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Molecular detection and hematological profile of *Trypanosoma evansi* in livestock

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Abstract. *Qudsiyati N, Nurcahyo RW, Priyowidodo D, Indarjulianto S. 2024. Molecular detection and hematological profile of* Trypanosoma evansi *in livestock. Biodiversitas 25: 3154-3159. Trypanosoma evansi*, which assaults a variety of hosts and is found around the world, is the causative agent of Surra sickness. The greatest Surra outbreak occurred in Indonesia in 2010-2012, primarily in Sumba, killing about 2,000 animals. The research purpose is to confirm *T. evansi* infection in livestock and determine hematological parameters in naturally infected livestock compared to non-infected (control). This study used molecular and parasitological testing techniques to identify Surra's illness in the field. The buffy coat technique, Polymerase Chain Reaction (PCR), and thin blood smear test are used in the research methodology. Hematological parameters (red blood cell count, hemoglobin concentration, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, red cell distribution width, and white blood cell count) were analyzed. According to the PCR results, out of 222 blood samples, only 1 horse blood sample from Sumba shows a positive result for *T. evansi*. PCR is the most effective diagnostic tool for trypanosomiasis. This was the first time *T. evansi* DNA was discovered in Konga Loko Hamlet, Karuni Village, Sumba, Indonesia, a finding that adds a new dimension to our understanding of the spread and impact of this disease. These findings could potentially lead to the development of more effective diagnostic and control measures, which could significantly benefit the livestock industry.

Keywords: Livestock, PCR, Surra, *Trypanosoma evansi*, zoonosis

INTRODUCTION

Indonesia, as a tropical country, has the ability to breed vectors of numerous parasitic diseases, such as malaria, filariasis, dengue hemorrhagic fever, Surra disease, etc. Surra, which has diverse hosts, is geographically widespread and is potentially zoonotic. Surra disease is caused by *Trypanosoma evansi*, which is transmitted by hematophagous vectors such as *Tabanus* and *Stomoxys* flies. Surra was originally discovered in the horse population in Java in 1897. Lockdowns on or outside the island of Java, as well as the presence of *T. evansi*, spreading vectors, are supporting factors of Surra. The worst cases on Sumba Island occurred between 2010 and 2012, killing almost 2,000 livestock. Trypanosomiasis cases in outbreak locations are more vulnerable to zoonoses because breeders whose communities are in close proximity to livestock pens are more likely to have Surra vectors (Sawitri et al. 2019).

In Indonesia, research on flies is still rare. Therefore, research related to Surra, such as vector research, which plays an important role in the propagation of Surra, needs to be improved. Research on the diversity of vectors with the capacity to transmit Surra will be updated, and various government initiatives will be conducted to prevent the spread of Surra. Information on economic losses due to flies still needs to be improved in Indonesia. Research regarding flies still needs to be done because there is no awareness of the losses caused by vectors. One of the studies related to flies *Tabanus* sp., *T. megalops*, *T. rubidus*, *Stomoxys calcitrans*, *S. sitiens*, *S. indica*, and *Haematobia irritans* are among the tabanid flies and hematophagous muscoids found in Kulon Progo and Bantul, Special Region of Yogyakarta (Qudsiyati et al. 2023). Research on fly diversity in Yogyakarta; Brebes contained (51.2%; 42.3%) *S. calcitrans*, (19.6%; 26.1%) *H. irritans*, (16.9%; 22.5%) *T. rubidus*, (6.9%; 5.4%) *Hippobosca* sp. and (5.4%; 3.6%) *T. striatus* (Nurcahyo et al. 2017). The diversity of flies in Tuatuka Village, Kupang was found to be *Musca domestica*, *Hippobosca equina*, *S. calcitrans*, *Fannia canicularis*, *H. irritans*, and *Tabanus striatus* (Oematan et al. 2019). Research related to vectors needs to be increased because vectors play a very important role in the transmission of Surra cases in Indonesia.

Surra detection on clinical symptoms, parasitological testing, and molecular tests will yield an accurate diagnosis, reducing the spread of Surra cases in the field. Surra is a main disease in camels, equines, and dogs. Without therapy, Surra disease causes abortion and/or death due to non-specific clinical indications such as anemia, loss of weight, and decreased primary output (meat, milk, energy, and fertility) (Desquesnes et al. 2013). Management and control of *T. evansi* infection and the spread of other Surra

to reduce the prevalence and the impact of weight loss in livestock with hematological parameters are biomarkers for anemia, neutrophilia, monocytosis, anisocytosis, and poikilocytosis (Ismail-Hamdi et al. 2022). This disease causes massive losses and is fatal if not detected early by breeders (Ereqat et al. 2020). PCR will enable a crucial tool, enabling more accurate and expeditious diagnosis of *T. evansi* infection in cases of low parasitemia. PCR diagnosis in the field will aid in the early detection and treatment of infections in reservoir animals and bloodsucking vectors present in the area. It is advised to employ vector traps to manage and lower the risk of illness (Javanshir et al. 2023).

Preventing a decline in cattle productivity is a key strategy in the fight against Surra. The government treats, prevents, and controls flies in addition to sanitizing the environment, providing a sense of reassurance and confidence in the battle against Surra instances (Nurcahyo et al. 2017; Nuryady et al. 2019; Oematan et al. 2019). This research, aimed at enhancing our knowledge and epidemiological data on Surra disease, is a significant step towards accurate identification and the best biomarker of hematological parameters for diagnosis and monitoring trypanosomiasis.

MATERIALS AND METHODS

The site selection, ethical approval, and sample collection

The study locations were chosen based on the number of cases (Owino et al. 2021) from September 2022 until December 2023. The Ethical Committee of LPPT UGM No: 00014/04/LPPT/V/2023 has approved this research. A total of 222 different types of blood samples were collected, including 179 cattle blood, 28 buffalo blood, and 15 horse blood.

Blood was collected via the jugular vein/coccygeal vein and was entered into an EDTA tube for microscopical and molecular examination. It was stored in a cool box in the field before being carried to the parasitology laboratory and preserved in the refrigerator at -20°C until laboratory analysis.

Thin blood smear and Giemsa staining

The blood sample was disseminated in the objective glass. The thin blood smear dried for about 5 minutes before being fixed with methanol and stained with Giemsa (pH 7.2) for 30 minutes. It was washed with water and air-dried before being examined under a microscope with a magnification of 400-1000x and oil immersion (WOAH 2021).

Buffy Coat Method (BCM)

Buffy Coat Method (BCM) modified from Chagas et al. (2020). Each blood sample was placed in a microhematocrit tube, sealed with plasticine at one end, and centrifuged for 5 minutes at 10,000 rpm. Under a microscope, the capillary tube was detected near the top of the buffy coat; if there was movement, the side of the capillary tube broke roughly 1 mm below the buffy coat layer. It was transferred on objectives glass slides and covered with an 18 x 18 mm coverslip. The preparation was left for around 2 minutes to allow the blood cells to settle on the slide before being inspected under a microscope at 400x magnification. Following observation, the coverslip was removed, and a thin smear was formed on the objective glass side. The film was then dried, fixed, stained, and inspected as a blood smear.

Hematological studies

A hematology analyzer measured horse blood samples in EDTA tubes. The hematological study included Red Blood Cell count (RBC) (10^{12}/L), Hemoglobin concentration (Hb) (g/dL), Mean Corpuscular Volume (MCV) (fL), Mean Corpuscular Hemoglobin Concentration (MCHC) (g/dL), Mean Corpuscular Hemoglobin (MCH) (pg), Red cell Distribution Width (RDW) (%), and White Blood Cell count (WBC) (10^{9}/L).

DNA extraction

Thermo Scientific GeneJet Genomic DNA Purification Kit for Mammalian Blood Genomic DNA Purification Protocol was modified for blood DNA extraction. To create a homogenous suspension, combine 200 µL of whole blood with 400 µL of lysis solution and 20 µL of proteinase K solution. Carefully mix by pipetting or vortexing the mixture. Use a shaking water bath or incubate the sample at 56°C for ten minutes until cells are totally lysed. Include 200 µL of ethanol (96-100%) and blend using a pipette or vortex. After the lysate is ready, transfer it to a collecting tube and insert the GeneJET Genomic DNA Purification Column into it. Centrifuge the column at 6,000 xg for 1 minute. The collecting tube holding the flow-through solution should be thrown away. Take a fresh 2 mL collection tube and put the GeneJET Genomic DNA Purification Column into it. Add 500 µL of the ethanol-infused Wash Buffer I. Centrifuge at 8,000 xg for 1 minute. Reinstall the purification column in the collection tube after discarding the flow-through. To the GeneJET Genomic DNA Purification Column, add 500 µL of Wash Buffer II (with ethanol added); centrifuge at full speed (12,000 xg) for three minutes. Discard the collection tube containing the flow-through solution and transfer the GeneJET Genomic DNA Purification Column to a sterile 1.5 microcentrifuge tube. Add 200 µL of elution buffer to the center of the GeneJET Genomic DNA Purification Column membrane to elute genomic DNA. After two minutes of room temperature incubation, one minute of 8,000 xg centrifugation is required. Discard the purification column. Purified DNA should be used right away for a downstream application or stored at -20°C. The concentration of DNA (ng/L) was determined using a Nanodrop spectrophotometer set at 260 nm.

PCR assay

DNA amplification with PCR used an ITS *T. evansi* specific primer (ITS1+5.8S+ITS2) with several primer modifications. The amplicon size of the PCR product was 556 bp. The forward primer 5'- `CGT TTG ACA TGG GAG ATG AG`-3' and reverse primer 5-'`GCC TTT CCC ATT TCT CTT CC`-3' were used. The forward and reverse primers were constructed using BioEdit,

PerlPrimer, and PrimerBlast based on the conserved section *T. evansi* nucleotide sequence (accession numbers D89527 and FJ416612) in the GenBank database. The primers anneal in the conserved region (Mohd Rajdi et al. 2021). Previous research provided positive control (Priyowidodo et al. 2023). PCR mixes with sterile distilled water/nuclease-free water were used as a negative control. The PCR was heated for 5 minutes at 94°C, followed by 35 cycles at 94°C for 30 seconds, 30 seconds at 56°C, and at 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. Electrophoresis with florosafe DNA on a 1.5% agarose gel dyed was used to visualize the amplicon DNA.

Sequencing and Basic Local Alignment Search Tool

PCR findings were sequenced at Universitas Gadjah Mada's Integrated Laboratory for Research and Testing. The nucleotide Basic Local Alignment Search Tool (BLAST) was utilized to confirm the sequences obtained from the PCR analysis.

Phylogenetic analysis

Molecular data (sequencing data) from chosen positive PCR amplification products were processed using BLAST and MEGA-X (Kumar et al. 2018). The neighbor-joining method using the Kimura 2 parameter distance matrix was applied to assess the genetic relationship of blood parasites from this research sequence with published sequences.

Statistical analysis

A descriptive study of hematological parameters was performed using Microsoft Excel.

RESULTS AND DISCUSSION

According to the findings of the thin blood smear and Buffy Coat Method (BCM) investigation, (222/0) 0% of blood was positive, or *T. evansi* was not found.

Polymerase Chain Reaction (PCR) results

PCR research results from 222 blood samples, including 179 cattle blood, 28 buffalo blood, and 15 horse blood, such as 36 blood from Pengasih Yogyakarta cattle, 27 blood from Kalibawang Yogyakarta cattle, 10 blood from Brebes cattle, 55 blood from Kudus cattle, 51 blood from Blora cattle, 10 blood from Brebes buffalo, 15 blood from Kudus Buffalo, 3 blood from Sumba buffalo and 15 blood from Sumba horses. It revealed one positive sample out of horse blood samples in the Sumba area with code (K12). The code of M (Marker/DNA ladder), + (positive control), - (negative control), K6, K9, K10, K11, K12, K13, K14, K15 (horse blood samples from Sumba), and K7, K8 (buffalo blood samples from Sumba) are shown in (Figure 1).

Phylogenetic tree

The phylogenetic tree in Figure 2 presents a novel finding: a set of samples (*T. evansi* strain Sumba) from horses is found in the same clade as the *T. evansi* from cattle and buffalo. The out group was chosen *T. equiperdum* and *T. brucei*. This study's *T. evansi* sequence was deposited in the NCBI database, receiving the accession number PQ066108.1.

Hematological parameters

The hematological parameters from blood samples were collected from 11 clinically healthy horses and 1 adult horse naturally infected with *Trypanosoma.* Hematological parameters between horses infected with *T. evansi* and uninfected horses are compared in Table 1.

Discussion

Trypanosoma evansi is the infectious agent that causes trypanosomiasis. It is brought on by these "Surra" trypanosomes, which are mechanically spread by the *Tabanus* and *Stomoxys* (Ramos et al. 2023). The potential zoonotic parasite's vectors are its means of Surra transmission. Surra is not immunized but uses both preventive and therapeutic methods to cure infected animals. Chemotherapeutic drugs include quinapyramine chloride/ quinapyramine sulfate combination, isometamidium chloride, diminazene diaceturate, suramin or melarsomine hydrochloride (Büscher et al. 2019). The prevention and control of vector-borne diseases (flies) are insecticides, smoke release to shield cattle from fly bites, and vavoua or Nzi traps used to catch flies.

Molecular, serological, and parasitological testing were utilized to diagnose Surra. When *T. evansi* was discovered in blood after host blood detection, the parasitological test was the gold standard diagnostic test (Suprihati et al. 2022). A serological test cannot distinguish acute infection and antibodies from prior infections. *T. evansi* can be found in the blood through Polymerase Chain Reaction (PCR) testing, which provides quick and precise result confirmation. The usage of specific primers affects the PCR's success rate. *Trypanosoma evansi* can be found by PCR using the ITS-1 primer (Suprihati et al. 2022).

Giemsa staining revealed that the trypanosomiasis prevalence in Sumba's equine population was 0% (0/211) and CATT 13.3% (28/211) (Nurcahyo et al. 2019). No positive sample was found by the Wet Blood Film (WBF), Micro Hematocrit Centrifugation Test (MHCT), and Giemsa-Stained Blood Smear (GSBS). The overall infection prevalence according to CATT/*T. evansi* and PCR were 7.2% (8/111) and 5.4% (6/111). This case might be related to most endemic hosts experiencing not only persistent infections with low parasitemia but also recurrent infections (Sawitri and Wardhana 2024). Thin blood smears stained with Giemsa 10% revealed that the prevalence of trypanosomiasis in East Sumba was 2.10% (6/286) and that the affected sub-districts were Lewa (3.65%), Waingapu City (1.72%), and Pahunga Lodu (7.4%) (Praing et al. 2023). Positive horses are typically maintained in stables. The incidence of trypanosomiasis in horses at the abattoir in Kelara sub-district, Jeneponto district, was reported to be 2 positive samples (65/2) 3.07% using the Giemsa staining method on thin blood films (Mursalim et al. 2017). Trypanosomiasis causes significant economic loss for breeders, such as lower yield and stunted growth, and if left untreated, it can lead to death.

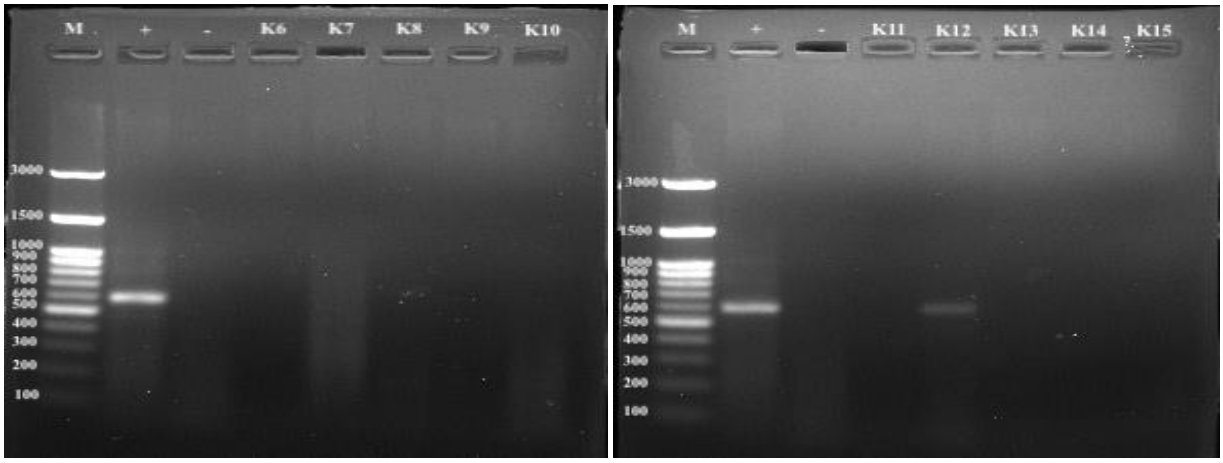

Figure 1. The Polymerase Chain Reaction (PCR) results of Sumba blood sample (K12)

Table 1. Hematological parameters in control/uninfected (n = 11), and *Trypanosoma* naturally infected in horses (n = 1)

Parameters	Reference range (Mohd Rajdi et al. 2021)	*T. evansi* negative (control)	*T. evansi* positive
WBC (10^9/L)	4.0-12.0	7.19±2.46	10.37
LYM (10^9/L)	1.5-5.5	4.10±1.83	6.84
MON (10^9/L)	0.1-0.8	0.17±0.24	0.23
NEU (10^9/L)		1.99±2.12	2.34
EOS (10^9/L)		0.82±0.49	0.81
BAS (10^9/L)		0.10±0.04	0.16
LYM (%)		58.61±21.71	65.9
MON (%)		2.01±2.59	2.2
NEU (%)		20.72±23.54	22.6
EOS (%)		13.58±13.43	7.8
BAS (%)		1.43±0.16	1.5
RBC (10^{12}/L)	6.0-12.0	6.76±1.54	3.62
HGB (g/dL)	11.0-17.0	10.04±2.20	9.8
HCT (%)	35.0-55.0	33.64±9.15	17.51
MCV (fL)	34.0-58.0	49.73±5.92	48
MCH (pg)	26.0-34.0	15.02±1.63	27.1
MCHC (g/dL)	31.0-35.5	30.32±2.58	56.1
RDWc (%)		25.55±4.56	19.4
RDWs (fL)		51.70±8.82	39.8

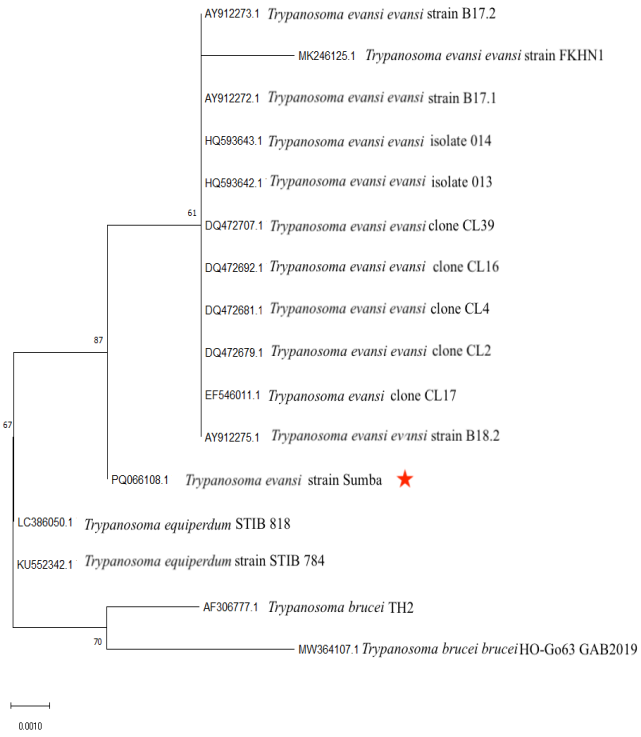

Figure 2. The analysis of phylogenetic tree

The microscope examination or blood smear examination is a straightforward, affordable, and highly specific diagnostic tool, although it is not very sensitive when parasitemia is low. *T. evansi* was found in very high parasitemia when the stained blood film was examined under a microscope (Ahmadi-hamedani et al. 2014). The process of microscopy examination requires a lot of time and effort because each slide must be looked at. Early stages of the illness are too sensitive for a blood smear screening to diagnose. Furthermore, microscopic analysis of blood cannot reliably distinguish between different types of trypanosomes. Hence, molecular testing is the most effective method for identifying species (Patel et al. 2022).

The blood of 222 different livestock animals was tested in this investigation. *T. evansi* infection was not found in the thin blood smear, and Buffy Coat Method (BCM) investigation and one positive sample PCR was found in a horse from Konga Loko Hamlet, Karuni Village, Sumba. Figure 1 depicts positive diagnostic results from a molecular test (PCR). Identification of Surra disease is difficult if only clinical symptoms are used because clinical symptoms such as anemia, weight loss, and decreased production (meat, milk, energy, and fertility) might induce miscarriage and/or death if not treated (Desquesnes et al. 2013). Therefore, identification in this study using molecular tests such as PCR will aid in disease elimination in reservoir

animals and blood-sucking vectors found in the area. This was the first time *T. evansi* DNA was discovered in Konga Loko Hamlet, Karuni Village, Sumba. A positive PCR result indicates that the horse has *T. evansi* infection. However, the PCR test only identifies parasite DNA in the blood, whether the parasite is dead or alive.

The PCR approach can discover more positive samples than the traditional parasitological method. PCR is the most effective diagnostic tool for trypanosomiasis (Setiawan et al. 2021). PCR is a more sensitive technique to diagnose *T. evansi* infection (Patel et al. 2022). When parasitemia is low, PCR is a rapid and reliable diagnostic method for trypanosomiasis detection (Sawitri and Wardhana 2024). The PCR test detects trypanosomiasis significantly more accurately than a Giemsa microscopic analysis of blood smears. The PCR process is extremely sensitive because very small amounts of parasite DNA can be amplified to detectable levels if enough cycles are performed. PCR diagnostics can be utilized in horses or other animals to diagnose trypanosomiasis rapidly and reliably, especially when parasitemia is low (Muieed et al. 2010). In this investigation, PCR diagnosis was more sensitive than thin blood smears and buffy coat techniques in detecting trypanosomiasis.

In the phylogenetic tree in Figure 2, it can be seen that sample K12 (PQ066108.1 *Trypanosoma evansi* strain Sumba) is in the same clade as the *T. evansi* in the host of buffalo or cattle. This is possible because the type of free (extensive) pen in Sumba or horse pens is close to the buffalo or cattle, so when the animal has high parasitemia. The presence of the Surra vector (flies) will make it easier for surrounding animals to be infected with *T. evansi* through the bite of flies that carry parasitic agents (*T. evansi*). Meanwhile, cattle and buffalo, as a reservoir for *T. evansi*, pose a high risk to horses when vector populations are abundant due to the virulence of Surra (Sawitri and Wardhana 2024).

In Table 1, we can observe that hematology parameters from blood samples were collected from 11 clinically healthy horses and 1 adult horse naturally infected with *Trypanosoma*; if *T. evansi* is positive, then there is a decrease in eosinophils, Red Blood Cells (RBC), HGB, HCT, MCV, and RDWc. The controls (negative *T. evansi*) experienced an increase in RBC. This is in line with research showing decreased Red Blood Cell (RBC) levels based on hematological data (Mohd Rajdi et al. 2021; Yamazaki et al. 2022). The RBC, HCT, and HGB/Hb levels were lower in infected horses, indicating the existence of anemia as reference ranged by Mohd Rajdi et al. (2021). Red cell indices such as MCV, MCH, and MCHC are used to determine the kind of anemia. The infected horses in this study had decreased levels of MCV and increased MCH and MCHC, which are signs of microcytic hyperchromic type anemia (Shoraba et al. 2024).

Hematological parameters are good biomarkers of various diseases, such as trypanosomiasis, a parasitic disease caused by *Trypanosoma* species. Hematologic findings in affected horses included normocytic normochromic anemia and erythrophagocytosis with leukocytosis due to lymphocytosis (Rodrigues et al. 2009). The main changes in camels are anemia, neutrophilia, monocytosis, anisocytosis, and poikilocytosis. Anemia is decreased Hb and PCV. Anisocytosis is increased RDW (Ismail-Hamdi et al. 2022). The clinical examinations of infected animals showed signs of loss of appetite, diarrhea, loss of weight with poor body condition, and the paleness of mucus membranes identified anemia. No abnormal clinical signs were observed in the healthy group; they had good body condition on the physical examinations (Eljalii et al. 2015).

In conclusion, out of 222 blood samples, only 1 horse blood sample from Sumba shows a positive result for *T. evansi*. It was detected by PCR, the most effective diagnostic tool for trypanosomiasis. This was the first time *T. evansi* DNA was discovered in Konga Loko Hamlet, Karuni Village, Sumba, Indonesia.

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