Trans R Soc Trop Med Hyg 2016; **110**: 286–293
doi:10.1093/trstmh/trw027

ORIGINAL ARTICLE

Detection of *Leishmania donovani* in peripheral blood of asymptomatic individuals in contact with patients with visceral leishmaniasis

Sultana S. Banu[a,b,c,d,*], Wieland Meyer[b,c], Be-Nazir Ahmed[d], Rady Kim[a] and Rogan Lee[a,c]

[a]*Parasitology Department, Centre for Infectious Diseases and Microbiology Laboratory Services (CIDMLS), ICPMR, Westmead Hospital, NSW, Australia;* [b]*Molecular Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, Marie Bashir Institute, Westmead Institute for Medical Research, NSW, Australia;* [c]*Discipline of Medicine, Sydney Medical School, University of Sydney, NSW, Australia;* [d]*Communicable Disease Control Unit, Directorate General of Health Services, Mohakhali, Dhaka, Bangladesh.*

*Corresponding author: Present address: Parasitology Department, Centre for Infectious Diseases and Microbiology Laboratory Services (CIDMLS), ICPMR, Westmead Hospital, Westmead, NSW, Australia. Tel: +61 469927964; E-mail: sban7118@uni.sydney.edu.au

Received 30 October 2015; revised 16 February 2016; accepted 28 March 2016

Background: The majority of individuals infected with *Leishmania donovani* complex remain asymptomatic. They may act as transmission reservoirs for visceral leishmaniasis (VL). We investigated sero-prevalence of *L. donovani* complex among those closely associated with patients with VL and whether these sero-reactive individuals had *Leishmania* parasites in their peripheral blood. Other risk factors were also investigated.

Methods: A total of 257 individuals in contact with patients with VL were tested for anti-*Leishmania* antibodies by rK39 immunochromatographic test (rK39 ICT), ELISA using promastigote antigen (p-ELISA) and indirect fluorescent antibody test (IFAT). Buffy coats of rK39 ICT positive individuals were cultured; sero-reactive buffy coats were tested for *Leishmania* DNA by ITS1 PCR. DNA obtained from culture was sequenced to confirm *Leishmania* species. Risk factors were evaluated for each sero-positive sample.

Results: The results showed 29.2% (75/257) prevalence by serological tests: 14.4% (37/257) were positive by rK39 ICT, 25.3% (65/257) by p-ELISA, 18.3% (47/257) by IFAT and 10.9% (28/257) by all three serological methods. Ten percent (3/30) of cultures were positive for *Leishmania* promastigotes. Only 3% (2/74) sero-reactive buffy coats were positive for DNA; sequence analysis revealed *L. donovani* species. Significant risk factors were age, working as farmers, domestic animals in household and proximity to animal shelters.

Conclusions: Asymptomatic family members of patients with VL can carry live *L. donovani* in peripheral blood and may act as potential reservoirs.

GenBank accession number: BankIt1863680 *Leishmania* KT921417 (DNA sequences of the ribosomal ITS1 region of *L. donovani*).

Keywords: Asymptomatic infection, Bangladesh, Culture, *Leishmania*, PCR, Serology

Introduction

Visceral leishmaniasis (VL) is a vector borne disease caused by the protozoan parasites of the *Leishmania donovani* complex.[1] An annual incidence of 0.2 to 0.4 million cases of VL with approximately 20 000 to 40 000 deaths are reported worldwide. Over 90% of these VL cases are from India, Bangladesh, Nepal, southern Sudan, north-east of Brazil and Ethiopia.[2] *Leishmania donovani* is the principal cause of VL in the Indian subcontinent and in Africa, while *L. infantum* causes VL in the Mediterranean basin and South America.[3]

The majority of infections with *L. donovani* complex remain asymptomatic.[4] Due to the intracellular existence within monocytes, parasites can be present in blood for an undefined period between the sand fly bite and their localization in organs to cause VL. During this period individuals remain asymptomatic and can carry parasites in their peripheral blood.[5] In endemic areas, only 10–20% of infected humans develop symptomatic VL while the other 80–90% remain asymptomatic and can either develop clinical disease months later or recover spontaneously in about one or two years.[6] Asymptomatic infections are not well defined. They are determined by a positive serological test

and/or leishmanin skin test (LST) and/or PCR and/or culture of blood buffy coats in apparently healthy individuals with no clinical history of either VL or post kala-azar dermal leishmaniasis (PKDL).[7–11] Several prospective serological studies calculate the incidence of asymptomatic infections compared to that of symptomatic cases as having a ratio of 4:1 in Kenya,[12] 18:1 in Brazil,[13] 4:1 in Bangladesh[9] and 8.9:1 in India and Nepal.[14] Mathematical modelling suggests that individuals with asymptomatic infection may act as potential reservoirs for sustained transmission of *Leishmania* in endemic areas.[15] In addition, several studies in endemic areas have revealed the presence of *Leishmania* parasites in peripheral blood of asymptomatic individuals by culturing sero-positive buffy coats and by microscopy.[7,8,16] However in Bangladesh, published data on parasite detection in peripheral blood of asymptomatic individuals are not yet available.

Approximately 80% of global individuals with VL live in remote rural areas of India, Bangladesh and Nepal[2] with about 200 million people at risk of VL.[15] Consequently, governments of these three countries launched a regional VL elimination program in 2005 with the aim of reducing the number of individuals with VL to less than one per 10 000 population per year at sub district level of endemic districts by the year 2015.[17] However, none of these countries has reached the elimination target yet.[18,19] Even though the numbers of VL patients are declining rapidly in Bangladesh, 16 districts still consistently report new cases every year.[18] The current elimination program is primarily focused on early detection and treatment of symptomatic VL cases and vector control activities. Therefore, many individuals with asymptomatic infection of *Leishmania* in the community may remain undetected. These individuals may carry *Leishmania* parasites in their peripheral blood and contribute to sustained transmission of VL in Bangladesh. We investigated the status of asymptomatic infection by serology and the presence of *Leishmania* parasites in peripheral blood of asymptomatic sero-positive individuals by culture and PCR targeting family members and adjacent neighbours of patients with VL. Other potential risk factors were also investigated.

Materials and methods

Study design and study population

A cross-sectional study was carried out amongst asymptomatic and apparently healthy contacts of 155 patients with VL recently diagnosed by parasitological methods. These patients with VL (index cases) attended the Surya Kanta Kala-azar Research Centre (SKKRC), Mymensingh district, Bangladesh, from May 2013 to May 2014 for diagnosis and treatment. We chose family members and adjacent neighbours as contacts of these patients with VL because sand flies have a short flight range of up to 200 meters.[20] Using a non-probability sampling technique, we selected a total of 257 contacts, of which 80.9% (208/257) were family members and 19.1% (49/257) were adjacent neighbours. Inclusion criteria were: age between 2 and 75 years old; either sex; clinically healthy with no previous history of VL or PKDL; family member residing in the same household as patients with VL or adjacent neighbours without VL cases in the household; and voluntary consent to participate in the study. Female participants who were pregnant or breastfeeding were excluded from the study.

Sample size calculation

In an endemic district of Bangladesh (Mymensingh), the prevalence of human VL was found to be about 3%.[21] Assuming a similar prevalence amongst contacts of infected individuals, a sample size of 200 was determined to be sufficient to ensure the estimated prevalence was correct within ±3%. According to this estimate, we surveyed 257 individuals.

Asymptomatic infection of *Leishmania*

Asymptomatic individuals with positive rK39 immunochromatographic test (ICT) (SD BIOLINE *Leishmania* Ab rapid test, SD Standard Diagnostics, Inc., Giheung-gu, Yongin-si, Gyeonggi-do, Korea) and/or positive p-ELISA (Human Pan-*Leishmania* Antibody CELISA kit, Cellabs Pty Ltd, Brookvale, Australia) and/or positive IFAT (*Leishmania*-Spot IF, bioMerieux, Marcy l'Etoile, France) results without clinical features and past history of either VL or PKDL were considered to have an asymptomatic infection of *Leishmania* in this study.

Collection of blood and relevant data

Blood samples and socio-epidemiological data were collected from the family members who accompanied index VL cases at the SKKRC. A house to house visit was done to collect data and blood samples from the adjacent neighbours. About 3–4 ml of venous blood was collected from each participant in a Vacutainer® containing lithium-heparin (BD, Franklin Lakes, NJ, USA). A complete medical history was obtained and a physical examination was done before specimen collection. Socio-demographic and epidemiological data collected in a structured questionnaire included: residential address, age, gender, educational level, occupation, medical history, family income, housing type, living with domestic animals, presence of pet/stray dogs and rats in the households. Other factors comprised of animal shelters, water sources, vegetation and accumulated garbage found within an approximate 100 meter radius of residences of study subjects. Written informed consent was obtained from each participant before collecting relevant information and biological specimens. In case of participants less than 18 years old, consent from parents or legal guardians was taken as proxy informant. Their contact numbers were also recorded to inform them of the test results. Data on samples and isolates were coded and anonymized.

Field testing, culture and storage of specimens

Field testing by rK39 ICT, and culturing and storing of specimens from family members were done at SKKRC. Blood samples from the adjacent neighbours were brought to the Microbiology Department of Mymensingh Medical College (MMC) for testing with rK39 ICT and for immediate inoculation into culture media. Plasma and buffy coat were separated after centrifugation at 3000 rpm for 15 minutes at ambient temperature. Plasma was tested for anti-*Leishmania* antibody by rK39 ICT. Buffy coats from samples showing positive reaction with rK39 ICT were immediately seeded into Novy-MacNeal-Nicolle (NNN) media. The plasma and buffy coat from each sample were stored at −20°C.

Duplicate vials of NNN media with antibiotics (penicillin and gentamycin) (Square Pharmaceuticals Ltd, Dhaka, Bangladesh) were prepared for each buffy coat (about 250 µl in each medium). Inoculated media were incubated at 24–26°C and examined weekly for up to 6 weeks. Positive isolates were sub cultured every 2 weeks for maintenance. Thirty buffy coats were inoculated from 37 individuals who tested positive by rK39 ICT. Seven other samples were insufficient for *Leishmania* culture, but their buffy coats were stored for further molecular analysis. Culture media harvested from positive isolates were stored at −80°C.

Frozen specimens were transported in dry ice to Australia for further serological and molecular testing.

Serological screening

Plasma specimens from study subjects were initially tested for anti-*Leishmania* antibodies by rK39 ICT in Bangladesh. The p-ELISA using mixed *Leishmania* promastigotes and IFAT using whole promastigotes of the *L. donovani* complex were performed in Australia on all samples following the manufacturer's instructions. Anti-*Leishmania* IgG against *Leishmania* promastigotes was detected by p-ELISA using 1:400 and by IFAT using 1:40 dilutions of assayed plasma. True negative and positive controls confirmed by clinical, parasitological and serological assays were included in all serological methods performed.

DNA extraction and PCR amplification

DNA was extracted from buffy coats obtained from sero-reactive individuals and cultured promastigotes using QIAamp DNA Mini Kit following the manufacturer's instructions (QIAGEN, Hilden, Germany). We extracted DNA from 74 out of 75 buffy coats; one buffy coat was lost during laboratory processing.

The ribosomal internal transcribed spacer 1 (ITS1) region (300–350 bp) was amplified using primers and methodology as previously described.[22] Amplicons were visualized and photographed under UV light in 1.5% agarose gels containing 2% ethidium bromide.

DNA sequence analysis

Each amplicon was bidirectionally sequenced by Macrogen, Korea, using respective forward and reverse primers. Sequences were assembled and edited using the Sequencher 5.3 software (Ann Arbor, MI, USA) and searched against GenBank using the Basic Local Alignment Search Tool (BLAST). An identity value greater than 99% was used to assign species identification.

Statistical analyses

Data were analyzed by using the statistical software IBM® SPSS version 21 (IBM® SPSS Statistics, Armonk, NY, USA). Two-tailed tests with a significance level of 5% were used throughout. Continuous variables were summarized using the mean, median and SD. Categorical variables were summarized by percentages. The 95% CI for a proportion was calculated as described previously.[23] Either χ^2 or Fisher's exact test was applied where appropriate for testing association between categorical variables.

Results

Study population

The study participants (n=257) were from 14 sub-districts and five metropolitan cities (Sadar) within eight different districts (Mymensingh, Tangail, Gazipur, Jamalpur, Pabna, Sirajganj, Shariatpur and Sherpur) of Bangladesh. Among them, 55.3% (142/257) participants were male and 44.7% (115/257) were female. The median age was 29 years (range 2–63) with the highest number of participants (35.8%, 92/257) in the 30–45 years age group (Table 1). About half (122/257, 47.5%) of the individuals in the study group had received only primary level education, 28.4% (73/257) were unschooled and 24.1% (62/257) either completed or were doing their secondary schooling. The distribution of socio-economic, epidemiological and environmental characteristics of the study population is shown in Table 2.

Table 1. Sero-prevalence of 257 individuals in contact with patients with VL divided by gender and age

Variables	Number examined n (%)	Positive by any of the serological tests n (%)	95% CI
Gender			
Male	142 (55.3)	48 (33.8)	26.5–41.9
Female	115 (44.7)	27 (23.5)	16.7–32.0
Age group (years)			
2–15	68 (26.5)	12 (17.7)	10.4–28.4
16–29	68 (26.5)	18 (26.5)	17.5–38.0
30–45[a]	92 (35.8)	37 (40.2)	30.8–50.4
46–60	27 (10.5)	7 (25.9)	13.2–44.7
61–75	2 (0.8)	1 (50.0)	9.5–90.6

[a] $p<0.05$, significant association of sero-reactivity with 30–45 years age group.

Table 2. Sero-prevalence of 257 individuals in contact with VL patients divided by socio-economic, epidemiological and environmental variables

Variables	Frequency n (%)	Sero-prevalence n (%)	p-value
Type of contact			
Family members	208 (80.9)	64 (30.8)	NS
Adjacent neighbours	49 (19.1)	11 (22.4)	NS
Educational status			
Nil	73 (28.4)	24 (32.9)	NS
Primary only	122 (47.5)	35 (28.7)	NS
Secondary or higher	62 (24.1)	16 (25.8)	NS
Occupation			
Farmers	70 (27.2)	29 (41.4)	0.002
Non-agriculture	32 (12.5)	10 (31.3)	NS
Student	79 (30.7)	11 (13.9)	NS
Housewife	68 (26.5)	24 (35.3)	NS
Dependent	8 (3.1)	1 (12.5)	NS
Family income			
Very poor[a]	100 (38.9)	34 (34.0)	NS
Poor[b]	118 (45.9)	29 (24.6)	NS
Medium income group[c]	39 (15.2)	12 (30.8)	NS
Type of housing			
Mud/thatched house	196 (76.3)	55 (28.1)	NS
Concrete/brick	61 (23.7)	20 (32.8)	NS
Domestic animals in household			
Yes	154 (59.9)	54 (35.1)	0.011
No	103 (40.1)	21 (20.4)	NS
Households close to animal shelter[d]			
Yes	207 (80.5)	68 (32.9)	0.009
No	50 (19.5)	7 (14.0)	NS
Dogs within domestic vicinity[d]			
Yes	175 (68.1)	52 (29.7)	NS
No	82 (31.9)	23 (28.1)	NS
Rats in household			
Yes	226 (87.9)	67 (29.6)	NS
No	31 (12.1)	8 (25.8)	NS
Use of bed-net			
Yes	216 (84.0)	64 (29.6)	NS
No	41 (16.0)	11 (26.8)	NS
Houses close to water bodies[d]			
Yes	165 (64.2)	55 (33.3)	NS
No	92 (35.8)	20 (21.7)	NS
Vegetation adjacent to household[d]			
Yes	253 (98.4)	74 (29.2)	NS
No	4 (1.6)	1 (25.0)	NS
Garbage close to household[d]			
Yes	247 (96.1)	73 (29.6)	NS
No	10 (3.9)	2 (20.0)	NS

NS: not significant.
[a] Income Bangladeshi (BD) Taka 2000–5000 (US$26–64)/month.
[b] Income BD Taka 5001–10 000 (US$ 65–128)/month.
[c] Income BD Taka 10 001–15 000 (US$ 129–192)/month.
[d] 'Close to', 'adjacent to', 'domestic vicinity' terms were used to indicate the area within an approximate100 meter radius of the residences.

Serology

Healthy contacts tested positive by any of the three serological methods were considered as asymptomatic cases. Of the 257 participants 75 were sero-positive by any of the tests performed showing a point prevalence of 29.2%. Of those, 14.4% (37/257) were positive by rK39 ICT, 25.3% (65/257) by p-ELISA, 18.3% (47/257) by IFAT and 10.9% (28/257) by all three serological methods (Table 3).

Culture, PCR and species identification

Parasites grew from 10% (3/30) of buffy coats inoculated into NNN media; the same individuals were also sero-reactive in all tests (Table 4). Direct PCR detection of parasite DNA from buffy coats was found in 3% (2/74) sero-reactive individuals; these two PCR positive individuals were also culture positive. ITS1 sequences obtained from three culture isolates showed 100% identity to *L. donovani* species. Since all generated sequences were the same, only one was deposited into GenBank with the accession number of KT921417.

Table 3. Prevalence of asymptomatic VL infections amongst 257 participants determined by different serological methods

Serological assays	Prevalence, n (%)	95% CI
rK39 ICT	37 (14.4)	10.6–19.2
p-ELISA	65 (25.3)	20.4–30.9
IFAT	47 (18.3)	14.0–23.5
rK39 ICT, p-ELISA and IFAT	28 (10.9)	7.6–15.3
Positive in at least one test	75 (29.2)	24.0–35.0

ICT: immunochromatographic test; IFAT: indirect fluorescent antibody test; p-ELISA: ELISA using promastigote antigen.

Risk factors

A significant number of individuals with asymptomatic infection was found amongst those aged 30–45 years ($p<0.05$) (Table 1), working as farmers ($p<0.01$), living with domestic animals in the same household ($p=0.01$) and living close to animal shelters ($p<0.01$) (Table 2). An increased tendency of sero-reactivity was also observed in individuals whose houses were close to water sources ($p=0.05$). No significant difference was observed by other socio-economic, cultural and environmental variables investigated in this study (Table 2).

Discussion

Due to the distinctive epidemiological features of VL in the Indian subcontinent, management of symptomatic cases and vector control activities are the principal objectives of the current elimination program of the participating countries.[17] Detection and follow up of asymptomatic cases for clinical disease development are expected to improve eradication of VL, because asymptomatic human carriers have been identified as

Table 4. Serological, molecular and socio-demographic details of three asymptomatic individuals from whom parasites were cultured

Variables	Participant 1	Participant 2	Participant 3
Personal details			
Age	30 years	25 years	42 years
Gender	Male	Female	Male
Educational status	Primary	Uneducated	Primary
Occupation	Service	Housewife	Agriculture
Family status	Poor[a]	Very poor[b]	Very poor[b]
Subdistrict/Upazila	Trishal	Gaffargaon	Fulbaria
District	Mymensingh	Mymensingh	Mymensingh
Type of contact	Family member	Family member	Family member
Duration of infection of Index case	8 months	7 months	4 months
Housing type	Mud-house	Mud-house	Mud-house
Test results			
rK39 ICT	Positive	Positive	Positive
p-ELISA	Positive	Positive	Positive
IFAT	Positive	Positive	Positive
ITS1 PCR (buffy coat)	Positive	Positive	Negative
DNA sequence analysis (culture)	*L. donovani*	*L. donovani*	*L. donovani*

ICT: immunochromatographic test; IFAT: indirect fluorescent antibody test; ITS1: internal transcribed spacer 1.
[a] Income Bangladeshi (BD) Taka 5000–10 000 (US$ 65–128)/month.
[b] Income BD Taka <5000 (US$<65)/month.

potential reservoirs for VL transmission in other endemic areas.[4,24] In Bangladesh, a few studies were conducted primarily to evaluate the immunological status of VL in asymptomatic individuals in an endemic district of Mymensingh.[9,25,26] Only one of these studies performed PCR on buffy coats from healthy blood donors who were sero-positive (0.3%), but no parasite DNA was detected in any of the sero-reactive donors.[26] The other two studies did not look for evidence of parasites in peripheral blood of sero-positive individuals. As such, our study was designed to investigate whether asymptomatic individuals could carry *Leishmania* parasites in their peripheral blood when they were found to be sero-reactive. We, therefore, cultured for live *Leishmania* promastigotes and tested for parasite DNA from extracted buffy coats from sero-reactive asymptomatic individuals who were in contact with index VL cases.

We found a point prevalence of 29.2% by serology indicating the presence of asymptomatic infection among individuals in contact with patients with VL. Follow up examination and testing of these sero-positive individuals at three months to one year could reveal disease progression. Others have shown that sero-positive individuals, who are asymptomatic, have disease conversion rates of 10 to 69%.[4,10]

Several studies used buffy coat as a means to culture parasites from asymptomatic sero-positive individuals.[7,8] Based on those studies, buffy coats from rK39 ICT reactive samples were inoculated into NNN media to detect live *Leishmania* parasites. We used rK39 ICT to identify individuals likely to be carrying *Leishmania* in their blood as it was a rapid screening test. Moreover, the test has been shown to have a 100% sensitivity and specificity for sero-diagnosis of VL in the Indian subcontinent.[27] Three (10%) of the inoculated cultures were found positive for *Leishmania* promastigotes in this study indicating that asymptomatic sero-positive individuals can harbour live parasites in their peripheral blood. Similar numbers of isolates of *L. infantum* were cultured from buffy coats in sero-positive asymptomatic individuals with 4% (3/67) occurring in Spain[8] and 12% (9/76) occurring in France.[7] Parasite detection in peripheral blood of asymptomatic individuals by PCR (2.7%) in this study was lower than that (4.2% to 25%) found in other studies conducted in neighbouring India.[10,11] These Indian studies were carried out in the highest endemic state of Bihar, while our study populations came from both endemic and less or relatively non-endemic districts of Bangladesh. Here we found that the three culture positive individuals were positive by all three serological methods and two of their buffy coats were positive for *Leishmania* DNA (Table 4). The failure of the PCR to detect *Leishmania* DNA from the third buffy coat, that was culture positive, might be due to insufficient sample. The majority of the buffy coat ($2 \times 250\,\mu l$) was used for culture. The remaining number of white blood cells present for DNA extraction might have been insufficient for molecular detection. However, the sensitivity of peripheral blood cultures and parasite detection by PCR could be improved with repeated attempts for isolating parasites and detecting parasite DNA from sero-positive individuals every 6–12 months.[8,10,24] Further studies are required to quantitate the number of parasites in the peripheral blood of these carriers and determine whether these levels are sufficient for transmission to the sand fly vector.

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. One study conducted in Bangladesh shows that living within a 50 meter radius of patients increases the risk of developing VL by threefold. The risk increases to 26-fold when living with a previously diagnosed case in the same household.[28] Furthermore, family members are genetically similar and this may predispose them to acquiring the infection.[29] In our study, the three individuals from whom live parasites were isolated were family members living in the same household. Therefore, screening of asymptomatic family members and household contacts of patients with VL by serology and monitoring sero-positive individuals for early case detection and management should be considered as part of the strategy for VL elimination.

Our study showed a higher sero-prevalence among adults of 30–45 years ($p<0.05$) as seen in other investigations.[24,30] This association with age might be explained by the longer period of exposure to the disease-carrying vector in endemic areas.[30] However, most of the family members accompanying patients with VL were adults (30–45 years age group) causing a selection bias in our study.

A significant number of asymptomatic cases were found amongst farmers ($p<0.01$). This supports a previous finding from an Italian study which has suggested that agricultural activities in rural environment are a significant risk for acquiring *Leishmania* infection.[31] Living with domestic animals in the same household was also significantly associated with asymptomatic infection in our study ($p=0.01$). This suggests that domestic animals may act as potential reservoirs for *Leishmania* as found in another study conducted in Nepal.[32] In addition, there was a significant association between asymptomatic infection and proximity of human residences to animal shelters ($p<0.01$) indicating that animal shelters and loose soil surrounding them are suitable breeding sites for *Phlebotomus argentipes*.[33] Since the flight range of the phlebotomine sand fly is estimated to be up to 200 meters,[20] the disease prevalence is likely to be enhanced when residing within the vector flight range from their breeding sites. The restricted flight range could also explain why there was a greater tendency of detecting asymptomatic sero-positive individuals whose houses were close to water bodies ($p=0.05$). An earlier study demonstrates that humidity provided from water sources favors sand fly survival.[34]

Most of the sero-positive participants in our study were either illiterate or poorly educated with very low socio-economic status. They live in mud/thatched houses often with cracked walls and floors which become damp during the rainy season, thus favoring sand fly breeding and survival.[28] Others also found poverty as a risk factor for VL spread.[35] However, this association was not found in our study ($p>0.05$).

This study did have a selection bias by targeting family members and adjacent neighbors for screening antibodies to *L. donovani* complex. The majority of the family members who accompanied patients with VL while attending SKKRC were adults. Hence the observed prevalence and age specific distribution of asymptomatic infection cannot be generalized among other strata of the population. Although serological tests cannot distinguish between relapses, re-infections, and past infections from individuals who have self-cured or received treatment for

VL, we selected those asymptomatic individuals who did not have a past history of VL or PKDL by scrutinizing their medical records and treatment history. The other limitation of serological tests is that they may miss those individuals who have a recent infection, but not yet developed antibodies at this early stage. We, therefore, suggest that a prospective study to follow up on this target population is required to minimize the possible underestimation in a single cross-sectional survey.

Conclusions

Asymptomatic infections were detected by rK39 ICT, p-ELISA and IFAT among apparently healthy family members and neighbours of patients with VL residing in various districts of Bangladesh. This study shows that healthy sero-positive individuals can be carriers of *L. donovani* and that detection from a single collection of peripheral blood underestimates the true number of asymptomatic carriers in an endemic community. Asymptomatic individuals who are sero-reactive should be monitored for early detection and managed under the VL elimination program, if clinical symptoms of disease appear. Further investigations are required to determine whether these carriers of live *Leishmania* parasites can act as reservoirs for this anthropophilic infection.

Authors' contributions: RL and SSB conceived and designed the study; B-NA supervised the data collection and field testing; SSB carried out all laboratory investigations, data analyses and writing of the manuscript; WM, RK and RL critically reviewed the manuscript for intellectual contribution; all authors read and approved the final version of the manuscript for publication. RL and SSB are the guarantors of the article.

Acknowledgements: The study was conducted in collaboration with the Communicable Disease Control Unit of the Directorate General of Health Services, Bangladesh. Help given by staff of the Surya Kanta Kala-azar Research Center and the microbiology department of Mymensingh Medical College, Bangladesh is acknowledged. Professor AKM Shamsuzzaman, IEDCR, Bangladesh assisted in culturing *Leishmania* parasites. Dr. Karen Byth Wilson helped in statistical analysis and John Clancy for providing technical assistance. Professor Gwendolyn Gilbert of CIDMLS provided constructive comments to this manuscript.

Funding: Funding support was obtained from the Center for Infectious Diseases and Microbiology Laboratory Services (CIDMLS), Public Health fund and Endeavour Foundation, Australia (ID: 3039_2012).

Competing interests: None declared.

Ethical approval: The study was approved by the Bangladesh Medical Research Council (BMRC: # BMRC/NREC/2010-2013/655(1-10) and Human Research Ethics Committee, Western Sydney Local Health District, Australia (LNR/13/WMEAD/173).

References

1 Herwaldt BL. Leishmaniasis. Lancet 1999;354:1191–9.

2 Alvar J, Velez ID, Bern C et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 2012;7:e35671.

3 Sharma U, Singh S. Insect vectors of *Leishmania*: distribution, physiology and their control. J Vector Borne Dis 2008;45:255–72.

4 Singh S, Kumari V, Singh N. Predicting kala-azar disease manifestations in asymptomatic patients with latent *Leishmania donovani* infection by detection of antibody against recombinant K39 antigen. Clin Diagn Lab Immunol 2002;9:568–72.

5 Otero AC, da Silva VO, Luz KG et al. Short report: occurrence of *Leishmania donovani* DNA in donated blood from seroreactive Brazilian blood donors. Am J Trop Med Hyg 2000;62:128–31.

6 Badaro R, Jones TC, Carvalho EM et al New perspectives on a subclinical form of visceral leishmaniasis. J Infect Dis 1986;154:1003–11.

7 le Fichoux Y, Quaranta JF, Aufeuvre JP et al. Occurrence of *Leishmania infantum* parasitemia in asymptomatic blood donors living in an area of endemicity in southern France. J Clin Microbiol 1999;37:1953–7.

8 Riera C, Fisa R, Udina M et al. Detection of *Leishmania infantum* cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. Trans R Soc Trop Med Hyg 2004;98:102–10.

9 Bern C, Haque R, Chowdhury R et al. The epidemiology of visceral leishmaniasis and asymptomatic leishmanial infection in a highly endemic Bangladeshi village. Am J Trop Med Hyg 2007;76:909–14.

10 Das VN, Siddiqui NA, Verma RB et al. Asymptomatic infection of visceral leishmaniasis in hyperendemic areas of Vaishali district, Bihar, India: a challenge to kala-azar elimination programmes. Trans R Soc Trop Med Hyg 2011;105:661–6.

11 Srivastava P, Gidwani K, Picado A et al. Molecular and serological markers of *Leishmania donovani* infection in healthy individuals from endemic areas of Bihar, India. Trop Med Int Health 2013;18:548–54.

12 Schaefer KU, Kurtzhals JA, Gachihi GS et al. A prospective sero-epidemiological study of visceral leishmaniasis in Baringo District, Rift Valley Province, Kenya. Trans R Soc Trop Med Hyg 1995;89:471–5.

13 Evans TG, Teixeira MJ, McAuliffe IT et al. Epidemiology of visceral leishmaniasis in northeast Brazil. J Infect Dis 1992;166:1124–32.

14 Ostyn B, Gidwani K, Khanal B et al. Incidence of symptomatic and asymptomatic *Leishmania donovani* infections in high-endemic foci in India and Nepal: a prospective study. PLoS Negl Trop Dis 2011;5: e1284.

15 Stauch A, Sarkar RR, Picado A et al. Visceral leishmaniasis in the Indian subcontinent: modelling epidemiology and control. PLoS Negl Trop Dis 2011;5:e1405.

16 Sharma MC, Gupta AK, Das VN et al. *Leishmania donovani* in blood smears of asymptomatic persons. Acta Trop 2000;76:195–6.

17 WHO. Regional strategic framework for elimination of kala-azar from the south-east Asia region (2005–2015). New Delhi: World Health Organization/South East Asia Regional Office; 2005.

18 Chowdhury R, Mondal D, Chowdhury V et al. How far are we from visceral leishmaniasis elimination in Bangladesh? An assessment of epidemiological surveillance data. PLoS Negl Trop Dis 2014;8:e3020.

19 Burki T. India's ambition to eliminate visceral leishmaniasis. BMJ 2014;349:g6671.

20 Moura GS, Santos AM, Aquino DM et al. Factors associated with asymptomatic infection in family members and neighbors of patients with visceral leishmaniasis. Cad Saude Publica 2012;28: 2306–14.

21 Bern C, Chowdhury R. The epidemiology of visceral leishmaniasis in Bangladesh: prospects for improved control. Indian J Med Res 2006;123:275–88.

22 Schonian G, Nasereddin A, Dinse N et al. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn Microbiol Infect Dis 2003;47:349–58.

23 Newcombe RG. Two-sided confidence intervals for the single proportion: comparison of seven methods. Stat Med 1998;17:857–72.

24 Topno RK, Das VN, Ranjan A et al. Asymptomatic infection with visceral leishmaniasis in a disease-endemic area in Bihar, India. Am J Trop Med Hyg 2010;83:502–6.

25 Kurkjian KM, Vaz LE, Haque R et al. Application of an improved method for the recombinant k 39 enzyme-linked immunosorbent assay to detect visceral leishmaniasis disease and infection in Bangladesh. Clin Diagn Lab Immunol 2005;12:1410–5.

26 Huda MM, Rudra S, Ghosh D et al. Low prevalence of *Leishmania donovani* infection among the blood donors in kala-azar endemic areas of Bangladesh. BMC Infect Dis 2013;13:62.

27 Mathur P, Samantaray J, Chauhan NK. Evaluation of a rapid immunochromatographic test for diagnosis of kala-azar & post kala-azar dermal leishmaniasis at a tertiary care centre of north India. Indian J Med Res 2005;122:485–90.

28 Bern C, Hightower AW, Chowdhury R et al. Risk factors for kala-azar in Bangladesh. Emerg Infect Dis 2005;11:655–62.

29 Fakiola M, Strange A, Cordell HJ et al. Common variants in the HLA-DRB1-HLA-DQA1 HLA class II region are associated with susceptibility to visceral leishmaniasis. Nat Genet 2013;45:208–13.

30 Romero HD, Silva Lde A, Silva-Vergara ML et al. Comparative study of serologic tests for the diagnosis of asymptomatic visceral leishmaniasis in an endemic area. Am J Trop Med Hyg 2009;81:27–33.

31 Biglino A, Bolla C, Concialdi E et al. Asymptomatic *Leishmania infantum* infection in an area of northwestern Italy (Piedmont region) where such infections are traditionally nonendemic. J Clin Microbiol 2010;48:131–6.

32 Bhattarai NR, Van der Auwera G, Rijal S et al. Domestic animals and epidemiology of visceral leishmaniasis, Nepal. Emerg Infect Dis 2010;16:231–7.

33 Dhiman RC, Shetty PS, Dhanda V. Breeding habitats of phlebotomine sandflies in Bihar, India. Indian J Med Res 1983;77:29–32.

34 Schenkel K, Rijal S, Koirala S et al. Visceral leishmaniasis in southeastern Nepal:a cross-sectional survey on *Leishmania donovani* infection and its risk factors. Trop Med Int Health 2006;11:1792–9.

35 Alvar J, Yactayo S, Bern C. Leishmaniasis and poverty. Trends Parasitol 2006;22:552–7.