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ORIGINAL RESEARCH

# Comparative diagnosis of parasitological, serological, and molecular tests in dourine-suspected horses

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**Abstract** Study on comparative sensitivity of parasitological, serological, and molecular tests on 237 horses originating from two dourine-suspected districts of Arsi-Bale highlands of Ethiopia was conducted to determine the prevalence of the disease and degree of agreement of the diagnostic tests. Accordingly, the prevalence of the disease was found to be 4.6%, 36.7%, and 47.6% by parasitological Woo test, RoTat 1.2 and 18S PCR tests, respectively. The seroprevalence of the disease was 27.6% in CATT/*Trypanosoma evansi* test. In Ethiopia, it was for the first time that trypanosomes from dourine suspected horses were demonstrated in 4.6% of the animals using Woo test. The findings of the present study disclosed that dourine is highly prevalent and one of the major diseases of horses in the area. There was no statistically significant difference ($P>0.05$) in prevalence of the disease between districts, sexes, and age groups of the animals. However, there was a statistically significant difference ($P<0.05$) in the prevalence of the disease between emaciated and animals with good body condition. Assessment of the degree of agreement of the diagnostic tests employed revealed low to fair ($k = 0.1 - 0.4$) with significantly higher sensitivity by PCR than other tests.

**Keywords** Dourine · Horses · Prevalence and test agreement

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## Introduction

Ethiopia has the largest equine population in Africa including 7 million horses, donkeys, and mules (CSA 2008). In Ethiopia, equines have their greatest contribution in agriculture and transport sector of the national economy. Equines are used for various works such as carting goods and people, carrying packs and bricks, and other construction materials, riding, tillage, weeding, and water carrying. Despite their tremendous contribution less attention has been paid to equines in terms of health care and husbandry managements (Maarten 2009). Of the various bottlenecks of equines in Ethiopia, the attribute due to trypanosomosis takes the lion share.

The subgenus *Trypanozoon* has diverse means of transmission, which includes a number of *Glossina* species, where they undergo a complex mode of development, mechanical transmission by blood sucking flies in which there is no development and during coitus (Zablotskij et al. 2003). These flagellates can be found in virtually every warm-blooded vertebrate species (Stuart et al. 2008).

Dourine caused by *Trypanosoma equiperdum*, a disease in equines, characterized by edematous lesions of the genitalia, fever, cutaneous eruptions, incoordination, facial-paralysis, ocular lesions, anemia, and progressive emaciation (Barrowman 1976; Luckins 1994). Diagnosis of equine trypanosomosis caused by the subgenus *Trypanozoon*

commence with the observation of clinical signs and symptoms however, further diagnosis requires demonstration of the parasite, serological, biochemical, and molecular tests. In Ethiopia little and fragmented studies were conducted with regard to Dourine based on clinical signs (Dagnachew et al. 1980), serologically by ELISA and CFT (Alemu et al. 1997), by ELISA, CATT/*T. evansi* and Latex/*T. evansi* (Hagos et al. 2010) and molecular by PCR (Clausen et al. 1999). However, none of the above studies isolated the trypanosomes in the blood of dourine suspected animals where the existence of the parasite was under doubt. Thus, the present study was initiated and designed to assess the comparative diagnosis of dourine involving various parasitological, serological, and molecular tests and thereby determine the degree of test agreement among the diagnostic tests used.

## Materials and methods

### Study area

The study was carried out in two dourine suspected districts namely Dodola and Assassa located in the Arsi-Bale highlands of Oromia regional state, Ethiopia. Dodola is located in the Bale Zone, 320 km away from the capital Addis Ababa, at 6.983°N latitude and 39.183°E longitude with an elevation ranging from 2,362 to 2,493 m above sea level. Assassa is located in Arsi Zone, about 300 km South from Addis Ababa with geographical coordinates are 7.2° N, 39.2° E, with an elevation 2,600 to 2,650 m above sea level. Agriculture is the mainstay of the livelihood of peoples and the leading economic activity of the area with a mixed farming system covering the highest percentage of the total agricultural activities with crop-livestock production (Arsi-Bale Zone Agricultural and Rural Development Office 2009).

### Study animals

A total of 237 local breed horses (120 female and 117 male) of age ranging from 2–20 years kept under traditional management system of free grazing were considered as study animals. Animals with history of recent trypanocidal treatment and castrated stallions were purposively excluded from sampling.

### Examination protocols

#### *Parasitology*

Blood collected from jugular vein of the horses was immediately examined by Woo test in which a capillary tube was filled up to three fourth (50 µl) and centrifuged for 5 min in micro-centrifuges at maximum rpm. Subsequently, the capillary tubes were mounted in a special holder, were examined microscopically at magnification of ×10×10 at the buffy coat as described in (Woo 1969; Reid et al. 2001) to look for live parasites.

#### *Serology*

Blood samples were collected from jugular vein of 237 horses using plain vacutainer tubes and venoject needles, after the site was wiped with cotton wool soaked in alcohol. The tubes were labeled and the blood was allowed to clot over night at room temperature and the serum was separated by centrifugation. Then sera were filled in to serum storage (polypropylene sterile cryogenic vials) and stored at −20°C until tested by Card Agglutination Test for Trypanosomiasis/*T. evansi* (CATT/*T. evansi*). The test CATT/*T. evansi*, a rapid direct agglutination test, which uses formaldehyde fixed, Coomassie stained, freeze-dried trypanosomes of *T. evansi* VAT RoTat 1.2. The test was conducted at field in Arsi-Bale highlands in Dodola and Assassa veterinary clinics. The positive results were determined at cut-off point dilutions 1:4. The test was checked with positive and negative controls before the whole samples were tested (Bajyana Songa and Hamers 1988; Verloo et al. 2000).

#### *Molecular tests*

The DNA extraction was carried out in molecular Parasitology laboratory at Addis Ababa University Faculty of Veterinary Medicine, Debre Zeit, Ethiopia. The molecular tests were conducted in the Catholic University of Leuven.

For DNA extraction 180 µl of blood was pipetted in a 1.5 ml Eppendorf tube from the blood collected in heparinized tube, then 180 µl of AS1 buffer was added to preserve the DNA. The DNA extraction was carried out according to the Qiagen DNA extraction protocol (QIAamp 2003). The collected pure DNA was stored at −20°C till transportation to Leuven and PCR tested.

The DNA samples were first subjected to *Trypanozoon* specific PCR such as 18S PCR (Claes et al. 2003), with 5′-3′ forward primer CGTAGTTGAACTGTGGGCCACGT and reverse primer ATGCATGACATGCGTGAAAGT GAG. The cycling condition of 18S PCR was initial PCR reaction at 94°C for 15 min, denaturation at 94°C for 30 min, 40 cycles of reaction at 60°C for 30 s, and annealing temperature of 72°C for 30 s and final extension at 72°C for 5 min. Then tested by *T. evansi* specific RoTat 1.2 PCR according to Claes et al. (2004) with forward primer GCGGGGTGTTTAAAGCAATA and reverse primer ATTAGTGCTGCGTGTGTTCG. The cycling condition of RoTat 1.2 PCR was initial PCR activation reaction 94°C for

15 min, denaturation at 94°C for 30 min, 40 cycles of reaction at 54°C for 30 s and annealing temperature of 72°C for 30 s and final extension at 72°C for 5 min. All amplifications were carried out in Biometra® thermocycler. The PCR products were run in 2% agarose gel electrophoresis then stained with SYBRsafe. After electrophoresis, the PCR products were visualized using ultra violet transilluminator. A red-orange band was observed in positive samples the molecular size of which depends on the type of PCR used

*Data analysis*

The data obtained from all the tests were stored in Microsoft excel spreadsheet. Statistical Package for Social Science (SPSS) version 17 was used for analysis. Prevalence of the disease was calculated by dividing the number of positive animals to the total number of animal tested. The degree of test agreement among the diagnostic tests was assessed using kappa statistics as described in Viera and Garrett (2005). Furthermore, Chi-square ($\chi^2$) test was applied to assess the association between the disease and districts, sex, age, and body condition of the animals; 95% confidence interval and $P<0.05$ was set for significance level.

## Results

Parasitology: Woo test

The parasitological prevalence of dourine was found to be 4.6%. For the first time trypanosomes causing the disease were demonstrated in the blood of examined horses (Fig. 1).

Serology: Card Agglutination Trypanosomiasis Test/*T. evansi* (CATT/*T. evansi*)

Despite the lower parasitological prevalence of the disease, 27% of the examined horse demonstrated antibodies against the parasite by CATT/*T. evansi* test (Fig. 1).

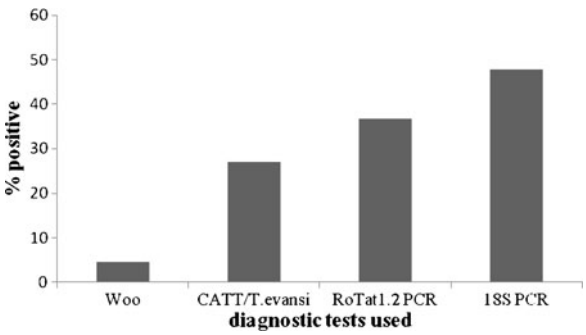


**Fig. 1** Prevalence of dourine in Arsi-Bale highlands of Ethiopia by different diagnostic tests

Molecular tests: RoTat 1.2 and 18S PCRs

Remarkably higher prevalence of 36.7% and 47.7% were recoded by RoTat 1.2 PCR and 18S PCR, respectively (Fig. 1) indicating the widespread of the disease in the area.

There was no statistically significant difference ($P>0.05$) in prevalence of the disease between the two dourine suspected districts, both sexes and age groups for all diagnostic parasitological, serological, and molecular tests (Table 1).

On the contrary, there was statistically significant difference ($P<0.05$) in the prevalence of the disease between emaciated and good body conditioned animals where relatively higher prevalence was recorded in emaciated animals (Fig. 2).

Assessment of the degree of test agreement of the employed diagnostic tests is explained in Tables 2, 3, 4. However, the degree of test agreement between all the tests were low to fair ($k = 0.1 - 0.4$).

## Discussion

The present study revealed the prevalence of dourine to be 4.6% (11 out of 237). Interestingly, it was for the first time that trypanosomes causing dourine were parasitologically demonstrated by Woo test in Arsi-Bale highlands from horses suspected of dourine and showing clinical signs. Previous studies conducted in the same area could not detect the parasite and the researchers were describing the diseases only based on clinical signs (Dagnachew et al. 1980; Alemu et al. 1997; Clausen et al. 1999; Hagos et al. 2010). Likewise, a study conducted in Mongolia has not shown the parasite by Giemsa® staining (Clausen et al. 2003). As there was no recent report on parasitological diagnosis of the causative trypanosomes of dourine, it was not possible to compare our findings with the prevalence of the disease in other countries. Isolation of the trypanosomes causing dourine would give an opportunity to conduct sexual transmission study, pathological, and pharmacological study of the parasite giving bright hope for the control of the disease in the area.

Relatively higher prevalence of dourine using serology CATT/*T. evansi* test (27%) and molecular tests (36.7% RoTaT 1.2 PCR and 47.6% 18S PCR) recorded in the present study. According to Claes et al. (2003), the VAT RoTat 1.2 antigen is expressed only in *T. evansi*. Hence, the result of presently used serological and molecular tests have dual implication, Firstly, the disease described as dourine, based on observed clinical signs, became positive for a test developed specifically for *T. evansi.* At this juncture, this finding has an implication of either the disease is surra caused by *T. evansi* causing similar clinical manifestation of dourine or it is dourine caused by *T. equiperdum* but the

**Table 1** The prevalence of dourine by Woo, CATT/*T. evansi*, 18S PCR and RoTat 1.2 PCR tests in different districts, sex, age groups of the animals

| | | Woo test | | CATT/*T. evansi* | | 18S PCR | | RoTat 1.2 PCR | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Negative (%) | Positive (%) | Negative (%) | Positive (%) | Negative (%) | Positive (%) | Negative (%) | Positive (%) | |
| District | Assassa | 54 (22.8) | 0 (0) | 37 (15.6) | 17 (7.2) | 27 (11.4) | 27 (11.4) | 38 (16) | 16 (6.8) | 54 (22.8) |
| | Dodola | 172 (72.6) | 11 (4.6) | 136 (57.4) | 47 (19.8) | 97 (40.9) | 86 (36.3) | 112 (47.3) | 71 (30) | 183 (77.2) |
| Sex | Female | 111 (46.8) | 9 (3.8) | 83 (35) | 37 (15.6) | 59 (24.9) | 61 (25.7) | 77 (32.5) | 43 (18.1) | 120 (50.6) |
| | Male | 115 (48.5) | 2 (0.8) | 90 (38) | 27 (11.4) | 65 (27.4) | 52 (21.9) | 73 (30.8) | 44 (18.6) | 117 (49.4) |
| Age group | ≤5 year | 48 (20.3) | 5 (2.1) | 40 (16.9) | 13 (5.5) | 27 (11.4) | 26 (11.0) | 36 (15.2) | 17 (7.2) | 53 (22.4) |
| | 6–10 year | 121 (51.1) | 4 (1.7) | 96 (40.5) | 29 (12.2) | 61 (25.7) | 64 (27.0) | 73 (30.8) | 52 (21.9) | 125 (52.7) |
| | 11–15 years | 41 (17.3) | 1 (0.4) | 27 (11.4) | 15 (6.3) | 24 (10.1) | 18 (7.6) | 25 (10.5) | 17 (7.2) | 42 (17.7) |
| | >15 years | 16 (6.8) | 1 (0.4) | 10 (4.2) | 7 (3.0) | 12 (5.1) | 5 (2.1) | 16 (6.8) | 1 (0.4) | 17 (7.2) |
| Total | | 226 (95.4) | 11 (4.6) | 173 (73) | 64 (27) | 124 (52.3) | 113 (47.7) | 150 (63.3) | 87 (36.7) | 237 |

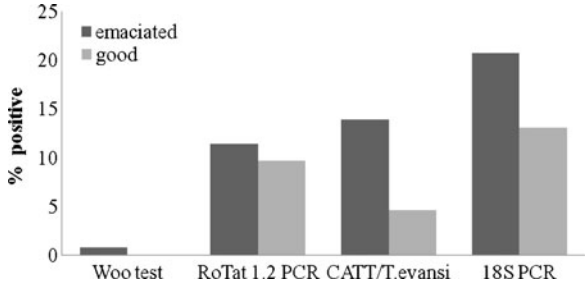


**Fig. 2** Prevalence of dourine between different body conditions by the diagnsostic tests

VAT RoTat1.2 antigen could also be expressed by *T. equiperdum*. Secondly, the disease is highly prevalent in that locality affecting a number of animals. This finding was in consent with previous studies in the same area by Clausen et al. (1999) who reported 28.3% seroprevalence using CFT. Similarly, Hagos et al. 2010 reported seroprevalence of 28%, 24.81%, and 19.26% using CATT/*T. evansi*, Latex/*T. evansi* and ELISA/*T. evansi*, respectively. Relatively lower seroprevalence of dourine was reported from Mongolia 7.65% and 6.7% by CFT and ELISA, respectively (Clausen et al. 2003). This difference is seroprevalence could be attributed to the difference in sensitivity and the specificity of the tests applied.

Furthermore, the study indicated that there was no statistically significant difference ($P>0.05$) in prevalence of the disease between the two districts and sexes of the animals. This could be explained by the ecological similarity and the close proximity of the two districts within the Arsi-Bale highlands. Animals in the study area freely move from one district to the other for transportation of goods to market and humans for social purpose that could increase the likelihood of disease transmission. The different sex and age groups can be equally exposed and affected by the disease as long as they are sexual active. Similarly, there was no statistically significant difference ($P>0.05$) in prevalence of the disease among different age groups. However, the prevalence of dourine was significantly ($P<0.05$) higher in emaciated animals as compared to animals of good body condition. This is in accordance with the findings of Clausen et al. (2003) in which higher concordance of poor body condition and higher seropositivity was recorded. This could be attributed to the fact that the disease was reported to cause loss of body condition. More importantly, animals with poor body condition could have compromised immune system due to poor nutrition and/or other calamity that may increase the likelihood of establishment of trypanosomosis in such animals.

The degree of test agreement between all the diagnostic tests used in the present study was found to be low to fair ($k = 0.1 - 0.4$). This could be associated to the difference in sensitivity and specificity the tests. In Woo test it

**Table 2** Degree of test agreement between Woo test and CATT/*T. evansi* and PCR tests

| | | CATT/*T. evansi* | | 18S PCR | | RoTat 1.2 PCR | |
|---|---|---|---|---|---|---|---|
| | | Positive | Negative | Positive | Negative | Positive | Negative |
| Woo test | Positive | 8 (72.7) | 3 (27.3) | 7 (63.6) | 4 (36.4) | 8 (72.7) | 3 (27.3) |
| | Negative | 56 (24.8) | 170 (75.2) | 106 (46.9) | 120 (53.1) | 79 (35) | 147 (65) |

depends on demonstration of the parasite. Reid et al. (2001) has revealed that the lowest level of detection of the Woo test is 31 *T. evansi* per ml of blood. However, in *T. equiperdum* the level of parasiteamia could be low, fluctuating from $10^6$ parasites/ml of blood to less than one parasite/1 unit of blood (0.000001 parasite/μl) (Desquesnes and Davila 2002) as the parasites are residing in tissues. Similar situation was encountered by Verloo et al. (2000), who reported none by Woo test out of eight of the water buffaloes *T. evansi* positive by mice inoculation.

In case of CATT/*T. evansi*, the test is dependent on detection of antibodies against the parasite surface glycoproteins. There could be possibility of examination of the animals before sufficient antibodies are produced. This was discussed by Verloo et al. (2000) that RoTat 1.2 specific antibodies always appeared within one month after infection in rabbits experimentally infected with *T. evansi* originating from different parts of the world and a VAT that appears early after infection and that is expressed by many if not all *T. evansi* serodemes. The CATT/*T. evansi* could on the other hand also overestimate the prevalence of the disease, as both animals with active infection and animals recovered from infection could be seroconvert. This finding is in agreement with observation of Hilali et al. (2004) reporting 24% prevalence of *T. evansi* infection in water buffaloes in Egypt while none of them were parasitologically positive. Ngaira et al. (2005) indicated in camels that there were PCR positive cases missed by CATT/*T. evansi*, explaining the case due to early infections where animals have not yet formed detectable antibody levels. There are also isolates that lack RoTat 1.2 gene and/or do not express RoTat 1.2 VSG (Ngaira et al. 2004). On the other hand, CATT/*T. evansi* may give false positive due to cross-reacting epitopes on the antigen, as it is crude extract of the parasite (Urakawa et al. 2001) giving higher prevalence of the case.

RoTat 1.2 PCR also depends on detection of a gene for the predominant surface glycoprotein of the *T. evansi*. As both RoTat1.2 PCR and CATT/*T. evansi* tests are dependent on the same factor the degree of test agreement between CATT/*T. evansi* and RoTat 1.2 PCR should have been better than the test agreement between the other diagnostic tests. However, the current study reported higher prevalence of the disease by RoTat1.2 PCR. This could indicate the higher sensitivity of PCR as compared to serological test. It is stated that the sensitivity threshold of the trypanosome detection by PCR generally ranges from 1 to 20 parasites/ml of blood (0.001–0.02 parasite/μl) (Desquesnes and Davila 2002). Nevertheless, it is lower than the case of 18S PCR; these could be associated to the number of copies of the genes. Nuclear DNA of trypanosomes bears the coding for the ribosomal RNA; the ribosomal DNA cistron genes occur in multiple copies in tandem arrays. The transcrpitonal unit of rDNA is made of 18S ribosomal subunit, 5.8S and 28S subunits that were indicated to be about 200 copies of each (Desquesnes and Davila 2002). There could also be those trypanosomes which are negative for RoTat 1.2 PCR. 18S PCR is a test which detects the rRNA gene of the *Trypanozoon*, thus it cannot differentiate between the different species of *Trypanozoon T. brucei*, *T. evansi*, and *T. equiperdum*. In addition, the strains of *T. evansi* which do not have RoTat 1.2 VSG gene can become positive for 18S PCR.

In conclusion the present study indicated that dourine is highly prevalent in Arsi-Bale highlands of Ethiopia. However, there was diagnostic sensitivity difference among parasitological, serological and molecular tests. In this regards PCR is the most sensitive followed by serological test. The isolation of trypanosomes from dourine suspected horses in the present study could create possibility for further molecular characterization of the parasites that will definitely answer the existing doubt whether dourine is caused by *T. equiperdum* or *T. evansi.*

**Table 3** Degree of test agreement between CATT/ *T. evansi* and 18S and RoTat 1.2 PCR tests

| | | 18S PCR | | RoTat 1.2 PCR | |
|---|---|---|---|---|---|
| | | Positive | Negative | positive | Negative |
| CATT/*T. evansi* | Positive | 38 (33.9) | 26 (21) | 27 (31) | 37 (24.7) |
| | Negative | 75 (66.4) | 98 (79) | 60 (69) | 113 (75.3) |

**Table 4** Degree of test agreement between RoTat 1.2 and 18S PCR tests

| | | 18S PCR | |
|---|---|---|---|
| | | Positive | Negative |
| RoTat 1.2 PCR | Positive | 63 (55.8) | 24 (19.4) |
| | Negative | 50 (44.2) | 100 (80.6) |

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