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Preliminary studies for development of an immunochromatographic test (ICT) for detection of antibody against salivarian trypanosomes by using recombinant ribosomal P0 protein

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ABSTRACT

The aim of the present study was to develop an immunochromatographic test (ICT) for the diagnosis of African trypanosomosis by using recombinant ribosomal P0 protein. The P0 C-terminal domain is highly antigenic and considered to be a major target of the antibody response in infected animals. Entire gene encoding the P0 protein was cloned, and expressed in *Escherichia coli* as a histidine-tagged recombinant protein (rP0). The rP0 was further purified by using nickel affinity chromatography under denaturing conditions. The rP0 was employed as a capture antigen in order to detect anti-P0 antibody in infected animal sera. Antigen-antibody reaction was detected by either colloidal-gold conjugated protein A or G. The results showed that colloidal gold-conjugated protein A and G were suitable for buffalo and cattle serum samples respectively. The rP0-ICT can detect specific antibody in 12,800-fold dilution serum. All sera from cattle or mouse infected with other protozoan parasites, such as *Toxoplasma* and *Babesia*, showed negative results. Field samples (150 serum samples from Uganda) were examined by rP0-ICT and rP0-ELISA showed that the positive rate is 48% (72/150) and 50% (75/150), respectively. The high agreement indicated that the rP0-ICT is reliable. This suggests that the rP0-based ICT can be used as a rapid on-site diagnostic method of salivarian trypanosomosis.

Key words: trypanosomosis; serodiagnosis; immunechromatography

INTRODUCTION

Animal African trypanosomosis (AAT) widely spread affecting 40 countries situated in regions that could potentially be the most productive. The main pathological symptoms of AAT are weight loss, anemia, and immunosuppression. It is estimated that 50 million cattle and 70 million small ruminants are at risk, costing the continent between 1.5 and 5 billion US dollars per annum (Coustou *et al*., 2010). *Trypanosoma brucei*, *T. congolense*, and *T. vivax* are transmitted by tsetse flies and cause AAT, while *T. evansi* is mechanically transmitted by biting flies such as tabanids, and cause surra in various animals (Artama *et al*., 1992; Ventura *et al*., 2002; Claes *et al*., 2004).

The ideal antigens for development of serodiagnostics should be immunogenic and invariable those are consistently expressed in the bloodstream forms of the parasite. In addition to cell surface antigens, cytosolic antigens can be diagnostic antigens, because they are released from trypanosomes as a result of immune mediated cell lysis. The ribosomal P0 protein, an ortholog of a bacterial ribosomal protein L10, is part of the eukaryotic ribosomal stalk, which is an elongated lateral protuberance of the large ribosomal subunit involved in the translocation step of protein synthesis. The P0 is the minimal portion of the stalk that is able to support accurate protein synthesis (Ban *et al*., 2000). The P0 C-terminal is highly antigenic and a major target of the autoantibody response in patients with systemic lupus erythematosus (Elkon *et al*., 1985), and patients suffering chronic heart disease caused by the *T. cruzi* infection (Bonfa *et al*., 1993). In protozoan parasites, the presence of the P0 on the surface of the sexual and asexual stages of the human

malarial parasite induced immunological responses against the parasites, since the anti-PfP0 antibodies inhibited the growth of merozoites in an *in vitro* culture and also conferred protection to mice against *Plasmodium yoelii* infection (Chatterjee *et al.*, 2000a; Chatterjee *et al.*, 2000b). Moreover, the vaccination with recombinant P0 or DNA carrying the P0 gene induced a protective immunity against cutaneous leishmaniasis and babesiosis in mice (Iborra *et al.*, 2003; Iborra *et al.*, 2005; Terkawi *et al.*, 2007). Previously we have established 7 clones of hybridoma cells producing monoclonal antibodies against procyclic forms of *T. congolense* (Inoue *et al.*, 2000). One clone, named 4D4, recognized cytosolic 37 kDa antigen, which was later clarified as ribosomal P0 protein (unpublished data).

Currently available diagnostic methods for AAT are light microscopy of blood samples, card-agglutination test for trypanosomes (CATT) (Bajyana Songa and Hamers, 1988), polymerase chain reaction (PCR) (Desquesnes *et al.*, 2001; Cortez *et al.*, 2009), and loop-mediated isothermal amplification assay (LAMP) (Kuboki *et al.*, 2003; Thekisoe *et al.*, 2007; Njiru *et al.*, 2008). Microscopy of thick blood films has low sensitivity (10,000 parasites per ml) (Chappuis *et al.*, 2005), and observation is laborious. Thus microscopy is not really practical as a large-scale screening method. The CATT to detect variant surface glycoprotein specific antibodies from the serum samples has proved their usefulness as convenient clinical diagnostics and seroepidemiology of trypanosomosis. However, variant surface glycoprotein-based diagnostic test have a problem of false negative due to antigenic variation (Holland *et al.*, 2005). Therefore, trypanosome invariable antigen-based serodiagnostic methods have to be developed. The immunochromatographic test (ICT) is simple, rapid (taking approximately 10 min), and affordable diagnostic method, which only needs small volume of the test serum. In addition, the ICT has been developed for a variety of applications over the past decade (De Saeger and Van Peteghem, 1996; Huang *et al.*, 2004; Kim *et al.*, 2007; Reithinger *et al.*, 2010). However, since no ICT test for African trypanosomosis has been developed, the objective of the present study is to develop an ICT assay for the specific diagnosis of AAT.

MATERIALS AND METHODS

Expression and purification of the recombinant P0 protein

Recombinant plasmid of pRSET-P0 was transferred to *E. coli* BL21 competent cells. A single colony of the transformed bacterial cell was cultivated in LB-ampicillin medium at 37^{o}C up to an optical density of 0.4-0.6 OD600 nm. The culture was treated with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) in order to induce recombinant P0 protein (rP0) expression and grown for an additional 3h at the same temperature. The bacterial cells were collected by centrifugation, resuspended in lysis buffer (0.1 M phosphate buffered saline pH 8.0, 10 mM Tris-HCl pH8.0), sonicated for 10 min, centrifuged at 10,000 xg for 30 min at 4^{o}C. The pellet was resuspended in CHAPS buffer (1% CHAPS, 0.1 M phosphate buffer pH 8.0, 10 mM Tris-HCl pH8.0), and centrifuged at 10,000 xg for 15 min at 4^{o}C, discarded the supernatant. The final pellet was resuspended in urea buffer (8 M urea, 0.1M phosphate buffer, 10 mM Tris-HCl pH8.0), rotated overnight, and then centrifuged at 10,000 xg for 15 min at 4^{o}C. The supernatant was mixed with the Ni-NTA agarose beads (QIAGEN Gmbh, Hilden). The mixture was incubated with gentle agitation for 1h at room temperature, and the beads were washed with the following 3 buffers, namely buffer 1 (0.1 M phosphate buffer pH 8.0, 10 mM Tris-HCl, 0.5% deoxycholic acid, 8 M urea), buffer 2 (10 mM Tris HCl pH8.0), and buffer 3 (60% isopropanol, 10 mM Tris-HCl pH8.0). Finally the Ni-NTA beads were resuspended in buffer 4 (0.1 M phosphate buffered saline pH 8.0, 10 mM Tris-HCl pH8.0, 8 M urea, adjust pH to 6.0 with HCl) and poured into column which had been previously equilibrated with buffer 4. The protein was eluted five times with elution buffer (0.1 M phosphate buffer, 10 mM Tris-HCl pH, 8 M urea, adjust pH to 4.0 with HCl) (Juri Ayub *et al.*, 2001). The eluted fractions were analyzed by SDS-PAGE (Laemmli, 1970; Juri Ayub *et al.*, 2001).

Five fractions of rP0 were put into a dialysis tube (Spectra/Por 16 mm regenerated cellulose membranes with a 10 kDa molecular mass cut-off), and dialyzed against 50x volume of dialysis buffer (10 mM Tris–HCl

pH8.0, stating from 1 M urea) at 4°C. Dialysis buffer was exchanged gradually until only Tris-HCl (pH 8.0). The sample was left overnight at 4°C and then concentrated to the original volume by filtration using Centricon (Amicon) with a 10 kDa cutoff. The protein concentration was determined from the absorbance at 280 nm.

Western blotting

Twelve percent gel SDS-PAGE was performed using a standard procedure (Laemmli, 1970). The rP0 was transferred to nitrocellulose membranes and blocked 1hr at 4°C with 5% skimmed milk in Tris-buffered saline (TBS-M). Monoclonal antibody 4D4 against rP0 diluted at 1:2,000 with TBS-T（0.05% Tween 20 in Tris-buffered saline）was used as the primary antibody. Conjugated HPR anti-mouse IgG (1:2,000) was used as the secondary antibody.

Conjugation of protein and colloidal gold

Colloidal gold (50 nm in diameter, Sigma) was used for the conjugation of protein A, protein G and goat anti-bovine IgG. The colloidal gold solution was adjusted to OD_{520}=1.0 by sterilized distilled water. Determination of optimal pH for conjugation was carried out by adding 10 μl each of KH_2PO_4 (pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5) into each well of 96-well plate, then add 90 μl colloidal gold and 10 μl protein A, protein G, or goat anti-bovine IgG at the concentration of 100 μg/ml. The reaction mix was agitated for 2 min, then added 10 μl of 10% NaCl into each well. The mixture was agitated for 5 min, and check on OD580 nm. Serial dilution of proteins (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mg/ml) and optimal pH of KH_2PO_4 were used to determine the optimal concentration of protein for conjugation. The procedure was same as described above. Conjugation was performed by mixing 9 ml of colloidal gold (OD_{520}=1), 1 ml of KH_2PO_4 (optimal pH) and 1 ml of protein A, protein G, or goat anti-bovine IgG (optimal concentration). The reaction mix was agitated for 10 min, added 0.5 ml of 1% PEG 20,000 and 1 ml of 10% BSA. The BSA was used to block the residual surface of the nanocolloidal gold particles. The conjugation reaction mix was agitated for 15 min, and was centrifuged at 8,000 xg for 15 min at 4°C. After the centrifugation, the pellet was suspended in 20 ml stock buffer (20 mM Tris-HCl pH 8.2, 0.05% PEG 20,000, 1% BSA, 0.1% sodium azide), then sonicated for a few minutes. The colloidal gold conjugate solution was then centrifuged for the second time under the same condition described above. The pellet was resuspended in 1 ml stock buffer and the optical density was adjusted to 6.0 at OD520 nm with the stock buffer. These proteins conjugated with colloidal gold were stored at 4°C until use. Colloidal gold conjugated Protein A (20 nm) was purchased from Sigma (USA).

Determination of optimal condition for control line

Prior to prepare rP0-ICT strip, dot blot assay was performed to determine optimal concentrations of the colloidal gold-conjugated protein A and anti-protein A antibody. The gold-conjugated protein A (Sigma) was diluted to OD=1.0, 1.5 and 2.0 with 10 mM phosphate buffer pH7.0, containing 0.15 M NaCl, 5 mg/ml BSA and 0.05% Tween 20. Anti-protein A antibody (Sigma-aldrich) was diluted with deionized water to 2.5 mg/ml, 2.0 mg/ml, 1.5 mg/ml, 1.0 mg/ml and 0.5 mg/ml. Gold conjugated-goat anti-bovine IgG and -protein G were diluted with deionized water to 1.0 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.1 mg/ml. One microliter each of anti-protein A antibody, goat anti-bovine IgG, or protein G was blotted onto the nitrocellulose (NC) membrane and reacted with series of diluted gold-conjugated protein A, protein G, or goat anti-bovine IgG.

Preparation of ICT strip

The gold-conjugated protein A, protein G, or goat anti-bovine IgG (67.5 μl/cm^2) was sprayed on glass fiber (0.8x300 mm, Schleicher and Schuell, Keene, NH) and dried in a vacuum overnight. The rP0 protein (1.0 mg/ml) containing 3% methanol and the anti-protein A antibody, goat anti-bovine IgG, or protein G (optimal concentration) were sprayed linearly on nitrocellulose (NC) membrane by using a Biojet 3050 quanti-dispenser (BioDot Inc., Irvine, Calif.) as test line and control line, respectively. The distance of test line and control line is 5 mm. The NC membrane was dried at 50°C for 1hr, and blocked by using 0.5%

casein in a 50 mM boric acid buffer (pH 8.5) for 30 min. After washing with 50 mM Tris-HCl (pH7.5) containing 0.5% sucrose and 0.05% mM SDS, the NC membrane was dried in air for overnight (or 50℃ 30 min). Sample pad was treated by buffer containing 100 mM Tris-HCl (pH 7.0) and 5 mM EDTA (pH 7.0) and dried at 50°C for 1hr. The NC membrane, the conjugate pad, sample pad, and absorbent pad, was assembled on an adhesive card, and then cut into 5 mm-wide strips using a CM4000 cutting module (BioDot Inc., Irvine, CA). The strips were stably stored with dehumidification in foil pouches and ambient temperature until use (Huang, X. *et al*., 2004; Kim, C. *et al*., 2008).

Evaluation of the ICT

The sensitivity of the ICT strip was determined by measuring the responses to bovine sera from rP0 protein immunized cattle, and sera from *T. evansi* experimentally infected water buffaloes. Serially diluted positive control sera were used to assess the sensitivity of the ICT. *Toxoplasma* and *Babesia* infected mouse and cattle sera were used to assess the specificity of the ICT. One hundred fifty field cattle sera samples collected in Uganda were examined both the ICT and crude antigen ELISA in order to assess performance of the ICT.

RESULTS

Recombinant P0

The rP0 was successfully expressed and purified as a 40 kDa His-tagged protein (data not shown). Specific anti-P0 monoclonal antibody 4D4 could recognize the rP0 by Western blotting (data not shown).

Conjugation of colloidal gold and proteins

The colloidal gold will aggregate and the OD580 nm will increase when add sodium chloride into it. This phenomenon does not occur if the colloidal gold is fully coated with protein. Taking advantage of the property of colloidal gold, we can determine the optimal pH and concentration for conjugation. In order to choose a more strong and sensitive conjugation of a gold colloid with protein, we conjugated 3 kinds of protein, namely, protein A, protein G and goat anti-bovine IgG, with colloidal gold (50 nm). The optimal condition for conjugation were 80 μg/ml protein in pH 7.0 KH_2PO_4 buffer for protein A, protein G and goat anti-bovine IgG (Fig. 1A and 1B).

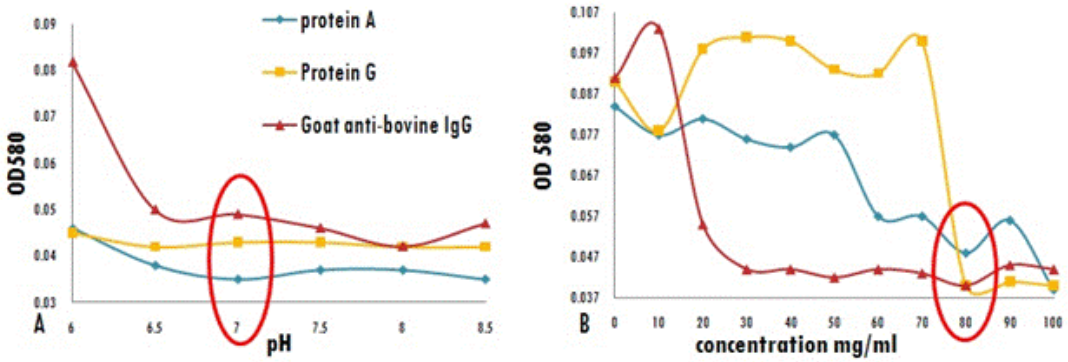

Fig. 1. Optimal pH and concentration for conjugation. The optimal pH is 7 (A), and optimal concentration of proteins is 80mg/ml (B).

Optimal condition for control line

The optimal concentration of anti-protein A and goat anti-bovine IgG was 1 mg/ml each, and the optimal OD520 nm value of protein A and protein G conjugated colloidal gold was 1.5 each (data not shown). The dots of protein G could not be seen reacting with goat anti-bovine IgG conjugated colloidal gold on NC membrane (data not shown).

Recombinant P0-based ICT

Methanol (3%) can increase the binding affinity of rP0 and NC membrane. The rP0-based ICT test

could detect anti-P0 antibody in infected buffalo and rP0 immunized cattle serum (Fig. 2A and 2B).

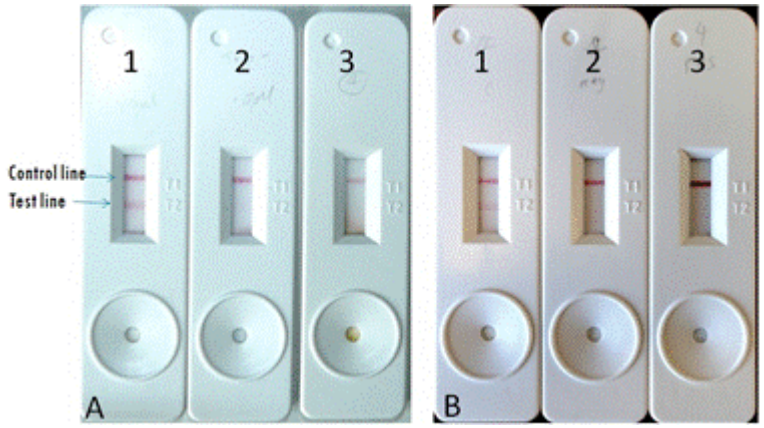

Fig. 2. Positive sera were tested by the rP0-based ICT. A (protein A conjugated colloidal gold): 1. *T. evansi* cpogz infected buffalo serum (49 days after inoculation); 2. Normal buffalo serum; 3. PBS control. B (protein G conjugated colloidal gold): 1. rP0 immunized cattle serum; 2. Normal cattle serum; 3. PBS control.

The sensitivity of ICT was evaluated using sera from buffalos experimentally infected with *T. evansi* and sera from rP0 immunized cattle. Recombinant P0-based ELISA showed that buffalo No.1 and No.2 were negative for anti-rP0 antibody responses, while buffalo No.3 was positive from day 8 of post-inoculation (Fig. 3A). Cattle No.1 was positive for anti-rP0 antibody from day 17 of post-immunization (Fig. 3B). In contrast to rP0-based ELISA, rP0-based ICT showed that all three buffalos sera were positive using protein A-gold (Fig. 3C). It indicated that ICT base on protein A-gold is more sensitive than ELISA in buffalos sera detection. The sera of cattle No.1 was positive for rP0 antibody at 17th day and 37th day post-infection, and the serum of cattle No.2 was negative throughout the experiment tested by ICT using protein G conjugated colloidal gold (Fig. 3D). Protein G-gold was suitable for cattle sera rather than buffalo sera. The ICT can detect 12,800-fold dilution serum (Fig. 4A).

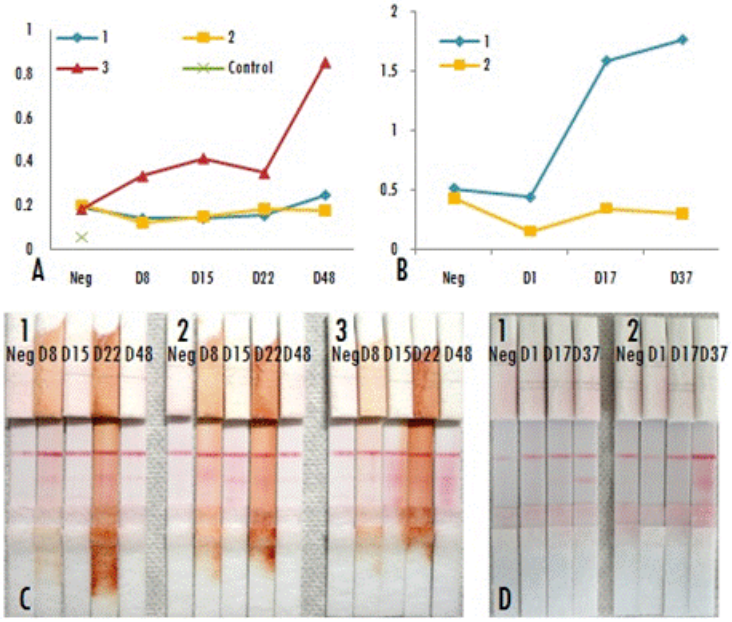

Fig. 3. Comparison of the results of rP0-based ELISA and ICT. A: Buffalos 1 and 2 were negative, while a buffalo 3 was positive for anti-rP0 antibody responses from day 8 of post-infection. B: Cattle 1 was immunized with rP0; Cattle 2 was negative control. C: Except negative control, all infected sera were positive. D: The immunized sera of cattle 1 at day17 and day 37 were positive, while cattle 2 was negative.

Specificity test showed that the rP0-ICT only detected anti-P0 antibody from rP0 immunized cattle serum but not from *Toxoplasma* and *Babesia* infected mouse and cattle sera, respectively (Fig. 4B). To evaluate the rP0-based ICT, 150 cattle sera from Uganda were examined. The result of rP0-based ICT was compared to those of rP0-based ELISA. There were 72 (48%) positive sera detected by rP0-based ICT, while 75 (50%) sera were positive detected by rP0-based ELISA (Table 1).

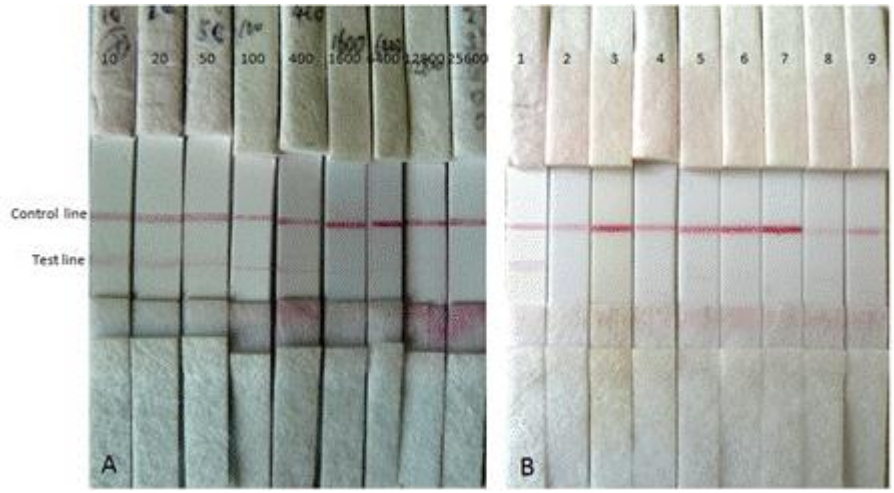

Fig. 4. A: Different fold dilution of serum from rP0 immunized cattle. 20-fold dilution was the optimal serum dilution rate in rP0-ICT. The ICT can detect 12,800-fold dilution serum. B: Specificity test. 1: rP0 immunized cattle serum; 2: normal cattle serum; 3: normal mouse serum; 4-7: *Toxoplasma* infected mouse serum; 8-9: *Babesia* infected cattle serum.

Table 1. Comparison of rP0-based ICT and ELISA

		ELISA		Total
		+	−	
ICT	+	49	23	72
	−	26	52	78
Total		75	75	150

DISCUSSION

Responding to market needs, many rapid diagnostic tests have recently come onto the market, especially ICT. For *Trypanosoma cruzi* infection, ICT diagnostic kit is commercially available (Reithinger *et al*., 2010). Thus the objective of the present study was to develop an ICT assay for the diagnosis of AAT. In this study, rP0 was employed as antigen because of its high antigenicity as compared to other antigens derived from trypanosomes (Juri Ayub *et al*., 2001). The P0 is the minimal portion of the eukaryotic stalk protein that is able to support accurate protein synthesis albeit at a lower rate than the complete pentameric complex (Remacha *et al*., 1995). Western blot analysis and ELISA test showed that rP0 could react with P0 specific monoclonal antibody and anti-serum. It's a good candidate for ICT device. But in ICT, the concentration of rP0 should be increased; because standard concentration (0.5 mg/ml) was not sensitive enough to detect anti-P0 antibody in serum from rP0 immunized cattle or from *T. evansi* infected buffalos. So the concentration of rP0 was increased from 0.5 mg/ml to 1 mg/ml, and the pH of rP0 was adjusted to isoelectric point (pH=8.85). At this condition, the test line can be seen, even though it was not strong. In order to improve the sensitivity of the rP0-ICT, we also adjusted the diameter of colloidal gold from 20 nm to 50 nm, examined several gold conjugated proteins, such as protein A, protein G and goat anti-bovine IgG,

and compared the several sample pad treatment. About sample pad, when we treated it with buffer of Tris-HCl and EDTA, the band of test line is clearer than not treated sample pad. Blocking NC membrane after spraying test line and control line, it can increase the specificity, and decrease the background, but it can decrease the sensitivity at the same time. When we make sure the test line clear enough, we can use blocking to make the test line better. According to the agreement of rP0-ICT and ELISA (96%) in this study, we concluded that the ICT based on rP0 can be used as a useful diagnostic tool in field detection. Furthermore, the use of a rapid detection test will improve surveillance and thus reduce the burden of disease estimates, especially in remote settings.

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