Performance of enzyme-linked immunosorbent assays for detection of antibodies against *T. congolense* and *T. vivax* in goats

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SUMMARY

Indirect ELISAs using denatured antigen preparations of *Trypanosoma (T.) congolense* (TcAGd) and *T. vivax* (TvAGd) for detection of anti trypanosome antibodies in bovine serum (I-TAB ELISAs), were adapted for serodiagnosis in goats. The diagnostic proficiency, the cross-reactivity with sera from heterologous trypanosome infections and the operational performance of the assays were evaluated on experimentally trypanosome-infected goats.

The I-TAB ELISA (TcAGd) detected antibodies in all *T. congolense* infected goats (100% overall sensitivity) from two to four weeks post-infection until the end of the experiments. Specificity tested on 92 uninfected goats was 96.7%. Extensive cross-reactions of I-TAB ELISA (TcAGd) with sera from *T. vivax* or *T. brucei* infected goats were observed. The I-TAB ELISA (TvAGd) detected antibodies in 5 of the 6 *T. vivax* infected goats, specificity tested on uninfected goats was 100%. Cross-reactivity with sera from *T. congolense* or *T. brucei* infected goats remained limited. Infecting species identification based on the highest percent positivity in both systems, correctly identified all *T. congolense* infections, but misidentified in 2/19 occasions a *T. vivax* infection as a *T. congolense* infection. In the absence of *T. brucei* specific antigen coated plates, *T. brucei* infections were identified in respectively 7/9 and 2/9 occasions as *T. congolense* or *T. vivax* infections. Acceptable inter-plate repeatability was observed. The implications of results and technical requirements for ongoing applied research are discussed.

Keywords: *Trypanosoma congolense*, *Trypanosoma brucei*, *Trypanosoma vivax*, I-TAB ELISA, goat, antibody, serum
The most important pathogenic trypanosomes of small ruminants in Africa are Trypanosoma (*T.*) congolense, *T.* vivax, and *T.* brucei. Although important differences in strain virulence occur, in general, *T.* congolense infections are highly pathogenic and often fatal whereas *T.* vivax infections rather result in production loss. Infections with *T.* brucei seem less prevalent and less pathogenic.

As a consequence of often low parasitaema, the sensitivity of parasite detection techniques is limited (Paris et al., 1982). Therefore, the use of serodiagnostic techniques seems appropriate. Antigen detection by direct sandwich enzyme-linked immunosorbent assays (ELISA) (Nantulya and Lindqvist, 1989) turned out to be unreliable (Eisler et al., 1998; Morzaria et al. 1998; Rebeski et al., 1999). The recommendations on the use of immunoassay methods (Morzaria et al., 1998) were followed when applied research activities were initiated on the validation of new ELISA methods for detection of *T.* congolense and *T.* vivax antibodies in bovine (IAEA 2000, Rebeski, 2001). The resulting ELISA kits, I-TAB ELISA (*TcAGd*) and I-TAB ELISA (*TvAGd*), contain all necessary reagents including ELISA plates, precoated with crude denatured antigen (*AGd*) preparations originating from *in vitro* cultured *T.* congolense (*TcAGd*) and *T.* vivax (*TvAGd*), respectively.

For the study described here, the FAO/IAEA I-TAB ELISA (*TcAGd*) and I-TAB ELISA (*TvAGd*) were adapted for detection of antibodies to *T.* congolense and *T.* vivax in goat serum.

**MATERIALS AND METHODS**

*Animals and design of study*

In total, 93 goats of different breeds were used in this study for controlled infections at the experimental farm of the Institute of Tropical Medicine (ITM) in Antwerp, Belgium and at the International Trypanotolerance Centre (ITC) in Banjul, The Gambia.

Group 1 consisted of six female Djallonké goats subcutaneously infected with the *T.* congolense ITC84 strain (1 ml, 10^6 parasites/ml) at ITC. Serum samples were collected before experimental infection (week 0), and at week 1, 2, 4, 8 and 12 (1 sample missing) post infection (p.i.).
Group 2 comprised six female Saanen goats subcutaneously infected with the *T. vivax* EATRO1185 strain (0.5 ml, $10^6$ parasites/ml) at ITM. Serum samples were collected before infection (week 0) and 1 (1 sample missing), 2, 4, 8, 12 and 16 weeks p.i..

Group 3 comprised six female Saanen goats subcutaneously infected with *T. brucei brucei* AnTat 1.1 clone (1 ml, $10^6$ parasites/ml) at ITM. Serum samples were collected before infection (week 0, 1 sample missing) and 1, 2, 4 and 6 weeks p.i..

Group 4 consisted of 36 non-infected (control group) and 39 *T. congolense* strain ITC84 infected goats (40 West-African Dwarf breed and 35 West African Dwarf X Sahelian cross-breeds; 41 female, 34 male). The animals were kept at the ITC. Serum samples were collected pre-infection (week 0) and 4, 8 and 12 weeks p.i.. When a low packed cell volume occurred, infected animals were withdrawn from the experiment (5 animals at week 4, 13 animals at week 8 and another 6 animals at week 12) and treated with diminazene aceturate (3.5 mg/kg). At week 12, serum samples of only 30/36 uninfected goats were available.

Parasitological techniques

The presence of circulating trypanosomes in blood was detected using the dark ground/phase contrast buffy coat technique (DG) as described by Murray et al. (1977).

ELISA protocol

For detection of *T. congolense* and *T. vivax* antibodies in goat serum, the standardised FAO/IAEA ELISA, developed for bovine, required some modifications. The optimum serum and conjugate dilutions were determined by checkerboard titration. Incubation steps were done at room temperature (20-24°C) throughout. Antigen-precoated plates, stored at room temperature until used, were re-hydrated with 300 µl PBS (0.01M phosphate, 0.14 M NaCl, pH 7.4) per well for 60 min. Plates were then washed three times with PBS-Tween (0.01M phosphate, 0.14 M NaCl, 0.05% v/v Tween 20, pH 7.4) using an automated plate washer (EL 402, Biotek). Each washing cycle included aspiration of the overall plate, immediate dispensing of 350 µl of PBS-Tween into each well followed by a soaking step for 1 second. Each washing step in the protocol was completed by a final aspiration step. Serum samples were diluted in sample diluent buffer (0.01M phosphate, 0.2 M NaCl, 0.05% w/v NaN₃, 1% w/v skimmed milk powder, pH 7.15, Régilait,) at a
dilution of 1:1000 for I-TAB ELISA (TcAGd) and 1:400 for I-TAB ELISA (TvAGd). The positive control serum was similarly diluted. It consisted of pooled serum from 5 goats with proven infection of *T. congolense* and *T. vivax*, respectively. Presence of circulating antibodies in these goats had been previously demonstrated by indirect immunofluorescence assay (Ndao, 1998). The diluted test sera and positive control serum were applied (150 µl/well) in triplicate (experiment 1) or in duplicate (experiment 2), and incubated for 60 min. Thereafter, wells were washed as described before. The horseradish-peroxidase donkey anti-goat IgG (H+L) conjugate (Jackson) was diluted at 1:2500 in PBS-Tween and incubated for 30 min (150 µl/well). After five washing cycles each well was incubated for 60 min with 150 µl of substrate-chromogen solution prepared from 50 mg ABTS (2,2’-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid, Boehringer) dissolved in 100 ml of ABTS-buffer (phosphate-citrate-sodium perborate solution, pH 4.6, Boehringer). The optical density values were measured at 415 nm wavelength using a multichannel spectrophotometer (Multiskan RC Version 6.0, Labsystems) and ranged between 0 and 4.0. Results were expressed as percent positivity (PP) values of the mean of duplicates or triplicates, relative to the optical density (OD) of the positive control serum samples (Wright et al., 1993), which had average ODs of 3.2 for I-TAB ELISA (TcAGd) and 2.4 for I-TAB ELISA (TvAGd).

**Cut-off values and standardization of PP values**

For the I-TAB ELISA (TcAGd), the cut-off value was calculated from the mean PP value plus 3 standard deviations (SD) obtained from sera taken at week 0 from 92 of the 93 goats used in this study. For the I-TAB ELISA (TvAGd), the cut-off value was calculated in a similar way from 17 goats of group 1-3. For comparison of PP values in the 2 types of ELISAs, results were standardized according to Desquesnes et al. (2001a). Values of PP<sub>TvAGd</sub> were multiplied by cut-off<sub>TcAGd</sub>/cut-off<sub>TvAGd</sub> to normalised PP values, in order to be able to compare them to the untransformed values of PP<sub>TcAGd</sub>.

**RESULTS**

**Cut-off values**
For I-TAB ELISA (TcAGd), the week 0 sera of 92 goats provided the mean PP value ± 1SD of 8.67% ± 2.27%. A PP value of 15% was therefore considered as the cut-off value. This resulted in a 96.7% specificity of I-TAB ELISA (TcAGd) on the 92 non-infected goats.

For I-TAB ELISA (TvAGd), PP$_{TvAGd}$ values of 17 non-infected goats provided the mean value ± 1SD of 14.54% ± 7.73%. A PP$_{TvAGd}$ value of 38% was therefore considered as the cut-off$_{TvAGd}$ value. All PP$_{TvAGd}$ were subsequently transformed to normalised PP values multiplying them by 15/38. The specificity of I-TAB ELISA (TvAGd) on the 17 non-infected goats was 100%.

**Experiment 1**

Both ELISA systems were evaluated on groups 1-3 for their potential 1°) to detect species-specific antibodies (homologous) 2°) to detect cross-reacting antibodies (heterologous) and 3°) to correctly identify the trypanosome species, based on the highest PP in one of the systems. All animals became parasitologically positive in DG on at least one occasion during infection. We therefore considered them all infected after week 0.

**Analysis of homologous reactions**

Using the I-TAB ELISA (TcAGd), seroconversion is demonstrated in each of the six T. congolense infected goats of group 1 at 2 weeks post-infection (Fig. 1A). All goats consistently tested positive during the whole 10 weeks infection period.

Results of the I-TAB ELISA (TvAGd) demonstrate variation of antibody response in the six T. vivax-infected goats of group 2 (Fig 1D). Three animals became positive after 2 weeks and one animal after 8 weeks. They remained positive during the rest of the infection. One goat was positive at week 12 post-infection only, and another goat remained sero-negative.

**Analysis of heterologous reactions**

Using I-TAB ELISA (TcAGd) antibodies were detected in 3 out of 6 T. vivax infected goats (Fig. 1C): a first goat tested positive during the pre- and post-infection period; a second goat was positive in week 0, 2, 4 and 8, and a third one in week 12. Antibodies reacting in I-TAB ELISA (TcAGd) were also detected in 5 out of 6
T. brucei infected goats (Fig 1E): three goats showed antibodies at week 4 and 6 post-infection; two goats showed weak immune response at week 4 and week 6 post-infection.

With I-TAB ELISA (TvAGd), antibodies were detected in 2 T. congolense infected goats at week 2 and 12 post-infection, respectively (Fig 1B) and in 2 of 6 T. brucei infected goats, one at week 2 and 4 and one at week 4 (Fig 1F).

Identification of species based on highest PP

According to Desquesnes et al. (2001a), specificity is increased if species identification is based on the highest PP of a seropositive animal.

The PP of T. congolense infected goats from week 2 p.i. onwards, was in all cases higher in the I-TAB ELISA (TcAGd) than in the I-TAB ELISA (TvAGd) (Fig. 1A versus 1B). Serum samples from T. vivax infected seropositive goats, however revealed a higher PP signal in the I-TAB ELISA (TcAGd) than the I-TAB ELISA (TvAGd) on 2 occasions: in one goat at week 2 and in another goat at week 16 (1D versus 1C).

T. brucei infected seropositive goats (Fig. 1E versus 1F), gave highest PP in I-TAB ELISA (TcAGd) at 7 instances (1 goat at week 6, 3 goats at week 4 and 6) and highest PP in I-TAB ELISA (TvAGd) at 2 instances (1 goat at week 2 and 4).

Experiment 2

The I-TAB ELISA (TcAGd) was further evaluated with a larger number of non-infected control goats and T. congolense infected goats of group 4, in function of time after infection. The average PP value was 9.4% ± 3.2% for the control sera (138 samples taken during the complete experiment) and 8.8% ± 1.4% for the 39 pre-infection sera. Four weeks post-infection with T. congolense, the average PP value was 69% ± 21% and remained high at week 8 (61% ± 27%) and 12 (73% ± 26%) for the untreated animals. All untreated goats tested positive at all occasions during the infection period. Therefore the estimated diagnostic sensitivity of the I-TAB ELISA (TcAGd) is 100%.

Repeatability of I-TAB ELISAs (TcAGd) and (TvAGd)
The inter-plate repeatability of both I-TAB ELISA (TcAGd) and I-TAB ELISA (TvAGd) was monitored as a measure of the precision of the assays. Optical density (OD) values of the positive serum control sample were plotted on a Levey-Jennings chart (Fig. 2). The OD values remained within ±2 standard deviations of the mean throughout, indicating acceptable inter-plate repeatability (Jacobson, 1998).

DISCUSSION

The FAO/IAEA I-TAB ELISA (TcAGd) and I-TAB ELISA (TvAGd) (Rebeski et al., 2000) validated for detection of antibodies against African bovine trypanosomosis were adapted for seromonitoring of African trypanosomosis in goats. The goat-adapted versions of the FAO/IAEA I-TAB ELISA systems were easy to use as demonstrated by acceptable plate-to-plate repeatability results.

From 2 weeks post infection on, the sensitivity of the I-TAB ELISA (TcAGd) for T. congolense infection was estimated at 100%. Seroscreening of uninfected goats revealed a diagnostic specificity of 96.7%. Therefore, this assay appears useful to complement parasitological diagnosis in goats.

The I-TAB ELISA (TvAGd) was 100% specific but failed to consistently identify T. vivax infected goats. This assay needs further improvement.

As with the original FAO/IAEA ELISA systems for bovine sera (IAEA, 2000), the detection of cross-reacting antibodies was also observed with ELISA systems adapted for goats. Considering PP values above cut-off as positive, cross-reactivity of I-TAB ELISA (TcAGd) with antibodies generated in T. vivax and T. brucei infected goats was extensive. Cross-reactivity of I-TAB ELISA (TvAGd) with T congolense sera was limited, but again considerable for T. brucei. Application of I-TAB ELISA (TcAGd) in the field, for epidemiological purposes disregarding the species, should therefore be further examined. In addition, the use of I-TAB ELISA is ideal for situations where decision on treatment needs to be taken, as appropriate therapeutic intervention does not rely on species-specific diagnosis.

According to Desquesnes et al. (2001a) species specificity should improve when PP values obtained in I-TAB ELISA (TcAGd) are compared to those in I-TAB ELISA (TvAGd). Thus the type of Ab-ELISA giving the highest PP value identifies the species in single infections. Based on highest PP value in I-TAB ELISA (TcAGd), all T. congolense infections were correctly identified. However, in 2/19 occasions a T. vivax infection was misidentified as a T. congolense infection. In the absence of T. brucei antigen coated plates, the majority of T. brucei infections in seropositive animals is classified as a T. congolense infection, based on
highest PP values. Species identification based on the I-TAB ELISAs is therefore not satisfactory and might need other techniques such as PCR (Desquesnes et al., 2001b) Moreover, data from goats with mixed infection are still lacking.

In addition to the need for improvement, the development of a robust ELISA-kit for screening of goats in the tropics requires further simplification. Standardisation and robustness of the ELISA method using internal control data charting methods as was done for detection of trypanosomal antibodies in bovine sera is necessary (Rebeski et al., 2001). Moreover, attention should be paid to the availability of the ELISA systems. At present, I-TAB ELISA (TvAGd) and (TcAGd) microtitre plates are precoated at the FAO/IAEA Agriculture and Biotechnology Laboratory and are available on demand, depending on funding and laboratory staff resources. To ensure the provision of I-TAB ELISA systems on a cost-recovery basis in the longer perspective, production and distribution within Africa should be established.

Acknowledgements

This study received financial support from the Directorate General for International Cooperation (DGIC, Brussels) and the International Trypanotolerance Centre, Banjul, The Gambia.

References


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Figure 1: Antibody response (percent positivity) in serum of 6 *T. congolense* (A, B), 6 *T. vivax* (C, D) and 6 *T. brucei* (E, F) infected goats measured by I-TAB ELISA (*TcAGd*) (A, C, E) and I-TAB ELISA (*TvAGd*) (B, D, F) respectively.
Figure 2: Levey-Jennings chart of the I-TAB ELISA (TcAGd) and I-TAB ELISA (TvAGd). For each assay the mean optical density (OD) value of the positive control is plotted. The solid horizontal line represents the overall mean OD of the positive control. The dashed and dotted horizontal lines represent the upper and lower ±1 and ±2 standard deviation of OD values from the overall mean OD value.