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Use of Polyclonal Antibody for the Diagnosis of Human African Trypanosomiasis

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Abstract

Human African trypanosomiasis (HAT), commonly known as sleeping sickness is one of the neglected tropical diseases (NTDs), which is fatal if left untreated. Its diagnosis is a challenge since the signs and symptoms of the primary phase are not specific, the existing diagnostic methods have low sensitivity and specificity, and the available drugs have some toxicity. New, robust, and cost-effective techniques are needed for the early identification of parasites. This study aimed to assess the sensitivity and specificity of two different types of polyclonal antibodies against *T. b. gambiense* using antigen detection ELISA. Polyclonal antibodies against the expressed proteins Tbg I2 and Tbg I17 were produced using New Zealand white rabbits. The antibody titer measured was greater than 32 g/L after the 3rd immunization for the expressed protein Tbg I2. For the expressed protein Tbg I17, the antibody titer meas-

ured was greater than 32 g/L after the 4th immunization. The sensitivity and specificity of the Tbg I2 polyclonal antibody confirmed with Polymerase Chain Reaction (PCR) as gold standard were respectively 89.5% and 80.6%, while for the Tbg I17 polyclonal antibody, the sensitivity and specificity were respectively 92.1% and 88.9%. The area under the curve for the Tbg I2 polyclonal antibody was 0.90 ± 0.032, while for the Tbg I17 polyclonal antibody, the area under the curve was 0.92 ± 0.0. The Tbg I17 polyclonal antibody produced in New Zealand white rabbits has good sensitivity and good specificity; it can be successfully used in the diagnosis of HAT.

Keywords

Human African Trypanosomiasis, Polyclonal Antibody, Tbg I2 Expressed Protein, Tbg I17 Expressed Protein, Sensitivity, Specificity

1. Introduction

Human African trypanosomiasis (HAT) commonly known as sleeping sickness, is one of the neglected tropical diseases (NTDs) and one of the causes of death in Africa. In Africa, two (2) subspecies of *Trypanosoma brucei* are known to be responsible for the disease: *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* [1]. The key vector for the transmission of trypanosomiasis is the tsetse fly of the genus *Glossina*, which during its blood meal, inoculates humans with the parasite [2].

Millions of people in 36 countries in sub-Saharan Africa are under the threat of sleeping sickness. The conditions of the disease differ between countries and within regions of a country. In 2009, several control measures were undertaken, bringing the number of reported cases below 10,000 for the first time in 50 years, then below 997 new cases in 2018, the lowest level in 80 years. The identification of sick people is based on specially trained screening teams that visit at-risk individuals or patients in quest of medical inspection. Diagnosing HAT in the field presents many challenges, including logistical problems for testing teams to reach communities in rural areas. Once screening teams are in communities, they face additional challenges in recruiting the entire local population into the HAT screening program, which can lead to underreporting and underestimation of infection rates [3] [4].

In 1972, environmental situation, clinical aspect, and host choice were used to differentiate and categorize *Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense*, and *Trypanosoma brucei brucei* [5]. *Trypanosoma brucei brucei* infects flora and fauna and domestic animals but not humans. *Trypanosoma brucei rhodesiense* is linked with acute sickness in humans in eastern and southern Africa. Its aptitude to infect human beings is controlled by a single genetic factor: the serum resistance antigen (SRA) gene. *T. b. gambiense* is observed to cause chronic disease in humans in Western and Central Africa, which often takes

months or years to develop into a severe disease [6].

Diagnosis and surveillance of HAT in the field presents many challenges, including logistical problems for testing teams to reach communities in rural areas. Once screening teams are in communities, they face additional challenges in recruiting the entire local population into the HAT screening program, which can lead to underreporting and underestimation of infection rates [7] [8].

Early detection of the disease is a challenge since the signs and symptoms of the primary phase are not specific and the diagnostic methods are not very sensitive and specific. The diagnosis requires validation of the existence of the parasite in any body fluid. With *T. b. gambiense*, it can be difficult to identify trypomastigotes in routine blood smears [7]. The classic method for identifying *T. b. gambiense* is microscopic detection of the parasite in a lymph node aspirate. With *T. b. rhodesiense*, the parasitaemia is often greater than with *T. b. gambiense* and symptomatic patients naturally have detectable parasites in the blood. The parasite can also be found in chancre fluid or bone marrow aspirates and its identification is an irrefutable diagnosis.

The most important advances in diagnostics were made during the late 1970s when the card agglutination trypanosomiasis test (CATT) became available for serological screening. Unfortunately, CATT is only applicable to *T. b. gambiense* infections and has low sensitivity (70.0%), specificity (95.9%), and stability [9]. The first rapid diagnostic tests (RDTs) for the diagnosis of HAT are presently being evaluated in the field. These tests utilise the native variable surface glycoprotein (VSG) LiTat 1.3 to test for anti-trypanosome antibodies that have been formulated by Standard Diagnostics (SD BIOLINE HAT) and Coris Bio-concept (Sero-K-SeT) [10]. These antigens undergo variation due to the ability of nature of the parasite to change the surface antigens. There is a need to improve the currently available diagnostic kits or to develop a diagnostic kit, using such as Tbg I2 and Tbg I17 expressed proteins that have been proven to have a better diagnostic value [7] [11] [12]. However, this study aimed to assess the specificity and the sensitivity of two (2) different types of polyclonal antibodies produced in rabbits against *T. b. gambiense* using antigen detection ELISA.

2. Materials and Methods

2.1. Determination of the Concentration of Tbg I2 and Tbg I17 Expressed Proteins

Tbg I2 and Tbg I17 expressed proteins were already produced and purified by Immobilized Metal Affinity Chromatography (IMAC) which was performed by TALON-Accept resin (Code#635503, Takara Bio, Inc, Japan) and then stored at −80˚C. The concentration of Tbg I2 and Tbg I17 expressed proteins was determined using the Pierce bicinchoninic acid (BCA) Protein Assay kit (Thermo Scientific, USA). The standards were measured from low to high concentration, followed by measurements of all samples within 10 minutes using NanoDrop 2000c.

2.2. Determination of the Molecular Weight, Specificity and Purity of the Tbg I2 and Tbg I17 Expressed Proteins

The expressed and purified proteins were separated on a 10% gel in one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting and Coomassie Brilliant Blue (CBB) staining. For Western blotting, the proteins were trans-blotted onto a polyvinylidene difluoride (PVDF) membrane in a Bio-Rad Trans Blot system. For detection of the antigens, PVDF (Polyvinylidene Fluoride)membrane incubated with 10,000 X diluted anti-His Tag HRP-Direct antibody (MBL, D291-7) diluted in 1X TBST for 30 minutes at room temperature. The PVDF membrane was washed five times, with an interval of 10 minutes in 100 mL of 1XTBST at room temperature. Then the PVDF membrane was incubated in chemiluminescence (ECL) (Lot# 9659209, GE Healthcare UK Limited) for five minutes at room temperature. For CBB, the gel was stained in Coomassie Brilliant Blue (40% Methanol, 10% Acetic Acid, 0.25% (w/v) CBB-R250) for protein purity visualization and gently shaken for two hours, then followed by gel destaining step overnight in destaining solution containing 10% Methanol, 7.5% Acetic Acid in water. The image was captured in Azure Biosystems 280, while the protein concentrations of the purified antigens were determined using the Pierce BCA protein assay kit (Thermo Scientific, USA).

2.3. Immunization of Rabbits

Three heads of New Zealand white rabbits (3 months old) were used for immunization, each rabbit was used for each expressed protein Tbg I2 and Tbg I17, and one remaining was used as negative control. A volume of 0.2 mg/mL of each expressed proteins was mixed with equal volume of Freund's Complete Adjuvant (MP Biomedicals, USA) for the first immunization, from the second immunization to fifth immunization, each expressed protein was mixed with equal volume of Freund's Incomplete Adjuvant (MP Biomedicals, USA). The mixture of expressed protein-adjuvant was inoculated to each rabbit by subcutaneous route. The control rabbit was inoculated with PBS by subcutaneous route. The immunization of the rabbits was repeated up to the fifth immunization with 2 weeks intervals.

2.4. Determination of Antibody Titers

An IgG indirect ELISA was performed to determine the antibody titer. An MaxiSorp ELISA plate was coated with 100uL of expressed proteins using ELISA coating buffer (pH 9.6). The sera collected from the rabbits after the fifth immunization were diluted with PBS-Tween 20 starting from 1000 times dilution up to 2^{14} times 1000 times dilution before being added to the wells. A volume of 100 uL of the standard sera (from 2° times 1000 times dilution to 2^{14} times 1000 times dilution) and 100 uL of 1000 times diluted hyper immune rabbit sera samples were added to the wells except for blank. Then the anti-rabbit IgG HRP Conjugate (American Qualex, CA, USA) was added in all wells except for blank,

followed by *O*-Phenylenediamine Dihydrochloride (OPD) (Sigma Chemical, St. Louis, MO, USA) substrate in all wells except for blank. The reaction was stopped by adding the stop solution (1N hydrochloric acid). The Optical Density (OD) was measured at 492 nm with an ELISA plate reader, then the OD values were plotted on the semi-log graph and the antibody titer of each rabbit serum was determined.

2.5. Determination of Sensitivity and Specificity of the Polyclonal Antibodies Produced Using ELISA

The two (2) different polyclonal antibodies which were against the two flagella antigens Tbg I2 and Tbg I17 were used as primary antibody in the ELISA assay. The ELISA plate was coated with 100 uL of the human samples to be tested. The well A1 (blank) of each plate was coated with 100 uL of the coating buffer, and the plates were incubated at 4˚C overnight. Then 100 uL of blockage was added to all the wells, and the plates were incubated at room temperature for 1 h, followed by washing three times with PBS-Tween 20. The two (2) polyclonal antibodies were diluted 10,000 times with PBS-Tween 20. And in the wells of the plate labelled I2, 100 uL of the Tbg I2 polyclonal antibody was added in all the wells, and in the wells of the plate labelled I17, 100 uL of the Tbg I17 polyclonal antibody was also added in all the wells. The plates were incubated at 37˚C for 1 h and then washed three times with PBS-Tween 20. At a volume of 100 uL, Anti-Rabbit IgG HRP Conjugate (American Qualex, CA, USA) diluted 10,000 times was added in all the wells, and the plates were incubated at 37˚C for 1 hour, then washed 3 times with PBS-Tween 20 then 100 uL of the substrate was added in all wells, and the plate was incubated at room temperature for 30 minutes. The reaction was stopped by adding 100 uL of the stop solution. The optical density was measured at 492 nm with an ELISA reader, and receiver operating characteristic (ROC) was used to determine optimal cut off value. IBM SPSS version 14.1 was used to analyze the data.

For this work, a local ethical approval was obtained from Kenyatta University's centre for research ethics and safety. The study was approved under the approval number PKU/2425/I1559.

3. Results

3.1. The Concentration of Tbg I2 and Tbg I17 Expressed Proteins

The concentration of Tbg I2 and Tbg I17 expressed proteins was determined using the Pierce bicinchoninic acid (BCA) Protein Assay kit. The concentration of Tbg I2 expressed proteins was 0.345 mg/mL and the concentration of Tbg I17 expressed proteins was 0.221 mg/mL as shown in Table 1.

3.2. Molecular Weight, Specificity and Purity of Tbg I2 and Tbg I17 Expressed Proteins

Western Blot was performed and its analysis confirmed that expressed proteins

Tbg I2 and Tbg I17 were effectively produced while the Coomassie Brilliant Blue analysis showed that all the two expressed proteins were of good purity. Expressed proteins Tbg I2 and Tbg I17 are respectively indicated by number 5 and number 10 in Figure 1.

In Figure 2 below, number 7 (Tbg I2 His-Elute) showed the size of the expressed protein I2 which is 52.2 KDa. Number 10 (Tbg I17 His-Elute) also showed the size of the expressed protein I17 which is 57.7 KDa.

3.3. Tbg I2 and Tbg I17 Polyclonal Antibodies Titer Determination

For both Tbg I2 and Tbg I17 polyclonal antibodies, the antibody titer was determined by drawing two semi-log graphs. Each graph had the optical density measured versus the dilution factors, and the optical density measured versus the antibody titer.

The antibody titers are shown in Table 2 below. The concentration of the antibody titer was greater than 32 g/L after the 3rd immunization for the expressed protein Tbg I2. For the expressed protein Tbg I17 the concentration of the antibody titer was greater than 32 g/L after the 4th immunization.

Table 1. Concentration of the expressed proteins Tbg I2 and Tbg I17 obtained by Pierce bicinchoninic acid (BCA) Protein Assay kit based on BCA for the colorimetric detection.

Antigens	Concentration (mg/mL)
Tbg I2	0.345
Tbg I17	0.221

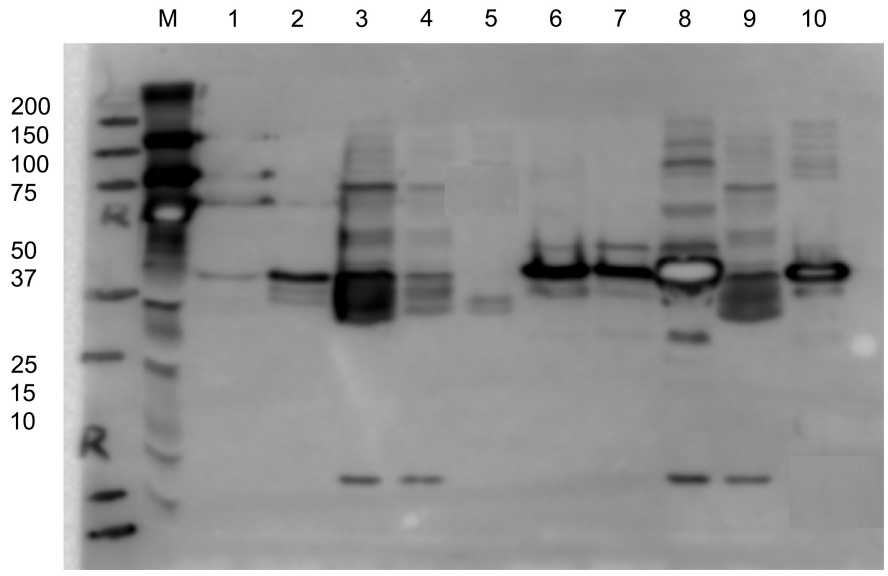

Figure 1. Western Blot Analysis in 10% SDS-PAGE. M: Precision Marker (KDa); 1: Tbg I2 before IPTG (Isopropyl ß-D-1-thiogalactopyranoside); 2: Tbg I2 after IPTG; 3: Tbg I2 soluble; 4: Tbg I2 His-FT (His Flow Through); 5: Tbg I2 His-Elute; 6: Tbg I17 before IPTG; 7: Tbg I17 after IPTG; 8: Tbg I17 soluble; 9: Tbg I17 His-FT; 10: Tbg I17 His-Elute.

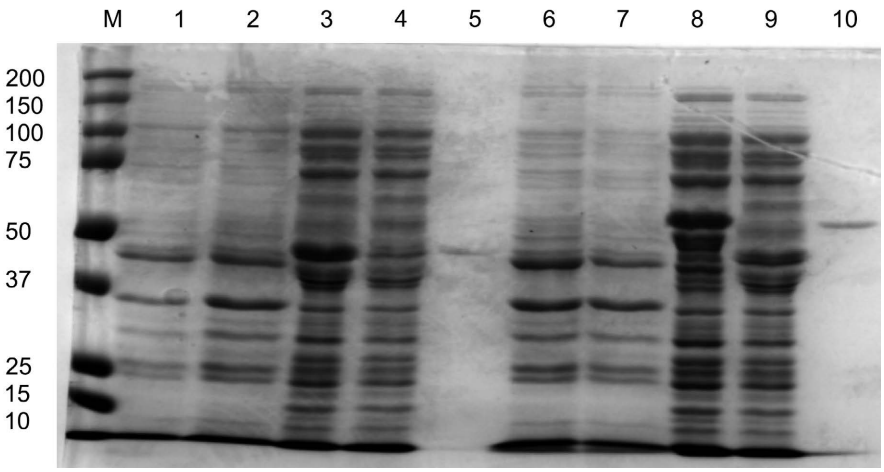

Figure 2. Gel stained in CBB. M: Precision Marker (KDa); 1: Tbg I2 before IPTG (Isopropyl ß-D-1-thiogalactopyranoside); 2: Tbg I2 after IPTG; 3: Tbg I2 soluble; 4: Tbg I2 His-FT (His Flow Through); 5: Tbg I2 His-Elute; 6: Tbg I17 before IPTG; 7: Tbg I17 after IPTG; 8: Tbg I17 soluble; 9: Tbg I17 His-FT; 10: Tbg I17 His-Elute.

Table 2. Antibody titer of Tbg I2 and Tbg I17 polyclonal antibodies.

Immunization	Pre	1st	2nd	3rd	4th	5th
Tbg I2	0.540 g/L	0.580 g/L	1.230 g/L	>32 g/L	>32 g/L	>32 g/L
Tbg I17	0.125 g/L	0.180 g/L	3.6 g/L	22 g/L	>32 g/L	>32 g/L

3.4. Sensitivity and Specificity Assessment of Tbg I2 Polyclonal Antibody

Among the 38 positive samples, 34 were positive, giving a sensitivity of 89.47% as shown in Table 3. And among the 36 negative samples, 29 were negative, showing a specificity of 80.56% as shown in Table 3.

In Figure 3 below, the arrow indicates the highest area under the ROC curve (AUC), which was 0.90 ± 0.032 for the Tbg I2 polyclonal antibody.

3.5. Sensitivity and Specificity Assessment of Tbg I17 Polyclonal Antibody

Among the 38 positive samples, 35 were positive, giving a sensitivity of 92.1% indicated in Table 4. And among the 36 negative samples, 32 were negative, showing a specificity of 88.9% indicated in Table 4.

In Figure 4 below, the arrow indicates the highest area under the ROC curve (AUC), which was 0.920 ± 0.036 for the Tbg I17 polyclonal antibody.

3.6. Sensitivity and Specificity Assessment of the Combination of Tbg I2 and Tbg I17 Polyclonal Antibodies

Among the 38 positive samples, 35 were positive, giving a sensitivity of 92.1% presented in Table 5. And among the 36 negative samples, 29 were negative, showing a specificity of 80.6% presented in Table 5.

As seen Figure 5 below, the arrow indicates the highest area under the ROC curve (AUC), which was 0.905 ± 0.022 for both Tbg I2 and Tbg I17 polyclonal antibodies.

Table 3. The reality crosstabulation of the Tbg I2 polyclonal antibody.

		PCR		Total
		Positive	Negative	
ELISA	Positive	34	07	41
	Negative	04	29	33
Total		38	36	74

*Sensitivity, specificty, positive predictive value (PPV), negative predictive value (NPV), and concordance of 74 samples were 89.5%, 80.6%, 82.9%, 87.88%, and 85.1%, respectively.

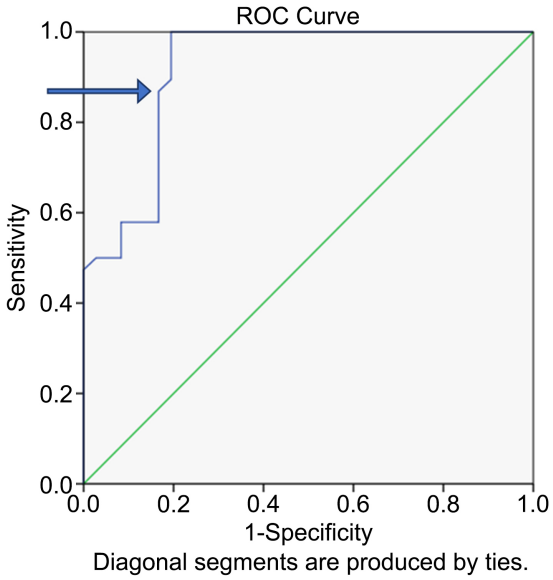

Figure 3. Receiver Operator Characteristic (ROC) Curve in SPSS for the Tbg I2 polyclonal antibody. The curve was generated by plotting the sensitivity and 1-specificity values on the y-axis and x-axis, respectively.

Table 4. The reality crosstabulation of the Tbg I17 polyclonal antibody.

		PCR		Total
		Positive	Negative	
ELISA	Positive	35	04	38
	Negative	03	32	36
Total		38	36	74

*Sensitivity, specificty, positive predictive value (PPV), negative predictive value (NPV), and concordance of 74 samples were 92.1%, 88.9%, 89.74%, 91.4%, and 90.5%, respectively.

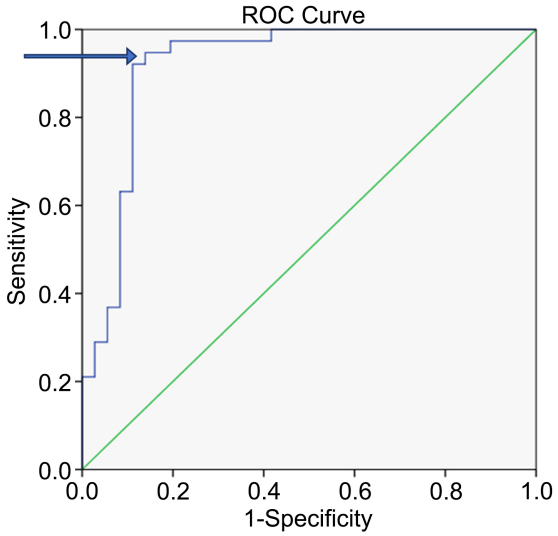

Figure 4. Receiver Operator Characteristic (ROC) Curve in SPSS for the Tbg I17 polyclonal antibody. The curve was generated by plotting the sensitivity and 1-specificity values on the y-axis and x-axis, respectively.

Table 5. The reality crosstabulation of both Tbg I2 and Tbg I17 polyclonal antibodies.

		PCR		Total
		Positive	Negative	
ELISA	Positive	35	07	42
	Negative	03	29	32
Total		38	36	74

*Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and concordance of 74 samples were 92.1%, 80.6%, 83.3%, 90.6%, and 86.5%, respectively.

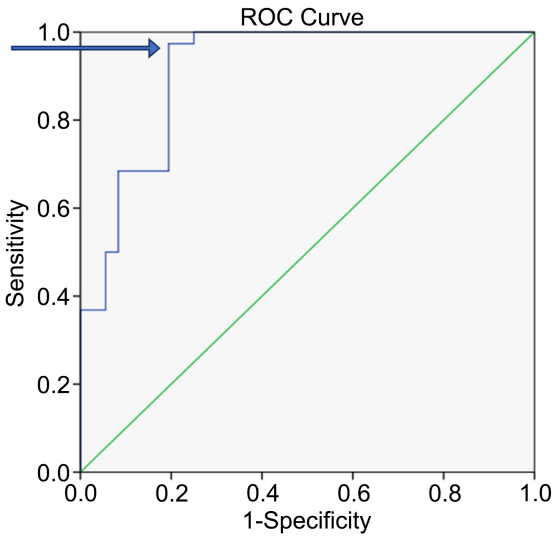

Figure 5. Receiver Operator Characteristic (ROC) Curve in SPSS for both Tbg I2 and Tbg I17 polyclonal antibodies. The curve was generated by plotting the sensitivity and 1-specificity values on the y-axis and x-axis, respectively.

4. Discussion

The concentrations of Tbg I2 and Tbg I17 expressed proteins were 0.345 and 0.221 mg/mL, respectively presented in the **Table 1**. The study conducted by Irumva *et al.*, 2019 reported a concentration for Tbg I2 and Tbg I17 expressed proteins of 1.299 mg/mL and 0.517 mg/mL, respectively [13]. These differences can be related to the short time given for the culture to grow in our study, whereas a long time was given for the culture to grow in Irumva *et al.*'s 2019 study. The sizes of Tbg I2 and Tbg I17 expressed proteins were 52.2 kDa and 57.7 kDa, respectively as described in **Figure 1** and **Figure 2** in our study as well as in Irumva *et al.* study of 2019. In both our study and the Irumva *et al.*, 2019 study, the sizes of Tbg I2 and Tbg I17 expressed proteins were 52.2 kDa and 57.7 kDa, respectively [13] [14].

The concentration of the antibody produced was quantified by ELISA in this study. After the 3rd immunization, the antibody was greater than 32 g/L for the Tbg I2 expressed protein, and, for the Tbg I17 expressed protein the concentration of the antibody titer was greater than 32 g/L after the 4th immunization showed in **Table 2**. The difference in the highest antibody titer reached with regard to the number of immunization, can be related to the structure of the Tbg I2 and Tbg I17 expressed proteins, as well as the pipetting errors during the preparation of the immunogen dose. The antibody titers increased gradually with the immunization period and also with the frequency of immunization. In the study conducted by Wang *et al.* in 2010 on Production of native bispecific antibodies in Rabbits, polyclonal antibodies against the corresponding haptens and carriers were successfully produced, and the maximum antibody titer was defined as a dilution giving an absorbance reading of 0.1 units over the blank signals [15]. In 1989, the study carried by Cooper and Paterson, showed that the amount of specific antibody present in polyclonal antiserum, ascites fluid, orhybridoma supernatant can be quantitated by either solid-phase radioimmunoassay (RIA)or by ELISA. The study also highlighted that, in the solid-phase assay, the amount of specific antibody in the antiserum is determined from a standard curve generated with a specific antibody of known concentration [16].

A total of 74 human samples were used to assess the reactivity of the Tbg I2 and Tbg I17 polyclonal antibodies. The sensitivity and specificity of the Tbg I2 polyclonal antibody were 89.5% and 80.6% respectively, while for the Tbg I17 polyclonal antibody, the sensitivity and specificity of were 92.1% and 88.9%, respectively indicated in **Figure 3** and **Figure 4**. The Tbg I17 polyclonal antibody showed better sensitivity and specificity compared to Tbg I2 polyclonal antibody. Due to the fact that the flagella antigens I2 and I17 are invariable and always present, the Tbg I17 polyclonal antibody can be used alone and give better diagnosis of HAT. The card agglutination trypanosomiasis test (CATT) that is been using since 1960 for the diagnosis of *T. b. gambiense* infections has been reevaluated by Bisser *et al.* in 2016, and the study showed a sensitivity of 70% and a specificity of 96% [9]. The first rapid diagnostic tests (RDTs) for the diagnosis of

HAT that were formulated by Standard Diagnostics (SD BIOLINE HAT) and Coris Bio-concept (Sero-K-SeT) and utilise the native variable surface glycoprotein (VSG) LiTat 1.3 to test for anti-trypanosome antibodies were also evaluated by Lumbala *et al.* in 2017 [10], the sensitivity of the test reported was 92% and its specificity was 97.1%. The genome of *T. b. gambiense* codes for a large number of VSG antigens that are expressed differentially during the course of infection and it has been reported that the VSGs LiTat 1.3 and LiTat 1.5 are predominantly expressed by *T. b. gambiense*. The difference in sensitivity that we report here between the RDT and CATT could be due in part to the inclusion of the LiTat 1.5 antigen, and as such, it could be assumed that a patient infected with trypanosomes that had expressed only the VSG LiTat 1.5 antigens might be missed by CATT and only detected using the RDT.

5. Conclusion

Diagnosing HAT in the field presents many challenges, including logistical problems for testing teams to reach communities in rural area. The present invention relates to the field of diagnostics in which polyclonal antibodies were used to detect Tbg I2 and Tbg I17 flagella antigens for Human African Tryoanosome. The polyclonal antibodies were successfully produced in rabbits with good sensitivity and good specificity; they can be successfully used in the diagnosis of HAT.

Ethics Statement

A local ethical approval was obtained from Kenyatta University's centre for research ethics and safety. The study was approved under the protocol number PKU/2425/I1559.

Credit Author Statement

Conceptualization, S.M.N., R.W.W., and D.K.O.; Methodology, S.M.N., R.W.W., D.K.O., S.I., M.M.M., and A.W.M.; Software, D.K.O., S.I.; Validation, S.M.N., S.I., M.M.M., and A.W.M.; Formal Analysis, S.M.N., S.I., and D.K.O.; Investigation, D.K.O.,S.M.N., A.W.M.,M.M.M., S.I., C.W.N., P.K.R.,T.T.N., J.J.Y., R.M.I., P.M.N., S.K.M., D.M.K., O.P.S., L.G., N.S.T., and G.N.K.; Resources, S.M.N., S.I; Data Curation, D.K.O., S.M.N., and S.I.; Writing-Original Draft Preparation, D.K.O., S.M.N., R.W.W.; Writing-Review and Editing, D.K.O., S.M.N., S.I., R.W.W., and R.M.I.; Visualization, S.M.N., M.M.M., S.I., and L.G.; Supervision, S.M.N., R.W.W.; Project Administration, S.M.N.; Funding Acquisition, S.M.N., and S.I.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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