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Prevalence and genetic diversity of *Trypanosoma evansi* infection causing abortion among Cattles and Buffaloes in Eastern border area of Thailand-Cambodia

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Abstract *Trypanosoma evansi*, a protozoan blood parasite in animals, causes surra disease and easily leads to abortion in cattle and buffaloes. The results demonstrated that the PCR product was 164 bp in length. The prevalence of *T. evansi* infection in cattle and buffaloes of Ta Phraya, Khok Sung, Aranyaprathet and Khlong Hat districts in Sa Kaeo province was 19.67% (12/61), 38.57% (27/70), 45.16% (70/155) and 35.45% (25/92), respectively. The satellite DNAs (TBR primer) were analyzed and revealed that it could demonstrate the genetic diversity of *T. evansi* of cattle and buffaloes. Tree construction based on the satellite DNAs in each district of border areas confirmed the close relationship between cattle and buffalo. The results found that trypanosome minor variations might be due to livestock system, a pasture or forest grazing. These feeding were difficult to get rid of insects that are disease vectors such as tabanidae, flies, and mosquitoes as well as easy to spread or transmission of trypanosome.

Keywords: *Trypanosoma evansi*, Cattle, Buffalo, Eastern border area of Thailand-Cambodia

Introduction

Livestock trypanosomosis caused by *Trypanosoma evansi*, a protozoon blood parasite in livestock animal. It is a major parasitic disease known as "surra" (Webster and MacDonald, 1995). which has a wide distribution in Asia. The main host species varies with the geographical region. Horses, cattle, and buffalo are most often affected in South-East Asia. It has been described in tigers in India (Bhaskararao *et al*., 1995), as well as in Thai elephants (Tuntasuvan and Luckins 1998). In Thailand, surra was first detected in mules in Rachaburi province in 1949 but now occurs throughout Thailand in horses, cattle, buffaloes, pigs, dogs and deer with varying clinical manifestations. Horses are most severely affected. Abortion at late stages of pregnancy or

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premature parturition has been reported in infected buffaloes, cattle and pigs. Evidence for *T. evansi* invasion of the central nervous system has been reported in cattle and more recently in hog deer (*Cervus porcinus*). Prevalence varies around 12.5, 20 and 4.6% in cattle, buffaloes and pigs, respectively (Rodtian *et al*., 2005). The surra symptoms are high fever, loss of appetite, paralysis and some animal have neurological symptoms, immunodeficiency, and death may occur in 2 weeks to 4 months. It can causes the economic losses, due to a wide range of pathological expressions of surra: weight loss and milk yield reduction, depreciated sale or slaughtering of sick livestock, infertility, mortality, diminution in animal work capacity and draught power, immune system impairment (increasing co-morbidity and decreasing in vaccination campaigns' efficacy), treatment costs (Holland *et al*., 2001; Reid, 2002). In addition, *T. evansi* is also the leading cause of animal birth abortion in late stage of pregnancy (Bhaskararao *et al*., 1995). Sa Kaeo province that has an area of the eastern border with Cambodia consists of Ta Phraya, Aranyaprathet, Khok Sung, and Khlong Hat district. Most of the population is farmer, crop production and livestock that the important livestock are dairy cattle, beef cattle and buffalo. The animal production system is pasture or forest grazing that it is poor management and care of animal. Farmers not focus on disease screening, vaccination, as well as disease control and pest control. The animal production in this system, it will reduce the yield of animals and cause the spread of the disease as well. Academic or research information on surra disease in the area is limited. In addition, the countryside is a border area; it may be a factor that facilitates the spread of the disease between the two countries. Therefore, it is necessary to check the epidemiology and genetic diversity of *T. evansi* of cattle and buffalo. The objective of this research was to investigate prevalence of *T. evansi* infection among cattle and buffalo from Sa Kaeo province, eastern border area of Thailand-Cambodia in order to local people benefits about the academic data of diseases transmitted from animals to animals and humans (zoonosis) which leads to the prevention of disease and the development of the animal production system.

Materials and methods

Study design and blood samples

Sa Kaeo province was divided into 9 regions consisting of the following 4 districts adjacent to the Cambodian border: (1) Ta Phraya; TP, (2) Khok Sung; KS, (3) Aranyaprathet; AR, (4) Khlong Hat; KH. A total of 378 cattle and buffaloes were randomly sampled from four border districts of Sa Kaeo

province (Figure 1). Blood samples were collected from the jugular vein and kept in the EDTA tubes to block clotting of the blood, for PCR examinations. Whole blood was stored at -20 ℃ until processing.

Figure 1. The four districts of Sa Kaeo province, eastern border area of Thailand-Cambodia

DNA extraction and PCR amplification

Total genomic DNA was extracted from whole blood by the acid phenol-chloroform method (Chomczynsky and Sacchi, 1987). The resulting DNA was amplified by polymerase chain reaction (PCR) using a specific forward primer TBR(F) 5'- GAATATTAAACAATGCGC AG -3' and a specific reverse primer TBR(R) 5'- CCATTTATTAGCTTTGTTGC -3'. Polymerase chain reaction was performed for 35 cycles at 94 ^{O}C for 1 min, 60 ^{O}C for 30 sec, and 72 ^{O}C for 30 sec and final extension at 72 ^{O}C for 2 min in a Primus 96 plus thermocycler. The PCR products were checked by gel electrophoresis technique.

Construct to cloning vector

The amplified satellite DNA of *T. evansi* was purified using QIA quick gel extraction kit (Qiagen, Hilden, Germany), and then it was ligated to

pGEMT easy cloning vector (Promega, Madison, WI, USA). This vector contains the amplicilin resistance gene for positive selection in *E. coli* (Invitrogen, Carlsbad, CA, USA). The ligated plasmids were used to transform *E. coli* strain DH5α competent cells. The positive clones were selected using colony screening in LB agar plates containing amplicilin (100 mg/m*l*) and positive clone was checked using PCR assay.

DNA sequencing and computer-assisted sequence analysis

A single colony of *E. coli* positive clone was selected and subcultured in LB media. After an overnight growth, plasmid DNA was purified from bacteria culture using QIAprep spin miniprep kit (Qiagen, Hilden, Germany) and confirmed by PCR technique. Nucleotide sequencing was performed by the 1st BASE, Malaysia. The satellite DNA diversity from salivary glands of each location cattle tick was analyzed by MEGA version 5.2 (http://www.megasoftware.net) using the maximum parsimony method. The different satellite DNA of *T. evansi* each location were compared using Clustal W program version 1.83.

Statistical analysis

The PCR-positive prevalence was calculated as the percentage of positive results out of the total number of the cattle and buffalo sampled. The percentage was calculated for the individual district of Sa Kaeo province, eastern border area of Thailand-Cambodia. Reporting for each region included the number and percentage of animal species with positive results.

Results

Blood samples and PCR amplification

A total of 281 cattle and 97 buffalo blood samples were collected from border area district of Sa Kaeo province including Ta Phraya, Khok Sung, Aranyaprathet, and Khlong Hat district. The whole bloods were used to DNAs extraction by phenol-chloroform method. Total DNAs were amplified by Polymerase Chain Reaction (PCR) using the primers TBR1 and TBR2. Gel electrophoresis was used to check PCR product in each district. The PCR products of the satellite gene of *T. evansi* are approximately 164 bps in size (Figure 2).

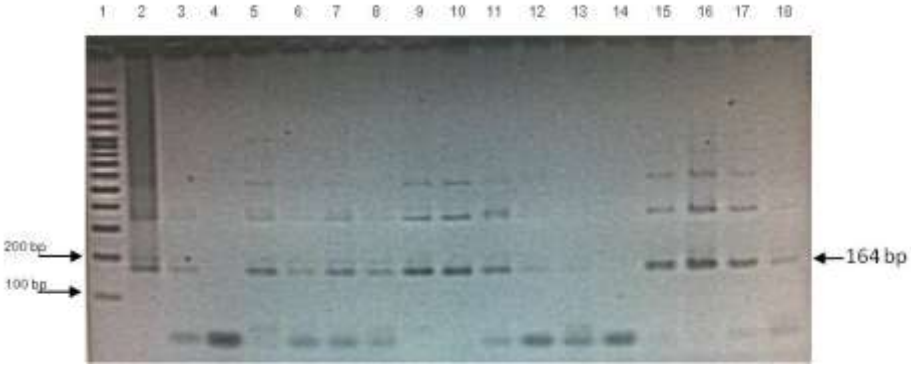

Figure 2. Analysis of PCR products of satellite gene of *T. evansi* from cattle and buffalo using 1% agarose gel electrophoresis. A 10 µl of PCR mixture was loaded onto each lane of agarose gel. Lane 1 = DNA marker (100 bp), Lane 2 = Positive control, lane 3-18 = PCR product of satellite gene from each district area

Prevalence rate of T. evansi

The prevalence of *T. evansi* was evaluated in the cattle and buffalo blood from border district, Sa Kaeo province of Thailand and Cambodia. Polymerase chain reaction method was detected for *T. evansi* infection in cattle and buffalo. The results showed that the prevalence of *T. evansi* infected cattle of Ta Phraya, Khok Sung, Aranyaprathet and Khlong Hat district was 20.93% (9/43), 41.67% (20/48), 46.85% (52/111), 27.85% (22/79), and prevalence of *T. evansi* infected buffalo was 16.67% (3/18), 31.82% (7/22), 40.91% (18/44), 23.08% (3/13), respectively. The total percentage of *T. evansi* infected cattle and buffalo was 34.33% (281/103), 28.12% (97/31), respectively (Table 1).

Eight satellite genes were cloned in pGEM-T easy vector and transformed to *E. coli* strain DH5α. Positive clones were confirmed using PCR and corrected for sequencing assay. A combination of 3' and 5' primer T7 and SP6 was used to amplify full-length DNA. All of satellite genes were 164 bp in length. Comparing the nucleotide sequence with the 8 satellite genes from each border district and each animal species, the nucleotide sequences of Ta Phraya Cattle were 100% identical to those from Khok Sung cattle, 99.39% identical to those from Khong Hat cattle, 98.78% identical to those from Khlong Hat and Aranyaprathet buffalo, and 97.56% identical to those from Khok Sung and Ta Phraya buffalo. There are 5 positions of base in satellite nucleotide as different base sequences; it consists of positions 54, 100, 118, 141, and 142 (Figure 3).

Table 1. Prevalence of *Trypanosoma evansi* isolated from cattle and buffalo blood of four border district of Sa Kaeo province

District	Sample (n)	TBR primer (positive)	Prevalence (%)
Ta Phraya	61	12	**19.67**
cattle	43	9	20.93
Buffalo	18	3	16.67
Khok Sung	70	27	**38.57**
cattle	48	20	41.67
Buffalo	22	7	31.82
Aranyaprathet	155	70	**45.16**
cattle	111	52	46.85
Buffalo	44	18	40.91
Khlong Hat	92	25	**27.17**
cattle	79	22	27.85
Buffalo	13	3	23.08
Net total	**378**	**134**	**35.45**
Total cattle	281	103	34.33
Total buffalo	97	31	28.12

According to the phylogenetic analysis of *T. evansi* nucleotide sequences of cattle and buffalo from each district area (Ta Phraya; TP, Khok Sung; KS, Aranyaprathet; AR and Khlong Hat; KH) were closely together and separated cluster from other trypanosome, (*T. congolense*; accession number X05769 *T. cruzi*; accession number K00393.1 and HQ335292 *T. brucei gambiense*; accession number FJ223603) and closely related to the *T. brucei* (accession number K00392). The trypanosome satellite DNA of TP buffalo was more closely to the KS buffalo and the TP cattle was more closely to the KS cattle too (Figure 4).

The phylogeny of trypanosome satellite DNA from each district area and other known organism related to *T. evansi* from the GenBank database was shown in Figure 5. As the result shown, they are evolutionary relationship with Trypanosoma sp. Satellite DNA, which can be grouped and separated cluster from other known organism, (Tsetse_flies; accession number AY220504, Camel1; accession number JX093552, Camel2; accession number JN845636, R_microplus; accession number FJ223603, Liver_fluke; accession number AF408147, Cestoda; accession number AY954521, Buffalo1; accession number AY956327, Buffalo2; accession number EU086340, Cattle; accession number AB471783).

Figure 3. Multiple alignments of the satellite DNA of *T. evansi* from each district area; TP, Ta Phraya; KS, Khok Sung; AR, Aranyaprathet and KH, Khlong Hat using CLUSTAL W (http://www.ebi.ac.uk/clustalw/). The different base regions of satellite DNA were in boxes

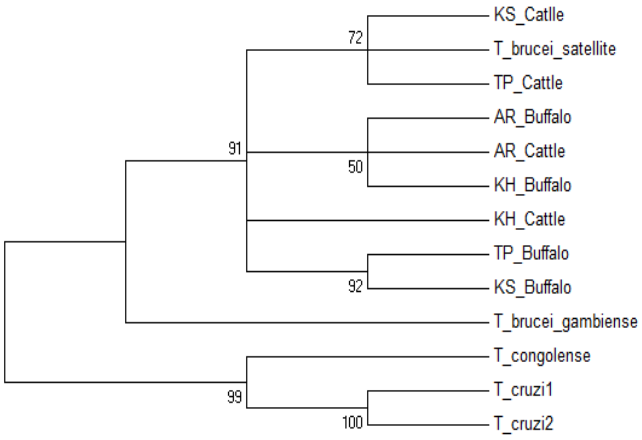

Figure 4. Phylogeny of *Trypanosoma evansi* satellite DNA (TP_Cattle, TP_Buffalo, KS_Cattle, KS_Buffalo, AR_Cattle, AR_Buffalo, KH_Cattle and KH_Buffalo) and other known *T. evansi* from the GenBank database (*Trypanosoma brucei*, *Trypanosoma brucei gambiense*, *Trypanosoma congolense* and *Trypanosoma cruzi*) using the maximum parsimony method and bootstrap support values higher than 50% are shown at the nodes (n = 2,000) and drawn using MEGA version

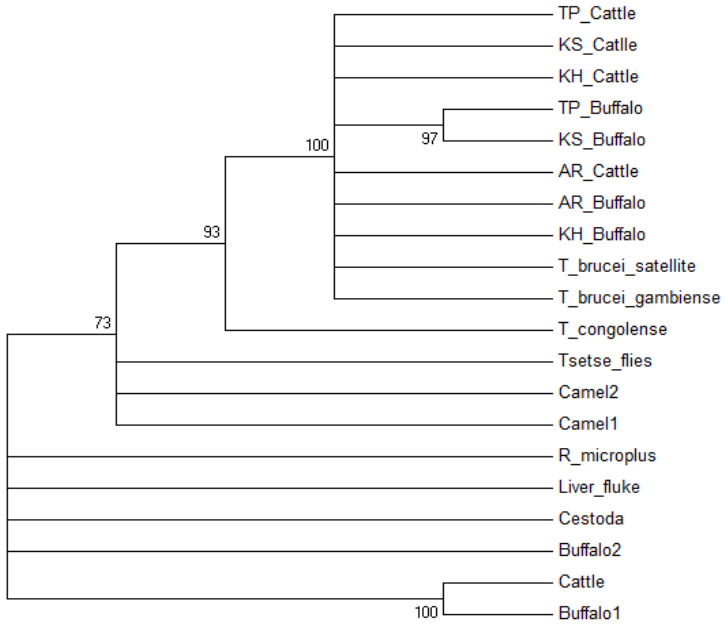

Figure 5. Phylogeny of *Trypanosoma evansi* satellite DNA (TP_Cattle, TP_Buffalo, KS_Cattle, KS_Buffalo, AR_Cattle, AR_Buffalo, KH_Cattle and

KH_Buffalo) and other known *T. evansi* from the GenBank database (*Trypanosoma brucei, Trypanosoma brucei gambiense, Trypanosoma congolense* and *Trypanosoma cruzi*) and and other known organism related to *T. evansi* from the GenBank database (tsetse flies, camel, *Rhiphicephalus microplus*, liver fluke, cestoda, buffalo and cattle) using the maximum parsimony method and bootstrap support values higher than 50% are shown at the nodes (n = 2,000) and drawn using MEGA version

Discussion

Previously, trypanozoon DNA has been isolated and checked by molecular technique using TBR primer for amplification and the PCR product was 164 bp in lengths (Kaewhom, 2014; Moser *et al*., 1989; Masiga, 1992). For our study, total DNAs were extracted from whole blood of cattle and buffalo and amplified by Polymerase Chain Reaction (PCR) using the primers TBR1 and TBR2. The PCR products of the satellite gene of *T. evansi* are approximately 164 bps in size. From this result, it is similar to previous reports, PCR condition can be used to determine the prevalence and genetic diversity of *T. evansi* in cattle and buffalo.

The PCR-positive prevalence was calculated as the percentage of positive results and reported in the individual district of Sa Kaeo province. The high prevalence of *T. evansi* in eastern border area of Thailand-Combodia was found, which differed from the result of 25 % in Loei province, Thailand (Kashiwasaki *et al*., 1998) 8.1 % in dairy cows in central Thailand (Jittapalapong *et al*., 2009) and the result of 20 % infection in buffaloes in north-east Thailand (Lohr, 1986) . The results showed that there are two border areas with high prevalence of *T. evansi* infected cattle and buffalo. The high percentage of trypanosome prevalence in Khok Sung and Aranyaprathet district might be due to a higher number of the live stock-producing areas, local livestock markets, and border crossing point than other districts. The forest grazing system (pasture or forest grazing) might be effect to spread or transmit trypanosome. In addition, the animals were transported in and out all the day at the border area which might be result in the prevalence of *T. evansi* infection increased.

The nucleotide sequences of the satellite gene were checked by the NCBI / BLAST / blastn. It was found to be Trypanosome nucleotide and 98% identity to *T. brucei* satellite DNA, 95% identity to *T. brucei gambiense*, and 97% identity to *T. brucei* Lister 427 surface glycoprotein. Multiple alignments of the satellite DNA of *T. evansi* in cattle and buffalo from each district area found that they are very similar, ranging from 97-100 percent. The trypanozoon satellite nucleotides from Ta Phraya district were 100% identity to trypanosome satellite nucleotides from Khok Sung district.

In a phylogenic analysis of *T. evansi* nucleotide sequences of cattle and buffalo from each district area indicated a close relationship. Thai satellite genes from *T. evansi* were a clearly separated cluster among the other trypanosome except *T. brucei* satellite DNA. From this result, it is are similar to previous reports, Sequencing of the rDNA complete internal transcribed spacer (ITS) region including the 5.8S subunit showed high similarity (99–100%) between Philippine isolates (*Bubalus bubalis*) and known *T. evansi* isolates in Genbank. Tree construction based on the same region confirmed the close relationship between Philippine and reported Thai isolates (Marjo *et al*., 2013). Tree construction base on trypanosome satellite DNA from each district area indicated the clearly separated group from other known organism related to *T. evansi*. In addition, the satellite DNA nucleotides of each Trypanosome have a few data reported in the Genbank database, using other primers, such as Internal Transcribed Spacing (ITS) to clone *T. evansi* genes from cattle and buffalo blood, can be seen more clearly evolutionary relationships because this gene is used for analyzing evolutionary relationships and for taxonomic identities as well. This suggests, there for, that border area location and animal species did not affect to evolutionary relationships of *T. evansi*. However, the border area district was affected by the prevalence of *T. evansi* because in two border districts with high prevalence, there are higher numbers of the livestock-producing, local livestock markets, and border crossing point than other districts.

In summary, the satellite DNA were isolated from *T. evansi* and amplified by polymerase chain reaction technique. The PCR product was approximately 164 bps in length. The prevalence of *T. evansi* infected cattle and buffalo in Ta Phraya, Khok Sung, Aranyaprathet and Khlong Hat district district were 20.93%(9/43) 16.67%(3/18), 41.67%(20/48) 31.82%(7/22), 46.85%(52/111) 40.91%(18/44) and 28.00%(22/79) 23.08%(3/13), respectively. The overall prevalence of *T. evansi* infection in cattle and buffalo in four border area districts was 35.45% (134/378). In a phylogenic analysis of the trypanosome satellite DNAs from each border district revealed a close relationship between cattle and buffalo and can be separated cluster from other trypanosome. Additionally, the animal species (cattle and buffalo) did not affect the evolutionary relationships of *T. evansi* in border area of Sa Kaeo province.

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