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Evaluation of buffered *Trypanosoma evansi* antigen and rapid serum agglutination test (BA/Te) for the detection of anti-*T. evansi* antibodies in horses in Brazil

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ABSTRACT

Surra is an infectious disease caused by *Trypanosoma evansi*, which affects a large number of domestic and wild animal species. Infection control is based on rapid diagnosis followed by treatment of sick animals. This study aimed to evaluate a buffered *T. evansi* antigen and rapid serum agglutination test (BA/Te) for the detection of anti-*T. evansi* antibodies in serum samples of horses. For this purpose, 445 serum samples from horses were evaluated and the results compared with the diagnosis by CATT/*T. evansi*. Our data show a sensitivity of 92%, specificity of 91% and a degree of agreement kappa (κ) of 0.82 (95% CI: 0.771–0.877, $P < 0.01$) between BA/Te and CATT/*T. evansi*. Antigen specificity was also evaluated against reactive serum for other infectious agents circulating in equine herds. In conclusion, our findings show that BA/Te has the potential to be a practical and quick screening method for the detection of anti-*T. evansi* antibodies in horses.

1. Introduction

Trypanosoma evansi is a flagellate haematoprotozoan that causes surra, which is a trypanosomosis infection that affects many domestic and wild animal species (Desquesnes et al., 2013a). This species is widely distributed in Africa, Asia, South America, and in some regions of Europe and Oceania, and its transmission occurs primarily *via* hematophagous insects, including *Tabanus*, *Culex* and *Stomoxys* (Desquesnes, 2004; Desquesnes et al., 2013b).

In equines, the disease is characterized by fever, abortion, subcutaneous edema, and neurological symptoms (Silva et al., 1995; Rodrigues et al., 2009). An acute fatal form has also been studied (Lun et al., 1993). The main strategy to control this infection is diagnosis followed by the treatment of sick animals and control of the vectors. Therefore, the use of a specific, rapid, and sensitive technique for the early diagnosis of *T. evansi* infection is essential in the acute, chronic, or subpatent stages of the disease (Claes et al., 2004) for effective treatment and to successfully achieve infection control.

Examination of stained blood films using microscopy frequently fails to detect patent infections and chronic forms of the disease (Zayed et al., 2010). Antigen and antibody detection and molecular methods (such as polymerase chain reaction (PCR) DNA amplification) are an alternative to complement parasite detection methods and improve diagnosis. This is especially relevant in the case of chronic or subclinical infection, in which parasitemia is low and intermittent, making diagnosis by direct microscopy difficult (Sharma et al., 2012).

Serological agglutination tests have been successfully used for both diagnostic and serum epidemiological studies of numerous diseases in several animal species (Laha & Sasmal, 2008; Singla et al., 2015). Rapid serological tests can be more practical as they enable screening large numbers of animals promptly and at a low cost. Thus, they can be easily introduced into laboratory routines and can be used in surveillance and

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control strategies for *T. evansi* infection (Hilali et al., 2004; Zayed et al., 2010; Singla et al., 2015).

Currently, the card agglutination test for *T. evansi* (CATT/*T. evansi*®; Institute of Tropical Medicine Antwerp, Belgium) is the reference serum agglutination test recommended by the World Organisation for Animal Health (OIE) for antibody detection of *T. evansi* and is known to detect mainly IgM. The antigen consists of fixed and dyed bloodstream-form trypanosomes of the variable surface antigen strain designated as RoTat1.2. However, the import and commercialization of this test prove to be difficult in many countries due to local legislation, restraint use, or high costs.

Crude sonicated antigens of *T. evansi* isolates from different host origins revealed a similar polypeptide profile, and whole cell lysate (WCL) antigens prepared from *T. evansi* isolated from a unique host might be useful for serodiagnosis of trypanosomiasis in several species of animals (Laha & Sasmal, 2008; Sivajothi et al., 2016). High levels of specificity for serum agglutination tests can be obtained by acidic buffering of the antigen (pH ~3.65), which decreases the activity of agglutinins with less specificity, such as IgM, thus favoring reactions based on IgG (Corbel, 1972; Patterson et al., 1976). These diagnostic methods are low-cost, provide fast results, and can be used as screening methods as they allow the testing of numerous animals. They can also be used alongside tests with higher specificity or for specific stages of infection.

This study aimed to evaluate a buffered *T. evansi* antigen and rapid serum agglutination test (BA/Te) for the detection of anti-*T. evansi* antibodies in serum samples from equines in Brazil.

2. Materials and methods

2.1. Production of T. evansi and buffered antigen

Female Wistar rats weighing on average 220 ± 10 g, were purchased from the Universidade Federal de Santa Catarina (Brazil). The animals were housed in standard cages (two animals per cage) in an experimental room with controlled temperature (23 °C) and relative humidity (70%) under a 12h light/dark photocycle. Commercial feed (Supra®, São Leopoldo, Brazil) and water were provided ad libitum. Six female Wistar rats (*Rattus norvegicus*) were inoculated intraperitoneally with *T. evansi* isolated from a naturally infected dog (Colpo et al., 2005). Parasitemia was regularly estimated through microscopic examination of tail blood smears. At the parasitemia peak 3–4 days post-infection (i.e. approximately 40 parasites per microscopic field at 100× magnification), the rats were anesthetized with xylazine (5 mg/kg) and ketamine (1 mg/kg), and blood was collected in EDTA *via* a cardiac puncture. Blood was initially separated using a Percoll® (GE Life Science, Chicago, USA) gradient (17,500× *g* for 25 min at 4 °C), and the supernatant enriched with parasites was washed twice with PBS containing 2% glucose (PBS-G) at 6,000× *g* for 10 min at 4 °C (Grab & Bwayo, 1982). Purified parasites were obtained through an additional separation step using DEAE-cellulose chromatography (Lanham & Godfrey, 1970).

Buffered WCL of *T. evansi* was obtained through three cycles of sonication at a frequency of 40 ± 2 kHz for 3.5 min with a 15-sec interval and 10 cycles of freezing in liquid nitrogen and defrosting (37 °C). Cell debris, resulting from lysis of parasites, was removed by centrifugation at 14,000× *g* for 30 min at 4 °C. The supernatant of the lysed trypanosomes was collected and used as WCL antigen. The final protein concentration in the supernatant was adjusted to 1.5 mg/ml using Nanodrop equipment (Thermo Fisher Scientific, USA). The antigen suspension was mixed with a buffer of glycerol (15%), bromophenol blue dye (0.01%), and phenol (0.5%) and adjusted to a pH of 5.3. The complete buffered WCL antigen *T. evansi* was agitated for 18 h at 120 *rpm* and then stored at 4 °C until use.

2.2. Serum agglutination tests

A rapid serum agglutination test (BA/Te) was performed using a transparent glass plate (dimensions: 200 mm × 400 mm × 3 mm) divided into fields (4 × 4 cm). In each field, 30 µl of test serum and 30 µl of the BA/Te antigen were added and homogenized by circular movements for 4 min. Then, the presence of agglutination was evaluated using a light source. Samples with granular agglutination were considered positive, while homogeneous suspensions were considered negative. For test validation, positive and negative control serum samples were used in each routine. Agglutination test results in different temperatures (ranging from 18 °C to 30 °C) were evaluated. Better agglutination intensity was observed at an ambient temperature of 22–24 °C.

The card agglutination test for *T. evansi* (CATT/*T. evansi*) was used as the gold standard and was performed according to the manufacturer's instructions. Briefly, 45 µl of the antigen reagent was transferred onto the card test and mixed with 45 µl of the equine test sera diluted to 1:4. The card was agitated for 5 min, and agglutination was observed using a clear light source (Bajyana Songa & Hamers, 1988).

In addition, the BA/Te antigen specificity was tested against serum samples from animals positive for other infectious agents such as *Babesia caballi*, *Leptospira interrogans*, *Theileria equi*, *Trypanosoma vivax*, *Brucella abortus*, *Neospora caninum*, *Toxoplasma gondii*, *Leishmania infantum*, viral encephalitis (EHV-I), equine infectious anemia virus (EIAv), and *Burkholderia mallei*. These supplemental serum samples were kindly provided by VERTÁ (Laboratory of Veterinary Diagnostic/Institute of Veterinary Research and Diagnostic, Brazil) and the Laboratory of Infectious Diseases of Animals of the Universidade Federal de Santa Catarina, Brazil. All samples tested negative for anti-*T. evansi* antibodies in the card agglutination test (CATT/*T. evansi*).

2.3. Statistical analysis

The degree of agreement between BA/Te and CATT/*T evansi* (gold standard test) was estimated using the kappa coefficient (κ) with 95% confidence intervals using Epi Info version 7. Values of $\kappa \leq 0.60$ indicate low agreement, $0.60 < \kappa < 0.80$ indicate moderate agreement, and $\kappa > 0.80$ indicates optimal agreement (Altman, 1990). The estimate of κ considers all samples analyzed. The sensitivity and specificity of BA/Te were estimated using the CATT/*T. evansi* reference test using the following formula: sensitivity (%) = TP/(TP + FN) × 100; and specificity (%) = TN/(TN + FP) × 100, where TN represents true negative, FN false negative, and FP false positive.

3. Results

Results of the analysis for anti-*T. evansi* antibodies using BA/Te and CATT/*T. evansi* are listed in Table 1.

Of the 445 equine serum samples analyzed, 233 were negative and 212 were positive for BA/Te. The estimated BA/Te sensitivity in this study was 92% (17 false-negative results), and the specificity was 91% (22 false-positive results), leading to a positive predictive value (PPV) and negative predictive value (NPV) of 90% and 93%, respectively.

Degree of agreement between BA/Te and CATT/T *evansi* was high ($\kappa = 0.82$; 95% CI = 0.771–0.877; $P < 0.01$).

BA/Te showed high specificity for the detection of anti-*T. evansi* antibodies in the supplementary study when serum samples positive for *B. caballi*, *L. interrogans*, *T. equi*, *T. vivax*, *B. abortus*, *N. caninum*, *T. gondii*, *L. infantum*, EHV-I, EIAv, and *B. mallei* were used (Table 2). The

Table 1
Sensitivity and specificity analysis of the buffered antigen of *T. evansi*, for detection of anti-*T. evansi* antibodies in positive and negative equine serum samples in CATT/*T. evansi* test

	CATT/*T. evansi* (Standard test)		Total
	Positive (true positive)	Negative (true negative)	
BA/Te			
Positive	190	22	212
Negative	17	216	233
Total	207	238	445

Table 2
Complementary study of the BA/Te specificity with positive equine serum samples to main infectious and protozoan agents in Brazilian equine herds

Equine serum control	Equine serum samples tested			
	Diagnostic method	No. of samples	No. of BA/Te-positive	No. of CATT/*T. evansi*-positive
Babesia caballi	PCR (+)/Microscopic analysis (+)/IFAT (+)	29	0	0
Leptospira interrogans	PCR (+)/MAT (+)/bacterial isolation (+)	26	0	0
Theileria equi	PCR (+)/Microscopic analysis (+)/IFAT (+)	21	0	0
Trypanosoma vivax	PCR (+)/IFAT (+)	18	0	0
Brucella abortus	PCR (+)/SAT (+)	17	0	0
Neospora caninum	PCR (+)/RIFI (+)/ELISA test (+)	15	0	0
Neospora hughesi	PCR (+)/IFAT (+)	12		
Toxoplasma gondii	PCR (+)/IFAT (+)	12	0	0
Leishmania infantum	PCR (+)/IFAT (+)	11	0	0
Viral encephalitis (EHV-1)	PCR (+)/Viral isolation (+)/ELISA test (+)	9	0	0
Equine infectious anemia virus (EIAv)	IDGA (+)/ELISA (+)	8	0	0
Burkholderia mallei	FC (+)/ELISA test (+)	5	0	0
Negative control serum[a]	Tested by standard methods for respective infectious agents	15	0	0
Total		198	0	0

Abbreviations: PCR, microscopic analysis, examination of stained blood films by microscopy; IFAT, indirect fluorescence antibody test; MAT, microscopic agglutination test; ELISA, enzyme-linked immunosorbent assay.

[a] Negative to *T. evansi*, *T. equiperdum*, *T. vivax*, *Toxoplasma gondii*, equine infectious anemia virus (EIAv), *Burkholderia mallei*, *Brucella abortus*, *Leishmania* sp., *Trypanosoma vivax*, *Theileria equi* and *Babesia caballi*.

specificity of the buffered antigen *T. evansi* was maintained when testing serum samples from animals with other infections.

4. Discussion

Our data show that BA/Te is a promising serodiagnostic tool that combines simplicity, speed, and high efficiency (92% sensitivity and 91% specificity). In addition, it can potentially be used in field conditions and surveillance programmes and to control *T. evansi* infection (VPP = 90% and VPN = 93%), thus facilitating the screening of many animals. BA/Te can be used as a complementary test along with other methods such as molecular techniques, parasitological analysis, and other serological techniques such as CATT/*T. evansi* and ELISA tests.

Antigens derived from the total lysate of *T. evansi* have been successfully used in seroepidemiological studies of infection in different animal species (Hilali et al., 2004; Laha & Sasmal, 2008; Zayed et al., 2010). In addition, the possibility of acidic buffering of the antigen (pH of ~3.65) increases the specificity of the test because it decreases the activity of agglutinins such as IgM and favors IgG-based reactions (Corbel, 1972; Patterson et al., 1976). BA/Te is a simple and accessible method that does not depend on specific *T. evansi* strains for antigen production. Thus, it allows laboratories in different countries to develop their diagnostic kits at a low cost, as importing, registering, and trading kits with biological components is difficult and expensive, and such limitations can jeopardize the diagnostic and control programmes for *T. evansi*.

The immune response against conformational epitopes of WCL antigens makes them suitable for serum agglutination tests. Similarly, polypeptides and common antigenic epitopes among antigens of WCL prepared from *T. evansi* isolated from different hosts can react with both homologous and heterologous sera, contributing to their successful use in trypanosomiasis serodiagnosis in different animal species (Laha & Sasmal, 2008; Sivajothi et al., 2016).

False-negative results in BA/Te (2/20) may be associated with a low titer of anti-*T. evansi* antibodies in the acute phase of the disease or with different levels of sensitization to the immune system. Therefore, for the diagnosis of acute infection in equines, it is important to account for the presence of parasitemia, clinical signs characteristic of trypanosomiasis, and hematological alterations. In the case of subclinical infection, the paired serology test 20–30 days after the first examination can also be used in a complementary way to diagnose the infection.

Regarding antigenic stability of BA/Te, periodic mutations in variable surface glycoproteins (VSGs), common in trypanosomes, may lead to a decrease in antibody titer considering a specific variable antigen. However, this does not result in a decrease in antibody titer against all the other *T. evansi* antigens (Jones & MckinnelL, 1985; Hilali et al., 2004), possibly maintaining the humoral response patterns against total lysate derivatives.

The BA/Te used in this study showed a high degree of agreement with the CATT/*T. evansi* ($\kappa = 0.82$) and can be used as a promising test to detect the presence of anti-*T. evansi* antibodies in naturally infected equines. Despite the degree of agreement, the greater specificity and sensitivity of CATT/*T. evansi* may be associated with its ability to detect antibodies to the variable surface antigen (VSA) type RoTat1.2.

Parasitological examinations based on microscopic analysis of blood smears or detection of *T. evansi* antigens (e.g. Ag-ELISA) are recommended only for the diagnosis of acute disease when parasitemia is severe. In contrast, serological tests allow for the detection of circulating anti-*T. evansi* antibodies and for the confirmation of animal contact with the parasite (Laha & Sasmal, 2008; Zayed et al., 2010). Serological tests allow for the diagnosis of subclinical or chronic infection, including when the parasitemia is low or intermittent (Bajyana Songa & Hamers, 1988; Hilali et al., 2004; Zayed et al., 2010; Singla et al., 2015). This is because anti-*Trypanosoma* spp. antibodies are usually detected 4–8 days post-infection and can persist for a long time even after the disease is cured or the parasite is eliminated from the bloodstream (Hilali et al., 2004; Aquino et al., 2010).

At the same time, PCR techniques and other techniques such as loop-mediated isothermal amplification (LAMP) (Thekisoe et al., 2005) can be considered sensitive and specific diagnostic tools recommended for the early diagnosis of *T. evansi* infection in the acute phase (with low or absent antibody production) and in the chronic phase or even during the subpatent period of the infection (Laha & Sasmal, 2008; Njiru, et al., 2010; Singla et al., 2015). In addition, these techniques allow evaluation of the efficiency of treatment protocols or differentiation of cured individuals from infected ones. However, they are laborious, time-consuming, and high-cost methods compared to the agglutination test (Singla et al., 2015).

Based on these findings, we can conclude that BA/Te has the potential to be used in the serodiagnosis of *T. evansi* infection in equines in Brazil. In addition, the simplicity, specificity, and sensitivity suggest that the test

can be used for the screening of suspect animals. The limitation of detecting the parasite or its antigens may be overcome by the use of one or more complementary techniques such as direct blood smear research or molecular tests (such as PCR) and clinical evaluation of the animals. Further studies will be carried out to improve the specificity of BA in order to promote the use of this test in programmes for the diagnosis and control of surra in Brazil.

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Ethical approval

The project was approved by the Animal Experimentation Ethics Committee of the Universidade do Estado de Santa Catarina (CEUA/UDESC), protocol number 9171110516. Equine serum from samples provided for routine analysis at the Laboratório Clínico Vertá, Curitibanos-Lages (Brazil) with the consent of horse owners were kindly donated and used in the present experiments.

CRediT author statement

Carolina Reck: Investigation, Resources, Formal Analysis. Álvaro Menin, Writing – Original Draft, Formal analysis. Franciane Batista: Investigation, Patricia Oliveira Meira Santos, Resources. Luiz Claudio Miletti: Conceptualization, Methodology, Supervision, Resources, Writing – Review & Editing.

Declaration of competing interests

The authors declare that they have no competing interests.

References

Altman, D.G., 1990. *Practical Statistics for Medical Research*. Chapman and Hall/CRC, London.

Aquino, L.P.C.T., Machado, R.Z., Lemos, K.R., Marques, L.C., Garcia, M.V., Borges, G.P., 2010. Antigenic characterization of *Trypanosoma evansi* using sera from experimentally and naturally infected bovines, equines, dogs, and coatis. Rev. Bras. Parasitol. Vet. 19, 112–118. https://doi.org/10.4322/rbpv.01902009.

Bajyana Songa, E., Hamers, R., 1988. A card agglutination test (CATT) for veterinary use based on an early VAT RoTat 1/2 of *Trypanosoma evansi*. Ann. Soc. Belg. Med. Trop. 68, 233–240.

Claes, F., Radwanska, M., Urakawa, T., Majiwa, P.A., Goddeeris, B., Büscher, P., 2004. Variable surface glycoprotein RoTat 1.2 PCR as a specific diagnostic tool for the detection of *Trypanosoma evansi* infections. Kinetoplastid Biol. Dis. 17, 3. https://doi.org/10.1186/1475-9292-3-3.

Colpo, C.B., Monteiro, S.G., Stainki, D.R., Colpo, E.T.B., Henriques, G.B., 2005. Infecção natural por *Trypanosoma evansi* em cães. Ciência Rural 35, 717–719.

Corbel, M.J., 1972. Identification of the immunoglobulin class active in the Rose Bengal plate test for bovine brucellosis. J. Hyg. (London) 70, 779–795. https://doi.org/10.1017/s0022172400022622.

Desquesnes, M., 2004. Livestock trypanosomoses and their vectors in Latin America. OIE (World Organisation for Animal Health). Paris, France.

Desquesnes, M., Dargantes, I., Lai, D.-H., Lun, Z.-H., Holzmuller, P., Jittapalapong, S., 2013b. *Trypanosoma evansi* and surra: A review and perspectives on transmission, epidemiology and control, impact, and zoonotic aspects. Biomed Res. Int. 321237. https://doi.org/10.1155/2013/321237.

Desquesnes, M., Holzmuller, P., Lai, D.H., Dargantes, A., Lun, Z.R., Jittaplapong, S., 2013a. *Trypanosoma evansi* and surra: A review and perspectives on origin, history, distribution, taxonomy, morphology, hosts, and pathogenic effects. Biomed Res. Int. 194176. https://doi.org/10.1155/2013/194176.

Grab, D.J., Bwayo, J.J., 1982. Isopycnic isolation of African trypanosomes on Percoll gradients formed *in situ*. Acta Tropica 39, 363–366.

Hilali, M., Abdel-Gawad, A., Nassar, A., Abdel-Wahab, A., Magnus, E., Büscher, P., 2004. Evaluation of the card agglutination test (CATT/*T. evansi*) for detection of *Trypanosoma evansi* infection in water buffaloes (*Bubalus bubalis*) in Egypt. Vet. Parasitol. 121, 45–51. https://doi.org/10.1016/j.vetpar.2004.02.009.

Jones, T.W., MckinnelL, C.D., 1985. Antigenic variation in *Trypanosoma evansi*: Variable antigen type development in mice, sheep and goats. Trop. Med. Parasitol. 36, 53–57.

Laha, R., Sasmal, N.K., 2008. Characterization of immunogenic proteins of *Trypanosoma evansi* isolated from three different Indian hosts using hyperimmune sera and immune sera. Res. Vet. Sci. 85, 534–539. https://doi.org/10.1016/j.rvsc.2008.02.011.

Lanham, S.M., Godfrey, D.G., 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp. Parasitol. 28, 521–534. https://doi.org/10.1016/0014-4894(70)90120-7.

Lun, Z.R., Fang, Y., Wang, C.J., Brun, R., 1993. Trypanosomiasis of domestic animals in China. Parasitol. Today 9, 41–45. https://doi.org/10.1016/0169-4758(93)90029-f.

Njiru, Z.K., Ouma, J.O., Enyaru, J.C., Dargantes, A.P., 2010. Loop-mediated isothermal amplification (LAMP) test for detection of *Trypanosoma evansi* strain B. Exp. Parasitol. 125, 196–201. https://doi.org/10.1016/j.exppara.2010.01.017.

Patterson, J.M., Deyoe, B.L., Stone, S.S., 1976. Identification of immunoglobulins associated with complement fixation, agglutination, and low pH buffered antigen tests for brucellosis. Am. J. Vet. Res. 37, 319–324.

Rodrigues, A., Fighera, R.A.,, Souza, T.M., Schild, A.L., Barros, C.S., 2009. Neuropathology of naturally occurring *Trypanosoma evansi* infection of horses. Vet. Pathol. 46, 251–258. https://doi.org/10.1354/vp.46-2-251.

Sharma, P., Juyal, P.D., Singla, L.D., Chachra, D., Pawar, H., 2012. Comparative evaluation of real-time PCR assay with conventional parasitological techniques for diagnosis of *Trypanosoma evansi* in cattle and buffaloes. Vet. Parasitol. 190, 375–382. https://doi.org/10.1016/j.vetpar.2012.07.005.

Silva, R.A., Arosemena, N.A., Herrera, H.M., Sahib, C.A., Ferreira, M.S., 1995. Outbreak of trypanosomosis due to *Trypanosoma evansi* in horses of Pantanal Mato-grossense, Brazil. Vet. Parasitol. 60, 167–171. https://doi.org/10.1016/0304-4017(94)00757-4.

Singla, L.D., Sharma, A., Kaur, P., Bal, M.S., 2015. Comparative evaluation of agglutination assay with microscopy and polymerase chain reaction for detection of *Trypanosoma evansi* in bovines of Punjab. Ind. J. Anim. Sci. 85, 1164–1166.

Sivajothi, S., Rayulu, V.C., Bhaskar Reddy, B.V., Malakondaiah, P., Sreenivasulu, D., Sudhakara Reddy, B., 2016. Polypeptide profiles of South Indian isolate of *Trypanosoma evansi*. J. Par. Dis. 40, 652–655. https://doi.org/10.1007/s12639-014-0552-1.

Thekisoe, O.M., Inoue, N., Kuboki, N., Tuntasuvan, D., Bunnoy, W., Borisutsuwan, S., et al., 2005. Evaluation of loop-mediated isothermal amplification (LAMP), PCR and parasitological tests for detection of *Trypanosoma evansi* in experimentally infected pigs. Vet. Parasitol. 130, 327–330.

Zayed, A.A., Habeeb, S.M., Allam, N.A.T., Ashry, H.M.Z., Mohamed, A.H.H., Ashour, A.A., Taha, H.A., 2010. A critical comparative study of parasitological and serological differential diagnostic methods of *Trypanosoma evansi* infections in some farm animals in Egypt. Am.-Euras. J. Agricul. Environ. Sci. 8, 633–642.