J. Bangladesh Agril. Univ. 10(1): 79–86, 2012 **ISSN 1810-3030**

Seroepidemiological study of visceral leishmaniasis and cattle as a possible reservoir host at Trishal Upazila in Bangladesh

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Abstract

To study the effect of months, seasons, age and sex on visceral leishmaniasis (VL) in human, data were collected during March 2010 through February 2011 from the register book of Upazila Health Complex (UHC) of Trishal, which is an endemic region for VL in the Mymensingh district. Besides, 70 blood samples were collected from suspected VL human patients to compare microscopy with rK39 immunochromatographic strip test. Additionally, 50 cattle blood samples were also collected from houses with active or recently-treated VL patients to determine the possible animal reservoir of VL through rK39 strip test. Of the suspected VL patients in the UHC registered book, 43.8% were seropositive. The percentages of seropositive cases were higher in September (73.3%), November (67.2%) and August (65.5%) than in May (18.5%), June (27.5%) and March (36.7%). The rainy season (58.4%) and the winter season (50.6%) showed higher seropositive than the summer (30.7%) season which was significant ($p \leq 0.001$). Hospital cases of VL were significantly ($p \leq 0.05$) higher in 3-14 years (49.3%) and 15-45 years (43.5%) than in children <3 years (32.1%) and adults aged >45 years (33.6%) people. The estimated sensitivity of rK39 strip test and microscopic examination were 100% and 58.8%, respectively and the specificity were 72% and 100%, respectively. Only two cattle blood samples were found positive to rK39 strip test. Our present study indicates that rK39 strip test is superior to the diagnosis of VL compared with parasitological confirmation by direct microscopy of the peripheral blood. Further works are needed to focus on molecular approaches for diagnosis and epidemiological study of VL, sand flies investigation and to confirm the cattle as animal reservoir for VL transmission.

Keywords: Visceral leishmaniasis, Epidemiology, rK39 strip test, Reservoir host, Trishal

Introduction

Visceral leishmaniasis is the most severe form of leishmaniasis and if left untreated, is usually fatal (Reithinger and Dujardin, 2007). *Leishmania donovani* complex *(Leishmania donovani and Leishmania infantum)* is the etiologic agent of VL or kala-azar (KA) which is characterized by prolonged fever, weight loss, hepatosplenomegaly, and pancytopenia (Jeronimo *et al*., 2006). The World Health Organization estimated an incidence of 500,000 VL cases per year, 90% of which occurs in developing countries like India, Nepal, Sudan, Bangladesh, and Brazil (Desjeux, 2004). Visceral leishmaniasis is an endemic disease of great public health importance in some rural communities in Bangladesh. Bangladesh experiences 40,000 to 45,000 cases yearly, with the total population at risk being around 20 million (Bern and Chowdhury, 2006). Mymensingh district accounts for more than 50% of the cases in Bangladesh (Bern and Chowdhury, 2006).

The laboratory diagnosis of VL is based on microscopic examination, culture, serological tests, and molecular methods. Field diagnosis of VL involves an immunochromatographic strip test (ICT) that detects immunoglobulin G (IgG) antibody to recombinant K39 (rk39), an antigen expressed by leishmanial species that produce VL. rK39 immunochromatographic strip test used with blood or serum proved sensitive (range, 99 to 100%) and specific (range, 95 to 100%) for the noninvasive serodiagnosis of VL in India **(**Sundar *et al*., 2005**)**. The rK39 dipstick is a rapid test for VL with very high sensitivity and moderately high specificity (Chappuis *et al*., 2003).

In South Asia, the disease is transmitted by the female sand fly *Phlebotomus argentipes* and humans are considered as the only reservoir of VL. Sand fly blood meal analysis in India confirms that *Phlebotomus argentipes* feed predominantly on bovines and depend on humans' blood as their second choice (Mukhopadhyay and Chakravarty (1987). As cattle are the preferred host for *P. argentipes*, the sand fly vector of VL in the Indian subcontinent, cattle may act as reservoir of VL. Although there are reports of VL in Bangladesh (Bern *et al.*, 2007) but the epidemiology of VL and the possible role of cattle for VL transmission has not been studied in Bangladesh so far.

The microscopic examination is still considered as gold standard for the diagnosis of VL especially in the areas of endemicity (Reithinger and Dujardin, 2007). The commonly used method for diagnosing VL has been the demonstration of parasites in splenic or bone marrow aspirate. Although the sensitivity of the bone marrow smear and splenic aspirate are about 60 to 85% and 95%, respectively, but collection of bone marrow is very much painful for the patients and splenic aspirate collection may be associated with fatal hemorrhage. These techniques require invasive procedures, which are difficult to use in large epidemiological surveys and difficult to repeat for follow-up of patients with the disease (Sundar and Rai, 2002). However, microscopy of peripheral blood is less invasive and it requires no special equipment and it is very easy to collect one drop of blood for preparation of microscopic slides.

Therefore, the present study was aimed with the objectives to perform a retrospective study with the epidemiological data of VL in suspected patients, to compare microscopy with rK39 strip test for the diagnosis of VL in human, and to investigate cattle as animal reservoir of VL in Trishal Upazila of Mymensingh district which is the most endemic area of VL in Bangladesh.

Materials and Methods

Study area

The study was done at the Trishal Upazila of the Mymensingh district. The data about the VL patients were collected from the register book of Upazila Health Complex (UHC) of Trishal. Human blood was collected from pathology laboratory of UHC. Cattle blood was collected from some active or recently-treated VL patients' house of Rampur union of Trishal upazila which were identified from the records of UHC Trishal. The parasitological examination was conducted in the laboratory of Parasitology, Bangladesh Agricultural University, Mymensingh.

Collection of data from UHC, Trishal

From March 2010 to February 2011, 826 suspected VL patients with one or more symptoms like fever for >2weeks, anemia, hepatomegaly, splenomegaly and who were referred by physicians to the pathology laboratory of UHC for VL diagnosis by rK39 strip test were recorded with their sex, age, etc. The whole period of data collection was divided into three seasons such as summer (March/2010-June/2010), rainy (July/2010-october/2010) and winter (November/2010-February/2011).

Collection of blood from human and performing rK39 strip test

Two to three drops of finger prick blood was taken from 70 suspected VL patients. One to two drops of blood followed by one or two drops of buffer solution provided with the test kit (Chase buffer solution) were placed on the absorbent pad at the bottom of the strip (In Bios kala-azar detect^{TM} rapid test, USA) and then observed for 8-10 minutes. In the positive cases, there were two bands, a control band and a positive test band appeared within 5 minutes. In few cases, positive band also appeared after 5 minutes (Fig. 1). Only the control band appeared in negative cases (Bern *et al.*, 2000).

Preparation of microscopic slides and examination of slides for the presence of amastigotes

Immediately after collection of blood from human, two thin smears were prepared for each sample. To prepare a thin smear a small drop of blood was placed on a clean, dry, grease free glass slide and applied even pressure with the help of another even edged glass slide. Then the smears were air dried and fixed with absolute methyl alcohol for 5 minutes and air dried. Afterwards, the smears were stained with Giemsa's stain and air dried (Cable, 1957). The slides were then examined under microscope at high magnification (100 ×).

Collection of blood from cattle and performing rK39 strip test

Blood samples were collected from 50 cattle by puncturing the jugular vein with the help of syringe and needle. About 0.5-1ml blood was taken and one drop of blood followed by one or two drops of buffer were placed on the absorbent pad at the bottom of the strip and waited for 10 minutes for observing the band.

Statistical analysis

Statistical analyses were carried out by using Statistical Package for Social Science (SPSS) software version, 13.0 (Coakes *et al.*, 2006). Z test for proportions was done to find out the significant differences in the seroprevalence of VL in human in terms of seasons and age. Chi-square test was also performed to test whether there existed any significant effect of sex on the seroprevalence of VL in human. A p value of ≤ 0.01 or ≤0.05 was considered as significant.

Results and Discussion

Seroprevalence of VL in human

The percentage of seropositive cases of VL in suspected patients from March 2010 to February 2011 were found 43.8% (Fig. 3) which supported the findings of El-Masum *et al.* (1995) who reported that seropositivity rate of VL ranged from 43.6% to 79.6% in different subdistricts of Bangladesh. Begum *et al.* (2002) also reported that 44.9% cases were seropositive among 9111 VL suspects from 39 endemic areas of Bangladesh. Slightly higher percentage of seropositive cases were observed by Zijlotra e*t al.* (1991) who reported 51% VL among 132 suspected cases in Southern Sudan. The possible reasons for the variations in this study from the previous findings might be due to differences in the geographical locations of study areas.

Monthly and seasonal seroprevalence of VL

In the present study, the higher rate of infection was observed in September (73.3%) followed by November (67.2%) and August (65.5%) and lowest infection was recorded in May (18.5%) followed by June (27.5%) and March (36.7%) (Fig. 4). Somewhat similar observations were found in the previous study by Bern *et al.* (2005) who reported that more cases of VL occurred from July-September and fewer cases from January-March in Fulbaria Thana of Mymensingh district in Bangladesh.

The proportion of VL was significantly higher in the rainy (58.4%) and winter (50.6%) season than the summer (30.7%) (Table 1) in the present study. Rukunuzzaman and Rahman (2008) found that the rainy season was significantly associated with VL (60%) and carried 2.4 times greater risk of developing VL than other seasons ($p<0.05$, OR=2.4). Ashkan and Rahim (2008) reported the seasonal variations with more cases presenting in late winter, spring and fewer in summer in southwest Iran. The highest percentage in rainy season may be due to high humidity, heavy rainfall and mostly due to the abundance of vectors because annual rainfall appeared to be the most important predictive variable affecting both the probability of presence and incidence of the disease and rainfall may affect the vector and reservoir hosts by affecting the vegetation, the temperature, and the relative humidity. The lower prevalence rate during summer may be due to the strong protective effect of bed net use in March-June.

Age related seroprevalence of VL

The present study demonstrated a significant association between VL and different age groups. Percentages of VL were higher in 3-14 years (49.3%) and 15-45 years (43.5%) than in children <3 years (32.1%) and adults aged >45 years (33.6%) (Table 2) which almost is supported by the findings of Bern *et al.* (2005) who reported that the risk of kala-azar was highest for people in the 3- to 14-year and 15- to 45-year age groups in Bangladesh. In a retrospective analysis of 965 patients worldwide, researchers found that 85.7 % VL cases were diagnosed in young adulthood (age 20-40 years) (WHO, 2000). Davies and Gavgani (1999) observed a declining seroprevalence with advancing age and suggested that cell-mediated immunity was associated with a reduction in the seroconversion rate and an increase in the serorecovery rate.

Table 1. Seasonal seroprevalence of VL

Season	No. of rK39 strip tests	No. of positive tests	Prevalence (%)
Summer (March/2010-June/2010)	368	113	30.7[a]
Rainy (July/2010-October/2010)	219	128	58.4[b]
Winter (November/2010-February/2011)	239	121	50.6[b]

Values with different letters within a column differ significantly at p≤0.001

Table 2. Age related seroprevalence of VL

Age groups	No. of rK39 strip tests	No. of positive tests	Prevalence (%)
< 3	28	9	32.1[a]
3-14	300	148	49.3[b]
15-45	379	165	43.5[ab]
> 45	119	40	33.6[a]

Values with different letters within a column differ significantly at p≤0.05

Sex related seroprevalence of VL

No significant difference was seen between the sexes in the present retrospective study. The percentage of VL was 44.6% in the female and 43.2% in the male (Table 3), which is almost similar and in agreement to the previous report of Rijal *et al.* (2006) in Nepal. In many studies it has been found that the disease is more common among male than the female (Masum *et al.,* 1990; Talukder *et al.*, 2003). In one explanation, women are sicker longer and die more often from the disease which may be due to women ignored the illness, deemed themselves powerless, women's lower status within the household. Moreover, the economic necessity gives priority of treatment seeking where children and income earners came before women.

Table 3. Sex related seroprevalence of VL

Sex	No. of rK39 strip tests	No. of positive tests	Prevalence (%)	Chi-square value	Level of significance
Male	449	194	43.2	0.153	NS
Female	377	168	44.6		

NS=Non significant

Comparison between rK39 strip test and microscopic examination for the diagnosis of VL

According to rK39 strip test, 34 (48.6%) of 70 blood samples were positive and the estimated sensitivity and specificity of the rK39 strip test compared to microscopic test were 100% and 72%, respectively (Table 4). Under microscope, *Leishmania* amastigotes appeared as round or oval bodies measuring 2 to 3µm in length and were found intracellularly in monocytes and macrophages as described by Sundar and Rai (2002) (Fig. 2). The result of microscopic examination was positive in 20 (28.6%) and the sensitivity and specificity were 58.8% and 100%, respectively compared to rK39 strip test (Table 4). Similar study was conducted by Ozerdem *et al.* (2009) who reported a sensitivity of 41.3% for rK39 dipstick test according to PCR while it was 90.9% to microscopic examination and specificity was 100% and 94.8%, respectively, the sensitivity of microscopic examination according to rK39 dipstick test and PCR was 76% and 34.5%, and specificity was 97.2% and 100%, respectively. Some researchers found that sensitivity

and specificity of rK39 dipstick test ranged from 92% to 100% (Veeken *et al.*, 2003) and from 59% to 97.9% (Veeken *et al.* ,2003; Fissore *et al.*, 2004), respectively. These observations suggest a regional limitation of the use of the rK39 test. There is limited research relating to the microscopic examination of peripheral blood for the diagnosis of VL. The sensitivity of microscopic examination of blood is low because of low parasite burden in the peripheral blood (Delgado *et al*., 1998).

Table 4. Comparative results between rK39 strip test and microscopic examination

Diagnostic assay	No. of positive samples	No. of negative samples	% of positive	Sensitivity	Specificity
rK39 strip test n=70	34	36	48.6	100	72
Microscopic examination n=70	20	50	28.6	58.8	100

Examination of cattle blood with rK39 immunochromatographic strip test

In the current study, 50 cattle blood samples were also examined with rK39 strip test and two samples (4.0%) gave positive band. Bhattarai *et al.* (2010) found *Leishmania* infections among persons (6.1%), cows (5%), buffaloes (4%), and goats (16%) by using PCR. There is no available data about the investigation of domestic cattle to identify them as reservoir host of VL in Bangladesh. As the rK39 strip test is not a confirmatory test to identify active case, more sensitive and reliable molecular method such as PCR is to be suggested to identify the parasitic DNA.

In conclusions, the present study supports the view that rK39 immunochromatographic strip test is superior to the diagnosis of VL compared to parasitological confirmation by direct microscopy of peripheral blood. Furthermore, our present study addresses several key epidemiological questions for VL in Trishal, Mymensingh and thus, creating a basis for further epidemiological investigation. Research work focused on molecular approaches (PCR, DNA sequencing) for diagnosis and epidemiological study of VL, the vector fly and to confirm the cattle as animal reservoir for VL transmission is warranted.

Fig. 1. rK39 strip test results (left one showing double band that reflects positive result and right one shows only control band means negative result)

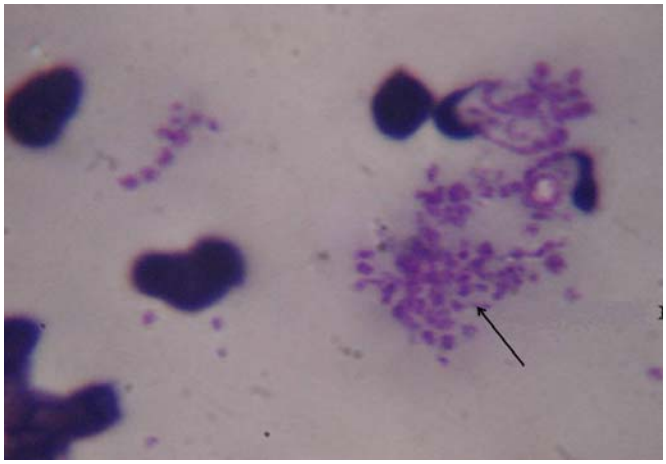

Fig. 2. Amastigote cluster in Giemsa's stained blood smear

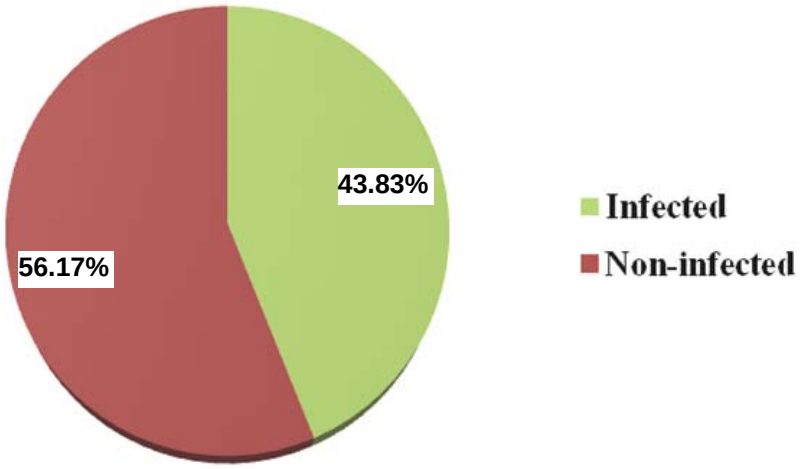

Fig. 3. Overall seroprevalence of VL

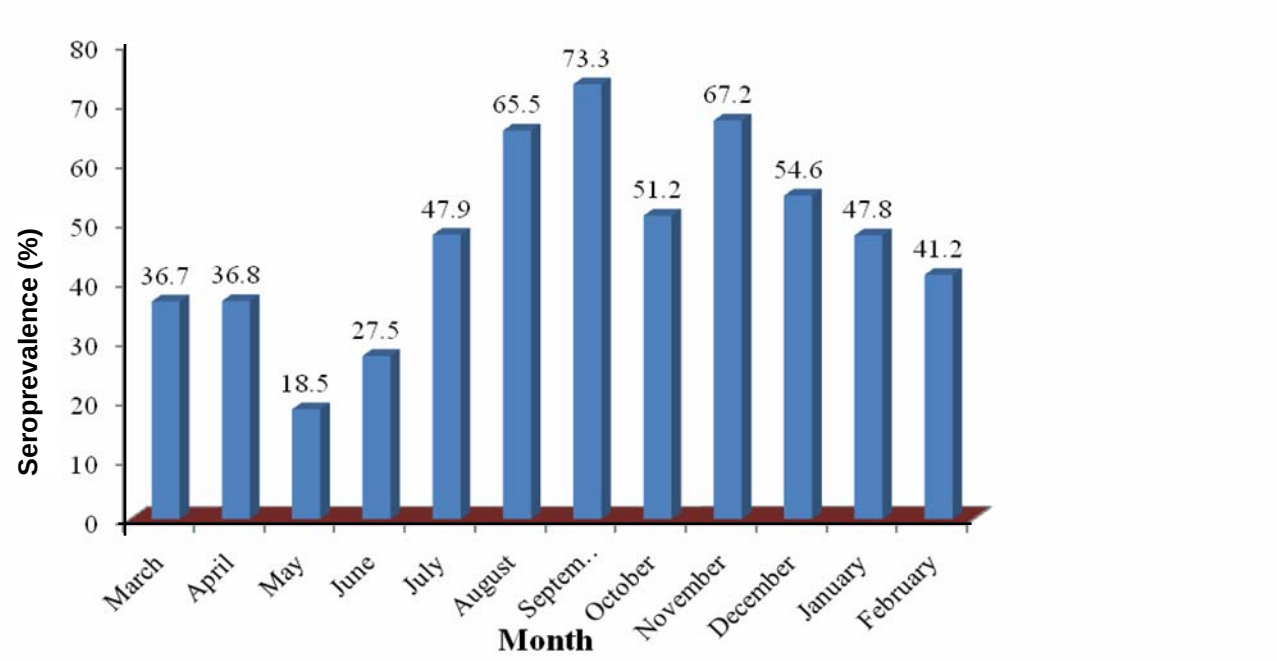

Fig. 4. Monthly seroprevalence of VL

Acknowledgement

The authors are very much thankful to the staff of the pathology laboratory of the Upazilla Health Complex of Trishal, Mymensingh for their kind help during collection of data and human blood samples. The authors are also grateful to Ministry of Science and Information and Communication Technology for providing financial support to conduct the research work through awarding Shirin Akter for National Science and Information and Communication Technology (NSICT) Fellowship (2010-2011).

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