# SHORT REPORT: PRODUCTION OF RECOMBINANT KINESIN-RELATED PROTEIN OF *LEISHMANIA DONOVANI* AND ITS APPLICATION IN THE SERODIAGNOSIS OF VISCERAL LEISHMANIASIS

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*Abstract.* To detect IgG antibody in the serodiagnosis of visceral leishmaniasis (VL), a recombinant antigen rK39, which is part of a *Leishmania chagasi* kinesin-related protein, has been used successfully and showed high sensitivity and specificity. We report production of a recombinant protein rKRP42, which is part of an *L. donovani* kinesin-related protein and a homolog of rK39, and its application in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of VL. When rKRP42 and rK39 were compared, amino acid sequence analysis showed 89.3% identity and 98.7% homology, with rKRP42 having 39 more amino acids than rK39. The ELISA using rKRP42 showed a sensitivity of 94.6% (70 positive samples among 74 from VL patients) and a specificity of 99.3% (148 negative samples among 149 samples from Japanese controls), whereas the sensitivity of the commercial rK39 dipstick test was 93.2% (69 positive samples among 74 from patients with VL). The rKRP42 is a promising new antigen in developing immunodiagnostic methods for VL.

Visceral leishmaniasis (VL) or kala-azar is caused by an intracellular protozoan parasite of the *Leishmania donovani* complex and is considered as one of the most neglected diseases.[1] More than 47 countries are currently affected, with at least 200 million people at risk.[2] Approximately 90% of the 500,000 estimated annual cases of VL occur in rural areas of Bangladesh, India, Nepal, Sudan, and Brazil in some of the world's poorest regions. This disease accounts for 75,000 deaths annually.[3]

Most VL cases in peripheral health facilities are still treated on the basis of clinical suspicion and/or the result of an inadequately sensitive and specific formol-gel test (aldehyde test).[4] However, classic clinical features of VL are shared by several other endemic diseases such as malaria, disseminated tuberculosis, and enteric fever, which are also common in many of the areas endemic for VL. Demonstration of the causative parasites in aspirates from lymph nodes, bone marrow, and spleen is the most specific diagnosis, with the sensitivities of 56.3%, 67.1%, and 93.3%, respectively.[5] These techniques are invasive and require skilled personnel and equipped facilities. Because of the high mortality if left untreated and the serious toxicity of the most widely used first-line drug, sodium stibogluconate, a highly sensitive and specific diagnostic method that is simple, inexpensive, and applicable in rural settings is urgently needed.

Several serologic tests, such as enzyme-linked immunosorbent assays (ELISAs) with crude or recombinant antigens[6–8] and the direct agglutinin test (DAT),[9,10] have provided useful diagnostic results. Recently, a recombinant antigen rK39, which is part of an *L. chagasi* kinesin-related protein, has been widely evaluated by ELISA or in a dipstick format.[11,12] Although the antigen has been reported satisfactory, results varied considerably in different disease-endemic areas. Thus, it was desirable to develop new antigens for comparison. We report the production of recombinant protein rKRP42, which is part of an *L. donovani* kinesin-related protein and a homolog of rK39 and evaluation of this antigen in an ELISA with serum samples for the diagnosis of VL.

*Leishmania donovani* strain DD8, isolated from a Bangladeshi patient, was used.[13] Promastigotes were cultured and harvested as described previously.[14] Genomic DNA was extracted from promastigotes by phenol extraction method. To obtain the gene coding the rK39 homolog, polymerase chain reaction (PCR) amplification was performed by using primers (rK39 sense, 5′-GAGCTCGCAACCGAGTGGGAGGAC-3′ and rK39 antisense, 5′-CTGGCTCGCCAGCTCCGCG-GCGCG-3′) with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and *L. donovani* genomic DNA. The amplified PCR product was subjected to electrophoresis on an agarose gel, purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and ligated into the *Eco*RV site of the pBluescript KS(-) vector (Stratagene). The PCR product was confirmed to have 1,011 basepairs (GenBank accession no. AB256033). Further PCR amplification was performed with the sequenced-confirmed plasmid containing the rK39 homolog gene, known as rKRP42, as a template by using primers (rK39 sense, 5′-GAGCTCGCAACCGAGTGG-GAGGAC-3′ and 6His/*Eco*RI-rK39 antisense, 5′-GTGAT-GGTGATGGTGATGGAATTGATCCTGGCTCGC-CAGCTC-3′) with *Pfu* DNA polymerase. The PCR product coding rKRP42 and a six-histidine amino acid (6His) tag was cloned into the in *Sma* I site of the pTYB12 expression vector (New England Biolabs, Beverly, MA). The rKRP42 was purified with QIAexpress kit (Qiagen) and IMPACT™-CN System (New England Biolabs) according to the manufacturer's protocols.

Briefly, *Escherichia coli* cells were cultured with Luria-Bertani medium containing 100 μg/mL of ampicillin. Protein expression was induced with isopropyl thio-β-galactoside at a final concentration of 0.4 mM at 16°C for 16 hours. The cells were then harvested and resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, and 0.1% Triton X-100). After sonication and centrifugation,

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the clarified cell extract was purified on an Ni-NTA column (Qiagen). The column was washed with the Ni-NTA wash buffer, and the extraction fraction was eluted with Ni-NTA elution buffer. The eluate was then purified on a chitin column (New England Biolabs) and washed with chitin column wash buffer. The column was kept with cleavage buffer containing dithiothreitol (DTT) at 16°C for 16 hours, and then rKRP42 was eluted with cleavage buffer without DTT (Figure 1). The rKRP42 antigen contains 337 amino acids and is one repeat (39 amino acids) longer than the rK39 antigen (Figure 2). The amino acid sequence of rKRP42 showed 89.3% identity and 98.7% homology with rK39 antigen.

We performed an ELISA with rKRP42 antigen as follows. Flat-bottomed 96-well microtiter plates (MaxiSorp™; Nunc, Roskilde, Denmark) were coated with 1 μg/mL (100 μL/well) of rKRP42 antigen and incubated overnight at 4°C. After blocking with casein buffer (1% casein in 0.05 M Tris-HCl buffer, 0.15 M NaCl, pH 7.6) for two hours at room temperature, 100 μL of serum (1:4,000 dilution in casein buffer) was added to the wells and incubated for one hour at 37°C. After four washes with phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20, peroxidase-conjugated goat anti-human IgG (Tago, Camarillo, CA), diluted 1:4,000 with casein buffer was added and incubated for one hour at 37°C. After four washes, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Kirkegaard and Perry Laboratories Inc., Gaithers-

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rKRP42   ELATEWEDALRERALAERDEAAAAELDAAASTSQNARESASERLTSLEQQLRESEERAVE   60
         ************************************************.*************.*
rK39     ELATEWEDALRERALAERDEAAAAELDAAASTSQNARESACERLTSLEQQLRESEERAAE   60

rKRP42   LASQLESTTAAKMSAEQDRENTRAALEQQLRESEERAAELASQLEATAAAKSSAEQDREN   120
         ******.*.*** *********** .********* .**************** *******
rK39     LASQLEATAAAKSSAEQDRENTRATLEQQLRESEARAAELASQLEATAAAKMSAEQDREN   120

rKRP42   TRAALEQRLRESEARAAELASQLEATAAAKSSAEQDRENTRAALEQRLRESEERAAELAS   180
         ***.***.**.**.**********.*.*** *******.***.***.**.**********
rK39     TRATLEQQLRDSEERAAELASQLESTTAAKMSAEQDRESTRATLEQQLRDSEERAAELAS   180

rKRP42   QLEATAAAKSSAEQDRENTRPALEQQLRESEERAAELASQLEATAAAKSSAEQDRENTRA   240
         ***.*.*** *******.**..****************** .*.*** *******.***
rK39     QLESTTAAKMSAEQDRESTRATLEQQLRESEERAAELASQLESTTAAKMSAEQDRESTRA   240

rKRP42   ALEQQLRESEERAAELASQLEATAAAKSSAEQDRENTRAALEQQLRESEERAAELASQLE   300
         .******.*************************************.***********
rK39     TLEQQLRDSEERAAELASQLEATAAAKSSAEQDRENTRAALEQQLRDSEERAAELASQ    298

rKRP42   ATAAAKSSAEQDRENTRAALEQRLRESEARGAELASQ                        337
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FIGURE 2.   Comparison of amino acid sequences of rKRP42 and rK39 antigens in a single-letter code. Asterisks indicate identical residues and periods indicate conservative amino acid substitutions.

burg, MD) was added and incubated for one hour at room temperature. The optical density was measured at 415 nm and at 492 nm as reference. Each sample was assayed in duplicate. If the absorbance values of the duplicate samples differed by > 40% from their average, the sample was retested. Antibody levels were expressed arbitrarily as units, which were was estimated from a standard curve constructed with serially diluted positive sera. The cutoff point for IgG to rKRP42 was calculated as the mean plus three standard deviations of log (unit + 1) values of the non-endemic healthy controls (NEHCs). The anti-logarithmic value was 109.4 units.

The rK39 antigen-based dipstick test (InBios International, Seattle, WA) was carried out according to the manufacturer's instruction. Briefly, 20 μL of serum was added to a test strip. The strip was placed in a well of 96-well microtiter plate, and two drops of chase buffer solution were added to each well. The test result was read within 10 minutes after addition of serum. Even a weak line was considered positive.

Seventy-four serum samples from defined VL patients collected from different medical college hospitals in Bangladesh were used to compute sensitivities of the rKRP42 ELISA and rk39 dipstick test. Among the 74 patients, 32 were confirmed parasitologically: Leishman-Donovan bodies were detected in smears of splenic aspirates (18 patients) or bone marrow aspirates (6 patients), and promastigotes were detected in 8 patients after inoculation of aspirate materials into Novy, MacNeal, and Nicolle medium. Of the other 42 clinically confirmed patients, 27, 7, and 8 were positive by conventional DAT, aldehyde test, and rK39 dipstick test, respectively. At the time of sample collection, all patients were being treated with sodium antimony gluconate at the recommended dose of the World Health Organization.[15] Sera containing preservative (NaN$_3$) at a concentration of 0.1% (w/v) were transported to Japan at ambient temperature and then stored at −40°C. Seventy-two samples from healthy Japanese individuals were used as NEHCs to determine the cutoff value. Another 149 NEHC samples were used to determine the specificity of the rKRP42 ELISA.

The study was reviewed and approved by the Ethics Committee of Aichi Medical University School of Medicine, Japan, and the Ethical Review Committee of the Bangladesh Medical Research Council.
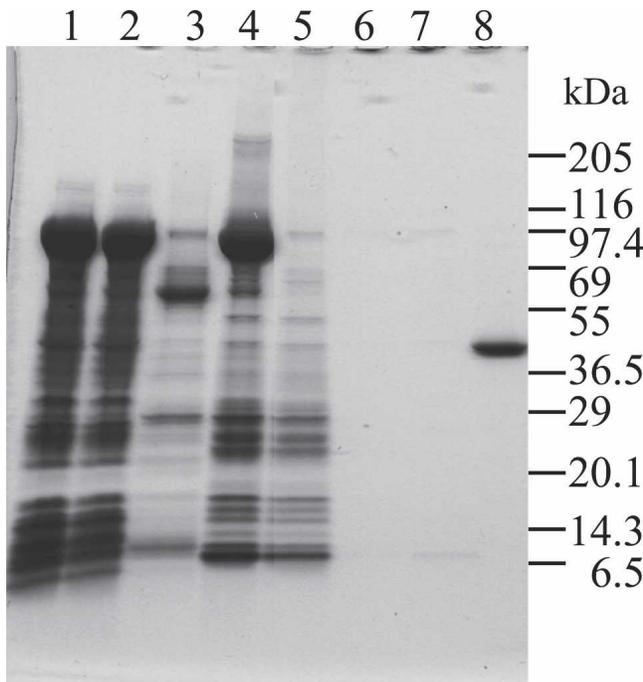


FIGURE 1.   Purification of rKRP42 from induced *Escherichia coli* clone cells by using Ni-NTA and chitin columns. Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized by staining with Coomassie brilliant blue. Lane 1, Crude extraction from induced cells; lane 2, Ni-NTA column flow through fraction; lane 3, Ni-NTA column wash fraction; lane 4, Ni-NTA column elution fraction with the elution buffer; lane 5, chitin column flow through fraction; lane 6, chitin column wash fraction; lane 7, chitin column rinse fraction with the cleavage buffer; lane 8, chitin column elution fraction after 16-hour cleavage reaction. kDa = kilodaltons.

The ELISA with rKRP42 antigen showed a sensitivity of 94.6% (70 positive samples among 74 VL samples) and a specificity of 99.3% (148 negative samples among 149 Japanese controls) (Figure 3). The sensitivity of the rK39 dipstick was 93.2% (69 positive samples among 74 VL samples). Because of the high specificities already reported for the rK39 dipstick test (97–100%),[11,16–19] Japanese controls were not tested. There are three parasitologically confirmed cases who were negative by both the ELISA and dipstick test. In a separate study, these three cases showed negative results with a conventional serum-based DAT, a urine-based ELISA with soluble antigen of acetone-treated promastigotes,[14] and a urine-based DAT.[20] One ELISA-positive, dipstick-negative sample had a relatively low antibody titer of 202.0 units. We could not determine the specificity for other diseases such as malaria, tuberculosis, and cutaneous leishmaniasis and for healthy controls from a disease-endemic area because of a lack of serum samples.

A variety of immunologic methods have been used to diagnose VL. Among others, the rK39 dipstick test is used because of its ease in handling, quick results, and high sensitivity and specificity. However, the sensitivity varied considerably in different disease-endemic areas. In India and Nepal,[16–18] the test showed the highest sensitivity (100%), but the sensitivity was significantly lower in Venezuela (88%),[21] southern Europe (71.4%),[19] and Sudan (67%).[22] This variation may be due to differences in the test accuracy between subspecies of *L. donovani* complexes, genetic differences in individual patients or in racial subgroups, and epidemiologic factors such as length or severity of diseases.[23] It would be worthwhile to test the new rKRP42 antigen in different geographic areas.

Some persons with VL do not show any clinical manifestations. Khalil and others[24] reported that in eastern Sudan the ratios of clinical and subclinical cases in 1994–1995 and 1995–1996 in Um-Salala village were 1.2:1 and 2.6:1, respectively, and in Mashrau Koka village were 1:11 and 1:2.5, respectively. In another study conducted in Bihar State, India, 69% of asymptomatic seropositve cases detected by the rK39 ELISA and dipstick test developed kala-azar within one year,[25] which suggested that that many of the asymptomatic cases were in a pre-clinical state. In predicting possible clinical cases, an ELISA that is quantitative would be more advantageous than a dipstick format; high antibody titers or an increase in antibody titers with time could be indicative of possible clinical cases. Such early diagnosis will have a practical importance now that oral treatment with miltefosine has become available.[26] Measurements of *Leishmania*-specific IgG, IgM, IgE, and IgG subclasses were also found to be useful as markers for active VL cases and for monitoring effective treatment.[27,28] The rKRP42 ELISA for IgG antibody can be used in clinical follow-up studies based on antibody titers and modified for various immunoglobulin classes.

Recently, the use of urine for blood has been considered valuable because of its ease in sample collection, and urine-based tests for the diagnosis of VL and other parasitic diseases have been reported.[14,20,29,30] Before establishing a urine-based immunodiagnostic method with a new antigen, the antigen must be first evaluated with serum samples. In a field study, when many borderline positive results can be expected, the serum-based rKRP42 ELISA can be a valuable reference.

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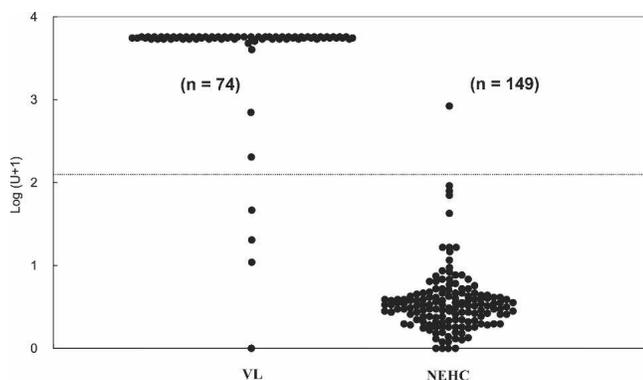


FIGURE 3. Detection of IgG antibody to rKRP42 in sera of patients with visceral leishmaniasis patients (VL) and non-endemic healthy Japanese controls (NEHC) by enzyme-linked immunosorbent assay. Each dot represents one serum sample. Thirty-three samples exceeded the highest measurable point (5,698 units) of the standard curve and were considered 5,698 units. The horizontal dotted line represents the cutoff value of 109.4 units.

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