Evaluation of recombinant K39 antigen and various promastigote antigens in sero-diagnosis of visceral leishmaniasis in Bangladesh

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ABSTRACT

Background: Definitive diagnosis of visceral leishmaniasis (VL) by demonstrating parasites in tissue smears or by culture involves invasive procedures, technical expertise and adequate laboratory facilities. Endemic countries rely mainly on serological tests to diagnose VL. Currently, the immunochromatographic test incorporating the recombinant K39 antigen (rK39 ICT) is the reference test for rapid diagnosis of VL in the Indian subcontinent. The performance of serological tests using rK39 and other promastigote antigens can vary due to differences in antigen expression, the various hosts and environmental factors. To achieve elimination of VL, diagnostic accuracy will be necessary for active case detection especially in those who carry asymptomatic infections. We evaluated the performance of rK39 ICT, enzyme linked immunosorbent assay using mixed *Leishmania* promastigotes from different *Leishmania* species (p-ELISA) and indirect fluorescent antibody test (IFAT) utilizing whole promastigotes from the *Leishmania donovani* complex for sero-diagnosis of VL in Bangladesh.
Methods: The sensitivity of each serological test was evaluated on 155 patients who were diagnosed to have VL by microscopy and/or by culture methods. Test specificities were calculated on 706 healthy blood donors, 91 diagnostic sera from patients with a febrile illness and sera from patients positive for malaria (n = 91) and Chagas disease (n = 91). All statistical calculations were at 95% confidence intervals.
Results: The sensitivities of rK39 ICT, p-ELISA and IFAT were 100%, 86.5% and 92.3%, respectively. All three serological methods had a pooled sensitivity of 82.6%. The specificities of rK39 ICT, p-ELISA and IFAT from combined control groups were 100%, 93.1% and 99.9%, respectively. The respective positive and negative predictive values of the tests were both 100% for rK39 ICT, 66.3% and 97.8% for p-ELISA and 99.3% and 98.8% for IFAT. The p-ELISA showed cross reactivity with 36.3% of sera positive for malaria and 28.6% of sera positive for Chagas disease while rK39 ICT and IFAT showed no cross reactivity.
Conclusion: This study confirms the efficiency of rK39 ICT for rapid diagnosis of VL. The p-ELISA using mixed promastigote antigens did not perform well as a serological test for VL in Bangladesh. Due to high sensitivity and specificity of whole promastigote antigen of *L. donovani* complex utilized in IFAT, this test can be considered in combination with rK39 ICT to confirm VL diagnosis when clinical diagnosis cannot distinguish between other diseases.

1. Introduction

In the Indian subcontinent, visceral leishmaniasis (VL) is an anthroponotic infection caused by *L. donovani* and transmitted by the vector, *Phlebotomous argentipes* (Sharma and Singh, 2008). Although the total number of cases and mortality due to VL has declined significantly over the last decade, India, Bangladesh and Nepal are yet to reach their elimination target. While Bangladesh has achieved elimination in 90% of endemic sub-districts (WHO, 2015a), there are 16 sub districts which still report 1.06 to 18.25 VL cases per 10,000 population per year (Chowdhury et al., 2014). The persistence of endemic foci in the country, even after extensive control interventions, may give rise to renewed outbreaks and spread to other non-endemic areas. Therefore, active case detection with reliable diagnostic methods used by first-line caregivers in rural communities should be ensured.

Visceral leishmaniasis is a progressive illness characterized by prolonged fever, enlarged spleen and liver, anaemia, weight loss and cachexia (Murray et al., 2005; Van Griensven and Diro, 2012). Clinical features of the disease can vary and are often confused with other infections and clinical conditions such as malaria, tuberculosis (TB), leprosy, typhoid fever, hyper reactive malarial splenomegaly (HMS), malnutrition, lymphoma and leukemia (Herwaldt, 1999; Bhargava and Singh, 2012; McGwire and Satoskar, 2014). Most of these disease conditions especially infectious diseases and malnutrition are common in Bangladesh (Naheed et al., 2010; Ahmed et al., 2012; Haque et al., 2014; WHO, 2015b). Malaria is endemic in 13 districts of the country, which overlaps with some districts endemic for VL such as Mymensingh (Haque et al., 2014). Furthermore, misdiagnosis can occur with patients showing less specific clinical manifestations. In addition, asymptomatic cases in the community may remain undetected and may act as potential reservoirs in VL transmission areas (Salotra and Singh, 2006; Bern et al., 2007; Mondal and Khan, 2011; Ostyn et al., 2011). For active case detection, the recombinant K39 immunochromatographic test (rK39 ICT) offers a simple, non-invasive and accurate test with increased patient compliance. Reliable tests are also required to estimate actual disease burden, track disease trends over time, improve diagnosis-treatment algorithms and to verify disease elimination within communities (Singh and Sundar, 2015). More importantly, serological tests are needed to identify asymptomatic cases of VL rather than by detection through invasive parasitological procedures which are not likely to be approved for ethical reasons (Singh and Sundar, 2015).

Although antibody based methods cannot rule out relapses, re-infections and past infections, serological techniques can help to identify active disease in conjunction with clinical symptoms of suspected individuals (Ostyn et al., 2011; Srivastava et al., 2013). The rK39 antigen, encoded by a kinesin-like gene found in *L. chagasi*, can be used either in ICT or in enzyme linked immunosorbent assays (ELISA) for detecting specific antibody against the *L. donovani* complex. Whole promastigote antigens are used in direct agglutination test (DAT) and indirect fluorescent antibody test (IFAT), while crude lysates or mixed *Leishmania* promastigotes are utilized in ELISA (p-ELISA) to detect anti-*Leishmania* antibodies (Maia et al., 2012).

In Bangladesh and other endemic areas, those individuals suspected of having VL by clinical examinations, are confirmed with rK39 ICT before initiating treatment (Ahmed et al., 2014; Ready, 2014). However, patients who have either atypical symptoms or are asymptomatic may not be detected, because invasive parasitological methods cannot be justified (Singh and Sundar, 2015). Furthermore, the rK39 ICT is approved for use with either serum or plasma only, it is however routinely performed on whole blood in the Indian subcontinent (Cunningham et al., 2012). A study in India shows that rK39 ICT may show a negative reaction to whole blood with low antibody titre against rK39 antigen even in individuals from endemic areas with clinical symptoms (Matlashewski et al., 2013). In addition, some cases could be missed because of kit failure due to either poor storage or improper use of the kit. In cases when the standard parasitological procedure cannot be justified, a second serological test using a different form of *Leishmania* antigen other than rK39, should be considered to confirm the result of the rK39 ICT. The elimination of VL will require definitive diagnosis with an accurate non-invasive serological test/s associated with better patient compliance.

Although, high sensitivity and specificity of rK39 ICT have been shown (Cunningham et al., 2012; Mathur et al., 2005), another study shows that these diagnostic parameters can vary due to extensive diversity of the kinesin gene encoding for the K39 antigen among strains of *L. donovani* (Bhattacharyya et al., 2013). Host, geographical and environmental factors could also influence the variation in test results (Cunningham et al., 2012; Bhattacharyya et al., 2013). Test performance using native antigens derived from *L. donovani* promastigotes are also shown to be affected by host and topographical factors (Abass et al., 2015). Furthermore, genetic variations in strains of *L. donovani* in neighbouring India and Nepal (Downing et al., 2012; Imamura et al., 2016) indicate that molecular divergence of *L. donovani* strains might exist in Bangladesh. A change in expression of promastigote molecules including that of the K39 protein could also occur. Consequently, use of a specific recombinant antigen in ICT may not detect variants of *L. donovani*, which might evolve during this elimination program. Furthermore, much of the research on the sensitivity and specificity of ICT and ELISA using rK39 antigen (Sarker et al., 2003; Kurkjian et al., 2005; Rouf et al., 2009) and IFAT using promastigotes of *L. enriettii* (Muazzam et al., 1992; Alam et al., 1996) were last done in Bangladesh about seven years ago. Since these serological tests are used to monitor population incidences of VL, regular monitoring and evaluation of serological tests based on these antigens are thus essential to ensure the integrity of their performance in the field. In the present study, we evaluated the efficiency of ICT using rK39 antigen, p-ELISA using mixed *Leishmania* promastigotes and IFAT utilizing whole promastigotes of the *L. donovani* complex. Their sensitivity, specificity and predictive values in diagnosing VL were assessed.

2. Methods

2.1. Study design

A cross-sectional study was carried out among VL patients who attended Surya Kanta Kala-azar Research Centre (SKKRC), Mymensingh, Bangladesh, for diagnosis and treatment from May 2013 to May 2014.

2.2. Study population

Clinically and parasitologically confirmed VL cases (n = 155) and 979 control individuals were included in this study. The control subjects included healthy blood donors from endemic and non-endemic areas of Bangladesh and Australian patients with other diseases. The study populations were divided into the following six groups.

2.2.1. Group I (VL cases)

This group comprised of 155 confirmed VL patients who resided in 13 different districts of Bangladesh. Inclusion criteria were: ages between 2 and 75 years old, either sex, clinical signs and symptoms of VL, no clinical or serological evidence of other acute or chronic illness and voluntary consent to participate in the study. Female participants who were pregnant or breastfeeding were excluded from the study.

2.2.2. Group II (endemic healthy control)

This group included 351 healthy blood donors who lived in districts of Mymensingh, Tangail, Jamalpur, Pabna, Sirajganj, Rajshahi, Panchagarh, Khulna and Gazipur. All of these Bangladeshi districts are known to be endemic for VL (Chowdhury et al., 2014).

2.2.3. Group III (non-endemic healthy control)

This group had 355 healthy blood donors residing in non-endemic areas of Bangladesh. Those who had lived in the districts known to be endemic for VL were excluded from this Group.

Inclusion criteria for healthy blood donors (Group II and III) were: ages between 18 and 60 years old, either sex, clinically healthy, no previous history of VL or PKDL, no clinical or serological evidence of acute or chronic illness and voluntary consent to participate in the study. Female participants who were pregnant or breastfeeding and menstrual women were excluded from the study.

2.2.4. Non-endemic diseased control

2.2.4.1. Group IV (patients with febrile illness). This group comprised of 91 diagnostic sera from individuals with a febrile illness obtained from the Institute of Clinical Pathology and Medical Research (ICPMR), Westmead Hospital, Australia. Australia is non-endemic for human leishmaniasis.

2.2.4.2. Group V (patients with antibodies to malaria) and Group VI (patients with antibodies to Chagas disease). Stored sera positive for *Plasmodium falciparum* (n = 91) and positive for *Trypanosoma cruzi* (n = 91) were obtained from ICPMR. In addition to calculating specificity, sera from Group V and VI were used to show cross-reactivity of these serological tests with other protozoan infections.

2.3. Confirmative diagnosis of VL

Suspect cases of VL were based on the clinical features of prolonged fever for two weeks or more, hepatosplenomegaly, anaemia, weight loss, weakness and cachexia (Van Griensven and Diro, 2012; Kumar and Nylen, 2012). These patients were admitted into SKKRC for confirmative diagnosis by parasite detection from blood buffy coats and/or splenic aspirates. The SKKRC is the only VL research centre in Bangladesh where splenic aspiration has been routinely performed to isolate parasites for research purposes. It provides facilities for diagnosis and treatment under the National VL Elimination Program. Routine investigations were carried out to exclude other possible causes of hepatosplenomegaly, fever and anaemia. All cases were screened for malaria using a rapid test kit (SD BIOLINE, Malaria Ag P.f/Pan, Standard Diagnostics, Korea). Individuals with previously mentioned clinical features and showing positive reactions to the Widal and Mantoux tests were not included in this study. Investigations such as total blood count, platelet count, haemoglobin levels, bleeding and clotting time were performed and severely anaemic patients were given blood transfusion. Informed written consent was taken from each participant and from parents or legal guardians of those <18 years old after they were explained the need for collecting blood and splenic aspirates for research. An experienced physician was designated to collect splenic aspirates by using a standard universal procedure. A few drops of aspirate were smeared on glass slides for microscopic examination and the rest was inoculated into Nicolle-Novy-McNeal (NNN) media (Lumsden and McMillan, 1996) with antibiotics (penicillin and gentamycin) (Square Pharmaceuticals Ltd., Dhaka, Bangladesh) and incubated at 24–26 °C. The VL diagnosis was confirmed by the demonstration of *Leishmania* amastigotes in Giemsa stained smears and/or presence of promastigotes in cultures prepared from either blood buffy coats

and/or splenic aspirates (when available). However, those patients under seven years of age did not undergo the splenic aspiration procedure. Confirmation of VL diagnosis in these subjects was done based on parasite detection from smears prepared from blood buffy coat. Splenic aspiration for parasitological diagnosis on control subjects could not be performed for ethical reasons (Singh et al., 2013). All 155 VL patients were smear positive and only 34 positive cultures could be obtained. These patients received anti-*Leishmania* treatment with liposomal amphotericin B (AmBisome) (Gilead, Foster, CA, USA) under the National VL Elimination Program.

2.4. Collection, processing, storage and shipment of blood specimens for Group I, II and III

Individuals included in each study population were assigned a unique identification number. A complete medical history was obtained and a physical examination was done before collection of blood from each subject in groups I, II and III. About 3–4 ml of venous blood was collected from every participant. Blood from Group I was collected in a Vacutainer® containing lithium-heparin (BD, Franklin Lakes, NJ, USA) when they attended SKKRC. Blood specimens from study Group II and III were obtained at the Transfusion Medicine Department of Mymensingh Medical College (MMC) and the Transfusion Medicine Department of Dhaka Medical College (DMC), respectively. Blood donors were routinely examined and screened serologically for other infections such as malaria, syphilis, human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) before collecting specimen. An aliquot (3–4 ml) of fresh blood was taken from the donor's transfusion bag containing anti-coagulant (Citrate, phosphate, dextrose and sodium citrate, USP) and then transferred to a sterile plain tube.

Plasma and buffy coats were separated from all blood samples after centrifugation at 3000 rpm for 15 min at ambient temperature. Collected plasma specimens were stored at −20 °C until required. Processing, storage and testing of specimens with rK39 ICT were done at the collection sites except for Group II, which were performed at the Microbiology Department of MMC. Frozen specimens from Bangladesh were transported in dry ice to Australia for serological testing with p-ELISA and IFAT.

Sera from Groups IV, V and VI were collected from a serum bank stored at −20 °C in the ICPMR, Australia.

2.5. Serological methods

Plasma and sera were tested for anti-*Leishmania* antibodies using three different serological methods. The tests were performed and interpreted according to the respective manufacturer's instructions.

The rK39 immunochromatographic test (rK39 ICT) (SD *Leishmania* Ab, BioLine, Standard Diagnostics, INC., Yongin-si, Gyeonggi-do, Korea) was performed for qualitative detection of anti-*Leishmania* antibody against *L. donovani* complex. Twenty microliters of each sample (plasma or sera) was used for testing. The result was read within 10–15 min. The National VL Elimination Program of the Peoples' Republic of Bangladesh supplied these kits.

An ELISA kit with mixed *Leishmania* promastigotes (p-ELISA) (Human Pan-*Leishmania* Antibody CELISA ELISA kit, CELLABS PTY LTD, Brookvale, Australia) was used to detect antibodies. Plasma or sera (5 μl) was diluted at 1:400 for testing. Specimens were considered positive for p-ELISA with optical density (OD) values over 0.22 (cut-off point) which represented the mean plus three standard deviations (SD) of absorbance obtained from sera and plasma negative for anti-*Leishmania* antibodies.

An indirect fluorescent antibody test (IFAT) kit (*Leishmania*-Spot IF, bioMerieux, Marcy l'Etoile, France) was used to detect antibodies against whole promastigotes of the *L. donovani* complex. Five microliters of either plasma or sera was diluted at 1:40 for testing. A positive reaction was recorded when promastigotes showed light green fluorescence of the cytoplasm, membrane and the flagellum.

The Parasitology Department, ICPMR, Westmead Hospital, Australia provided the p-ELISA and the IFAT kits.

2.6. DNA extraction and PCR amplification

DNA was extracted from buffy coats obtained from sero-reactive blood donors using QIAamp DNA Mini Kit following the manufacturer's instructions (QIAGEN, Hilden, Germany). The ribosomal internal transcribed spacer 1 (ITS1) region (300–350 bp) was amplified using primers and methodology as previously described (Schonian et al., 2003). Amplicons were visualized and photographed under UV light in 1.5% agarose gels containing 2% ethidium bromide.

2.7. Statistical analysis

Data were analysed by using the statistical software IBM® SPSS version 21 (IBM® SPSS Statistics, Armonk, NY, USA). Sensitivity, specificity, positive (PPV) and negative predictive values (NPV) were calculated. Sensitivity was determined on Group I, and the specificity was calculated on Group II, III, IV, V and VI. All calculations were estimated at a 95% confidence interval (95% CI) as described elsewhere (Newcombe, 1998).

2.8. 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). Informed written consent was obtained from each participant and from parents or legal guardians of minors before collecting relevant information (personal details and medical records) and clinical specimens in Bangladesh. The stored sera from Australian patients were obtained from the ICPMR, Westmead Hospital, Australia. All samples were used for research purposes only and the data were anonymously analysed. No competing interest was declared by any of the authors. Funding sources were not involved in designing the study; in the collection, analysis and interpretation of data; in the writing of the report; nor in the decision to submit the article for publication.

3. Results

According to medical records and personal interview, all 155 (Group I) were primary VL cases as none of these patients had previous history of treatment for VL. The respective sensitivity of rK39 ICT, p-ELISA and IFAT were 100%, 86.5% and 92.3% in Group I. The three methods gave a pooled sensitivity of 82.6% (Table 1). All control plasma and sera in Group II, III, IV, V and VI were non-reactive by rK39 ICT showing specificity of 100%. The p-ELISA and IFAT showed respective specificities of 93.1% and 99.9% among these 979 control individuals. Only nine samples from blood donors (five from Group II and four from Group III) were positive by p-ELISA. One from Group II was positive by both p-ELISA and IFAT. DNA extracts from buffy coats of these nine seropositive blood donors were negative for *Leishmania* DNA by ITS1 PCR. The positive and negative predictive values of rK39 ICT were both 100%, p-ELISA were 66.3% and 97.8% and IFAT were 99.3% and 98.8% (Table 2). The p-ELISA showed 36.3% (33/91, 95% CI = 27.1–46.5%) cross reactivity with sera positive for malaria and 28.6% (26/91, 95% CI = 20.3–38.6%) with sera positive for Chagas disease. Neither rK39 ICT nor IFAT showed cross-reactivity with these 182 sera.

4. Discussion

Demonstration of parasites in either tissue aspirates or buffy coat from peripheral blood is the recognized gold standard method (Srivastava et al., 2011a; Srividya et al., 2012). Since the sensitivity of parasitological diagnosis from peripheral blood is poor (Elmahallawy et al., 2014), collection of aspirates from either spleen or bone marrow are therefore the preferred specimen. Testing with splenic aspirates has 93–99% sensitivity, while aspirates from other body sites have a suboptimal sensitivity of 50–86% (Srivastava et al., 2011a; Chappuis et al., 2007). Apart from being cumbersome, expensive, time consuming and needing skilled personnel, collection of splenic aspirates has significant risks of introducing other infections and causing intra-abdominal

Table 1
Sensitivity of three serological methods in 155 cases of confirmed VL.

Serological test	Positive	Sensitivity	95% CI[a]
	n	%	%
rK39 ICT	155	100	97.6–100
p-ELISA	134	86.5	80.2–91.0
IFAT	143	92.3	87.0–95.5
rK39 ICT, p-ELISA & IFAT	128	82.6	75.8–87.7

[a] CI = Confidence interval.

Table 2
Specificity and predictive values of three serological methods when tested against control sera or plasma.

Serological test	Specificity in control groups, % (95% CI[a])						Predictive value (95% CI)	
	Group II (n = 351)	Group III (n = 355)	Group IV (n = 91)	Group V (n = 91)	Group VI (n = 91)	All groups (n = 979)	PPV[b]	NPV[c]
rK39 ICT	100 (99.9–100)	100 (98.9–100)	100 (96–100)	100 (96–100)	100 (96–100)	100 (99.6–100)	100 (97.6–100)	100 (99.6–100)
p-ELISA	98.6 (96.7–99.4)	98.9 (97.1–99.6)	100 (96–100)	63.7 (53.5–72.9)	71.4 (61.4–79.7)	93.1 (91.3–94.5)	66.3 (59.6–72.5)	97.8 (96.6–98.5)
IFAT	99.7 (98.4–100)	100 (98.9–100)	100 (96–100)	100 (96–100)	100 (96–100)	99.9 (99.4–100)	99.3 (96.2–99.9)	98.8 (97.9–99.3)

[a] CI = Confidence interval.
[b] PPV = Positive predictive value.
[c] NPV = Negative predictive value.

hemorrhage. Parasitological techniques involving invasive procedures are therefore not suitable for field investigations and contribute to poor patient compliance (Sundar and Rai, 2002). While molecular detection by PCR is a highly sensitive method (Srivastava et al., 2011b), it is not available for routine diagnosis of VL in endemic countries. Serological tests can offer a better alternative for VL diagnosis with the added advantage of detecting anti-*Leishmania* antibodies when parasites are not found either by microscopy or by culture (Kilic et al., 2008).

It has been proposed that asymptomatic human cases can act as potential reservoirs for transmission of VL (Ostyn et al., 2011; Sundar et al., 2008; Mondal et al., 2010). In a recent study in Bangladesh, 29.2% sero-prevalence of asymptomatic infections were detected and 10% of these sero-positive individuals had parasites cultured from blood buffy coats. These individuals from whom parasites were cultured were also sero-reactive to rK39 ICT, p-ELISA and IFAT (Banu et al., 2016). A successful elimination program will therefore need to accurately detect and manage asymptomatic individuals who can carry live *Leishmania* parasites in their peripheral blood and have the potential of being hidden reservoirs. Since serological reaction to the rK39 antigen can vary (Cunningham et al., 2012; Bhattacharyya et al., 2013; Abass et al., 2015; Monno et al., 2009), performance of the rK39 ICT should be monitored regularly for any deviation of sensitivity and specificity since its first introduction for the regional elimination program. In addition, other serological tests using alternative promastigote antigens also need to be evaluated as their performance has also been shown to vary with different geographical areas and host immune status (Abass et al., 2015). Studies assessing the efficiency of different serological tests have recently been conducted in India, Nepal and other countries (Cunningham et al., 2012; Abass et al., 2015; Singh et al., 2013; Kumar et al., 2013), but there is no recent published data on performance of serological tests in Bangladesh. Furthermore, the use of mixed promastigote antigens derived from various *Leishmania* species in ELISA for sero-diagnosis of VL have not been assessed in this country. We chose mixed promastigote antigens in ELISA (p-ELISA) expecting high sensitivity in disease diagnosis. In this study, we evaluated the rK39 antigen together with different promastigote preparations for testing on VL patients and blood donors from Bangladesh and control sera from Australian patients.

4.1. rK39 ICT

The sensitivity and specificity of the rK39 ICT in this study corroborate with results of an earlier Indian study where 100% sensitivity and 100% specificity of the test were shown (Mathur et al., 2005). In the Indian subcontinent, other studies show 93–100% sensitivity and 94–100% specificity (Cunningham et al., 2012; Sarker et al., 2003; Rouf et al., 2009; Singh et al., 2013; Kumar et al., 2013), while respective lower sensitivity and specificity are observed in Brazil (61.5%–91% and >95%) and East Africa (36.8%–87.2% and 90.8%–98%) (Cunningham et al., 2012). A comparison of sensitivities and specificities of rK39 ICTs for VL in the Indian subcontinent show that the performance of the test kits of different brands are similar. However, the Onsite *Leishmania* Ab (RevB) rapid test demonstrated lower specificities in India (42%) and Nepal (48.7%) (Kumar et al., 2013) (Table 3). Lack of published data did not allow us to compare our results with others using the same rK39 ICT kit in this region.

4.2. p-ELISA

The sensitivity of p-ELISA in our study compared to estimated values by meta-analysis was similar (87%), but the specificity was higher than the estimated 77% (Maia et al., 2012). A study from India demonstrated a sensitivity of 100% and specificity of 87% with ELISA using promastigote antigen from *L. infantum* (Mandal et al., 2008). Other studies in Bangladesh using the rK39 antigen in ELISA (Kurkjian et al., 2005; Alam et al., 1996) had higher sensitivity and specificity than the p-ELISA in our study which used mixed promastigotes from various *Leishmania* species. This demonstrates that the rK39 antigen has better sensitivity and specificity when incorporated either in ELISA or in ICT compared to the mixed promastigote antigens used in the p-ELISA. Unfortunately, no published data could be found to compare our results using mixed *Leishmania* promastigotes in Bangladesh.

4.3. IFAT

Combined sensitivity of 88% and specificity of 90% with IFAT calculated in the meta-analysis (Maia et al., 2012) were slightly lower than that found in our study. Although our study showed a similar specificity to two other studies in Bangladesh, the sensitivity in our study was however lower than 100% found by these same two studies (Muazzam et al., 1992; Alam et al., 1996). This variation of test sensitivity between studies in Bangladesh could be due to the different species used as antigen. *Leishmania infantum*, which is a species of the *L. donovani* complex, was used in a commercial kit in our study. The other two studies used a laboratory preparation of *L. enriettii* promastigotes which are an enigmatic *Leishmania* species of neotropics.

4.4. Cross-reactivity with other protozoan diseases

Several studies demonstrate that promastigote antigens of *Leishmania* cross-react with sera showing positive reactions against antigens of malaria and Chagas disease (Romero et al., 2009; Pinedo-Cancino et al., 2013; Toledo-Machado et al., 2015). In this study, sera positive for malaria and Chagas disease showed considerable cross reactivity in the p-ELISA while

Table 3
Comparison of sensitivities and specificities of rK39 ICT kits used in the Indian subcontinent.

Country	Product	Manufacturer	Sensitivity n (%) (95% CI)	Specificity n (%) (95% CI)	Reference
India	Kala-azar Detect	InBios International Inc., Seattle, WA	57 (100) (93.7–100)	116 (100) (96.8–100)	Mathur et al. (2005)
	IT-LEISH	Diamed AG	221 (99.0–100) (97.5–99.9)	365 (95–100)	Sundar et al. (2006)
	Onsite *Leishmania* Ab (RevA) rapid test	CTK Biotech Inc., San Diego, CA, USA	145 (96.7) (92.4–98.9)	112 (94.1) (88.3–97.6)	Kumar et al. (2013)
	Onsite *Leishmania* Ab (RevB) rapid test	CTK Biotech Inc., San Diego, CA, USA	148 (98.7) (95.3–99.8)	50 (42.0) (33.0–51.4)	
	Kala-azar Detect	InBios International Inc., Seattle, WA	149 (99.3) (96.3–100)	118 (99.2) (95.4–100)	
	Diamed-IT LEISH	Bio-Rad Laboratories	148 (98.7) (95.3–99.8)	118 (99.2) (95.4–100)	
	Kala-azar Detect	InBios International Inc., Seattle, WA	365 (100) (98.9–100)	421 (94–100) (94.5–100)	Singh et al. (2013)
Nepal	Onsite *Leishmania* Ab (RevA) rapid test	CTK Biotech Inc., San Diego, CA, USA	26 (96.3) (81.0–99.9)	137 (90.1) (84.3–94.4)	Kumar et al. (2013)
	Onsite *Leishmania* Ab (RevB) rapid test	CTK Biotech Inc., San Diego, CA, USA	27 (100) (87.0–100)	74 (48.7) (40.5–56.9)	
	Kala-azar Detect	InBios International Inc., Seattle, WA	26 (96.3) (81.0–99.9)	143 (94.1) (89.1–97.3)	
	Diamed-IT LEISH	Bio-Rad Laboratories	26 (96.3) (81.0–99.9)	141 (92.8) (87.4–96.3)	
ISC (Bangladesh, India and Nepal)	Diamed-IT LEISH	Bio-Rad Laboratories	250 (98.8) (96.5–99.6)	249 (97.6) (94.8–98.9)	Cunningham et al. (2012)
	Kala-azar Detect	InBios International Inc., Seattle, WA	250 (99.6) (97.8–99.9)	249 (96.0) (92.8–97.8)	
	Onsite *Leishmania* Ab rapid test	CTK Biotech Inc.	250 (99.6) (97.8–99.9)	249 (96.8) (93.8–98.4)	
Bangladesh	NA[a]	NA	60 (96.6) (88.6–99.1)	120 (98.3) (94.1–99.5)	Sarker et al. (2003)
	NA	NA	60 (95) (86.3–98.3)	60 (98.3) (91.1–99.7)	Rouf et al. (2009)
	SD *Leishmania* Ab, BioLine	Standard Diagnostics, INC. Yongin-si, Gyeonggi-do, Korea	155 (100) (97.6–100)	979 (100) (99.6–100)	Current study

[a] Information not available.

the rK39 ICT and IFAT did not. These findings are in agreement with other researchers (Monno et al., 2009; Romero et al., 2009; Carvalho et al., 2003; Flores-Chavez et al., 2010). Although Chagas disease is not endemic in the Indian subcontinent, the parasite is a related flagellate with which antibodies generated against this organism cross react with antigens in the p-ELISA.

Nine samples from blood donors in this study showed positive reactions to p-ELISA (n = 9) and IFAT (n = 1) but their blood buffy coats were tested negative for *Leishmania* DNA. This was also seen in an earlier study in Bangladesh where none of the sero-reactive donors were found to have *Leishmania* DNA in their buffy coats (Huda et al., 2013). These nine sero-positive blood donors may be at an early stage of an asymptomatic infection when parasites are not detected in the peripheral blood. Two studies in the Mediterranean region showed that sero-positive blood donors carry *L. infantum* in their peripheral blood in low numbers (4.5–11.4%) (le Fichoux et al., 1999; Riera et al., 2004). These findings indicate that follow up of sero-reactive donors should be included as part of the elimination program, because clinical disease can occur weeks or months later (Singh et al., 2002; Das et al., 2011). While this study and a previous study in Bangladesh did not show parasites present in the blood of donors, routine screening of blood donors for VL should be considered to prevent potential transmission of *Leishmania* through blood transfusions.

As the disease declines in prevalence, serological tests are expected to become the method of choice for early detection and disease surveillance in the Indian subcontinent. The success of the elimination program has lowered the number of VL cases, this drop in numbers will in turn affect test efficiency and cause a decline in the positive predicative values of diagnostic tests (Singh and Sundar, 2015). Several studies recommend a combination of antibody based methods for accurate diagnosis of VL to avoid invasive parasitological procedures, although most of them prefer DAT and rK39 ICT for combined testing (Maia et al., 2012; Chappuis et al., 2007; Boelaert et al., 2007; ter Horst et al., 2009). The rK39 ICT along with IFAT in this study still show high sensitivity and specificity with no cross reactivity against a related flagellate and malaria when using serum or plasma. Furthermore, testing for VL in Bangladesh using whole blood on the rK39 ICT is common practice and has not been evaluated for sensitivity and specificity so far. Others have also reported false negative results of ICT tests using whole blood (Matlashewski et al., 2013). We are recommending a combination of two tests using different antigens such as rK39 and promastigotes of *L. donovani* complex for IFAT to diagnose VL with declining case numbers. In addition, antibody titres measured by the IFAT can be used to monitor the patient's long-term recovery (Akin et al., 2009; Proverbio et al., 2014). Although not covered in our study, the

collection of filter paper specimens that are mailed back to a reference laboratory for testing will overcome major logistical problems for further specimen testing (Panteleeff et al., 1999; Smit et al., 2014). Optimizing tests such as the IFAT for screening of antibodies eluted off filter paper will widen the choice of testing methods.

5. Conclusion

The performance of the rK39 ICT, currently in use in the elimination program in Bangladesh, remains consistent with earlier studies and has overall advantages compared with p-ELISA and IFAT. Although p-ELISA shows reasonable sensitivity and specificity in detecting antibodies to VL, the performance is below that seen with rK39 ICT and IFAT. In addition, antigen containing mixed *Leishmania* promastigotes used in p-ELISA cross reacts substantially with malaria and Chagas disease and hence is not suitable for detecting VL in areas where these diseases are known to be endemic. Although, rK39 ICT still performs well in the field in Bangladesh, regular monitoring for deviation from its expected performance are required as test efficiency can vary. Although IFAT may not be field friendly, optimizing the IFAT using specimens collected on filter paper will allow this test to be used as an adjunct test with the rK39 ICT. In the later stages of the elimination program, these tests will become more important to detect asymptomatic infections and to confirm diagnosis especially when clinical features cannot distinguish between other diseases. However, a cost-effective analysis to show whether serology alone can replace invasive parasitological procedures has yet to be done.

Authors' contributions

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