

Evaluation of antigen detection and antibody detection tests for *Trypanosoma evansi* infections of buffaloes in Indonesia

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SUMMARY

Two Ag-ELISAs, an IgG-specific antibody detection ELISA (IgG ELISA) and a card agglutination test (CATT) for the detection of *Trypanosoma evansi* infections in buffaloes in Indonesia, were compared. Diagnostic sensitivity estimates were obtained by testing sera from 139 Indonesian buffaloes which had been found to be infected by parasitological tests. Diagnostic specificity was estimated by testing sera from 263 buffaloes living in Australia. Response-operating characteristic curves were constructed, and optimal ELISA cut-off values, which minimized the number of false-negative and false-positive results, were chosen. The IgG ELISA had the highest sensitivity (89%) and the CATT had the highest specificity (100%). There was a significant difference between the sensitivities (71 and 81%), but not between the specificities (75 and 78%), of the two Ag-ELISAs. The four tests were further compared by calculation of post-test probabilities of infection for positive and negative test results using a range of prevalence values, and likelihood ratios. The results suggested that the CATT was the best test to 'rule-in' infection (i.e. the highest probability of infection in test-positive animals) and the IgG ELISA was the best test to 'rule-out' infection (i.e. the lowest probability of infection in test-negative animals).

INTRODUCTION

Trypanosoma evansi is the most widely geographically distributed pathogenic trypanosome. It can infect several species of animals, including cattle, buffaloes, horses and camels, causing trypanosomosis – commonly known as surra [1]. Surra is endemic in many areas of Southeast Asia, where buffaloes and cattle are important for draught power, meat and milk production, and as financial investments of low-income

farmers. Typically, surra is a chronic disease characterized by weight loss [2, 3] and infertility, including abortion [4, 5]; however, epidemics of acute trypanosomosis with high case-fatality rates have been reported in Vietnam, China and the Philippines [1, 6].

Despite the application of a variety of diagnostic tests, the diagnosis of surra remains problematic, and commonly-used tests have important limitations. *Trypanosoma evansi* infections can be detected using parasitological techniques [e.g. the microhaematocrit test (MHCT) and mouse inoculation (MI)] and

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various antibody-detection tests [7]. However, these tests cannot always detect current infections because the level of parasitaemia fluctuates (particularly during chronic stages of infection), and antibodies, which are not present during the first few weeks of infection, can persist after chemotherapy [8]. Antigen-detection ELISAs have been developed more recently [9, 10]. These have the potential to improve the detection of trypanosome infections, and can be more readily standardized [7]. Several studies have reported the use of *T. evansi* Ag-ELISAs to test camels [11, 12], horses [13] and buffaloes [14]. However, the diagnostic sensitivity and specificity, and other key diagnostic parameters of *T. evansi* Ag-ELISAs, have not yet been evaluated rigorously.

The prevalence of infection and of disease – in this case trypanosomosis – in the target population will affect both the probability that an animal that is test-positive is truly infected [the positive predictive value (PPV)] and the probability that an animal that is test-negative is not infected [the negative predictive value (NPV)] [15]. The influence of the cut-off value and prevalence on these test parameters of the four *T. evansi* tests are presented, together with likelihood ratios which are a function of diagnostic sensitivity and specificity, and the suitability of the tests for different applications is discussed.

The aim of this study, therefore, was to use appropriate buffalo populations to evaluate two *T. evansi* Ag-ELISAs (2G6 Ag-ELISA and Tr7 Ag-ELISA), based on different capture monoclonal antibodies, and two antibody-detection tests [IgG ELISA and card agglutination test (CATT)].

METHODS

Samples

A total of 2387 swamp buffaloes, which were chosen by convenience sampling for prevalence and incidence studies (to be reported subsequently), were blood sampled in Central Java, Indonesia. Of these, 139 buffaloes were found to be naturally infected with *T. evansi* either by the MHCT ($n = 39$) or by MI ($n = 100$). Standard MHCT and MI techniques [16] were conducted using whole blood collected earlier the same day.

Briefly, in the MHCT, 30 μ l blood was centrifuged in a microhaematocrit tube which was then examined microscopically for the presence of live trypanosomes near the buffy layer. Laboratory-bred mice were

inoculated intraperitoneally with 0.5 ml blood and then monitored for parasitaemia by examination of tail blood every 3 days for 30 days. For estimation of specificity, 263 sera were obtained from a large-scale survey of buffaloes living in the Northern Territories, Australia, where *T. evansi* is not reported to occur [17], because it was not possible to identify a population of naive buffaloes in Indonesia. All serum samples were stored at -20°C .

Diagnostic tests

All sera were tested with two *T. evansi* antigen-detection ELISAs (2G6 Ag-ELISA and Tr7 Ag-ELISA), as previously described [18]. The Ag-ELISAs use monoclonal antibodies that recognize different *T. evansi* antigenic determinants: a 70 kDa antigen (2G6 Ag-ELISA) and a 15 kDa antigen (Tr7 Ag-ELISA). In addition, the sera from the parasitologically-positive Indonesian buffaloes, and 114 of the 263 Australian buffalo sera, were tested with a *T. evansi* IgG ELISA [19] and CATT (Institute of Tropical Medicine, Laboratory of Serology, Nationalstraat 155, B-2000 Antwerp, Belgium). The CATT uses formaldehyde-fixed trypanosomes (RoTat 1.2) to detect primarily IgG-specific antibodies against *T. evansi*, and a sample was considered positive if agglutination was observed, as recommended by the supplier.

Data analysis

The ELISA optical densities (ODs) were expressed as a percentage of the high-positive control OD within each ELISA (percent positivity: PP). Test sensitivity was calculated as the number of test-positive buffaloes divided by the number of *T. evansi*-infected buffaloes in the following groups: (1) MI-positive buffaloes ($n = 39$), (2) MHCT-positive buffaloes ($n = 100$) and (3) MI-positive and MHCT-positive buffaloes ($n = 139$). Test specificity was calculated as the number of test-negative buffaloes divided by the number of uninfected buffaloes tested (Ag-ELISAs: $n = 263$; IgG ELISA and CATT: $n = 144$). Associated 95% confidence intervals (CIs) were calculated using the computer software Confidence Interval Analysis (CIA; © British Medical Journal, London) with either the exact binomial method or the Normal approximation [when nP or $n(1-P) > 10$, where n = sample size, and P = estimated proportion] [20]. Differences between estimates of sensitivity and

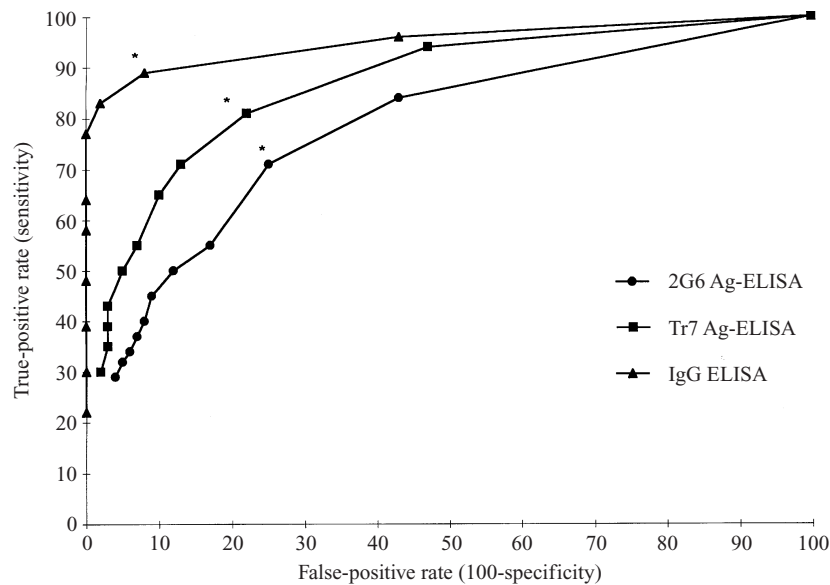


Fig. 1. Response-operating characteristic curves for two *Trypanosoma evansi* Ag-ELISAs and an IgG ELISA. (Data points, left to right, are shown for cut-off values of 100, 90, 80, 70, 60, 50, 40, 30, 20, 10 and 0 PP; optimum cut-off values (20 PP) are marked with *).

specificity were also calculated, with 95% CIs, using CIA, and were considered significant at the 5% level if the 95% CI excluded the value zero.

Response-operating characteristic curves (ROCs) [15] were constructed for the two Ag-ELISAs and the IgG ELISA, using cut-off values of 0 PP to 100 PP at 10 PP intervals. The optimum cut-off value for each ELISA was chosen as the PP value that gave the highest combined sensitivity and specificity values (i.e. highest value of sensitivity plus specificity, divided by two). To compare the CATT (which has a positive/negative outcome) with the ELISAs using their optimum cut-off values, the post-test probabilities of a buffalo having a *T. evansi* infection if either the test result was positive (equivalent to PPV) or the test result was negative (equivalent to $1 - \text{NPV}$) were calculated for prevalence values (pre-test probabilities) of 0 to 100% [15].

Likelihood ratios for a positive test result, defined as the probability of a positive test result in infected buffaloes divided by the probability of a positive test result in uninfected buffaloes, were calculated using: $\text{sensitivity}/(1 - \text{specificity})$ [15].

RESULTS

A cut-off value of 20 PP was found to give the highest values of diagnostic sensitivity and specificity for the two Ag-ELISAs and the IgG ELISA. The effects of

different cut-off values on diagnostic sensitivity and false-positive rate ($1 - \text{specificity}$) are shown by the ROCs presented in Figure 1. The IgG ELISA had higher estimates of sensitivity with lower associated false-positive rates than both Ag-ELISAs.

The diagnostic sensitivities of the two Ag-ELISAs, IgG ELISA and CATT were estimated using 139 buffaloes found to be parasitologically-positive with either the MHCT and/or the MI (Table 1). The point estimate of sensitivity of the IgG ELISA (89%) was significantly higher (differences and 95% CI are shown in parentheses) than the estimates of the 2G6 Ag-ELISA [71, 18% (10, 27%)], Tr7 Ag-ELISA [81, 8% (1, 16%)] and CATT [78, 11 (3, 18%)]. Also, the point estimate of sensitivity of the Tr7 Ag-ELISA (81%) was significantly higher [10% (1, 19%)] than the sensitivity of the 2G6 Ag-ELISA (71%).

Point estimates of diagnostic specificity are also shown in Table 1. The specificity of the CATT (100%) was significantly higher than the specificities of the 2G6 Ag-ELISA [75, 25% (20, 30%)], Tr7 Ag-ELISA [78, 22% (17, 27%)] and the IgG ELISA [92, 8% (3, 8%)]. There was not a significant difference [3% (-4, 10%)] between the estimates of specificity of the 2G6 Ag-ELISA (75%) and Tr7 Ag-ELISA (78%). Of the 263 uninfected buffaloes, 82 buffaloes gave a false-positive result with either the 2G6 Ag-ELISA or Tr7 Ag-ELISA and 20 buffaloes gave a false-positive result with both Ag-ELISAs. Only 9 of 114 uninfected buffaloes tested gave a false-positive result with the

Table 1. Estimates of diagnostic sensitivity (%) and diagnostic specificity (%) of four *Trypanosoma evansi* diagnostic tests

Test	Sensitivity (%)*			Specificity (%)
	MHCT-positive buffaloes (n = 100)	MI-positive buffaloes (n = 39)	MHCT-positive and MI-positive buffaloes (n = 139)	Uninfected buffaloes (n = 263)
2G6 Ag-ELISA†	67 (57, 76)	79 (64, 91)	71 (63, 79)	75 (70, 80)
Tr7 Ag-ELISA†	80 (71, 87)	82 (67, 93)	81 (75, 88)	78 (73, 83)
IgG ELISA†	90 (82, 95)	87 (73, 96)	89 (84, 94)	92 (86, 96)‡
CATT	79 (70, 87)	77 (61, 89)	78 (72, 85)	100 (97, 100)‡

* 95% CI are given in parentheses.

† Using a 20 PP cut-off value.

‡ 114 of 263 sera were tested by the IgG ELISA and CATT.

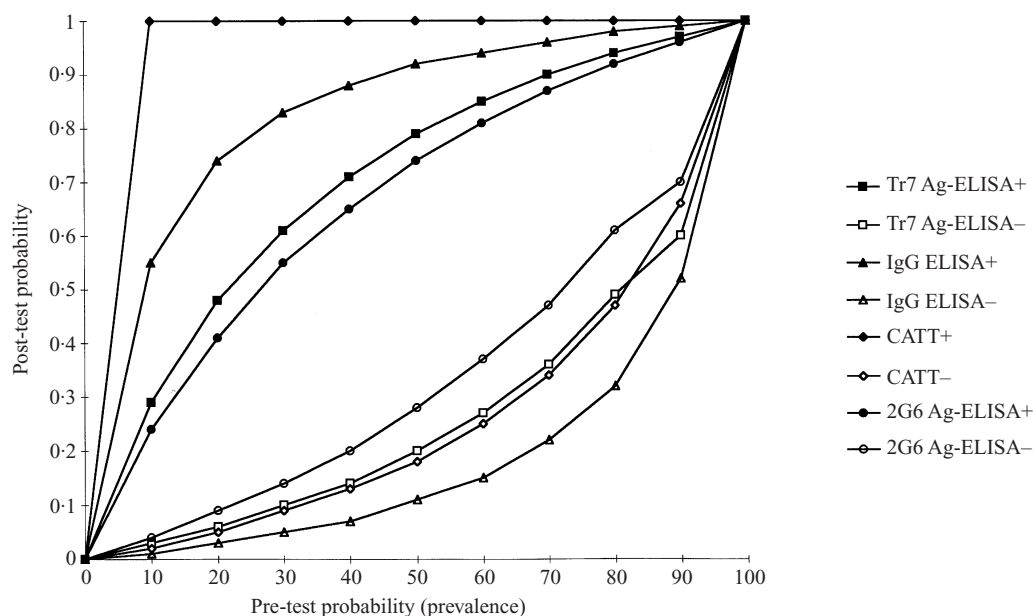


Fig. 2. Comparison of pre-test and post-test probabilities of *Trypanosoma evansi* infection in buffaloes for four diagnostic tests. (Solid symbols indicate values for positive test results and open symbols indicate values for negative test results.)

IgG ELISA, and of these all were positive with the 2G6 Ag-ELISA, and 5 were positive with the Tr7 Ag-ELISA.

To compare all 4 tests, post-test probabilities of *T. evansi* infection in buffaloes with either a positive or negative test result were calculated for different prevalence values (Fig. 2). The highest probability (PPV = 1.0) of a buffalo being infected with *T. evansi* was obtained for positive CATT results with prevalence values from 10 to 100%, and PPV increased with increasing prevalence with the other tests. In contrast, post-test probabilities of *T. evansi* infection were lower for buffaloes found to be negative with the IgG ELISA than with the other tests. For example, with a

prevalence of 50%, the probability that a test-negative buffalo was truly infected was 0.11, 0.18, 0.20 or 0.28 when tested with the IgG ELISA, CATT, Tr7 Ag-ELISA or 2G6 Ag-ELISA, respectively.

Point estimates of likelihood ratios (Table 2) suggested that a buffalo found to be positive with the CATT would be 'infinitely' more likely to be *T. evansi*-infected than uninfected, whereas a buffalo found to be positive with the 2G6 Ag-ELISA or Tr7 Ag-ELISA would be only about three to four times as likely to be truly infected than uninfected. Also, a buffalo found to be negative with the IgG ELISA would be less likely to be truly infected than if found to be negative with one of the other tests.

Table 2. Point estimates of likelihood ratios of four *Trypanosoma evansi* diagnostic tests

Test	Likelihood ratio*	
	Positive test	Negative test
2G6 Ag-ELISA	2.84	0.39
Tr7 Ag-ELISA	3.68	0.24
IgG ELISA	11.13	0.12
CATT	∞ (infinity)†	0.22

* Calculated using the estimates of diagnostic sensitivity and specificity in Table 1.

† Derived from testing 114 buffaloes – all buffaloes were negative with the CATT.

DISCUSSION

Trypanosoma evansi is endemic throughout Southeast Asia where large numbers of buffaloes are at risk of infection, yet the diagnosis of *T. evansi* infections commonly depends on parasitological tests with low sensitivities [8]. Therefore, diagnostic tests with a high sensitivity and specificity are needed. This study was conducted to estimate diagnostic validity parameters of two *T. evansi* Ag-ELISAs and two *T. evansi* antibody-detection tests to assess their suitability for wider application in Indonesia and other countries.

The point estimate of sensitivity obtained for the IgG ELISA (89%) was significantly higher than the estimates obtained for the other three tests, and the point estimates of specificity of the IgG ELISA (92%) and CATT (100%) were significantly higher than the estimates obtained for the Ag-ELISAs. The sensitivities of the two Ag-ELISAs were similar to previously reported estimates that were obtained by testing parasitaemic camels (83%) [12] or horses (74%) [13]. In early infections, animals may not have a detectable antigenaemia because insufficient antigens have been released from dying trypanosomes into the circulation. Negative Ag-ELISA results may also be obtained due to the inability of antigen present in serum to bind to capture monoclonal antibodies [21]. For example, antigens may be bound within antibody complexes [22] and other serum components may interfere with antigen binding [23].

The reasons for the false-positive results obtained with the Ag-ELISAs, which utilize monoclonal antibodies to capture specific trypanosomal antigens, are not known. Cross-reactions with homologous antigenic components may have occurred, but *T. theileri* is the only other trypanosome of buffaloes in Indonesia, and these Ag-ELISAs have not been found

to bind antigens of this trypanosome [10, 24]. Heterophilic antibodies are known to occur in trypanosomal infections [25] and this type of antibody can cross-link monoclonal antibodies in sandwich ELISA systems [23]. However, it is likely that the majority of observed false-positive results were due to specific cross-reactions because serum titration and inclusion of different blocking agents did not eliminate these reactions [16]. It is also possible that the absence of anti-trypanosome antibodies early in infection, mentioned in the Introduction, would reduce sensitivity in animals tested early in infection (with the corollary that persistence of antibodies after effective treatment would decrease test specificity).

For the evaluation of diagnostic tests, the selection of appropriate truly positive and truly negative animal populations can be problematic because sufficient numbers of animals that are representative of the target population need to be identified to produce estimates of the validity parameters with a reasonable degree of precision [26, 27]. The diagnostic purpose of a test, or the target population, may change. For example, a *T. evansi* test may be evaluated initially as a screening test, but later used to test diseased animals during an outbreak of trypanosomosis when more animals would have acute infections. In the present study, the positive subpopulation consisted of Indonesian buffaloes found to be positive parasitologically, but it was not possible to identify a negative Indonesian buffalo subpopulation because a ‘gold standard’ test, with 100% sensitivity and 100% specificity, is not available. The positive subpopulation would not have included buffaloes with a full range of *T. evansi* infection stages, because parasitological tests do not detect all infected buffaloes and are more likely to detect buffaloes with early infections than buffaloes with chronic infections (which are typically aparasitaemic). Therefore, estimation of test sensitivity would have been affected by the distribution of the test variable (e.g. ELISA PP values) in the subpopulation of buffaloes tested. The influence of the heterogeneity of the distribution of a test variable in a population is particularly important for tests with continuous values which are interpreted using a cut-off value [28].

The 4 *T. evansi* tests were compared by calculation of post-test probabilities of infection, which are dependent on prevalence, and estimated likelihood ratios, which are generally considered to be independent of prevalence [15]. Predictive values and likelihood ratios are useful measures of test validity,

and the parameters obtained for the four *T. evansi* tests suggested that the IgG ELISA would be more likely to correctly classify uninfected buffaloes and that the CATT would be more likely to correctly classify truly infected buffaloes.

In Indonesia and other countries in Southeast Asia, buffaloes are often transported between regions; for example, buffaloes are bred on Sumbawa island, Indonesia, and then moved to other islands to be worked in rice fields. Outbreaks of trypanosomosis have been attributed to the introduction of different strains of *T. evansi* into an area by the movement of animals [29]. The IgG ELISA would be suitable for testing buffaloes to check that they were not infected either prior to movement or during quarantine after arrival in new area. ELISAs also have the advantage that large numbers of tests can be rapidly conducted, and procedures can be readily standardized. When clinical signs of trypanosomosis are observed in Indonesian village buffaloes, government veterinary officers often give trypanocidal treatment to affected buffaloes and, sometimes, in-contact buffaloes. However, trypanocidal drugs are costly and are not widely available, and so diagnostic tests could be used to identify infected buffaloes for targeted chemotherapy. The CATT would be a useful test for this purpose because it could rapidly be performed by field veterinarians in simple laboratories, whereas ELISAs require more sophisticated equipment and better trained personnel. In conclusion, for the diagnosis of *T. evansi* infections in buffaloes, the two antibody-detection tests had higher measures of validity than either of the Ag-ELISAs, and the selection of an appropriate test would also depend on the specific diagnostic purpose of the test and the associated costs of diagnostic misclassification.

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