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Surra Cases in East Sumba Examined by Parasitological, Serological and Molecular Methods

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ABSTRACT

Trypanosoma evansi infection (Surra) is the most important disease *Trypanosoma evansi* infection (Surra) is the most important disease attacking livestock in Indonesia. In 2010-2012, outbreak of Surra occurred in Sumba Island where was previously reported as free region from Surra. It generated thousands livestock dead, mostly horses and buffaloes. The aim of the study was to use the parasitological (Giemsa Stained Blood Smears), serological (CATT *T. evansi*) and molecular techniques (PCR) for detection and confirmation of Surra cases in both horses and buffaloes occurred in Pahunga Lodu Distrit, East Sumba Regency. Blood samples of 20 horses and 20 buffaloes from traditional farm raised on extensive system were used in the study. Those three methods were applied. For molecular technique, ITS-1 primers were employed for 480 bp of DNA fragment from *T. evansi*. The results revealed that 4 samples (25.0%) of horses and 4 samples (33.3%) of buffaloes were positive microscopically and molecularly, while 10 samples (50%) of horses and 9 samples (75%) of buffaloes were found positive through CATT *T. evansi* test. It indicated that all positive horses and buffaloes might be in the active phase of Surra. The numbers of parasites found in the blood smears were relatively high, suggesting high parasitemia level. All livestock were treated with *diminazene aceturate* after investigation. It is concluded that Surra in East Sumba Regency remains a primary problem and PCR technique can be applied in the field to detect and confirm *T. evansi*, specially horses and buffaloes when the parasitemia is low. In addition, this technique is able to detect minute amounts of trypanosomal DNA in infected and apparently healthy livestock.

Key Words: Surra, East Sumba, CATT *T. evansi*, Parasitological Test, Molecular Technique

INTRODUCTION

Sumba Island is one of central livestock regions located in the Eastern Indonesia. Most Sumbanese live primarily as farmers, cattle breeders, rice-field farmers and trader. The livestock population is relatively higher compared to other islands in Indonesia. In addition, livestock industry in East Sumba significantly contribute to the income of local government. The farmers raise livestock not only for economic status but also traditional cultural ceremonies, family pride and social status. The more livestock they own, the higher their social status in the communities. Accordingly, livestock particularly horses and buffaloes has an important role in the life and culture of Sumbanese (Ndiha *et al.* 2018).

Before 2010, Sumba was claimed as the only island in Indonesia which has been reported free from trypanosomosis (Surra). The disease is caused by blood protozoan parasite, *Trypanosoma evansi*. It is believed that Surra was introduced into Sumba due to frequent livestock movement from endemic surra area. Additionally, an annual traditional racing horse might contribute into larger surra distribution. Many horses would be brought from many regions around Sumba or Sumbawa Island. They will be pooled together during the event. It would act as an entry point for *T. evansi* to get into Sumba Island (Laporan Dinas Peternakan Sumba Timur 2012).

In March 2010, the first case of Surra in Sumba Island was detected in extensively raised horses in West Sumba Regency. Two months later, other cases were found in South West Sumba Regency and killed horses and buffaloes. Because of lack natural immunity, unavailability of effective drug and abundance of biting flies (Tabanidae and Stomoxys species), the disease spread rapidly in East Sumba Regency and caused thousands livestock death particularly horses and buffaloes. In 2010-2012, Surra became outbreak in East Sumba Regency. During in 2011, the disease had attacked 4268 animals including 1,608 horses, 2,464 buffaloes and 196 cattle. As a result, 1,760 animals died, 1159 horses, 600 buffaloes and 1 cattle (Laporan Dinas Peternakan Sumba Timur 2012).

Following the prepatent period, Surra occurs both in acute and chronic form (Ali *et al.* 2011; Ramirez-Iglesias *et al.* 2011; Ghattas & Helmy 2016). The acute form is characterized by high level of parasitemia and noticeable clinical signs of the disease, and the chronic form is marked by low parasitemia which can be generate emaciation or become unapparent clinically. In the latest phase of chronic form, the animal may become a parasite reservoir (Fernandez *et al.* 2009). Moreover, the chronic form of surra is the most common and is likely to be associated with secondary infection (Njiru *et al.* 2004). According to Baticados *et al.* (2011), trypanosomosis in cattle and buffaloes frequently causes marked suppression of the immune system leading to increased vulnerability to other opportunistic bacterial or viral diseases.

It would be necessary to get a good epidemiologic data including availability of sensitive and specific diagnostic techniques capable of detecting the infectious agent particularly in the prepatency and/or the chronic phase of surra (Fernandez *et al.* 2009). Accordingly, study of *T. evansi* in East Sumba is fundamentally required.

There are several diagnostic tests developed to detect and confirm *T. evansi*, which have both advantages and limitations. The parasitological technique is widely applied in many laboratories in Indonesia. This technique is a simple and of low cost, but has low sensitivity and requires skilled technicians; therefore it is only effective in the acute phase of surra when high levels of parasitemia are present (Ramirez-Iglesias *et al.* 2011). Serological technique such as Card Agglutination Trypanosome Test – *T. evansi* (CATT *T. evansi*) is a quick and easy test which can be conducted under field condition. This technique is reliable for seroprevalence study (Ghattas & Helmy 2016). Another diagnostic method for Surra is molecular technique, Polymerase Chain Reaction (PCR). This technique has been developed and applied in many countries in order to overcome the problems of conventional and serological test. Accordingly, diagnosis of Surra requires at least two diagnostic methods to confirm *T. evansi* in the body of the host (Gonzales *et al.* 2007; El-Metanawey *et al.* 2009).

Current study of Surra in East Sumba Regency has been conducted by Ndiha *et al.* 2018 in Rindi District. However, the study had limitation because it only used parasitological method to detect and confirm the cases. As a result, the prevalence of surra in horse relatively was low (8%). The aim of the present study was to use the parasitological (Giemsa stained blood smears), serological (CATT *T. evansi*) and

molecular techniques (PCR) for detection and confirmation of Surra cases in both horses and buffaloes occurred in Pahunga Lodu District, East Sumba Regency in 2017.

MATERIAL AND METHODS

Blood samples

Blood samples were randomly collected from 30 horses and 12 buffaloes in May 2017. The livestock were raised extensively in Kaliuda Village, Pahunga Lodu District, East Sumba Regency, East Nusa Tenggara Province. The blood samples were drawn from the livestock through jugular vein aseptically using a sterilized disposable needle. The blood were placed into two vacuum clean tube *i.e.* 10 ml vacuum tube containing heparin as anticoagulant for blood smear examination and PCR tests. Another 10 mL vacuum tube was non-heparin (plain) tube for collecting serum. Each tube was labelled and put into cold container (4°C) for further analyses (Sawitri & Wardhana 2017). All livestock were treated with diminazene aciturate after investigation.

Giemsa-stained blood smears

A drop of blood (5 µl) was placed on the one end of a clean microscopic slide and a thin film was drawn. The film was air-dried briefly followed by fixation step in absolute methanol for about 2 minutes and allowed to dry. The blood smears were then stained by Giemsa (one drop giemsa + 1 ml PBS, pH 7.2) for 25-30 minutes. The remaining Giemsa solution on the slide was poured off, and then the slide was washed in tap water and dried (Sigh *et al.* 2017). The parasites were identified according to the characters described by Desquesnes *et al.* (2013). *Trypanosoma evansi* presents the characteristics of slender Trypanozoon parasites, extracellular, thin posterior extremity, free flagellum, active movement, highly visible undulating membrane and monomorphic parasites.

Card agglutination trypanosome test (CATT *T. evansi*)

The CATT *T. evansi* method was described by Bajyana & Hammers (1988). All sera were tested following a protocol provided by Institute of Tropical Medicine, Antwerp, Belgium. The test samples (sera) were diluted 1:8 in CATT buffer. On the test area of card was put 25 µl of diluted serum and then vertically added one drop (about 45 µl) of the well homogenised CATT antigen in each circle test area, without touching the card. The sera and antigen were gently mixed in the individual circles test area using a plastic stirrer that was wiped clean between each sample. The test card was placed on a flat bed orbital rotator and rotated for 5 minutes at 70 rpm. Furthermore, blue granular agglutinations indicated a positive reaction between sera and antigen of *T. evansi* which can be read immediately before removing the card from the rotator (El-Metanawey *et al.* 2009).

DNA extraction

DNA of *T. evansi* was extracted from whole blood sample using Genomic DNA Mini Kit (Geneaid, Taiwan) following the manufacturer's instruction. The extracted DNA of each isolate was put in the 1.5 mL labeled Eppendorf tube. All tubes were stored at -20°C for further analysis (Sawitri & Wardhana 2017).

Polymerase chain reaction and gel electrophoresis analysis

PCR amplification was carried out in ABi GeneAmp Thermal Cycler 9700 machine using ITS 1 primer *i.e.* forward (F): 5'-CCGGAAGTTCACCGATATTG-3' and reverse (R): 5'-TGCTGCGTTCTTCAACGAA-3' (Njiru *et al.* 2005). Each reaction has total volume by 25 µl using KAPA2GTM Fast PCR kit (KAPA BIOSYSTEMS, USA). The optimized PCR condition was programmed to a temperature-step cycle of pre-denaturation (95°C, 3 minutes, 1 cycle); denaturation (95°C, 10 seconds, 35 cycles); primer attachment (58°C, 15 seconds, 35 cycles); DNA fragment extension (72°C, 15 seconds, 35 cycles) and the last DNA fragment extension (72°C, 10 minutes, 1 cycle). The PCR products were fragmented on 1.5% TAE (Tris-acetate-EDTA) agarose gel together with 1000 bp DNA ladder as a standard molecular weight. Following SYBR® Safe gel staining (InvitrogeneTM), the gels were electrophorised (100 volt 20 minutes) and visualized by transillumination machine, GelDoc Transluminator (Cleaver). The DNA fragment with 480 bp indicated that the test sample was positive (Baticados *et al.* 2011; Sawitri & Wardhana 2017).

RESULTS AND DISCUSSION

Livestock management system in Sumba and clinical signs of Surra

All livestock sampled in this present study were collected from traditional farms. Livestock in Sumba Island mostly are extensively raised in the field. Normally, the farmers do not have a proper stable to keep all livestock. They are freely released to the pasture in groups for grazing. The farmers will put them into a stable if there are mass treatment conducted by local livestock agency or the farmers will sell them. At that time, the farmers just realize whether the number of their livestock increase by having foals or decrease by death. The study was conducted in the same time with a program of Surra monitoring carried out by local livestock agency of East Sumba Region. During the study, some of the clinical symptoms of Surra such as fever, emaciation and oedema on the abdomen and testicular were recorded in one horse (Ndiha *et al.* 2018). The remaining livestock sample did not show any clinical manifestation of Surra during collecting blood samples.

Parasitological test

The result of parasitological test in both horses and buffaloes can be seen in Table 1. Based on Giemsa-stained blood smears demonstrated that 5 of 20 horses (25.0%) and 5 of 12 (41.6%) were found to be positive trypanosomosis. The parasites had specific shape, monomorphic thin trypomastigote, long free flagellum and thin posterior extremity with sub terminal small kinetoplast indicating as *T. evansi* (Figure 1). The surra prevalence of horse in the present study was higher (25.0%) than that reported by Ndiha *et al.* (2018) investigating horses in Rindi District, East Sumba Regency. They detected 8% of horses infected by *T. evansi* based on parasitological test. Their study also revealed that there was no significant difference in prevalence of *T. evansi* attacking mares or stallions. A lower prevalence of Surra in horse in Pakistan was reported by Muieed *et al.* (2010) and Tehseen *et al.* (2017). They reported that 0.5% of horse in Paskitan were positive Surra based on microscope examination and Woo'e test, respectively. The low percent positivity of *T. evansi* by Giemsa-stained blood smear can be attributed to the test's

limited sensitivity and ability to detect livestock with early and acute infections only (Tehseen *et al.* 2017).

Table 1. Parasitological, Serological and molecular results for detection of *T. evansi* in both horses and buffaloes in East Sumba Regency

Livestock	Samples (n)	Diagnostic method	Positive (%)	Negative (%)
Horse	20	Giemsa-stained blood smear	5 (25.0%)	15 (75.0%)
		CATT	9 (45.0%)	11 (55.0%)
		PCR	5 (2.05%)	15 (75.0%)
Buffaloes	12	Giemsa-stained blood smear	4 (33.3%)	8 (66.7%)
		CATT	9 (75.0%)	3 (25.0%)
		PCR	4 (33.0%)	8 (66.7%)

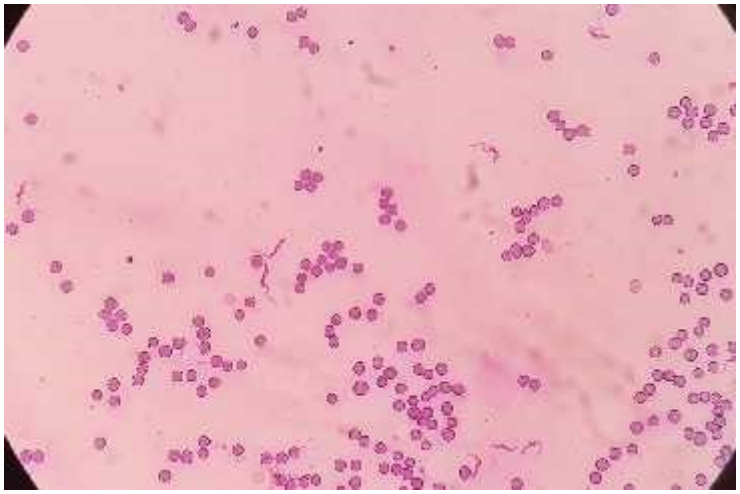
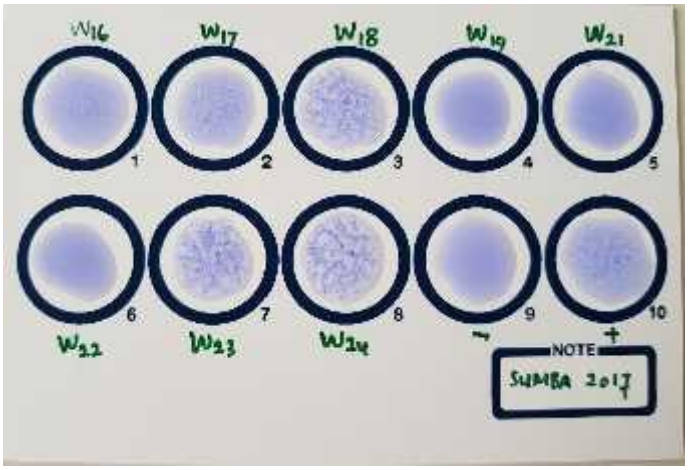


Figure 1. Morphology of *T. evansi* in thin Giemsa-stained blood smear collected from infected horse in East Sumba Regency, observed under light microscope (400×)

The occurrence of Surra in buffaloes (33.3%) was higher than horses (25.5%). This finding was nearly similar to the study conducted by Holland *et al.* (2001) and Sigh *et al.* (2017). They found 38.2% samples positive based on Giemsa-stained blood smears method. All samples positive for parasitological method were also positive for PCR assay. According to Baticados *et al.* (2017) that parasitological and PCR techniques may show very comparable sensitivity in early infections. Conservely, during the chronic infections, the parasitological examination exhibits very low sensitivity (<10%), however PCR technique is 2-3 times more sensitive (Desquesnes & Davila 2002). In this study, it is likely that both horses and buffaloes detected positive for Surra based on Giemsa - stained blood smears were in the active phase of infection. In addition, the number of parasite observed under microscope relatively high (>10 parasites) indicating that the infection might be in the active phase.

Serological test

Table 1 showed that the CATT *T. evansi* test giving seropositive results were relatively high in both horses (45.0%) and buffaloes (75.0%). In term of granular agglutination levels, all positive samples were in strong agglutination and very strong agglutination categories (Figure 2). It also indicated that the infection of surra might be in the active phase. Tehseen *et al.* (2017) stated that a high percent positivity of antitrypanosomal antibodies indicates an endemic area and there is a high threat for livestock raised in the area, particularly in the extensive management systems. Vectors of surra (biting flies) have high chance to transmit the disease among flock of livestock.



1-2: Strong agglutinations; 3, 7 & 8: Very strong agglutinations; 4-6: Seronegative; 9: Negative control; 10: Positive control

Figure 2. The result of CATT *T. evansi* test. Blue granular agglutinations indicated seropositive

Molecular test

The result of PCR assays can be seen in Table 1 and Figure 3. The size of single amplified DNA fragments was 480 bp indicating that the livestock were infected by *T. evansi*. In horse and buffaloes samples, PCR assays gave the same results to parasitological test *i.e.* 25.0% and 33.3%, respectively. All samples positive for Giemsa-stained blood smears were also positive by PCR test. It also indicated that the infection of Surra in horses and buffaloes sample might be in the active phase. Sample negative through PCR were also negative by Giemsa-stained blood smears revealed that specificity of PCR was satisfactory (Shahzad *et al.* 2012).

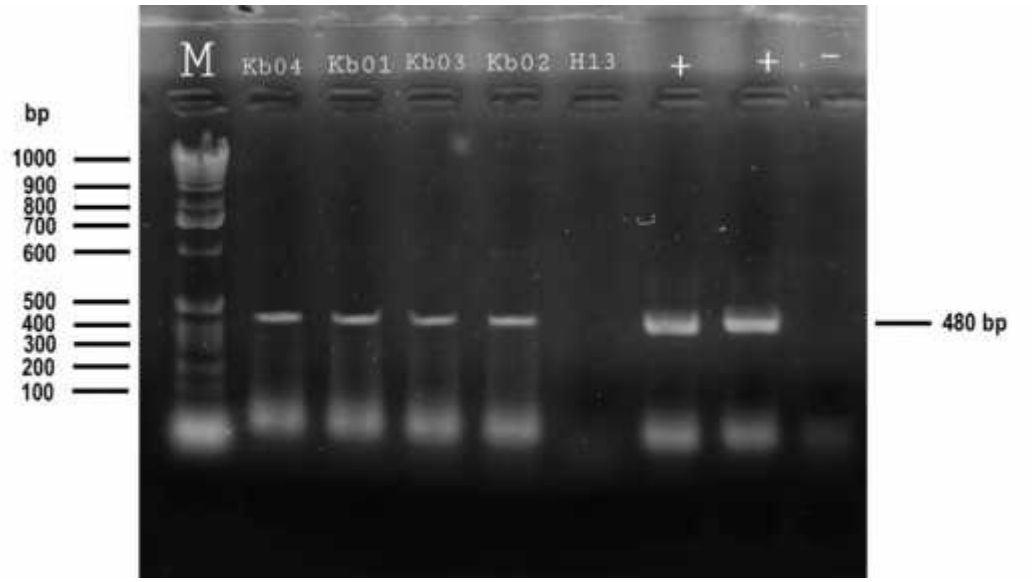


Figure 3. The result of PCR technique from buffaloes and horse samples amplified using ITS 1 primers. The DNA fragments appeared as white band on 480 base pairs (bp). Kb04, Kb01, Kb03 and Kb02 (buffaloes samples) were found positive. Hb13 was horse sample (negative); +: Positive control; -: Negative control

According to Desquesnes *et al.* (2001), the primers amplify the internal transcribed spacers, which are present in the ribosomal DNA of various trypanosomes. In addition, Njiru et al, (2005) demonstrated that the ITS – 1 primers are able to amplify DNA of *T. evansi*, *T. congolense* and *T. vivax* from blood mice infected with those parasites. It was also supported by Fernandez *et al.* (2009) reporting that the use of the ITS-1 primers could be helpful to identify multiple infections in the same host, which will reduce the cost and the time of analyses, Hence, the ITS 1 primers were used to confirm whether the livestock sampled in the study were only infected by *T. evansi* or there are mix infection with other species of trypanosomes.

Among those diagnostic methods, Ghattas & Helmy (2016) suggested that the use of PCR beside parasitological and serological methods is highly recommended for exact diagnosis in survey and control programmes. This technique is able to detect minute amounts of trypanosomal DNA in infected and apparently healthy livestock.

CONCLUSION

Surra caused by *T. evansi* in Sumba Island remains a primary problem. Based on the parasitological, serological and molecular test in the present study indicates that surra attacking horses and buffaloes is in the active phase. Accordingly, the livestock should be treated using effective drugs. In term of chronic phase, PCR could be used as a quick and reliable test for the detection of trypanosomiasis, particularly when the parasitemia is low.

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DISCUSSION

Question

What is the best technique to detect Trypanosoma evansii?

Answer

PCR method is the best method to detect T.evansii since it is very sensitive.