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Detection of Leishmania Antigen from Buccal Swabs in Kala-azar Patients Using KATEX Method

Alfred Muia[1*] Margaret Mbuchi[1] Jesca O. Wesongah[4] Juma Rashid[1] Charles Magiri[1]
Rukia Kibaya[1] Samwel Odiwuor[3] Robert Kimutai [1] Raymond Omollo[2] Monique Wasunna [1, 2]
1. Kenya Medical Research Institute – Centre for Clinical Research, P O box 54840-00200, Nairobi, Kenya
2. Drugs for Neglected Diseases *initiative* (DND*i*) Nairobi, Kenya
3.Mt. Kenya University, Thika, Kenya
4.Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

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Abstract

Background: Visceral leishmaniasis (VL) or kala-azar is a chronic protozoan infection in humans that is fatal unless treated and is associated with significant global morbidity and mortality. Confirmation of visceral leishmaniasis (VL) diagnosis requires microscopic examination to visualize the causative agent *Leishmania Donovani* usually in spleen or bone marrow aspirate. Tissue aspiration is invasive, potentially risky and require skilled personnel. KATEX (Kalon biologicals) antigen test for VL in buccal swabs has not been evaluated locally and could offer a significant advantage in screening patients suspected of VL. **Method:** A cross sectional study was conducted after receiving approval from KEMRI SERU. We obtained buccal swabs from patients presenting at Kimalel Health Centre, Baringo County, Kenya from VL cases and controls. VL was defined as patients meeting VL case definition with positive splenic aspirate microscopy while endemic controls were defined as patients presenting to the health center with no fever and no prior history of Kala azar but living in VL endemic. Latex agglutination based test (KATEX) was used to detect parasite antigen in buccal swabs. It was a proof of principle study carried out to explore the ability to use KATEX, a simple non-invasive diagnostic test to detect leishmania antigens in buccal swabs, determine the ability of the kit to detect leishmania antigens in buccal cells of kala-azar patients and compare the sensitivity and specificity of KATEX - buccal assay using microscopy as the gold standard. **Results:** 88 patients were analyzed, including 44 VL and 44 non-VL patients. The median age of VL patients was 18 years with predominance of males (68.2%). None of the tested VL patients were co-infected with HIV. KATEX kit was able to detect visceral leishmaniasis antigens from the buccal swabs giving a sensitivity of (81.8%; 95%CI: 67.3% to 91.8%, specificity of (79.5%; 95%CI: 64.7 – 90.2%), Positive predictive value n= 36(80.0%); 95%CI: 65.4% to 90.4% and Negative predictive values n = 35(81.4%); 95% CI: 66.6% to 91.6%. **Conclusion and Recommendation:** Buccal swab test assay using KATEX is an easy test to perform and promising non-invasive based antigen detection test which may be useful for screening kala-azar patients and could be applied in the diagnosis of VL. It is a functional assay that warrants a larger study with a larger sample size for the purpose of evaluating the utility of the test in diagnosing visceral leishmaniasis. There is dire need to identify non-invasive, less risky and field adapted point of care diagnostics for VL.

Keywords: Kala-azar; Visceral Leishmaniasis; Diagnosis; KATEX; Latex; Buccal swab antigen detection.

1. Introduction

Visceral leishmaniasis (VL) or kala-azar is a chronic protozoan infection in humans associated with significant global morbidity and mortality. The etiological agent is a haemoflagellate protozoan *Leishmania donovani*, an obligate intracellular parasite that resides and multiplies within macrophages of the reticulo-endothelial system. Over 90% of cases are found in three regions, Sudan /Ethiopia/ Kenya, India /Bangladesh Nepal, and Brazil with as many as 100,000 deaths every year. In Kenya, Visceral leishmaniasis (VL) is endemic in Kitui, Machakos, West Pokot, Meru, Turkana and Baringo Counties. In Baringo, the disease has a focal distribution in the dry, hot areas (Schafer *et al.,* 1995).The symptoms of VL include prolonged fever, anaemia, splenomegaly, pancytopenia, and weight loss/wasting, and are often mimicked by malaria, enteric fever, and tuberculosis, all widely prevalent in the VL-endemic regions. Therefore proper diagnostic methods for VL must be available, accurate, simple and affordable for the population for which they are intended.

1.2 Diagnosis of Visceral Leishmaniasis

VL is diagnosed by direct Microscopy of splenic aspirate, Direct Agglutination Test and rK39-Based Immunochromatographic Test which detects presence or absence of antibodies and Molecular Detection Test which detects parasite DNA. According to Kenyan national guidelines for VL, demonstration of parasite amastigotes by microscopic examination of smears from splenic aspirates is needed to confirm diagnosis and initiate treatment. Splenic aspiration is an invasive procedure that is highly sensitive (93.1–98.7%) but carries a small but significant risk of major bleeding. It should be performed by experienced clinicians in reference

hospitals or research centers, and is therefore not suitable for use in first-line health services or district hospitals. Blood transfusion must be locally available in case of need. In addition, splenic aspiration is not possible in non-cooperative children, difficult in those with mild splenomegaly, and contra-indicated in persons with active bleeding, thrombocytopenia, severe anemia, as well as in pregnant and moribund patients. Identification of amastigotes and grading in stained smears of splenic aspirates requires expertise and well-trained microscopists (Jane Mbui *et al*.2013). Direct Agglutination Test, a semi-quantitative test for detection of antibodies showed sensitivity and specificity estimates of 94.8% (95%CI: 92.7-96.4) and 97.1% (95%CI: 93.9-98.7), respectively (Chappuis *et al*. 2006b). rK39-based ELISA showed excellent sensitivity (93 - 100%) and specificity (97-98%) in numerous VL endemic countries (Badaro *et al*.1996, Braz *et al*. 2002, Burns, Jr. *et al*. 1993, Kurkjian *et al*. 2005, Maalej *et al.* 2003). Further validation studies of rK39 ICT showed sensitivity and specificity estimates of 93.9% (95%CI: 87.7-97.1) and 95.3% (95%CI: 88.8-98.1), respectively (Chappuis *et al.* 2006b). Recently, the excellent diagnostic performance of rK39 ICT was confirmed in India and Nepal (Chappuis *et al*. 2006a; Sundar *et al*. 2006a; Sundar *et al*. 2006b; Sundar *et al*. 2007). KATEX test, the only leishmanial antigen detection-based test currently available which detects leishmanial antigen in urine was developed. Several studies conducted in East Africa and the Indian subcontinent showed good specificity but low-to-moderate sensitivity (48-87%) (Chappuis *et al*. 2006a, Rijal *et al*. 2004, Sundar *et al*. 2005, Sundar *et al*. 2007).

However, these tests have their limitation which includes invasiveness in obtaining Splenic Aspirate, Antibody based tests cannot differentiate active from past infection in addition to rK39 ICT being less accurate as shown in studies in East Africa (Boelaert M *et al*. 2008;Diro et al. 2007;Ritmeijer *et al*. 2006;Veeken *et al*. 2003;Zijlstra *et al*. 2001).,antigen detection in urine has low sensitivity while Molecular tests remain restricted to referral hospitals and research laboratories due to their complexity, cost and need of skilled personnel therefore need to evaluate non-invasive and easy to use diagnostic tests such as leishmania antigen detection in buccal cells which can serve as an alternative test.

3.0 Materials and Methods.

3.1 Study Objectives

This was a pilot study to explore the ability to use KATEX, a simple non-invasive diagnostic test to detect leishmania antigens in buccal swabs, determine the ability of the kit to detect leishmania antigens in buccal cells of kala-azar patients and compare the sensitivity and specificity of KATEX - buccal assay using microscopy as the gold standard.

3.2 Study site

The study was conducted at Kimalel Health Centre in Baringo county, treatment center, located in the Rift Valley province of Kenya. It is situated 280 km north of Nairobi County and located 10 km from Marigat township along Marigat-Kabarnet road. The area is endemic for Kala-azar, a fatal disease if untreated. Rainfall is sparse and the area is semi-arid low land**.** The Ethnicity comprises of Tugens, Njemps and pokots. The villages on the borders of Kimalel include Koriema, Patkawanini on the north and Kimorok on the south. The physical facility comprise outpatient lounge, kala-azar ward, delivery/maternity ward, kitchen, newborn ward, laboratory, minor theatre, pharmacy, store, staff houses and an ambulance.

3.3 Study population

The study population comprised of patients who were evaluated at the KEMRI clinical research station at Kimalel Health Centre in Baringo County, a treatment Centre for VL in Kenya. VL clinical suspect patients, defined by a history of fever 14 days with clinical splenomegaly and endemic controls, defined as patients presenting to the health center with no fever and no prior history of Kala azar but living in VL endemic aged between 5 to 60 years were enrolled between March 2015 to July 2016. For precision purposes, cases and controls were matched.

3.3.1 Inclusion

Male / female aged 5 to 60 years patients presenting with VL symptoms and a positive splenic aspirate test by microscopy, patients with no fever and no prior history of Kala azar but living in VL endemic and willing to sign informed consent.

3.3.2 Exclusion criteria

Male / female aged below 5 years and above 60 years and unwilling to sign informed consent.

3.4 Study design

This was a cross-sectional study of individuals who were newly diagnosed of *L. donovani* by microscopy of graded splenic aspirate and those presenting to the health centre with no fever and no prior history of Kala azar but living in the endemic area.

3.5 Sample size

The Sample size was attained by the formula; n= Z2 p (1-p)/e2 (Bernard Roster: Fundamentals of Biostatistics, Sixth Edition, pages 416-420) where Z is the value (1.96) corresponding to 95% confidence level, assuming p is the estimated proportion (50%) of confirmed VL patients by microscopy who test positive with the buccal swab kit, and e is the acceptable margin of error (5%). Therefore the sample size n=384. Considering that, the maximum number of VL cases by microscopy seen at Kimalel (N~90) in a year is less than 10 times the calculated sample size of 384 which is 3,840, a corrected maximum sample size to be attained was calculated using the finite population correction (FPC) and the new maximum sample size n' given by the formula n'=n/ (1+ (n/N) was approximately 73VL cases and 73 VL controls in ratio of 1:1. For precision purposes and according to the sample size calculation, the ratio of cases to controls was 1:1. ; thus the study targeted a maximum of 73 VL cases and 73 VL controls aged between 5 and 60 years of age in order to achieve at least 30 participants each for cases and controls.

3.6 Specimen Collection

3.6.1 Buccal swab collection procedure.

The buccal swabs were collected from confirmed VL cases by microscopy and controls using a sterile buccal swab according to standard operating procedure (SOP) following manufacturers' instructions. Briefly it was removed from the packaging without handling the actual swab then cheek cells collected from the subjects mouth by rolling the tip of the collection swab firmly for about 20 seconds on the inside of one cheek several times in each direction. The Specimens were briefly air dried then placed directly into the receptacle which contained a desiccant for proper drying of the swab to avoid other microbes.

3.6.2 Testing procedure.

This entailed, two step methods that involved the harvesting of the antigens from the buccal swab and their detection using the KATEX kit as described by *Attar et al., 2000* as described below:

1. **Heat Induced Epitope Retrieval.**

To expose the leishmania antigens from the buccal cells, 100 - 500 µL of citrate buffer PH 6.0 was dispensed in a 2 mL eppendorf tube; the swab was placed in the tube and rotated at least five times. The swab was pressed against the side of the tube to ensure that most of the liquid remained in the tube. The tube with retrieved antigens was then vortexed for 60 seconds and heated at 98°C for 5 minutes then allowed to cool at ambient temperature before carrying out the test.

2. **KATEX kit testing procedure.**

All reagents stored at 4°C were brought to ambient temperature. The latex particles were shaken immediately before use. Fifty (50) µL of the retrieved antigens were dispensed on to a reaction zone on the glass slide and one drop of test latex added .This was stirred to obtain a homogenous mixture which covered the whole surface of the reaction zone. The glass slide was tilted with a rotating action in clockwise and anticlockwise directions continuously for two minutes, and the results (agglutination or no agglutination) read.

Quality assurance

A one day protocol training workshop was organized at the Kimalel Health Centre in Baringo during the study preparation phase. Standard Operating Procedures were prepared and clinicians trained on collection of buccal swabs .Laboratory technicians had refresher training on reading splenic aspirates. Leishmania culture supernatant diluted in saline as a positive control and buffered saline as a negative control (both were preserved with sodium azide and ready to use) were incorporated when carrying out the Katex test.

Ethical considerations

The study was conducted after Ethical clearance was sought through the KEMRI Ethics Committee. Detailed information was made available for potential study participants in their language. A consent form was completed only after the patient had understood the points enumerated in the information sheet. Eligible patients were included after signing the informed consent form (or parent/guardian's for minors).Patients diagnosed with VL were treated according to the Kenyan VL national guidelines. Standard clinical care and free provision of VL diagnostic tests and drugs to all patients (whether or not enrolled in the study) were guaranteed by the participating centers.

Data management and statistical analysis

The Results of both the microscopic examination of spleen aspirate and buccal swabs and demographics were recorded in log books and later entered in Microsoft excel. A review on completeness of the data was performed with consistency checks done on the data set. Any deviations were counter checked against the log books which were the source documents. Data analyses was done using STATA software version 13.2 standard edition (*http://www.stata.com*). Continuous data have been summarized using mean and standard deviation or median and inter-quartile range where appropriate while binary data have been summarised using proportions. The calculations for specificity (defined as the number of individuals with a negative test among the total number of individuals with a negative reference standard) and sensitivity (defined as the number of individuals with a

positive test among the total number of individuals with a positive reference standard) were also calculated. The usefulness of these tests (sensitivity and specificity) was estimated using positive diagnostic likelihood ratio (DLR+) which is calculated as (Sensitivity/1-Specificity) as a proxy determinant of how useful a test is with a value further away from +1 implying more usefulness of the test while a value of +1 indicating that the test is not useful. The parameter estimates for both the specificity and sensitivity have been presented alongside their binomial exact 95% confidence intervals.

All the buccal swabs from both cases (VL splenic aspirate positive patients by microscopy) and endemic controls were tested using KATEX. The results were recorded as positive when agglutination was observed or negative when no agglutination was observed.

4.0 Results

A total of 88 patients aged between 5–60 years, including 44 VL and 44 non-VL patients were included in the study between March 2015 to July 2016. All the buccal swabs from both cases (VL splenic aspirate positive patients by microscopy) and endemic controls were tested using KATEX. Of the 44 cases, 34 were male and out of this, 27 samples were true positive (79.4%) while 7 were False Negative (20.6%) compared to 10 females who had 9 true positives (90%) and 1 false negative (10%) reflecting 75 % of males who were true positive against 25% females and 87.5% of males who were false negatives against 12.5% of females. Of the 44 endemic controls, 26 were male and out of this, 21 were true negative (80.8%) with 5 samples turning to be false positive (19.2%) compared to 18 females who had 14 true negatives (77.8%) and 4 false positives (22.2%). By gender, overall male group n = 60(68.2%) with VL cases representing 34(77.3%) and Controls 26(59.1%) while females n= 28(31.8%) with VL cases representing 10(22.7%) and Controls 18(40.9%). By age, the VL cases; Mean (SD) was 17(11) with the median (IQR) being 15(10 to 22) while for Controls, Mean (SD) was 26(15) with the median (IQR) being 28(13 to34) with overall Mean (SD) of 22(14) and IQR Mean (SD) of 18(11 to 28) as summarized in table 4.1.

Table 4.1: Comparison of Demographics between VL Cases and Controls.

		Overall, n=88	VL cases n=44	Controls n=44
Age (years)	Mean (SD)	22 (14)	17 (11)	26 (15)
	Median (IQR)	18 (11 to 28)	15 (10 to 22)	28 (13 to 34)
Gender	Male, n (%)	60 (68.2%)	34 (77.3%)	26 (59.1%)
	Female, n (%)	28 (31.8%)	10 (22.7%)	18 (40.9%)

Abbreviations: IQR – Interquartile Range; SD – Standard deviation; VL – Visceral leishmaniasis.

Of the 44 VL Cases included in the analysis, 36 had a positive splenic aspirate (WHO parasite grading: 1–2: n= 4; 3–4: n= 20; 5–6: n =12) detected by KATEX while 8 (WHO parasite grading 1-2: n=5, 3-4: n=3 and 5-6: n=0) were not detected by KATEX as summarized in table 4.2.

Table 4.2: Comparison between grading and KATEX

		KATEX		
		+ve(Agglutination)	-ve(No-Agglutination)	
Grading	1+	0	5	5 (11.4%)
	2+	4	0	4 (9.1%)
	3+	10	3	13 (29.5%)
	4+	10	0	10 (22.7%)
	5+	7	0	7 (15.9%)
	6+	5	0	5 (11.4%)

Compared to microscopy, the buccal swabs from 44 cases of confirmed VL patients were positive by KATEX in 36 patients (sensitivity = 81.8%; 95%CI: 67.3% to 91.8%).Out of 44 endemic controls, 35 were negative (specificity = 79.5%; 95%CI: 64.7 –90.2%) with a Positive predictive value (Defined as the number of individuals with a positive reference standard among the total number of individuals with a positive test) n= 36(80.0%); 95%CI: 65.4% to 90.4% and Negative predictive values (Defined as the number of individuals with a negative reference standard among the total number of individuals with a negative test) n = 35(81.4%); 95% CI: 66.6% to 91.6%. The results are summarized in Table 4.3

Table 4.3 Sensitivity, Specificity, Positive and Negative predictive values of KATEX Screening in Buccal Swabs for VL and Non VL in Kimalel health center, Baringo County, Kenya.

	Index test result	VL cases, n=44	Non VL n=44	Sensitivity [95% C.I]	Specificity [95% C.I]	PPV [95% C.I]	NPV [95% C.I]	DLR+
KATEX	Positive	36	9	81.8% [67.3% to 91.8%]	79.5% [64.7% to 90.2%]	80.0% [65.4% to 90.4%]	81.4% [66.6% to 91.6%]	4.0
	Negative	8	35					

Abbreviations: PPV = positive predictive value, NPV = negative predictive value, CI = Confidence Interval, DLR+ = positive Diagnostic Likelihood Ratio; VL – Visceral leishmaniasis.

5.0 Discussion

The lack of access to good quality diagnostic tests for infectious diseases contributes to the enormous burden of ill health in the developing world, where infectious diseases are the major causes of death. Good diagnostic tests that are fit for purpose and provide accurate results are therefore of paramount quality importance in reducing the burden of infectious diseases (Rosanna *et al.,* 2010). Demonstrating the presence of the infecting organism, or a surrogate marker of infection, is often crucial for effective clinical management and for selecting other appropriate disease control activities. To be useful, diagnostic methods must be accurate, simple and affordable for the population for which they are intended. They must also provide a result in time to institute effective control measures, particularly treatment. For some infections, early diagnosis and treatment can have an important role in preventing the development of long-term complications or in interrupting transmission of the infectious agent. In a broader context, diagnostic tests can have multiple uses, including: patient management, especially when clinical symptoms are not specific for a particular infection (as is often the case); screening for asymptomatic infections; surveillance; epidemiological studies (for example, rapid assessments of disease burden or outbreak investigations); evaluating the effectiveness of interventions, including verification of elimination; and detecting infections with markers of drug resistance (TDR Diagnostics Evaluation Expert Panel.,2010).The commonly used method for diagnosing visceral leishmaniasis (VL) has been the demonstration of parasites (amastigotes) in splenic or bone marrow aspirate, which is invasive and examining by direct microscopy. Detection of antigens in patient serum is also done but this is made complex by the presence of high levels of antibodies, circulating immune complexes (CIC) and auto-antibodies, all of which may mask immunologically important antigenic determinants or competitively inhibit the binding of antibodies to free antigen. This may explain why no antigen detection assay for VL is routinely in use to-date, despite a number of reports describing the existence of circulating antigens and immune complexes in VL (Sehgal *et al.,* 1982; Galvao-Castro et al., 1984; Azazy *et al*., 1994., 1997). Antibody based tests may yield positive results long time after complete cure. The KATEX test is based on the detection of a leishmanial antigen in urine. This test is useful in difficult field conditions because it is simple, easy to perform, does not require any equipment, and is read visually (Attar *et al.,* 2001). In this study, KATEX kit was used to detect VL antigens in buccal swabs and the results compared using microscopic examination of spleen aspirate, one of the recommended reference standards for VL diagnostic evaluation studies (Boelaert *et al.,* 2007). In its present format, the test is simple to use, economical and robust: its two main advantages are the fact that the performance of the test does not require sophisticated electric appliance and that most technicians can perform the test with ease (Zamil *et al.,* 2001). After the swab was placed in the tube, rotated for at least five times before pressing the swab against the side of the tube to ensure that most of the liquid remained in the tube, the tube was then vortexed for 60 seconds and heated at 98^{o}C for 5 Minutes to ensure specificity. The KATEX kit was able to detect Visceral Leishmania antigens from the buccal swabs giving a sensitivity of (81.8%; 95%CI: 67.3% to 91.8%, specificity of (79.5%; 95%CI: 64.7 –90.2%), Positive predictive value n= 36(80.0%); 95%CI: 65.4% to 90.4% and Negative predictive values n = 35(81.4%); 95% CI: 66.6% to 91.6% as shown in table 4.3. The test being evaluated in this study performed literally well as microscopy, the accepted gold standard for the diagnosis of leishmaniasis.

5.1 Conclusion

The diagnosis of Visceral Leishmaniasis (VL) remains a great challenge, especially in endemic areas due to lack of skilled personnel and limitation of the available methods. The demonstration of the amastigote forms of leishmanial parasites from splenic aspirations is considered to be the reference standard for VL diagnosis, due to its high sensitivity and specificity; nevertheless, it is an invasive life threatening method. Molecular methods have been successfully used in VL diagnosis, demonstrating high sensitivity and specificity. However, their implementation poses a challenge because they are not cost-effective; they need well-established laboratories, trained and skilled staff. The sensitivity and specificity of serological methods, such as DAT, IHA, IFAT, ELISA and ICTs may be variable in different endemic areas worldwide. However, their main drawback is that, antileishmanial antibodies may be detectable for a long period after recovery thus the need to use non-invasive and cost-effective antigen diagnostic test which can be used by less skilled personnel in the diagnosis of VL in the endemic areas. KATEX kit was used as a screening tool to detect Visceral Leishmania antigens from buccal swabs and compared to microscopy in a group of 44 participants with clinical manifestations suggestive of Visceral leishmaniasis and 44 endemic controls. KATEX kit was able to detect Visceral Leishmania antigens from the buccal swabs giving a sensitivity of 81.8% and specificity of 79.5%. This pilot study suggests that, after further evaluation, buccal swab test using KATEX a non-invasive antigen detection test may be useful for screening active kala-azar patients.

5.2 Recommendations

Based on the results of this study, further validation and evaluations using ‘convenience’ samples, followed by evaluations in populations of intended use needs to be carried out. These trial results can be used to demonstrate

the utility and potential impact of the diagnostic test and obtain data for regulatory submission and approval so that the test can be recommended for use in the country.

5.3 Study Limitations

Difficult to distinguish between weakly positive from negative results, affecting the test's reproducibility.

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References

Attar, A.J., et al. (2001). Latex test for the detection of urinary antigens in visceral leishmaniasis. *Acta Tropica*, 78,11-16.

Azazy, A.A., Devaney E., Chance M.L. (1994). A PEGELISA for the detection of Leishmania donovani antigen in circulating immune complexes. *Trans. R. Soc. Trop. Med. Hyg*, 88 ,62–66.

Azazy, A.A., Chance M.L., Devaney E. (1997). A time-course study of circulating antigen and parasite-specific antibody in cotton rats infected with Leishmania donovani. *Ann. Trop. Med. Parasit*, 91,153–162.

Badaro, R., Benson, D., Eulalio, M.C. et al. (1996). rK39: A cloned antigen of Leishmania chagasi that predicts active visceral leishmaniasis. *Journal of Infectious* Diseases, 173, 758-761.

Boelaert, M., Bhattacharya, S., Chappuis, F., El Safi, S., Hailu, A., et al. (2007). Evaluation of rapid diagnostic tests: Visceral leishmaniasis. *Nature Microbiology Reviews*, 5, 30–39.

Braz, R. F., Nascimento, E. T., Martins, D. R. et al. (2002). The sensitivity and specificity of Leishmania chagasi recombinant K39 antigen in the diagnosis of American visceral leishmaniasis and in differentiating active from subclinical infection. *Am J Trop Med Hyg*, 67,344-348.

Burns, J. M., Jr., Shreffler, W. G., Benson, D. R., Ghalib, H. W., Badaro, R. and Reed, S. G. (1993). Molecular characterization of a kinesin-related antigen of Leishmania chagasi that detects specific antibody in African and American visceral leishmaniasis. *Proc Natl Acad Sci U S A*, 90,775-779.

Chappuis, F., Rijal, S., Jha, U. K. et al. (2006a). Field validity, reproducibility and feasibility of diagnostic tests for visceral leishmaniasis in rural Nepal. *Tropical medicine & international health*, 11, 31-40.

Chappuis, F., Rijal, S., Soto, A., Menten, J. and Boelaert, M. (2006b). A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. *BMJ*, 333,723.

Diro, E., Techane, Y., Tefera, T., et al. (2007). Field evaluation of FD-DAT, rK39 dipstick and KATEX (urine latex agglutination) for diagnosis of visceral leishmaniasis in northwest Ethiopia. *Trans R Soc Trop Med Hyg*, 101,908-914.

Galvao-Castro B., Ferreira J.A., Marzochi K.F., Marzochi M.C., Coutinho S.G., Lambert P.H. (1984). Polyclonal B cell activation, circulating immune complexes and autoimmunity in human American visceral leishmaniasis. *Clin. Exp. Immunol* ,56, 58–66.

http://www.stata.com

Jane, M., Monique, W., Manica, B., Adrian, L., Rashid, J., Simon, N., George, K., Mark, R., Roberto, T., and Joke, P., Raymond, O., Francois, C., (2013). Validation of Two Rapid Diagnostic Tests for Visceral Leishmaniasis in Kenya

Kurkjian, K.M., Vaz., L. E., Haque., R. et al. (2005). Application of an improved method for the recombinant k39 enzyme-linked immunosorbent assay to detect visceral leishmaniasis disease and infection in Bangladesh. *Clin Diagn Lab Immunol*, 12, 1410-1415.

Maalej, I. A., Chenik, M., Louzir, H. et al. (2003). Comparative evaluation of ELISAs based on ten recombinant or purified Leishmania antigens for the serodiagnosis of Mediterranean visceral leishmaniasis. *Am J Trop Med Hyg*, 68,312-320.

Ritmeijer, K., Melaku, Y., Mueller, M., Kipngetich, S., O'keeffe, C., and Davidson, R.N. (2006). Evaluation of a new recombinant K39 rapid diagnostic test for Sudanese visceral leishmaniasis. *American Journal of Tropical Medicine and Hygiene*, 74, 76-80.

Rosanna, W. P., Peter, G. S., Patrick, M. M., and Bossuyt. (2010). A guide for diagnostic evaluations, S2.

Schafer, K. U., Kurtzhals, J. A., Sherwood, R. D., Noyes, H., Ponce, C., Belli, A., Arana, B., Zeledon, R., and Sousa, O. E.(1995). Molecular techniques in the characterization of leishmaniasis isolate from central. *America Annual tropical medicine and parasitology,* 89(1), 31-36.

Sehgal S., Aikat., B.K., Pathania A.G.S. (1982). Immune complexes in Indian kala-azar. Bull. World Health Organization, 60: 945–949.

Sundar, S., Agrawal, S., Pai, K., Chance, M. and Hommel, M. (2005). Detection of leishmanial antigen in the urine of patients with visceral leishmaniasis by a latex agglutination test. *American Journal of Tropical Medicine and Hygiene*, 73,269-271.

Sundar, S., Singh, R. K., Bimal, S.K. et al. (2007). Comparative evaluation of parasitology and serological tests in the diagnosis of visceral leishmaniasis in India: a phase III diagnostic accuracy study. *Tropical Medicine and International Health*, 12,284-289.

Sundar, S., Maurya, R., Singh, R. K. et al. (2006a). Rapid, noninvasive diagnosis of visceral leishmaniasis in India: comparison of two immunochromatographic strip tests for detection of anti-K39 antibody. *J Clin Microbiol*, 44,251-253.

Sundar, S., Singh, R. K., Maurya, R. et al. (2006b). Serological diagnosis of Indian visceral leishmaniasis: direct agglutination test versus rK39 strip test. *Trans R Soc Trop Med Hyg*, 100,533-537.

The TDR Diagnostics Evaluation Expert Panel. (2010). Evaluation of diagnostic tests for infectious diseases: general principles. *Nature Reviews for Microbiology*, S17.

Veeken, H., Ritmeijer, K., Seaman, J. and Davidson, R. (2003). Comparison of an rK39 dipstick rapid test with direct agglutination test and splenic aspiration for the diagnosis of kala-azar in Sudan. *Tropical Medicine and International Health*, 8,164-167.

Zamil J. Attar., Michael L. Chance., Sayda el-Safi., James Carney., Ahmed Azazy., Maha El-Hadi.,Cibele Dourado., and Marcel Hommel (2001). Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. *Acta Tropica*, 78, 11–16.

Zijlstra, E. E., Nur, Y., Desjeux, P., Khalil, E. A., el Hassan, A. M. and Groen, J. (2001). Diagnosing visceral leishmaniasis with the recombinant K39 strip test: experience from the Sudan. *Tropical Medicine and International Health*, 6,108-113.