Brief Original Article

Comparative evaluation of rK9, rK26 and rK39 antigens in the serodiagnosis of Indian visceral leishmaniasis

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Abstract

Background: This study was designed for comparative evaluation of two relatively newer recombinant hydrophilic antigens, rK9 and rK26 of *Leishmania chagasi* along with rK39 (a 39-aminoacid-repetitive immunodominant B-cell epitope of kinesin-related antigen from *L. chagasi*) and crude soluble antigen (CSA) for the serodiagnosis of Indian visceral leishmaniasis (VL) patients by quantitative ELISA.

Methodology: In the present study a total of 80 subjects comprising of 55 confirmed VL cases and 25 endemic controls (EC) were subjected to ELISA using four different antigens, namely rK9, rK26, rK39 and CSA (derived from *Leishmania donovani* promastigotes).

Results: Sensitivity was as follows: 78% (95%CI 63-100%) for rK9, 38% (95%CI 28-59%) for rK26, 100% for rK39, and 80% (95% CI 65-100%) for CSA. The specificity of rK9, rK26, rK39 and CSA was found to be 84% (95%CI 61-100%), 80% (95%CI 56-100%), 96% (95%CI 75-100%) and 72% (95%CI 49-100%), respectively.

Conclusions: rK39 was observed to be the most suitable antigen as compared to rK26 and rK9 whereas rK9 performed better than rK26. Hence rK9 antigen may either be used as an adjunct to rK39 for accurate diagnosis of VL or may be used in the absence or non-availability of rK39 antigen for the serodiagnosis.

Key words: rK9, rK26, rK39, visceral leishmaniasis, crude soluble antigen, serodiagnosis

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Introduction

Visceral leishmaniasis (VL) is a potentially fatal disease caused by *Leishmania donovani* complex comprised of *L. donovani* (Indian subcontinent and Africa), L. *chagasi* (South America) and *L. infantum* (Mediterranean countries). The disease is endemic in 88 countries on 5 continents with a total of 350 million people at risk and12 million cases [1]. More than 90% of cases are from India, Nepal, Bangladesh, southern Sudan and northeast Brazil.

Detection of the parasite is definitive as well as the gold standard for the diagnosis of VL. But the procedure is cumbersome, risky and very difficult to apply in field conditions. Conventional methods of antibody detection have become obsolete as the sensitivities and specificities of most of the tests have been the limiting factors [2-4]. In addition, various serological tests such as IFAT, DAT, KATEX and ELISA have been developed, evaluated and tried [5-10]. ELISA is the best choice for the development of a rapid and reliable diagnostic method, because it is more practical, easy to standardize and suitable for mass screening. Specificity and sensitivity of the ELISA based immunoassay strictly depends on antigen quality and can be improved by use of recombinant technology [6], which drives the expression and purification of diagnostically relevant proteins in large amounts [11].In the last decade, several Leishmania antigens have been genetically and antigenically characterized. Recombinant K39 antigen (rK39) is a 39-amino-acid repetitive immunodominant B-cell epitope of the 230 kDa kinesin related protein of *L. chagasi* [12, 13]. The rK39 ELISA has been demonstrated to be suitable for detection of human VL and canine (both clinical and asymptomatic) VL [12-14].

Recently, reported cloning and characterisation of two new related hydrophilic antigens of *Leishmania chagasi*, rK9 and rK26, have led to an increase in the list of recombinant antigens for serodiagnosis of VL. K9 and K26 differ in the presence of 11 copies of a 14-amino-acid repeat in the open reading frame of K26 [15].

The aim of the present study was to assess the performance of rK9 and rK26 antigens for the serodiagnosis of VL and to compare the results with rK39 and CSA by quantitative ELISA.

Materials and methods

Sera

Sera from 55 clinically diagnosed as well as parasitologically confirmed VL cases were collected from Sir Sunder Lal Hospital, Banaras Hindu University, and KAMRC, its field site at Muzaffarpur, Bihar. Informed consent was obtained prior to blood collection. None of the VL patients had received any treatment before the collection of blood. The sera were separated and preserved at -70˚C until use.

Selection Criteria

Power analysis was utilized for calculation of sample size. Only those clinically suspected patients whose diagnosis of VL was confirmed by demonstration of LD bodies in splenic aspirates were included in the study. Endemic controls (EC) were selected as healthy volunteers or relatives of VL patients (no history of VL/relapse of VL) living in the area of endemicity (Muzaffarpur). All the subjects were screened for HIV infection by tri-dot test and found to be negative.

ELISA

The recombinant antigens (rK9, rK26 and rK39) were provided by Dr. S. G. Reed of Corixa Corporation, Seattle, WA, U.S.A. The tests were performed following the method described by Kumar *et al.* [16]. Briefly, 96-well microtitre plates were coated with rK9 (25 ng) or rK26 (25 ng) or rK39 (25 ng) or 5 µg of CSA per well. Protein (crude soluble fraction) of promastigotes [MHOM/IN/01/BHU36] isolated from an Indian patient with VL was used as CSA. After coating, plates were kept overnight at 4ºC then aspirated, blocked with PBS containing 1 or 5% (wt/vol) bovine serum albumin (BSA) for 2 hours at room temperature and then washed six times with PBS containing 0.1% Tween 20 (PBS-T). Sera were serially diluted in PBS containing 0.1% BSA (1% when tested for CSA). Next 0.1% Tween 20 was added to the wells, and plates were incubated for 30 minutes at room temperature for rK9, rK26 and rK39 and for one hour at 37ºC for CSA. The wells were then washed six times with PBS-T and incubated for 30 minutes with protein A-horseradish peroxidase (1/2000 dilution; Bangalore Genei, India) in PBS containing 0.1% BSA and 1.1% Tween 20 for one hour at 37ºC for rK9, rK26 and rK39. The conjugates antisera for CSA was goat anti-human immunoglobulin G conjugated with horseradish peroxidase (1/5,000 dilution**)**. Plates were then washed six times in PBS-T and incubated with tetramethylbenzidine (TMB) substrate for a further 15 minutes for recombinant antigens and 30 min for CSA. The reaction was stopped by the addition of 1N sulphuric acid, and the optical density at 450 nm was determined. The cutoff points for rK9, rK26, rK39 and CSA were determined from the serum dilution with optical densities of 0.102, 0.106, 0.101 and 0.346, respectively, calculated as the mean of the negative controls plus 3 standard deviations. All the sera were tested on single dilution (1:512) and OD was estimated. Sera having cutoff values more than that of the respective antigen as described earlier were further tested for quantitative ELISA. To quantitatively assess the result for each serum, the highest dilution giving the positive reaction were read by the naked eye and expressed as titers. Each serum was assayed in triplicate with negative (healthy individual from non-endemic area) and positive serum samples used as standards.

Results

Sera from 43 out of 55 patients (78%) showed high titers (2.5×10^3-5.2×10^5) with rK9. Only 21 out of 55 patients (38%) showed high titer (1.2×10^3-1.6×10^4) with rK26. All patients (100%) showed very high titers (6.5×10^4-1.0×10^6) with rK39 and 44 out of 55 (80%) patients showed high titers (2.7×10^3 to 4.0×10^4) with CSA. The range of antibody titers from the controls was 1.0×10^3 – 1.5×10^4 for rK9, 0.8×10^3 – 5.1×10^3 for rK26, 3.5×10^3 – 1.7 x, 10^4 for rK39 and 0.9×10^3 – 4.0×10^3 for CSA. The mean titer for detecting antibodies to different antigens is shown in table 2. Statistical evaluation was done after entry into the EPI-info (version-6) and SPSS statistical packages for calculation of sensitivity and specificity. Sensitivity was found to be 78% (95% CI 63-100%) for rK9, 38% (95% CI 28-59%) for rK26, 100% for rK39, and 80% (95% CI 65-100%) for CSA. Specificity was found to be 84% (95%CI 61-100%) for rK9, 80% (95% CI 56-100%) for rK26, 96% (95% CI 75-100%) for rK39 and 72% (95% CI 49-100%) for CSA and, as shown in table 1.

Discussion

Classic symptomatic VL is almost always fatal if left untreated. Definitive diagnosis of VL still

Table 1. Sensitivity and Specificity of Antigens.

Antigens	Sensitivity	Specificity
CSA	80 % (95% CI 65-100%)	72% (95%CI 49-100%)
rK9	78 % (95%CI 63-100%)	84% (95%CI 61-100%)
rK 26	38 % (95%CI 28-59%)	80% (95%CI 56-100%)
rK 39	100%	96% (95%CI 75-100%)

Table 2. Mean Titer of CSA, rK9, rK26, and rK39 for detection of antibody by ELISA.

Subjects	**Total**	**Mean Titer**			
	no.	CSA	rK9	rK26	rK39
VL	55	1.8×10^3	6.2×10^3	1.4×10^3	3.1×10^5
EC	25	1.1×10^3	1.4×10^3	1.0×10^3	6.5×10^3

depends on parasite detection in a smear or culture of aspirate from the spleen, bone marrow or lymph node. These tests can be insensitive or generate equivocal results [17]. In addition, the procedure must be performed by individuals with the required expertise or the tests may result in serious consequences [17]. Noninvasive tests, such as conventional detection of IgG antibodies of *Leishmania,* are often not sensitive enough to detect asymptomatic or subclincial individuals [12]. Though serological tests such as IFAT, ELISA using CSA, and CIEP have become available during last few years, due to various reasons none of them have proved to be as reliable as the splenic smear [18]. Consequently, there is a need for a specific as well as sensitive serological test/s to diagnose the disease.

A total of 55 VL cases and 25 EC were investigated for antibody response to rK9, rK26, rK39 and CSA to determine the most suitable antigen for diagnostic purposes. The sensitivity and specificity of rK9 and rK26 detected in this preliminary study are supported by another study conducted in India which reported 21.3% sensitivity by the rK26 strip test [19]. The difference in sensitivity might be that a quantitative method was used in the current study whereas the rK26 strip test used in the other study is a qualitative method of antibody detection. Other studies conducted in symptomatic canine VL cases in Brazil showed higher sensitivity by ELISA using rK9 and rK26 antigen [20-22]. The lower sensitivity in this study may be due to the fact that dog is a different species than human. Another explanation may be that there are different leishmanial species (other than *L. donovani*) causing canine Leishmaniasis; hence difference in the antibody response. In this study, rK39 antigen showed a sensitivity of 100% and specificity of 96%. Similar findings have also been observed by other researchers [23]. Another investigation showed great utility of rK39 in unambiguous diagnosis and prognosis of VL which was much superior to crude soluble antigen [14]. On analysis, though the specificities of both rK9 and rK26 were comparable (84% & 80%, respectively), the sensitivity of rK9 was much higher (78%) than rK26 (38%).

This study was conducted on limited cases; hence no definite conclusion can be drawn. From the preliminary study, it appears that for the serological diagnosis, rK39 is the most suitable antigen followed by rK9 and rK26. Pending detailed serological evaluation of rK9 with large samples and well-matched controls, we believe that this antigen could be used as an adjunct to parasitological diagnosis or in the absence/unavailability of rK39 for an effective, non-invasive and accurate diagnosis of VL in remote endemic areas.

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Conflict of interest: No conflict of interest is declared.