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# Evaluation of whole fresh blood and dried blood on filter paper discs in serological tests for *Trypanosoma evansi* in experimentally infected water buffaloes

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**Abstract**

In this study we investigated if whole blood could substitute for serum in the direct card agglutination test (CATT/*Trypansosoma evansi* ) and the indirect card agglutination test (LATEX/*T. evansi* ) for the sero-diagnosis of *T. evansi* in buffaloes. Likewise blood spots on filter paper were compared with sera for use in the indirect enzyme-linked immunosorbent assay/*T. evansi* (ELISA) and immunotrypanolysis test (T.L./*T. evansi* ). Samples were collected weekly from experimentally *T. evansi* infected- and non-infected water buffaloes. To estimate test agreement between serum and respectively whole fresh blood and dried blood spots on filterpaper of the tests, $\kappa$ values with 95% confidence intervals were calculated, $0.75 \pm 0.11$ for the CATT/*T. evansi*; $0.80 \pm 0.11$ for the ELISA/*T. evansi*; $0.84 \pm 0.11$ for the LATEX/*T. evansi* and $0.93 \pm 0.11$ for the T.L./*T. evansi*. In addition $\kappa$ values with 95% confidence intervals were computed to assess agreement between results obtained in the reference T.L./*T. evansi* test and those obtained in the other assays; $0.70 \pm 0.10$ for the CATT-Serum; $0.75 \pm 0.11$ for the LATEX-Blood; $0.77 \pm 0.11$ for the LATEX-Serum; $0.81 \pm 0.10$ for the CATT-Blood; $0.81 \pm 0.11$ for the ELISA-Serum and $0.84 \pm 0.11$ for the ELISA-Confetti. Based on the high $\kappa$ values as calculated, we conclude that serum can be replaced by fresh whole blood for the agglutination assays or blood on filter paper for the ELISA/*T. evansi* and T.L./*T. evansi*. © 2002 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

The water buffalo (*Bubalus bubalis*) is an important livestock species in the northern half of

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Vietnam with 2.5 million heads, providing draught power for tillage, manure and a supply of meat for local consumption or sale. Trypanosomosis (Surra), caused by *Trypanosoma evansi* is an important endemic disease affecting buffaloes in the area (Lohr et al., 1986; Luckins, 1988; My et al., 1998; Thu et al., 1998). Until 20 years ago, Surra occurred mostly as outbreaks due to the habit of farmers to transfer their animals after the cultivation season to the forest where the animals grazed freely until the next cultivation season. Due to stress induced in animals when they returned to their villages and the continuous commingling, outbreaks occurred particularly at the start of the cultivation season. With changes in the husbandry system over the last years, animals now remain in the villages for the entire year and *T. evansi* infections manifest themselves more as a chronic ailment (Thu et al., 1998).

The clinical diagnosis of chronic Surra is difficult as symptoms vary and are non-specific. Due to intermittent low parasitaemia levels in the chronically infected host, the sensitivity of parasitological diagnostic tests is often limited (Nantulya, 1990). Moreover, fresh blood is required. Conversely, serological diagnosis can be performed on stored samples and on a larger scale but has some disadvantages as antibodies may not be detectable in early infection and otherwise may persist long after cure (Luckins, 1992). Several antibody detection techniques have been developed such as the immunofluorescent antibody test (IFAT, Katende et al., 1987), the immune trypanolysis test (T.L., Verloo et al., 2000) and various enzyme-linked immunosorbent assay's (ELISA; Davison et al., 1999; Verloo et al., 2000), all to be conducted under laboratory conditions. A card agglutination test (CATT/*T. evansi*, Bajyana Songa and Hamers, 1988) and a latex agglutination test (LATEX/*T. evansi*, Verloo et al., 1998, 2000) have been developed for use in field conditions. Both assays are easy to perform and robust and are highly sensitive and specific (Davison et al., 1999; Verloo et al., 2000). Until now, they have only been applied on serum samples. Similar tests, CATT/*T.b. gambiense* (Magnus et al., 1978) and LATEX/*T.b. gambiense* (Büscher et al., 1999) exist for sleeping sickness or human African trypanosomiasis caused by *T.b. gambiense*. Their application is greatly facilitated by the testing of whole blood instead of serum, the latter not being readily available in the field. Furthermore, when large numbers of samples have to be collected, e.g. for epidemiological surveys, testing in ELISA/*T. evansi* of blood spots on filter paper instead of serum may be advantageous because collection of blood on filter paper is relatively easy and cost-effective, particularly in sub-optimal field conditions (Esposito et al., 1990; Hopkins et al., 1998).

Therefore, the present study was undertaken to compare the use of blood-dotted filter paper disks (confetti) versus serum in the indirect antibody ELISA and the T.L. and the use of whole blood versus serum in CATT and LATEX. All studied tests contain the variable surface glycoprotein (VSG) of the predominant variable antigen type (VAT) RoTat 1.2 (Bajyana Songa and Hamers 1988) as major antigen. Blood and serum test samples were obtained from experimentally infected water buffaloes.

Accuracies of these tests were estimated according to two parameters for infection status: the parasitological status of the animal, assessed by mouse inoculation (MI) and the presence of RoTat 1.2 VAT lytic antibodies as determined by T.L. on serum which is considered as a 100% specific reference antibody test for the presence of antibodies against this specific VAT (Van Meirvenne et al., 1995; Verloo et al., 2000).

## 2. Material and methods

### 2.1. Animals and experimental design

Eighteen water buffaloes, 4–8 months old, were purchased from an area with no recent history of *T. evansi* outbreaks. The absence of *T. evansi* parasites was verified by MI while the absence of specific antibodies was confirmed with ELISA/*T. evansi* (Verloo et al., 2000). Upon arrival, all animals were treated with 10 mg/kg mebendazole (Havetco–Hanoi) and received an injection of 3.5 mg/kg diaminazene aceturate (Berenil–Internet). The water buffaloes were housed in fly proof

stables and fed on grass. After 1 week of acclimatization, the buffaloes were randomly divided into two equal groups. Group B remained uninfected while animals of group A were intravenously injected with $1 \times 10^5$ trypanosomes of WHITMAS 101298, isolated in 1998 from a water buffalo in Ha Tay province, Vietnam. In weeks 1–4 animals were bled twice per week while onwards only weekly. MI and both LATEX and CATT on whole blood were conducted within 2 h after sample collection. For the ELISA and T.L. with blood-spots, whole blood was collected onto 7.5 cm discs of Whatman No. 4 filter paper which were air dried and stored in a cool place in sealed plastic bags with silica gel desiccant. Blood for serum collection was allowed to clot at room temperature and kept overnight at 4 °C. The next day, blood samples were centrifuged and serum was collected and stored at $-20$ °C for further analysis.

### 2.2. Diagnostic tests

#### 2.2.1. Mouse inoculation (MI)

To confirm the presence of parasites in infected buffaloes and to verify the absence of parasites in non-infected buffaloes, blood samples were checked with the MI technique by injecting 0.25–0.5 ml of heparinized blood intraperitoneally into mice. All mice were checked three times a week for 1 month by wet blood film examination of tail blood.

#### 2.2.2. Immune trypanolysis (T.L.)

Immune trypanolysis was performed according to Verloo et al. (2000) using *T. evansi* VAT RoTat 1.2. Sera were tested at a 1:4 dilution in guinea pig serum (GPS, GIBCO 19195-015).

The test with blood-dotted filter-paper was a modification of the test with serum. One filter-paper disk of 6 mm diameter was punched out from blood dotted filter-paper. The filter paper disk was put per well in a flat-bottom plate and 20 μl GPS per well was added. The plate was covered and incubated for 1 h at 4 °C with continuous shaking. A trypanosome-suspension (5 trypanosomes/microscopic field, 400 ×) in GPS was prepared and 10 μl were added to each well, with the confetti's still in place. Plates were shaken for 1 min and incubated for 90 min at room temperature and again shaken after 30, 60 and 90 min. If RoTat 1.2 variant specific antibodies are present in the serum, lysis of the RoTat 1.2 trypanosomes is observed under the microscope. When 50% or more of the trypanosomes are lysed, the sample is considered positive for the presence of anti RoTat 1.2 antibodies, indicating current or past infection

#### 2.2.3. CATT/*T. evansi*

The CATT/*T. evansi* is a rapid direct agglutination test which uses freeze-dried trypanosomes of *T. evansi* VAT RoTat 1.2, fixed with formaldehyde and stained with Coomassie-blue (Bajyana Songa and Hamers, 1988). According to Verloo et al. (2000), who determined the cut-off value for the CATT/*T. evansi* on serum with optimal combination of sensitivity and specificity, the test is considered positive when macroscopic agglutination occurs at 1:8 or higher serum or blood dilutions.

#### 2.2.4. LATEX/*T. evansi*

The LATEX/*T. evansi* is a rapid indirect agglutination test in which the antigen consists of purified VSG of *T. evansi* VAT RoTat 1.2 covalently coupled to latex particles and was performed as described previously (Verloo et al., 2000). According to the latter the test is considered positive when macroscopic agglutination occurs at 1:8 or higher serum dilutions. The same cut-off was used for the LATEX on blood.

#### 2.2.5. ELISA/*T. evansi*

In ELISA/*T. evansi*, serum samples were tested at 1:400 dilution in PBS-Blotto (0.01 M; pH 7.4; NaCl 11.7 g/l; $NaH_2PO_4 \cdot H_2O$ 0.2 g/l; $Na_2HPO_4 \cdot 2H_2O$ 1.44 g/l; $NaN_3$ 0.5 g/l; skimmed milk powder 10 g/l) while blood collected on filter paper was first eluted as follows. From each sample filter paper, two confetti of 6 mm diameter were punched out and added to 2.0 ml of PBS-Blotto/Tween (PBS-Blotto with Tween-20 0.5 ml/l) for overnight incubation at 4 °C. Either 100 μl of diluted serum or 100 μl of confetti elution was added in duplicate to an ELISA plate which was coated overnight with 2 μg/ml of purified VSG of

*T. evansi* VAT RoTat 1.2 in PBS. Test procedures and tests reading were conducted as described by Verloo et al. (2000). The corrected OD's were expressed as percentages of the O.D. obtained with the strong positive control (percent positivity, P.P.) included in each plate. For both ELISA's the same positive control was used with mean corrected optical density of 1.14 (S.D. $\pm$ 0.30) and 1.04 (S.D. $\pm$ 0.29) for Serum-ELISA and confetti-ELISA, respectively. Cut-off values for both ELISA's: ELISA-Serum P.P. = 32 and ELISA-Confetti P.P. = 26, were calculated by adding two times the standard deviation to the mean of the P.P. values obtained in the non-infected group

### 2.3. Statistics

For each sampling round, the proportion of test positive animals per test was calculated for the infected group and the proportion of test negative animals for the non-infected group. For the non- infected group the mean of the proportions was calculated for the whole period.

Test agreement between the serum and blood versions of CATT, LATEX, ELISA and T.L. was estimated by calculation of the $\kappa$ values with associated 95% confidence intervals.

In addition agreement between results obtained in T.L., which is considered as a reference test for the presence of anti RoTat 1.2 antibodies, and those obtained in the other assays was estimated by calculation of the $\kappa$ value.

## 3. Results

### 3.1. Parasitological results

All infected water buffaloes were positive in the MI on at least one occasion. All mice inoculated with the blood of non-infected buffaloes remained negative during the 1 month follow-up.

### 3.2. Serological results

In total, 147 and 171 samples (blood and serum) were collected from respectively non-infected and infected buffaloes and were subsequently tested in all assays. The proportion of sero-positive animals in the infected group per sampling round is presented in Table 1. The proportion of sero-negative animals in the non-infected group per sampling round is presented in Table 2.

Table 1
Proportion of sero-positive animals for the different diagnostic tests in the infected group during the experiment

| Week | T.L.-serum | T.L.-confetti | CATT-serum | CATT-blood | LATEX-serum | LATEX-blood | ELISA-serum | ELISA-confetti |
|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 72 | 0 | 28 | 11 | 0 | 6 |
| 2 | 0 | 0 | 22 | 0 | 22 | 28 | 17 | 11 |
| 3 | 0 | 0 | 33 | 11 | 22 | 17 | 11 | 11 |
| 4 | 22 | 0 | 72 | 28 | 50 | 28 | 22 | 17 |
| 5 | 89 | 67 | 67 | 56 | 56 | 56 | 44 | 56 |
| 6 | 100 | 89 | 89 | 89 | 89 | 89 | 67 | 78 |
| 7 | 100 | 100 | 89 | 89 | 89 | 89 | 78 | 78 |
| 8 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 9 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 10 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 11 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 12 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 13 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 14 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 15 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

Table 2
Proportion of sero-negative animals for the different tests in the non-infected group during the experiment

| Week | T.L.-serum | T.L.-confetti | CATT-serum | CATT-blood | LATEX-serum | LATEX-blood | ELISA-serum | ELISA-confetti |
|---|---|---|---|---|---|---|---|---|
| 1 | 100 | 100 | 100 | 80 | 80 | 100 | 100 | 100 |
| 2 | 100 | 100 | 90 | 100 | 90 | 70 | 80 | 90 |
| 3 | 100 | 100 | 90 | 90 | 90 | 90 | 100 | 100 |
| 4 | 100 | 100 | 80 | 80 | 80 | 80 | 100 | 100 |
| 5 | 100 | 100 | 90 | 90 | 100 | 90 | 100 | 100 |
| 6 | 100 | 100 | 100 | 70 | 100 | 100 | 100 | 100 |
| 7 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 8 | 100 | 100 | 100 | 100 | 100 | 100 | 89 | 89 |
| 9 | 100 | 100 | 100 | 89 | 100 | 100 | 100 | 100 |
| 10 | 100 | 100 | 100 | 100 | 100 | 67 | 100 | 100 |
| 11 | 100 | 100 | 100 | 100 | 100 | 78 | 100 | 89 |
| 12 | 100 | 100 | 100 | 89 | 100 | 89 | 100 | 89 |
| 13 | 100 | 100 | 100 | 100 | 100 | 100 | 88 | 100 |
| 14 | 100 | 100 | 100 | 100 | 100 | 88 | 88 | 100 |
| 15 | 100 | 100 | 100 | 100 | 100 | 75 | 88 | 100 |
| Mean | 100 | 100 | 96.6 | 92.5 | 96 | 88.5 | 95.5 | 97.1 |

*3.2.1. Infected group*

During the first 3 weeks none of the animals tested positive in T.L.-Serum. In all other tests, except for T.L.-Confetti the number of positive results fluctuated during this period (Table 1). The T.L.-Serum test, at week 6 P.I. was the first that tested all animals positive, the T.L.-Confetti followed at week 7 P.I. while the rest of the tests followed at week 8. From that point on, all animals remained positive in all tests (Table 1).

*3.2.2. Non-infected group*

Both T.L. tests were the only tests that never yielded false positive results during the experiment. With all other tests, some false positive results were obtained throughout the whole experiment period (Table 2). The mean proportion values calculated over the whole period indicate that, when applied on blood, the agglutination assays yielded slightly more false positives than on serum. In contrast the ELISA on serum yielded more false positives compared with confetti.

*3.2.3. Agreement between the serum and blood versions of a test*

The $\kappa$ values with their 95% confidence interval for test agreement between serum and blood (confetti) versions of the tests were in increasing order $0.75 \pm 0.11$ for the CATT; $0.80 \pm 0.11$ for the ELISA; $0.84 \pm 0.11$ for the LATEX and $0.93 \pm 0.11$ for the T.L.

*3.2.4. Agreement between T.L.-serum and the other serological tests*

The $\kappa$ values with their 95% confidence interval for test agreement between the T.L.-Serum and the other tests were in increasing order $0.70 \pm 0.10$ for the CATT-Serum; $0.75 \pm 0.11$ for the LATEX-Blood; $0.77 \pm 0.11$ for the LATEX-Serum; $0.81 \pm 0.10$ for the CATT-Blood; $0.81 \pm 0.11$ for the ELISA-Serum and $0.84 \pm 0.11$ for the ELISA-Confetti.

## 4. Discussion

The main objective of this study was to investigate whether whole blood could substitute for serum in the CATT and the LATEX for the sero-diagnosis of *T. evansi* in buffaloes. Likewise blood spots on filter paper were compared with sera for use in the indirect ELISA and T.L.

Well aware of the inherent limitations, it was decided to use experimentally infected animals

due to the lack of suitable reference material and the absence of an accurate 'golden standard' (Luckins, 1992). Since the subsequent observations are not independent and experimental infection studies often suffer from small numbers of tested animals, the individual animal variation might become important and observed $\kappa$ values should be interpreted with caution. However, it was chosen to use the $\kappa$ values to compare the performance of the tests during the infection as all test were executed in parallel during the same period. Further limitations of experimental infections are the short monitoring period compared with long-standing natural infections that can evolve from acute to chronic stages. Finally, severity of infection and associated clinical signs may depend on the strain of parasite chosen (Jacobson, 1998).

The studied animals originated from an area with no recent history of *T. evansi* and tested negative in the MI and the ELISA-Serum (data not shown). Moreover, the *T. evansi*-infected buffaloes only became sero-positive with the T.L. test after 3 weeks, suggesting the absence of a previous exposure to *T. evansi*. Although T.L. remained negative during the first 3 weeks CATT, LATEX and ELISA positive results were observed in various degrees during that period. This can partially be explained by trypanosome specific VSG epitopes, which are not exposed at the surface of living trypanosomes as used in T.L. (Verloo et al., 2000).

In *T. evansi* experimentally infected dogs and goats Bajyana Songa et al. (1987) described CATT/*T. evansi* positive results after 2 weeks of infection. In the present study, however, the CATT-Serum test yielded an unexpected high number of sero-positives (72%) during the first week decreasing to 22% the second week. Therefore, it is more likely that we observed some unexplained aspecific reactions during that first week which is also the reason why the $\kappa$ value for the agreement between CATT-Serum and T.L.-Serum was less than for the other tests. All tests however, except the T.L.-Serum and T.L.-Confetti, tend to give some false positive results in the non-infected population at different time and for different animals (individual data not shown).

The agreement between the serum and blood (confetti) versions of the T.L. is best followed by the LATEX, ELISA and CATT (respectively $\kappa$ values 0.93, 0.84, 0.80 and 0.75). The above described aspecific reaction of the CATT-Serum during the first week of infection is responsible for the lower $\kappa$ value. For the ELISA-Confetti the overall results (best agreement with T.L.-Serum) are even better than the ELISA-Serum which is in line with a previous study of Hopkins et al. (1998) who compared eluted bloodspots and serum in an antibody detection ELISA for tsetse transmitted trypanosomosis and who demonstrated a lower cut-off value and a higher specificity and sensitivity using eluted bloodspots. It appeared that during weeks 4–6, which is the period antibody levels rapidly increase, the (analytical) sensitivity of the T.L.-Confetti is slightly lower than that of T.L.-Serum.

We conclude that serum can be replaced by fresh blood for the agglutination assays or blood on filter paper for the ELISA and T.L., which facilitates the work on the field.

As was done for the CATT/*T.b. gambiense* for human sleeping sickness (Laveissière and Penchenier, 2000) further simplification of the agglutination test protocols will allow 'pen side' testing even under sub-optimal conditions as they exists in large parts of the region in which surra is endemic.

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