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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

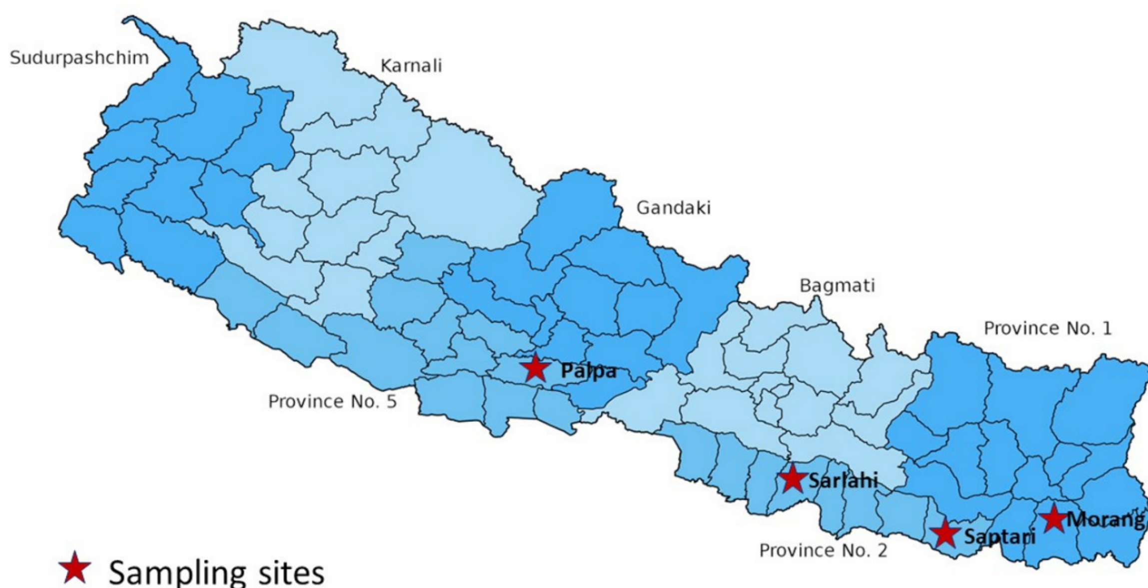

Figure 1: Location of sampling sites.

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.

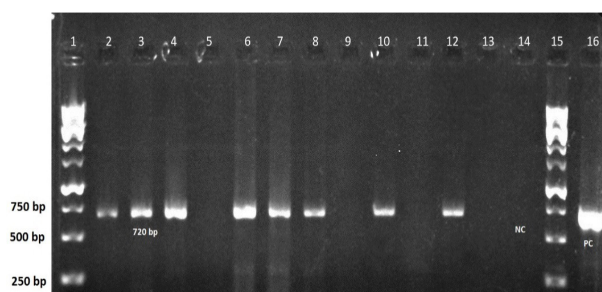

Figure 2. Representative PCR amplification of the 720bp kDNA of *L. donovani* from pooled female P *argentipes* Morang district.Lanes 1and 15,DNA size marker; lane14,negative control; lane16,positive control.

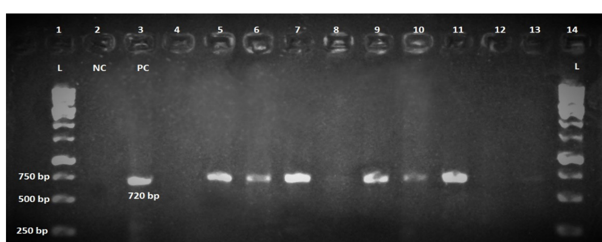

Figure 3. Representative PCR amplification of the 720bpkDNA of *L. donovani* from human blood samples derived from the Ishworpur village, Sarlahi district. Lane 1, negative control; lane2, positive control; lane15, DNA size marker.

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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