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Original papers

# Characterization of drug resistance-associated TevAT1 gene of *Trypanosoma evansi* from Philippine water buffaloes (*Bubalus bubalis*)

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**ABSTRACT.** This study detected and characterized the TevAT1 gene of *Trypanosoma evansi* isolates from Philippine water buffaloes (*Bubalus bubalis*). A total of 68 blood samples from Philippine water buffaloes were subjected to DNA extraction and PCR assay was performed using RoTat 1.2 gene to detect *T. evansi*. Those samples positive for *T. evansi* subsequently underwent another PCR assay to detect the presence of TevAT1 gene. *Trypanosoma evansi* was detected in 26.47% (18/68) blood samples in which distributed throughout the main islands of the country (4 from Luzon, 2 from Visayas and 12 from Mindanao). However, only 10 of these samples were positive for TevAT1 gene. Sequence alignment of the TevAT1 gene from local isolates showed no single nucleotide polymorphisms when compared to other strains in various countries. Those *T. evansi* without the gene of interest could be possibly resistant to some trypanocidal drugs but this needs to be further investigated in-vitro or in-vivo.

**Keywords:** *Trypanosoma evansi*, drug resistance, water buffalo

## Introduction

*Trypanosoma evansi*, the causative agent of surra, can infect almost all mammals. Horses are the most severely affected, however, in the Philippines, water buffaloes are the most generally infected [1]. Surra is present in 13 regions of the country particularly in regions II, III and IV in Luzon and regions IX, X and XI in Mindanao [2]. Tabanid flies are the primary transmitters of the disease and its bite is the most important mode of transmission of the disease in the Philippines [3].

Since its introduction in 1901, surra had pestered the Philippines causing economic losses amounting to 44.8 million pesos due to death, excluding losses from reduced reproductive performance, milk yield, loss of weight, and draught power [4]. As animals do not show pathognomonic signs and lesions, several techniques have been developed to detect the parasite, but these methods are time consuming, impractical and have low sensitivity [5]. Polymerase chain reaction (PCR) has now been widely used for trypanosome detection in many countries due to its high sensitivity and specificity. One of the several primer sets used is RoTat 1.2 gene encoding the variable surface glycoprotein (VSG) expressed during early, middle and late stages of the infection [6,7].

Because of possible lapses in parasite detection, preventive measures have been done to prevent further losses and avoid outbreaks of surra. Vaccines are not an option due to the large collection of

variable surface antigens of the protozoa. The nearly complete absence of extensive vector control programmes had led farmers to depend greatly on the few available drugs present in the market [8]. Resistance to drugs have now reached a severe level and is posing a serious problem to livestock productivity, not only in the Philippines but also in countries where it has been reported [9]. Resistance to drugs like diaminazene, suramin, quinapyramine and melarsomine ensues due to a lot of factors such as extensive use of drugs [10,11], changes in the drug concentration of the target site or alteration in the target or both [11,12], underdosing unsystematic program of treatments [9,13], and improper use of the few available trypanocides [11]. Through decades of use and misuse, *T. evansi* had started to develop resistance to drugs used commonly against it.

The loss or down-regulation of certain genes also plays a role in drug resistance of the *T. evansi*. The TevAT1 genecodes for P2 adenosine transporter which facilitates the uptake of trypanocidal drugs [14-16]. Drugs that accumulate in this transporter are diminazene aceturate (berenil and diamidine) and melaminophenyl arsenicals.

The objective of this study is to characterize the drug resistance-associated gene of *T. evansi* Philippine isolates. Through the objective of this study, the information obtained would allow the understanding as to why efficacies of the drug, not only in the Philippines but around the globe, are declining. Ultimately, knowledge on the presence of resistance genes to common drugs used by farmers and veterinarians in the Philippines against surra can possibly lead to innovative ways to prevent the disease and therefore, lessen its economic impact on livestock production.

## Materials and Methods

**Sample collection.** A total of 68 blood samples were collected from water buffaloes regardless of age, sex and breed from Luzon, Visayas and Mindanao islands. About 5 ml of blood was aseptically collected from the jugular vein and placed in heparinized vacutainers. The collected blood samples were then stored in an ice box and transported to the laboratory until use.

**Mouse inoculation.** A total of 4 Balb/c mice were used for inoculation of *T. evansi* isolates. Heparinised blood (0.25 mL) was inoculated intraperitoneally. After 48 hr, a drop of blood was collected from the tail of the inoculated mouse and examined by microscopy under ×400 magnification. Once the parasitemia level of the mouse reached a log of 9.0, the mouse was sacrificed and the liver and spleen were removed.

**DNA extraction and PCR**. DNA was extracted from the whole blood samples using Wizard Genomic DNA extraction kit (Promega, USA) according to the manufacturer's instructions. Extracted DNA was placed in 1.5 microcentrifuge tube and was stored at 4°C until use. For tissue samples, it was pre-treated by washing in 1× PBS twice, and centrifuged. Spin column protocol was employed for the DNA extraction (DNeasy Blood and Tissue Extraction Kit).

**RoTat 1.2 and TevAT1 Gene**. The DNA extract of each sample was subjected to PCR assay. Specific primers used for the detection of *T. evansi* and the drug resistance-associated gene are shown in Table 1. Template DNA (2µl) and PCR mix (8µl, double distilled water (3.4 µl), 5× PCR buffer (2µl), $MgCl_2$ (1µl, 25mM), dNTP (0.5µl), RoTat 1.2/TevAT1 primer pair (0.5µl) and *Taq* polymerase (0.1µl) was added. Thermal cycler was used to carry out PCR amplification. Cycling conditions of RoTat 1.2 gene were as follows: denaturation for 4 min at 94°C, followed by 40 amplification cycles of denaturation for 1 min at 94°C, primer-template annealing for 1 min at 59°C and polymerization for 1 min at 72°C followed by a final elongation step for 5 min at 72°C. For the TevAT1 gene, the cycling conditions were as follows: initial denaturation at 95°C for 3 min followed by 35 cycles composed of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and elongation at 72°C for 10 sec. After completion of PCR cycles, another elongation followed at 72°C and final cooling to 4°C. PCR products (3µl) were stained with GelRed® (CA, USA) and electrophoresed together in 2% agarose gel undertaken for 30 minutes using an electrical current of 120 volt and 400 mA with a 100 bp DNA marker.

**Sequencing and phylogenetic analysis.** PCR products were submitted for purification and sequencing at the First Base Laboratories, Malaysia. The obtained sequence of TevAT1 gene was compared with the available sequences in the GenBank using Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Center (NCBI).

Nucleotide sequences were deduced using genetic information processing software (MEGA 5). Phylogenetic analysis was performed using the

Table 1. Primers of RoTat 1.2 and TevAT1 genes

| Genes | Primers (5'>3') | Product Length (bp) | Reference |
|---|---|---|---|
| RoTat | F: GCGGGGTGTTTAAAGCAATA | 205 | [7] |
| 1.2 | R: ATTAGTGCTGC GTGTGTTCG | | |
| TevAT1 | F:GGATCCATGCTCGGG TTTGACTCAGCCAATG | 625 | [16] |
| | R:CTCGAGCCTGC ATAAACATGA CCAATCCA | | |

CLUSTALX program.Bootstrapping values were calculated using the modules SEQBOOT (random number seed: 123; 100 replicates), DNADIST (distance estimation: maximum likelihood; analysis of 100 data sets), NEIGHBOR (neighbor joining and UPGMA method; random number seed: 99; analysis of 100 data sets) and CONSENSE from the PHYLIP package, version 3.573.

## Results and Discussion

Using the PCR assay, *T. evansi* infection was detected in 26.47% of blood samples using the RoTat 1.2 primer set with 205 bp amplicon size (Fig. 1). Positive samples were found throughout the main islands of the country (4 from Luzon, 2 from Visayas and 12 from Mindanao) (Fig. 1).

Fig. 1. Geographical location of the blood sample collection sites shown in the Philippine map

Figure 2 shows the amplified PCR products of

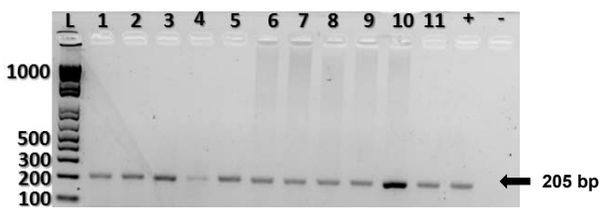


Fig. 2. Agarose gel stained with GelRed showing the PCR amplification products of *T. evansi* RoTat 1.2-specific primers with amplicon size of 205 bp. Lane L (100 bp ladder); lanes 1 to 11 (PCR products of *T. evansi* in Philippine water buffaloes); lane + (positive control) and lane – (negative control)

TevAT1 primer set with an amplicon size of 625 bp. Only ten out of 18 *T. evansi* isolates were found to be positive.

The obtained sequences of TevAT1 isolates of Luzon and Mindanao isolates were respectively composed of 582 to 585 and 514 to 580 nucleotides, respectively.The Philippine isolates were 100% homologous to Indian strain (Accession no. KF280206.1) and 99% homologous to the Thailand strain (Accession no. AB124588.1) as shown in the nucleotide sequence analysis. Further phylogenetic analysis revealed the similar relationship between the Philippine and Indian *T. evansi* isolates substantiated by their location in a single cluster.

Trypanocidal drug resistance is becoming a major problem in various countries with high rates of surra transmission and high levels of drug utilization [9,11]. However, it is unknown whether this increase is due to a higher incidences of drug-resistant strains or improper use of the limited trypanocidal drugs [9,11–13]. One of the most studied genetic reasons for resistance is the adenosine transporter gene [18]. TevAT1 gene codes for the P2 transporter that transports adenosine,

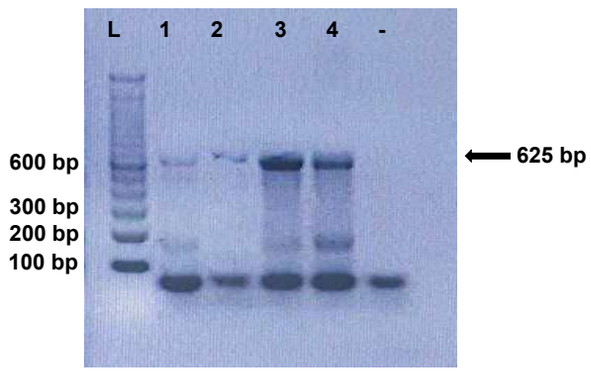


Fig. 3. Agarose gel stained with Gel red showing the PCR amplification products of *T. evansi* TevAT1-specific primers with amplicon size of 625 bp. Lane L (100bp ladder); lanes 1 to 4 (TevAT1 positive *T. evansi*) and lane – (negative control)

adenine, melaminophenyl arsenicals and diamidines [14–16,19–21]. It was actually the first drug transporter identified in trypanosomes [15,16,20,21] with the same identity (99.7%) as that of TbAT1 found in *T. brucei brucei* [16]. Isolates from Luzon and Mindanao were compared to both Indian and Thailand isolates and single nucleotide polymorphisms were not observed in the Philippine strains. This is in contrast with its counterpart gene in *T. brucei brucei*, the TbAT1 gene, where in single nucleotide polymorphisms were present [14,21,23]. Moreover, despite the wide geographical distances of collection sites in the Philippines, the isolates still produced the same monophyletic cluster in the phylogenetic analysis which explains the persistence of a particular strain of the parasite in these regions of the country [1].

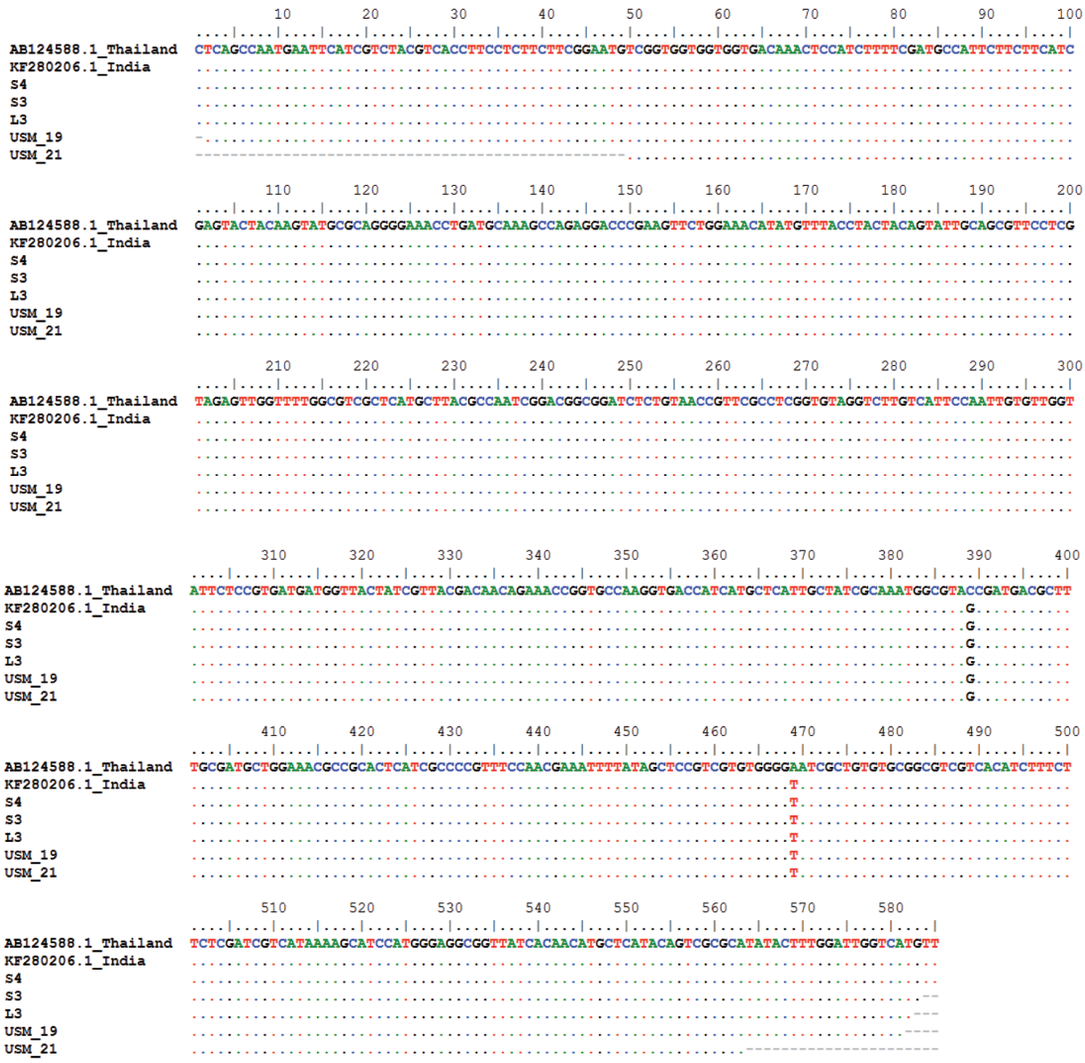


Fig. 4. Nucleotide sequence analysis of TevAT1 genes from *T. evansi.* The sequences of TevAT1 genes from Philippine isolates S4, S3, L3, USM_19 and USM_21 were aligned and compared with *T. evansi* from Thailand (AB124588.1) and India (KF280206.1)

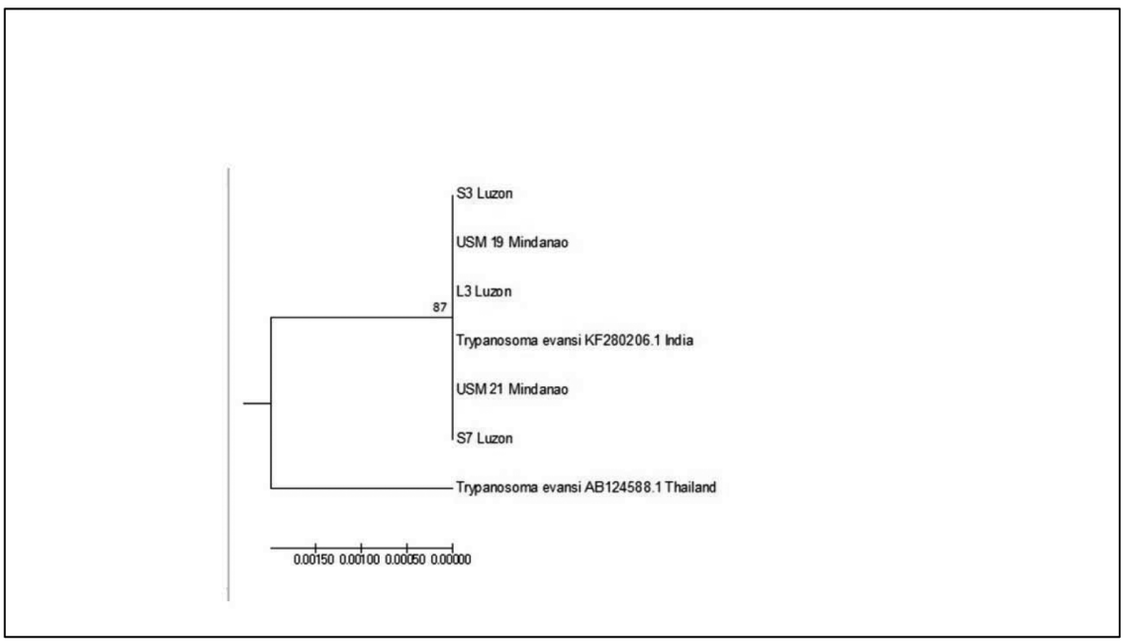


Fig. 5. The phylogenetic relationship of the five *T. evansi* isolates from Luzon (S3, L3 and S7) and Mindanao (USM19 and USM21), Philippines and other strains registered in GenBank (KF280206.1 from India and AB123588.1 from Thailand) based on the nucleotide sequence of TevAT1 genes. The length of horizontal bar indicates the number of nucleotide substitution per side.

To date, there are no studies regarding single nucleotide polymorphisms on TevAT1 but the down-regulation or knockout of this gene in some strains has been associated by other workers with the high resistance in melaminophenyl arsenical [15,19] and diminazene [16]. Thus, in the present study, the 8 isolates of *T. evansi* that failed to amplify TevAT1 gene could be possibly resistant to the aforementioned groups of drugs which form the most commonly used drugs in the Philippines along with naganol, suramin, isometamidium and quinapyramine [4]. The same findings were also found in TbAT1, wherein loss of this gene corresponds to drug resistance [22].

In conclusion, this study involved the detection of TevAT1 gene whose absence or downregulation is linked to resistance of *T. evansi* to trypanocidal drugs. Close association of the Philippine and Indian strains of *T. evansi* has also been found because of the nucleotide sequence homology of the TevAT1 gene from isolates in both countries.

It is recommended to examine more isolates from the different regions of the Philippines to further characterize *T. evansi*. It is highly suggested to perform an in-vivo or in-vitro assessment of the effects of commonly used trypanocidal drugs against *T. evansi* to provide an in-depth correlation with the gene detected. The use of other genes such as TeDR40 linked to drug resistance in *T. evansi* should also be explored.

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