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Environmental Science

PAPER • **OPEN ACCESS**

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To cite this article: D H Sawitri and A H Wardhana 2024 *IOP Conf. Ser.: Earth Environ. Sci.* **1292** 012039

Detection of *Trypanosoma evansi* in healthy horses, cattle and buffaloes in East Sumba: eight years after outbreak

D H Sawitri[1*] and A H Wardhana[1,2]

[1] Research Centre for Veterinary Science, The National Research and Innovation Agency, Bogor, Indonesia
[2)] Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia

*Corresponding author's e-mail: dyah021@brin.go.id

Abstract. Trypanosoma evansi is a widespread hemoflagellate parasite that causes surra (trypanosomiasis) in a variety of mammal species, including horses, cattle, and buffaloes. In 2010-2012, the greatest outbreak of surra in Indonesia occurred on one of Indonesia's most remote islands, Sumba. This parasite has killed thousands of animals, particularly horses. The purpose of this study was to determine the prevalence of *T. evansi* infection in healthy livestock (buffaloes, cattle, and horses) after the 8th outbreak in East Sumba using different methods, including serological test (CATT/T. evansi), parasitological test (WBF, Giemsa-stained blood smears (GSBS), and Microhematocrit Centrifugation Test (MHCT), and molecular method by PCR. The survey was conducted during the arid season, when the population of vectors was at its lowest. Blood samples were taken from 111 animals in three subdistricts in East Sumba (Lewa, Wulla Waijelu, and Kota Waingapu). Results revealed that the infection rate of livestock with *T. evansi* varies by animal species and detection methods. No positive sampel found by Micro Hematocrit Centrifugation Test (MHCT), wet blood film (WBF), and Giemza-stained blood smears (GSBS). The overall infection prevalence according CATT/*T.evansi* and PCR were 7.2%(8/111) and 5.4% (6/111); respectively. The infection rates by CATT *T.evansi* and PCR were as follows: horse, 0/57 (0%) and 1/ 57(1.75%); buffalo 5/23 (21.7%) and 13.04% (3/23); cattle, 3/31 (9.7%) and 2/31(6.45%); respectively. Buffalo and cattle have a higher prevalence of *T. evansi* compared to horses. The results of the present study indicate that cattle and buffalo may serve as a reservoir for *T. evansi*, which poses a high risk to horses due to the virulence of Surra when vector populations are abundant.

1. Introduction

Surra, caused by *Trypanosoma evansi*, is a flagellated blood protozoa that affects a wide range of animals, causing high morbidity and mortality in multispecies animals, including wild and domesticated mammals [1]. The parasite is widely distributed in tropical and subtropical areas, including Indonesia, and manifests different symptoms in different breeds [2].There are two forms of surra. An acute form (occurs mostly in horses, dogs, and camels) and a chronic form (mostly seen in cattle, water buffalo, etc.). The symptoms of acute infections is fever (41.5◦C up to 44◦C), weakness, lethargy, anaemia, severe weight loss, abortion, and alteration of locomotion, with nervous sign. Death may occur in 2 weeks to 8 months. In chronic infections, subsequent waves of fever are associated with parasitemia peaks, oedema (mainly of the distal limbs), urticarial plaques, and petechial hemorrhages of the mucous membranes., and chronic infections may last up to 2 years [3]. *T. evansi* can have a variety of origins, habitats, hosts, and clinical traits. It also has numerous, intricate transmission methods, the relative importance of which varies depending on the host, parasite virulence and the geographical region [4]

During 2010 - 2012, Sumba Island experienced the greatest surra outbreak in Indonesia in the previous ten years. Surra became a serious threat that might have potentially wiped out the cattle population in Indonesia, especially in Sumba Island, one of the country's main hubs for livestock development. [5] Besides that, Sumba has sandalwood horse germplasm, which has high social and economic functions [6] The Surra outbreak was reported to have caused the deaths of more than 2000 horses, cattle, and buffalo on Sumba in 2010 (direct communication with East Sumba livestock services, 2012). Outbreaks occurred sporadically and were localized, marked by the deaths of thousands of livestock. According to Payne *et al*. [7,8], the availability of surra-positive animal transportation to surra-free areas and the existence of surra-free livestock transportation into surra-endemic areas are two contributing factors to surra outbrake.

This outbreak occurred due to the entry of surra-positive animals into surra-free areas. Previously, Sumba was a Surra-free area until 2010. This incident began in 2010, when horses were transported from Sumbawa to West Sumba for traditional horse racing events. Surra then spread to Southwest Sumba, Central Sumba, and East Sumba in 2012 (direct communication with East Sumba livestock services, 2012). Horse mortality rates peaked at 7.6% in 2010 and water buffalo mortality rates reached 2.7% in 2011 in east Sumba. Horse mortality rates peaked at 11.27% in 2010 and water buffalo mortality rates peaked at 7.1% in 2011 [9].

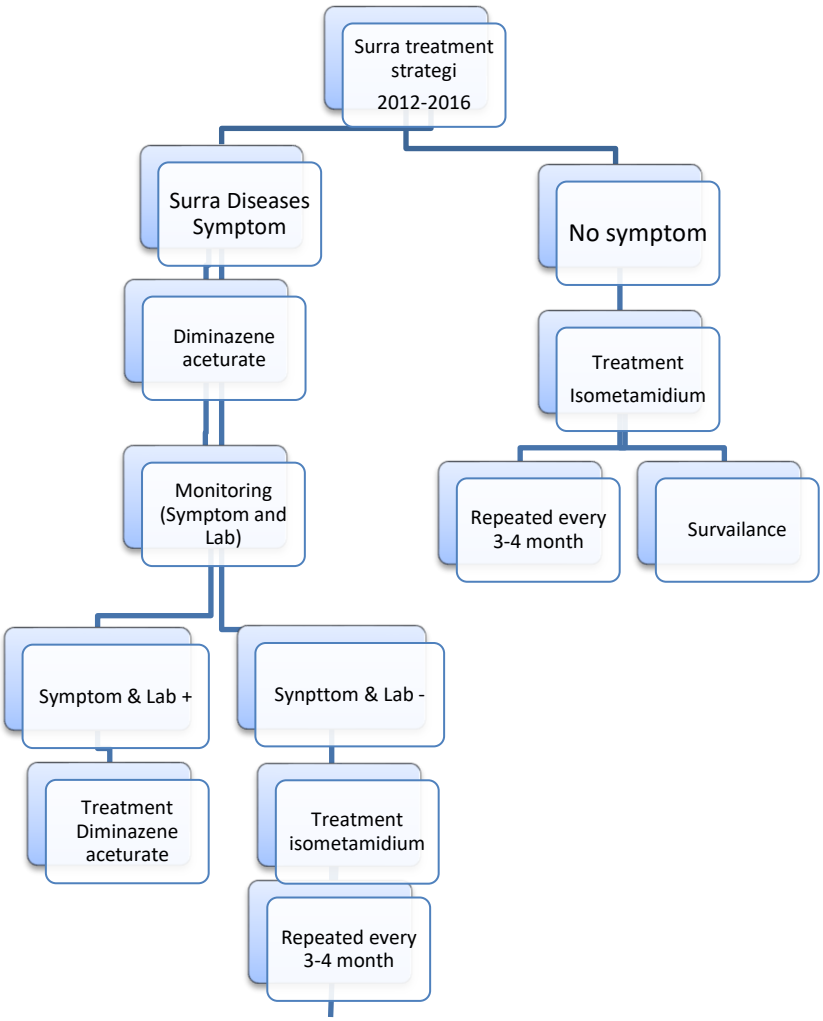

Figure 1. Surra Treatment Strategy in Sumba (2012-2016). Source: East Sumba livestock services

The East Sumba Livestock Services carried out a treatment strategy with 2 doses of diminazene aceturate (DA) after the outbreak in 2012. In conditions of severe infection or high parasitaemia, DA can be administered at 3.5 mg/kg BW on the first day to reduce parasitemia and at 5 days at a double dose (7 mg/kg body weight) to eradicate all parasites. After 1 month, if the result is still positive, it will be repeated with 1 dose of DA. All negative animals were treated with isomethamidium chloride (1 mg/kg BB) as prevention. Treatment with isomethamidium chloride is repeated every 3–4 months. This strategy successfully decreased Surra cases on Sumba (Figure 1).

Trypanosomosis control requires the ability to identify the infectious agent and the immune response of the host. Furthermore, all epidemiological investigations and the monitoring of control programs are founded on host diagnostic testing. A reliable diagnosis may be achieved by combining appropriate diagnostic tests. The lack of pathognomonic Surra symptoms necessitates a laboratory investigation to confirm the infection. Therefore, it is not recommended to establish a diagnosis of Surra and determine a treatment plan based solely on parasitological techniques; rather, it must be confirmed with other more precise and accurate diagnostic techniques that have a higher level of specificity and sensitivity [10].

This can be done using microscopy, genetic methods to show the parasite and thus an ongoing infection, or serological tools to show antibodies produced by a current or prior infection. Desquesnes *et al*. [11] stated that parasitological techniques like GSTBS are simple and low-cost, but have low sensitivity and require skilled technicians; therefore, they are only effective if high levels of parasitemia are present. According to [12]Card Agglutination Trypanosome Test—T. *evansi* (CATT/*T. evansi*) is a quick and easy test that is reliable for seroprevalence studies. Polymerase chain reaction (PCR)-type DNA amplification techniques are frequently used as a substitute due to the inadequate sensitivity of parasitological exams in chronic infections [13]. Animals with persistent infections may false negative in both parasitological and molecular assays when parasite burdens are extremely low[14]. Additionally, trypanocides can cause antibodies to persist in the circulation for several months following a successful cure, resulting in animals false positive for the disease in herds[15].

Since an outbreak of Surra was reported in 2010-2012 in Sumba, East Nusatenggara, there are few reports of the prevalence of Surra in Sumba after outbreak. The objective of this study was to investigate the prevalence of *T. evansi* in healthy animals (buffaloes, cattle and horses) after 8 years outbrake employing parasitological, serological and molecular technique in order to support appropriate control programmes against the disease in Sumba.

2. Materials and methods

2.1. Ethical statement

The experimental design was approved by the Ethics Committee on animal use at the Indonesian Agricultural Research and Development Agency no Balitbangtan/BB litvet/Rd_Rm/01.01/2019.

2.2. Study areas

The study was conducted in three subdistricts of East Sumba district, namely Lewa, Wulla Waijelu and Kota Waingapu during the dry season (November 2019). The East Sumba is 7,000.5 km2 wide, with a semiarid to arid environment. East Sumba's districts are drier than Sumba Island's other 3 districts. In East Sumba, the rainy season is brief and only lasts from January through March or December through April.

2.3. Field samples

The blood samples taken from buffaloes, cattle and horses which were proven to be clinically healthy. All livestock sampled in this present study were collected from traditional farms kept extensively. One hundred and eleven beef cattle, buffalo and horse serum and heparinized blood samples were collected from smallholder farmer. Blood samples were collected from jugular vein of cattle, buffalo and horse using vacutainer tubes and venoject needles. After labeling the tubes, the blood was allowed to coagulate over the course of one night at room temperature, and the serum was separated. Furthermore, heparinized blood samples sample was processed for the preparation for Wet Blood Film (WBF), Giemsa Stain Thin Blood Smear (GSTBS), Microhematocrit Centrifugation Test (MHCT); Polymerase Chain Reaction

(PCR) and serum sample for CATT/*T.evansi*. All remaining sera and blood were stored at -20ºC for further analysis.

2.4. Wet blood film (WBF)
A small drop of EDTA blood (2–3 µl) is placed on a clean glass slide, and then a cover-slip is placed over the blood to form a monolayer of cells. Trypanosomes are examined under a 200x light microscope to determine whether or not any are motile [15].

2.5. Giemza stained thin blood smear (GSTBS)
The end of a clean microscope slide was placed with a little drop of blood (2–5 l) containing EDTA, and a thin film was pulled out. Before being fixed in methyl alcohol for two minutes, the slide was left to air-dry. The smears were stained with Giemsa (1:10 Giemsa and PBS, pH 7.2) for 30 minutes, after which the slide was rinsed with running water and dried. Using oil immersion and a magnification of 1000, the slides were examined [15].

2.6. Microhematocrit Centrifugation Test (MHCT)
The cattle's or buffalo's blood from the jugular vein, as much as 60 l each, was immediately placed into two heparinized capillary tubes (75 x 1.5 mm each). Plasticine was used to seal the tube's extremities, and then it was spun in a hemocrit centrifuge for 5 minutes at 12,000 g. After that, the capillary tubes were put in a separate container and magnified 10X10 at the buffy coat to be examined microscopically (15). Under a microscope at a magnification of 100–200 x, trypanosomes are big cells that collect at the junction of the buffy coat and plasma. The light environment must be changed to cause the cells to exhibit refringency in order to improve the visibility of moving trypanosomes.

2.7. Card agglutination test for T. evansi (CATT/T. evansi)
The card agglutination test for trypanosomosis/T. evansi (CATT/T. evansi®, Institute of Tropical Medicine «Prince Leopold», Laboratory of Serology, Nationalestraat 155, B-2000 Antwerp, Belgium) is a quick direct agglutination test by using freeze-dried T. evansi VAT RoTat 1.2 trypanosomes. Using a serum dilution of 1:4, it was carried out in accordance with the manufacturer's instructions [16]. Briefly, 45 µl of the antigen was poured into the circular reaction zones of the provided plastic card and mixed with 25 µl of the test serum. Before testing the entire sample, the test was validated using positive and negative controls. The antigen/serum mixture was thoroughly mixed and disseminated to a distance of about 1 mm from the edge of the test surface using a clean plastic stirring rod. After each use, the stirring rod was cleaned with sterile filter paper. A flat electric orbital rotator bed was used to agitate the card for five minutes at a speed of 70 rpm. Before the card was removed from the rotator, the results were read after five minutes. A reaction is considered favorable when agglutination is visible to the unaided eye. The findings showed various levels of agglutination. When agglutination was scored as +++ (strong positive), ++ (medium positive), + (weak positive), doubtful agglutination, and - (no agglutination, negative).

2.8. DNA extraction
Trypanosoma evansi DNA was extracted from a complete blood sample using the Genomic DNA Mini Kit (Geneaid, Taiwan) in accordance with the manufacturer's instructions. Each isolate's DNA was put into a 1.5 mL tube with a label. All tubes were stored at - 20°C for the ensuing examination [17].

2.9. Polymerase chain reaction and gel electrophoresis analysis
PCR amplification conducted by using the TR3/4 primer (257 bp), which is forward (F):5’-GCGCGGATTCTTTGCAGACGA-3’ and reverse R: 5’-TGCAGACACTGGAATGTTACT-3 [18] .PCR was performed in an ABi GeneAmp Thermal Cycler 9700 equipment (Njiru *et al*. 2005). Each reaction contains a total volume of 25 µl utilizing the My Taq TM HS Mix Bioline (Meridian Bioscience, UK), The PCR condition were Pre-denaturation (95°C, 3 minutes, 1 cycle); denaturation (95°C, 15 seconds, 35 cycles); primer attachment (56°C, 15 seconds, 35 cycles); DNA fragment extension (72°C, 15 seconds, 35 cycles); and final DNA fragment extension (72°C, 10 minutes, 1 cycle). On a 1.5% TAE

(Tris-acetate-EDTA) agarose gel, the PCR products were fragmented using a 1000 bp DNA ladder as a standard molecular weight. The gels were electrophorized (100 volt, 20 minutes) after Fluoro® Safe gel staining (1stBase), and then observed using the GelDoc Transluminator (Cleaver) device. The test sample's positive status was revealed by the DNA fragment of 480 bp [17].

2.10. Assessment of diagnostic efficacy

Diagnostic efficacies of GSBS, MHCT; CATT/*T.evansi* and PCR were evaluated on the basis of % positivity shown by individual diagnostic test.

$$(\%)\ \textbf{positivity} = \frac{\textbf{number of pisitive samples given by particular diagnostic}}{\textbf{total no suspected cases}} \times \textbf{100}$$

3. Results and discussion

This study was undertaken to investigate the epidemiological situation of T. evansi infection in healthy cattle, horses, and buffalo from the subdistricts of Lewa, Wulla Waijelu, and Kota Waingapu, East Sumba district, East Nusatenggara Province. A total of 111 blood samples were examined by parasitological examination (WBF, MHCT, GSBS, CATT/T. evansi, and PCR) (Table-1). During the study, the animals were found to be in healthy conditioan and they did not exhibit any of the clinical symptoms associated with surra. The examination by microscopically WBF, GSTBS and MHCT detected no sample positive from all sample tested (0%). The result of CATT/T. evansi test showed that 17 out of 111 (15.3 %) examined animal (buffalo, cettle and horse) found to be seropositive to T. evansi with a weak-medium grade. The incidence rates for horses, cattle, and buffaloes were 0; 9.7%; and 21.7%, respectively. Likewise, using PCR showed that 7.2% (8/111) of animals were positive for surra, with incidence values in horses, cattle, and buffaloes of 1.75, 6.45, and 13.04, respectively. The occurrence of Surra in buffaloes and cattle was higher than in horses, either by serology or molecular examination.

Table 1. Examination result of WBF, MHCT, GSBS, CATT/T.evansi and PCR for *T. evansi* in East Sumba regency

No	Sub district	District/Province	Breed	No of sample (n)	Detection Methode (% positive)				
					WBF	MHCT	GSBS	CATT	PCR
1	Lewa	East Sumba/ East Nusa Tenggara	Horse	7	0/7	0/7	0/7	0/7	0/7
			Buffalo	21	0/21	0/21	0/21	5/21	3/21
2	Wulla Waijelu	East Sumba/ East Nusa Tenggara	Cattle	21	0/21	0/21	0/21	3/21	2/21
3	Kota Waingapu	East Sumba/ East Nusa Tenggara	Horse	50	0/50	0/50	0/50	0/50	1/50
			Buffalo	2	0/2	0/2	0/2	0/2	0/2
			Cattle	10	0/10	0/10	0/10	0/10	0/10
		TOTAL		111	0%	0%	0%	7.2 % (8/111)	5.4% (6/111

By using the parasitological method, no positive sample was found. This case might be related to the fact that most endemic hosts not only have persistent infections with low parasitaemia but also relapsing infections, and under these circumstances, treatments like MHCT have the same result as GSTBS (Tabel-1). However, compared to parasitological and molecular prevalence estimations, the seroprevalence values from CATT/T. evansi (7.2%) were higher than PCR (Table-1). This can be

explained by the possibility of false-negative results in parasitological and molecular tests in animals with chronic illnesses when parasite burdens are extremely low. Additionally, it is well known that antibodies can persist in the bloodstream for several months after a successful cure, which can result in animals in trypanocide-treated herds testing falsely positive for the disease [15,19]. The incidence rate in buffaloes (21.7%) are higher than cattle (13.4%). According to Rudramurthy [20], buffaloes have a more sensitive CATT T. evansi antibody than cows. According to the OIE [15] this syndrome is caused by an abundance of antibodies produced during an active infection that protect against agglutination interactions between antigens and antibodies.

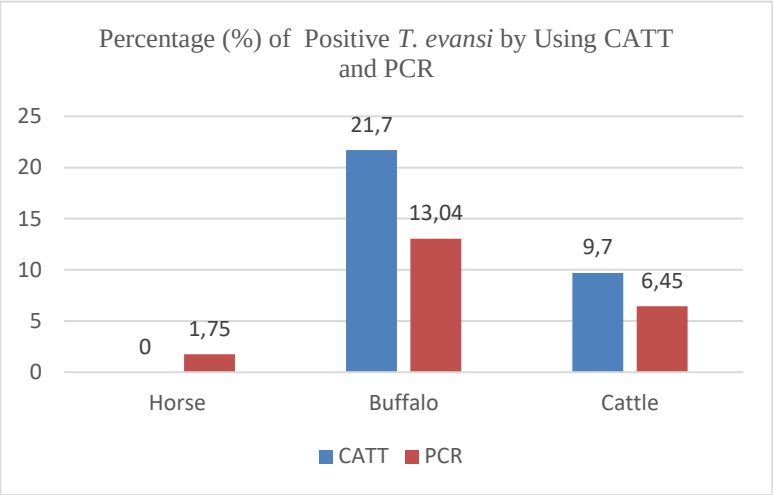

Figure 2. Percentage CATT/*T.evansi* and PCR positive for *T. evansi* according to different breed

This study carried out during the dry season when the fly population is minimal, so positive cases of surra (active infection) are rare. The diagnosis of T. evansi was made using parasitological techniques that are regarded as simple, quick, and affordable. However, they are insufficient to identify all trypanosome-infected animals, particularly when parasitemia is low and in the chronic form of the disease .The prevalence of T. evansi by microscopic examination in this study is lower than the report of Ndiha *et al*. [21] in horses in Kabaru sub-district, East Sumba district, which is 8% positive by GSBS. The similar result showed with study T.evansi in Sumba island conducted by Nurcahyo *et al*. [22], which found negative result 0.0% in 211 samples of blood smears examined using Giemsa staining. The study conducted by Praing *et al*. [23]on horse blood samples examined with GSBS showed higher results than this study, 6 out of 286 (2.1%) horses from East Sumba Regency were positive for T. evansi with the distribution of events spreading in Lewa District (3.65%), Kota Waingapu District (1.72%), and Pahunga Lodu District (7.4%). However the results of horse serologis examination in this study were lower than the study conducted by Nurcahyo *et al.* [22] and Dewi *et al.* [24] which The CATT results showed that 13.3% (28/211) and 45% (9/20) of the samples were seropositive for antibodies to T. evansi respectively. The seropositivity grade in animal in this study is weak to medium indicating that they may have been exposed to the parasite in the recent past. Although CATT/T. evansi cannot differentiate between a current infection and a previous infection, but it can be utilized successfully in conjunction with the clinical signs and symptoms of the animal illness. It features a pen-side test has a benefit over traditional parasitological and molecular methods because it is easy and affordable [25].

Furthermore, the PCR incidence rate in buffaloes in this study was higer than 7.7% reported in buffaloes from Pakistan [26]. However the PCR incidence rate in this study was lower in horse (8.2%) and cattle (36.49%) reported in horse from Marajo island Brazil [27] and cow in India [28] respectively. The variation in the T. evansi prevalence could be attributed to sample size, sampling technique and vector density in the study area [29]. The horse incidence rate was lowest in this study (Figure-1)

According to Camoin [1] Such a low seroprevalence in horse should be anticipated that Surra in horses is acute generates severe clinical symptoms, which are frequently followed by mortality if

animals are not diagnosed and treated early enough. Information obtained from the veterinarians of the East Sumba Animal Husbandry Service that Investigations into outbreaks revealed T. evansi's strong pathogenicity in horses, where lethality extremely high levels (near 100% in one case). So there is a very low probability that healthy carriers remaining in the horse population.

This low seroprevalence is also due to serum samples were taken during the dry season (November 2019) where the fly population was minimal and there were no reports of surra cases in the last 6 months. Luckins (1988) stated that the spread of infection of T. evansi occured when many animals are stabled together or close herded and when the biting fly population is abundant, especially during the wet season. The immunological response that horses have been able to establish, as documented in India, allows them to survive the infection, either as chronically ill animal or even healthy carriers [30]. Since the parasite can resurface later as a result of stress, the animals that survived might infect other horses. Polymerase chain reaction examination results revealed that the prevalence of Surra in cattle (9.7%) and buffalo (13%) was higher than in horses (1.75%). According to Desquesnes *et al.* [4], surra in cattle and buffalo frequently results in a decline in the immune system, increasing the risk that the animals could contract bacteria or other diseases.

All samples negative for Giemsa- stained blood smears but many of them positive by PCR test. It also indicated that the infection of Surra in that catlles,buffaloes, and horse in the chronic phase. The PCR result in this study showed lower result than Surra study in East Sumba conducted by Dewi *et al.* .[24] which in buffalo and horses PCR assays gave the result 25 and 33% respectively. According to Desquesnes & Dávila [31] during the chronic infections, the parasitological examination exhibits very low sensitivity (<10%), however PCR technique is 2-3 times more sensitive. Ghattas & Helmy [12] suggested using PCR in addition to parasitological and serological approaches for precise diagnosis in survey and control programs among those diagnostic techniques. This methods can detect small amounts of trypanosomal DNA including in healthy animal.

The low positivity in East Sumba is suspected because East Sumba has implemented the Surra control program as shown in (Figure 2).According to Dewi [9] that the Surra prevalence decreased significantly from 13.6 % in the year 2010 to 0,32% in the year 2015. Trypamidium® and Tryphonyl® (diminazene aceturate) were used as preventive and curative treatments from 2012 to 2016, respectively. After 2012, both mortality and morbidity experienced a significant reduction, with the number of cases and fatalities dropping to 1% or less.

The Surra control program includes a public awareness campaign, curative and preventive treatments, monitoring and surveillance, laboratory diagnostics, livestock traffic management, vector control, and the destruction of deceased animals.

In 2021 Surra outbreak was reported again in Sumba with the deaths of more than 500 horses (direct communication with East Sumba livestock services 2021). This circumstances happened because the surra drug "isothamidium chloride" (IC), which is commonly used as a prevention, has been removed from circulation in Indonesia. Thus the usual precautions taken with these drugs can no longer be taken. According to Dewi [9] The lack of a control program and the lack farmers' knowledge of Surra at the beginning of the outbreak were to blame for the outbreak's high costs.

The findings of this study also demonstrate that the Surra control program in East Sumba from 2012 to 2019 was successful, as seen by the Surra prevalence decreasing in the area. However, since there are still active diseases in the field, control measures must still be taken. The condition of all livestock must also be maintained so that the animal health remains good and does not have the potential to become an epidemic for sensitive animals such as horses. This needs to be aware that cattle and buffalo can become Surra reservoirs for horses that are very sensitive to T. evansi. Water buffalo and cattle that are infected with T. evansi are frequently asymptomatic, but they can serve as a reservoir for the parasite and a route of transmission [32].

4. Conclusion

Even during the arid season, serological and molecular testing indicates that surra continues to infect buffaloes, cattle, and horses in a chronic phase. Buffaloes and cattle are more susceptible to *T. evansi* than horses. This result suggests that cattle and buffalo may serve as a reservoir for *T. evansi*, which poses a significant risk to horses when vector populations are abundant due to the virulence of Surra.

Suitable Surra control programs combine by incorporating curative therapy, preventative treatment, surveillance and monitoring, laboratory diagnostics, livestock traffic management, vector control, and a public education program. PCR could be utilized as a quick and accurate diagnostic for the detection of trypanosomiasis when the parasitaemia is low.

Acknowledgments
The author would like to thank drh. Okto Rohi, drh. Rambu Peristiwati Pandjukang and the entire staff of the local livestock agency in East Sumba District for great cooperation during the study.

The study was supported by The Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture funded this study in part through the 2019 National Budget.

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