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PARASITOLOGY

Evaluation of Cysteine Protease C of *Leishmania donovani* in Comparison with Glycoprotein 63 and Elongation Factor 1α for Diagnosis of Human Visceral Leishmaniasis and for Posttreatment Follow-Up Response

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ABSTRACT Visceral leishmaniasis (VL) is a threat in many developing countries. Much effort has been put to eliminating this disease, for which serodiagnosis remains the mainstay for VL control programs. New and improved antigens as diagnostic candidates are required, though, as the available antigens fail to demonstrate equal optimum performance in all areas of endemicity. Moreover, these diagnoses are dependent on invasive serum sampling. In the current study, we cloned and expressed *Leishmania donovani* cysteine protease C (CPC) and evaluated its diagnostic and test-of-cure possibilities by detecting the antibody levels in human serum and urine through ELISA and immunoblot assays. Two immunodominant antigens, recombinant glycoprotein 63 (GP63) and elongation factor 1α (EF1α), identified earlier by our group, were also assessed by employing human serum and urine samples. Of these three antigens in ELISAs, CPC demonstrated the highest sensitivities of 98.15% and 96% positive testing in serum and urine of VL patients, respectively. Moreover, CPC yielded 100% specificity with serum and urine of nonendemic healthy controls compared to GP63 and EF1α. Urine samples were found to be more specific than serum for distinguishing endemic healthy controls and other diseases by means of all three antigens. In all cases, CPC gave the most promising results. Unlike serum, urine tests demonstrated a significant decrease in antibody levels for CPC, GP63, and EF1α after 6 months of treatment. The diagnostic and test-of-cure performances of CPC in the immunoblot assay were found to be better than those of GP63 and EF1α. In conclusion, CPC, followed by GP63 and EF1α, may be utilized as candidates for diagnosis of VL and to assess treatment response.

KEYWORDS immunology, infection, diagnosis, leishmaniasis, recombinant antigens, parasitology

Leishmaniasis is a set of diseases manifested by the infection of parasites belonging to the genus *Leishmania*. The parasites are carried to the mammalian hosts by the bite of an infected female sandfly of either the genus *Lutzomyia* (New world) or the genus *Phlebotomus* (Old world) (1). More than 20 species of *Leishmania* are responsible for infecting mammals, resulting in a wide spectrum of clinical manifestations. This includes visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL). The most serious of all clinical forms is VL, also known as kala-azar,

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which can be fatal if not treated in time. The disease is rampant in 88 countries of the world, with around 350 million people at risk of getting the infection (2).

During VL, the infected macrophages in the bloodstream invade the visceral organs such as liver, bone marrow, and spleen, leading to enlargement of these organs (3). The routine diagnosis of VL is done by the microscopic examination of tissue aspirates. However, the presence of amastigotes can be mistaken as *Histoplasma*, *Toxoplasma*, or artifacts, leading to misdiagnosis (4). Culturing the tissue aspirates has been employed to increase sensitivity and specificity of the diagnosis (5, 6). Again, the growth rates of all *Leishmania* strains are different and the parasite load in all tissues is not the same. Molecular diagnosis techniques, such as PCR and quantitative PCR (qPCR), have shown better sensitivity and specificity (7–11). However, these techniques are time consuming and require skilled technicians and advanced laboratory setups.

As the visceral disease progresses, large amounts of antibodies are generated in the host, leading to a condition known as hypergammaglobulinemia (12). Many of these circulating antibodies demonstrate specific reactivity against various leishmanial antigens. Therefore, this observation has been exploited in various serological methods, such as the direct agglutination test (DAT), the immunofluorescent antibody test (IFAT), and enzyme-linked immunosorbent assay (ELISA) for VL diagnosis (13–15). However, these serological tests rely on advanced laboratory instruments and skilled personal, thus lacking field adaptability.

A major advancement in VL diagnosis has come with the development of rapid diagnostic tests (RDTs), such as the rK39 antigen-based immunochromatographic test, especially in the Indian Subcontinent. However, the suboptimal sensitivity of the rK39 antigen in Brazil and East Africa remains a matter of concern (16). Another limitation of the rK39 test is that it cannot differentiate between active and past infection. In the last decade, many other recombinant antigens apart from rK39, such as rKE16, rK28, and rKLO8, have been developed for serological diagnosis (17–19). Since antibodies persist in the blood even after a complete cure, it is difficult to use serological testing as a test of cure post therapy. Therefore, samples other than serum, such as saliva and urine, are being tested for antibody detection (20, 21). Several urine-based diagnostic tests have been described in recent years, detecting antigen or antibodies in the samples through ELISA, immunoblot, dipstick immunochromatographic tests, and qPCR. However, these assays have been found to be variable in sensitivity and specificity (22–24). The identification of newer antigens in VL diagnosis, with better sensitivity and specificity across all endemic regions and for test of cure that can be implemented easily during and after the course of treatment, is highly desirable.

Earlier, our laboratory demonstrated the immunogenicity of *leishmania* membrane antigens (LAg), isolated from *L. donovani* promastigotes (25), and soluble leishmanial antigens (SLA) (26) in *Leishmania* infection. Further, the diagnostic potential of LAg to detect antibodies in *Leishmania*-infected serum and urine samples was reported (27–29). Additionally, it was shown that, unlike serum, levels of antibodies in urine declined after 6 months of active VL infection. However, the major limitation of using crude antigens is the high risk associated with culturing the pathogenic organism, along with the uncertainty of maintaining homogeneity during the isolation procedure of crude antigens. These limitations highlight the need to explore the diagnostic potential of novel immunogenic and defined recombinant proteins.

The recombinant antigens glycoprotein 63 (GP63), elongation factor 1 alpha (EF1α), and cysteine protease (CP) were extensively studied earlier for their immunogenicity in mice (30–32). In the current study, we evaluated the diagnostic potential of the newly cloned CPC with two recombinant antigens, GP63 and EF1α, through ELISA and immunoblot assays. In serum and urine, antibodies specific to these antigens were compared for diagnosis of VL and to assess treatment response.

MATERIALS AND METHODS

Sampling. A panel of 113 human sera and 107 urine samples were collected from the School of Tropical Medicine (STM), Kolkata, the Rajendra Memorial Research Institute of Medical Sciences (RMRIMS),

Patna, and the CSIR-Indian Institute of Chemical Biology (IICB), Kolkata. *Leishmania* infection for 54 sera and 50 urine samples was proven by parasitology and rK39-RDT (InBios Int. Inc., USA). Noninfected samples were collected from healthy individuals of VL regions of endemicity and nonendemicity. These samples included 18 serum and 20 urine samples of endemic healthy controls (EHC) and 21 serum and 19 urine samples of nonendemic healthy controls (NEHC). Also, 20 serum and 18 urine samples were collected from symptomatically similar diseases other than VL (OD). The OD serum samples included 5 each from malaria, viral fever, typhoid, and tuberculosis. The OD urine samples included 5 each from malaria and viral fever, and 4 samples each from typhoid and tuberculosis. Serum and urine samples were also collected from seven VL patients at three time points as follows: before the treatment started (day 0), 1 month after treatment (day 30), and after at least 6 months from when first sampling was done (day >180). After obtaining blood by venepuncture, it was kept undisturbed at room temperature (RT) to coagulate and centrifuged at 1,400 × *g* for 10 min. The sera thus obtained were stored at −20°C until further use. Random urine samples were obtained and preserved by adding 0.1% sodium azide immediately to each sample followed by storage at 4°C.

Ethics statement. This study was approved by the Ethical Committee on Human Subjects of IICB, Kolkata; STM, Kolkata; and RMRIMS, Patna. Informed written consents in the local languages were taken before participation in this study.

Cloning and expression of *L. donovani* cysteine protease C. The cysteine protease C (CPC) gene (GenBank accession number JX968801.1) from *L. donovani* strain AG83 (ATCC PRA-413) was cloned into the pET28a vector and expressed in *Escherichia coli* Rosetta (DE3). The sequence of the CPC gene of *L. donovani* was obtained from the TriTrypDB genome database. Primers containing restriction sites for NcoI and HindIII were designed using Oligotech software. Codons for six histidine residues were added on the C terminus. Genomic DNA from *L. donovani* promastigotes was isolated and followed by PCR using the above-mentioned primers. The PCR conditions at which the gene was amplified were as follows: one cycle of 5 min at 94°C, 35 cycles of 30 sec at 94°C, 20 sec at 70°C, and 1 min 40 sec at 72°C, followed by a final extension step of 7 min at 72°C. The PCR-amplified fragments were cloned into the bacterial expression vector pET28a (Novagen, Madison, WI, USA) at the NcoI/HindIII restriction sites. Colonies found to be positive were grown overnight in LB broth containing 50 mg/ml kanamycin at 37°C. Plasmid DNA was extracted using the QIAprep Spin miniprep kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. Plasmid DNA was subjected to double digestion with NcoI and HindIII to confirm the size of the double-digested products of plasmid digestion. For expression, the cloned plasmid was transformed into the expression strain *E. coli* Rosetta (DE3). The expression conditions were optimized by growing the cells at different conditions of temperature and isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration. The expression-induced cell lysate was compared with the uninduced cell lysate by running a 12% SDS-PAGE followed by Coomassie brilliant blue staining.

Purification and identification of recombinant proteins. Newly cloned recombinant antigen CPC, along with antigens GP63 and EF1α that were cloned in previous studies (31, 33), were purified as follows. *E. coli* Rosetta (DE3) transformed with previously cloned pET16b-GP63 and pET15b-EF1α and freshly cloned pET28a-CPC constructs were grown in LB medium until the optical density at 600 nm (OD_{600}) reached 0.6. Expression of protein was induced by adding IPTG to a final concentration of 0.5 mM. The bacterial cultures were incubated further at their optimum inducing temperatures for 4 h. The cultures were then harvested and the cell pellets were suspended in bacterial lysis buffer (25 mM Tris-HCl, 300 mM NaCl, 1 mg/ml of lysozyme [Roche], and 1 mM phenylmethylsulfonyl fluoride [PMSF] [pH 8.0]). The cell lysates were subjected to sonication using an ultrasonicator (Misonix, Farmingdale, NY, USA). The lysates were then centrifuged at 12,000 rpm for 30 min. The pellets containing inclusion bodies were subjected to purification under denaturing conditions. Upon confirmation of purification of urea-denatured proteins, the eluted fractions were subjected to dialysis with a gradually decreasing concentration of urea to achieve proper folding of the proteins. These protein fractions were further concentrated using Amicon Ultra centrifugal filter devices (Millipore Corporation, USA) of 30 kDa, 30 kDa, and 10 kDa thresholds for GP63, EF1α, and CPC, respectively. The purification of proteins was confirmed by performing a 12% SDS-PAGE followed by Coomassie brilliant blue staining. The amount of protein in each case was estimated by the method of Lowry et al. (50). All three proteins, GP63, EF1α, and CPC, were identified through Western blotting by probing with anti-GP63 IgG raised in rabbit, anti-EF1α IgG raised in mouse, and anti-CPC IgG raised in rabbit, respectively.

ELISA. Levels of IgG antibodies (specific for each of the three antigens) were detected by enzyme-linked immunosorbent assay (ELISA). The experiment was performed in 96-well ELISA plates (Nunc). Each well was coated overnight at 4°C with 1 μg of antigen/well of GP63, EF1α, or CPC diluted in carbonate-bicarbonate buffer (pH 9.6). The next day, the wells were blocked with 1% bovine serum albumin (BSA) (200 μl/well) at 37°C for 1 h. Serum (100 μl/well) and urine samples (100 μl/well) were added at a dilution of 1:2,000 (27) and 1:10 (29), respectively, and incubated at 37°C for 2 h followed by 1:3,000 dilution of horseradish peroxidase (HRP)-conjugated anti-human IgG. The presence of bound IgG was detected by adding 3,3′,5,5′-tetramethylbenidine (TMB) (Sigma-Aldrich) as the substrate. The reaction was stopped by the addition of 2 N H_2SO_4. Optical density values were obtained at 450 nm using a Multiskan microplate spectrophotometer (Thermo Fisher Scientific).

Immunoblot assay. Immunoblot assay was carried out by using 12% SDS-PAGE with 0.5 μg of antigen/lane, either GP63, EF1α, or CPC. The separated proteins were then electrophoretically transferred onto nitrocellulose membranes in a transblot apparatus (Bio-Rad Laboratories, USA) at 1.5 A constant current for 15 min. Transfer of proteins onto membranes was confirmed by staining with Ponceau S. Each lane was then cut into strips to be incubated with different samples of serum and urine. Strips were then blocked with 5% BSA in 1× Tris-buffered saline (TBS) for 90 min. After blocking was done, strips were

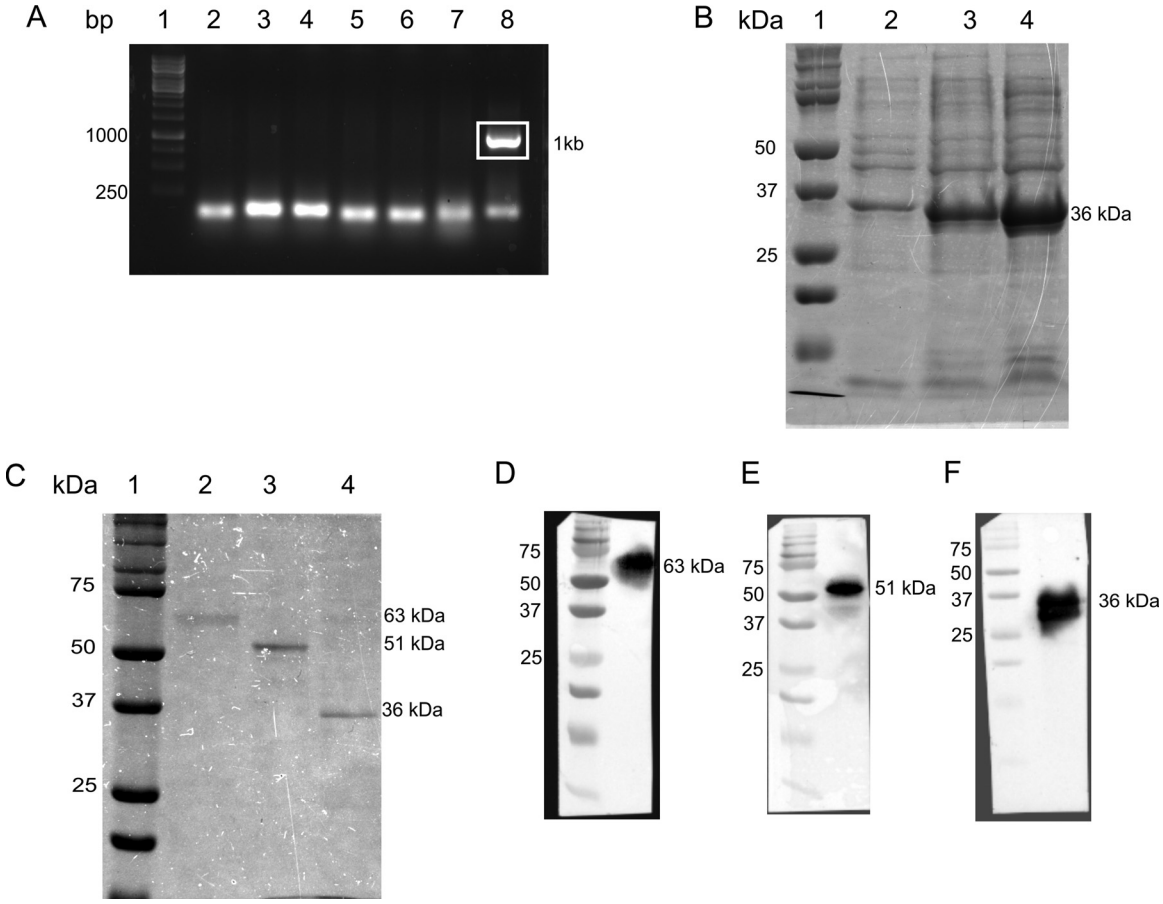

FIG 1 Cloning and expression of cysteine protease C (CPC) and identification of purified recombinant proteins GP63, EF1α, and CPC through SDS-PAGE and Western blotting. (A) CPC (GenBank accession number JX968801.1) was PCR amplified and cloned into pET28a. Lane 1 contains base pair markers, lanes 2 to 7 correspond to different PCR standardization conditions, lane 8 depicts the 1-kb PCR product corresponding to the gene length of CPC. (B) Expression of CPC upon induction with variable concentrations of IPTG. Lane 1 contains molecular weight markers, lanes 2 to 4 represent uninduced lysate, lysate of culture induced with 0.25 mM IPTG, and 0.5 mM IPTG, respectively, corresponding to a 36-kDa protein. (C) Purification of GP63, EF1α, and CPC. Lane 1 contains molecular weight markers, lanes 2 to 4 depict recombinant proteins GP63, EF1α, and CPC, respectively. (D to F) GP63, EF1α, and CPC were identified by their respective antibodies, anti-GP63 (D), anti-EF1α (E), and anti-CPC (F), corresponding to molecular weights 63 kDa, 51 kDa, and 36 kDa, respectively.

incubated with serum (1:2,000) and urine (1:5) samples with 2% BSA in 1× Tris-buffered saline with Tween 20 (TBST) (0.1% Tween 20) and incubated overnight at 4°C with constant rocking. The next day, HRP-conjugated goat anti-human IgG antibody diluted (1:3,000) in wash buffer was added and incubated for 1h at RT with constant rocking. Strips were washed 5 times for 5 min each with wash buffer, and a last wash was done with 1× TBS (without Tween 20). After washing, the strips were incubated for 5 min in freshly prepared substrate solution of 0.05% DAB and 0.05% H_2O_2 in 1× TBS. The reaction was stopped with distilled water and strips were air dried. Images of the membrane blots were captured using the Bio-Rad Gel Doc system and analyzed in Image Lab software (version 54.2.1).

Statistical analysis. Statistical analysis was performed using the GraphPad Prism 8.01 software. Receiver operating characteristic (ROC) curves were obtained to determine the cutoff values for each ELISA where maximum sensitivity and specificity were achieved at 95% confidence intervals. A two-tailed, Mann-Whitney U test was performed to calculate the statistical significance between VL positive- and VL negative-control groups. A paired *t* test was done to compare the level of antibodies at different time points. Differences were considered statistically significant when the *P* value was <0.05.

RESULTS

Cloning, expression, and purification of recombinant antigens. The nucleotide sequence of CPC was used to generate the recombinant antigen (as described in the Materials and Methods section). The genomic DNA of the targeted protein from a clinical isolate of *L. donovani* strain AG83 was PCR amplified using gene-specific primers. CPC was cloned successfully after PCR amplification of a band of approximately 1 kb corresponding to a gene size of CPC in lane 8 (Fig. 1A). The protein was expressed

by inducing with 0.25 mM IPTG and 0.5 mM IPTG in comparison with uninduced lysate in expression strain *E. coli* Rosetta (DE3) (Fig. 1B). The expression of CPC was found to be maximal at a 0.5 mM concentration of IPTG. CPC, along with the two recombinant proteins GP63 and EF1α, obtained from the previous studies, were expressed and purified according to the protocol discussed in the Materials and Methods. The integrity and molecular weights of the purified recombinant proteins were confirmed with SDS-PAGE as shown in Fig. 1C. The proteins were identified through Western blotting. The corresponding molecular weights of GP63, EF1α, and CPC were found to be 63 kDA (Fig. 1D), 51 kDa (Fig. 1E), and 36 kDa (Fig. 1F), respectively.

ELISA to detect serum antibodies against recombinant proteins. To assess the reactivity of recombinant antigens GP63, EF1α, and CPC, ELISA was carried out with the serum of VL-positive patients as well as controls. There were 54 VL-positive serum samples and 59 serum samples from control groups comprising both endemic (18 samples) and nonendemic healthy individuals (21 samples), along with patients having diseases other than VL (as described in the Materials and Methods). The presence of antigen-specific IgG antibodies was determined through ELISA and presented as optical density values. Cutoff values for each ELISA were determined from the ROC curve, where maximum sensitivity and specificity were observed. For GP63, 92.59% of the confirmed VL cases were detected as positive and a specificity of 95.23% was observed, with nonendemic healthy individuals as negative controls (Fig. 2A). With endemic healthy controls and other diseases, the specificities were found to be 88.89% and 60%, respectively. Moreover, statistically significant differences were found with respect to the level of antibodies on comparing VL with endemic healthy controls and other diseases. EF1α as a diagnostic candidate showed 96.29% sensitivity and 100% specificity in comparison to nonendemic healthy controls (Fig. 2B). With endemic healthy controls, 33.34% of the samples cross-reacted with the antigen. However, the mean optical density value was below the cutoff, with significant differences compared to confirmed VL cases. EF1α exhibited 90% cross-reactivity with other diseases, and very little significant difference was found with active VL. The CPC antigen showed 98.15% sensitivity and 100% specificity with nonendemic healthy controls (Fig. 2C). The mean optical density values of endemic healthy controls and other diseases were comparable to the set cutoff, demonstrating significant differences from active VL cases. The ROC curves obtained for the antigens GP63, EF1α, and CPC are shown in Fig. 2D to F, respectively.

ELISA to detect urine antibodies against recombinant proteins. On determining the presence of antigen-specific antibodies against all the three recombinant proteins in the sera of VL-positive patients, we further investigated their reactivity with urine samples. For this, 50 urine samples of VL-positive patients, 19 samples of nonendemic healthy controls, 20 samples from endemic healthy controls, and 18 samples obtained from other diseases (as described in the Materials and Methods) were studied. ROC curves were used to determine the cutoff values which represent the performance of the antigen. ELISA with the GP63 antigen showed 90% sensitivity and 100% specificity with nonendemic healthy controls (Fig. 3A). The endemic healthy control urine samples showed 85% specificity. In the case of other diseases, only 11.11% of the samples were above the cutoff line, depicting very little cross-reactivity with other diseases. Moreover, the mean of the optical density values was below the cutoff and the differences in the antibody levels were highly significant in comparison to VL, with 88.89% specificity. EF1α in ELISA showed 84% sensitivity and 94.73% specificity considering nonendemic healthy individuals as negative controls (Fig. 3B). The specificity of EF1α with endemic healthy controls and other diseases were found to be 85% and 55.55%, respectively. ELISA with CPC antigen resulted in 96% sensitivity and 100% specificity with nonendemic healthy controls (Fig. 3C). ELISAs with urine samples of endemic healthy controls showed 90% specificity compared with VL samples. Urine samples from other diseases showed minimum cross-reactivity, with only one sample above the cutoff line, thus demonstrating 94.44% specificity. The ROC curves obtained for all three antigens, GP63,

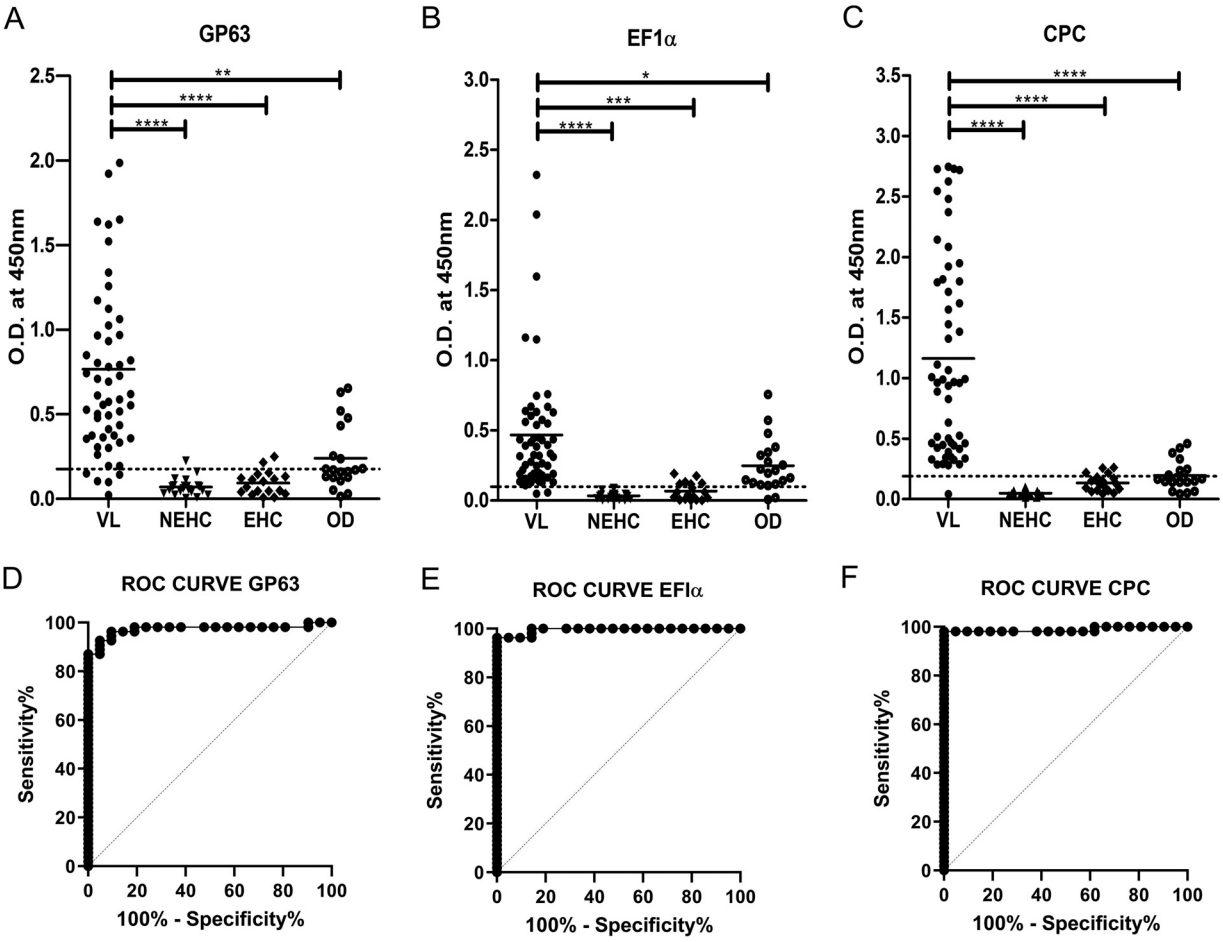

FIG 2 Serum-based ELISAs with recombinant antigens GP63, EF1α, and CPC. (A to C) Three recombinant antigens, GP63 (A), EF1α (B), and CPC (C), were tested by ELISA for anti-GP63, anti-EF1α, and anti-CPC antibodies in serum samples. Sera used in this study were collected from active visceral leishmaniasis patients (VL, $n = 54$), nonendemic healthy controls (NEHC, $n = 21$), endemic healthy controls (EHC, $n = 18$), and other diseases (OD, $n = 20$), which included 5 samples each from malaria, viral fever, typhoid, and tuberculosis patients. Each dot in the figure represents the mean of optical density values of a single sample tested in duplicate. The solid horizontal lines represent the means of the absorbance values of different groups. The dotted horizontal lines (A to C) represent the cutoff values for each ELISA calculated using ROC curves where the highest sensitivity and specificity were obtained. (D to F) ROC curves obtained from ELISAs using antigens GP63 (D), EF1α (E), and CPC (F) for detection of antigen-specific antibodies in serum samples. Two tailed, Mann-Whitney U tests were performed to calculate the statistical significances.

EF1α and CPC, are shown in Fig. 3D to F, respectively. The overall results of ELISA with serum and urine samples are depicted in Table 1.

ELISA as a test of cure. Levels of disease-specific IgG antibodies in urine are short term in comparison to the serum, and can therefore serve as a test of cure. To assess the treatment response, paired serum and urine samples were used for the longitudinal study with seven *Leishmania*-infected patients before the treatment started, after 1 month of treatment, and after at least 6 months of treatment with AmBisome. Antibodies levels against all the three antigens were all retained in the serum after 6 months of treatment. Thus, no significant differences were found in the three different stages of treatment (Fig. 4A to C). However, level of antibodies in urine against all the three antigens declined sharply after 30 days of treatment, and decreased to low levels after 180 days post treatment (Fig. 4D to F).

Immunoblot assay of recombinant proteins with serum samples. After evaluating the recombinant antigens GP63, EF1α, and CPC in serum and urine ELISAs, we screened the antigens' reactivity in the immunoblot assay to identify the best candidate among the three for serodiagnosis of VL, as well as to assess treatment response. Antigens were evaluated in two separate immunoblot assays. First was to differentiate active VL patients from different sets of controls, including nonendemic healthy

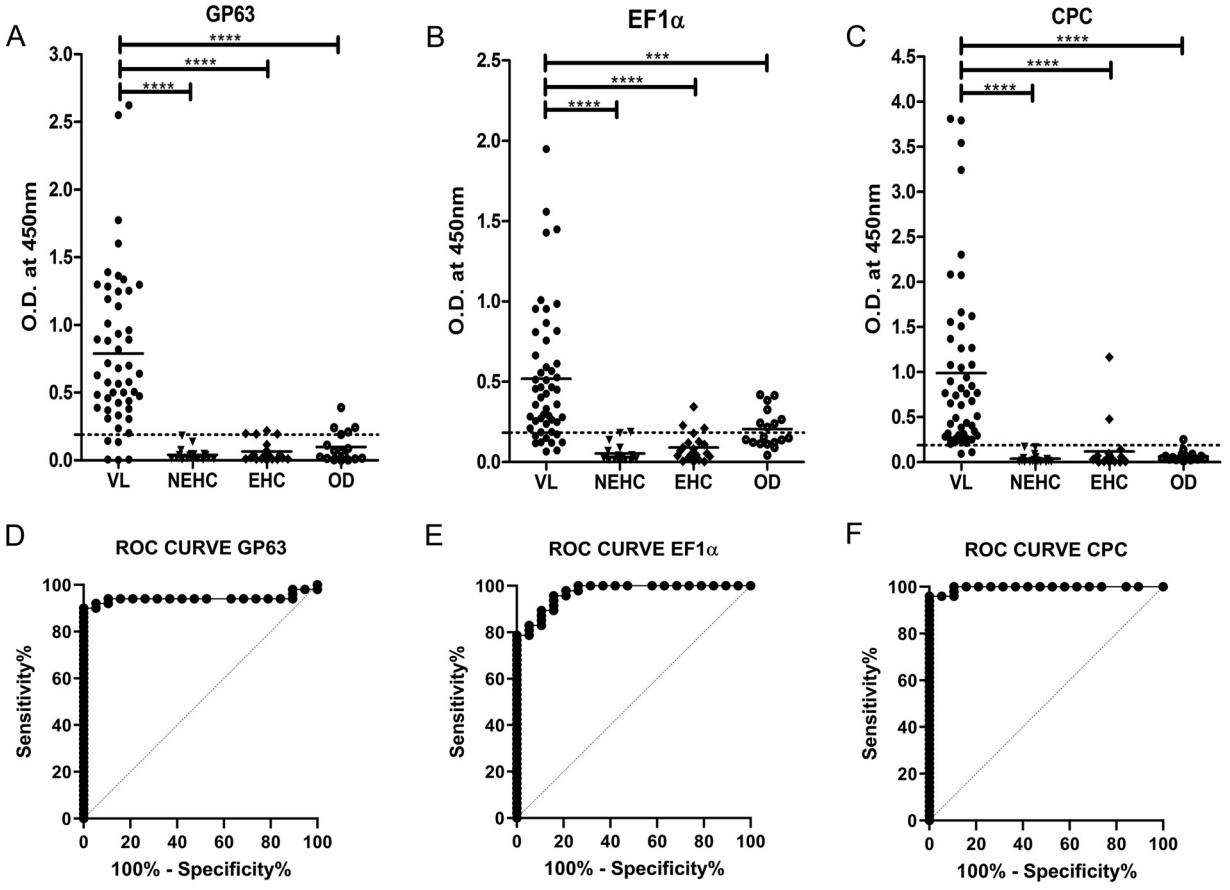

FIG 3 Urine-based ELISAs with recombinant antigens GP63, EF1α, and CPC. (A to C) Three recombinant antigens, GP63 (A), EF1α (B) and CPC (C), were tested by ELISA for antigen-specific antibodies in urine samples. Urine used in this study were collected from active visceral leishmaniasis patients (VL, $n = 50$), nonendemic healthy controls (NEHC, $n = 19$), endemic healthy controls (EHC, $n = 20$), and other diseases (OD, $n = 18$), which included 5 samples each from malaria and viral fever patients, and 4 samples each from typhoid and tuberculosis patients. Each dot in the figure represents the mean of optical density values of a single sample tested in duplicate. The solid horizontal lines represent the mean of the absorbance values of different groups. The horizontal dotted lines (A to C) represent the cutoff values for each ELISA calculated using the ROC curves where the highest sensitivity and specificity were obtained. (D to F) ROC curves obtained from ELISAs using antigens GP63 (D), EF1α (E), and CPC (F) for detection of antigen-specific antibodies in urine samples. Two tailed, Mann-Whitney U tests were performed to calculate the statistical significances.

controls, endemic healthy controls, and other similar diseases for diagnosis. Another was to see the treatment response of paired VL serum samples before treatment started (day 0), after 1-month treatment (day 30), and after at least 6 months posttreatment (day >180). All three antigens, GP63, EF1α and CPC, showed positive reactivity with VL sera and negative with endemic and nonendemic healthy individuals (Fig. 5A, C, and E). With other diseases, GP63 demonstrated cross-reactivity with malaria, viral fever, and

TABLE 1 Sensitivity and specificity of the three antigens under study with serum and urine samples

Sample type	Recombinant antigen	% Sensitivity with active VL patients (n/N)[a]	% Specificity with nonendemic healthy controls (n′/N)[b]	% Specificity with endemic healthy controls (n′/N)[b]	% Specificity with other diseases (n′/N)[b]
Serum	GP63	92.59 (50/54)	95.23 (20/21)	88.89 (16/18)	60 (12/20)
	EF1α	96.29 (52/54)	100 (21/21)	66.67 (12/18)	10 (2/20)
	CPC	98.15 (53/54)	100 (21/21)	77.78 (14/18)	65 (13/20)
Urine	GP63	90 (45/50)	100 (19/19)	85 (17/20)	88.89 (16/18)
	EF1α	84 (42/50)	94.73 (18/19)	85(17/20)	55.55 (10/18)
	CPC	96 (48/50)	100 (19/19)	90 (18/20)	94.44 (17/18)

[a]n, no of positive samples in each group; N, total number of samples tested in each group.
[b]n′, no of negative samples in each group; N, total number of samples tested in each group.

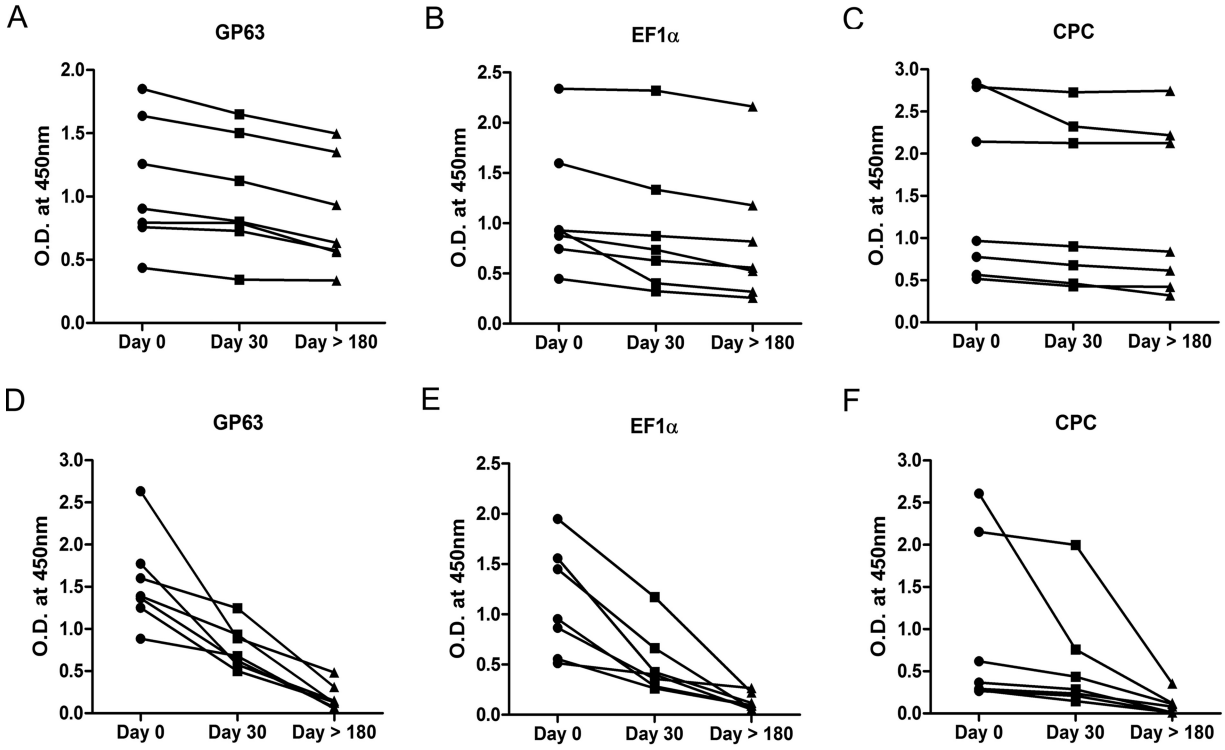

FIG 4 Comparison of levels of antibodies in serum and urine of VL patients before treatment started and posttreatment. Absorbance values of antibodies specific to GP63 (A), EF1α (B), and CPC (C) in serum and antibodies specific to GP63 (D), EF1α (E), and CPC (F) in urine before the treatment of VL started at day 0, 1 month after treatment at day 30, and at least 6 months after treatment at day >180. Paired *t* tests were done to compare the antibodies at different time points.

tuberculosis, whereas EF1α and CPC were found to show faint positive reactions only with malaria.

In the longitudinal study at three different time points after treatment, antibodies specific to antigens GP63, EF1α, and CPC showed resemblance in intensity at day 30 of treatment compared to day 0. After 6 months of treatment, the intensity of the bands for GP63 and EF1α decreased but persisted in all three paired samples (Fig. 5B and D). In contrast, only one sample was positive against CPC at up to 180 days after treatment, and the other two showed slight decline in the antibody response against CPC at this time point (Fig. 5F).

Immunoblot assay of recombinant proteins with urine samples. Immunoblot assays were also conducted with urine samples using recombinant antigens for VL diagnosis and to examine treatment response. Antigens GP63, EF1α, and CPC all showed reactivity with VL urine, whereas endemic and nonendemic healthy controls did not show cross-reactivity against any of the antigens, except for one endemic sample which showed a faint false-positive band with GP63 (Fig. 6A, C, and E). Again, GP63 demonstrated cross-reactivity with malaria and viral fever, whereas EF1α and CPC antigens showed no cross-reactivity with urine samples for any of the other diseases.

Comparing the urine reactivity of VL patients (1 month and 6 months after therapy started) against all three antigens showed mixed responses. After 1 month of treatment, urine antibodies remained the same for two patients against GP63, whereas one patient showed a significant decrease (Fig. 6B). However, after 6 months of treatment, urine samples from two out of three patients did not show any reactivity against GP63. For antigen EF1α, no change in band intensity was observed after a 1-month treatment, whereas after 6 months only one out of three patients showed faint but positive reactivity against the antigen (Fig. 6D). Urine antibody reactivity against CPC after 1 month of treatment showed considerable decreases in antibody levels and became

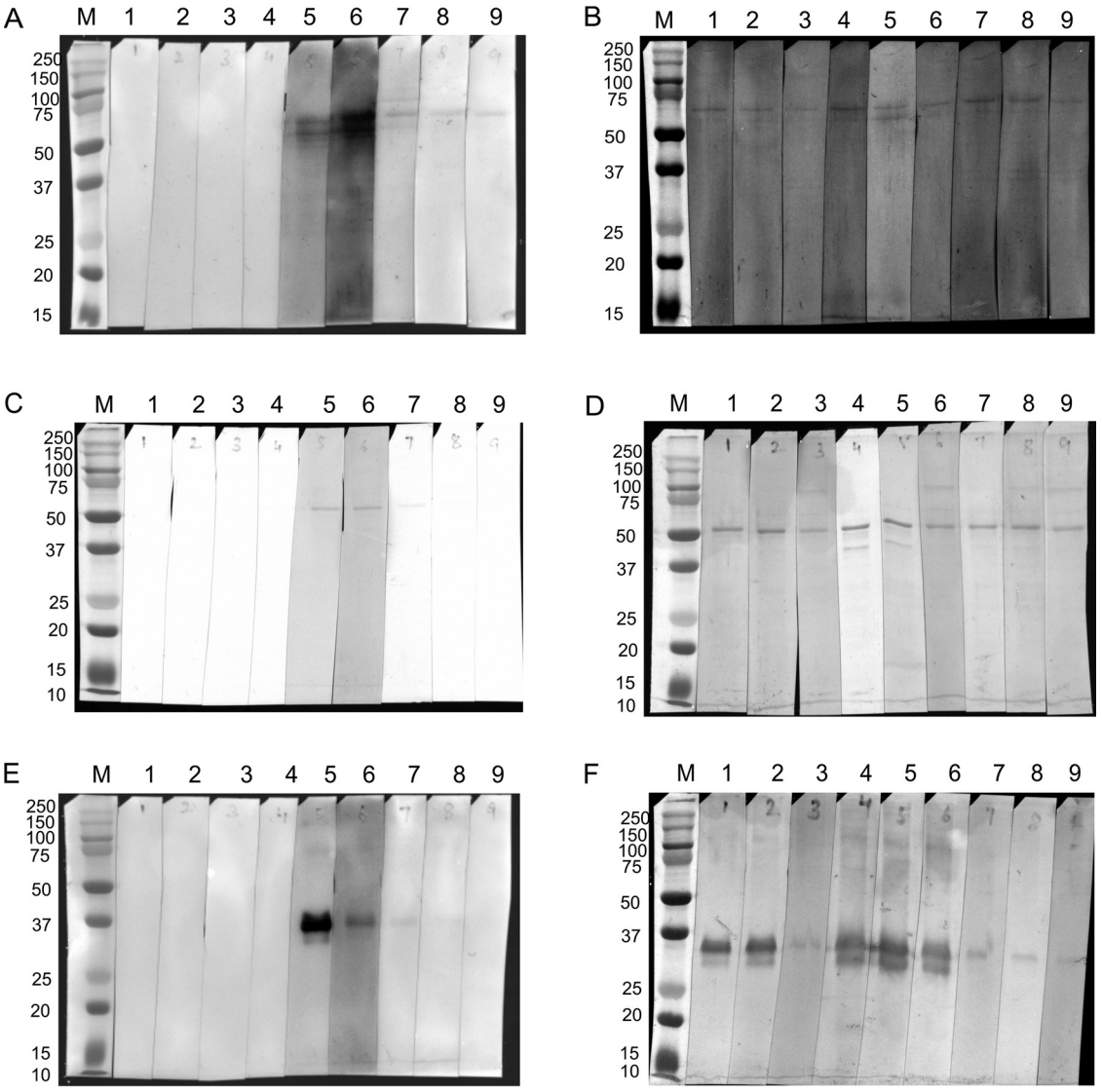

FIG 5 Immunoblot assays using sera against three recombinant antigens. (A, C, and E) Immunoblot assays of recombinant *Leishmania* proteins GP63 (A), EF1α (C), and CPC (E) with serum samples of nonendemic healthy controls (lanes 1 and 2), endemic healthy controls (lanes 3 and 4), VL-positive samples (lanes 5 and 6), and other diseases, including malaria, viral fever, and tuberculosis (lanes 7, 8, and 9). (B, D, and F) In another set of experiments, three paired VL serum samples were used before treatment started (day 0, lanes 1, 4, and 7), 1 month after treatment (day 30, lanes 2, 5, and 8), and 6 months posttreatment (day $>$180, lanes 3, 6, and 9) against GP63 (B), EF1α (D), and CPC (F).

negative in two out of three patients. After 6 months of treatment, the sample that was faintly positive at day 30 also became negative (Fig. 6F).

DISCUSSION

In our previous reports, we have shown the diagnostic potential of crude membrane antigen, LAg, isolated from strain AG83 (ATCC PRA-413) of *L. donovani* promastigotes. LAg exhibited high sensitivity and specificity in VL diagnoses using serum and urine samples (27, 29). Several serum- and urine-reactive antigens of LAg were identified and electroeluted to demonstrate their diagnostic potential (5, 34). However, uncertainty associated with the consistency of the isolation procedure of crude membrane antigens demands the need to explore the diagnostic potential of recombinant proteins. In the present study, we evaluated the diagnostic potential and test of cure possibilities of the antigen CPC, along with two previously identified recombinant antigens, GP63 and EF1α, by detecting antigen-specific antibodies in serum and urine samples. We found

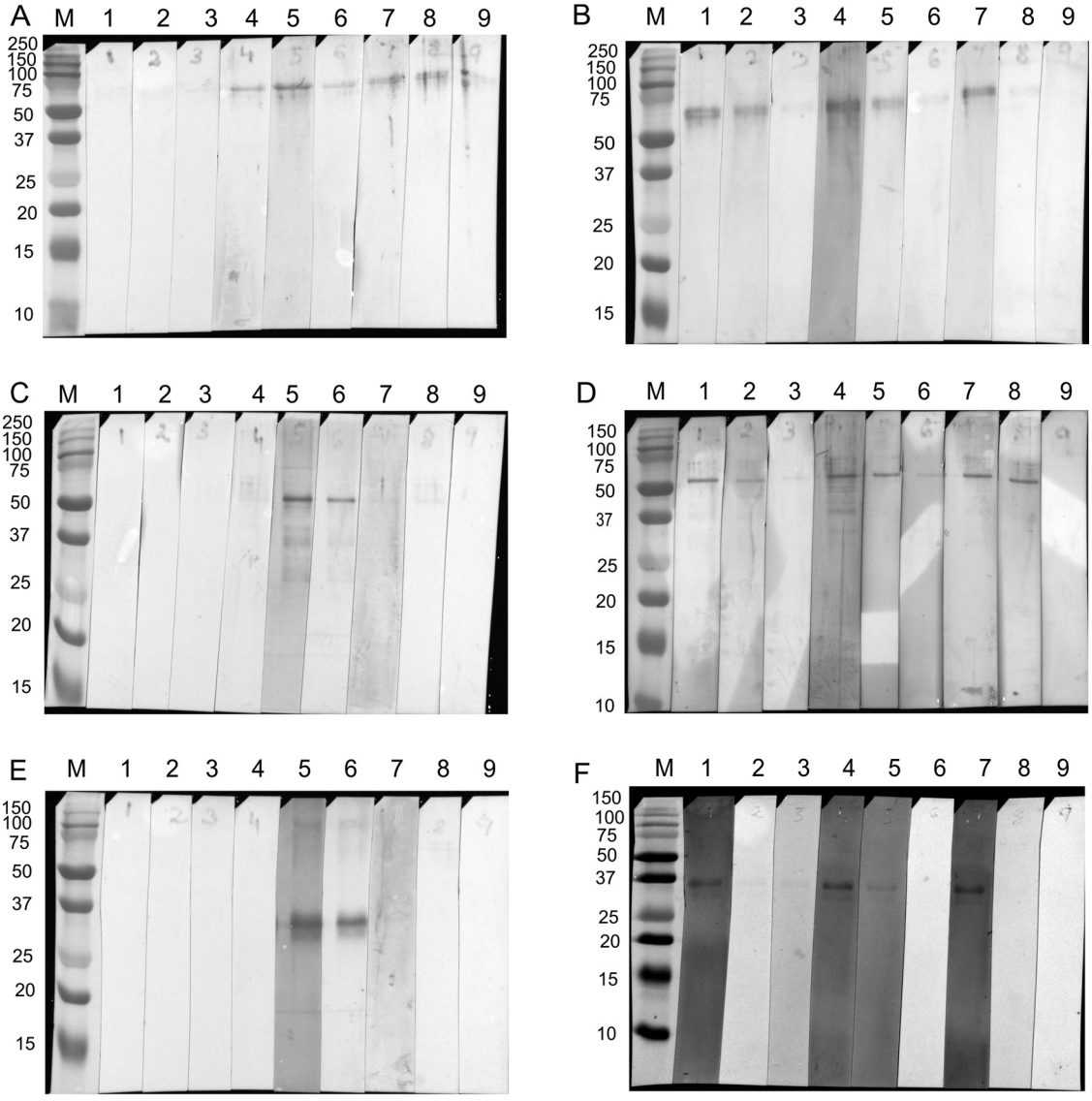

FIG 6 Immunoblot assays using urine against three recombinant antigens. (A, C, and E) Immunoblot assays of recombinant *Leishmania* proteins GP63 (A), EF1α (C), and CPC (E) with urine samples of nonendemic healthy controls (lanes 1 and 2), endemic healthy controls (lanes 3 and 4), VL-positive samples (lanes 5 and 6), and other diseases, including malaria, viral fever, and tuberculosis (lanes 7, 8, and 9). (B, D, and F) In another set of experiments, three paired VL urine samples were used before treatment started (day 0, lanes 1, 4, and 7), 1 month after treatment (day 30, lanes 2, 5, and 8), and 6 months posttreatment (day $>$180, lanes 3, 6, and 9) against GP63 (B), EF1α (D), and CPC (F).

that urine samples are better than serum samples for distinguishing active VL from healthy controls and other diseases. Moreover, among all three recombinant antigens tested, CPC was found to be the best diagnostic and test-of-cure candidate for VL.

It has been observed that high levels of antileishmanial antibodies are generated during VL (35). Thus, serology has been utilized as an effective diagnostic tool for detecting VL owing to the lower invasiveness of the process in comparison with the biopsy specimens used for the gold standard parasitological examination. A range of antibody detection assays like DAT, ELISA, and rapid immunochromatographic tests have been developed. A variety of antigens, including whole-parasite promastigotes and crude (27) or recombinant antigens (36) have been extensively used for developing immunodiagnostic assays for VL. DAT is a semiqualitative test that uses Coomassie-stained whole-promastigote antigens. It has been validated in many VL countries of endemicity, such as India, Nepal, Bangladesh, Sudan, Ethiopia, Kenya, and Brazil (37).

Performance of DAT varies, with 70.5% to 100% sensitivity and 53% to 100% specificity (37, 38). DAT has major shortcomings in requiring multiple pipetting steps, a long incubation time, batch-to-batch variability of the antigen, and cross-reactivity with other trypanosomatids. Application of ELISA to detect antileishmanial antibodies is frequently performed where the sensitivity and specificity is largely dependent on the antigen used. Cell lysate and crude antigens of promastigotes or amastigotes have been used in ELISA in many previous studies, but cross-reactivity with other diseases has resulted in giving it the lowest priority for diagnosis. Moreover, maintenance of the quality of the crude antigens is limited in batch-to-batch preparations. Therefore, more defined recombinant antigens were developed for VL diagnosis, with rK39 being on the top with 67% to 100% sensitivity and 93% to 100% specificity. However, due to its low sensitivity in African regions, a fusion antigen, rK28, was developed with improved sensitivity (92% to 100% in Sudan) (18) without any changes in its sensitivity in the Indian subcontinent (39). In India, rK39 strip tests showed false positivity in about 15% to 32% of healthy individuals living in the region of endemicity, which is a major drawback of this test. Also, cross-reactivity of the rK39 test with patients having illnesses like malaria and enteric fever, mimicking symptoms of VL, might receive toxic antileishmanial therapy (40). The variability of performance of the existing antigens in different areas of endemicity demands newer recombinant antigens to diagnose VL.

In the current study, the protein CPC was cloned, purified, and evaluated as a candidate for VL diagnosis and to assess treatment response in comparison to two other recombinant antigens, GP63 and EF1α. Lysosomal cysteine proteases (CPs) of *Leishmania* are conserved proteins which regulate cell cycle and host-parasite interactions through their proteolytic activities. They have also been found to be immunogenic in experimental VL (32, 41). GP63, conserved in all *Leishmania* species, is the major zinc metalloprotease of *L. donovani* (42). EF1α has been reported as a virulence factor in *Leishmania* that penetrates into the infected macrophages of hosts and deactivates several related pathways (43). In our earlier studies, GP63 and EF1α were identified and found to be the most immunodominant and protective components of soluble leishmanial antigens against experiment VL (30, 31). Herein, we show that antigen-specific antibody levels in serum samples exhibited good sensitivities of 92.59%, 96.29%, and 98.15% for GP63, EF1α, and CPC, respectively. The overall specificities of GP63, EF1α, and CPC were found to be 81.35%, 59.32%, and 81.35%, respectively. Thus, diagnostic performance of CPC was the best among all three antigens (Table 1). However, the common disadvantage for all serology-based antibody detection systems is that antileishmanial antibodies persevere for 2 to 30 years after successful cure and thus cannot be used as tests of cure or relapse (44). The present study strengthens the above observations, as we found the persistence of antibodies against all three antigens in samples collected from seven VL patients at three time points, before the treatment started (day 0), 1 month after treatment (day 30), and after at least 6 months when first sampling was done (day $>$180).

To curb the limitations of serodiagnostic tests, attempts have been made to find a noninvasive biological source for diagnosis of VL, such as urine and/or saliva samples. The rK39 RDT has been employed to test anti-*Leishmania* antibodies in saliva, but the results showed only 58.6% sensitivity (45). The rK39 has also been tested for VL diagnosis with urine. The serum-based rK39 RDT test with urine samples demonstrated sensitivities of 96.1% to 100% in India and sensitivity of 95% in Bangladesh (46, 47). In another study, urine samples were used to evaluate the performance of rK39 and compared with blood samples. The urine testing was found 100% sensitive and 86.33% specific for the diagnosis of VL. However, urine showed more false-positive results in comparison with blood testing (13.67% versus 9.45%) (48).

In this study, on testing three recombinant antigens for noninvasive diagnostic potential, we found their sensitivities to be 90%, 84%, and 96% for GP63, EF1α, and CPC, respectively, with the positive urine samples. Both GP63 and CPC showed specificities of 100% with the nonendemic healthy controls. In the case of endemic healthy controls, the specificities were 85% and 90%, respectively. On studying the cross-

reactivity with other diseases, CPC came up as the best candidate, showing specificity of 94.44%, followed by 88.89% and 55.55% specificities for GP63 and EF1α with urine samples. Therefore, CPC can be considered for noninvasive diagnosis using urine to differentiate VL patients from healthy controls and other diseases like malaria, tuberculosis, and viral fever. The study of antigens GP63, EF1α, and CPC for test of cure suggested that, unlike serum, levels of antibodies in the infected urine fell significantly after 6 months posttreatment with AmbiSome. Particularly, urine samples against CPC antigen did not show any reactivity after 6 months of treatment, suggesting CPC can be a good candidate for test of cure as indicated from ELISA.

ELISA has its own limitations, as it can be performed only in well-equipped hospitals and diagnostic centers, not in the field. Therefore, chromatography-based strip tests were developed using immunoblot antigen-antibody binding to the nitrocellulose membrane, which gave results as visual bands within a few minutes. One such strip used rK39 antigen for the serodiagnosis of VL (5). The performance of the rK39 strip test was, however, moderate in Latin America (Brazil and Venezuela), where sensitivities and specificities varied from 86% to 100% and 82% to 100%, respectively (49). The World Health Organization (WHO) evaluated five immunochromatographic tests using either rK39 or rKE16 in the regions of endemicity of the Indian subcontinent, East-Africa, and Brazil. The study showed variable results, with sensitivities ranging between 36.8% and 100% and specificities between 90.8% and 100% (7). None of the tests were found to be optimum across all regions and conditions. Therefore, the need for field-adaptable diagnostic and prognostic testing remains.

Toward development of field-applicable strip tests, we transformed our experiment from ELISA to membrane-based immunoblot assay, which is a step forward toward membrane-based lateral flow or dipstick tests for rapid diagnosis of VL in field settings. Herein, we subjected the three recombinant antigens GP63, EF1α, and CPC to immunoblot assays against both serum and urine samples. All three antigens showed distinct positivity with VL sera and no reactivity with endemic and nonendemic healthy control sera. However, some false-positive bands were found with malaria serum samples against EF1α and CPC, and GP63 cross reacted with all the sera from other diseases. Thus, EF1α and CPC demonstrated acceptable diagnostic potential to differentiate VL sera from all sets of controls. Since the antibodies persist in serum samples after treatment, serum samples of VL patients before treatment started, after 1 month, and after 6 months of treatment showed more or less similar positivity in immunoblot assays. Thus, similar to the ELISA results, none of the antigens in the immunoblot were able to assess treatment response after therapy using serum samples.

Immunoblot assays with urine samples against EF1α and CPC demonstrated clear distinction of VL from endemic controls and other diseases. However, GP63 showed cross-reactivity with a subset of urine samples, one each from endemic healthy individuals, malaria, and viral fever patients. The antibody response in VL urine after treatment demonstrated no difference in reactivity after 1 month of treatment against GP63 and EF1α. However, after 6 months antibodies disappeared completely except for one case with both GP63 and EF1α. CPC demonstrated the best results for monitoring treatment response, as all the VL-positive urine samples showed decline in positivity and became completely negative after 1 month, which persisted up to 6 months after treatment.

Early and correct diagnosis of the disease is a primary requisite for proper treatment. Over the past two decades, much effort has been devoted to designing and developing an accurate, easily performable, and field-applicable diagnostic test for VL. The available tests based on PCR are expensive, lack field adaptability, and require skilled personnel. On the other hand, tests based on antibody detection in serum and urine are not optimum. New-generation tests based on new recombinant antigens are needed. In this regard, the present study has come up with a *L. donovani* antigen CPC which has shown promising results both as a diagnostic tool and a test-of-cure antigen using both serum and urine samples.

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N.A., N.D., and S.A.E. conceptualized and designed the experiments. N.D., M.K., and R.K. performed the experiments. N.A., N.D., and S.A.E. analyzed the results and prepared the manuscript. A.S., R.C., and S.M. provided the expression clones of GP63 and EF1α. K.P., V.N.R.D., P.D., M.R., and R.P.G. contributed to clinical sample collection, conducted confirmatory tests, and obtained ethical clearance from the hospitals.

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We declare no conflict of interests.

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