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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.

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 FCaBP, 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.

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, FCaBP, 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).

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
T. b. gambiense	FCaBP	PCR	MASWSHPQFEKGALEVLFQGPGYQ	1–230	ELALVPRGSSAHHHHHHHHHH	XP_011775881
	GM6	Synthetic	MASWSHPQFEKGALEVLFQGPGYQDP	(1962 -2029) ×3	VDAAAELALVPRGSSAHHHHHHHHHH	Tbg972.11.1200
	Hypothetical 2120	PCR	MASWSHPQFEKGALEVLFQGPGYQDP	1–415	VDAAAELALVPRGSSAHHHHHHHHHH	XP_011774551
	Hypothetical 3020	PCR	MASWSHPQFEKGALEVLFQGPGYQDP	1–288	VDAAAELALVPRGSSAHHHHHHHHHH	XP_011774088
	ISG64	PCR	MASWSHPQFEKGALEVLFQGPGYQDP	24–365	VDAAAELALVPRGSSAHHHHHHHHHH	XP_011773802
	ISG65	PCR	MASWSHPQFEKGALEVLFQGPGYQDP	20–385	VDAAAELALVPRGSSAHHHHHHHHHH	XP_011771746
	MARP	Synthetic	MASWSHPQFEKGALEVLFQGPGYQDP	(70–107) ×3	VDAAAELALVPRGSSAHHHHHHHHHH	Tbg972.10.12630
	Tbg6	Synthetic	MASWSHPQFEKGALEVLFQGPGYQDP	(1193–1254) ×3	VDAAAELALVPRGSSAHHHHHHHHHH	Tbg972.7.4640

* xTR indicates number of tandem repeat in antigen.