

Comparative seroprevalences of bovine trypanosomiasis and anaplasmosis in five states of Malaysia

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Abstract. A comparative seroprevalence study on bovine trypanosomiasis and anaplasmosis was conducted. Sera of adult cattle and buffaloes of different breeds from farms from five different states in Malaysia were collected and tested for the presence of *Trypanosoma evansi* antibodies by CATT and *Anaplasma marginale* antibodies by c-ELISA. Of the 116 samples, 14.7% tested positive for bovine trypanosomiasis and 77.6% for bovine anaplasmosis.

INTRODUCTION

Trypanosoma evansi is a causal agent of bovine trypanosomiasis and surra. A protozoan parasite, it lives in blood plasma and tissue fluids of its hosts, especially cattle, buffalo, horse and camel. It is the largest geographically distributed pathogenic trypanosome that is found in Asia, Africa, South and Central America (Li *et al.*, 2008). In cattle, the disease is often chronic but may be asymptomatic. Common symptoms of infection by *T. evansi* include fever, anaemia, emaciation, lachrymation, paresis of hind legs with terminal oedema of brisket (Cheah *et al.*, 1996). The main vector for this disease is the tabanid fly that transmits the protozoa mechanically (Nurulaini *et al.*, 2007). Others include *Lyperosia*, *Haematopota*, *Chrysops* and *Stomoxys* spp. (Luckins, 1999). In Malaysia, the symptoms reported were poor body condition, as well as eczematous eruptions at the forequarters and back of the infected animal (Cheah *et al.*, 1996). According to other reports, lactating cattle are more susceptible than other cattle (Cheah *et al.*, 1996). Principally, all blood-sucking flies are able to transmit the disease, but

Tabanus spp. and *Stomoxys* spp. are the most common (Holland, 2002). It is also reported that in South America, vampire bats are important as reservoirs as well as vectors of this parasite (Maudlin *et al.*, 2004).

Trypanosoma evansi infections cause specific antibody responses and a variety of serological tests are used in the laboratory for diagnosis. Those frequently used tests are Enzyme-Linked Immunosorbent Assays (ELISA) and Card Agglutination Test for Trypanosomiasis (CATT). CATT is preferred between the two because in overt disease outbreaks, CATT can target specific animals for treatment with trypanocidal drugs. However, serial testing, which refers to ELISA followed by CATT, is recommended to help declare a disease-free condition (Office International Epizooties, OIE 2008).

Anaplasma marginale, an intra-erythrocytic parasite, is the etiological agent of bovine anaplasmosis that is found in tropical and subtropical locations worldwide (Ristic, 1960). It is considered an important disease in bovines as it causes haemolytic anaemia (Ribeiro *et al.*, 1997) resulting in economic loss in cattle production (Centro Panamericano de Zoonosis, 1976). It is

transmitted by several tick species of the genera *Boophilus*, *Rhipicephalus* and *Hyalomma* (James, 1979). Transmission by other blood-sucking insects includes *Stomoxys calcitrans*, *Haematobia irritans* and *Tabanus sp.* (Wagner *et al.*, 1991). Anaplasmosis has a worldwide distribution and is dispersing further into temperate regions (Brandt, 2009). It is a tick-borne zoonosis transmitted mainly by the Ixodidae and the dominant species is *A. marginale* which has a significant impact on cattle production and related world economy (McCallon, 1973; Lonibardo, 1976; De Waal, 2000; Kocan *et al.*, 2004).

The current diagnostic tests and quarantine methods are successful in containing the disease. The Complement Fixation Test (CFT) has proved to be reliable, but its complex technicalities are time-consuming and this raises the need for a faster and more sensitive technique. *Anaplasma marginale* could also be detected through ELISA. When dealing with experimental calf infections, major antibody levels were detected by ELISA and CFT with almost similar results. The only difference was that the antibodies against *A. marginale* were measurable for longer periods using ELISA as opposed to CFT (Nakamura *et al.*, 1988).

This study was carried out to determine the prevalence of *T. evansi* and *A. marginale* in both local as well as imported species of cattle throughout Malaysia. By using CATT for the detection of *T. evansi* and competitive ELISA for the detection of *A. marginale*, a comparison of the infection rates among locations within the country was made possible.

MATERIALS AND METHODS

Blood sample collection

Sera of cattle nationwide were collected and kept in a serum bank at the Serological Unit in the Veterinary Research Institute (VRI), Ipoh, Perak, Malaysia. Cattle sera were collected from the following states in Malaysia: Perak, Terengganu, Melaka, Johor and Sabah.

Cattle and buffalo breeds included in the study were the Kedah-Kelantan, Yellow Cattle, Brahman, Lid Cross, mixed breed, and *Bubalus bubalis*.

The sera were kept at -6°C until analysis was carried out using the CATT kit for the detection of *T. evansi* and c-ELISA test kit for the detection of *A. marginale*.

Card Agglutination Test (CATT)

Materials

A card that contains 10 test areas was used. The buffer consisted of phosphate buffered saline (pH 7.2) with 0.1% sodium azide for preservation. The antigen was made of freeze dried suspension of purified, fixed and stained trypanosomes of VAT RoTat 1.2 with 0.1% sodium azide for preservation. Positive and negative sera were also included in the test. The positive control consisted of freeze dried goat antiserum while the negative control consisted of freeze dried solution of bovine albumin. In both controls 0.1% of sodium azide was used for preservation.

Procedure

A drop of the homogenized CATT antigen (about 45µl) was added to the antigen suspension. Then, 25µl of diluted test samples, according to the ratio 1:8, were dropped in each test area of the card. The dropper was held vertically and drops were allowed to fall freely without touching the card to get drops of constant volume.

The reaction mixture was mixed and spread properly using a 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 positioned on a flat bed orbital rotator for 5 minutes at 70 rpm. After 5 minutes had elapsed, the results were read before removing the card from the rotator. A positive reaction is when agglutination is visible with the naked eye (Verloo *et al.*, 2000).

Competitive Enzyme-Linked Immunosorbent Assay (c-ELISA)

Materials

A microwell plate with 8 by 12 wells, and each well holding 350µL of sample each was

coated with immunoglobulin fraction of the antiserum before use. The coating buffer used was 0.01M Phosphate Buffer and 0.15M Sodium chloride, pH 7.2. Detection of *Anaplasma* was by colour development, using 100µL of chromogenic substrate that was added to each well. The plate was covered and incubated for 15 minutes, or until a blue color had developed. Washing was done using the washing or dilution buffer which consisted of 0.01M Phosphate Buffer, 0.50M Sodium chloride, 0.1% Tween 20 at pH 7.2.

Procedure

100µL of antibody diluted in coating buffer was added to each well. The plate was covered with aluminium foil and was incubated at 4°C overnight.

Wells were washed with washing buffer using a microplate washer. The test sample diluted in diluting buffer was added to each well. The plate was covered and incubated at room temperature for 2 hours. 100µL of peroxidase-conjugated antibody diluted with dilution buffer was added to each well. The plate was covered again and incubated at room temperature for 1 hour.

Washing was done before 100µL of chromogenic substrate was added to each well and was incubated for 15 minutes, or until a blue colour had developed. The reaction was stopped by adding 100µL of 0.5M sulphuric acid, H₂SO₄ to each well.

Results were read directly through the bottom of the microwell plate using an automated photometer ELISA-reader (Model 240, Gilford Instrument Laboratories, UK) at wavelength 800 nm (±2 nm).

RESULTS

When using CATT, positive results were indicated by agglutination on the test area. The cattle and buffalo that tested positive showed that they were infected with *T. evansi*. As for c-ELISA, positive results were indicated by the colour change from blue to clear. Clear wells always indicated that the cattle or buffalo was tested positive for *A. marginale* infection.

14.7% of the 116 samples tested positive for bovine trypanosomiasis (Table 1). The prevalence of disease was highest in Melaka (20%) and the lowest rate was seen in Johor and Sabah (10%).

For bovine anaplasmosis, 77.6% of the 116 sera tested positive (Table 2). The lowest prevalence rate was 28% as seen in Sabah, with only seven positive samples. The state with the highest prevalence rate was Terengganu, where all samples tested positive for *A. marginale*.

According to the results obtained, mixed infections were apparent in a number of the samples, where the cattle or buffalo was infected simultaneously with *T. evansi* and *A. marginale*.

DISCUSSION

The sera samples were obtained from Perak, Terengganu, Melaka, Johor and Sabah and samples were not available from the other states of Malaysia. The serological test

Table 1. Bovine trypanosomiasis incidences in all five states

State	Number of Samples	Positive Results	Percentage (%)*
Perak	21	3	14.29
Terengganu	30	5	16.67
Melaka	25	5	20
Johor	20	2	10
Sabah	20	2	10
TOTAL	116	17	14.7

*All percentages were calculated based on the number of samples from each state

Table 2. Bovine anaplasmosis incidences in all five states

State	Number of Samples	Positive Results	Percentage (%)*
Perak	21	20	95.24
Terengganu	30	30	100
Melaka	25	17	68
Johor	20	16	80
Sabah	20	7	28
TOTAL	116	90	77.6

*All percentages were calculated based on the number of samples from each state

results show that both anaplasmosis and trypanosomiasis are prevalent in all the five states. However, bovine trypanosomiasis has a lower prevalence rate as compared to bovine anaplasmosis. Generally, the definitive host of *T. evansi* is the horse, where cattle and buffaloes are mostly reservoirs of the disease, which explains why the prevalence is low for this disease. On the other hand, *A. marginale* infections are much more prevalent, because cattle and buffalo are the definite hosts of this intraerythrocytic parasite and are more susceptible to bovine anaplasmosis (Palmer *et al.*, 1998).

The livestock industry in Malaysia is small, however the *T. evansi* is endemic as shown by Cheah *et al.* (2000), Rahman *et al.* (2002) and Buniamin *et al.* (2002). Therefore a sensitive versatile test such as the CATT has its advantage and the present study proves it.

Based on this study, Johor and Sabah both recorded the lowest percentage of prevalence with respect to bovine trypanosomiasis, although the reasons for this are not clear. However, according to OIE World Animal Health Information Database (WAHID), outbreaks of bovine trypanosomiasis in Sabah have been reported in the first half of 2005, in March 2007, July 2008 and January 2009 while reports on outbreaks in Johor were reported in January 2009. No other reports have been filed to the database from other states. For that reason, it is possible that Johor and Sabah were more well-informed about this disease than the other three states, and had taken necessary precautionary steps to prevent severe outbreaks.

Trypanosoma evansi infections in human like the ones observed in Africa and South America are not seen in Asia, except for one case in Chandrapur district in Maharashtra, India. The patient was an adult male farmhand who was an unqualified veterinary practitioner, who had a history of febrile episodes on and off and drowsiness for five months. Based on serological evidence, the causal agent was identified as *T. evansi*. This was a peculiar case because of the unique circumstances of the patient who was not immunosuppressed, and yet was infected with a non-human parasite (Powar, 2006).

Competitive ELISA is used for the detection of *A. marginale*. It is a diagnostic tool that has been proven to be very sensitive and specific for the detection of *Anaplasma*-infected animals (Visser *et al.*, 1992; Ndung'u *et al.*, 1995; Knowles *et al.*, 1996; Strik *et al.*, 2007). Reports showed that the test is 100% specific using negative sera from a non-endemic region that detected acutely infected cattle as early as sixteen days after experimental tick or blood inoculation. Detection of *A. marginale* was also possible in cattle that have been experimentally infected for as long as six years ago (Knowles *et al.*, 1996).

No outbreaks were documented on bovine anaplasmosis in the World Animal Health Information Database (WAHID). Unfortunately, extensive and valid studies on bovine anaplasmosis outbreaks in Malaysia are lacking, and annual epidemiological distribution is unsystematically reported.

Based on this study, Terengganu has the highest prevalence rate as all of the samples (100%) were diagnosed with bovine anaplasmosis. The high number of cases may indicate relaxed tick control measures with misuse of acaricides. Studies reported that farmers may have wrongly diluted stock solutions or failed to apply them correctly (Barnett, 1961; Drummond *et al.*, 1974). In fact, a general misuse of acaricides may also cause its efficacy to reduce, causing a high degree of tick resistance (Swai, 2002). Rural practices such as bleeding, tagging and vaccination that are carried out frequently may also cause iatrogenic transmission. Other factors such as location, herd management and vector incidences also affect the disease prevalence rate (Buniamin *et al.*, 2002). According to studies, anaplasmosis showed a higher incidence during rainy season and lower incidences during dry season (Kudi & Kala, 2001). Cattle age and husbandry system also affects the prevalence rate of bovine anaplasmosis (Melendez & Forlano, 1997).

Proper and valid documentations regarding nationwide infections of bovine trypanosomiasis and bovine anaplasmosis in Malaysia are scarce. Unfortunately the present study was confined to just 5 states,

therefore the results cannot be used to reflect on the prevalence of these 2 diseases at a national level, to have any significant impact on the situation. Therefore, it is suggested that future studies on the subject be carried out nation-wide, so as to have a better understanding of the dynamics of transmission cycles and the genetic diversity of *T. evansi* and *A. marginale* in our Malaysian farms.

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