Antibody-ELISA for Trypanosoma evansi: Application in a serological survey of dairy cattle, Thailand, and validation of a locally produced antigen
Antibody-ELISA for Trypanosoma evansi: Application in a serological survey of dairy cattle, Thailand, and validation of a locally produced antigen

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ABSTRACT

Trypanosoma evansi is generally considered a mild pathogen in bovines. However, in Asia, acute and chronic signs have been observed in cattle, with high levels of parasitaemia, abortion and death. Investigations in Asian cattle are needed to better understand this epidemiological situation. To generate comparable data at a regional level, development and standardization of an antibody-enzyme linked immunosorbent assay for T. evansi (ELISA/T. evansi) was initiated and applied in an epidemiological survey carried out in dairy cattle in Thailand. A batch of 1979 samples was collected from dairy farms located throughout the country’s four regions. Soluble T. evansi antigens initially produced in France were also produced in Thailand for comparison and technology transfer. Screening of 500 samples allowed us to identify reference samples and to determine the cut-off value of the ELISA. Seropositive animals – some of them confirmed by PCR – were found in the four regions, in 12 out of 13 provinces, in 22 out of 31 districts, in 56 farms out of 222 (25%, 95%CI 6%) and in 163 animals out of 1979 (8.2, 95%CI 1.2%). Estimated seroprevalence in 35 farms ranged between 1% and 30%, and in 21 farms it was >30%. Approximately 25% of survey cattle were exposed to the infection, in various situations. A sub-sample of 160 sera was tested on both antigens. Wilcoxon’s (Z = 1.24; p = 0.22) and McNemars’s tests (CH2 = 3.55; p = 0.09) did not show any significant differences, showing that the locally produced antigen is suitable for further evaluation in the surrounding countries. Use of this standardized serological method will broaden knowledge of the prevalence and impact of the disease at the regional level in South-East Asia. Further validation of this ELISA will be necessary in other host species such as buffalo, horse and pig.

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1. Introduction

Trypanosoma evansi was identified for the first time in 1880 by Griffith Evans in Indian equines and camels (Stephen, 1986). It originated, however, in Africa where it probably developed from the tsetse-transmitted Trypanosoma brucei brucei, by deletion of kinetoplastic maxicircles (Lun and Desser, 1995; Lai et al., 2008). In Africa it is principally a parasite of camels and horses, present in the
Saharan area, above the tsetse belt, where it is transmitted mechanically by biting insects such as tabanids and stomoxes (Hoare, 1972). The presence of healthy carriers allows the parasite to easily spread through populations (Luckins, 1988). T. evansi has spread into the Middle East and Asia regions, where its geographical distribution is still increasing; it has been observed in Pakistan, Iran, Bhutan, Nepal, India, China, Mongolia, Myanmar, Laos, Viet-Nam, Cambodia, Thailand, Malaysia, the Philippines and Indonesia (Luckins, 1988; Reid, 2002).

It appears that the medical and economic impact of T. evansi is highly variable in the different geographical locations. In Africa, it principally affects camels and horses and is generally considered a mild or negligible pathogen in cattle. In Latin America, it principally affects horses, dogs and buffalo (Bubalus bubalis); it is also considered a mild or negligible pathogen in cattle. However, in Asia, it affects horses, water buffalo, but also cattle and pigs (Tuntasuvan et al., 1997; Tuntasuvan and Luckins, 1998; Holland et al., 2005). Asia is the first region where cattle disease caused by T. evansi appears to be medically and economically important and therefore requires further investigations (Stephen, 1986; Tuntasuvan and Luckins, 1998; Reid, 2002). The recent development of a model in The Philippines (Dobson et al., 2009) could be adapted and validated in other Asian countries. However, to generate results that can be compared for the different Asian countries, diagnostic tests should be developed, standardized and applied at the regional level.

In the present study, we describe and apply an antibody–enzyme linked immunosorbent assay for T. evansi (ELISA/T. evansi) in an epidemiological survey carried out in Thailand, using random sampling of cattle, to establish the seroprevalence of antibodies directed against T. evansi. Antigen preparation, the ELISA procedure, selection of reference samples and determination of the cut-off value are described. Reagents and protocols will be made available in a network of researchers inside Thailand and South-East Asia to increase the quality and quantity of data on this emerging disease.

2. Materials and methods

Dairy farming is a recent development in Thailand; it was initiated in 1956, when a Dutch company started producing plain and flavoured milk in Bangkok; approximately one-third of the dairy farms are located in the Central region of Thailand, one-fourth is located in the North-East region, 10% are located in the Northern region and the remainder are located in the Southern region (Riethmueller and Chalermpao, 2002). Holstein type breed, mainly introduced by artificial insemination, are mostly kept in 10-20 head units organized in cooperatives. The study was confined to dairy farms which are mainly from two types: (i) small units of 5–20 adults, generally with dairy cattle only, which represent 55% of the dairy cows and 85% of the dairy units, or (ii) larger farms of 20–50 dairy cattle, generally including heifers and calves, representing more than 35% of the dairy cows and nearly 15% of the dairy units (Poapongsakorn et al., 2003).

2.1. Sampling

Sampling was stratified geographically by the country’s four regions, and sample size was based on a mean expected infection prevalence of 10%. With an alpha of 5% and an accuracy of 3%, the design required collecting at least 384 samples per region. As a first step in this survey, sampling focused on the main provinces for dairy cattle breeding (in the North, North-East and Center). Dairy farms were selected by local veterinary services (Department of Livestock Development) insuring that both small and large units would be represented. Animals were sampled regardless of age and even very young animals were included (upon the farmer’s agreement). In order to guarantee an overall proportional sampling, all animals were sampled, when the total number of animals in the farm was less than 31, and, only the first 30 animals presented were sampled when total number was more than 30. The sampling in a region was stopped when the target number was approximately reached. Cattle blood was collected from the jugular vein in 5-ml dry and citrated tubes for serology and PCR examinations, respectively. Serum samples collected in dry tubes were allowed to clot for 24 h at 4 °C; serum was collected and stored in 1.5-ml microtubes at −20 °C until ELISA processing. Blood samples collected in 5-ml citrated tubes and kept at −20 °C until DNA preparation procedure for PCR.

Throughout the manuscript, means and percentages are expressed with 95% confidence intervals and 5% risk error as (Ancelle, 2002): μ = m ± 1.96Sm (with Sm = S/√n), and P = p ± 1.96Sp (with Sp = √((p(1 − p))/n)).

2.2. Preparation of T. evansi soluble antigens

A cryopreserved isolate of T. evansi from Canary Island camels, previously confirmed for species identification (Desquesnes et al., 2008), was inoculated intraperitoneally in two Wistar rats. When parasitaemia reached 10⁸ parasites/ml (days 4–6), rats where anaesthesised with chloroform and bled to death. The blood was collected with citrate/glucose buffer and centrifuged at 10,000 × g for 10 min. Buffy-coat and the lower part of the plasma were collected and deposited on a diethylaminoethoxy-cellulose (DEAE-cellulose®; D9099, Sigma–Aldrich, Saint Louis, MO, USA) for separation from blood cells, as previously described (Lanham and Godfrey, 1970). Parasites were washed twice in phosphate saline glucose (PSG, pH 8) by centrifugation at 10,000 × g for 10 min, and the supernatant was discarded. The pellet was measured and resuspended 1/20 (v/v) in distilled water and added to an anti-enzyme cocktail (Complete Protease Inhibitor cocktail®, ref 11697498001, Roche Diagnostic, France) at the rate of one tablet/8.5 ml of lysate. The parasite lysate was exposed to five cycles of 2 min freezing in liquid nitrogen and 5 min thawing in an incubator at 37 °C. The lysate was sonicated on ice three times for 2 min at a 60% active cycle, output power 7, with Sonificateur 130W® (Fisher Bioblock Scientific, Belgique), and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was collected and the pellet discarded. The protein concentration of the supernatant was estimated
by UV readings at 260 and 280 nm, using Adams normograph, derived from Warburg and Christian (Warburg and Christian, 1942). The soluble antigen was aliquoted and stored at −80 °C for long-term storage, or −20 °C for short-term storage. It was transferred to Thailand under continuous cold chain on dry ice.

A cryopreserved T. evansi from Rusa deer, isolated in 2001 in Ratchaburi, Thailand, was inoculated intraperitoneally in two Wistar rats to produce soluble trypanosome antigens using the same procedure in Bangkok, Thailand.

2.3. ELISA/T. evansi procedure

The ELISA procedure is derived from a previously described technique (Desquesnes et al., 2007). Briefly, Microtest 96-well Polysorp Nunc® immunoplates (Nunc, Roskilde, Denmark) were coated with 100 μl/well of T. evansi soluble antigen at 5 μg/ml protein concentration in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plates were rinsed once with phosphate buffer saline (PBS) and blocked with 150 μl/well of blocking buffer, PBS–7% skim milk powder (ref: 190-12865, Wako Pure Chemical Industries Ltd., Osaka, Japan) with permanent shaking (200 rpm) for 45 min at 37 °C. The blocking buffer was discarded. Sera diluted 1:100 in blocking buffer were transferred in duplicate on the ELISA plate. After 30 min in a shaker-incubator at 37 °C, 200 rpm, the plates were washed five times with PBS 0.1% Tween 20® (Sigma–Aldrich) (washing buffer, WB). Then 100 μl of peroxidase-conjugated anti-bovine IgG (A5295, Sigma–Aldrich), diluted 1:10,000 in blocking buffer, was added and the plates incubated for 30 min at 37 °C with permanent shaking (200 rpm). After washing five times with washing buffer, 100 μl of the complex substrate/chromogen 3,3’,5,5’-tetramethylbenzidine (TMB) (K blue®, TMB substrate, Neogen Europe Ltd., Scotland, UK) was added. The plates were shaken and then incubated in a dark room for 30 min. Optical density (OD) was measured at 630 nm in an ELISA reader (Dynex Technologies®, VA, USA).

2.3.1. Selection of reference samples

A batch of 47 serum samples from cattle living in Aveyron, France, negative to PCR (see Section 2.4) and CATT T. evansi (Bajyana Songa and Hamers, 1988), was used to evaluate the specificity of the ELISA.

In Thailand, reference samples were selected amongst the local cattle population. To select positive and negative reference samples, a first batch of 500 samples was analysed by ELISA and the results were expressed in OD. Based on previous results of the test, an arbitrary and temporary value of the OD less than 0.250 was considered negative. In order to constitute a pool of approximately 300 presumed negative samples, we defined non-infected farms as farms in which no animal exhibited an OD greater than 0.250. A batch of negative samples was constituted with animals from presumed non-infected farms. The mean OD of these negative samples (MeanN) was calculated and three negative controls (NC), representative of the negative population (low, medium and high responses), were selected such that their OD values were equal or 10% below or above the MeanN:

\[ \text{NC}_1 = \text{Mean}_N; \quad \text{NC}_2 = 0.9 \times \text{Mean}_N; \quad \text{NC}_3 = 1.1 \times \text{Mean}_N \]

Similarly, based on previous results obtained in experimentally infected cattle in Burkina Faso (Dia and Desquesnes, 2007a), an arbitrary and temporary OD value greater than 0.500 was considered positive. The mean OD of all positive samples (MeanP) was calculated and three positive controls (PC) representative of the positive population (low, medium and high responses) were selected such that their OD values were equal to or 10% below or above the MeanP:

\[ \text{PC}_1 = \text{Mean}_P; \quad \text{PC}_2 = 0.9 \times \text{Mean}_P; \quad \text{PC}_3 = 1.1 \times \text{Mean}_P \]

The ELISAs were rerun for all samples in duplicate with three PC and three NC on each plate. The blank OD value was automatically deducted from each sample value. The results were expressed in relative percentage of positivity (RPP), as described previously (Desquesnes, 1997), according to the following ratio:

\[ \text{RPP of a sample} = \frac{\text{mean OD of the sample} - \text{mean OD of NC}}{\text{mean OD of PC} - \text{mean OD of NC}} \]

The cut-off value (COV) was determined based on the mean RPP of the batch of samples from presumed non-infected farms:

\[ \text{COV} (%) = \frac{\text{mean RPP}_N(\%)}{3 \times \text{standard deviation} (\%)} \]

The sample status was positive when the RPP of a sample was greater than the COV.

2.4. PCR analysis

In order to confirm the species identification and the presence of the parasite in blood samples, 119 seropositive samples (from two farms with seroprevalence >60%) and 84 seronegative samples (from three farms considered non-infected) were submitted to DNA preparation and PCR analysis with specific primers for Trypanozoon (Masiga et al., 1992).

DNA preparation was carried out with DNA preparation kits (Flexigene DNA kit®, cat. no. 51204, Qiagen, Germany) on 100 μl of citrated blood samples that had been kept at −20 °C. The procedure was prepared according to the protocol provided by the manufacturer. PCR was done in 10-μl master mixed volumes with 1 μl of sample or according to the technique described by Masiga et al. (1992). PCR products were migrated 1 h at 120 V in 2% agarose gels, together with Generuler® 100 bp DNA ladder plus (Fermentas, Ontario, Canada), stained with ethidium bromide and visualized under UV light.

2.5. Validation of the local antigen for ELISA

To ensure that the antigen produced with the local strain of T. evansi gave similar results to the antigen produced in France (parasite stock from the Canary Islands), a batch of 160 cattle samples containing approximately 40% positive samples was tested with ELISA on both antigens. Two statistical tests were applied...
to these ELISA results. To compare the global proportion of positive samples obtained with each antigen, a McNemar’s test for paired sample was applied. Then, to assess the semi-quantitative equivalence between ELISA with the two different antigens, the Wilcoxon signed-rank test was applied to the two paired series of RPP to determine whether there was a significant difference.

Other statistical analyses were performed using Excel, Epilinfo and SAS softwares. For each geographic level (region, province, district), prevalences and their 95% confidence interval were estimated considering the total number of sampled animals in the area and the total number of infected animals among them. At the regional level, comparison between estimated prevalence was globally made with a Chi square test. A Tukey-like multiple comparison test (Zar, 1999) was then applied using a SAS macro named «compprop» (Elliott and Reisch, 2006).

3. Results

Preliminary work on 47 samples from non-infected cattle (bred in Metropolitan France) provided negative results to all tests (PCR, CATT and ELISA). Results of ELISA *T. evansi*; provided optical densities below 0.200. Results expressed with reference samples from cattle experimentally infected in Burkina Faso (Dia and Desquesnes, 2007a) indicated a mean of −1% ± 1% RPP.

### 3.1. Sampling

A total of 1979 blood samples were collected from 222 dairy farms in Thailand, located in 31 districts throughout the country in 13 provinces of the four regions: Udorn Thani, Khon Khaen, Sakon Nakhon, Nakorn Radcha Srima, Chiang Mai, Lum Pang, Chiang Rai, Pat ta lung, Kanjanaburi, Radchaburi, Nakorn Patom, Saraburi and Lopburi. The animals were aged between 6 days and 15 years, with a mean of 4.93 years (95%CI ± 5.02 years). The target number of samples was reached and exceeded in the North (642), North-East (460) and Central regions (690), but it was not reached in the South region where only 187 samples were collected (Table 1).

### 3.2. Preparation of *T. evansi* soluble antigens

Antigen preparation in IRD-CIRAD (France) and Kasetsart (Thailand) provided protein concentrations of 1 and 6.8 mg/ml, respectively. In both cases, the antigens were coated at 5 μg of protein per milliliter in carbonate buffer (pH 9.6).

### 3.3. ELISA results

In the first step, a batch of 500 samples from 59 farms was tested for ELISA. Thirty-nine of these farms were presumed non-infected (all 306 samples with OD values <0.250) and 20 farms (for a total of 194 animals) were presumed infected with a total of 23 samples exhibiting OD greater than 0.50. The frequency distribution of the OD-values of these samples is presented in Fig. 1. The 306 negative samples provided a Mean of 0.136 OD. Negative controls were selected for values close to: NC1 = 0.136; NC2 = 0.122; NC3 = 0.149.

The 23 positive samples provided a Mean of 0.834. Positive controls were then selected for their values close to: PC1 = 0.834; PC2 = 0.750; PC3 = 0.917.

In the second step, all 1979 samples were ELISA tested in duplicate, together with the six control sera; the results were expressed in RPP. Based on the mean and standard deviation of the previous batch of 306 negative samples, COV = 0% + (3 × 6%) = 18%.

A sample was positive when its RPP was greater than 18%.

Of the 1979 samples originating on 222 farms, 163 were positive (8.2%, 95%CI ± 1.2% positive samples), belonging to 56 farms (25%, 95%CI ± 6% of the farms were infected); a total of 597 animals sampled were exposed to infected animals.

<table>
<thead>
<tr>
<th>Region</th>
<th>N</th>
<th>Np</th>
<th>P (%) ± CI</th>
<th>PF (%) ± CI</th>
<th>mPFi (%) ± CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>North</td>
<td>642</td>
<td>72</td>
<td>11 ± 2</td>
<td>46 ± 22</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>North-East</td>
<td>460</td>
<td>43</td>
<td>9 ± 3</td>
<td>24 ± 11</td>
<td>33 ± 8</td>
</tr>
<tr>
<td>Center</td>
<td>690</td>
<td>44</td>
<td>8 ± 2</td>
<td>19 ± 8</td>
<td>39 ± 8</td>
</tr>
<tr>
<td>South</td>
<td>187</td>
<td>4</td>
<td>2 ± 2</td>
<td>21 ± 8</td>
<td>7 ± 7</td>
</tr>
<tr>
<td>Total</td>
<td>1979</td>
<td>163</td>
<td>8 ± 1.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*N* = number of serum sample tested. *Np* = number of positive samples by ELISA (prevalence). *P* = apparent seroprevalence rate. CI = confidence interval. PF = percentage of farms having at least one seropositive sample. mPFi = mean seroprevalence rate in infected farms.

### Table 1

Results of dairy cattle seroepidemiological survey by ELISA *Trypanosoma evansi* at the Regional level in Thailand.

![Fig. 1. Frequency distribution of the OD-values of reference seronegative (306) and seropositive samples (23) from dairy cattle located in 59 farms in Thailand.](image-url)
(30% of the population sampled). The prevalence on 56 infected farms was 27.3% (95%CI ± 3.6%) (163/597).

RPP obtained with 47 cattle from France were all below 3%, with a mean of −0.6 ± 0.3%.

3.3.1. Age groups

Seroprevalence increased in the three age groups from 4.7% (<2 years) to 8.9% (2–5 years) and 11.0% (>5 years), but the difference in age-specific seroprevalences was not statistically significant (P < 0.05). On infected farms where young and adult animals had been sampled, with two groups defined (young <1 year of age and adults >1 year), seroprevalence was nil in the subgroup aged less than 1 year (61 animals) and 30% in adults.

3.3.2. Geographical distribution

The details of the results for each province and district are presented in Fig. 2 and respectively in Tables 2 and 3, where for each level (region, province or district) P is the percentage of positive samples, PF the percentage of farms infected (having at least one seropositive sample) and mPFi the mean seroprevalence in the infected farms. Positive samples were found throughout the country.

At the regional levels, seroprevalences in Northern, North-Eastern, Central and Southern region were 11.2%, 9.3%, 6.4% and 2.1%, respectively (χ² = 20.64, p = 0.0001). A Tukey-like test was used to perform a multiple comparison of proportions (Zar, 1999; Elliott and Reisch, 2006); it indicated that there were three seroprevalence groups: Northern and North-Eastern; North-Eastern and Central; and Central and Southern.

At the provincial level, only the province of Nakorn Radcha Srima did not show serological evidence of infection. The highest prevalences were observed in the northern province of Chiang Mai (21%) (bordering Myanmar), the North-Eastern provinces of Udorn Thani and Sakon Nakhon (16% and 19%) (bordering Laos), and in the central province of Saraburi (17%). In the 12 provinces

![Fig. 2. Geographical representation of the results of a serological survey using antibody-ELISA/ Trypanosoma evansi in dairy cattle farms in Thailand.](image-url)
presenting serological evidence of *T. evansi*, 5%–75% (PF) of the farms were infected (Table 2). The mean prevalence in infected farms (mPFi) in each province ranged from 7% to 60% (mean, 28.1%, 95% CI ± 4.1%).

At the district level, 22 out of 31 districts (71%) had seropositive animals, and prevalences ranged from 0% to 46%. Three provinces showed high ranges of prevalences at the district level: Chiang Rai (North), with five districts below 5% and two over 40%; Udorn Thani (North-East) with two districts at 0% and one with 26%, and Ratchaburi (Center), with two districts below 3% and two over 20%.

At the farm level, 56 farms out of 222 (25%, 95% CI ± 6%) had seropositive animals. Seroprevalences ranged from 0% to 100%. Infected and non-infected farms were present throughout Thailand. Some districts (such as Sri That District/Udorn Thani), had a high range of farm prevalence which was >60% on two farms, while it was <5% on three other farms. The highest prevalences were observed in Maeung (Sakon Nakhon) and Muak lek (Saraburi), ranging from 7% to 100% (mean, 31.6%, 95% CI ± 3.8%).

To assess the potential impact of the infection, based on the representative sampling design, exposed animals were defined as animals sampled on the same farm as a seropositive animal. The number of exposed animals reached 30% of the sampled animals. Approximately one-third of the population could then be considered exposed to the risk.

3.4. PCR results

Of the 119 seropositive samples tested (from 27 farms), 55 were positive by PCR, confirming the presence of the parasite in these farms.

<table>
<thead>
<tr>
<th>Region</th>
<th>Province</th>
<th>PF (%) ± CI</th>
<th>mPFi (%) ± CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>North</td>
<td>Chiang Mai</td>
<td>75 ± 30 %</td>
<td>31 ± 9 %</td>
</tr>
<tr>
<td></td>
<td>Lum Pang</td>
<td>40 ± 43 %</td>
<td>9 ± 9 %</td>
</tr>
<tr>
<td></td>
<td>Chiang Rai</td>
<td>39 ± 18 %</td>
<td>26.7 ± 6.7%</td>
</tr>
<tr>
<td>North-East</td>
<td>Udorn thani</td>
<td>23 ± 23 %</td>
<td>44 ± 12 %</td>
</tr>
<tr>
<td></td>
<td>Khon Khaen</td>
<td>10 ± 11 %</td>
<td>29 ± 18 %</td>
</tr>
<tr>
<td></td>
<td>Sakon Nakhon</td>
<td>57 ± 26 %</td>
<td>31 ± 12 %</td>
</tr>
<tr>
<td></td>
<td>Nakorn Radcha S</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Center</td>
<td>Kanjanaburi</td>
<td>5 ± 9 %</td>
<td>20 ± 35%</td>
</tr>
<tr>
<td></td>
<td>Radchaburi</td>
<td>25 ± 19 %</td>
<td>28 ± 15 %</td>
</tr>
<tr>
<td></td>
<td>Saraburi</td>
<td>29 ± 18 %</td>
<td>60 ± 13 %</td>
</tr>
<tr>
<td></td>
<td>Lopburi</td>
<td>5 ± 10 %</td>
<td>20 ± 35 %</td>
</tr>
<tr>
<td>South</td>
<td>Pat ta lung</td>
<td>21 ± 22 %</td>
<td>7 ± 7 %</td>
</tr>
</tbody>
</table>

PF = percentage of farms infected (having at least one seropositive sample) mPFi = mean seroprevalence rate in infected farms.

<table>
<thead>
<tr>
<th>Region</th>
<th>Province</th>
<th>District</th>
<th>P (%) ± CI</th>
<th>NF</th>
<th>NFi</th>
<th>PFi (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>North</td>
<td>Chiang Mai</td>
<td>Chai Pra Karn</td>
<td>21 ± 3</td>
<td>8</td>
<td>6</td>
<td>75%</td>
</tr>
<tr>
<td></td>
<td>Lum Pang</td>
<td>Hang Chut</td>
<td>3 ± 2</td>
<td>5</td>
<td>2</td>
<td>40%</td>
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<tr>
<td></td>
<td>Chiang Rai</td>
<td>Maeung</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mae lao</td>
<td>10 ± 6</td>
<td>5</td>
<td>3</td>
<td>60%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phan</td>
<td>2 ± 2</td>
<td>5</td>
<td>1</td>
<td>20%</td>
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<tr>
<td></td>
<td></td>
<td>Terng</td>
<td>2 ± 1</td>
<td>9</td>
<td>2</td>
<td>22%</td>
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<tr>
<td></td>
<td></td>
<td>Pa Ya Meng Rai</td>
<td>46 ± 9</td>
<td>2</td>
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<tr>
<td></td>
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<td>Koon Tan</td>
<td>43 ± 8</td>
<td>3</td>
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<td>4 ± 3</td>
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<td>100%</td>
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<td>Udorn thani</td>
<td>Sri That</td>
<td>26 ± 5</td>
<td>5</td>
<td>3</td>
<td>60%</td>
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<td>North-East</td>
<td>Udorn thani</td>
<td>Kud Jab</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maeung</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>Khon Khaen</td>
<td>Maeung</td>
<td>1 ± 1</td>
<td>10</td>
<td>1</td>
<td>10%</td>
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<tr>
<td></td>
<td></td>
<td>Nam Pong</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ubounrat</td>
<td>4 ± 3</td>
<td>11</td>
<td>1</td>
<td>9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kra Nuan</td>
<td>12 ± 7</td>
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<td>1</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>Sakon Nakhon</td>
<td>Maeung</td>
<td>19 ± 4</td>
<td>14</td>
<td>8</td>
<td>57%</td>
</tr>
<tr>
<td></td>
<td>Nakorn Radcha S</td>
<td>Pak tong chai</td>
<td>0</td>
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<td>0%</td>
</tr>
<tr>
<td></td>
<td>Kanjanaburi</td>
<td>Ta Moang</td>
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<td>10</td>
<td>1</td>
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</tr>
<tr>
<td>Center</td>
<td>Ta ma ka</td>
<td>0</td>
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<td>0</td>
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<tr>
<td></td>
<td>Lao Kwan</td>
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<tr>
<td></td>
<td>Non</td>
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<td>0</td>
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<tr>
<td></td>
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<td>Ban pong</td>
<td>2 ± 2</td>
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<td>1</td>
<td>13%</td>
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<tr>
<td></td>
<td></td>
<td>Potaram</td>
<td>21 ± 6</td>
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<td>3</td>
<td>43%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chom bung</td>
<td>0</td>
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<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Muang</td>
<td>20 ± 18</td>
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<td>1</td>
<td>100%</td>
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<tr>
<td></td>
<td>Nakorn Patom</td>
<td>Kampang saen</td>
<td>6 ± 2</td>
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<td>5</td>
<td>38%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Muang</td>
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<td>1</td>
<td>14%</td>
</tr>
<tr>
<td></td>
<td>Saraburi</td>
<td>Muak lek</td>
<td>17 ± 3</td>
<td>32</td>
<td>7</td>
<td>29%</td>
</tr>
<tr>
<td></td>
<td>Lopburi</td>
<td>Pattananikom</td>
<td>1 ± 1</td>
<td>19</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td>South</td>
<td>Pat ta lung</td>
<td>Muang</td>
<td>2 ± 1</td>
<td>14</td>
<td>3</td>
<td>21%</td>
</tr>
</tbody>
</table>

P = apparent seroprevalence rate (*N*$_p$/*N*). CI = confidence interval. NF = number of farms tested. NFi = number of farms infected (having at least one seropositive sample). PF = percentage of farms infected.
Of the 84 seronegative samples from farms presumed non-infected, none was positive by PCR, which was compatible with the presumed absence of infection in these farms.

3.5. Validation of the local antigen

Among the 160 samples tested by ELISA, 52 were positive and 90 were negative with both the foreign and the local antigen, 5 were positive with the local antigen but positive with the foreign, whereas 13 were positive with the foreign and negative with the local antigen. There was no significant difference between results of these two tests performed on the same samples (McNemar test: $\chi^2 = 3.55$, $p = 0.09$). The Wilcoxon signed-rank test applied on RPP paired distributions indicated a value of $Z = 1.24$ ($p = 0.22$), confirming that there was no significant difference between the two matched distributions of RPP. These results confirmed a global equivalence in classifying a sample as positive or negative, but also an equivalence in semi-quantitative results of RPP given by ELISA with these two antigens.

4. Discussion and conclusion

The ELISA method for diagnosing animal trypanosomes has been used for a long time (Gray and Luckins, 1977) and is sensitive and specific. Considerable effort has been made to improve standardization and reproducibility and to validate these tests (Bocquentin and Duvallet, 1990; Diall et al., 1992; Wright et al., 1993; Desquesnes, 1997; Dia and Desquesnes, 2007b). The ELISA/T. evansi initially developed for camels (Zweygarth et al., 1986) was adapted to horses (Reyna-Bello et al., 1998; Monzon et al., 2003), buffalo (Holland et al., 2004), cattle (Reid and Copeman, 2002) and pigs (Holland et al., 2005). The protocol used in the present study, initially developed in sheep (Desquesnes, 2004), was adapted and partially validated in experimentally infected cattle (Dia and Desquesnes, 2007a).

Negative ELISA observed with cattle from France, and negative PCR results observed in sero-negative samples from Thailand confirmed the species specificity of the ELISA and its high negative predictive value.

The antigen produced in Thailand, with a parasite isolated from a wild deer, gave very similar results to the antigen produced in France with a parasite originating from the Canary Islands and isolated from a camel. This observation confirmed the high homogeneity of the parasite’s soluble antigen, already observed (Laha and Sasmal, 2008), and thus allowing comparable and worldwide use of the test, providing the same protocols are used.

There is evidence of an immune reaction against T. evansi, and presence of the parasite has also been confirmed by PCR results, proving the active circulation of this parasite in Thailand dairy cattle. The lower PCR-prevalence versus ELISA was expected for two main reasons: (i) the persistence of antibodies for 4–5 months after elimination of the parasite in treated or naturally cured animals (Desquesnes et al., 2003; Dia and Desquesnes, 2007a), and (ii) the fact that infected animals harboring antibodies may exhibit parasitaemia below the PCR detection level (1–20 trypanosomes/ml) (Desquesnes and Davila, 2002). This low sensitivity of the PCR has been demonstrated in experimental conditions (Bengaly et al., 2001; Desquesnes and Davila, 2002). In some farms, PCR and ELISA prevalence were very close, reflecting active circulation of the parasite. In others, prevalence by PCR was lower than by ELISA, reflecting chronic or sub-clinical low parasitaemic infections. These observations highlight the benefit provided by the combination of these diagnostic tools to describe various epidemiologic situations.

The sampling design of the serological survey was completed in the Central, North and North-East regions of Thailand where the numbers of samples were all higher than the target number (384 samples); in these areas the seroprevalence observed provided an accuracy of more than 3% and suggested a decreasing prevalence of the infection from north to south. However, given the small number of districts sampled this observation requires further confirmation, especially for the South region, where the target was not reached (187 samples collected) and provided an accuracy of only 4%. Further investigations could be necessary in this region to obtain a more accurate estimation. However, it is important to note that T. evansi is present throughout the country. The results showed a wide geographic range of seroprevalence. Indeed, heterogeneity increased with the size of the entity observed, from region, to province, district and farm. This confirms the variety of epidemiological situations: some areas are free of the parasite (for example Nakorn Radcha Srima province) or have some sporadic cases, as was observed in Lopburi province where only one infected animal was found out of 19 farms sampled. Enzootic situations can be observed with many infected farms distributed over several districts, whether with low prevalence as in Pat Ta Lung province where three farms out of 14 were infected, with a mean prevalence of 7%, or with high prevalence (enzootic–epizootic situation) as was observed in Chiang Mai province where six out of eight farms were infected, with a mean prevalence of 30%. Very high seroprevalences were observed in a limited number of farms, most probably reflecting epizootic situations, as in Saraburi where seven out of 24 farms were infected, with a mean prevalence of 60%. However, concerning geographical distribution, it is still difficult to draw firm conclusions since only a few districts and provinces were sampled; consequently, a fully representative geographical picture of T.evansi distribution in Thailand is not yet possible.

Despite a moderate overall prevalence (8.2%, 95%CI ± 1.2%), 25% of the farms sampled were infected and showed wide ranges of prevalences, 0%–21%, 0%–46% and 0%–100%, respectively, at the province, district and farm levels. At a local level (irrespective of district boundaries), some farms were severely burdened by T. evansi infections, with prevalences over 80%. The impact in these areas could be considerable, and the number of exposed animals highlights the potential threat of this parasite. This study showed higher seroprevalences in older animals. Animals had no serological evidence of infection before 1 year of age, most probably because of poor exposure of young animals to insects and the role of adults acting as a screen protecting young animals.
been demonstrated (Pholpark et al., 1999); seropositive to provide more information on seasonal variation but they sampling season. Only longitudinal studies can contribute in Thailand, cattle-breeding is characterized by a very high level of animal movement and exchange between farms and areas, and even between countries (importation from Myanmar and Laos). These controlled or uncontrolled movements could explain the number of sporadic cases observed, as well as the wide distribution of T. evansi infection. This is particularly important in cattle or buffalo where infections are often asymptomatic or even cryptic. However, stress caused by transportation could contribute to disseminating the infection from these carriers in their new location. It is essential to carry out longitudinal surveys to improve our understanding of infection dynamics.

These results are consistent with previous observations in the North-Eastern part of Thailand, in 1984–1989, with seroprevalences of 13% in cattle and 20% in buffalo (Kashemsant et al., 1989). In a countrywide survey carried out in 1990 using the indirect fluorescent antibody test, Nishikawa observed higher prevalences: 50% in cattle and 38.6% in buffalo (Tuntasuvan and Luckins, 1998). For buffalo, prevalence was higher in the North (57.4%) than in the South (28.7%), but for cattle it was higher in the Central region (64.7%) than in the North (28.9%). Results of a survey in cattle, buffalo and pigs indicated seroprevalence of respectively 12.5%, 20% and 4.6% (Tuntasuvan and Luckins, 1998), which is consistent with the seroprevalence of 20% observed by complement fixation test in another study (Löhr et al., 1985). In this study, a peak of infection during the rainy season was observed. However, in our study, samples were collected over a 15-month period, so the prevalence level of each district or province could be influenced by the sampling season. Only longitudinal studies can contribute to provide more information on seasonal variation but they are time consuming and need a significant budget.

The economic impact of surra in Thai dairy cattle has been demonstrated (Pholpark et al., 1999); seropositive dairy cattle may produce 20–35% less milk (Sarataphan et al., 1989). Moreover, abortion, stillbirth and placenta retention are additional effects (Tuntasuvan and Luckins, 1998). In The Philippines, cross-sectional surveys and models were developed for estimating the impact of surra in buffalo (Dobson et al., 2007, 2009).

Diagnostics is a very basic preliminary step for any investigation of a disease. This ELISA has proved to be one of the important tools available for such studies. It is now proposed for general validation and use in South-East Asian countries, most particularly Cambodia and Laos, for which little if any information is available on the prevalence of surra infection. Application of this ELISA could help us to evaluate the potential impact of surra in South-East Asian dairy cattle, especially if the models recently developed (Dobson et al., 2009) can be adapted to other countries and host-species.

Conflict of interest

All authors declare that they have no financial and personal relationships with other people or organization that could inappropriately influence (bias) their work, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent application/registration, and grant or other funding.

Ethics

All authors certify that animal experiments conducted in the present work meet the International Guiding Principles for Biomedical Research Involving animals.

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