



Development of multiplex serological assay for the detection of human African trypanosomiasis



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ABSTRACT

Human African trypanosomiasis (HAT) is a disease caused by Kinetoplastid infection. Serological tests are useful for epidemiological surveillance. The aim of this study was to develop a multiplex serological assay for HAT to assess the diagnostic value of selected HAT antigens for sero-epidemiological surveillance.

We cloned loci encoding eight antigens from *Trypanosoma brucei gambiense*, expressed the genes in bacterial systems, and purified the resulting proteins. Antigens were subjected to Luminex multiplex assays using sera from HAT and VL patients to assess the antigens' immunodiagnostic potential. Among *T. b. gambiense* antigens, the 64-kDa and 65-kDa invariant surface glycoproteins (ISGs) and flagellar calcium binding protein (FCaBP) had high sensitivity for sera from *T. b. gambiense* patients, yielding AUC values of 0.871, 0.737 and 0.858 respectively in receiver operating characteristics (ROC) analysis. The ISG64, ISG65, and FCaBP antigens were partially cross-reactive to sera from *Trypanosoma brucei rhodesiense* patients. The GM6 antigen was cross-reactive to sera from *T. b. rhodesiense* patients as well as to sera from VL patients. Furthermore, heterogeneous antibody responses to each individual HAT antigen were observed. Testing for multiple HAT antigens in the same panel allowed specific and sensitive detection. Our results demonstrate the utility of applying multiplex assays for development and evaluation of HAT antigens for use in sero-epidemiological surveillance.

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1. Introduction

Human African trypanosomiasis (HAT) causes chronic and acute trypanosomiasis with an approximate 70 million people estimated to be at various levels of risk of infection. At least, 57 and 12.3 million people are at risk of *T. b. gambiense* and *T. b. rhodesiense* infection [1,2]. Moreover, over 5 million people are living in high or very high HAT risk areas [2]. In an attempt to reduce or contain the risk, 24 out of the 36 listed endemic countries received exclusive support from WHO to assess either the epidemiological status or establish control and

surveillance activities [3]. There have been efforts to control HAT and other NTDs [3,4] and this has led the focus into elimination of the disease [5–7]. However, challenges have been reported [8,9]. This calls for efficient control and surveillance systems which will form the basis for planning, implementation, monitoring and evaluation for such intervention.

HAT is predominantly characterized by an early blood infection stage, which is followed by a late brain infection stage. During both stages, the parasite expresses a set of variable surface glycoproteins (VSGs). VSGs protect the parasite from the host's immune response, with the resulting antigenic variation leading to cyclical waves of parasitemia as each glycoprotein is replaced by a new VSG. The variable nature of the VSG means that HAT has properties converse to those usually required for detection of a sero-diagnostic target [10].

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Several studies have documented the development of recombinant antigens for use in the diagnosis of HAT [11–13], but direct comparison through simultaneous detection of the diagnostic ability of these antigens has not been previously documented.

Sero-epidemiological surveys are important in estimating disease burden and evaluating the efficacy of interventions. The use of multiplex serological assays in such surveys will enable the detection of multiple antigens for a given disease and the simultaneous detection of multiple diseases. Additionally, multiplex assays can incorporate multiple antigens encoded by a given disease, providing potential to increase sensitivity and specificity.

We had previously described the development of a multiplex immunoassay system for several pathogens detection using a single limited volume of human sample and its use in sero-epidemiological surveys [14]. In this study, we report proof of principle for the development and evaluation of the diagnostic ability and suitability of selected HAT antigens in a multiplex format. To provide stable diagnosis, we selected proteins without high sequence variability.

2. Materials and methods

2.1. Sources of human sera

A total of 139 human sera were used in this study. Of these, 119 human sera corresponded to HAT reference sera that were obtained from the Human African Trypanosomiasis Specimen Biobank, World Health Organization (WHO)/Organization Mondiale De La Sante/NTD-IDM. The sera represented different stages of disease progression as follows: *T. b. gambiense*, 20 negative (Cg), 20 stage-one positive (Pg1), and 20 stage-two positive sera (Pg2); *T. b. rhodesiense*, 20 negative (Cr), 3 stage-one positive (Pr1), and 36 stage-two positive sera (Pr2). The remaining 20 human sera were derived from visceral leishmaniasis patients and were obtained from the Rajshah Medical College in Bangladesh. All sera were stored frozen at -80°C .

2.2. cDNA cloning

T. b. gambiense (Kenyan strain) was kindly provided by NEKKEN NBRP with the support of the Institute of Tropical Medicine, Nagasaki University, which is partly supported by National Bio-Resource Project of MEXT, Japan. The cDNAs encoding FcCaBP, hypothetical 2120, hypothetical 3020, ISG64, and ISG65 were amplified by PCR from genomic DNA of the Kenyan strain. PCR primers were designed based on genomic sequence for DAL972 strain. The DNA sequences of the clones were confirmed using an ABI 3730 DNA Analyzer (Applied Biosystems). The cDNAs encoding GM6, Tbg6 and MARP were chemically synthesized by Integrated DNA Technologies, Inc. Each cDNA was (individually) cloned into the pET52b vector, providing expression of the proteins with N-terminal Strep tags and C-terminal 10xHis fusion tags. Structure of the recombinant antigens, number of tandem repeats, and accession numbers for the reference sequences in TriTrypDB or National Centre for Biotechnology Information (NCBI) database are shown in Table 1.

Table 1
Structure of recombinant antigens expressed in pET52b vector.

Pathogen	Antigen	cDNA	Fusion tag on N-terminus	Antigen region xTR*	Fusion tag on C-terminus	Protein ID
<i>T. b. gambiense</i>	FcCaBP	PCR	MASWSHPQFEKGALEVLFGPGYQ	1–230	ELALVPRGSSAHHHHHHHHHH	XP_011775881
	GM6	Synthetic	MASWSHPQFEKGALEVLFGPGYQDP	(1962–2029) × 3	VDAAEALALVPRGSSAHHHHHHHHHH	Tbg972.11.1200
	Hypothetical 2120	PCR	MASWSHPQFEKGALEVLFGPGYQDP	1–415	VDAAEALALVPRGSSAHHHHHHHHHH	XP_011774551
	Hypothetical 3020	PCR	MASWSHPQFEKGALEVLFGPGYQDP	1–288	VDAAEALALVPRGSSAHHHHHHHHHH	XP_011774088
	ISG64	PCR	MASWSHPQFEKGALEVLFGPGYQDP	24–365	VDAAEALALVPRGSSAHHHHHHHHHH	XP_011773802
	ISG65	PCR	MASWSHPQFEKGALEVLFGPGYQDP	20–385	VDAAEALALVPRGSSAHHHHHHHHHH	XP_011771746
	MARP	Synthetic	MASWSHPQFEKGALEVLFGPGYQDP	(70–107) × 3	VDAAEALALVPRGSSAHHHHHHHHHH	Tbg972.10.12630
	Tbg6	Synthetic	MASWSHPQFEKGALEVLFGPGYQDP	(1193–1254) × 3	VDAAEALALVPRGSSAHHHHHHHHHH	Tbg972.7.4640

* xTR indicates number of tandem repeat in antigen.

2.3. Expression and purification of the recombinant proteins

The recombinant proteins were expressed and purified as described previously [14] except for the use of BL21 (DE3) chemically competent cells in place of BL21Star (DE3) pLysS (Invitrogen). Among *T. b. gambiense* antigens, the hypothetical 2120, ISG64, ISG65, FcCaBP, GM6, and MARP proteins were produced as part of the soluble fractions, whereas the hypothetical 3020 and Tbg6 proteins formed inclusion bodies. Protein concentrations of the purified antigens were determined using the Pierce BCA protein assay kit (Thermo Scientific).

2.4. Coupling of antigens to magnetic microspheres

Individual antigens were dialyzed against phosphate-buffered saline (PBS (–)) (for soluble proteins) or 0.1% N-lauroylsarcosine/PBS (–) (for insoluble proteins) prior to coupling onto MagPlex microspheres (Luminex). Briefly, uncoupled beads were suspended in wash buffer (PBS (–), 0.02% Tween 20) and the concentration of beads was determined by counting under a microscope. For each antigen, 25 µg of purified protein was coupled with 1.25×10^6 microspheres. Carboxyl groups on the microspheres were activated by exposure to EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; Thermo Scientific Inc.) and S-NHS (N-hydroxysulfosuccinimide; Thermo Scientific Inc.) in activation buffer (0.1 M NaH_2PO_4 , pH 6.2) with rotation for 30 min at room temperature. The beads were then subjected to two washes with coupling buffer (50 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.0), and antigen coupling was performed by incubation for 2 h at room temperature with gentle rotation. Blocking of the free carboxyl group was performed using 50 mM ethanolamine pH 8.5 (Wako) for 30 min at room temperature with gentle rotation. StabilGuard Immunoassay Stabilizer (SurModics) was used to wash the microspheres twice and then to adjust the concentration to 1000 beads/µL. Coupled beads that showed aggregation under the microscope were sonicated for 20 s at 10% power limit (Q500 Sonicator, QSONICA LLC.). The coupled beads were then stored at 4°C . Coupling of antigen to the beads was confirmed using anti-His-tag mouse monoclonal antibody (MBL, Code#D291-7), with detection using phycoerythrin-conjugated anti-mouse IgG as the secondary antibody (Rockland Inc., Code#710–1822) on a Bio-Plex 200 system (Bio-Rad).

2.5. Multiplex assay

The multiplex assay was performed as described previously [14]. MagPlex beads coupled (separately) to each of eight *T. b. gambiense* antigens were used for the reactions. Briefly, 2.0 µL of serum was diluted by adding in 98.0 µL of staining buffer (0.1% bovine serum albumin, 0.05% Tween 20, 0.05% sodium azide in PBS (–), pH 7.5) in each well of a 96-well microtiter plate. Binding reactions were performed for 30 min at room temperature in the dark with shaking at 750 rpm. The plate was then washed three times in 100 µL/well staining buffer using an EL×405 microplate washer (BioTek).

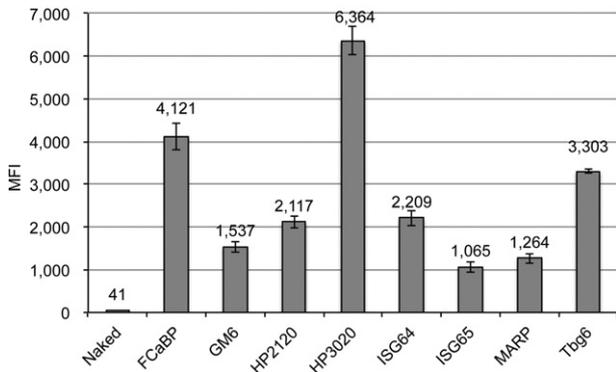


Fig. 1. Coupling confirmation by multiplex format staining of recombinant antigen-coupled beads with anti poly-histidine tag antibody. Y axis: median fluorescent intensity of the coupled beads while the X axis represents the antigens.

Phycoerythrin-conjugated goat anti-human IgG (Rockland Inc.) (diluted 1:250 in staining buffer) was added at 100 μ L/well and the plate was incubated for 30 min at room temperature in the dark with

shaking at 750 rpm. Washing was then performed as previously described and 125 μ L of wash buffer was dispensed into each well. The microspheres were suspended by shaking at 750 rpm for five minutes in the dark. Fluorescence was monitored using a Bio-Plex200 system as above.

2.6. Statistics

Receiver operating characteristics (ROC) analysis and the generation of box plots and graphs were performed using XLSTAT software (Addinsoft). To measure diagnostic ability of the antigens, area under the curve (AUC) was used. Tree diagram of maximum likelihood phylogeny was produced using CLC main workbench (CLC bio).

2.7. Ethics statement

Sampling protocol for visceral leishmaniasis patients was approved by Ethical Committee of Rajshahi Medical College. All adult subjects provided informed consent and a parent or guardian of any child participant provided informed consent on their behalf. For this study, patient signature consent was obtained.

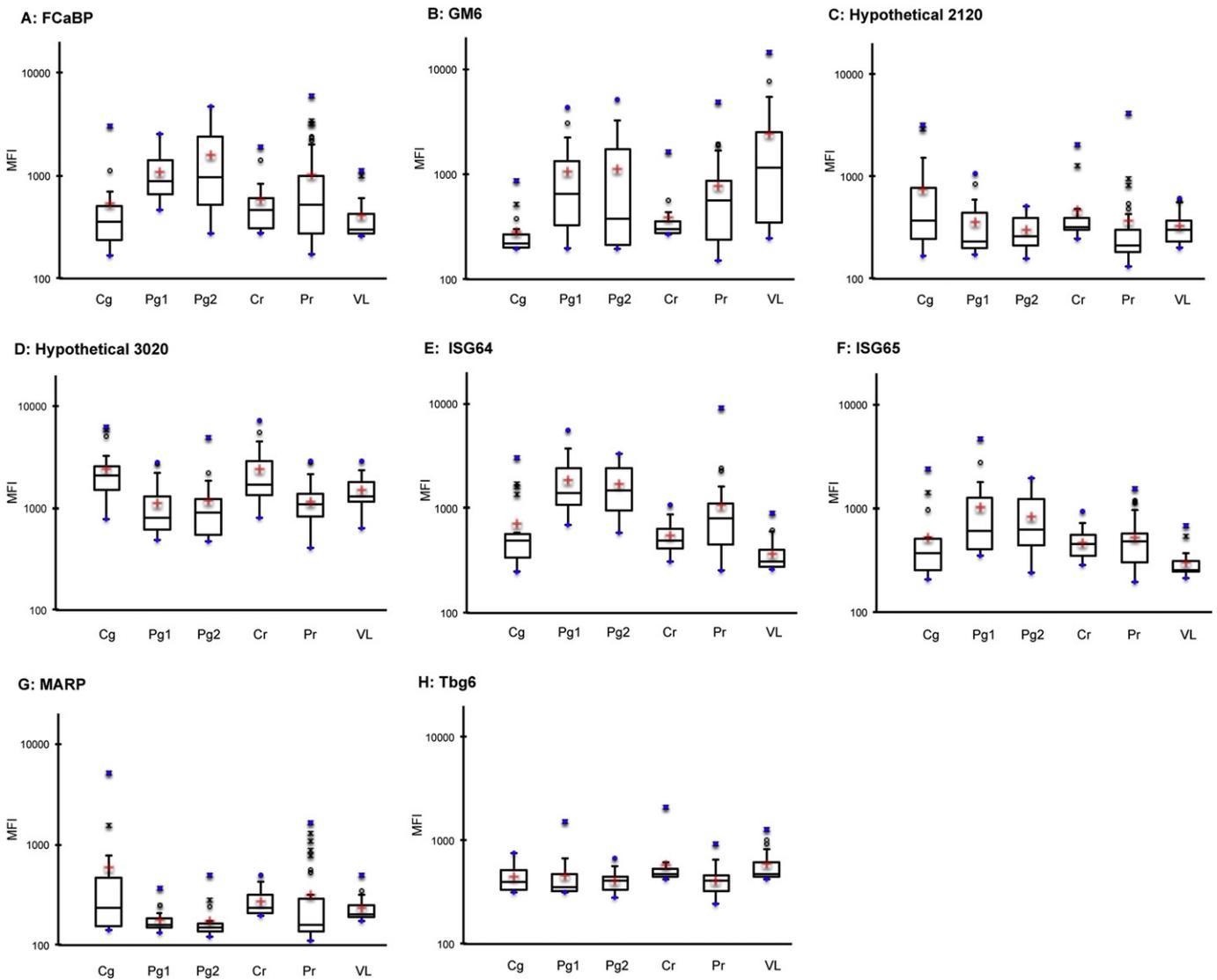


Fig. 2. Box-plot graph for *T. b. gambiense* antigens. Box plots represent fluorescence signals for antigens. A–H: *T. b. gambiense* antigens. The boxes extend from the 25th percentile to the 75th percentile boundaries; the line within each box indicates the median; the red cross indicate the average; the whiskers indicate the 10th and the 90th percentiles. Sera are indicated by pathogen and disease stage; from *T. b. gambiense* patients, Pg1 = stage1, Pg2 = stage 2, and Cg = control; from *T. b. rhodesiense* patients, Pr = stage 1 plus stage 2, and Cr = control; from *L. donovani* patients, VL = visceral leishmaniasis.

Table 2
ROC analysis of *T. b. gambiense* recombinant antigens.

Infectious species	Antigen	AUC*	Cut off**	Sensitivity	95% CI		Specificity	95% CI	
					Low	High		Low	High
<i>T. b. gambiense</i>	ISG64	0.871	587.0	0.975	0.857	1.000	0.800	0.577	0.923
	ISG65	0.737	525.5	0.575	0.422	0.715	0.850	0.629	0.954
	FCaBP	0.858	567.0	0.775	0.622	0.878	0.850	0.629	0.954
<i>T. b. rhodesiense</i>	ISG64	0.694	738.0	0.615	0.459	0.751	0.850	0.629	0.954
	ISG65	0.494	490.0	0.487	0.339	0.638	0.700	0.478	0.855
	FCaBP	0.535	559.0	0.462	0.316	0.614	0.750	0.526	0.890

*AUC; area under the curve.

**Cut off values were determined at MFI values which gave maximum sensitivity plus specificity.

3. Results

3.1. Antigen selection

A total of eight antigens from *T. b. gambiense* were selected for assessment of their diagnostic utility. We selected antigens without high sequence variability to provide stable diagnosis. Two of the invariant surface glycoproteins (ISG64 (XP_011773802) and ISG65 (XP_011771746)), and two of the hypothetical proteins (hypothetical protein 2120 (XP_011774551) and hypothetical protein 3020 (XP_011774088)) were selected because these proteins were identified by proteomics as being reactive to IgG in *T. b. gambiense* infection [11]. GM6 (Tbg972.11.1200), MARP (Tbg972.10.12630), and Tbg6 (Tbg972.7.4640) are tandem-repeat-containing proteins that were identified in animal models by screening of a cDNA library (GM6, MARP) [13] or by bioinformatics (Tbg6) [12]. The flagellar calcium-binding protein (FCaBP, also referred to as Tb-17; XP_011775881), is *T. b. gambiense* homolog of *T. cruzi* antigen used for diagnosis of Chagas disease [15], which was also identified by proteomics as being reactive to IgG in *T. b. gambiense* infection [11].

We cloned the genes encoding FCaBP, hypothetical 2120, hypothetical 3020, ISG64, and ISG65 by PCR amplification from a *T. b. gambiense* cDNA library; cDNAs encoding the other antigens were chemically synthesized. DNA sequencing revealed that our ISG64 and ISG65 clones encoded proteins with multiple sequence changes compared to the *T. b. gambiense* genomic sequence (DAL972 strain); ISG64 included 11 amino acid substitutions (N236D, V241T, K242N, M244L, E245K, R293K, K294R, F298I, T308A, H310Q, E348G); ISG65 included 15 amino acid substitutions (N86G, G87D, P88R, G90Q, R91Y, K92N, G108E, V148Q, V150L, S151D, E153D, T154R, N155D, S158T, G172E) as well as an extra amino acid (i.e., one-codon) insertion after 155 N. Additionally, in our 2120 clone, hypothetical 2120 protein was encoded with a single substitution (P205S) compared to that encoded by the *T. b. gambiense* genomic sequence (DAL972 strain). The sequences of the clones encoding FCaBP and hypothetical 3020 were identical to those of the *T. b. gambiense* genomic sequence (DAL972 strain). The structures of the antigens used in this study are summarized in Table 1.

3.2. Coupling confirmation

To determine the coupling efficiency, polyhistidine tags on the antigen-coupled beads were detected in multiplex format (Fig. 1). The resulting Median Fluorescence Intensity (MFI) values, which reflect the molecular number of antigens per bead, varied from 1065 (for ISG65) to 6364 (hypothetical protein 3020).

3.3. Reactivity of antigens

To clearly describe the reactivity of the antigens for disease-stage determination and cross reactivity between *T. b. gambiense* and *T. b. rhodesiense*, eight *T. b. gambiense* antigens were incorporated into a multiplex assay. We assessed the diagnostic potential of the antigens using a total of 119 HAT reference sera (from patients Cg: 20, Pg1: 20,

Pg2: 20, Cr: 20, Pr1: 3, and Pr2: 36). For *T. b. rhodesiense*, disease stage analysis was not performed due to the minimum number (3 samples) of stage one *T. b. rhodesiense* sera obtained. To access the cross reactivity of the antigens to Leishmaniasis, which is also caused by Kinetoplastid, 20 of visceral leishmaniasis positive sera were tested. Fig. 2 provides box-plot summaries of the resulting data.

Among the eight *T. b. gambiense* antigens, ISG64, ISG65, and FCaBP were specifically reactive to sera from HAT patients; in contrast, GM6 reacted to sera from both HAT and VL patients. No positive reaction was observed for the remaining *T. b. gambiense* antigens (hypothetical 2120, hypothetical 3020, MARP, and Tbg6). Notably, the ISG64, ISG65, and FCaBP antigens were not disease stage specific because they were not able to differentiate *T. b. gambiense* stages Pg1 and Pg2. Reactivity of these antigens (ISG64, ISG65, and FCaBP) to sera from *T. b. rhodesiense* patients was weaker than that observed with sera from *T. b. gambiense* patients. Molecular number of coupled antigens on beads was not a determinant of the reactivity (Figs. 1 and 2).

3.4. Sensitivity and specificity of *T. b. gambiense* antigens

To establish the level of reactivity of *T. b. gambiense* antigens, receiver operating characteristics (ROC) analysis was performed by grouping sera exhibiting positive (Pg1 + Pg2 or Pr1 + Pr2) reactivity compared to those with negative (Cg or Cr) reactivity (Table 2). None of the antigens was disease stage specific (Fig. 2), hence analysis was done based on the *T. b. species*. For *T. b. gambiense* patients, ISG64 showed the best area under the curve (AUC) value at 0.871, followed by FCaBP at 0.858. AUC values obtained for *T. b. rhodesiense* patients were lower than those for *T. b. gambiense* patients. Cut-off values were determined at MFI values which gave maximum sensitivity and specificity in ROC analysis (Table 2).

In multiplex assays, positive definition by multiple antigens is possible. We evaluated positive definition by comparing sera exhibiting single-antigen positive, multiple-antigen positive and triple-antigen positive reactivity against *T. b. gambiense* specific antigens only (ISG64, ISG65, and FCaBP) (Table 3). For *T. b. gambiense* patients,

Table 3
Sensitivity and specificity of *T. b. gambiense* antigens in multiplex assay.

Infectious species	Positive definition	Sample		ISG64 + ISG65 + FCaBP		
		Profile	Number	# positive	Sensitivity	Specificity
<i>T. b. gambiense</i>	Single	Cg	20	6	0.975	0.700
		Pg	40	39		
	Multiple	Cg	20	3	0.850	0.850
		Pg	40	34		
	Triple	Cg	20	1	0.500	0.950
		Pg	40	20		
<i>T. b. rhodesiense</i>	Single	Cr	20	8	0.769	0.600
		Pr	39	30		
	Multiple	Cr	20	5	0.564	0.750
		Pr	39	22		
	Triple	Cr	20	1	0.231	0.950
		Pr	39	9		

3.5. Heterogeneous antibody response in HAT

To understand the meaning of the multiplex assay, the profile of reactivity for each serum was determined (Fig. 3). In *T. b. gambiense* patients (Fig. 3A), double-antigen-positive sera were observed from a combination of ISG64 + FCaBP and ISG64 + ISG65 positivity. In *T. b. rhodesiense* patients (Fig. 3B), similar combinations were observed but sensitivity by definition of multiple-positive was low because of the low sensitivity of the individual antigens. In all sample groups, strong positive reactions (those with MFI values at least three-fold higher than the cut-off value) showed a variety of profiles. These results suggest that HAT patients produce heterogeneous antibody against these invariant antigens following infection.

Through inclusion of the three antigens (ISG64, ISG65 and FCaBP) in a multiplex assay, positive definition by double antigen definition provided an acceptable level of sensitivity and specificity for sero-epidemiological surveillance, hence, reducing false positive and covering heterogeneous antibody response.

4. Discussion

There is a worldwide campaign from WHO and HAT specialists to control and eliminate HAT, but majority of the population at risk still lives in poverty with all ages being vulnerable to the disease. Case detection and management are the existing primary interventions, but suffer from inadequate surveillance, under detection, limited access to essential medicines and drug toxicity [4,16,17]. Surveillance of HAT is key in planning, monitoring, and policy making in prevention, control and eradication of this disease. There are studies which have attempted to map and express the burden of this disease [18–21], but the under reporting and estimation has limited its true burden in the African continent. Indeed, there is need to enhance the existing surveillance tools if control and eradication are to be achieved. The development of a sero-epidemiological method for the determination and mapping of HAT cases by use of advanced multiplex bead-based technology might be a good choice for enhancement of HAT surveillance. Efficacy of the multiplex immunoassay system is crucial and is key to the development, hence, we utilized a literature-guided approach in antigen panel selection. We identified candidate antigens, expressed them in *E. coli*, purified, and assessed for their diagnostic potential using positive and negative patient sera.

Variable surface glycoproteins (VSGs) cover nearly all the membrane of trypanosomes and are the pre-dominant surface antigen in trypanosomes making these proteins to be used as diagnostic tools such as LitTat 1.3 and 1.5 because of their antigenicity. The variable nature of VSGs which is intrinsically related to the surviving strategy of African trypanosome will be cause for poor reactivity. However, there are intracellular proteins that can also be used for diagnosis and do not have the variable nature as the VSGs. FCaBP is a conserved intracellular protein that is part of the Kinetoplastid flagellum. For diagnostic purposes, intracellular conserved proteins might be suitable to consistent diagnosis, although such antigens (being intracellular) may not be appropriate targets for vaccine development.

As demonstrated by our results, ISG64 was better than ISG65 for the detection of IgG against *T. b. gambiense*. This observation contrasts that of an earlier study, which showed that ISG65 gave the best results for diagnosis of HAT [11]. Searches of the NCBI protein database revealed the presence of 39 kinds of ISG64 and ISG65 (Fig. S1). In addition, our clones for ISG64 and ISG65 had sequence variations compared to the respective reference; identified as the best-matching sequences by BLASTP search. Although ISG64 and ISG65 are considered “invariant” (in contrast to the variability inherent to the VSGs), the results suggest that ISG proteins nonetheless exhibit sequence variation among isolates. Further studies using a higher number of individual ISGs are required to demonstrate the relation between ISG variability and antigenicity.

In a multiplex assay, we can evaluate the diagnostic ability of several specific antigens to a given disease simultaneously. Our data suggest that the inclusion of several antigens for a given disease can help eliminate false positive results by use of double (or more)–antigen-positive status definition; the combination of antigens also improves sensitivity by overcoming the heterogeneity of antibody production in separate hosts.

Identification of new *T. b. rhodesiense* antigens is needed to improve the sensitivity of our multiplex assay for use in *T. b. rhodesiense* diagnosis. For the FCaBP, ISG64 and ISG65 antigens, further evaluation is recommended using a higher number of negative and positive *T. b. gambiense* sera.

Furthermore, the multiplex assay format would be a more effective tool than conventional ELISA for the determination and selection of useful antigens amid candidates. In the present report, we selected three antigens from an original set of eight *T. b. gambiense* antigens. Single-reaction testing of the given serum for reactivity against multiple candidate antigens is expected to reduce errors while facilitating comparison of the diagnostic ability. Such an assay is also expected to reduce the amount of serum and time required for such tests.

5. Conclusion

We demonstrated the utility of multiplex assay in determining the diagnostic potential of HAT antigens and combination of three *T. b. gambiense* antigens (FCaBP, ISG64 and ISG65) for possible use in HAT serological surveillance.

Nevertheless, this study needs to be further improved because of the following limitations. There was a limited number of negative and positive sera for *T. b. gambiense* and *T. b. rhodesiense*. Only two ISG antigens were evaluated, hence antigenicity determination between the ISGs was limited. The lack of VL positive sera from the African population led us to use the available Bangladesh VL sera for determination of cross reactivity between HAT and VL.

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