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Investigation into *Trypanosoma evansi* Infection in Horses in East Sumba-Indonesia

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ABSTRACT

Trypanosoma evansi (*T. evansi*) is a blood parasite, the causative agent of Trypanosomiasis (Surra) in many animal species primarily horses. In Indonesia, surra is a major disease of horses causing a catastrophic outbreak in Sumba Island killed thousands of horses during 2010-2012. Diagnosis of the disease is frequently based on parasitological technique. The aims of this study were to investigate *T. evansi* infection using both serological, parasitological and hematological techniques in horses in East Sumba. A total of 270 blood samples were randomly collected from both sexes of the healthy-looking animals for serum tested with Card Agglutination Test for Trypanosomiasis (CATT), and blood smear for detecting the presence of the parasite and for the leukocyte sub-population respectively. The results showed that 24.81% (67/270) of the samples were positive antibody to *T. evansi* antigen, and only 2.2% (6/270) of the serologically positive samples were also confirmed positive with the parasite. The leukocytes sub-population of the parasite-positive animals consisted of lymphocytes 60-98% (82.17 ± 14.43%), monocyte 0-4% (1.5 ± 1.76%), neutrophiles 0-40% (16.17±14.57%), eosinophils 0% and basophils 0-1% (0.17±0.41%) respectively. It was concluded that the seroprevalence of investigated animals were almost 25%, 2.2% of them suffered with parasitemia, lymphocytosis and neutropenia. This data suggesting a positive correlation between the applied tests, and it was considered as a novel diagnostic confirmation regarding Surra infection in the region.

Key words: *Trypanosoma evansi* seroprevalence, Surra, CATT, Leukocyte sub-population

INTRODUCTION

Trypanosoma evansi (*T. evansi*) the causative agent of Surra is a protozoan of the flagellate class that has a predilection for blood, infecting its host via the fly intermediate host. Generally, horses are most often affected by the disease compared to other animals, causing economic losses mainly due to morbidity and mortality (OIE 2013; Aregawi et al. 2019). Moreover, horses are the most susceptible to *T. evansi* infection, followed by camels and dogs, and buffaloes that are referred to as reservoir animals but in pet animals, dogs are considered to be the most susceptible to *T. evansi* infection (Aregawi et al. 2019; Khan et al. 2022). The incubation period of Surra in horses is 1-4 weeks followed by common but not specific clinical signs such as fluctuating fever, weakness, lethargy, anemia, severe weight loss, petechial hemorrhages sometime with nervous signs which are different from one host and one place to another associated with its immunosuppressive effects (Desquesnes et al. 2013; Wardhana and Savitri 2018). The grates catastrophic outbreak of Surra in Sumba Island, as the largest source of horse breeding in Indonesia, was reported during 2010-2012 causing more than 2000 animal death, primarily horses followed by cattle (Sawitri and Wardhana 2024). This condition was very detrimental to horse breeders from economic and cultural aspects (Anggung et al. 2019). With the high mortality, as no vaccine for Surra, suggesting no effective anti surra treatment was applied during the outbreak, although prolonged survival of the infected animals may helped by the increased production of anti-inflammatory IL-10 (Nguyen et al. 2023). Therefore, the only appropriate prevention strategy was suggested by understanding and implementing epidemiological approach, including an appropriate control vectors (Kim et al. 2024).

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Clinical signs of Surra are not always sufficiently specific for confirmation clinical diagnoses, so that laboratory tests are required to support definitive diagnosis (Desquesnes et al. 2022). The diagnosis of trypanosomiasis can be based on finding the parasite itself, although with limited sensitivity and specificity compared to serological assay (Apsari et al. 2024), so that detecting its molecular properties with more sensitivity and high specificity were advised (Desquesnes et al. 2022). The specific potency of every test should be evaluated carefully for effective application based on the different epidemiological conditions. Many researchers have compared several diagnostic techniques such as using PCR, CATT and serological assays (Tehseen et al. 2017; Kim et al. 2024). Detection of *T. evansi* through blood smear is very dependent on the amount of parasitemia that occurs. Serologically, ELISA and CATT both give good results in cattle, buffalo and horses (Laha and Sasmal 2009; Apsari et al. 2024). For field investigations in horses, it was suggested to use serological diagnosis with Card Agglutination Test for *T. evansi* (CATT) because it provides more sensitive, it also showed a higher prevalence for a wide range of many animal species than other traditionally used diagnostic methods (Tehseen et al. 2017; Junco et al. 2024). However, no single test could be used to detect active infections and/or trypanosome species or subspecies and further corrections are required to find specific gaps in diagnostic methods and the sustainable control or elimination of the disease, mainly the use of molecular based diagnostic tests (Desquesnes et al. 2022; Villena et al. 2023). For this reason, in this study, the developed diagnostic method namely CATT and microscopy were coupled with the analysis of leukocyte sub-papulation, to get a comparative diagnosis.

MATERIALS AND METHODS

Ethical clearance

This research was approved by Ethical Commission of The Faculty of Veterinary Medicine Udayana University with letter No. B/26/UN14.2.9/PT.01.04/2023.

Blood samples collection

A total of 270 blood samples were originated from nine purposively selected districts in East Sumba including Lewa, Katala Hamu Lingu, Waingapu, Pandawai, Kahaungu Eti, Rindi, Pahunga Lodu, Ngadu Ngala, and Wula Waijelu. The samples were collected randomly from both sexes of apparently healthy horses, using K3 EDTA vacutainer tubes (Arkan Medical) for microscopy examination and Plain tube for serological assays respectively. The CATT/ *T. evansi*) kit was purchased commercially (htt://www.itg.be/production@itg.be).

Microanalysis

The microanalysis of blood samples were based on a published-hematological method (Deshpande et al. 2021) with a minor modification. Briefly, a drop of blood sample (3–5µL) from an animal was placed on one end of a clean glass object for thin blood, air dried for 5 minutes, fixed with absolute methanol and immediately stained with 10% Giemsa. The blood smear preparations were washed under running water and dried and examined under a microscope with a magnification of 400 – 1000x with immersion oil. Counting of leukocyte-sub populations was done using the automatic hematology analyzer Abaxis HM 5, counting each type of leukocyte per 100 leukocyte cells and analyzed statically using published methods (Conboy and Zajac 2012).

Serological testing

The Surra serological test was carried out using the card agglutination technique for *T. evansi* (CATT/ *T. evansi*) (CATT kit) based on a standard method (Schlenker 1997) with a slight modification. Briefly, serum was diluted in CATT kit buffer with a ratio of 1: 8. Serological testing was carried out by mixing 20µL of diluted serum samples with one drop (45µL) of *T. evansi* antigen on the circle on the test card. Each serum sample and antigen were homogenized using a disposable-plastic stirrer. After each sample in the circle was mixed, CATT /T card was placed in a rotating machine at a speed of 70rpm for 5min. The positive reactions were scored based on a publish method (Hagos 2010), indicated by the presence of blue agglutination (sand-like sediment) and scored into five categories namely +3 (very clumpy), +2 (a clear sand-like precipitate), +1 (a sand-like precipitate that can be seen evenly dotted on the circle), +/- (the visible reaction is very faint and almost invisible, - (no agglutination reaction).

RESULTS AND DISCUSSION

Of the total 270 serum samples tested, 24.81% (67/270) were positive using the CATT/ *T. evansi* kit. Only one of the tested samples 1.5% (1/67) showed very strong agglutination (+++), 18% (12/67) with strong agglutination (++), and majority (80.6%; 54/67) of samples classified as weak agglutination (+) (Table 1).

Table 1: Serological test result of 67 samples tested using CATT

Total samples	Agglutination with CATT			
	(+++)	(++)	(+)	(-)
67	1	12	54	-

Note: +++ (very strong agglutination), ++ (Strong agglutination), + (weak agglutination, - (no agglutination)

The microcopy of blood smear examination revealed that only 6 of 270 (2.2%) tested samples were positive parasite of interest (Fig. 1), and these samples were coincidently positive antibody using CATT ranged from + to +++. It has been proven that CATT was very sensitive to demonstrate the presence of positive antibody to *T. evansi* in buffalo (Farida et al. 2022). However, CATT results cannot confirm whether the infection is active or not, in some cases the animal had infected by *T. evansi* and the agent was no longer present in the animal, therefore the CATT should be confirmed with PCR for an eradication program purpose (Tejedor-Junco et al. 2023). Likewise, antibodies can last for 2.3-22.6 months after trypanocide treatment, so that serological reactions are not necessarily the result of an active *T evansi* reaction at that time (Monzon et al. 2003; Elshafie et al. 2013). The positive CATT test results have also been associated with the quantity of parasite circulating in the blood, during parasitemia phase, when trypanosomes exceed 2.5x 106par/ml of blood (Chappuis et al. 2005). Similarly, in this study the serological-positive

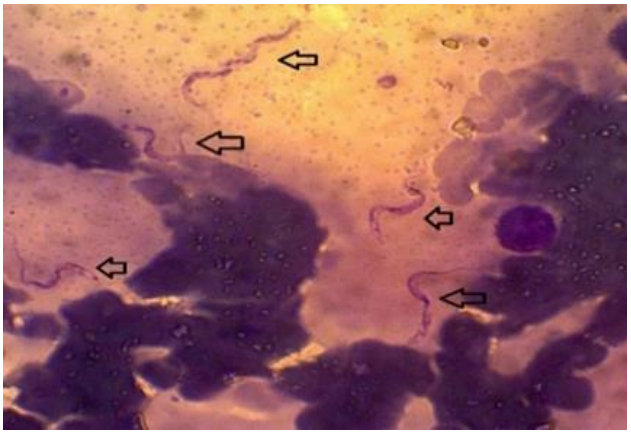

Fig. 1: An example of blood smear sample of a horse positive for *Trypanosoma evansi* with strong agglutination status (100x, the parasites are pointed with black arrows).

horses with CAAT were also found positive to contain trypanosomes microscopically, suggesting the horses under study had infected with huge number of detectable trypanosomes. Moreover, the Giemsa staining of thin blood smear method can detect *T. evansi* during parasitemia if 105 trypanosomes/ml blood are circulating in the blood (Reid et al. 2001), although microscopy observation was considered less sensitive than serological assay using CATT and real-time PCR (Nurcahyo et al. 2019; Habeeba et al. 2022). Several risk factors are believed to influence the high or low prevalence of *T. evansi* in horses such as location of sampling, gender, age, horse breed, season when samples were collected, rainy season - summer, when the number of vectors and blood-sucking activity by vectors increases, so that such condition should be considered when doing investigation (Sumbria et al. 2017).

The seroprevalence of *T evansi* in horses in different regions was found to vary, also depending on the presence of risk factors that influence the region. In this study, which was conducted from March to July (dry season), and a seroprevalence of 24.81% was found. This result was slightly higher than those reported previously (Nurcahyo et al. 2019) when the study was done during wet season (January to March) who found 12.9%. The two different seroprevalence within the same location of study and using the same assay of CATT, may be associated with seasonal reason, as one factor, although no association mainly between sex and age was reported (Benaissa et al. 2020; Sana et al. 2022). Using several methods for diagnosing *T. evansi* in horses, the CATT method was considered the most appropriate choice and sensitive for serological surveys, it can give the highest rate (14.4%) compared to PCR (1.3%) and WOOS test (0.5%), suggesting the PCR and the WOOS test were more specific, so that the seropositive status of animals should be further confirmed using the PCR method on satellite DNA targets (Tehseen et al. 2017; Kim et al. 2024). However, in this study there was 100% association between the microscopy and the CATT analysis, indicating that the animals under investigated were in acute parasitemia phase with a high quantity of *T. evansi* in the circulating blood, and the CATT predominantly detect IgM during this stage of disease progression (Chandu et al. 2021).

The results of the leukocytes-sub population originated from the serology and microscopy positive horses were lymphocytes (82.17±14.43%), neutrophils (16.17±14.57%), monocytes (1.5±1.76%), basophils ~~and~~ (0.17±0.41%) and eosinophils (0%) respectively. The significance of results with the negative animals is presented in Table 2.

The leukocyte sub-population investigation becomes a reference for diagnosing the cause of the disease for monitoring the course of it. The results of this study (Table 2) showed that only basophils had a significant difference between positive and negative parasitemia. There was a significant decrease in basophils ($P \leq 0.05$) during parasitemia, compared with the non-parasitemia animals. This condition was strongly associated with the function of the cells to protective immunity against parasites infection including helminths, ticks, mites, and protozoan parasites (Eberle and Voehringer 2016). So that the significant reduction of basophils reported here suggesting strong immune responses against the parasites. Interestingly, blood glucose observed from the same animal with parasitemia was significantly lower ($P<0.01$) than the non-parasitemia horses, but other blood biochemical components were in normal ranges (unpublished data). The reduction of blood glucose during acute infection of Surra as reported elsewhere (Garba and Mayaki 2018), may be associated with the increased utilization of host glucose and depletion by horse body cell during febrile condition.

A relatively high lymphocytosis during parasitemia compared to the normal values was observed, although with no significant difference with $P \geq 0.05$ as demonstrated (Table 3). This phenomenon indicating that the animals with lymphocytosis were in the acute phase of disease progression as reported in experimentally *T. evansi* infected rabbit and sheep (Sivajothi et al. 2015; Olatunde et al. 2021). However, in normal condition, lymphocytosis/leukocytosis in young horses frequently was due to the release of adrenaline as a result of fear, excitement or physical exercise that triggers an increased blood pressure and heart rate (Rossdale et al. 1992). The neutrophil value observed in parasitemia animals was lower compared to the normal value (Table 3), but no significant different ($P \geq 0.05$) illustrated in Table 1.

Table 2: Leukocyte sub-populations (% mean±SD) and significance of positive and negative serological examination.

Leukocyte sub-population	Positive	Negative	Significance	Level of significance
Lymphocyte	82.17±14.43	76.30±11.90	0.392NS	P≥0.05
Neutrophil	16.17±14.57	15.70±12.96	0.948NS	P≥0.05
Basophils	0.17±0.41	3.60±3.24	0.023*	P≤0.05
Monocyte	1.50±1.76	4.40±3.37	0.073NS	P≥0.05
Eosinophils	0	0	-	-

Note: NS (no significance), *(Significance), Positive and Negative: detected with serological and microscopic analysis.

Table 3: Comparison values (%) leukocyte-subsets during parasitemia with normal condition

Leukocytes subset	Parasitemia	Values[1]	Values[2]	Values[3]
Lymphocyte	60-98	21-42	18-55	5.0-33.1
Neutrophil	0-40	52-70	36-79	56.19-65.49
Basophils	0-1	0-2	0-3	0
Monocyte	0-4	0-6	0-7	3.97-8.69
Eosinophils	0	0-7	0-16	3.56-6.44

[1]Merck's Manual (2010); [2]Advia (2010); [3]Data of local horses (Radityas 2013).

The reduction of neutrophil in this study, although was not significant was accordance with a condition that when parasitemia by *T. evansi* occurs, neutropenia occurs (Olatunde et al. 2021). This condition is associated with severe acute inflammatory phase and infection as endotoxemia (Mun et al. 2010). Moreover, the neutropenia occurs due to the effect of endotoxemia, 25-35% of horses that show symptoms of colic (as a symptom of surra in horses) experienced endotoxemia, the marginalization of neutrophils in small diameter blood vessels, resulting in a decrease in circulating neutrophils in the peripheral blood (Cuervo 2019). So that the reduction of the neutrophiles during the acute phase of *T. evansi* infection may indicate a typical sign of leukocyte subsets.

Conclusion

The investigation into *Trypanosoma evansi* infection in horses in East Sumba, the largest source of horse breeding in Indonesia was done using serological, parasitological and hematological techniques, although additional data regarding significant reduction of blood glucose in horses with parasitemia was not reported here. The seroprevalence of the samples tested was 25 and 2.2% of the seropositive animals suffered with parasitemia, which were also coincidently experienced lymphocytosis and neutropenia. This data suggesting a positive correlation between the applied tests, and it was considered as a novel diagnostic confirmation regarding Surra infection in the region. One limitation of this study is that the use of molecular technique primarily real-time PCR was not applied to further confirmed diagnostic purposes that may be used to build national policy in controlling Surra in the region.

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Author's contribution

All authors were actively involved with different responsibilities. Ida Ayu Pasti Apsari, Ida Bagus Ngurah Swacita and Nyoman Sadra Dharmawan: preparing research proposal. Ida Bagus Oka Winaya and Umbu Yabu Anngung Praing: Sample collection and conducted laboratory works. Kadek Karang Agustina and I Wayan Masa Tenaya: statistical analyses and write manuscript.

Conflict of Interest: None

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