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Application of Dried Blood Sample on Whatman Filter Paper for Detection of *Trypanosoma evansi* from Cattle in Central Kalimantan by Internal Trascriber Spacer-1 Polymerase Chain Reaction

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ABSTRACT

*Trypanosoma evans*i is a hemoflagella parasite that infects cattle as agents of Surra. The disease causes great economic losses due to decrease in production and death. Rapid and accurate method for early detection is necessary so that the appropriate control can be implemented. In recent years, PCR has been widely applied for detection of *Trypanosoma* with higher sensitivity. *Trypanosoma*-species-specific-DNA-sequences multi copy can be used as a target parasite identification. Internal transcribed spacer regions 1 (ITS-1) is a useful marker for descriminating trypanosomes species. The aims of this study was to detect *T. evansi* in cattle from Central Kalimantan using ITS-1 PCR. A total of 44 bovine blood samples were colleted using Whatman filter paper from Lamandau District of Central Kalimantan Province. PCR was carried out using ITS-1 primers on DNA templete that was isolated from dried blood. The results showed that 27 out of 44 samples (61.4%) were positive for *T. evansi*. Lamandau District was suspected to be endemic area of Surra and therefore requires a special attention.

Key Words: *Trypanosoma evansi*, Internal Transcriber Spacer-1, PCR

INTRODUCTION

Trypanosoma evansi, a flagellated kinetoplastid haemoprotozoa, is the most prevalent pathogenic trypanosome in Asia, Africa, South, and Central America (Laha & Sasmal 2008; Desquesnes et al. 2009). It causes a major problem and known as 'Surra'. The parasite infects a wide range of domestic and wild animals owing to its ability of to be mechanically transmitted by biting flies such as *Tabanus* and *Stomoxys* (Luckins 1988; Desquesnes et al. 2009). Several cases of human trypanosomiasis by *T. evansi* have been reported recently, and therefore it is a new emerging disease in human. In Indonesia, a severe Surra outbreak occurred in Sumba in 2010-2012 causing the deaths of more than 2,000 head of livestock (Ditjennak 2012). In 2011 outbreaks of Surra in Dharmasraya districts of West Sumatra Province were also reported (Diseases Investigation Centre Bukittinggi 2014). In addition, Surra cases in South and East Kalimantan were increasing from 2011-2013 but there was a few Surra case in Central Kalimantan (Disease Investigation Centre Banjarbaru 2014).

Effects of the infection in different geographical locations vary according to the strain of parasites and species of the host (Desquesnes et al. 2013a). Clinical signs are variable and non-specific (Desquesnes et al. 2013b). In Indonesia, buffalo is apparently more susceptible than bovine (Payne et al. 1991). The disease may manifest as an acute or chronic form, and in the latter case could persist for several months, possibly years. Accutely infection in cattle or buffalo usually end in death, whereas the chronic form result in unproductive animas (Partoutomo 1996). The disease in bovines is characterized by severe anaemia, nervous complications, weight loss, reduced productivity, infertility and abortion and death in some animals during the early phase of the disease (Juyal 2002). The

disease also interferes with immune system and causes immunosuppression paving the way for bacterial and viral infections (Holland et al. 2003; Jittapalapong et al. 2009; Singla, Juyal, and Sharma 2010).

The economic impact of surra, result from a wide range of pathological expression (e.g. weight loss, milk yield reduction, draught power reduction, immune system impairment, abortion, infertility, mortality). Reid (2002) is often under-estimated as a result of an insufficient detection of the parasite. The inability to detect *T. evansi* infections may also increase the risk of spreading through the movement of healthy carriers (Luckins 1988).

Clinical symptoms of this disease are not pathognomonic so that the clinical diagnosis relies on blood tests to detect parasites and do serological tests such as ELISA and CATT/*T. evansi* (Dia et al. 1997) including to perform a molecular test by Polymerase Chain Reaction (OIE 2012). The PCR method has a high sensitivity and specificity. In addition, the method is able to detect down to 1-10 trypanosomes/ml of blood and distinguish different species of Trypanosomes (Davila et al. 2003; Desquesnes et al. 2001). Variations in the sensitivity and specificity of the diagnosis by PCR depends on the primer used and the number of repetitions of the target sequence in the trypanosome genome (Njiru et al. 2005; Pruvot et al. 2010; Ahmed et al. 2013). In some determining molecular markers used to detect, differenciate and study *Trypanosoma* species, the internal transcribed spacers (ITS) are versatile genetic markers and have been used for phylogenetic analysis, evaluation of the evolutionary process, and for taxonomic identities (Powers et al. 1997).The ITS are located between the repeating array of nuclear 18S, 5.8S, and 28S ribosomal RNA genes; a locus with 100-200 copies per genome (Hernandez et al. 1993). Generally, the ITS region has been used extensively in characterization of *T. evansi* (Beltrame-Botelho et al. 2005; Khuchareontaworn et al. 2007; Areekit & Singhaphan 2008). The ITS1 region has been successfully used as target for PCR-based detection to discriminate trypanosomes species (Salim et al. 2011). Specific PCR product length of ITS 1 region corresponding to each Trypanosoma species, which was the base of differentiation among *Trypanosoma* species. For example, *T. congolense* savannah, an ITS1 PCR product is 700, 400 bp for *T. simiae* and 250 bp for *T. vivax, T. evansi*, and *T. brucei* subspecies was the same size, 480 bp (Salim et al. 2011).

Surveillance for trypanosomiasis in Indonesia sometimes is problematic because of the need to work in remote area where provision of a cold-chain for traditional blood storage is difficult. Blood sampling is usually done with a blood-EDTA collection and stored cool. This method is less practical when applied to blood sampling in remote areas with minimal facilities and away from the laboratory diagnosis. Storage of dried blood on filter paper at room temperature could be a more practical option. Chompoochan et al. (2007) stated that blood samples on filter paper (dried blood) can be use for PCR analysis to detect *T. evansi* DNA. The use of filter paper besides faster also convenient and easier to delivery especially in remote areas (Handy et al. 2004; Dargantes et al. 2010). Similar results were reported by Ekawasti et al. (2014) that the storage of flies blood on filter paper did not affect the *T. evansi* DNA so it can be used for multiplex PCR analysis.

The present study was designed to investigate the case of Surra in cattle at the District of Lamandau of Central Kalimantan province by using ITS-1 PCR.

MATERIAL AND METHODS

Sample collection

Forty four blood samples were collected from cattle at Lamandau District of Central Kalimantan Province. Blood was collected from the jugular vein from each cattle using 5 ml disposable syringe for blood spotted on Whatman filter paper and the rest was placed in 5 ml plastic tube for other analysis.

Blood spotted on Whatman filter paper

One drop of fresh blood (5 µl) was placed into the Whatman filter paper no. 1 (Whatman Ltd, England) and was allowed to dry in the shade. Dried paper were later put separately into a tightly closed small plastics bag individually and were transported to the laboratory and then kept at refrigerator until DNA extraction was done.

DNA extraction

Extracted genomic DNA of 44 samples were conducted by using Genomic DNA Mini Kit (Geneaid). A piece of 5×5 mm dried blood in Whatman filter paper was placed into 1.5 ml eppendorf tube. The paper was incubate with 300 ml lysis buffer at 60°C for 10 minutes. After blood dissolved, paper was taken and continue to the next step DNA extraction as manufactured guidance. Genomic DNA was stored at -20 until use.

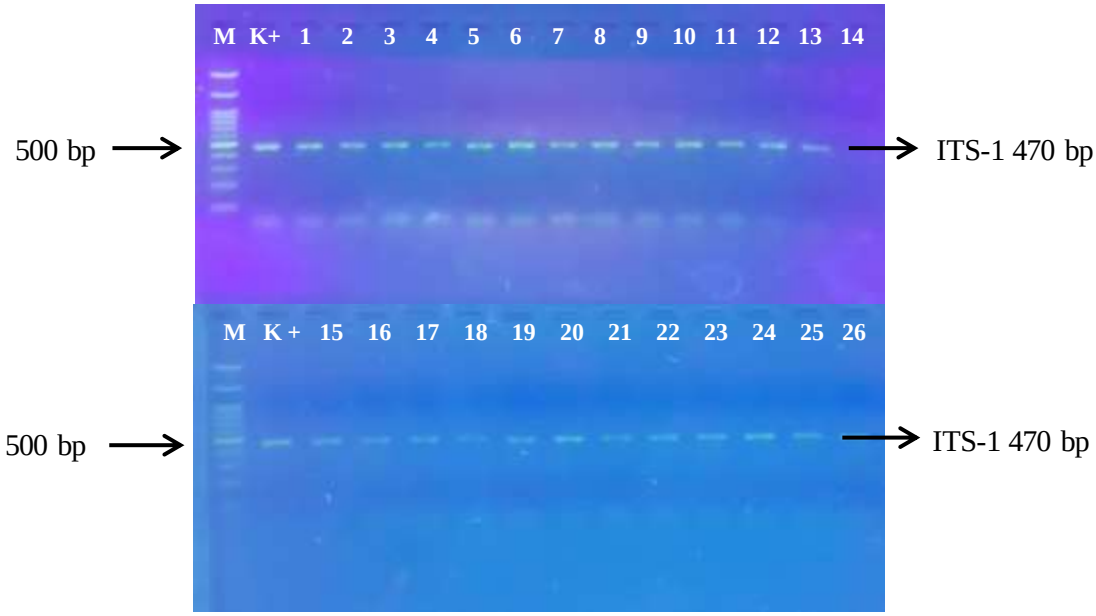
PCR amplification

PCR amplifies the ITS1 region of the rDNA gene by using forward primers ITS1F:5'CCGGAAGTTCACCGATATTG'3 and reverse primer ITS1R:5'TGCTGCGTTCTTCAACGAA'3. The 480 bp PCR product was amplified using KAPA 2G Fast PCR kit (KAPA) in a 25 µl total volume. Each reaction included 5 µl (5 unit/µl) 2 G Fast Master Mix, 0.1 (5 unit/µl) KAPA 2G Fast DNA polymerase, 0.5 µl (10 µM/dNTP) dNTP mix; 2 µL (10 mM) of each primer, 2 µl (50ng/µl) extracted DNA; 17.44 µl RNase-free water. Thermocycling profile started with initial hold for 3 min at 95°C, followed by 35 cycles of 95°C for 10 sec, 58°C for 10 sec, and 72°C for 15 sec and final extension step of 10 min at 72°C. Five microlitres of PCR products of approximately 470 bp in size was analysed by using 1.5% agarose gel electrophoresis in (Tris-acetate EDTA) buffer and stained using fluorosafe (Geneaid), before being visualized under UV translluminator.

RESULTS AND DISCUSSION

PCR amplification was carried out on targeting 470 bp ITS-1 fragmen of 44 cattle blood collected from Lamandau District of Central Kalimantan Province (Figure 1). The result indicated that 33 of 44 (61.4%) samples were positive for ITS-1. These result showed high case Surra in that district.

In the survey area we didn't find any clinical sign of Surra and no case of animal death. The absence of pathognomonic clinical signs, with the evasive nature of *T. evansi*, especially in chronic infections, make detection of surra in cattle very challenging (Nantulya 1990; Ngaira et al. 2003). The disease pathology varies with the host, chronic and latent disease occure in ruminants (Kundu et al. 2013).



Lane M: Molecular size marker 100 bp; Lane K+: Positive control; Lane 1-13,15-26: Positive samples; Lane 14: Negative sample

Figure 1. Agarose gels of PCR products of 470 bp fragment obtained with ITS-1 from cattle blood sample from Lamandau district of Central Kalimantan Province

According to the farmers, the cattle have been raising in the area implementing the integration cow-palm oil program. Surra has not been reported in Lamandau District. Nevertheless this district is very closed to West Kotawaringin District which is endemic Surra area. Distribution of Surra disease is influenced by local topography and livestock management (Mastra 2011). Surra has multiple and complex means of transmission, which vary in terms of relative significance depending on the hosts and the geographical area. Indeed, *T. evansi* is transmitted in several ways, via biting insects, sucking insects, and vampire bats, transmission can also be vertical, horizontal, iatrogenic, and per-oral with various epidemiological significances, depending on the season, the location, and host species (Desquesnes et al. 2013a).

In this area, livestock are kept in a certain pasture near oil palm plantations. There is no contact between cattle in this location with other cattle from different locations. Suspected cattle have been infected from their place of origin. According to the farmer's information cattle derived from Kotawaringin Barat (Central Kalimantan) and Malang (East Java), so far West Kotawaringin known as Surra endemic (DIC Banjarbaru, unpublised). The existence of hematofagus flies like T*abanus* and *Stomoxys* sp in the area might to spread Surra. As many cases remain undetected and untreated even after the completion of parasitological investigations. This situation is disappointing for the infected individual as well as the other animals in the herd. These individuals might remain being the reservoir of the infection and contribute to further distribution of the disease in the animal population.

However, because the cattle were on good condition, their immunity did not show any clinical symptoms. According Desquesnes et al. (2013) Trypanosomosis due to *T. evansi* has long been considered of the cattle, they as a mild, chronic, or asymptomatic disease in Bovinae (*Bos, Bubalus, Syncerus,* and *Poephagus*). The immune response to *T. evansi* infection in buffaloes and cows are relatively better than in horses so that ruminants are more resistant to Surra disease. The chronic incidence of the disease showing no clinical symptoms but later the animals become useless (Partoutomo 1996). While the sub-clinical

Surra is generally not easy to diagnose based on clinical examination and conventional laboratory tests, as a result the incidence of the disease tends to undetected and neglected without any receiving proper treatment (Mastra 2011).

The results of this study indicated that Surra at Lamandau District need serious attention. Although this district is not an endemic area but this area bordered by the district of West Kotawaringin which Surra is endemic. So that livestock transportation between the two district can caused the introduction of Surra in Lamandau district. According to Partoutomo (1996) if the cattle are not on good conditions and the hematophagus flies population are abundant, it would be potential Surra transmission showing clinical symptoms and causing epidemics. Surra control in this region is urgently needed to prevent the spread to another farm site. Naturally endemic areas of *T. evansi* characterized by the outbreak with high mortality due to stable enzootic *T. evansi* in some areas in Indonesia (Payne et al. 1991). However, to make sure where *T. evansi* isolate in Lamandau distict come from need further analyzed by using microsatellite marker. According to Sawitri (2016) the origin of isolates that causing Surra outbrake can be traced by using molecular analysis with microsatellite markers. Sawitri (2016) has succesfully identify and trace *T. evansi* isolates that cause outbreak at Pandeglang in 2014 with eight microsatellite loci markers.

Surveillance for *T. evansi* in Indonesia sometimes face some problem due to difficulty in collecting suitable samples from potential host species in remote areas. There is limited access to many of these areas and no permanent facilities for storing samples that require refrigeration, which means that sample collection is an expensive operation and is only done infrequently by trained operators. By using a spotted blood on filter paper method, this study provides several advantages. Firstly, the method is practically applied in the field where refrigeration facilities are limited. Secondly, the samples can be stored at room temperature, so that the transportation of samples will be easier, especially for samples collected from remote areas which have long distance from laboratory. Thirdly, the method is easier handling and can be sent by courier to another laboratory. According to Chompoochan et al. (2007) that the blood spotted in filter paper method of preservation is very easy to implement and suitable for use in epidemiological surveys for trypanosomiasis control programs in endemic areas.

CONCLUSION

Surra livestock has occured in the Lamandau District, Central Kalimantan Province. Mass treatment is needed to treat, prevent, anticipate and control of potential outbreak. PCR positive status should be treated using antitrypanosoma drugs with recommended dose and negative livestock could be given a prophylactic dose. Surra examination need to be recommended as one of the delivery requirements of livestock from endemic or outbreaks areas to nonendemic area.

REFERENCES

Ahmed HA, Picozzi K, Welburn SC, MacLeod ET. 2013. A comparative evaluation of PCR-based methods for species-specific determination of African animal trypanosomes in Ugandan cattle. Parasit Vectors. 6:316.

Areekit S, Singhaphan P. 2008. Genetic diversity of *Trypanosoma evansi* in beef cattle based on internal transcribed spacer region. Infect Genet Evol. 8:484-488.

Beltrame-Botelho I, Gaspar-Silva D, Steindel M, Davila A, Grisard E. 2005. Internal transcribed spacers (its) of *Trypanosoma rangeli* ribosomal DNA (rDNA): A useful marker for inter-s DNA (rDNA): A useful marker for inter-specific differentiat. Infect Genet Evol. 5:17-28.

Chompoochan K, Mohkaew S, Ngamjiaue N, Sarataphan. 2007. Application of dried blood sample on FTA® paper for detection of *Trypanosoma evansi* by PCR. In: Developing methodologies for the use of polymerase chain reaction in the diagnosis and monitoring of trypanosomosis. Vienna: Technical Support of the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture. p. 103-108.

Davila A, Herrera H, Schlebinger T, Souza SS, Traub-Cseko YM. 2003. Using PCR for unraveling the cryptic epizootiology of livestock trypanosomosis in the Pantanal. Braz Vet Parasitol. 117:1-13.

Desquesnes M, Biteau-Coroller F, Bouyer J, Dia ML, Foil L. 2009. Development of a mathematical model for mechanical transmission of trypanosomes and other pathogens of cattle transmitted by *Tabanids*. Int J Parasitol. 39:333-346.

Desquesnes M, Dargantes A, Lai D, Lun Z, Holzmuller P, Jittapalapong S. 2013. *Trypanosoma evansi* and Surra: A review and perspectives on transmission, epidemiology and control, impact, and zoonotic aspects. Biomed Res Int. 2013:321237.

Desquesnes M, Holzmuller P, Lai D, Dargantes A, Lun Z, Jittaplapong S. 2013. *Trypanosoma evansi* and surra: A review and perspectives on origin, history, distribution, taxonomy, morphology, hosts, and pathogenic effects. Biomed Res Int. 2013:194176.

Desquesnes M, McLaughlin G, Zoungrana A, Davila A. 2001. Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. Int J Parasitol. 31:610-614.

Dia M, Van-Meirvenne N, Magnus E, Luckins A, Diop C, Thiam A, Jacquiet P, Harmers D. 1997. Evaluation of four diagnosis tests: blood smears, CATT, IFAT and ELISA-Ag in a study of the epidemiology of *Trypanosoma evansi* camel trypanosomosis in Mauritania. Revue Elev Med Vet Pays Trop. 50:29-36.

Disease Investigation Centre Banjarbaru. 2014. In: International Seminar on Biting Flies and *Trypanosomes* and the Role One Health in Animal Health. Unpublised.

Diseases Investigation Centre Bukittinggi. 2014. In: International Seminar on Biting Flies and *Trypanosomes* and the Role One Health in Animal Health. Unpublised.

Ditjennak. 2012. Pedoman pengendalian dan pemberantasan penyakit Trypanosomiasis (Surra). Jakarta (Indonesia): Direktorat Jenderal Peternakan.

Ekawasti F, Sawitri D, Wardhana AH. 2014. Perbandingan metode penyimpanan darah vektor Surra (lalat Haematophagus) untuk analisis *multiplex polimerase chain reaction*. Dalam: Pamungkas D, Widiawati Y, Noor SM, Purwantari ND, Widiastuti R, Brahmantiyo B, Herawati T, Kusumaningsih A, Handiwirawan E, Puastuti W, penyunting. Teknologi Peternakan dan Veteriner Mendukung Pertanian Bioindustri Berkelanjutan. Prosiding Seminar Nasional Teknologi Peternakan dan Veteriner. Malang, 12-14 Agustus 2014. Bogor (Indonesia): Puslitbangnak. hlm. 209-217.

Hernandez R, Martinez-Calvillo S, Hernandez-Rivas S, Gomez E. 1993. *Trypanosoma cruzi* ribosomal RNA genes: A review. Biol Res. 26 109-114

Holland W, Do TT, Huong NT, Dung NT, Thanh NG, Vercruysse J, Goddeeris BM. 2003. The effect of *Trypanosoma evansi* infection on pig performance and vaccination against classical swine fever. Vet Parasitol. 111. 115-123.

Jittapalapong S, Pinyopanuwat N, Inpankaew T, Sangvaranond A, Phasuk C, Chimnoi W, Kengradomkij C, Kamyingkird K, Sarataphan N, Desquesnes M, Arunvipas P. 2009.

Prevalence of *Trypanosoma evansi* infection causing abortion in dairy cows in Central Thailand. Kasetsart J. 43:53-57.

Juyal P. 2002. Newer perspectives in the diagnosis and control of trypanosomosis (Surra) in domestic livestock in India. In: Department of Veterinary Parasitology, College of Veterinary Science PAU.

Khuchareontaworn S, Singhaphan P, Viseshakul N, Chansiri K. 2007. Genetic diversity of trypanosoma evansi in buffalo based on internal transcribed spacer (ITS) regions. J Vet Med Sci. 69:487-493.

Kundu K, Tewari AK, Kurup SP, Baidya S, Rao JR, Joshi P. 2013. Sero-surveillance for surra in cattle using native surface glycoprotein antigen from *Trypanosoma evansi*. Vet Parasitol. 196:258-64.

Laha R, Sasmal NK. 2008. Characterization of immunogenic proteins of *Trypanosoma evansi* isolated from three different Indian hosts using hyperimmune sera and immune sera. Res Vet Sci. 85:534-539.

Luckins AG. 1988. *Trypanosoma evansi* in Asia. Parasitol Today. 4:137-142.

Mastra I. 2011. Seroprevalensi Trypanosomiasis di Pulau Sumbawa, Provinsi Nusa Tenggara Barat. Bul Vet. 23:131-138.

Nantulya VM. 1990. Trypanosomiasis in domestic animals: The problems of diagnosis. 9:357-367.

Ngaira J, Bett B, Karanja S, Njagi E. 2003. Evaluation of antigen and antibody rapid detection test for *Trypanosoma evansi* infection in camels in Kenya. Vet Parasitol. 114:131-141.

Njiru N, Constantine C, Guya S, Crowther J, Kiragu J, Thompson R, Dávila A. 2005. The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. Parasitol Res. 95:186-192.

OIE. 2012. *Trypanosoma evansi* infection (SURRA). Rome (Italy): FAO.

Partoutomo S. 1996. Patogenesis *Trypanosoma evansi* pada kerbau yang diberi ransum bermutu tinggi dan rendah. J Ilmu Ternak Vet. 2:137-144.

Payne RC, Sukanto IP, Djauhari D, Partoutomo S, Wilson AJ, Jones TW, Boid R, Luckins AG. 1991. *Trypanosoma evansi* infection in cattle, buffaloes and horses in Indonesia. Vet Parasitol. 38:109-119.

Powers T, Todd TC, Burnell AM, Murray PCB, Fleming CC, Szalanki AL, Adams BA, Harris TS. 1997. The internal transcribed spacer region as a taxonomic marker for nematodes. J Nematol. 29:441-450.

Pruvot M, Kamyingkird K, Desquesnes M, Sarataphan N. 2010. Veterinary parasitology a comparison of six primer sets for detection of *Trypanosoma evansi* by polymerase chain reaction in rodents and Thai livestock. Vet Parasitol. 171:185-193.

Reid SA. 2002. *Trypanosoma evansi* control and containment in Australasia. Trends Parasitol. 18:219-224.

Salim B, Bakheit MA, Kamau J, Nakamura I, Sugimoto C. 2011. Molecular epidemiology of camel trypanosomiasis based on ITS1 rDNA and RoTat 1. 2 VSG gene in the Sudan. Parasit Vectors. 4:31.

Sawitri DH. 2016. Virulence study of *Trypanosoma evansi* isolates from Indonesia and identification of molecular marker based on microsatellite DNA and cytokine profile analyses in mice (*Mus musculus*) [Thesis]. Jakarta (Indonesia): Universitas Indonesia.

Singla LD, Juyal PD, Sharma NS. 2010. Immune responses to haemorrhagic septicaemia (HS) vaccination in *Trypanosoma evansi* infected buffalo-calves. Trop Anim Health Prod. 42:589-95.