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

Sultana Shahana Banu [a,b,c,*], Be-Nazir Ahmed [c], Abul Khair Mohammad Shamsuzzaman [c], Rogan Lee [a,b]

[a] Parasitology Department, Centre for Infectious Diseases and Microbiology (CIDM), ICPMR, Westmead Hospital, Westmead, NSW, Australia
[b] Discipline of Medicine, Sydney Medical School, University of Sydney, NSW, Australia
[c] Directorate General of Health Services (DGHS), Ministry of Health and Family Welfare (MOHFW), Dhaka, 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.

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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.