Evaluation of rK39 strip test for the diagnosis of visceral leishmaniasis in infants

A. Alborzi,[1] M. Rasouli,[1] Z. Nademi,[1] M.R. Kadivar[1] and B. Pourabbas[1]

تقييم اختبار أشرطة rk 39 لتشخيص داء الليشمانيات الحشوي لدى الرضَّع

عبد الوهاب البُرزي، منوجهر رسولي، زهرة نديمي، محمد رحيم كديَور، بهمن بور عباس

الخلاصة: تم في هذه الدراسة تقييم حساسية ونوعية أشرطة الاختبار rk 39 بالمقارنة مع اختبار الأضداد المناعية المتألقة والفحص المجهري للُّطاخات المهيَّأة من رُشَافات نقي العظم (المعيار الذهبي) لدى 47 طفلاً يشتبه بإصابتهم بداء الليشمانيات الحشوي. كما تم اختبار مجموعة شاهدة من الأطفال الذين شُخِّصت حالتهم بأمراض أخرى (السل، أو داء المقوسات، أو الذئبة الحمامية المجموعية، أو الملاريا، أو داء الليشمانيات الجلدي) للتحقُّق من النتائج الإيجابية الكاذبة. وقُدِّرت حساسية اختبار الأشرطة بنحو 82.4٪ ونوعيتها بـ 100٪، بالمقارنة بنحو 100٪ لحساسية اختبار الأضداد المناعية المتألقة و92.7٪ لنوعيتها، مما يشير إلى موثوقية اختبار أشرطة rk 39 في حالة صعوبة الوصول للمرافق المختبرية.

ABSTRACT This study estimated the sensitivity and specificity of the rK39 strip test compared with the immunofluorescent antibody test and microscopy of bone marrow aspirate smears (the gold standard) in 47 children with suspected visceral leishmaniasis. A control group of children with other diagnoses (tuberculosis, toxoplasmosis, systemic lupus erythematosus, malaria or cutaneous leishmaniasis) were also tested to check false positive results. The sensitivity and specificity of the strip test were 82.4% and 100% and that of immunofluorescent antibody were 100% and 92.7%. The rK39 strip test is reliable where there is no access to laboratory facilities.

Évaluation du test sur bandelette réactive au rK39 pour le diagnostic de la leishmaniose viscérale chez le jeune enfant

RÉSUMÉ Cette étude a estimé la sensibilité et la spécificité du test sur bandelette au rK39 par rapport à la recherche des anticorps par immunofluorescence et à l'examen microscopique de frottis médullaires (la méthode de référence) chez 47 enfants suspects de leishmaniose viscérale. Un groupe témoin d'enfants ayant d'autres diagnostics (tuberculose, toxoplasmose, lupus érythémateux systémique, paludisme ou leishmaniose cutanée) a également été soumis à un test pour vérifier les résultats faux positifs. La sensibilité et la spécificité des bandelettes réactives étaient de 82,4 % et de 100 % et celles de la recherche des anticorps par immunofluorescence étaient de 100 % et de 92,7 %. Le test sur bandelette au rK39 est fiable en l'absence de moyens de laboratoire.

[1]Clinical Microbiology Research Centre, Nemazee Hospital, Shiraz, Islamic Republic of Iran (Correspondence to A. Alborazi: cmrc@sums.ac.ir).
Received: 01/03/04; accepted: 06/07/04

Introduction

Visceral leishmaniasis is caused by various strains of *Leishmania* spp. [*1*]. While a minority of infected individuals develops full-blown visceral leishmanaisis, characterized by fever, hepatosplenomegaly, anaemia, neutropaenia and hypergammaglobulinaemia, most of them remain asymptomatic with the disease self-limiting [*2,3*]. Visceral leishmaniasis is endemic in the south of the Islamic Republic of Iran, especially in Fars province where it involves mostly infants [*4*]. The disease is observed mainly among nomads and in rural areas that are far from equipped medical centres. Therefore, there is a need for a non-invasive, cost-effective, reliable, easily available and fast method of diagnosis of visceral leishmaniasis in these regions.

For decades, definite diagnosis of visceral leishmaniasis has required invasive procedures to find the parasite in the organs, such as spleen, bone marrow, lymph nodes and liver [*5*]. Non-invasive serological methods such as immunofluorescent antibody (IFA) and enzyme-linked immunosorbent assay (ELISA) are sensitive methods of diagnosing visceral leishmaniasis [*6–8*], but they require fluorescent microscopy and ELISA equipment. Samples for direct agglutination test (DAT) can be easily obtained, but must be sent to distant medical centres [*9*]. Polymerase chain reaction (PCR) amplification testing for leishmania DNA is also not practical in the field [*10,11*].

It has been shown that detection of circulating antibody to *Leishmania* antigen rK39 had both high sensitivity and specificity for active Indian visceral leishmaniasis, which mostly affects adults [*12,13*]. The rK39 strip-test is a simple and cost-effective test that uses chromatographic strips impregnated with rK39 antigen and can be easily used in the field. While previous studies have shown the efficacy of this test, their study groups were mainly adults [*14*]. The present study was made on infants, who are more commonly affected by visceral leishmaniasis in the Islamic Republic of Iran due to infection with *L. infantum*. The aim was to compare the sensitivity and specificity of the rK39 strip test with that of the IFA test (the usual laboratory method at this centre) in the detection of visceral leishmaniasis.

Methods

The study group consisted of 47 children, age range 3 month to 5 years old, who were admitted to 2 teaching hospitals at Shiraz University of Medical Sciences over a 1-year period (2003). The inclusion criteria were patients with hepatosplenomegaly, fever, anaemia (with or without neutropaenia) or hypergammaglubolinaemia who had been referred from endemic regions. The following data were recorded: age, sex, place of residence, history of visceral leishmaniasis in the area, adjacent health centre and grandfather's name. Informed consent was obtained from the parents of all patients before the study started.

The study group was tested for visceral leishmaniasis by 3 different methods: light microscopy of bone marrow aspirate smears, and IFA and rK39 strip tests on serum samples. Bone marrow aspiration was taken as the gold standard.

We followed all 47 patients monthly to find out the time of the disappearance of the anti-rK39 antibody after treatment, but only 13 of them finished the 6-month follow-up. We repeated the test for those who were positive with the rK39 strip test in order to identify the time of the disappearance of anti-rK39 antibody.

A control group of 161 children with other diagnoses were selected: tuberculosis (positive sputum test for mycobacterium tuberculosis); toxoplasmosis (clinically and serologically positive); systemic lupus erythematosus (clinical evaluation and laboratory findings from antinuclear antibody and LE cell test); malaria (positive peripheral blood smear); or cutaneous leishmaniasis (smear-positive for leishman bodies). The control group were patients with a positive record of those diseases who did not have visceral leishmaniasis and did not live in an endemic area of leishmaniasis. The IFA test was used on serum samples of all 161 control children: 40 with toxoplasmosis, 56 with systemic lupus erythematosus, 25 with malaria and 40 with tuberculosis. Due to supply difficulties, the rK39 strip test was used on samples from only 137 of the children: 32 with tuberculosis, 40 with toxoplasmosis, 20 with systemic lupus erythematosus, 5 with malaria and 20 with cutaneous leishmaniasis.

Laboratory methods

For the confirmatory tests, bone marrow aspirated smears were stained with Giemsa and examined using light microscopy.

For IFA, the promastigotes of *Leishmania infantum* were coated onto slides and after drying at room temperature, preserved at –70 ºC. Serum at various dilutions was added and the slides were incubated at room temperature. After washing with phosphate-buffered saline, conjugated anti-human globulin was added and after further incubation and washing, the slides were studied under fluorescent microscope. A titre ≥ 1:128 was considered as positive.

For the rK39 strip test, nitrocellulose strips impregnated with recombinant rK39 antigen (InBios International, Seattle, Washington, USA). One drop of peripheral blood (or finger-stick blood), serum or plasma was applied at the base of nitrocellulose strips. After being air-dried, 3 drops of the test buffer (phosphate-buffered saline, plus bovine serum albumin) were added and the strip was placed upright for 60 seconds. The appearance of a lower red band (control) indicated the proper functioning of the test, while the appearance of an upper red band indicated the presence of anti-rK39 IgG signifying a positive test.

Analysis

The data were analysed using *SPSS* software and compared using Fisher's exact test. The sensitivity of the tests was calculated as 100 × [TP/(TP +FN)] and specificity as 100 × [TN/(FP +TN)], where TP = true positives, TN = true negatives, FP = false positives and FN = false negatives.

Results

Visceral leishmaniasis was confirmed in 17 out of 47 study patients who were positive using bone marrow aspiration. All 17 patients were also positive by the IFA test, giving a sensitivity of 100% for IFA (Table 1). Of the 17 patients, 14 (82.4%) were positive for rK39 strip test and 3 were negative,

Table 1 **Comparison of the immunofluorescent antibody test and the rK39 strip test for visceral leishmaniasis in 17 patients with confirmed visceral leishmaniasis and 161 control patients with other diseases**

Group	rK39 positive	rK39 negative
VL confirmed cases		
IFA positive	14	3
IFA negative	0	0
Controls		
IFA positive	0	10
IFA negative	0	127

giving a sensitivity for the rK39 strip test of 82.4%.

The control group was used to test for false positives. Ten out of the 137 control group patients were positive for IFA test (9 of them had tuberculosis involvement and 1 had systemic lupus erythematosus). No positives cases were detected with the rK39 test. Thus, the specificities of the rK39 strip test and IFA were 100% and 92.7% respectively.

Statistical analysis did not show any significant differences between the sensitivity of rK39 and IFA test (Fisher's exact test, $P = 0.227$) while there was a significant difference between the specificity of rK39 and IFA test (Fisher's exact test, $P = 0.002$).

All positive patients were treated with meglumine antimoniate, as amphoteracin B is not routinely used in our centre except for special cases such as jaundice, ascitis and patients in a poor condition. We performed follow-up rK39 strip tests for several months on 13 patients who completed the treatment and showed signs of being cured (i.e. were afebrile after 6 months). Out of them, 8 patients had a negative rK39 strip test before 3 months, 3 cases had a positive rK39 test after 3 but before 6 months and in only 2 patients (15.3%) the test remained positive for more than 6 months.

Discussion

The present study shows that the rK39 strip test is a reliable test for the diagnosis of visceral leishmaniasis. Our data showed no false positives by the rK39 strip test that would not also have been false positive by IFA, which is the currently used test in our centre. Thus the specificity for the diagnosis of visceral leishmaniasis using rK39 was 100%. This specificity figure is the same as figures reported by Bern et al. [*15*] (100%), Iqbal et al. [*16*] (100%) and Sunder et al. [*14*] (98%) who were working in Nepal, Kuwait and India, respectively. However, Sundar et al. [*17*] reported 99% specificity of rK39 in 100 cases in Indian patients. The specificity of IFA in our study was 92.7%, which is similar to the results of a study conducted in Kuwait (93%) [*16*]. The estimated sensitivity of the rK39 strip test was 82.4%, which is in agreement with Iqbal et al. [*16*] (80%) from Kuwait but not with Bern et al. [*15*] (100%), and Sundar et al. [*14*] (100%).

The striking similarities between our results and those of Iqbal et al. [*16*] may be explained by the causative agent of visceral leishmaniasis. *L. infantum* is responsible in most of the cases of visceral leishmaniasis seen in the Middle East, while *L. donovani* is the major cause of visceral leishmaniasis in India. The sensitivity of IFA in our study (100%) was higher than that of Iqbal [*16*] (86.6%).

We tested the 13 apparently cured visceral leishmaniasis patients with strip test during their monthly follow-up. A positive strip test after 6 months was only seen in 2 patients while the rest of them showed a negative strip test before 6 months. Our study group showed a negative strip test after successful treatment earlier than Zijlstras et al. [*18*], suggesting that anti-rK39 antibodies persist for several months. Hence, the rK39 strip test is not suggested for following up the disease.

In many developing countries, visceral leishmaniasis occurs in distant urban and tribal regions, where there are no proper laboratory facilities.

Conclusion

This study shows that the rK39 strip test is a sensitive and specific indicator of visceral leishmaniasis in children. About 18% of infected patients showed false negative results of rK39 and for those suspected patients with negative results other sensitive diagnostic methods should be performed in a well-equipped medical centre. However, the specificity of the rK39 strip test was 100%, which means that all positive results can be considered as visceral leishmaniasis.

The rK39 strip test can be readily used under field conditions and needs only one drop of peripheral blood. Clinicians can perform it independently without laboratory facilities and treat patients without any requirement to refer them to equipped hospitals or clinics. Our results confirm that the rK39 strip test is suitable for use in this region and other developing countries for the diagnosis of visceral leishmaniasis in infants.

References

1. *Control of the leishmaniases. Report of a WHO Expert Committee.* Geneva, World Health Organization, 1990 (WHO Technical Report Series, No. 793).
2. Badaro R et al. New perspectives on a subclinical form of visceral leishmaniasis. *Journal of infectious disease*, 1986, 154: 1003–11.
3. Evans T et al. American visceral leishmaniasis (kala-azar). *Western journal of medicine*, 1985, 142:777–81.
4. Alborzi A, Torab Jahromi F. Autoantibodies and complement levels of kala-azar patients in the south of Iran. *Iranian journal of medical sciences*, 1993, 18 (3&4): 94–8.
5. Pearson RD, Sousa AQ. Clinical spectrum of Leishmaniasis. *Clinical infectious disease*, 1991, 22:1–13.
6. Choudhry AP et al. Enzyme linked immunosorbent assay in the diagnosis of kala-azar in Bhadoni (Varanasi), India. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 1994, 84: 363–6.
7. Gupta S et al. Evaluation of enzyme linked immunosorbent assay in the dignosis of kala-azar in Malda district (West Bengal). *Indian journal of medical research*, 1993, 97:242–6.
8. Choudhary A et al. An indirect fluorescent antibody (IFA) test for the serodiagnosis of kala-azar. *Journal of communicable disease*, 1992, 24:32–6.
9. El-Harith A et al. Improvement of a direct agglutination test for field studies of visceral leishmaniasis. *Journal of clinical microbiology*, 1988, 26:1321–5.
10. Piarroux R et al. Comparison of PCR with direct examination of bone marrow aspiration, myeloculture and serology for diagnosis of visceral Leishmaniasis in immunocompromised patients. *Journal of clinical microbiology*, 1994, 32:746–9.
11. Nuzum E et al. Diagnosis of symptomatic visceral leishmaniasis by the use of the polymerase chain reaction on patient blood. *Journal of infectious diseases*, 1995, 171:751–4.
12. Singh S et al. Diagnostic and prognostic value of K39 recombinant antigen in Indian Leishmaniasis. *Journal of parasitology*, 1995, 81:1000–3.
13. Badaro R et al. rK39: a cloned antigen of *Leishmania chagasi* that predicts active visceral leishmaniasis. *Journal of infectious disease*, 1996, 173:758–61.
14. Sundar S et al. Rapid accurate field diagnosis of Indian visceral leishmaniasis. *Lancet*, 1998, 351:363–5.

15. Bern C et al. Use of recombinant K39 dipstick test and the direct agglutination test in a setting endemic for visceral leishmaniasis in Nepal. *American journal of tropical and medical hygiene*, 2000, 63: 153–7.

16. Iqbal J et al. Imported visceral leishmaniasis: diagnostic dilemmas and comparative analysis of three assays. *Journal of clinical microbiology*, 2002, 40:475–9.

17. Sundar S et al. Immunochromatographic strip-test detection of anti-K39 antibody in Indian visceral leishmaniasis. *Annals of tropical medicine and parasitology*, 2002, 96:19–23.

18. Zijlstra EE, El-Hassan AM. Leishmaniasis in Sudan. Visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 2001, 95 (suppl. 1):S27–58.

Leishmania/HIV co-infection

Leishmania/HIV co-infection is emerging as an extremely serious, new disease and it is increasingly frequent. There are important clinical, diagnostic, chemotherapeutic, epidemiological and economic implications of this trend.

Although most people bitten by sandflies infected with *Leishmania* protozoa do not develop the disease, those who are immunosuppressed quickly evolve to a full clinical presentation of severe leishmaniasis. Furthermore, AIDS and VL are locked in a vicious circle of mutual reinforcement. This duo of diseases produces cumulative deficiency of the immune response since *Leishmania* parasites and HIV destroy the same cells, exponentially increasing disease severity and consequences. Visceral leishmaniasis is considered a major contributor to a fatal outcome in co-infected patients. Use of tri-therapy, where it is available, has improved the prognosis for *Leishmania*/HIV cases.

Source: WHO. Fact sheet No.116. Revised May 2000 (http://www.who.int/mediacentre/factsheets/fs116/en/)