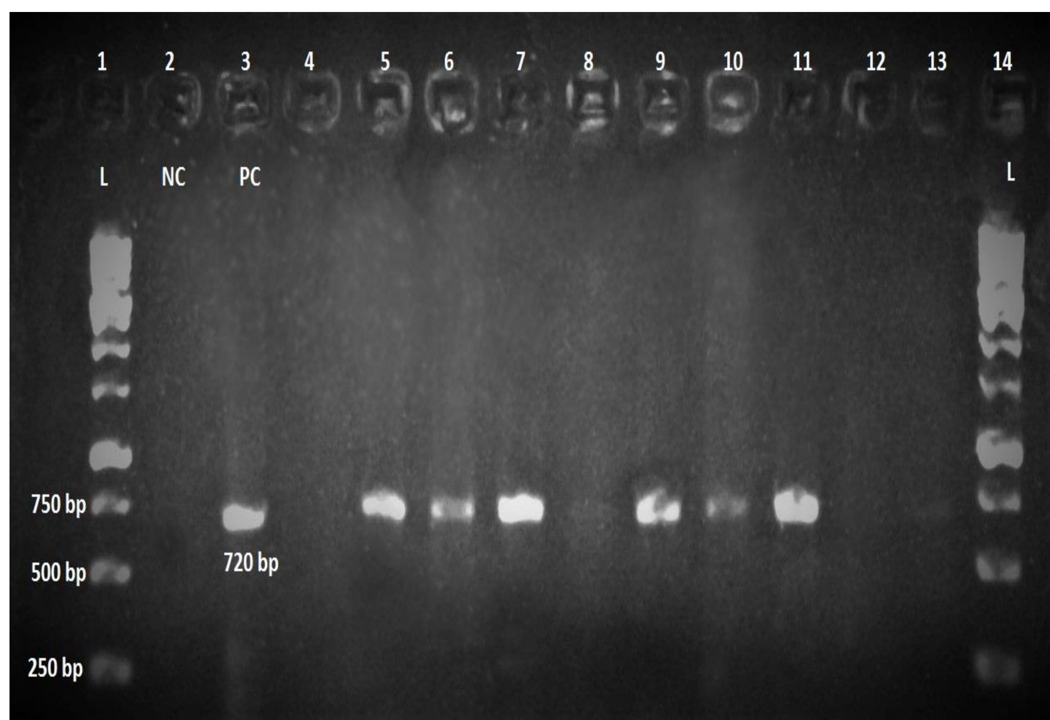


Figure 4: Minicircle kDNA, PCR amplification of L. donovani in human blood samples from the Ishworpur village, Sarlahi district.

Lanes: 1 and 14, DNA size marker; lane 2, negative control; lane 3, positive control.

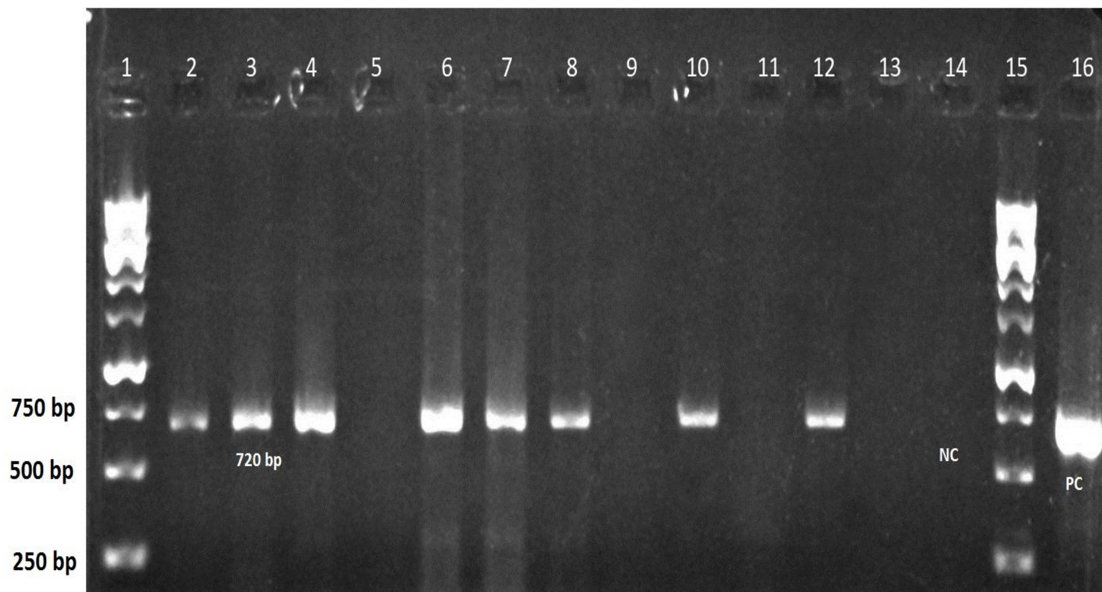


Figure 5: Minicircle kDNA PCR amplification of L.donovani in pooled female P. argentipes from Morang district

Lanes: 1 and 15, DNA size marker; lane 14, negative control; lane 16, positive control.

4.5 rK39 and PCR positivity in initial and follow up

In Palpa district, 1 rK39 test was found also PCR test positive and in Sarlahi district, all 10 rK39 test positives were also found PCR test positive in initial blood collection. In Saptari district, out of 3 rK39 test positives only 2 were found PCR test positves. In Morang district, out of 4 rK39 test positives only 3 were found PCR test positive in the initial blood collection. We were not able to do further follow up the study cases in Morang district due to COVID-19 pandemic.

Table 7: rK39 and PCR positivity in initial and follow up

District (Villages)	First collection			Follow up collection		
	Individuals No.	**rK39 positive No. (%)**	**PCR positiveNo. (%)**	**Individuals No.**	**rK39 positive No. (%)**	**PCR positive No. (%)**
Palpa (Rampur & Pipaldanda)	60	1 (1.7)	1 (1.7)	62	1 (1.6)	0 (0.0)
Morang(Jahada-7)	96	4 (4.2)	3 (3.1)	-	-	-
Sarlahi (Ishworpur)	28	10 (35.7)	10 (35.7)	15	10 (66.7)	0 (0.0)
Saptari (Belhichapena)	50	3 (6.0)	2 (4.0)	30	3 (10.0)	0 (0.0)

4.6 rK39 and PCR positive persons with or without symptoms

Among 32 persons with rK39 seropositive tests, 23 (71.8%) had clinical symptoms of VL, 7 (21.8%) were asymptomatic and 2 (6.3%) were relapse cases. Among 16 PCR positives, 10 (62.5%) had clinical symptoms of VL, 4 (25.0%) were asymptomatic and 2 (12.5%) were relapse cases.

Table 8: rK39 and PCR positivity in symptomatic and asymptomatic individuals

Test	rK39	PCR
No. of symptomatic person (%)	23 (71.8)	10 (62.5)
No. of asymptomatic person (%)	7 (21.8)	4 (25.0)
No. of relapse cases (%)	2 (6.3)	2 (12.5)
Total	32	16

4. 7 Detection of *Leishmania* infection in sandflies

Infected sandflies were detected in 1HH in Pipaldanda in pools of VL HH of Palpa and in 2HH in Argali, 4 HH in Pipaldanda, 1HH in Phoksingkot, 3HH in Khanichap and 1HH in Kachal in pools of non-VL HHs. In Morang district, infected sandflies were detected in 2HH in Jahada-7 in pools of VL HH and 2HH in Jahada-5, 1HH in Sundarharaicha, 1HHin Sunbarsha and1HH in Rangeli in pools of non-VL HHs. Infected sandflies were detected in 4HH in Ishworpur in pools of VL HH of Sarlahi and in 2HH in Ishworpur in pools of non-VL HHs.In Saptari district, infected sandflies were detected in 2 HH in Belhichapena in pools of VL HH and in 3HH in Daulatpur, 2HH in Malhaniya and 3HH in Westpipra in pools of non-VL HHs.

Table 9: Infection of sandflies collected from different villages

Districts	No. of HHs	No. of villages	Total tested (Pool of 2HHs each)	No. of PCR positive tests	Infected sandflies in VL HHs pools	Infected sandflies in pools of non-VL HHs
Palpa	60	10	30	12	1in Pipaldanda	2 in Argali 4in Pipaldanda 1in Phoksingkot 3 in Khanichap 1 in Kachal
Morang	36	9	18	10	2in Jahada-7	3 in Jahada-7 2 in Jahada-5 1in Sundarharaicha 1 in Sunbarsha 1 in Rangeli
Sarlahi	10	1	10	6	4 in Ishworpur	2 in Ishworpur
Saptari	36	6	18	10	2 in Belhichapena	3 in Daulatpur 2 in Malhaniya 3in Westpipra

Parasite positivity in sandfly pools

In Khanichap village of Palpa, parasite positivity in sandfly pools was 75% and 50% in Pipaldanda and Argali villages. But the parasite positivity rate in sandfly pools is low in Kachal and Khanichap villages of Palpa. In Jahada-7 village of Morang, parasite positivity in sandfly pools was 62.5%. In Sunbarsha and Sundarharaicha villages of Saptari, the parasite positivity rate in sandfly pools were positive in all tested sandfly pools.In Ishworpur village of Sarlahi districts,the parasite positivity rate in sandfly pools was 60%. In Daulatpur and Westpipra villages of Saptari, parasite positivity in sandfly pools were 75%. But the parasite positivity rate in sandfly pools of Belhichapena village was low.

Table 10: Village wise parasite positivity in sandfly pools

District	Village	No. of pools	No. of PCR positives	Positive prevalence (%)
Palpa	Argali	4	2	50.0
	Pipaldanda	10	5	50.0
	Phoksingkot	6	1	16.7
	Kachal	6	1	16.7
	Khanichap	4	3	75.0
Morang	Jahada-5	6	2	33.3
	Jahada-7	8	5	62.5
	Sunbarsha	1	1	100.0
	Sundarharaicha	1	1	100.0
	Rangali	2	1	50.0
Sarlahi	Ishworpur	10	6	60.0
Saptari	Belhichapena	6	2	33.3
	Daulatpur	4	3	75.0
	Westpipra	4	3	75.0
	Malhaniya	4	2	50.0

Density of sandfly

In this study sandfly density was found high in Ishworpur village of Sarlahi district.In the same village of Sarlahi, seropositivity of VL was found also high in human. In Palpa district the density of female sandfly was found high than in Morang district. This may be due to lack of spraying activity in Palpa which is a new foci but Morang is one of endemic district with VL control program from many years.

Table 11: Female P.argentipes density in study districts

Districts	Sandfly collection method	No. of HHs	No. of female *P. argentipes*	Sandfly density
Palpa	Sandfly collection by CDC light trap	60	364	0.50
Morang		36	294	0.21
Saptari		36	266	0.62
Sarlahi	Sandfly collection by Mouth aspiration method	10	40	1

No. of sandflies /HHs/night/CDC light trap for CDC light trap collection method

No. of sandflies / man/HHS/night for mouth aspiration method

4.8 *L. donovani* in domestic animals

All PCR tested for animal blood samples were found negative.We did not found *Leishmania* infection in blood samples of domestic animals.

4.9 Risk factors of VL

The risk factors of VL were calculated using the data of only three districts-Palpa, Sarlahi and Saptari.

In this study seropositivity for VL are seen more in children and above 60 age group there were no seropositive VL cases. However, males were found more seropositive than female (p-value=0.043) (Table 12).

Table 12: VL risks factors in VL seropositive individuals in age groups and gender

Age (years)	rK39 tests done	VL seropositive No. (%)	VL seronegative No. (%)	p- value
1-15	56	10 (17.9)	46 (82.1)	0.116
16-30	76	5 (6.5)	71 (93.4)	0.081
31-45	48	8 (16.7)	40 (83.3)	0. 255
46-60	43	5 (11.6)	38 (88.4)	0.948
61-75	5	0 (0.0)	5(100.0)	-
76-90	7	0 (0.0)	7 (100.0)	-
Gender				
Male	101	17 (16.8)	84 (83.2)	0.043
Female	134	11 (8.2)	123 (91.8)	

In case of occupation, students were found statistically significant for VL seropositivity than other occupations. However farmers were significantly less likely to be sero-positive for VL (Table 13).

Table 13: VL risks factors in VL seropositive individuals in different occupation

Occupation	rK39 tests done	VL seropositive No. (%)	VL seronegative No. (%)	p-value
Farmers	120	4 (3.3)	116 (96.7)	<0.001
Daily labour	38	8 (21.1)	30 (78.9)	0.057
House wife	29	1 (3.4)	28 (96.6)	0.231
Others	20	1 (5.0)	19 (95.0)	0.524
Student	28	14 (50.0)	14 (50.0)	<0.001

The individuals are likely to be seropositive for VL, living in mud house.There is less risk of VL for those who live in mixed cemented house (Table14).

Table 14: VL risks factors in VL seropositive individuals with type of house

House type	rK39 tests done	VL seropositive No. (%)	VL seronegative No. (%)	p-value
Mud	100	25(25.0)	75(75.0)	<0.001
Thatched	67	2 (3.0)	65 (97.0)	<0.144
Mixed cement	68	1 (1.5)	67 (98.5)	<0.003

Individuals of HHs without domestic animals are significantly higher VL seropositive than the individuals of HHs with domestic animals (Table15).

Table 15: VL risks factors in VL seropositive individuals with owing domestic animals

Owing domestic animals	rK39 tests done	VL seropositive No. (%)	VL seronegative No. (%)	p-value
Yes	192	6 (3.1)	186 (96.9)	<0.001
No	43	22 (51. 2)	21 (48.8)	

The individuals are likely to be seropositive for VL in sleeping on floor and sleeping on cot (Table16).

Table 16: VL risks factors in VL seropositive individuals with sleeping behavior

Sleeping behavior	rK39 tests done	VL seropositive No. (%)	VL seronegative No. (%)	p-value
Sleeping on floor	86	24 (27.9)	62 (72.1)	<0.001
Sleeping on cot	142	4 (2.8)	138 (97.2)	<0.001
Sleeping under bed net	7	0 (0.0)	7 (100.0)	-

4.10 Discussion

Nepal has successfully achieved the elimination target of <1/10000, although this threshold has been exceeded in hilly district demonstrating ongoing transmission. So, it is crucial to continue to investigate new foci where epidemics of VL occurs This study was conducted with the main aim to investigate transmission of VL endemic in new foci of hilly district of Nepal. Study sites selected were Morang, Saptari and Sarlahi districts which are considered to be endemic for VL and running control program from many years wheras hilly district Palpa is recently confirmed as endemic.

Humans are the only established reservoir for *L. donovani* in the Indian subcontinent, including Bangaladesh, Nepal and India. However, it becomes important to know the transmission of VL in acute cases and subclinical infections. The selection of the villages for human study were done by tracing past VL cases obtained from the District Health Office's record and from VL patient's record from EDCD, Kathmandu.

VL has both zoonotic and anthroponotic etiologies, but VL due to the parasite *L. donovani* is considered to be anthroponotic. In Nepal it is considered to be anthroponotic, human to human transmission. (Desjeux, 2004).

In this study 32 out of 331 people tested were rK39 RDT positive, with the majority coming from the same Ishworpur village, Sarlahi district. The recombinant 39-amino acid antigen (rK-39) RDT can be used to detect subclinical, recent or active infection in a VL endemic area (Bern et al., 2000). The seropositivity of VL in different villages of four endemic districts were detected, 1.8% in Palpa, 4.2% in Morang, 46.5% in Sarlahi, and7.5% in Saptari districts respectively. It was remarkable to identify 10 out of 28 people tested in the first sample collection were rK39 positive in Ishworpur village, Sarlahi where tests were performed in the laboratory. Within 15 days of the tests being performed, 9 out of 10 rK39 positive cases developed VL, indicating a significant level of transmission in this village. We did not perform microscopy for the parasite in the collected blood samples. All these VL positive cases were from daily labour of Ishworpur village, Sarlahi. In a study conducted in Bangladesh showed the risk of developing VL increases by threefold in people living within a 50 meter radius of VL patients (Bern et al., 2005).In Sarlahi district, family members of same HHs were found seropositive for VL and their neighbors were also found VL seropositives.

In Belichapena village of Saptari, 3/50 samples were found rK39 positive in the sample collected for the first time from different HHs. In Rampur and Pipaldanda villages, 1/60 sample tested were found rK39 RDT positive. In Jahada-7 village, Morang 4/96 were found rK39 seropositive. Among, 4 serpositives, 1case had symptoms of VL, 2 were relapse and 1 was asymptomatic cases of VL. 2 people of Jahada- 7 village of Morang, who had completed the full course of VL treatment six years earlier but demonstrated signs and symptoms of VL and were found rK39 positive on screening of VL cases. It is unknown whether these were relapsed or new infections, although they were treated 6 years ago suggests they could be re-infection.

All family members of VL patients were found rK39 tests negative in Palpa, Saptari and Morang districts. In Sarlahi, Palpa and Saptari districts, all asymptomic cases of VL but positive for rK39 test were followed up for six to twelve months to observe seroconversion. In Sarlahi, 10 out of 15 people, in Saptari, 3 out of 30 people and in Palpa, 1 out of 62 people were found rK39 seropositive in the follow- up. In Palpa, we were able to detect 1 known VL case during follow up. In Sarlahi, we observed 2 out of 20 rK39 positive remained asymptomatic upto 6 month and in Saptari, 4 out of 6 rK39 positive were asymptomatic upto 12 months.

We review from our data that significant proportion of asymptomatic cases can be a reservoir of VL in endemic areas. Moreover, the asymptomatic cases can be the source of infection to sandfly vector thus can contribute for the transmission cycle of VL. But during the study period, no asymptomatic cases were found in Palpa district.The asymptomatic VL cases can be identified by serological tests, though they have a low titer of antibodies and therefore test as seronegative.

A high level of seropositivity and a high level of conversion to symptomatic cases were observed in Ishworpur village of Sarlahi. In this maintenance phase of VL elimination, investigation of both symptomatic and asymptomatic cases is particularly important to reduce VL transmission. In this study, rK39 positivity of VL was found quite high in Sarlahi district in comparison to Saptari, Palpa and Morang districts. With 9.7% (32/331) positivity, the serological analysis showed that there is presence of transmission of VL in these districts.

The rK39 positivity was found high in 1-15 years and 31-45 years of age group in the Sarlahi districts. More proportion of males were rK39 positive than females. In Saptari district, rK39 positivity was found high in females and rK39 positive cases were found up to 61 years of age. In Palpa and Morang districts, there was no differences in rK39 positivity according to gender. In all 4 districts, there was no rK39 positive cases above 61 years age group. The seropositivity of VL was found higher in males than in females which correlates with the findings of Masum et al. (1990) and Talukder et al. (2003).

Since 2000, VL has been reported in increasing number from the hilly areas of Nepal but none of these reported VL case were considered as local transmission. We found 2 symptomatic cases of VL, one in Pipaldanda village another in Rampur village Palpa, were without travel history. Palpa is a hilly district with cold climate in winter. The houses in Palpa district are scattered and sandflies can hop only up to 50 meters. So, there is limited infections of human by infected sandflies. However, we were able to detect infected female *P. argentipes* sandflies from known VL HHS and also fom non VL HHs in Palpa. This shows that there is definitely local transmission in Palpa district, so VL control activities should be extended in this village. In Pipaldanda and Rampur villages of Palpa symptomatic cases of VL was also detected in local residents. We also found *L. donovani* DNA in collected *P. argentipes* sandflies in the villages of Palpa. Based on our findings we accomplish that there is local transmission of *L. donovani* in Papla district.

We collected human blood samples in two different points of time. So after first sample collection, 9 out of 10 cases became asymptomatic to symptomatic before collection of the second sample in Sarlahi district. Those samples were treated in the district hospital, therefore when we collected blood samples in the follow up none of them were positive in PCR but became positive by rK39 test positive.

In PCR amplfications done for initially collected blood samples in Ishworpur, village of Sarlahi, kDNA of *L. donovani* was amplified in 35.7 % (10/28) human blood samples which actually shows large reservoir of infected people that can transmit parasites in sandflies.

The PCR positivity in humans from villages of the Saptari, Palpa and Morang districts were 4%, 1.7% and 3.1% respectively which indicates lower transmission of VL in these districts.

In molecular analysis of human blood samples collected from 4 VL endemic districts, 4.8% (16 /331) were found PCR positive which is almost similar with the findings of Bhattarai et al. (2010) who found 6.1% (17/278) PCR positive. Shrestha et al. (2019) found only 2 (33.3%) rK39 positive cases positive by PCR. But, we found out of 32 rK39 test positives, only 16 cases were positive by PCR.

For vector study, sandfly collection sites included 6 villages in Saptari, 10 villages in Palpa, 9 villages in Morang, and 1 village in Sarlahi.

In Khanichap village of Palpa, parasite positivity in sandfly pools was 75% and 50% in Pipaldanda and Argali villages. But the parasite positivity rate in sandfly pools is low in Kachal and Khanichap villages of Palpa. In Jahada-7 village of Morang, parasite positivity in sandfly pools was 62.5%. In Sunbarsha and Sundarharaicha villages of Saptari, the parasite positivity rate in sandfly pools were positive in all tested sandfly pools.In Ishworpur village of Sarlahi districts,the parasite positivity rate in sandfly pools was 60%. In Daulatpur and Westpipra villages of Saptari, parasite positivity in sandfly pools were 75%. But the parasite positivity rate in sandfly pools of Belhichapena village was low.

Though we captured *P. argentipes* from houses of all 4 VL endemic districts, sandfly density was higher in the HHs of Sarlahi which belongs to poorer wealth quintiles. This finding was similar to the findings of Malaviya (2014) who also found sandfly density higher in the HHs belonging to poorer wealth quintiles. In this study, sandfly density was found low in in Morang district than in Palpa district. This may be due to lack of spraying activities in Palpa, which is recently confirmed as endemic to VL and Morang district is VL endemic district with VL control program from many years.

In this study, seropositivity of VL was found higher in children but people above 61 years were found VL seronegative. In this study seropositivity of VL was found higher among age group 1-15 and 31-45 years while in a study Bern et al., found seropositivity of VL high among age group 15-40 years (Bern et al., 2000). The risk of VL was found highest for persons 3–45 years of age in a survey done in a Bangaladeshi community (Bern, et al., 2005). In this study males were found more seropositive than female, similar findings has been observed in study done in Nepal (Rijal et al., 2010). The result was also consistent with the findings of study done in Bangaladesh (ICDDRB, 2003).

In case of occupation, students were found statistically significant for VL seropositivity than other occupations. However, the prevalence of VL was found high in agriculture farmers in a study done in Bangladesh (Khanum et al., 2017).

The individuals are likely to be seropositive for VL in sleeping on floor and sleeping on cot than sleeping under bed net, similar findings was observed in a study conducted in Morang district by Mandal et al., 2020. The individuals are likely to be seropositive for VL, living in mud house and mixed houses. In a study done by Mandal et al. (2020) showed that high risk for VLwere people living in thatched house, sleeping in ground floor, and ownership of animal. But in this study, individuals of households without domestic animals are significantly higher VL seropositive than the individuals of households with domestic animals.

In PCR analysis of blood samples of domestic animals, from known VL patients and their neighbors, all of the tests were negative. We did not detected *Leishmania* infection in domestic animals blood collected from the villages of Palpa and Saptari districts. The absence of *Leishmania* DNA is indicates that domestic animals are not reservoirs of VL in these endemic focus.

This shows that the human remains the major reservoir host of VL in these villages. Thus, from our finding we can conclude that human are the reservoir host for *L. donovani* in these VL endemic district.

The results of human and sandfly findings strongly point towards ongoing local transmission of VL in these villages. Thus, surveillance is needed to identify new outbreaks or transmission in the new foci and previous endemic districts.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

- All of the four studied districts showed the VL cases, *L. donovani* DNA and anti-k39 antibodies in human blood depicting ongoing transmission of VL. VL transmission was detected higher in Ishworpur village, Sarlahi district.
- Transmission of VL was high in Sarlahi district, demonstrating a potentially large reservoir of infected individuals that can potentially transmit parasites to sandflies. It will be essential to maintain strong surveillance in this village and nearby villages in the Sarlahi district.
- *P. argentipes* is the most common species in these VL endemic areas, and was also found infected with *Leishmania* parasites. Therefore, it can be assumed that this species plays an important role in the transmission of leishmaniasis in these endemic areas.
- There was no evidence found that domestic animals represent a reservoir for *L. donovani* in these villages. Thus, persons are still considered the only reservoir host for *L. donovani* in Nepal.
- This study showed that healthy seropositive asymptomatic individuals can be carriers of *L. donovani* based on PCR analysis and follow up result of rK39 test. Therefore, they should be observed for early detection of symptoms and managed under the VL elimination program.
- This study showed, there is still ongoing transmission of VL in endemic districts-Saptari, Sarlahi and Morang though there is control program for many years. Therefore, the maintenance of the elimination target of 1 case in 10,000 will be difficult in Nepal unless a strong surveillance program is in place in the endemic districts.
- Asymptomatically infected persons in VL endemic areas may alter transmission dynamics of VL and lead to a high incidence of disease. Thus, assessment of the magnitude of asymptomatically infected persons by using serologic screening

and follow-up for at least 6 months to detect seroconversion can help in early detection of VL cases in areas endemic for VL.

- Male gender, students, people living in mud and mixed cemented houses, without domestic animals in house, sleeping on floors and cot were found with the significant risk factors of VL in this study.

5. 2 Recommendations

1. At present VL incidence in Nepal, is reduced by 90%, compared to 2005. In spite of this progress, VL has now been reported from hilly districts, where there is no VL control programme. Therefore, screening of VL in new foci and previously identified VL endemic should be compared.
2. Surveys including combination of Leishmanin skin test and rK39 rapid immunochromatographic test should be done to screen transmission in a low incidence country like Nepal.
3. Active case detection by serological method should be expanded as Nepal is in the maintenance phase of VL elimination.
4. Screening of VL cases should be done in other animals besides domestic animals although, human are the main reservoir of VL in these endemic districts.
5. Role of other vectors if present should be explored besides *P. argentipes* vector.

CHAPTER 6

SUMMARY

Visceral leishmaniasis (VL), remains a significant public health problem in Nepal. As human are the only reservoir of VLin Nepal, it is essential to investigate the role of symptomatic and asymptomatic cases in VL transmission. The role of domestic animals in the maintenance and transmission of VL infection is not clear. Therefore, the main aim of this study was to investigate the transmission dynamics of VL in 4 VL endemic districts of Nepal.

331 human blood samples, 100 domestic animals blood samples and 3976 sandflies samples from 142 HHs were collected from the villages of Palpa: Morang, Saptari and Sarlahi districts from 2016 to 2019.

The seropositivity of VL as measured by antibody response to rK39 antigen were 46.5% in Sarlahi,1.8% in Palpa, 4.2% in Morang and 7.5% in Saptari districts of Nepal. The seropositivity of VL was found high in males (15.4%) than in females (6.1%) in the villages of these four districts.

The seropositivity for VL was found high in 1-15 years of age groups and above 61 years of age group there was no rK39 positivity in all these districts. In case of occupation, students were found statistically significant than in other occupations. The individuals were likely to be seropositive for VL, living in mud house, and in mixed house than other types of houses. The individuals were likely to be seropositive for VL in sleeping on cot and in floors.

With 9.7% (32/331) serpositivity, showed there is ongoing transmission of VL in these districts with the high level of seropositivity, 46.5% (20/43) in the Ishworpur village, Sarlahi district. In Belhichapena village, Saptari district, among 6 rK39 positives, 2 were symptomatic and 4 were asymptomatic cases of VL. Among 112 people tested, one in each two villages Rampur and Pipaldanda from Palpa district, were found symptomatic VL cases. Among 4/96 rK39 RDT test positives, 2 were relapse cases of VL, 1was symptomatic case of VL and another 1 was asymptomatic case of VL in Jahada-7 village of Morang. In Ishworpur village, Sarlahi district, different HHs, 6 seropositive cases were from the same HH, 8 seropositive cases were from and 6 seropositive cases were from

another same HH. In Belichapena village, Saptari district, all 6 seropositive cases were from different HHs. In Jahada -7 village, Morang district all the 4 seropositive VL cases were from different HHs. In Palpa, Saptari and Morang districts all family members of diagnosed VL cases were found seronegative for VL. The PCR tests done in human blood samples were found 35.7% (10/28) positives in Sarlahi, in 4% (2/50) in Saptari, in 1.7% (1/60) in Palpa and in 3.1% (3/96) in Morang districts of Nepal. In comparison to Sarlahi district, there were much fewer PCR test positives from Saptari, Palpa and Morang districts but each of these districts did have positive tests. Among 16 persons with PCR positive test, 10 (62.5%) had clinical symptoms of VL. Among 32 persons with rK39 positive test, 23(71.8%) had clinical symptoms of VL. In this study amplification of *L. donovani* infected sandflies were detected in 55.6% of pooled samples in villages in the Saptari and Morang districts; in 40 % the pooled samples from the HHs in the villages in the Palpa district and 60.0 % for the pooled samples from the HHs in Ishworpur village, Sarlahi district. Thus, we were able to detect kDNA of Leishmania within female *P. argentipes* sandflies collected both from HHs of known VL cases as well as from non-VL cases in Morang, Palpa, Sarlahi and Saptari districts. This indicates the potential for significant outbreaks in these districts arguing that surveillance must remain vigilant. We are not able to detect the *Leishmania* parasites in animal blood samples.

The seropositivity for VL was found high in 1-15 years of age group above 61 years there was no VL seropositive cases in villages of 4 disticts. In this study, males were found more seropositive than females. In case of occupation, students were found statistically significant than other occupation. The individuals were likely to be seropositive for VL living in mud houses and mixed houses. The individuals are likely to be seropositive for VL who slept on floor and on cot. Households without domestic animals are significantly higher VL seropositive than the households with domestic animals. This study showed, there is still ongoing transmission of VL in endemic districts- Saptari, Sarlahi and Morang though there is control program for many years.

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Annex I

Investigation form for active case detection of VL

Date: …………………….   House No. ………………………………

1. District:………………..   2. VDC/Municipality:……………………

3. Ward No. ………………   4. Village/ Tole / Street: …………………

5. Name of the head of the household: ………………Contact No. …………….

6. Occupation: i) Farming   ii) Government Employee

iii) Teaching   iii) Factory worker

iv) Business   v) Daily Labour   vi) Housewife

7. No. of Family Members:……….   House No. ………

8. Uses bed net: a)Yes ……   b) No ……

9. Keeps cattle: a)Yes ……   b) No……

10. Sleeping behavior: Only on cot ....   Only on Floor……. Outside house……

11. Use of insecticide for last 1 year: a) Yes…..   b) No……..

SN	Name	Age	sex	Fever for more than 2Weeks	Travel history for last 4months	Diagnosis & diagnosed in Health Institution Date of diagnosis

Annex II

Investigation form for animal study

Code: A

SN	District/ VDC/ Contact Number	No.of animals	No. of Cows	No.of buffaloes	No.of goats	Keeping animal in shed	Distance of animal shed from house (in Meter)	Keeping animal inside the house at night

……………………………………….

Name & signature of sample collector

Date:

Annex III

Investigation form for sandfly study

Code No.	District (Village)	House number	Sandfly collection method: CDC light trap /mouth aspiration	Female *P. argentipes* No.

……………………………………..

Name & signature of sample collector

Date:

Annex IV

मन्जुरी पत्र

अन्वेशक –शैला बस्नेत

शोध अध्ययनको शिर्षक – Transmission Dynamics of Visceral Leishmaniasis in Nepal

<u>प्रिय सहभागीहरु:</u>

तपाईलाई यो अनुसन्धानमा सहभागीगराइएको छ । सहभागी हुनु भन्दा पहिला तपाईहरुलाई यो महत्वपूर्ण कुरा थाहा हुनु पर्यो कीतपाई यो अनुसन्धानमा किन हुनुहुन्छ । क्रिपया समय दिएर सबै जानकारीहरु राम्ररी बुझ्नुहोस र केही कुरा स्पष्ट नहुदा वा अरु जानकारी चाहिमा मलाई सोध्नुहोस ।

यो सबैलाई थाहाभएको कुरा हो हाम्रो नेपालजस्तो विकासशील देशमा तराई लगायत केही पहाडीजिल्लामा समेत कालाज्वर रोगको प्रकोप देखा परेको छ। कालाज्वर क्रोनिक रोग भएकोले यस रोग समयमा नै पहिचानभई यसको उपचार हुनु जरुरी छ । यस अनुसन्धानले Active cases of VL पहिचानहुने हुदा समयमा नै उपचारको लागि सम्वन्धित अस्पतालमा सिफारीस गरिने हुदा यहाँहरुको सहभागीताकोलागि विनम्र अनुरोध गर्दछु। यस अनुसन्धनकोलागी चाहिने रगतको नमूना दक्ष प्राविधिकबाट संकलन गरिने जानकारी गराउछु। यसको साथै तपाई सहभागीहरुको नाम, ठेगाना तथा अन्य सूचनाहरु पनि गोप्य राखीने छ। तपाईको सहभागीता एउटा स्वयंम सेवकको रुपमा हुनेछ। यस अनुसन्धनामा सहभागीभई कुनै हद सम्म कालाज्वर रोगको उम्मूलन कार्यक्रममा सहयोग गर्नको लागी अध्ययनमा भागलिन चाहनुहुन्छ भने कृपया मन्जुरी पत्रमा हस्ताक्षर गरिदिनुहोला ।

शैला बस्नेत

फोन नं ९८४१३३१५६८

Annex V

मन्जुरीनामा

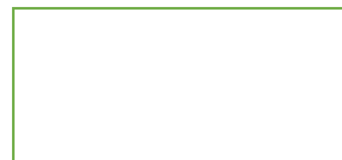
सोध अध्ययन शिर्षक Transmission Dynamics of Visceral Leishmaniasis in Nepal ।

सहभागीलाई अनुरोध छ कि माथी उल्लेखित अनुसन्धानमा सहभागी हुन अनुरोध गरिएको छ । यो मन्जुरी पत्रमा हस्ताक्षर गरेपछि, मैले सबै कुरा पढेको र बुझेको छु । मलाई थाह छ कि मेरो सहभागिता एउटा स्वयम सेवकको रुपमा हुनेछ र मलाई थाह छ कि म कुनै बेला पनि यो अनुसन्धानबाट बाहिरिन सक्छु । विना कुनै कारण देखाई कुनै खर्च गरी मलाई थाह छ कि मलाई मञ्जुरी पत्र दिइसकेको छु र म यो अनुसन्धानमा स्वयम् सेवकको रुपमा सहभागी हुन तयार छु ।

हस्ताक्षर........................ मिति ....

निरक्षकका लागी

मलाई स्पष्टसंग भनिएको छ कि म यो अध्ययनमा सहभागी छु । म यो स्पष्ट गर्न चाहन्छु कि मलाई कुनै जोर जबरजस्ती नगरी सहभागी गराएको छ ।



दाहिने औठाको छाप

अन्वेषकले यो फारम प्रष्ट भाषामा पढेर सुनाएको तथा सहभागिलाई नबुझेको कुरा सोध्ने पूर्ण अवसर दिएको सत्य हो । त्यसैले सहभागीले स्वेच्छाले यो मन्जुरीनामा दिएको प्रमाणित गर्दछु ।

साक्षीको नामः ....

साक्षीको हस्ताक्षरः .... मितिः ....

Annex VI

InBios Kalazar Detect Rapid Test (Seattle, WA)

Immunochromatographic Strip Test for Detection of Anti-K39 Immunoglobulin G Antibodies for Diagnosis of Visceral Leishmaniasis

Principle: The InBios Kalazar Detect rapid test utilizes the recombinant *L. chagasi* antigen rK39, which is a 39-amino-acid repeat section in the 230-kDa LcKin protein (1). It has previously been reported that *L. chagasi, L. donovani*, and *L. infantum* all contain the gene encoding the LcKin protein (1). A membrane strip which also contains a conjugate dye region is coated with this protein. Through capillary action, the patient serum will react with the dye and antigen to quickly indicate the presence of anti-rK39 immunoglobulin G (IgG) in a patient sample.

Test Procedure:

1. Remove the Kala-azar dipstick strip from the pouch.

2. Place a drop of patient serum on the assigned location on the immunochromatographic strip

3. Place the strip in a tube containing InBios chase buffer solution.

4. Following a 10-minute incubation at room temperature, the strips were analyzed. The presence of a visible band in the designated location indicated anti-rK39 IgG in the sample and therefore a VL-positive patient.

Interpretation:

A Positive Result: The test is positive when a control line and test line appear in the test area. A positive result indicates that the Kala-azar dipstick detected antibodies to *L.donovani*.

A Negative Result: The test is negative when only the control line appears. A negative result indicates that the Kala-azar dipstick did not detect antibodies to *L.donovani.*

Reference:

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Annex VII

Procedures for extraction Genomic DNA from animal blood

(EZ-10 Spin Column Blood Genomic DNA Minipreps Kit, Animal)

Before use, add 1ml of sterilized water to the tube containing 20mg of proteinase K. Keep solution at -20 ℃. Before use, add 48ml of 100% ethanol to 12ml Wash solution (volume of added ethanol: volume of wash solution= 4:1). Elution buffer is 2.0mMTris-HCL pH 8.0.

1. Place about 150 µl of EDTA blood into 1.5ml centrifuge tube.

2. Add 300µl of ACL solution and 20 µl of Proteinase K. Mix well.

3. Incubate at 55 ° C for 10min.

NB: The solution should appear clear after complete lysis. If solution still appears cloudy, extend incubation time until lysis is complete &solution is clear.

4. Vortex for 20 seconds and centrifuge at 12,000 rpm for 5mins.

5. Pipette 300µl of supernatant to a new Eppendorf tube, add 300 µl of AB solution .Mix by occasionally inverting tube and keep for 2mins.Then load all the solutions into an EZ-10 spin column.

6. Centrifuge at 4,000 rpm for 2 mins and discard the flow through.

7. Add 500 µl of wash solution and spin at 10,000 rpm for 2 mins.

8. Repeat step 7.

9. Discard flow through. Spin at 10,000 rpm for 1 min to remove residual amount of Wash solution.

10. Place the column into a clean 1.5ml Eppendrof tube. Add 30-50 µl Elution Buffer in to the center part of membrane in the column. Incubate at RT for 2 or 3 mins. Incubating the tube at37 ° C or 50 ° C for 2mins may increase recovery yield.

11. Spin at 10,000 rpm for 1min to elute DNA from the column.

12. For long term storage, keep aliquots of purified genomic DNA at -20 ℃

Annex VIII

Procedures for extraction Genomic DNA from human blood

(EZ-10Column Blood Genomic DNA Miniprep Kit)

Before use, add 34ml of 100% ethanol to 26 ml CW1solution concentrate and 42ml of 100% ethanol to 18ml of CW2 solution concentrate.

1. Harvest about 100µl of EDTA blood into 2ml centrifuge tube.Then add100µl PBS solution .Vortex gently and let the tube stand for 1 min at room temperature.
2. Add 20µl of proteinase K. Mix well. Add200µl of Buffer CL. Vortex gently. Incubate at56 ° C for 10min.

   NB: The solution should appear clear after complete lysis.If solution still appears cloudy, extend incubation time until lysis is complete & solution is clear.
3. Add 200µl of 100% ethanol to the mixture and mix thoroughly.

   NB: small cloudy insoluble material may be seen after addition of ethanol.
4. Transfer entire components and apply onto EZ-10 column that is in a 2ml collection tube. Let it stand at room temperature for 1-2 min.Spin at 10,000 rpm for 2 min.Discard the flow-through in the collection tube.
5. Add 500µl of CW1 solution and spin at 10,000 rpm for 1 min .
6. Add 500µlof CW2 solution and spin at 10,000 rpm for 1 min.
7. Discard the flowthrough.Spin at 10,000 rpm for 1 min to remove any residual amount of wash solution.
8. Place the column into a clean 1.5ml Eppendrof tube.Add 30-50µl CE Buffer in to the center part of membrane in the column.Incubate at RT for 2 or 3 mins.

NB: Incubating the tube at 37 ° C or 50 ° C for 2min may increase recovery yield.

9. Spin at 10,000 rpm for 1min to elute DNA from the column.
10. For long term storage, keep aliquots of purified genomic DNA at -20 ℃

Annex IX

Procedure for Genomic DNA extraction from sandflies

(QIAamp® DNA Micro Handbook, Edition December 2014)

Procedure

1. Transfer a tissue sample of less than 10 mg in weight to a 1.5 ml microcentrifuge tube.

2. Immediately add 180 µl Buffer ATL, and equilibrate to room temperature (15–25°C).

3. Add 20 µl proteinase K and mix by pulse-vortexing for 15 s.

4. Place the 1.5 ml tube in a thermomixer or heated orbital incubator, and incubate at 56°C overnight until the sample is completely lysed. For small amounts of tissue, lysis is complete in 4–6 h, but best results are achieved after overnight lysis.

5. Add 200 µl Buffer AL, close the lid, and mix by pulse-vortexing for 15 s. To ensure efficient lysis, it is essential that the sample and Buffer AL are thoroughly mixed to yield a homogenous solution. Note: If carrier RNA is required (see page 13), add 1 µg dissolved carrier RNA to 200 µl Buffer AL. Note that carrier RNA does not dissolve in Buffer AL. It must first be dissolved in Buffer AE and then added to Buffer AL.

6. Add 200 µl ethanol (96–100%), close the lid, and mix thoroughly by pulse-vortexing for 15 s. Incubate for 5 min at room temperature (15–25°C). Note: If room temperature exceeds 25°C, cool the ethanol on ice before adding to the tube.

7. Briefly centrifuge the 1.5 ml tube to remove drops from inside the lid.

8. Carefully transfer the entire lysate from step 7 to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim. Close the lid, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through. If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute column is empty.

9. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW1 without wetting the rim. Close the lid, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

10. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW2 without wetting the rim. Close the lid, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through. Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol, coming into contact with the QIAamp MinElute column. Take care when removing the QIAamp MinElute column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute column.

11. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min to dry the membrane completely. This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.

12. Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube (not provided) and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column and apply 20–100 µl Buffer AE or distilled water to the center of the membrane. If high pH or EDTA affects sensitive downstream applications, use water for elution.

13. Close the lid and incubate at room temperature (15–25°C) for 1 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min.Incubating the QIAamp MinElute column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield.

Government of Nepal
Nepal Health Research Council (NHRC)

Ref. No.: 1398

16 February 2017

Ms. Shaila Basnyat
Principal Investigator
Central Department of Microbiology
Tribhuvan University

Subject: Approval of research proposal entitled **Transmission dynamics of visceral leishmaniasis in Nepal**

Dear Ms. Basnyat,

It is my pleasure to inform you that the above-mentioned proposal submitted on **23 January 2017 (Reg.no. 16/2017** please use this Reg. No. during further correspondence) has been approved by NHRC Ethical Review Board on **15 February 2017**.

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol before the expiration date of this approval. Expiration date of this study is **December 2019.**

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission. The researchers will not be allowed to ship any raw/crude human biomaterial outside the country; only extracted and amplified samples can be taken to labs outside of Nepal for further study, as per the protocol submitted and approved by the NHRC. The remaining samples of the lab should be destroyed as per standard operating procedure, the process documented, and the NHRC informed.

Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their research proposal and submit progress report and full or summary report upon completion.

As per your research proposal, the research amount is **NRs. 326,000** and accordingly the processing fee amount to **NRs. 1000.** It is acknowledged that the above-mentioned processing fee has been received at NHRC.

If you have any queries, please feel free to contact the Ethical Review M & E section of NHRC.

Thanking you,

Dr. Khem Bahadur Karki
Member-Secretary

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List of published articles

1. Basnyat, S., Banjara, M. R., Ghimire, P., Matlashewski, G., & Singh, A. (2021).Seropositivity of Visceral leishmaniasis on people of VL endemic three districts of Nepal. Parasitology International Elsevier, 80:102236.

   https:// doi.org/10.1016/j.parint2020.102236

2. Basnyat, S., Banjara, M. R., Ghimire, P., Matlashewski, G., & Singh, A.(2022). Investigation of visceral leishmaniasis transmission in selected districts of Nepal. This article has been accepted to (Jan-March 2022 Issue) by JNHRC.

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List of presentation on national conference:

Shaila Basnyat, Megha Raj Banjara, Prakash Ghimire, Greg Matlashewski, Anjana Singh Seropositivity of Visceral leishmaniasis on people of VL endemic three districts of Nepal for poster presentation at the Fifth National Summit of Health and Population Scientists in Nepal, Kathmandu, Nepal 10-12 April, 2019.

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Seropositivity of Visceral leishmaniasis on people of VL endemic three districts of Nepal

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ABSTRACT

Visceral leishmaniasis (VL) is a life-threatening vector borne disease caused by the *Leishmania donovani* species complex. In Nepal, it is transmitted to humans by *L donovani* infected *Phlebotomus argentipes* sand flies [12]. The pathogenesis of VL is complex, and the clinical presentation ranges from asymptomatic infection to severe and fatal disease. Asymptomatic infection may act as potential reservoirs for sustained transmission of VL in endemic areas. We investigated the sero-prevalence of symptomatic and asymptomatic infection of VL in people of three endemic districts of Nepal by serology targeting family members and neighbors of VL patients. Sero-survey was conducted among 189 people of villages endemic to VL from Palpa, Sarlahi and Saptari districts during 2016 to 2018 using the rK39 rapid diagnostic test (InBios International, Seattle, WA) to detect anti-*Leishmania* antibodies. Sero-positivity was 35.7% (10/28) in people tested from Sarlahi districts, 6% (3/50) in Saptari district and 1.7% (1/59) from the Palpa district. In Sarlahi, sero-positivity was found to be highest among the age group below 15 years (44.5%). All family members of diagnosed VL cases in Saptari and Palpa districts were found to be rK39 test negative. In Sarlahi district, among the ten sero-positive cases, nine were febrile and became symptomatic VL cases after few days and one case remained asymptomatic during the six month follow up. Asymptomatic cases in VL endemic districts of Nepal were found to be sero-positive, screening of people in VL endemic districts would be important for prevention of VL transmission.

1. Introduction

Visceral leishmaniasis (VL) or kala-azar is the most severe form of leishmaniasis and if left untreated, is usually fatal. The World Health Organization estimated an incidence of 50,000 to 90,000 new cases of VL occur worldwide each year [15]. VL remains a significant public health problem in Nepal where the first cases were reported as early in 1960s in the 12 districts of central and eastern terai region. In 2016 six new districts were added to the endemic list, including hilly districts. VL has been expanded to new districts previously non endemic to VL. The highest number of kala-azar cases in Nepal were reported in 2003 and since then the cases are in decreasing trend [7]. In 2005, Nepal formulated a national plan to eliminate kala-azar defined as less than 1 case per 10,000 population at district level with a target of achieving elimination by 2015. Nepal reached this target in 2013, although sporadic cases are being consistently reported from other districts which are considered as non-endemic and this threatens to reverse the maintenance of the elimination target, particularly in sparsely populated districts [11].

In an endemic area, most of L. *donovani* infections remain asymptomatic [5]. Asymptomatic cases of VL are considered as potential reservoirs of VL and thus can play a major role in transmission of the disease in VL endemic areas. The antigen-antibody based recombinant 39-amino acid antigen (rK-39) rapid diagnostic test (RDT) can be applied to assess the proportion of asymptomatic infections in an endemic area as it can detect subclinical, recent or active infection [1,8]. The rK39 dipstick is a rapid test for VL with very high sensitivity and moderately high specificity [6,13].

This study aimed to investigate the sero-prevalence of symptomatic and asymptomatic infection of VL in people from villages in three endemic districts of Nepal by serology testing family members and neighbors of VL patients. We have identified a recent outbreak cluster in a Ishworpur, VDC (Village Development Committee) of the Sarlahi district where there is a high incidence of conversion from asymptomatic to symptomatic cases.

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Fig. 1. Location of study districts.

2. Materials and methods

2.1. Ethical issues

The study was approved by Nepal Health Research Council (NHRC Reg.No.16 /2017). Before taking blood samples, written informed consent was obtained from each study participant and assent from guardian for all child participants.

2.2. Study sites

Sero-survey was conducted among 189 people of villages reported of VL cases from Rampur and Pipaldanda VDC's of Palpa, Ishworpur, VDC of Sarlahi and Belhichapena, VDC of Saptari districts during 2016 to 2018. The villages were selected based on the record of past VL cases obtained from District Public Health office (DPHO) of the districts. Further, any person from whom blood samples were collected were asked of previous history of VL.

2.3. Data collection

A structured questionnaire was developed to collect data on demographic information, clinical symptoms and past VL treatment.

2.4. Blood sample collection

2 ml blood sample from each household member was collected by a vein puncture in a 3.2% sodium citrate vials by a trained laboratory technician. All samples collected were transported within 24 h in an ice box with proper labeling to Central Department of Microbiology laboratory for further processing.

2.5. Screening of VL cases by rK39 test

Serum samples were separated by centrifugation at 3000 rpm for 15 min. Then sera samples were tested for anti-*Leishmania* antibodies using the rK39 RDT kit (InBios International, Seattle, WA) [3,4].

2.6. Follow-up

All the identified asymptomatic cases were followed up serologically on 6th and 12th month of the study to know the rate of progression to clinical disease and sero-conversion. At the end of follow-up, the rK39 antigen test was performed to assess serologic status of persons who did not show any signs and symptoms of VL.

Table 1
Sero-positivity (%) of VL in three endemic districts of Nepal.

Districts(VDC)	Individuals with rK39 tests done	No. of positive tests	Sero-positivity (%)
Sarlahi (Ishworpur)	28	10	35.7
Saptari (Belhichapena)	50	3	6.0
Palpa (Rampur & Pipaldanda)	111	2	1.8

2.7. Data analysis

Data were entered into SPSS version 21 and analyzed. Descriptive analysis was performed.

3. Results

A sero-survey was conducted among 189 people from four villages from three VL endemic districts of Nepal. (Fig. 1) In the village of Ishworpur located in the Sarlahi district, there was a high level of VL sero-positivity (10/28). These 10 cases were from 11 households of Sarlahi districts. In Belichapena, VDC of Saptari district, the level of rK39 positive individuals was also relatively high (3/50). In two separate Rampur and Pipaldanda VDC's of the Palpa district, 2/111 tests were positive on the rK39 RDT (Table 1) (See Fig. 1).

It was interesting to identify one village Ishworpur, VDC in the Sarlahi where there was 10 out of 28 people tested that were positive when the rK39 tests were performed in the lab. Out of these 10, 9 developed VL within 15 days of the tests being performed, asymptomatic infections revealing a very high level of transmission in this village. All these VL positive cases were from Ishworpur, VDC and their occupation were daily labour, living in low socioeconomic conditions (Data not shown).

In Ishworpur VDC of Sarlahi district, 4 sero-positive cases were from different households, 3 sero-positive cases were from same household and 3 sero-positive cases were from another same household. Among 111 blood specimen tested, one each in two VDCs of Palpa district was found to be sero-positive. All family members of diagnosed VL cases in two villages of Palpa district were found to be rK39 tests negative

Table 2
Age related sero-prevalence of VL in three endemic districts of Nepal.

Age group (years)	Palpa			Saptari			Sarlahi		
	No. of rK39 tests	No. of positive tests (%)	Prevalence (%)	No. of rK39 tests	No. of positive tests (%)	Prevalence (%)	No. of rK39 tests	No. of positive tests (%)	Prevalence (%)
1–15	14	0	0.0	20	1	5.0	9	4	44.4
16–30	46	1	2.2	13	0	0.0	8	2	25.0
31–45	16	0	0.0	13	1	7.7	5	2	40.0
46–60	26	1	3.8	4	1	25.0	6	2	33.3
61–75	4	0	0.0	0	0	0.0	0	0	0.0
76–90	5	0	0.0	0	0	0.0	0	0	0.0

Table 3
Follow up results of rK39 positive cases within twelve months.

Districts	Village Development Committee (VDC)	Individuals with rK39 tests done	Total number of rK39 test positive	Number of symptomatic person in follow up	Number of asymptomatic person in follow up
Sarlahi	Ishworpur	28	10	9	1
Saptari	Belhichapena	50	3	1	2
Palpa	Rampur&Pipaldanda	111	2	2	0

(Table 2). In Belichapena, VDC of Saptari district, all 3 sero-positive cases were from three individual households.

Healthy individuals having no signs and symptoms of VL but positive for rK39 test were followed up for 12 months to observe disease progression. In the present study, we observed 1 out of 10 and 2 out of 3 rK39 positive from Sarlahi and Saptari districts respectively remained asymptomatic upto twelve months. But in Palpa district, no asymptomatic cases of VL detected during the study period. In Sarlahi district 9 asymptomatic individuals out of 10 rK39 positive developed the signs and symptoms of VL during follow up period while one in Saptari (Table 3).

4. Discussion

In VL endemic areas, the majority of the *L donovani* infected persons remains asymptomatic and fewer than 10% typically will convert to VL cases [5,10]. It is remarkable that there was such a high conversion rate in Ishworpur, VDC of Sarlahi. The asymptomatic cases can be identified by serological tests, although many asymptomatic cases have a low titer of antibodies and therefore test as sero-negative. It was again remarkable that there was a very high proportion of cases from 11 households of sarlahi districts that tested positive on the rK39 RDT. It is interesting that there was both a high level of sero-positivity and a high level of conversion to symptomatic cases in this cluster. Three months ago, there was one VL case each in nearby two other villages of that outbreak village. This is an example of a very aggressive outbreak occurring over one month. In this maintenance phase of VL elimination, surveillances of symptomatic VL as well as asymptomatic cases is particularly important to reduce transmission.

In this study, rK39 positivity of VL was found relatively high in Sarlahi district as compared to Saptari and Palpa district. We collected blood samples in Sarlahi while there was report of VL cases. After fifteen days of collection of blood samples, outbreak of VL occurred in the same place. In Ishworpur VDC of Sarlahi district, 4 seropositive cases were from different households, 3 sero-positive cases were from same household and 3 sero-positive case were from another same household. Therefore, there was an active transmission of VL infection ongoing during the sample collection time. In Sarlahi district, among ten VL seropositive cases, nine became symptomatic after few days but one case remained asymptomatic during follow up to six months. All family members of diagnosed VL cases in Saptari and Palpa districts were found to be rK39 tests negative. In Sarlahi district, the rK39 positivity of VL was found higher in 1-15 years which almost is supported by the findings of [2] who reported that the risk of Kala-azar was highest in the 3 to 14 years and 15 to 45 year of age groups in Bangladesh. In this study rK39 positivity of VL was found higher in males than in females in Sarlahi and Saptari districts, which is almost similar and in agreement to studies done by [9,14].

5. Conclusion

This study describes an active outbreak of VL in Nepal. This provides an opportunity for further investigation why this outbreak has occurred. This further indicates that surveillance must continue to identify such outbreaks to reduce transmission to other regions of Nepal.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interestsor personal relationships that could have appeared to influence the work reported in this paper.

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Investigation of Visceral LeishmaniasisTransmission in Selected Districts of Nepal

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ABSTRACT

Background: Visceral leishmaniasis is transmitted to humans by *Leishmania donovani* infected *Phlebotomus argentipes* sandflies. Nepal has successfully met the elimination target of less than 1 case per 10,000, although recently this threshold has been surpassed demonstrating ongoing transmission. The main objective of the present study was to investigate transmission of visceral leishmaniasis in 4 visceral leishmaniasis endemic districts of Nepal including Palpa, Morang, Saptari and Sarlahi.

Methods: Human blood samples (331), domestic animals blood samples [goats (n =67), dogs (n =1), cows (n = 6), buffaloes (n = 16), and ox (n = 10)] and sandflies samples (3976 from 142 households) were collected from the villages of these 4 districts. Human blood samples were tested for VL antibodies using the rK39 rapid diagnostic test (InBios International, Seattle, WA). kDNA of *L.donovani* was amplified by PCR from DNA extracted from human blood, animal blood and sandfly samples.

Results: Out of 331 screened across 4 districts,32 were positive on rK39 serology and 16 were positive by PCR amplification of kDNA from *L. donovani*. The majority of the positive serology and PCR tests were from the Ishworpur village in the Sarlahi district where there was an outbreak of 18 cases of VL. This study also revealed the presence of *L. donovani* DNA in female *P. argentipes* sandflies collected from the Ishworpur village of Sarlahi, 6 villages in the Saptari,10 villages in the Palpa, and from 9 villages in the Morang. Blood samples from domestic animals in the same villages were negative for kDNA detection by PCR.

Conclusions: The results of human and sandfly findings strongly point towards local transmission of visceral leishmaniasis in these 4 districts of Nepal. Notably, there is a significant level of transmission in the Ishworpur village in the Sarlahi district. The observations from this study suggest that domestic animals are not a reservoir host for *L. donovani* in these districts in Nepal. Ongoing surveillance is needed to identify new outbreaks such as in the Sarlahi district.

Keywords: Domestic animals; Nepal; *phlebotomus argentipes*; transmission; visceral leishmaniasis.

INTRODUCTION

In Nepal over 8.6 million people are at risk of visceral leishmaniasis with 23 districtsendemic.[1, 2] VL has both zoonotic and anthroponotic etiologies, but in Nepal it is considered to be anthroponotic.[3,4] VL is caused by the protozoan parasite *Leishmania donovani* and transmitted by the sand fly *Phlebotomus argentipes*.[5,6] VL has a transmission cycle that is based upon the dynamic interaction between the female sandfly and the human population. As humans are the only proven reservoir for *L. donovani*, it is necessary to understand the role of acute visceral leishmaniasis cases and asymptomatic infections play in VL transmission.

One of the important criteria towards the incrimination of *Leishmania* vectors as per WHO guidelines is the detection of *Leishmania* DNA within sandflies.[7,8] In this study, we therefore further investigated the presence of infected sandflies in villages where blood samples were analyzed.

The role of domestic animals as a risk factor of VL is still not clear. In the villages of Nepal, domestic animals are kept in close proximity to the houses. There is a common believe that sleeping in the same room with domestic animals can increase the risk for VL as it attracts more sandflies into the house.[9]We therefore investigated whether *L. donovani* DNA could be detected in the blood

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of domestic animals living in close proximity to infected people in these districts. Domestic animals blood samples collected from households of known VL patients and their neighbors were tested by PCR to detect kDNA of *L. donovani*.

METHODS

The study was approved by Nepal Health Research Council (NHRC Reg.No.16 /2017. Before taking human blood sample, consent was obtained from each study participant and assent from guardian for all child participants. Before taking domestic animal blood samples, consent was obtained from the owners of the domestic animals for the collection of blood samples by a veterinarian. Household consent was obtained to install CDC light traps in the house for the collection of sandflies.

Study sites were Jahada-7 village of Morang, Pipaldanda and Rampur villages of Palpa, Belhichapena village of Saptari and Ishworpur village of Sarlahi districts of Nepal, known to be endemic for VL. The villages were selected based on the record of past VL cases obtained from District Public Health office (DPHO) of the districts. The pVL (past VL treated) cases were traced out from District Public Health office's (DPHO) record and from EDCD, Teku, Kathmandu.

The study area consisted of ten villages of Palpa, nine villages of Morang, six villages of Saptari and one village of Sarlahi districts of Nepal. For sand fly collection, 6 households each were selected from each villages of Saptari and Palpa districts, 4 households each from nine villages of Morang and 10 households of one village of Sarlahi districts for two consecutive nights.

A structured questionnaire was developed to collect data on demographic information, clinical symptoms and past VL treatment (*i.e.* date of onset of VL, health seeking behavior, place of treatment, date of treatment, drug of choice, dosage and duration, clinical setting, hospitalization during treatment) were collected.

For animal study, data were collected using pre-tested questionnaires to evaluate the involvement of potential risk factors, such as a history of kala-azar in the owners (treated for the disease or not) and sharing the house with domestic animals at night.

Case definitions: VL case was defined by following national guideline as a person from an endemic area with fever for more than 2 weeks, splenomegaly and a positive rK39 test; past case of VL: history of treatment for VL, corroborated by prescriptions and/or case records from the health facility. Asymptomatic leishmanial infection is not well defined, but is usually ascertained by a positive serological test, PCR or Leishmanin Skin Test in individuals who are otherwise in a healthy condition

The study population selection was done targeting family members and neighbours of VL patients. Both symptomatic and asymptomatic cases of VL were included in this study.

2ml blood sample from each household member was collected by a vein puncture in a 3.2% sodium citrate vials by a trained laboratory technician. All samples collected were transported within 24 hours in an ice box with proper labeling to Central Department of Microbiology laboratory for further processing.

About 2ml blood samples were collected aseptically from the jugular vein of cow, buffaloes, Ox and goats in a 3.2% sodium citrate vials by a trained veterinary technician. All samples collected were transported within 24 hours in an ice box with proper labeling to Central Department of Microbiology laboratory for further processing.

Sandflies were collected from the villages of these endemic districts for two consecutive nights by using CDC miniature light traps method (Centers for Disease Control and Prevention light traps) and by mouth aspiration method. CDC light trap set-up and collection were carried out by trained insect collectors supervised by the entomologist. Two CDC light traps were used per night per household; one light trap was installed indoor and one in the door. The light traps were set before sunset and sandflies were collected early morning in the next day. The collected sandflies were then placed in a Petridish with chloroform-soaked cotton balls. Then sandflies were transferred to 1.5ml collection vials for identification. Further, sand flies were differentiated using sand fly identification keys, such as morphology of the maxillae and the hairs of the abdominal tergites and terminalia under a stereomicroscope, and identified as *P.argentipes*.[10] All sandflies were further differentiated as either male or female on the basis of morphology of the reproductive organs as assessed using the stereomicroscope. Only morphologically confirmed female *P. argentipes* by the entomologist were transferred into the vials, filled with 80% ethanol selected for the detection of *Leishmania* infection. All specimens were transferred to the laboratory of the Central Department of Microbiology and were stored at −20°C, for the further investigations.

Human serum samples and domestic animals serum

samples were separated by centrifugation at 3000 rpm for 15 minutes. Then sera samples were tested for anti-*Leishmania* antibodies using the rK39 RDT kit (InBios International, Seattle, WA).

All the identified asymptomatic cases were followed up serologically on 12thmonth of the study to know the rate of progression to clinical disease and sero-conversion. At the end of follow-up, the rK39 antigen test was performed to assess serologic status of persons who did not show any signs and symptoms of VL.

Genomic DNA extraction from human blood specimens were done by using Bio Basic, EZ-10Column Blood Genomic DNA Miniprep Kit, following the manufacturer's instructions. DNA was quantified using a Nano Drop (Thermo Scientific).

DNA was extracted from domestic animals blood samples by using Bio Basic, EZ-10 Column Blood Genomic DNA Miniprep Kit, Animal (Canada), following the manufacturer's instructions. DNA was quantified using a Nano Drop (Thermo Scientific). All the samples were aliquoted in duplicate to avoid DNA damage during repeated freeze thawing and stored at −80°C.

Pools of females of *P. argentipes* were homogenised using a disposable pestle. The number of female *P. argentipes* in each pool varied from 12 to 16. DNA from each pool of female *P. argentipes* was extracted using QIAmp the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), following the manufacturer's instructions. DNA was quantified using a NanoDrop (Thermo Scientific).

PCR amplifications were performed on human blood samples, domestic animals blood samples and from female sandflies by using Mini-circle primers: LIN4 and LIN19 which amplifies a 720bp fragments in *L. donovani* species- specific kDNA.[11]

The amplification reaction was carried out in a volume of 25 μL using the pair of primers LIN4 (forward) and LIN19 (reverse),the QIAGEN Multiplex PCR Master Mix and template DNA . An initial denaturation of 15 minutes at 95°C, followed by PCR amplification for 40 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 90 seconds and extension at72°C for 90 seconds with final extension at 72°C for 10 min in a thermocycler (Gene Amp PCR System 9700).The DNA of reference strain *L. donovani* 1S2D strain, was used as a positive control, and molecular-biology-grade water was used as the negative control. A 1KbDNA Ladder, RTU (50 μg /500 μL) marker was used. Twenty microliters of the amplification reaction products were resolved in a1.5% agarose gel stained with ethidium bromide and visualized under UV transillumination (Azure Biosystem).

Data were entered into SPSS version 21 and analyzed. Descriptive analysis was performed.

RESULTS

In this study, 32 out of 331 people from the four study districts were serologically positive by the rK39 rapid diagnostic test (RDT)and rK39 positivity of VL was found

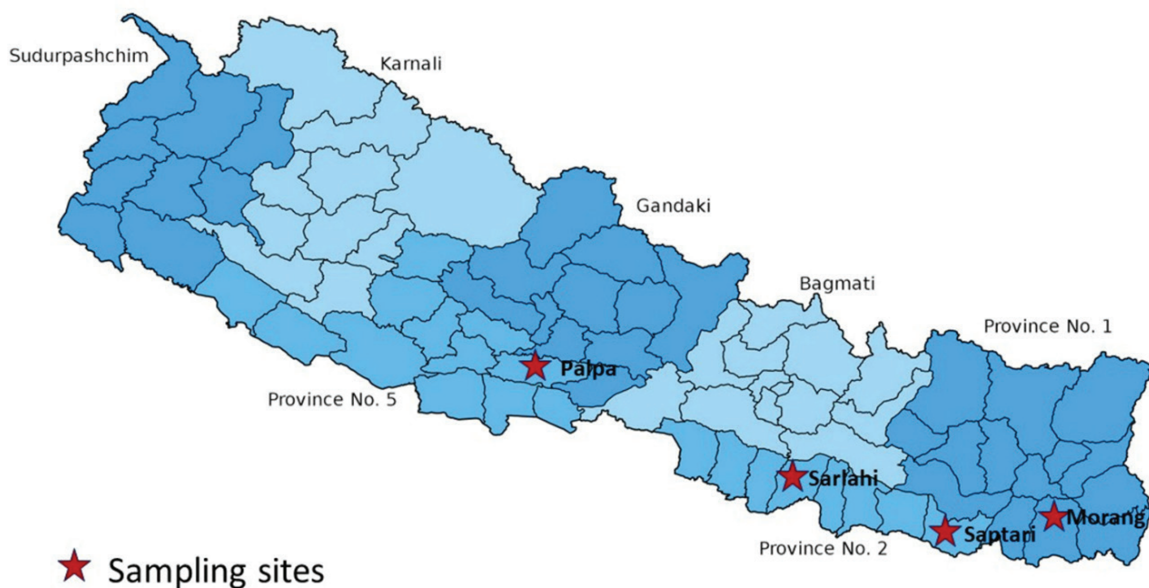

Figure 1: Location of sampling sites.

higher in males than in females and in 1-15 years of age group (Table 1).

Table 1. Gender and age-specific distribution of sero-prevalence of VL.

Age (years)	rK39test done	rK39 positives test No. (%)	rK39 negatives test No. (%)
1-15	74	10 (13.5)	64 (86.5)
16-30	117	8 (6.8)	109 (93.2)
31-45	67	8 (11.9)	59 (88.1)
46-60	58	6 (10.3)	52 (89.7)
61-75	8	0 (0.0)	8 (100.0)
76-90	7	0 (0.0)	7 (100.0)
Total	331	32 (9.7)	299 (90.3)

Table 1. Gender and age-specific distribution of sero-prevalence of VL.

Age (years)	rK39test done	rK39 positives test No. (%)	rK39 negatives test No. (%)
Gender			
Male	133	20(15.4)	115(86.5)
Female	198	12 (6.1)	187(94.4)

Among the 32 seropositive cases, 23 were symptomatic, 7 were asymptomatic and 2 were relapse cases of VL. All of the identified symptomatic cases were reported to District Hospitals of the district and sent for treatment. During the screening of Morang district, two previously treated people who had successfully completed a course of standard VL treatment 6 years previously demonstrate clinical signs of VL and were rK39 test positive (Table 2).

Table 2. Distribution of rK39 positive persons with or without symptoms and relapses in villages of four VL endemic districts of Nepal.

Districts (Village)	No.of Individuals tested	No. rK39 test Positive	No. Symptomatic cases	No. Asymptomatic cases	No. Relapse cases
Sarlahi (Ishworpur)	43	20	18	2	0
Saptari (Belichapena)	80	6	2	4	0
Palpa(Rampur &Pipaldanda)	112	2	2	0	0
Morang (Jahada-7)	96	4	1	1	2
Total	331	32	23	7	2

Table 3. rK39 and PCR positives among individuals tested in first and follow up collection.

District (Villages)	First collection			Follow up collection		
	Number of Individuals	No. of rK39 positive tests (%)	No. of PCR positives (%)	Number of Individuals	No. of rK39 positive tests (%)	No. of PCR positives (%)
Sarlahi (Ishworpur)	28	10 (35.7)	10 (35.7)	15	10 (66.7)	0 (0.0)
Saptari (Belhichapena)	50	3 (6.0)	2 (4.0)	30	3 (10.0)	0 (0.0)
Palpa (Rampur & Pipaldanda)	60	1 (1.7)	1 (1.7)	62	1 (1.6)	0 (0.0)
Morang (Jahada-7)	96	4 (4.2)	3 (3.1)	-	-	-

Note: Because of Covid-19 pandemic, we were not able to do follow up in Morang district.

Table 4. Infection of sandflies collected from households (HH) from different villages intheVL endemic districts of Nepal.

Districts	No. of HHs	No. of Villages	Total tested (Pool of 2HHs each)	No. of PCR positive tests	Infected sandflies in pools of VL HHs	Infected sandflies in pools of non-VL HHs
Saptari	36	6	18	10	2HHs in Belhichapena	3 HHs in Daulatpur
						2HHs in Malhaniya
						3HHs in Westpipra
Palpa	60	10	30	12	1HHs in Pipaldanda	2HHs in Argali
						4HHs in Pipaldanda
						1HHs in Phoksingkot
						3 HHs in Khanichap
						1 HHs in kachal
Morang	36	9	18	10	2HHs inJahada-7	3 HHs in Jahada-7

Table 5. Density of female *P.argentipes* in four VL endemic districts.

Districts	Sandfly collection method	No. of households	No. of female *P. argentipes*	Sandfly density*
Palpa	CDC light trap	60 HHs	364	0.50
Morang		36 HHs	294	0.21
Saptari		36 HHs	266	0.62
				Sandfly density**
Sarlahi	Mouth aspiration method	10 HHS	40	1

*No. of sandflies/HHs/night/CDC light trap

**No. of sandflies/man/HHS/night

In the molecular analysis of human blood samples done in first sample collection, kDNA of *L. donovani* was amplified in 35.7 % (10/28) human blood samples from the Ishworpur village of Sarlahi district, in 4% (2/50) of human blood samples from the Belichapena village of Saptari district, in 1.7 % (1/60) of blood samples from the Rampur and Pipaldanda villages of Palpa district and in 3.1% (3/96) of human blood samples from the Jahada-7 village of Morang district (Table 3). In Sarlahi 10 PCR positive cases,inSaptari 2 PCR positive cases and in Palpa 1 PCR positive case were also positive in rK39 tests(Table 3).

We further determined whether infected sandflies could be detected in the villages households (HH) under investigation (Table 5). The amplification of *L. donovani* kDNAwas performed on pools of sandflies collected from households as detailed in methods.*L.donovani* kDNA was detected in 10/18 HHs (55.6%) of pooled samples (2HH/pool) from the households in villages in the Saptari and Morang districts. *L. donovani* kDNA was detected in 12/30 HHs (40 %) for the pooled samples from the households in the villages in the Palpa district and 10/18 (55%) for the sandflies from the HHs from the Sarlahi district. (Table 4).

We further investigated whether *L. donovani* kDNA could be detected in the blood samples from any domestic animals living in close proximity to infected people in these districts.Blood samples were therefore collected from goats (n =67), dogs (n =1), cows (n = 6), buffaloes (n = 16), and ox (n = 10) and PCR performed to detect kDNA of *L. donovani*. All of the tests were negative.

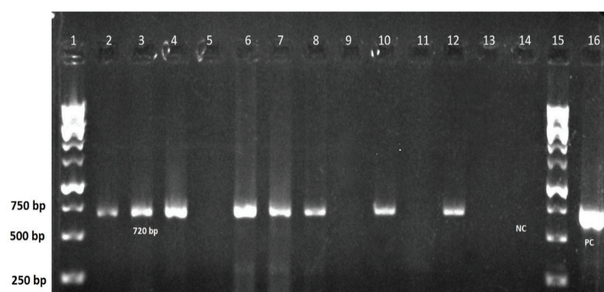

Figure 2. Representative PCR amplification of the 720bp kDNA of *L. donovani* from pooled female P *argentipes* Morang district.Lanes 1and 15,DNA size marker; lane14,negative control; lane16,positive control.

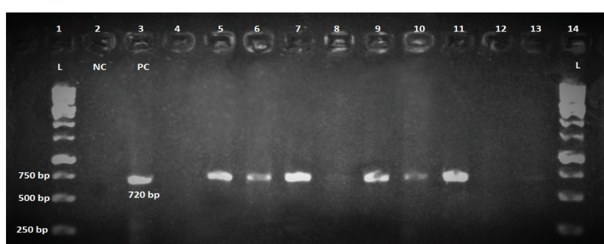

Figure 3. Representative PCR amplification of the 720bpkDNA of *L. donovani* from human blood samples derived from the Ishworpur village, Sarlahi district. Lane 1, negative control; lane2, positive control; lane15, DNA size marker.

DISCUSSION

Nepal has successfully met the elimination target of less than 1case per 10,000,although recently this threshold has been surpassed demonstrating ongoing transmission in hilly district. It is therefore necessary to continue to investigate where new outbreaks of VL occur. To investigate transmission of VL in endemic districts and hilly new foci, Morang, Saptari and Sarlahi districts were selected as endemic districts with control program for many years and Palpa as hilly district which is new foci and recently confirmed as endemic to VL.

The seropositivity of VL was found1.8%,4.2%,46.5% and 7.5% in villages of Palpa, Morang, Sarlahi and Saptari districts respectively. In this study seropositivity of VL in previously identified VL endemic districts was found higher than in new foci (Palpa). The Ishworpur village ofSarlahi district however demonstrated a more active area of transmission with 18 VL cases and 2 asymptomatic cases identified that were positive by the rK39 RDT and among 10 people with PCR positive tests, 9 had clinical symptoms of VL and 1was asymptomatic. Among these seropositive cases of Sarlahi district, 4 sero-positive cases were from different households, 3 sero-positive cases were from same household and 3 sero-positive cases were from another same household. Family members and adjacent neighbors may have an increased chance of infection as they share similar socio-cultural, economic and environmental conditions as the index VL cases. In a study conducted in Bangladesh shows that living within a 50 meter radius of patients increases the risk of developing VL by threefold.[12] Therefore, screening of asymptomatic family members and household contacts of patients with VL by serology and monitoring seropositive individuals for early case detection and management should be considered as part of the strategy for VL elimination. In Belichapena, village of Saptari district, 3out of 50 people from different households were found rK39 seropositive in the initial sample collection time. In two separate Rampur and Pipaldanda villages of the Palpa district, 1 out of 60 people tested were found positive on the rK39 RDT. In Jahada-7 village of Morang district 4 out of 96 were found rK39 seropositive, among which 1 case was symptomatic,2 cases were relapse and another 1 was asymptomatic. During the screening in Morang district, two previously treated cases who had successfully completed a course of standard VL treatment 6 years previously demonstrate clinical signs of VL and were rK39 positive. All family members of diagnosed VL cases in two villages of Palpa, Belichapena village of Saptari and Jahada-7 village of Morang were found to be rK39 tests negative. Healthy individuals having no signs and symptoms of VL but positive for rK39 test were followed up for 6 to 12 months to observe disease progression. In the follow up 10 out of 15 people from Sarlahi, 3 out of 30 people from Saptari and 1 out of 62 people from Palpa districts were found rK39 seropositives. In this study, we observed 1 out of 10 rK39 positive from Sarlahi district remained asymptomatic upto six month and 2 out of 3 rK39 positive from Saptari districts remained asymptomatic upto twelve months. But in Palpa district, no asymptomatic cases were found during the study period.

With 9.7% positive (32/331), the serological analysis revealed the presence of ongoing transmission in these districts with the highest level in the Ishworpur village in the Sarlahi district. In a cross-sectional study, done by Cloots and colleagues provided evidence that the seroprevalence of VL in Nepal had reduced significantly from 8·4% in 2006 to 4·7% in 2016 ($p<0·0001$) indicating a very low transmission of visceral leishmaniasis in Nepal.[1]

Sporadic cases of VL has been reported from the hilly districts of Nepal since 2000 and are considered the result of *L. donovani* infection during travel. But in our study we found 2 symptomatic cases of VL, one eachin Pipaldanda and Rampur village of Palpa, without

travel history Moreover, we managed to capture female *P. argentipes* sandflies from different households in Palpa districts and found DNA of leishmania parasites inside. This proves that there is indeed ongoing local transmission in Palpa district and that surveillance and control activities should be extended. Entomological and serological findings meanwhile strongly point towards local transmission. We conclude there is local transmission of VL ongoing in the hilly district, based on the arguments that symptomatic cases of VL was detected in permanent residents of Pipaldanda and Rampur villages of Palpa and found DNA of leishmania parasites inside *P. argentipes* sandflies collected in these villages of Palpa.

In this study, *L. donovani* KDNA was amplified in 35.7 % (10/28) human blood samples collected in the first collection, from the Ishworpur village of Sarlahi district. This demonstrates a potentially large reservoir of infected individuals that can potentially transmit parasites sandflies. It will be important to maintain strong surveillance in this village and surrounding villages in the Sarlahi district. In contrast.,4%, 1.7% and 3.1% were positive from the investigated villages in the Saptari, Palpa and Morang districts suggesting ongoing but lower transmission in these districts. The presence of *L donovani* DNA in the human blood samples collected in these areas indicates that the *P. argentipes* vectors in these districts may have also been infected.

In our study, we found 4.8% (16 /331) PCR positive for *L donovani* infection in human blood samples collected from four VL endemic districts which is almost similar with the findings of Bhattarai et al who found 6.1%(17/278) PCR positive.[13] In a study done by Shrestha et al found 6 out of 14 VL suspected cases positive by rK39 test and only 2(33.3%) rK39 positive cases positive by PCR.[14] But in our study, we found 16 rK39 positive cases positive by PCR.

In this study, *L. donovani* kDNA was detected in 10/18 households (55.6%) of pooled samples (2HH/pool) from the households in villages in the Saptari and Morang districts. *L. donovani* kDNA was detected in 12/30 households (40 %) for the pooled samples from the households in the villages in Palpa district and 10/18 (55%) for the sandflies from the HHs from the Sarlahi district. Thus, we were able to detect *L. donovani* infected sandflies in the households of different villages of four VL endemic districts. In this study, sandfly density was more in Palpa district than in Morang. This may be due to lack of spraying activities in Palpa.

The amplification of kDNA of *L. donovani* was seen in sandflies collected from different households of Argali, Pipaldanda, Phoksingkot, Khanichap and Kachal villages of Palpa district. In Saptari district, amplification of kDNA was seen in sandflies collected from different households of Daulatpur, Malhaniya, Tikuliya and Westpipra villages. The observation of *L.donovani* DNA among collected sandflies from these areas may indicate a higher circulation rate of the parasite among vectors in these areas.

We also investigated whether *L. donovani* DNA could be detected in the blood of domestic animals living in close proximity to infected people in these districts. Domestic animals blood samples collected from goats (n =67), dogs (n =1), cows (n = 6), buffaloes (n = 16), and ox (n = 10) from households of known VL patients and their neighbors were tested by PCR were found negative. The absence of Leishmania **DNA is suggestive of no role of domestic animals as reservoirs of VL** in these endemic focus. In PCR analysis of blood samples from these animals, we did not detect the presence of *L. donovani* kDNA, yet there was positive human samples in the same villages. This continue to argue that the major reservoir in these villages is the human population, although future studies could include non-domestic animals. Thus, human are still considered the only reservoir host for *L. donovani in these VL endemic district*.

CONCLUSIONS

The presence of VL cases, *L donovani* DNA and anti-k39 antibodies in human blood in these districts demonstrates that there is ongoing transmission with the highest transmission detected in Ishworpur village in the Sarlahi district. Highest transmission of VL in Sarlahi demonstrates a potentially large reservoir of infected individuals that can potentially transmit parasites sandflies. It will be important to maintain strong surveillance in this village and surrounding villages in the Sarlahi district. The sandfly species *P. argentipes* is the most common species in these VL endemic areas, and was also found to harbor *Leishmania* parasites. Therefore, it can be presumed that this species plays an important role in the transmission of leishmaniasis in these endemic areas. There was no evidence that domestic animals represent a reservoir for *L. donovani* in these villages. This study shows that healthy sero-positive asymptomatic individuals can be carriers of *L. donovani* based on PCR analysis should be monitored for early detection of symptoms and managed under the VL elimination program. Due to the ongoing transmission, the maintenance of the elimination target of 1case in 10,000 will be difficult in Nepal unless a strong surveillance program is in place in the endemic districts.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

None

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List of presentation on national conference:

Shaila Basnyat, Megha Raj Banjara, Prakash Ghimire, Greg Matlashewski, Anjana Singh Seropositivity of Visceral leishmaniasis on people of VL endemic three districts of Nepal for poster presentation at the Fifth National Summit of Health and Population Scientists in Nepal, Kathmandu, Nepal 10-12 April, 2019.

Certificate of Poster Presentation

This is to certify that

Ms. Shaila Basnyat

has made a poster presentation in the Fifth national summit of health and population scientists in Nepal

10-12 April 2019, Kathmandu, Nepal.

Anjanijha

Prof. Dr. Anjani Kumar Jha

Executive Chairperson

Organized by

Nepal Health Research Council (NHRC)

Ramshah Path, Kathmandu, Nepal

2.3.2 Seropositivity of Visceral Leishmaniasis on People of VL endemic three districts of Nepal

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Background

Visceral leishmaniasis is a life-threatening vector borne disease caused by the *Leishmania donov* species complex. In Nepal, it is transmitted to humans by *Leishmania donovani*-infected *Phleboto argentipes* sand flies. The pathogenesis of VL is complex, and the clinical presentation ranges fi asymptomatic infection to severe and fatal disease. Asymptomatic infection may act as poter reservoirs for sustained transmission of VL in endemic areas. We investigated the sero-prevale of symptomatic and asymptomatic infection of VL in people of three endemic districts of Nepa serology targeting family members and neighbours of VL patients.

Methodology

Sero-survey was conducted among 137 people of villages endemic to VL from Palpa, Sarlahi Saptari districts during 2016 to 2018. Sera samples were tested for anti-*Leishmania* antibodie rK39test kits (InBios International, Seattle, WA). The study was approved by NHRC Ethical Rev Board. Before the start of screening activities, written informed consent was obtained from study participant and from guardian for all child participants.

Results

35.7% (10/28) VL seropositivity were seen in people of Sarlahi district, 6% (3/50) seroposit were seen in people of Saptari district and 1.7% (1/59) VL seropositivity were seen in people of P district. In Sarlahi district, seropositivity of VL was found to be highest among the age group belo years (44.4%). All family members of diagnosed VL cases in Saptari and Palpa districts were four be VL seronegative. One asymptomatic person of Sarlahi district had no history of VL was foun seropositive.

Conclusion

Since asymptomatic case of VL in people of VL endemic districts of Nepal was found to be VL positive, all febrile cases in VL endemic districts should be screened for VL for prevention transmission.

Keywords

VL,febrile cases, asymptomatic

Certificate of Participation

This is to certify that

Ms. Shaila Basnyat

participated in the fifth national summit of health and population scientists in Nepal
10-12 April 2019, Kathmandu, Nepal.

Anjanijha

Prof. Dr. Anjani Kumar Jha
Executive Chairperson

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