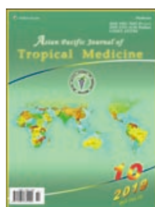

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Detection of *Trypanosoma* spp. in *Bandicota indica* from the Thai-Myanmar border area, Mae Sot District Tak Province, Thailand

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ABSTRACT

Objective: To investigate the prevalence of trypanosome infection and their phylogeny in *Bandicota indica* rats from the cadmium-contaminated area of Mae Sot and the Myanmar border.

Methods: Blood samples were taken from 100 animals, and parasite infection was examined by light microscopy observation and polymerase chain reaction (PCR) studies.

Results: *Trypanosoma* spp. infection was found in 20% of the thin blood smear samples. PCR showed positive 623 bp DNA bands in 21 samples (21%). The sequencing analysis showed that all of the samples (100%) had the *Trypanasoma lewisi* 18S ribosomal RNA gene. Phylogenetic analysis confirmed that these 16 isolates of *Trypanosoma* spp. were closely related to *Trypanasoma lewisi*.

Conclusions: Molecular detection using PCR is as effective as conventional light microscopy analysis. This study confirms that trypanosomal infection in rodents is still high; therefore, fleas as their vectors need to be controlled in order to prevent transmission to humans.

1. Introduction

Trypanosoma spp. is a parasitic protozoon living in the blood and tissues of vertebrates and mammals including humans. *Trypanosoma* spp. can be transmitted from animals to people *via* insect vectors bites, causing trypanosomiasis with the pathogen in a metacyclic trypomastigotes form. All 44 species[1,2] that can be transmitted from animals to humans (Zoonoses) contribute to various diseases depending on the strain and route of infection. For example, in Africa, *Trypanosoma* (*T.*) *brucei* causes African trypanosomiasis or sleeping sickness. The disease is restricted to tropical areas of Africa with the insect vector, the tsetse fly. While *T. cruzi* found in North and South America causes American trypanosomiasis (Chagas' disease) with the insect killer (Reduviid bugs) as vectors[5]. Study on infectious *Trypanosoma* spp. in blood samples from mice using the polymerase chain reaction (PCR) reported that in Southeast Asia, infection of *Trypanosoma* spp. is mainly from two species that can be transmitted from animals to humans: *T. lewisi*[6,7] and *T. evansi*[8,9] in Malaysia, Thailand, Sri Lanka and India. These two species cause disease in humans, and have different carriers: fleas living on rats and voles as the carriers for *T. lewisi*, and blood-sucking insects, such as midges as the main vectors for *T. evansi*[3,10]. The assumed life cycle of *T. lewisi* (Figure 1) begins with rat fleas taking a blood meal from vertebrate hosts, and then

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the metacyclic trypomastigotes penetrate various cells at the bite wound site. Amastigotes multiply by binary fission in the infected cells and form a pseudocyst. When the pseudocysts rupture, the trypomastigotes are released into the bloodstream where they infect other cells, and transform into amastigotes, or are taken with a blood meal by rat fleas and transformed into epimastigotes. Inside the rat flea vector, the epimastigotes multiply in the midgut, then migrate to the hind gut and differentiate into the infective metacyclic trypomastigotes stage.

Most infections in Thailand are caused by *T. lewisi* with infection rates as 48.8%, 22.3%, 13.9%, 8.3% and 2.2% in Bangkok, Kanchanaburi, Buriram, Nan and Loei, respectively[2]. The first case of *T. lewisi* infection in humans was reported in a premature Asian child[11,12] who lived in a rat-infested dwelling. Other human infections caused by this parasite were described mainly in Asia[7,13-15] including fatal cases[6], and also in Africa[15]. In the North of Thailand, a report about a child with the symptoms as high fever, anemia, coughing, anorexia and depression found *T. lewisi* as the causative agent through hematological analysis[7]. *Bandicota* (*B.*) *indica* is one of the important reservoir hosts of the zoonotic trypanosome. *T. lewisi*-like parasites were first reported in rats from Chiang Mai Province[16]. *T. lewisi* and other Herpetosoma are commonly found in the blood of rats worldwide, and members of this subgenus are generally considered to be non-pathogenic and rarely found in humans[3]. *T. lewisi* was found in 14.3% of *Rattus* spp. and 18.0% in *B. indica* from the rodents trapped in urban and rural areas of three Thailand provinces between 2006 and 2007[17]. Thus, *Rattus* and *Bandicota* species are possible sources for human exposure to these parasites[17]. Since Thailand joined the Association of Southeast Asian Nations Economic Community, members and animals have moved freely throughout Asia. Monitoring parasites in the *B. indica* should be regularly performed because the pathogens can spread from animals to people. The aim of this study was to investigate the prevalence of the trypanosome infection in *B. indica* from the cadmium-contaminated area of Maesot and the Myanmar border by microscopic analysis and polymerase chain reactions detection. Besides, the phylogenetic relationship was analyzed using the bioinformatics program. The identification of the *Trypanosoma* spp. species infecting the *B. indica* rats should be helpful for controlling and prevention of the infection.

2. Materials and methods

2.1. Animals

One hundred (*B. indica*) rats trapped in Mae Sot, Tak Province, Northern Thailand were selected. The geographical location of Mae Sot District, Tak Province, Thailand in the Thai-Myanmar border area is shown in Figure 2. The animals were housed and acclimatized in cages containing food and water as appropriate. The animals were euthanized using the cervical dislocation method. Following

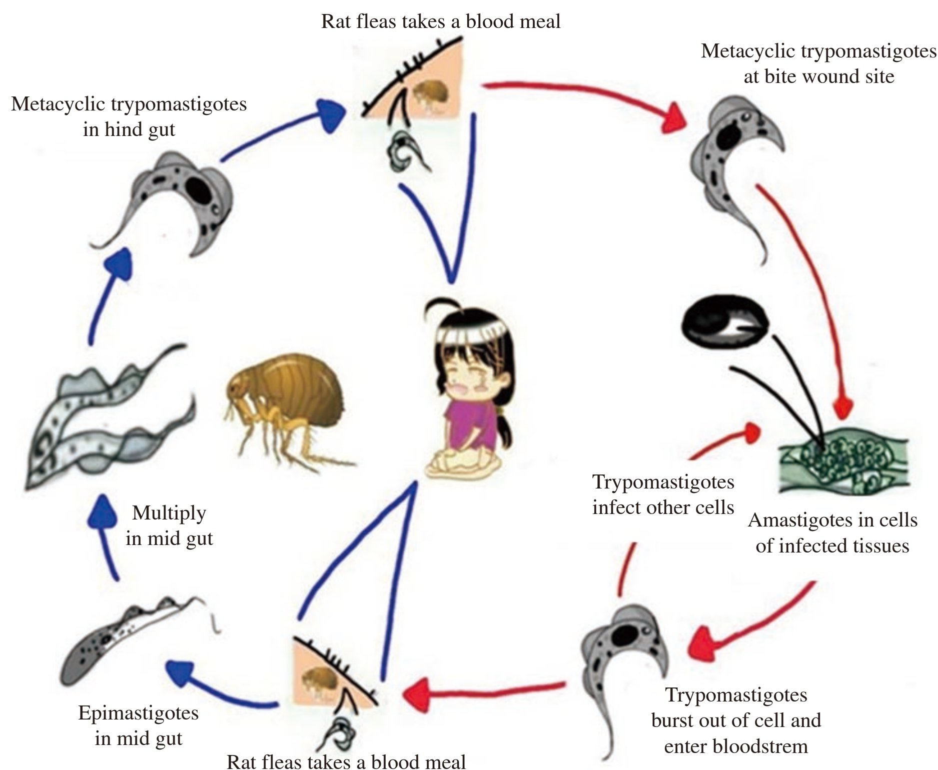

Figure 1. Life cycle of *Trypanosoma lewisi*.

euthanasia of small rodents (NUCAR SOP No.05-14), the carcasses were collected in a red bag, and placed in a freezer at -20 ℃ before being incinerated.

This study was approved by the Naresuan University Animal Care and Use on June 26th, 2015, with certificate No. 5802003.

Figure 2. Geographical location of Mae Sot District, Tak Province, Thailand in the Thai-Myanmar border area.

2.2. Methods

2.2.1. Blood sample collection

The rats were dissected following standard operating procedures to reveal their abdomen and chest cavity, and 2 mL of blood was removed from the left ventricle using a syringe. A drop of the blood sample was collected for the smear test, and the remainder was preserved in citrate salt tubes, and stored at -20 ℃ for the PCR detection.

2.2.2. Microscopic diagnosis (thin blood film)

Blood films were made by placing a drop of blood on one end of a slide, and then smeared using a spreader in order to obtain a thin film of blood for counting and differentiation. The smeared slides were left to air-dry, fixed in methanol, and then subjected to the process of Giemsa's staining. After this process, the slides were examined for *Trypanosoma* spp. infection under a light microscope.

2.2.3. Molecular diagnosis

All 100 samples were detected by the PCR technique. Following the extraction of DNA by a NucleoSpin blood DNA kit, the concentrations were measured with a Nanodrop. Primers TRYP1S (5'CGT CCC TGC CAT TTG TACA CA-3') and TRYP1R (5'-GGA AGC CAA GTC ATC CAT CG-3') were used for the amplification of the internal transcribed spacer 1 (ITS)-1 fragment[18]. The PCR was carried out in 20 μL solution, which consisted of Master mix 10 μL containing Buffer $MgCl_2$ dNTPs (dTTP, dGTP, dCTP, and dATP) and Taq polymerase, 0.4 μL of each primer, 2 μL DNA template and 7.2 μL deionized water (18 M). PCR was run with an initial denature at 94 ℃ for 2 min, followed by 30 cycles of 30 s at 94 ℃, 30 s at 61.5 ℃ and 30 s at 72 ℃ with a final extension of 72 ℃ for 10 min. The PCR products were then electrophoresed on a 2% agarose gel and stained with nucleic acid staining solution (Red SafeTM, iNtRON Biotecnology, Inc.) for analysis. The visualization of a 623 bp was considered as a positive result.

2.2.4. Phylogenetic analysis

The PCR products were purified using NucleoSpin Gel and a PCR Clean-up kit (Macherey-Nagel, Germany) as recommended by the manufacturer. The DNA sequencing was performed by Macrogen Inc. service in Korea. The gene sequences were analyzed using a nucleotide BLAST search *via* NCBI. The nucleotide sequences were aligned using Clustal W. A phylogenetic tree was reconstructed using the maximum likelihood method with the MEGA Version 7.0 program[19].

Table 1. BLAST results showing identity of the pathogenic isolates of *Trypanosoma lewisi*.

No.	Code sample	Maximum identity	BLASTN				
			Accession number	Total score	Query coverage (%)	E value	Identity (%)
1	RE2	*T. lewisi* 18S ribosomal RNA gene	FJ011095.1	680	95	1e-38	88
2	RE5	*T. lewisi* 18S ribosomal RNA gene	EU861192.1	363	94	2e-96	98
3	RE47	*T. lewisi* 18S ribosomal RNA gene	HQ437158.1	584	88	9e-163	87
4	RE56	*T. lewisi* 18S ribosomal RNA gene	EU861192.1	773	95	6e-63	91
5	RE63	*T. lewisi* 18S ribosomal RNA gene	EU861192.1	800	95	7e-62	92
6	RE64	*T. lewisi* 18S ribosomal RNA gene	FJ011095.1	67	96	1e-38	90
7	RE68	*T. lewisi* 18S ribosomal RNA gene	HQ437158.1	592	67	6e-165	93
8	RE69	*T. lewisi* 18S ribosomal RNA gene	FJ011095.1	708	66	0	91
9	RE71	*T. lewisi* 18S ribosomal RNA gene	EU861192.	636	95	3e-178	87
10	RE73	*T. lewisi* 18S ribosomal RNA gene	EU861192.1	780	95	0	91
11	RE75	*T. lewisi* 18S ribosomal RNA gene	FJ011095.1	713	92	0	90
12	RE78	*T. lewisi* 18S ribosomal RNA gene	EU861192.1	680	96	0	88
13	RE82	*T. lewisi* 18S ribosomal RNA gene	EU861192.1	754	95	0	90
14	RE83	*T. lewisi* 18S ribosomal RNA gene	EU861192.1	732	95	0	90
15	RE85	*T. lewisi* 18S ribosomal RNA gene	HQ437158.1	601	86	7e-168	93
16	RE86	*T. lewisi* 18S ribosomal RNA gene	FJ011095.1	763	95	0	91

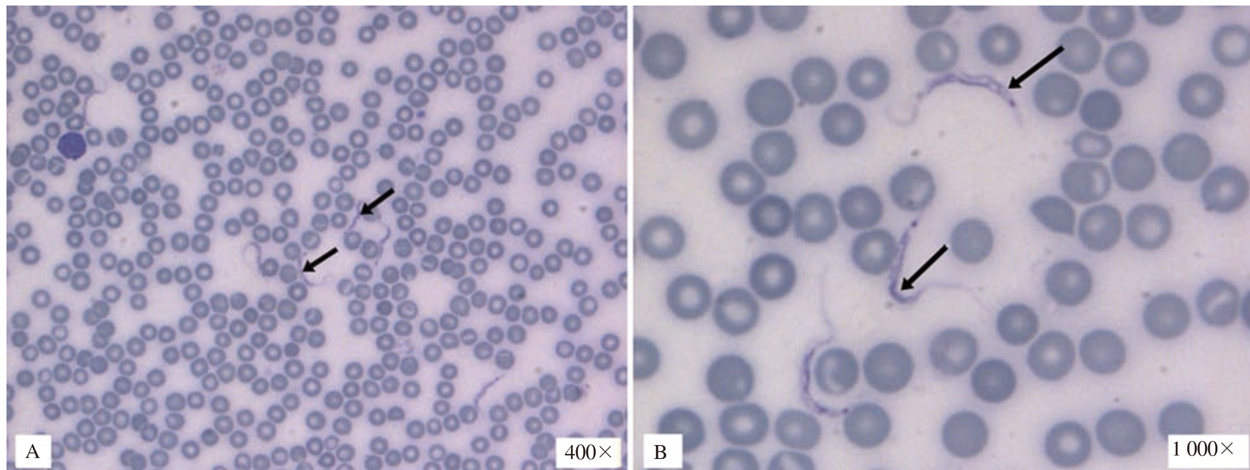

Figure 3. Light microscopic images of thin blood films showing presence of *Trypanosoma* spp. (A) The trypomastigote stage under a magnification of 400×(arrows), and (B) under a magnification of 1 000× in oil immersion (arrows).

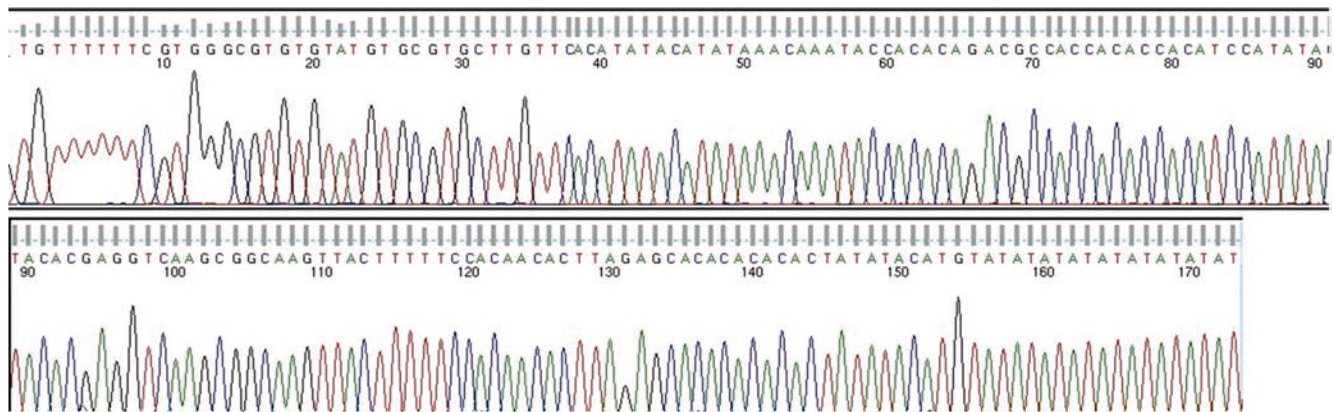

Figure 4. Pathogenic sequencing of *Trypanosoma lewisi*.

Trypanosoma lewisi isolate TryCC internal transcribed spacer 1, complete sequence

Sequence ID: gb|GU252219.1| Length: 423 Number of Matches: 1

Range 1: 229 to 401 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
320 bits(173)	5e-84	173/173(100%)	0/173(0%)	Plus/Minus

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Query  1    TGTTTTTTCGTGGGCGTGTGTATGTGCGTGCTTGTTCACATATACATATAAACAAATACC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  401  TGTTTTTTCGTGGGCGTGTGTATGTGCGTGCTTGTTCACATATACATATAAACAAATACC  342

Query  61   ACACAGACGCCACCACACCACATCCATATACACGAGGTCAAGCGGCAAGTTACTTTTTCC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  341  ACACAGACGCCACCACACCACATCCATATACACGAGGTCAAGCGGCAAGTTACTTTTTCC  282

Query  121  ACAACACTTAGAGCACACACACACTATATACATGtatatatatatatatatat  173
            |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  281  ACAACACTTAGAGCACACACACACTATATACATGTATATATATATATATATAT  229
```

Figure 5. Sequencing compared to the NCBI database for *Trypanosoma lewisi*.

3. Results

3.1. Prevalence of Trypanosoma spp. infection

Microscopic analysis showed a prevalence of 20% for *Trypanosoma* spp. infection (Figure 3), while PCR analysis demonstrated a prevalence of 21%.

3.2. Phylogenetic results

Sequence trace files identified the *Trypanosoma* spp. as *T. lewisi* (Figure 4). The BLAST search (87%-98%) *via* NCBI showed that all of them (100%) were *T. lewisi* (Figure 5). From 100 samples, 21 were PCR-positive. We could sequence only 16 samples of these PCR-positive samples because of failure to obtain enough PCR products for sequencing RE38, RE58, RE77, RE90 and RE93. Sixteen of the 21 PCR positive samples were analyzed by the BLAST sequencing application. All of them (100%) contained *T. lewisi* 18S ribosomal RNA gene (Table 1).

Based on phylogenetic analysis, 16 of the 21 samples were *Trypanosoma* spp. positive, and were closely related to *T. lewisi* (Figure 6).

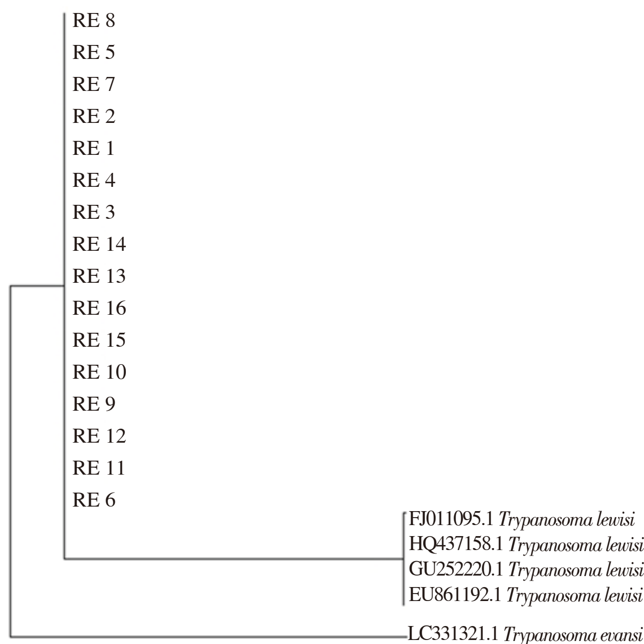

Figure 6. Phylogenetic analysis from the samples sequencing.

4. Discussion

In our study, the microscopic analysis revealed infection of *Trypanosoma* spp. in 20 of the specimens: RE2, RE5, RE38, RE47, RE56, RE58, RE63, RE64, RE68, RE69, RE71, RE73, RE75, RE77, RE78, RE82, RE83, RE85, RE86 and RE90. Our results were similar to a comparative study of the techniques using 423 mice blood samples in Peninsular, Malaysia, which reported the prevalence rate of *Trypanosoma* spp. infection in the quantitative buffy coat was 20.8% and in the Giemsa-stained technique was 22.0%[20]. In fact, different geographic locations in Thailand seemed to have a wide-range of infections of *T. lewisi*. Five regions in Thailand, Bangkok, Kanchanaburi, Buriram, Nan and Loei were found to have an infection rate of 48.8%, 22.3%, 13.9%, 8.3% and 2.2%, respectively[2]. Interestingly, a similar prevalence rate was found in Kanchanaburi, a province bound to Myanmar.

We also estimated the prevalence of this parasitic protozoon by PCR using genus-specific primers, TRYP1S and TRYP1R. These have also been used to detect and diagnose *T. lewisi* infection in an infant living in Lampang, Thailand[7] and *T. lewisi* infection in rodents Thailand[17]. This molecular technique could amplify the ITS1 sequence (623 bp) between 18S and 5.8S rDNA region of trypanosome in all the blood smear positive samples with one more sample (RE93) not detected by the light microscopy. The prevalence rate detected by this molecular technique was 21%. Detection of the trypanosome infection with PCR was previously reported to be more sensitive than the thin blood film method[1]. The possible reason may be due to the total number of protozoa in the blood samples. If it is too low, it may not be detected by the blood smear method.

Although the right size of the PCR product could ensure trypanosomal infection, the analysis of the sequence inside the ITS1 is essential to identify the species. The specific and the variable sequence inside ITS1 could define *T. lewisi*[21]. In this study, we sequenced all the PCR products and the analysis was performed with BLAST-search in the database, which was also used to find out the type of infection in the rodents in Thailand[18]. Our result showed that the species of all the positive samples were *T. lewisi*. High prevalence of *T. lewisi* was previously observed in rodents living near human settlements and in areas with high cover of built-up habitat[22]. Different species of *Trypanosoma* spp. were transmitted by various kinds of vectors. The *T. brucei* which causes African trypanosomiasis or sleeping sickness was transmitted by tsetse flies[3]; while *T. brucei* which causes Chagas' disease was transmitted by triatomine bugs[4]. From our findings and former work, we assumed that *T. lewisi* might be transmitted by rat fleas. Thailand has joined the Association of Southeast Asian Nations Economic Community that allows people and animal populations to move freely. Therefore, the trypanosome infection should be monitored as the transmission of pathogens from animals to humans can cause disease.

This study reveals the prevalence of *Trypanosoma* spp. detected by PCR to be 21% in the Thailand-Myanmar border region of Mae Sot, Tak Province. The parasite species is confirmed as *T. lewisi* by PCR and nucleotide sequences. This dominant rodent pathogen can be transmitted to humans and cause disease. Our finding can provide reference for the prevention and control of outbreaks of the disease caused by *T. lewisi*.

Conflict of interest statement

We declare that we have no conflict of interest.

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Authors' contribution

P.W.M. and S.S developed the theoretical formalism, performed the analytic calculations and performed the numerical simulations. P.W.M, S.S., and N.S. authors contributed to the final version of the manuscript. P.W.M supervised the project.

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