Detection of T. evansi Using Parasitological, Serological, and Biological Test in Cattle and Buffalo at Surra Endemic Area (District of Pemalong and Brebes), Central Java

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ABSTRACT

Hemoparasitic diseases like Surra are present as major constraints to the development of the livestock production in developing countries such as Indonesia. Definitive diagnosis according to the demonstration of the parasites in the blood are not always successful as the level of parasitaemia is often low and fluctuates during the chronic stage. Besides, the clinical sign of Surra suspected animals is usually not specific in endemic area. District of Pemalong and Brebes, are the areas with the biggest population buffalo in Central Java which endemic of Surra. The aim of the study was to assess the efficacy of five diagnostic techniques by using Wet Blood Film (WBF), Microhematocrit Centrifugation Test (MHCT), Giemsa Stain Thin Blood Smear (GSBS), CATT/T. evansi, and Mice Inoculation Test (MIT) in natural host of Surra in endemic area. Fifty nine buffalo and thirty three beef cattle from traditional farm raised semi intensively system was used in the study. Blood samples were collected from jugular vein of the livestock and applied for those five types of test The result of the study showed that 10 samples (9.8%) of buffalo were positive microscopically test by WBF and GSBS, and MIT. Examination by MHCT and CATT/T. evansi show 11.2 and 46% positive, respectively. The number of parasites found in the blood smears was relatively low. The order of decreasing diagnostic efficacy during this study was found as: CATT/T.evansi (46%) > MHCT (11.6%) > GSBS (9.8%) = WBF (9.8%) = MIT (9.8%). Detection microscopically by using MHCT and serology by CATT/T. evansi can be applied together in order to obtain a powerful diagnostic conclusion for T. evansi.

Key Words: Trypanosoma evansi, Parasitological Test, Serological Test, Biological Test

INTRODUCTION

Parasitic diseases are the major limitation to the development the livestock industry as they globally affect human health, trade and economy (Razzaq et al. 2015). Haemoparasitic disease is one of the major constraints of livestock industry in developing countries (Shahzad et al. 2012). Blood protozoa especially Trypanosoma evansi has wide geographical distribution among pathogenic blood protozoa found in tropical and subtropical regions (America, Africa, Asia, and Eropa) (Konai et al. 2009). Indonesia is one of endemic countries of South-East Asia (Luckins 1988). T. evansi as a cause of Surra in many developing countries of the world, affects a wide range of wild species and livestock population (horse, cattle, buffalo, camel) (Sengupta et al. 2010). In water buffalo, Surra is a chronic disease characterized by weight loss and infertility including abortion (Luckins 1988; Davison et al. 1999). Water buffaloes and cattle are important power for rice cultivate, meat and milk production and as investment of small holder
farmers (Davison et al. 1999). The costs of Sura are connected with mortalities, abortions, decreased meat and milk production, control measures, losses of potential production, and cattle trade restrictions (Reid 2002).

Pemalang and Brebes are the two districts with the biggest population buffalo in Central Java which T. evansi have been reported endemic in there. They have 8825 and 7725 head buffalo population respectively. It is estimated that 25% total buffalo populations in Central Java exist in the two districts. The remaining population are spread in 33 other districts in Central Java (BPS Jateng, 2016). In 1995, survey in buffalo from Pemalang and Brebes showed seropositif of Sura (Davison et al. 2000). Until now that two district are still Sura endemic area (Suryanto & Parmini 2016).The incidence of Sura in buffalo in Central Java is 6.3% (67 positive from 1063 samples (Suryanto & Parmini 2016). However, the diagnosis of T. evansi infection sometimes is problematic, because of its non-specific clinical manifestations in endemic areas.

Parasite detection techniques by examination of GSBS are suitable in acute cases, when the parasitaemia is high, but not always reliable during the chronic stage of infection when the level of parasitaemia remains low and fluctuates (Laha & Sasmal 2009). Serological assays for the detection of circulating antibodies have high validity, economical and applicable at large scale screening (Lejon et al. 2003). CATT/T. evansi is one of serological tests for T. evansi but not suitable for active case detection (Buscher 2014). The method is sensitive and an easy test which can be performed under field conditions for detection antibodies against T. evansi in domestic animals (Gutierrez et al. 2000). Assessed with CATT/T.evansi showed higher seroprevalence than molecular prevalence but it is not unexpected reasons as cannot distinguish current or chronic infection as detectable level of antibodies can persist for 2-22.6 month after trypanocidal treatment (Eyob & Matios 2013). CATT/T. evansi is which many cases remain undetected and untreated after the completion of parasitological investigations. This situation is disappointing as the infected individual might maintain as the reservoir and contributes to further spread of the disease in the animal population. In more chronic cases generally when the parasitaemia is low, method of Microhematocrite Centrifugation Test as well as Mouse Inoculation Test (MIT) are required. In case of healthy carriers (animals without clinical signs), parasites are rarely observed and mouse inoculation gives the best results (OIE Terrestrial Manual 2012)

True incidence of Sura, therefore, are commonly underestimated in studies that use only parasitological tests. Therefore, in the present study, we assess five alternative methods of diagnosis of suspected cases of Sura using GSBS, WBF, MHCT, CATT/T. evansi and MIT for the detection of Sura in cattle and buffaloes. This study was conducted on samples from Pemalang and Brebes district were suspected of T. evansi infection as observed in endemic areas. Sura suspected animals which the clinical sign is usually not specific in endemic area remains a main problem as reservoir animal if the livestock distributed to non endemic area (Sawitri 2016). There is a need to detect parasite in livestock before being distributed to another area especially to non endemic area. The prevalence of the disease in endemic area of Sura is necessary in order to define appropriate control programmes against the disease.
MATERIAL AND METHODS

Survey area

Survey area was carried out in Surra endemic area in Central Java (Pemalong and Brebes) in 2017. The livelihood of farmer in these areas is a mixed farming system (crop-livestock production). Buffalo and beef cattle used in this study were raised semi intensively system in traditional farm.

Field samples

One hundred and two buffalo and beef cattle serum and heparinized blood samples were collected from smallholder farmer around the area of Pemalong and Brebes. Blood samples were collected from jugular vein of cattle and buffalo using vacutainer tubes and venoject needles. The tubes were labeled and the blood was allowed to clot over night at room temperature and the serum was separated. All sera and blood were kept at -20°C until uses for the analysis. This study was approved by the Ethics committee: Balitbangtan/BBLitvet/Rm/01/2017.

Examination protocol

Wet blood films(WBF)

A small drop of blood (2-3 µl) was place on a clean glass slide and placed over it a cover-slip to spread the blood as a monolayer of cells. Examine by light microscopy (200×) to detect any motile trypanosomes. Improved visualisation can be obtained with dark-ground microscopy (200-400×). The sensitivity of this method is low, approximately 10 trypanosomes per µl, which is frequent in early or acute infections only (OIE 2012)

Giemza Stained Thin Blood Smear (GSBS)

A small drop of EDTA blood (2-5 µl) was placed at one end of a clean microscope slide and draw out a thin film. Air-dried and fixed in methyl alcohol for 2 minutes and allowed to dry. The smears are Giemsa staining in (1:10 Giemsa and PBS, pH 7.2 ) for 30 minutes then washed the slide in running water and dried. Examine at a magnification of 1000× with oil immersion (OIE 2012).

Microhematocrit Centrifugation Test (MHCT)

Blood collected from jugular vein of the cattle/buffalo was immediately examined by MHCT test in which a capillary tube was filled up to 60 µl and centrifuged for 5 minutes in micro-centrifuges at 12000 g. Subsequently, the capillary tubes were applied in a special holder and were examined microscopically at magnification of ×10×10 at the buffy coat to look for live parasites ( Reide et al. 2001).

Card agglutination test for T. evansi (CATT/T. evansi)

The test CATT/T. evansi, a rapid direct agglutination test, which used freeze-dried trypanosomes of T. evansi VAT RoTat 1.2. Approximately 45 µl of the antigen was
poured into the circular reaction zones of the supplied plastic card and mixed with 25 µl of the test sera according to the manufacturer’s instructions. The test was checked with positive and negative controls before the whole samples were tested (Bajyan & Hamers 1998; Verloo et al. 2000) The antigen/serum mixture was mixed and spread properly using a clean plastic stirring rod to approximately 1mm from the rim of the test area and the stirring rod was wiped with sterile filter paper after each use. The card was agitated for 5 minutes at 70 rpm after positioning on a flat bed of electric orbital rotator. After five minutes, the results were read before removing the card from the rotator. A reaction was considered positive when agglutination is visible with the naked eye. Cattle/buffalo positive for anti-trypanosome antibodies by CATT showed different degrees of agglutination graded as +++ (very high positive), ++ (high positive), + (moderate positive), ± (mild positive) and – (negative).

**Mice Innoculation Test (MIT)**

Heparinised blood (0.5 ml) was inoculated intraperitoneally into two DDY mice. The inoculated mice were tail bled and a wet blood film was prepared and examined after every 48 hours to detect parasitaemia. Mice were checked at periods of 2 weeks. Virulence depends on the strain of trypanosomes and the strain of mice (4 -14 days) (OIE Terrestrial Manual 2012)

**Assessment of diagnostic efficacy**

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

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\text{Positivity} = \frac{\text{Number of positive samples given by particular diagnostic}}{\text{Total no suspected cases}} \times 100
\]

**RESULTS AND DISCUSSION**

A total of 102 animals (59 buffaloes and 43 cattle) from two districts of Central Java Province (Pemalang and Brebes) were examined by Wet Blood Film, GSBS, MHCT and MIT to determine the prevalence of the disease. Examination by Wet blood film showed long slender trypanozoon parasites and active movement. The morphological features of *T. evansi* observed in fresh blood samples, has the characteristics of long slender trypanozoon parasites, small size, free flagellum, active movements but limited displacements in the microscope field with visible undulating membrane (Desquesnes et al. 2013). The examination by microscopically (WBF, GSBS) and biologically (MIT) have the same result which detected 9.8% positive in buffaloes and no samples positive in cattle. This finding in GSBS method has similar result with study by Laha et al. (2009) which detected 5.3, 9.4, and 40.6% infections in cattle, buffalo and horses by examination of GSBS. Using MHCT examination in suspected cases of trypanosomiasis in buffaloes revealed the presence of *T. evansi* with efficacy detected 11.8% and 0% infections in buffaloes and cattle respectively (Table-1). This finding was different from earlier reported by Hollanda (2001) found the case sensitivity of the buffycoat technique (BCT) to be 38.6%. The buffy coat technique detected more number of cases of *T. evansi* infection compared with giemsa stained blood smears examination. It could attribute to
the reason that in most of the hosts in endemic area, they are generally have chronic infections with low parasitaemia and in such conditions, concentration methods like MHCT become necessary (Singh et al. 2017). Dwivedi (2017) stressed the importance of MHCT for the identification of subclinical or carrier state of T. evansi infection in bovines.

Parasitological methods used in the diagnosis of T. evansi considered easy, rapid and economic. However, they are not sufficient to detect all trypanosome infected animals, especially in case of low parasitaemia and also in the chronic form of the disease (Ahmed 2008).

The Wet blood film is a technique which permits detailed morphological studies and identification of the Trypanosoma species, but it has very low sensitivity (it can only detect parasitaemia >500,000 trypanosomes/ml of blood). In this research MHCT have better result than another third tests (WBF,GSBS and MIT) (Table 1). The samples positive for WBF, GSBS and MIT were also positive in MHCT but out of 10 samples, 2 samples positive by MHCT test were not detected by microscopic examination and MIT. This finding indicated that MHCT had the higher efficacy. In this study, the MHCT test was more sensitive than MIT, probably due to the mice infestation carried out directly from positive buffalo blood not with a buffy coat, thus trypanosome might not grow because the parasite concentration was too low. Sensitivity inoculation in mice can be increased by injecting the buffy coat (Monzon et al. 1990). This method can detect 1.25 T. evansi/ml blood (Reid et al. 2001).

**Table 1.** Examination result of WBF, MHCT, GSBS and MIT for T. evansi

<table>
<thead>
<tr>
<th>District</th>
<th>Sub-district</th>
<th>Animal species</th>
<th>No samples</th>
<th>T. evansi examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pemalang district</td>
<td>Pemalang</td>
<td>Buffalo</td>
<td>37</td>
<td>5 5 5 5</td>
</tr>
<tr>
<td></td>
<td>Taman</td>
<td>Beef cattle (PO)</td>
<td>12</td>
<td>- - - -</td>
</tr>
<tr>
<td>Brebes district</td>
<td>Bantarkawung</td>
<td>Buffalo</td>
<td>22</td>
<td>5 7 5 5</td>
</tr>
<tr>
<td></td>
<td>Tonjong</td>
<td>Beef cattle (Jabres)</td>
<td>31</td>
<td>- - - -</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>102</strong></td>
<td><strong>10(9.8%) 12(11.8%) 10(9.8%) 10(9.8%)</strong></td>
</tr>
</tbody>
</table>

The conventional methods of MHCT and MIT are recommended method by OIE besides PCR (OIE Terrestrial Manual 2012) to know the existence of parasites in the host. MHCT technique can detect about 50-200 trypanosoma / ml blood (Desquesnes & Tresse 1996). In buffalo, the diagnostic sensitivity of mouse inoculation was 78% similar to PCR (Holland et al. 2001).

The result of CATT/T. evansi test showed that out of 102 examined cattle serum 46 (46%) heads found to be seropositive to T. evansi with different grade of agglutination (Tabel 2). This examination detected 8.8; 17.6; and 19% antitypanosoma antibody in buffaloes from Pemalang, buffaloes and cattle from Brebes District, respectively (Table 2). This result indicated that animal has been infected by T. evansi in the past or present time. Low antibody titre described the chronicity of the infection. High antibody titre
described present infection. Twelve heads animal with strong and very strong agglutination were positively having parasite which examined by MIT (9.8%).

Interesting results obtained in cow blood samples in Pemalang. The cow was in one location with buffalo which had positive T. evansi. However, the cow showed negative result from all the tests. This event is possibly of being influenced by season factors and distribution of biting flies. The current study was conducted during the biting flies population is low on the dry season. Luckins (1988) stated the spread of infection of T. evansi occurred when many animals are stabled together or close each other and when the biting flies population is abundant, especially during wet season.

Table 2. The result of examined serologically using CATT/T. evansi

<table>
<thead>
<tr>
<th>District</th>
<th>Animal</th>
<th>CATT/T. evansi Category</th>
<th>Negative</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strong</td>
<td>Medium</td>
<td>Weak</td>
<td></td>
</tr>
<tr>
<td>Pemalang</td>
<td>Buffalo</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brebes</td>
<td>Buffalo</td>
<td>16</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td>3</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>18</td>
<td>8</td>
<td>56</td>
</tr>
</tbody>
</table>

The CATT/T. evansi test is dependent on detection of antibodies against the parasite surface glycoproteins may give false positive due to cross-reacting epitopes on the antigen (Urakawa et al. 2001). Besides, there could be possibility of examination of the animals before sufficient antibodies are produced. The CATT/T.evansi test was highly sensitive but was not strictly species specific (Babeker and Elrasoul 2014). The CATT/T. evansi could also overestimate the prevalence of the disease, as animals with active infection and animals recovered from infection could not be differentenc in seroconvert. In this research, we found 46% prevalence of T. evansi infection in buffalo and cattle in Pemalang and Brebes district with 11.8% of them parasite positive. This result indicated that serology positive not always positive parasite but serology positive by CATT/T. evansi with high agglutination category sometimes found circulation parasite. Circulating antigen detection in blood or serum is also a way to detect active infection (Monzon 2006). CATT/T. evansi is suitable for detection of early or late infections with recent circulation of parasites in the blood, and can detect active infections with a high positive predictive value (OIE Terrestrial Manual 2012). Microscopic examination by Giemsa-stained thin blood smear from the host, or from a mouse inoculation test are recommended methods by OIE (OIE Terrestrial Manual 2012). The final diagnosis of surra will depend on epizootiological information, laboratory results and observations. In order to detect trypanosomes and avoid false positive results, it is suggested to combine 2 detection methods CATT/T. evansi and MHCT. CATT/T. evansi technique is sensitive and suitable for mass screening, however is not always able to distinguish current from past infection so two or more techniques should be used to diagnose reservoir host.
Figure 1. *T. evansi* in mice blood from Pemalong district

In conclusion, in endemic area both healthy and suspected animals showed non specific clinical sign. Parasitological investigations should be done in endemic area to prevent further spread of the disease in the animal population and in order to define appropriate control programmes against the disease. Two techniques by CATT/*T. evansi* and MHCT can be done together in order to obtain a powerful diagnostic conclusion for *T. evansi*. No test is 100% sensitive or specific, more studies should be carried out to assess the current disease status and comparative diagnostics applying multiple tests (Tehseen *et al.* 2017)

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REFERENCES


