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Molecular Identification of *Trypanosoma theileri* and Biology of Trypanosomes

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ABSTRACT

Trypanosoma theileri (*T. theileri*) is a non-pathogenic, cosmopolitan, and commensal protozoa of cattle. The main objective of the current study was to investigate the biology and feasibility of *T. theileri* as a model candidate for the discovery of a novel drug. In the present study, the isolates of *T. theileri* obtained from the Institute of Tropical Medicine (ITM) in SDM 79 were cultivated at 26^{o}C. Eight experiments with different inoculum and different times were grown. The growth curve was plotted to check the growth trends. The doubling time in the logarithmic phase was determined to be 17.43 hours. In addition, an experimental infection was done on a 3-month-old Holstein Friesian calf to isolate the blood-streaming shape; however, it was not successful after the blood buffy coat smear and PBMC culture in RPMI 1640 and HMI 9. Furthermore, the viability was determined by quantitative colorimetric Resazurin assay in 96-well fluorescence Microplates containing 0.4 to 2.4 mM of Resazurin. On the other hand, the response to Pentamidine (1-100 ng/mL) showed a strong negative correlation between the fluorescence signal and the highest Pentamidine concentration. IC_{50} was 9.25 ng/mL. Genomic DNA was extracted using the phenol-chloroform method. The gradient PCR amplification using *T. theileri* specific PCR (Tth625-PCR) primers was detected at 465 base pair (bp). In addition, the full-length 18S rDNA sequence was detected at 730 bp. In the silico analysis using common anti-trypanosome drug targets, no significant similarity could be found on either the DNA or the protein level. Nevertheless, homologous sequences have been identified among the drug targets for Ornithine decarboxylase. Therefore, the analysis might show the possibility of using *T. theileri* as a model for the search of new drugs once they have entire genome sequences. Analysis of the whole genome and transcriptome indicated a phylogenetic relationship between *T. theileri* and other pathogenic trypanosomes which can be the basis for novel drug development.

Keywords: Drug model, Novel drug, PCR, Resazurin, SDM 79, *Trypanosoma theileri*

INTRODUCTION

The trypanosomatid parasites cause one of the most notorious human and animal trypanosomiasis in all parts of Africa and South America. Even if trypanosomes are the main cause of diseases in humans (sleeping sickness) and animals (Nagana), many other species are not pathogenic (Mott et al., 2011). Such pathogenic trypanosomatids occur globally and infect a large number of hosts. Among these, *Trypanosoma theileri* (*T. theileri*) is ubiquitous, 'truly cosmopolitan' cattle protozoan commensal found worldwide (Mott et al., 2011 and Lee et al., 2013).

Natural infections could be found in all age groups of cattle although they are rare in cattle younger than one year old. Neither its life cycle nor its host relationship is fully understood in the mammalian host. The main vector responsible for the transmission of the parasite is Tabanidae. However, ticks including *Hyalomma anatolicum* and *Boophilus microplus* were also later reported as vectors (Latif et al., 2004). Hence, *T. theileri* is typically characterized by a stercorarian type of transmission (Latif et al., 2004). After ingesting infected blood, trypanosomes develop in the vector's hindgut. The infection is then transmitted to new hosts through fecal contamination of the mucus membrane or abrasions of the skin (Lukes, 2009). In the newly infected host, the epimastigotes multiply in the bloodstream by binary fission. Besides epimastigotes and large trypomastigotes in the peripheral blood, flagellates have also been found in extra-vascular sites of lymph nodes, kidneys, spleen, and brain (Braun et al., 2002).

In order to isolate pathogenic African Trypanosomes, a kit called KIVI (Kit for In Vitro Isolation of trypanosome) was designed (Aerts et al., 1992). Similarly, Verloo et al. (2000) proved that this kit can be used as an excellent device for isolating *T. theileri* with much higher sensitivity than the Roswell Park Memorial Institute (RPMI) medium. On the other hand, there was evidence that the growth of *T. theileri* on the RPMI medium could be easily confirmed (Lee et al., 2013). Many trypanocidal drugs are available in the market. Among these drugs, pentamidine, diminazene aceturate (Berenil), isometamidium chloride (Samorin), and ethidium bromide are important anti-trypanosomal drugs (Shapiro and Englund, 1990).

High-Throughput Screening (HTS) and virtual screening are used as a standard means in drug discovery to identify novel lead compounds that target a biomolecule of interest. However, the latter is considered a cost-effective means

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(Ekins et al., 2007; Fatumo et al., 2013). Editing of trypanosomatid RNA could be used to identify the drug target for protozoal parasites that cause diseases, such as trypanosomiasis. Amaro et al. (2008) reported that RNA-Editing Lgase-1 (REL-1) could be used as drug-like inhibitors of a key enzyme in the editing machine. The identification of inhibitors was done through a strategy employing molecular dynamics to account for protein flexibility (Amaro et al., 2008). New parasitic inhibitors had been identified due to the availability of an automated approach to high content microscopy (Alonso-Padilla and Rodriguez, 2014).

For better pharmacology hypotheses and tests, the development of computational (In Silico) methods plays a significant role. This methodology comprises pharmacophores, databases, quantitative structure-activity relationships, homology models, and other molecular modeling approaches, machine learning, network analysis tools, and data analysis tools using a computer.

Although *T. theileri* was not naturally pathogenic, it can cause disease in stressed cattle. Moreover, little is known about *T. theileri*. Recently, however, it has become an area of interest and is viewed as a tool and a vector for treating pathogenic microorganisms, particularly protozoan parasites (Mott et al., 2011). Furthermore, the mixed infection of *T. theileri* cause pathogenic trypanosome on the same host (cattle), and the presence of homologous sequences with specific sequences of anti-trypanosomal drug targets from pathogenic trypanosomes could lead to the use of this parasite (*T. theileri*) as a model candidate for the development of new drugs for the treatment of pathogenic trypanosomes. To this end, the basics of the parasite should be studied to manipulate the parasite as a tool to combat pathogenic trypanosomes. Furthermore, little is known about the biology, cell growth pattern, doubling time, and viability of the parasite. There is little convincing data to determine whether *T. theileri* could be used as a model for discovering new drugs for the treatment of pathogenic trypanosomiasis.

The general aim of the present research was to provide fundamental insights into the biology of *T. theileri* to verify its feasibility as a model organism for the discovery of the new drug. The specific objectives were cultivation of *T. theileri* both *in vitro* and *in vivo*, comparing and analyzing the growth pattern with others to check viability with Resazurin assay analysis and response to the drug, and determining the presence of homologous sequences between its genome and the specific target (conserved) sequence of anti-trypanosome drugs.

MATERIALS AND METHODS

Ethical approval

During the entire experimental period, the care and maintenance of the calf in its pen was performed based on the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. Furthermore, the experimental protocols were used after the approval by the Animal Research and Ethical Review Committee at the KU Leuven University (Permit No: P024/2017)

Parasites and *in vitro* culture

The sample used in the current study was *T. theileri*, kindly donated by the Diagnostic Parasitology Department of the Institute of Tropical Medicine, Belgium. These cryogenically preserved trypanosomes in culture were isolated from a small farm nearby Antwerp, Belgium by Verloo et al. (2000). Different growth media and conditions, under which other trypanosomes were grown in the institute, were assessed to determine the optimal conditions and favorable growth media for culturing the parasite. After identifying the appropriate medium (SDM 79) and the growth condition (at 26^{o}C, without CO_2), the cryostablate was seeded (1: 10 ratio) and propagated three times with SDM 79 medium (BioConcept AMIMED company, Switzerland) in a cell culture flask (25cm^2, Thermo Scientific™ Nunc™ Cell Culture Flasks). It was counted daily with a hemocytometer and recorded in the logarithmic table. A growth curve was then plotted to determine the growth pattern. In addition, the minimum and the maximum number of cells in the eight-cell culture flasks (eight experiments) that could serve as a potential indicator of the logarithmic phase and considered for both the experimental infection and Resazurin assay development were stored. Moreover, *T. theileri* was sub-passaged every three days. Epimastigotes were harvested in the exponential growth phase. Epimastigoteswas were centrifuged (1500 rpm for 10 minutes to sediment, washed, and re-suspended with phosphate buffer saline (PBS, pH = 7.2) before inoculation to the experimental calf while it was being used for DNA extraction.

Experimental animal and infection

A Holstein Friesian calf (aged three months) was randomly selected from the zootechnical Centre, KU Leuven, Belgium. It was confirmed to be trypanosome-negative by taking the blood sample and smear and culture in HMI-9 medium. After confirmation, it was inoculated with the sub-cultured epimastigotes of *T. theileri* (4.5 ×10^6/mL to 7.3 ×10^6/mL), intravenously (IV) through the jugular vein in a volume of 5-9 mL. In addition to parenteral inoculation, the calf was orally drenched with the same amount of inoculation. The calf was monitored and examined for parasitemia for three consecutive weeks. Blood samples (10 mL) were collected using Ethylenediaminetetraacetic Acid (EDTA) coated

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vacutainer tubes. The samples were collected two times after experimental infection (every week after experimental infection). After the blood samples were transported to the Host-Pathogen Interaction Laboratory of KU Leuven, Belgium, the samples were examined for the presence of trypanosomes using the standard parasitological methods of the wet and thin blood smear (stained with Giemsa), PBMC, and buffy coat technique within two to three hours of sampling per day (Murray et al., 1977).

The buffy coat/PBMCculture

After extracting the buffy coat (Murray et al., 1977) and PBMC (Ficoll-Paque method), the buffy coat samples were transferred to two cell culture flasks (25cm^2, Thermo Scientific™ Nunc™ Cell Culture Flasks) containing HMI-9 medium and RPMI 1640 supplemented with 10% fetal calf serum (FCS, Sigma) and 200 IU/mL of penicillin and 100µg/Ml streptomycin (Invitrogen, Carlsbad, CA) according to Hirumi and Hirumi (1989). A ratio of 1:10 (1 ml of buffy coat sample to 10 ml of the HMI-9 medium) was used. The inoculated cell culture flasks containing HMI-9 medium were then incubated at 37^{o}C and exposed to 5% CO_2. The tests were checked daily under an inverted microscope at 40 × magnification to monitor the progress of growth in both cell culture flasks inoculated for a maximum of two weeks.

***In vitro* sensitivity assays (Resazurin assay)**

A reagent, Resazurine obtained from Sigma-Aldrich, was used as a quantitative colorimetric assay based on the oxidation (blue) and reduction (pink) indicators to measure *T. theileri* viability and its response to pentamidine. Resazurin stock solution (0.4 to 2.4 mM) was prepared in PBS withpH 7and filter-sterilized (Miriam et al., 2006).

Optimization of Resazurin to Trypanosoma theileri viability

To reduce the background signal for a better sensitivity assay and also to avoid light piping between wells, the Black Microtiter® 96-Well Fluorescence microplates were used. Cells ranging from 5.2 ×10^5/mL to 8.5 ×10^6/mL with a logarithmic phase were obtained after incubation in cell culture flasks at 26°C for 48 hours. Then, 120 µL cells of *T. theileri* were removed from this cell culture flask into wells of Microtiter® 96-Well Fluorescence Microplates (Thermo Scientific) and incubated again at 26^{o}C for 48 hours. After 48 hours of incubation, 20 µl of different concentrations of Resazurin solution (0.4 to 2.4 mM) was added to each inoculum while an equivalent amount of SDM 79 medium was added for blank wells. The plates were returned to the incubator for 24 hours for optimal oxidation and reduction. The Fluorescence signal was read 1, 3, 5, 7, 18, and 24 hours after the addition of Resazurin by dual-wavelength using a GFP protocol-VICTOR™ X Series Multi-label Plate Reader (Perkin Elmer Instruments Inc.) at λ_{exc}485, and λ_{em} 535 nm. A single micro-titer plate was used for three different concentrations of Resazurin per experiment. The background was subtracted from each reading. The experiments were performed three times and an average was taken.

Standard curves

Following incubating epimastigotes in the range of 1.3 ×10^6 to 5.3 ×10^6 epimastigotes/mL for 48 hours, 120 µL of epimastigotes /mL per well were seeded to a 96 well microtiter plate for further 48 hours of incubation at 26^{o}C. Then, 20 µL of 2.4 mM Resazurin was added followed by fluorescence signal reading after 7 hours of incubation. This procedure was performed twice in all three times.

Drugs sensitivity assay

Pentamidine was dissolved in concentrations from1.0 to 80ng/ml. In each wellofCostarTM 96-well microtitre plates, 120µl of epimastigotes of *T. theileri* in the logarithmic phase (1.4-5.3 ×10^6/mL) was seeded with 20 µL of different concentrations of pentamidine (1.0 to 80ng/ml). Three drug concentrations were tested per plate (column 1-3 with 1.0ng/ml, column 4-6 with 40 ng/ml, column 7-9 with 80 ng/ml pentamidine, column 10 without the drug, and column 11SDM 79 medium used as control medium). The plates were then incubated with pentamidine for 48 hours. The trypanosome density was counted with a hemocytometer up to 48 hours of incubation before the addition of Resazurin. Afterwards, the plates were incubated by adding a 20 µL Resazurin to each well and incubated at 26°C for an additional seven hours. IC_{50} values were also calculated at concentrations 1-100 ng/mL using this assay and microscopic counting. Columns 1-10 were tested for drugs, column 11 was without the drug, and column 12 included only SDM 79 as the control medium.

Data Evaluation and analysis

The plate was read at an excitation wavelength of 485 nm and an emission wavelength of 535 nm in a fluorescence/microplate reader (GFP protocol-VICTOR™ X Series Multi-label Plate Reader (PerkinElmer Instruments Inc.). The data were transferred into a graphics program (Excel) and analyzed using the GraphPad Prism 7.0. Descriptive

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statistics and Pearson r^2(r) correlation coefficient were also calculated. To measure the anti-epimastigotes activity (%AE), the following formula according to Miriam et al. (2006) was used.

$$\mathbf{AE}\ (\%) = \frac{(\mathbf{Gc-Gp})}{\boldsymbol{Gc}} \times \mathbf{100} \quad \text{(Equation 1)}$$

Where, Gc represents the mean number of parasites per milliliter in the control, and Gp shows the mean number of parasites per milliliter according to the different doses of drugs.

Furthermore, the doubling time was calculated based on the following equation used for *T.b.brucei* by Sykes and Avery (2009) and Melissa et al. (2009).

$$\mathrm{TD} = (\mathrm{t2} - \mathrm{t1})\frac{\log(2)}{\log(\mathrm{q2/q1})} \text{(Equation 2)}$$

Where, Td refers to doubling time (q1), t1 is the first quantity for the first time, and (q2) at the time (t2) denotes the second quantity at the second time.

Genomic DNA extraction and PCR amplification

A *T. theileri* culture in a high density of the logarithmic phase ranging from 3.6 $\times 10^6$ cells/ml to 7.8 $\times$ 10^6 epimastigotes/mL was utilized to extract genomic DNA. Both the phenol-chloroform method and DNeasy®Blood and Tissue Kit (Qiagen, Hilden, Germany) were used. The concentration and purity of the DNA were determined with NanoDrop ™Spectrophotometer (Thermo Fisher Scientific) and agarose gel electrophoresis (1%).

DNeasy®Blood and tissue kit

The genomic DNA extraction was conducted according to the protocol recommended by the manufacture Purification of Total DNA from Animal Blood or Cells (DNeasy 96 Protocol), DNeasy®Blood and tissue kit (Qiagen, Hilden, Germany).

Phenol-chloroform method

Cultivated *T. theileri* cells (3.6$\times$ 10^6 cells/ml to 7.8 $\times$ 10^6 cells/ml) in 2ml Eppendorftubeswere lysed by centrifugation at 13000 rpm for a minute. The supernatants were removed and 500 µL of T10N150E10wereadded and mixed thoroughly by pipetting several times. The content was then centrifuged at 13000 rpm for 10 minutes and the supernatant was removed, followed by the addition of500 µL of $T_{10}N_{150}E_{10.}$ This step was repeated twice.

Eight hundred microliter of freshly prepared Glouton-Buffer (10mM Tris HCl, 10 mM EDTA, 100mM NaCl, 10% SDS, 3.9Mm DTT) were added and mixed well by pipetting up and down. It was then incubated at 65^{o}C for one hour. Afterwards, 20µl of 10 µg/mL proteinase K was added and incubated at 56^{o}C overnight.

An equal volume(1000 µL) of phenol/chloroform/isoamyl alcohol in the ratio of 25:24:1 was added and mixed gently. After centrifuging at 13000 rpm for 10minutes, the supernatant was transferred to a new tube. The same amount of phenol/chloroform was added to a new tube, mixed, and centrifuged as above-mentioned. The supernatants were transferred to a new tube again and the same amount of chloroform was added, mixed, and centrifuged as indicated above. The aqueous layer (500 µl) was transferred to a new tube and mixed gently with 1166 µL 100 % Ethanol. The supernatants were removed after centrifugation at 13000 rpm for 1 minute. The tubes were left open to dry DNA for 3 hours, then it was resuspended with 30 µL of Mili-Q water.

PCR amplification and gel electrophoresis

Species-specific PCR identification was performed using the following primers in Table 1 as described by Rodrigues et al. (2003) and Lee et al. (2013). Amplification was conducted using 25 µL reaction mixture with 200 ng template DNA (genomic DNA), 2.5 IU (International Unit) TagDNA polymerase (Promega, U.S.A), 0.2 mM dNTP, PCR buffer, 1.5 mM $MgCl_2$, (Promega, U.S.A), and 0.3µM of primer, according to the manufacturer's instructions. For gradient PCR Amplification, the procedure involved 95°C for 5 minutes, followed by 20 cycles of 94°C for 10 seconds, 53°C for 15 seconds, and 67°C for 1 minute, with a final extension at 72°C for 3 minutes.

Finally, PCR products were run on 1% agarose gel (Sigma) electrophoresis using 1× TBE buffer and stained with 7µL ethidium bromide in 50 mL agarose (0.5 ng/ mL) and made visible using UV transillumination (Vilmar Lourmat).

Table 1. Primers used to detect *Trypanosoma theileri*

	Primer (Forward and Reverse)	Purpose
1	Tth625a (5'-CCG CTG GAG CTA AGA ATA GA-3') and Tth625b (5'-AAT TGC ATA AAC ACA GCT CCC-3')	For species-specific PCR amplification (Tth625-PCR)
2	Forward primer 18STnF2 (5'-CAA CGA TGA CAC CCA TGA ATT GGG GA-3') and Reverse primer 18STnR3 (5'-TGC GCG ACC AAT AAT TGC AAT AC-3')	The full length 18S ribosomal DNA sequence Analysis
3	Kin1 reverse (5'- GCG TTC AAA GAT TGG GCA AT-3') and Kin2 forward (5'-CGC CCG AAA GTT CAC C-3')	For single PCR amplification to anneal internal transcribed spacer of ribosomal genes (ITS) sequence

Source: Rodrigues et al. (2003) and Lee et al.(2013)

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***In silico analysis by* multiple sequence alignments**

Multiple Sequence Alignments (MSA) were conducted with commonly used anti-trypanosomal drug target sequences (Table 1) from pathogenic *Trypanosoma* species and *T. Theileri* genome/proteome using FFT in ExPASy tool, Switzerland (Rodrigues et al., 2003). In addition, phylogenetic trees were created to study the relationships between the different drug targets and the *T. theileri* genome/proteome, as well as between the drug target sequences.

Risk analysis

The *T. theileri* is a non-pathogenic parasite and does not cause disease in either livestock or humans. There is no biological hazard to the calf from parasite inoculation. Therefore, there are no biochemical hazards for the farm and environment when carrying out such an experiment.

RESULTS

Growth medium and conditions

Different types of cell culture media commonly used to culture other types of *Trypanosoma* in ITM were screened to assess the optimal and conducive conditions to culture *T. theileri* epimastigotes. Cryogenically preserved 2.5× 10^6 epimastigotes/mL were seeded into a cell culture flask (25cm^2, Thermo Scientific™ Nunc™ Cell Culture Flasks) in four different media and growth conditions (Figures 1 and 2). It was seeded at a 1:10 ratio and sub-passaged every three days for two consecutive weeks. Finally, the optimal conditions and favorable growth media for *T. theileri* epimastigotes were identified. As a result, significant growth of the *T. theileri* could be observed in SDM 79 at 26°C without CO_2, and RPM'3I 1640 at 37°C with 5 % CO_2 with 10 % Fetal Calf Serum (FCS), as shown in Figures 1 and 2, respectively. However, relatively slower growth was observed for the later third day. On the other hand, there was a prominent growth in RPMI 1640 with 10 % FCS than other serum types used (Figure 2).

Growth pattern and doubling time

After inoculation (2.5 × 10^6 epimastigotes/mL) of *T. theileri* in eight flasks containing SDM 79 with10 % FCS, the growth pattern was determined by counting 10 µL from each flask daily with a Haemocytometer. As indicated in Figure 3, maximum growth was observed on the sixth day in the entire eight-cell culture flask, except for the seventh experiment, which took place on the seventh day. The maximum number of epimastigotes that could be grown among the eight flasks was estimated to be 1.7 × 10^7cells/mL (Experiment 2). The doubling time was calculated per day assuming they were in the logarithmic phase from the third to sixth day as indicated in Figure 3. Since there was a significant growth of the parasite in this period, from 3.2× 10^4 cells/mL, which was the lowest on the third day from the second experiment to the highest 1.3×10^7 cells/mL from the same flask on the sixth day. The calculation was based on the manipulation of the doubling time equation used for *T.b.brucei* from Sykes and Avery(2009) and Melissa et al. (2009) as described in materials and methods. Therefore, the doubling time calculated in the logarithmic growth phase averaged 17.43 hours (0.73 day) for eight experiments.

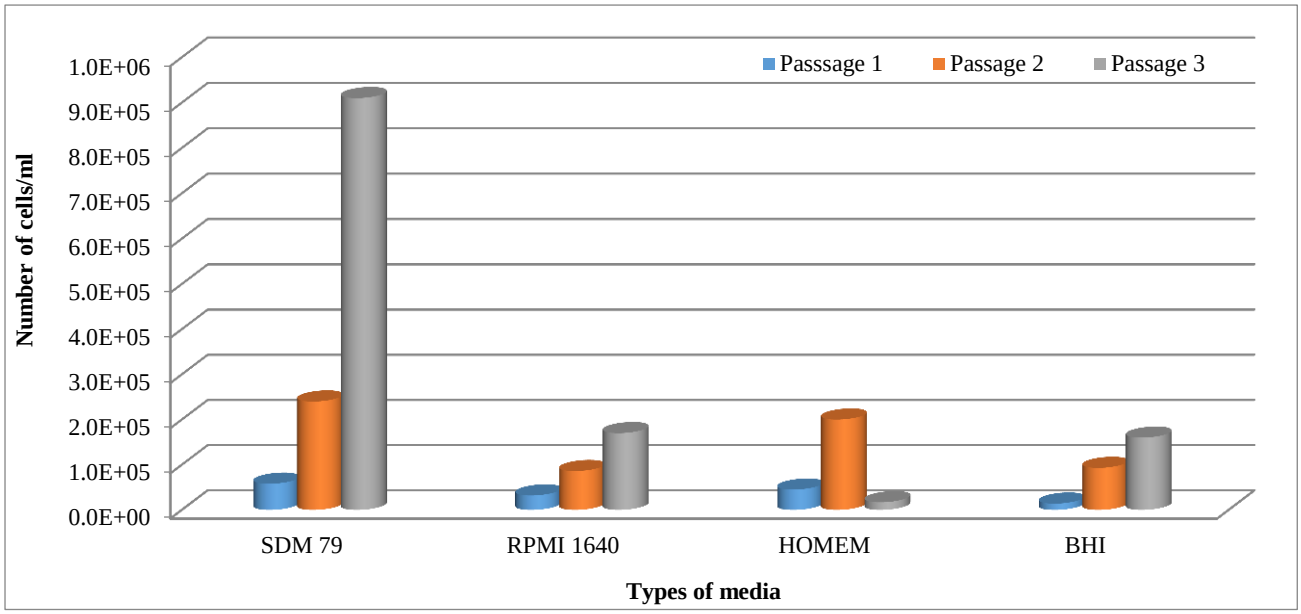

Figure 1. Growth of *Trypanosoma theileri* with various media at 26°C without CO_2 in cell culture flasks. It was performed at the Institute of Tropical Medicine Antwerp, Belgium for two consecutive weeks

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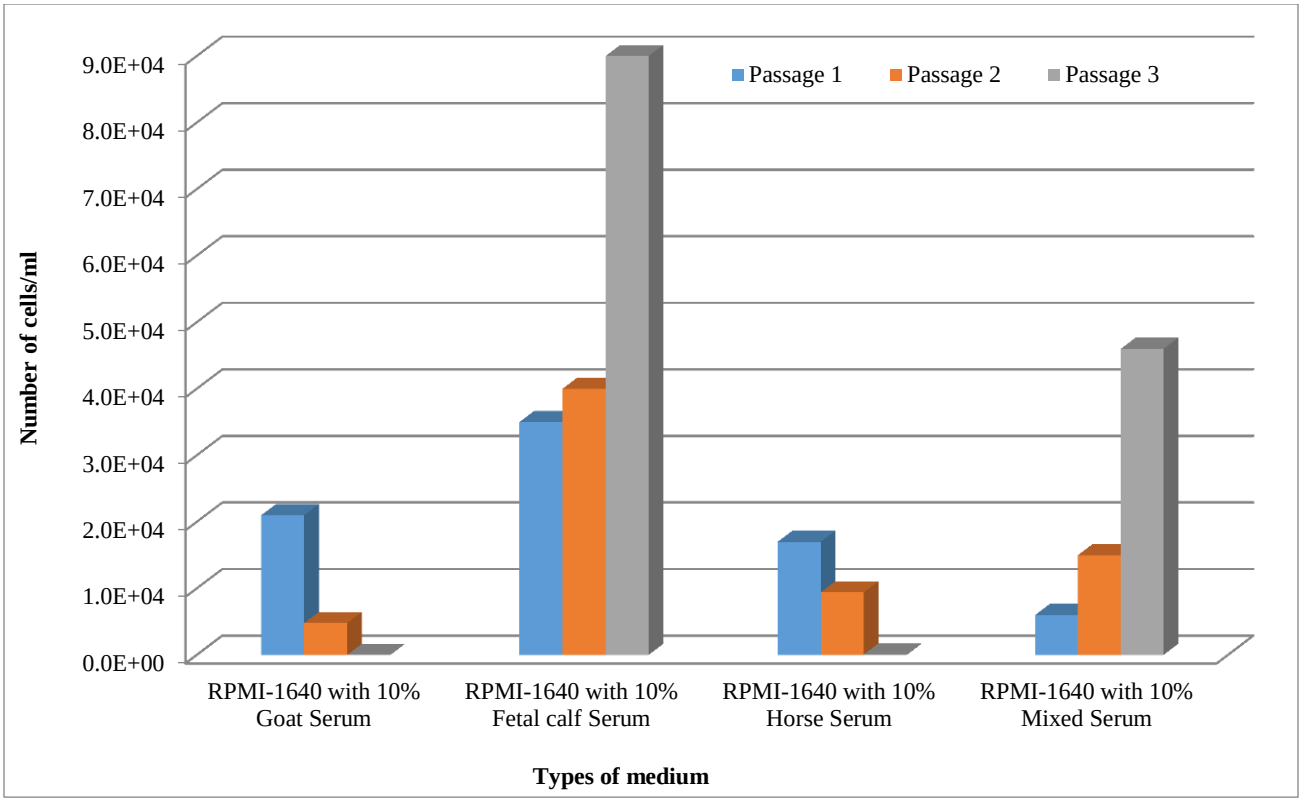

Figure 2. Growth of *Trypanosoma theileri* with RPMI 1640 in different serum types at 37°C with 5 %CO_2 in cell culture flask at ITMor two weeks

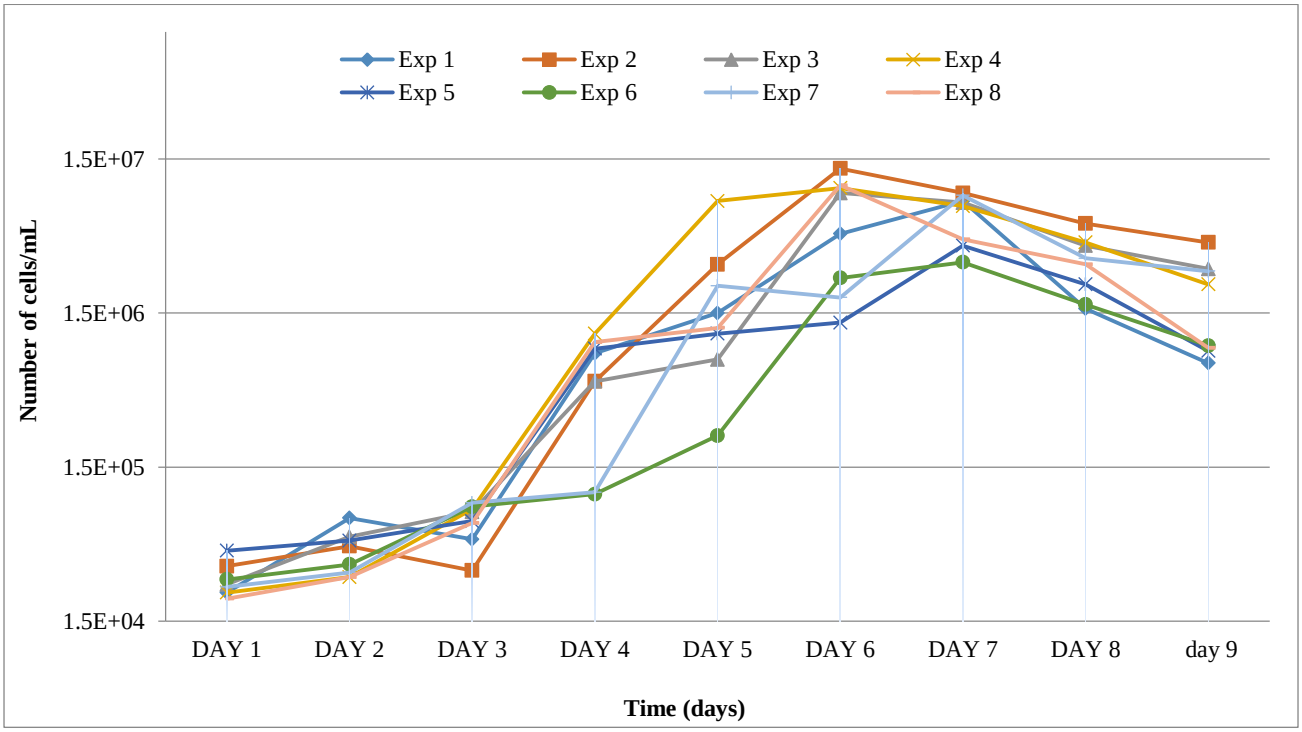

Figure 3. The growth pattern of *Trypanosoma theileri* in eight tissue culture flasks considered as experiments for nine days incubated in SDM 79at 26°C without CO_2. Exp: Experiment

To assess the morphology and the relative difference between other stages, Giemsa and DAPIstains (4′,6-diamidino-2-phenylindole) were performed on slides. Some of the slides were stained at ITM and some others in the Host Interaction lab, KU Leuven, Belgium (Figure 4).

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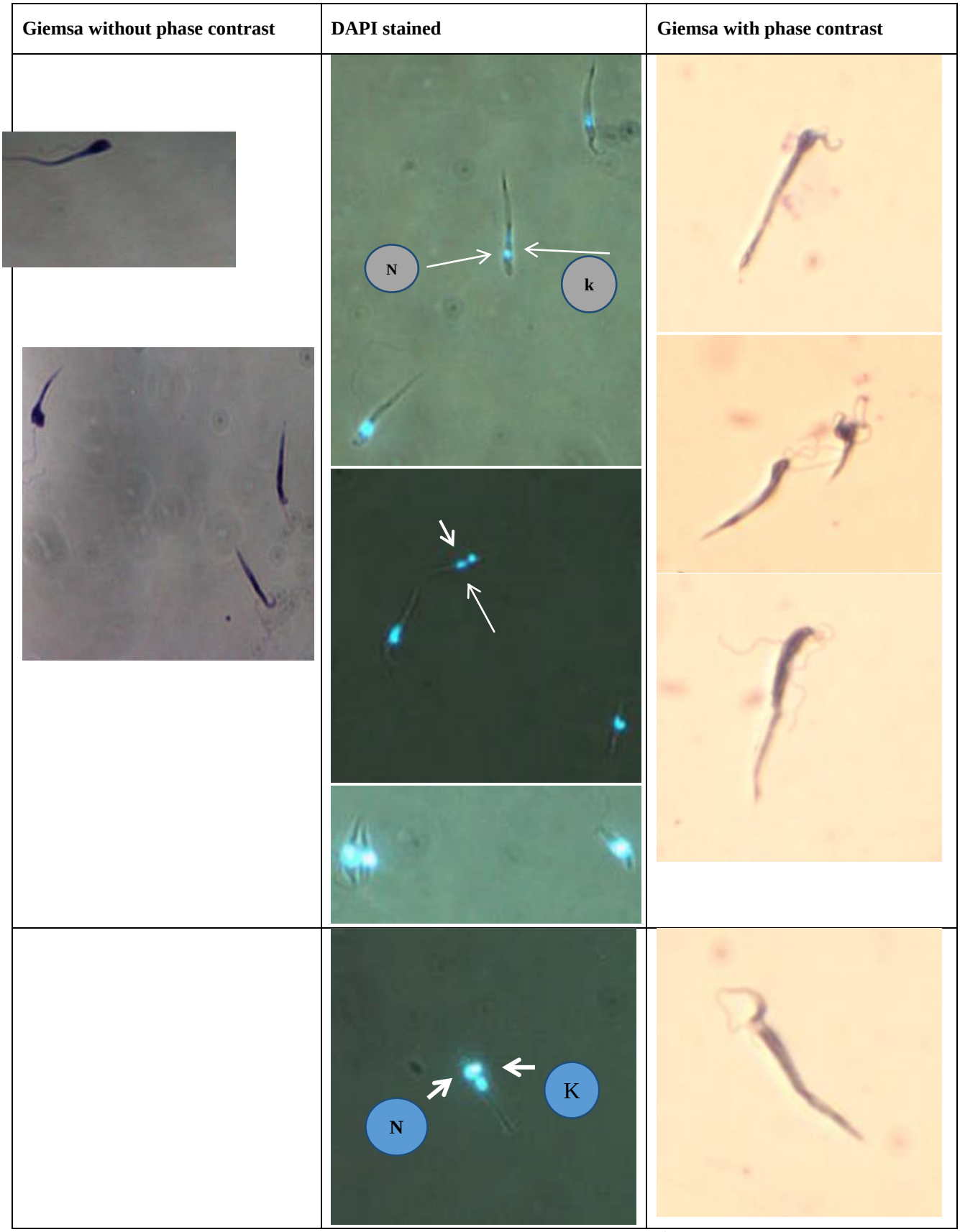

Figure 4. Light microscopy of *Trypanosoma theileri* epimastigotes cultured at 26°C (Giemsa and DAPI stained, 400 × magnifications). A kinetoplast is anterior to the nucleus, unlike a trypomastigote which has a kinetoplast posterior to the nucleus. N: Nucleus, K: Kinetoplast.

Resazurin assay

After optimization, *T. Theileri* was grown in microtiter plates and reached the level to produce a detectable fluorescence signal by incubation with 20 µL of each Resazurin solution. The magnitude of fluorescence increased remarkably up to7 hours, after which the fluorescence saturation occurred, particularly at 3.5×10^6 epimastigotes /mL, which had the highest density and then gradually decreased (Figure 5).

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A statistically significant difference in the fluorescence of Resazurin solution at three concentrations (0.4 mM to 2.4 mM) was observed ($p < 0.05$) with an increase in the number of parasites and the incubation time (7 hours) during the assay (Figures 5 and 6). There was a positive correlation (r=0.75 to 0.925) between the magnitude of fluorescence of various cell densities and the three Resazurin concentrations (Table 6). However, the growth declined from 3.5×10^6 to 7.9×10^6 epimastigotes/mL, which indicated that the epimastigote was reaching the stationary phase. Hence, the upper limit of the assay was a plating density of 3.5×10^6 epimastigotes/ml (Figure5).

Trypanosoma theileri were seeded with 1.6×10^6 epimastigotes/ml, which resulted in a low level of fluorescence on the first reading (in the first hour after the addition of Resazurin) at the beginning of the assay process. Then, it gradually increased and a maximum signal was received seventh hours after adding Resazurin, and then significantly declined until the last reading (24 hours after adding Resazurin, Figure 6). Overall, the fluorescence signal of Resazurin solutions had a positive correlation (r = 0.75 to 0.925) with cell density and incubation time. There was a statistically significant difference between Resazurin fluorescence and the number of epimastigotes and also with reading time after incubation ($p < 0.05$, Table 6).

There was a positive, and linear correlation between the fluorescence and density of epimastigote at 1.6×10^6 cells/mL for a 2.4 mM Resazurin concentration at7hours of incubation (r = 0.8297), compared to the other two concentrations of Resazurin (Table 3). After incubation of 2.4 mM Resazurin at 26°C for 7 hours, a very high and linear association (r = 0.9876) in the range of 1.3×10^6 to 5.3×10^6 was observed in the fluorescence signal of the parasite (Table 3). For a comparative demonstration of the Resazurin based colorimetric assay, 1.8×10^6 epimastigotes/Ml (300 µL/well) with 2.4 mM (25 µL) of Resazurin was seeded in a Corning® 24-well culture plate (Sigma Aldrich) containing to appreciate the color changes from blue to pink. The test showed a gradual colorimetric change in the three concentrations of Resazurin and one control (Figure 8).

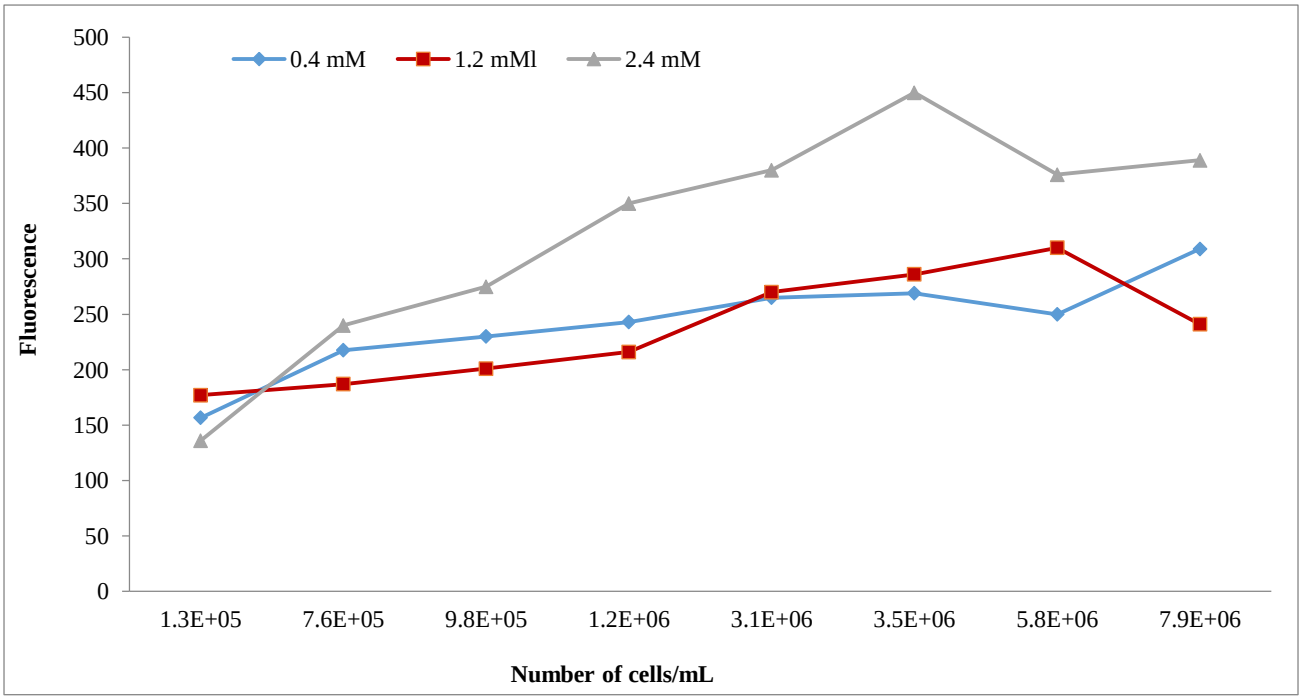

Figure 5. The relationship between the different fluorescence concentrations of Resazurin and the growth of epimastigote cultures after 7 hours of incubation time

Table 2. Statistical values of fluorescence for different cell concentrations concerning Resazurin concentrations

	Cells/ml and their respective fluorescence								r-value	P-value
Resazurin	$1.3X10^5$	$7.6X10^5$	$9.8X10^5$	$1.2X10^6$	$3.1X10^6$	$3.5X10^6$	$5.8X10^6$	$7.9X10^6$		
0.4 mM	156.63	217.6	230	243	280	292	268	290	0.752	0.0315
1.2 mM	145	187	201	216	255	310	298	341	0.925	0.0010
2.4 mM	184	240	275	341	380	489	450	490	0.865	0.0056

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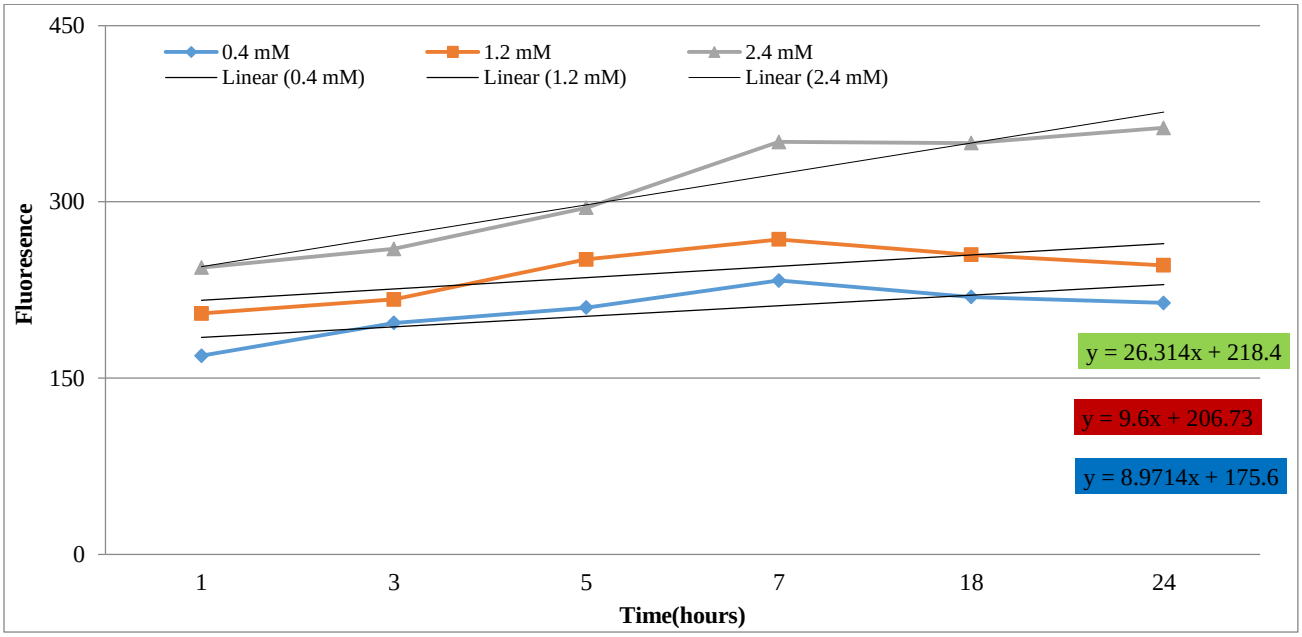

Figure 6. Fluorescence of Resazurin concerning incubation time and cell density of 1.6×10^6 epimastigotes/mL of *Trypanosoma theileri*. All experiments were performed two times each in three duplicates, and average values were taken.

Table 3. Correlation between fluorescence signal and an incubation time of *Trypanosoma theileri* epimastigote

	Time (Hours)						r -value	P -value
Resazurin	1	3	5	7	18	24		
0.4 mM	169	197	210	233	219	214	0.5066	0.3051
1.2 mM	205	217	251	268	257	246	0.4851	0.3295
2.4 mM	244	260	295	351	350	363	0.8297	0.0410

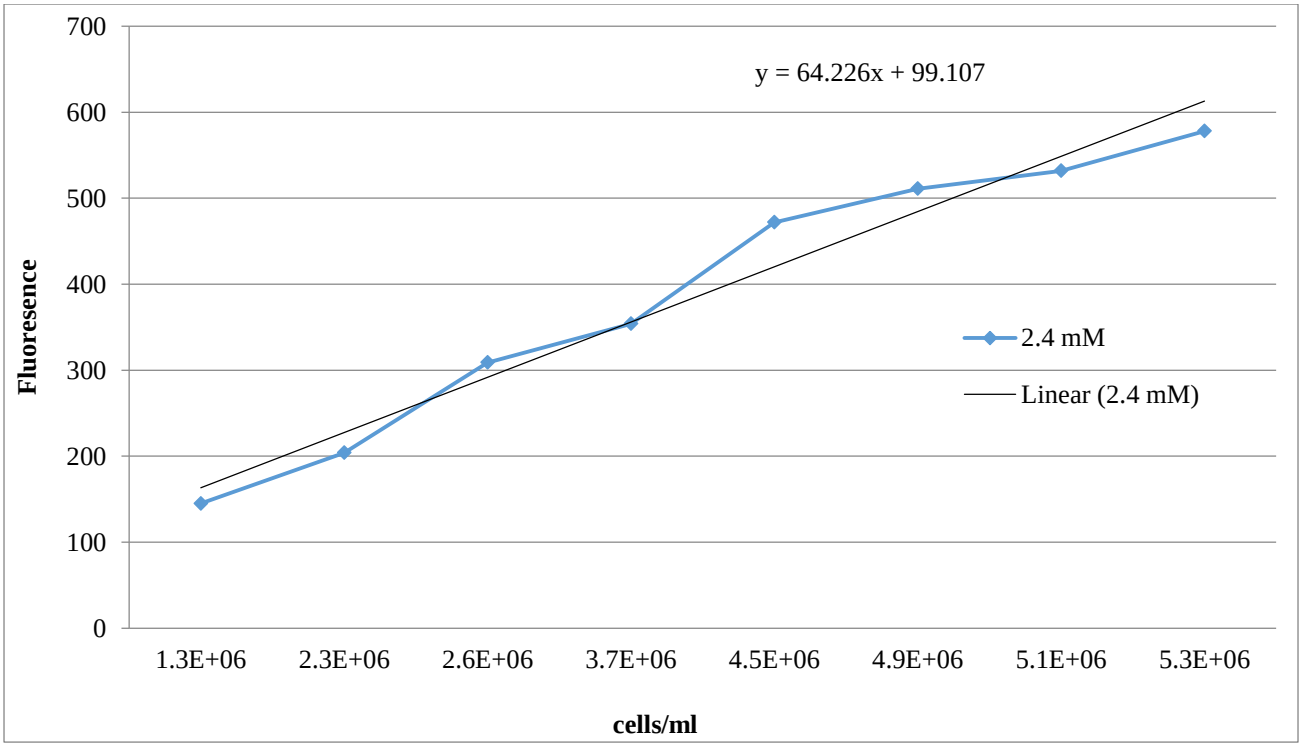

Figure 7. Standard curves for Resazurin fluorescence and the number of epimastigotes in the logarithmic growth phase, r = 0.9876, $p < 0.05$

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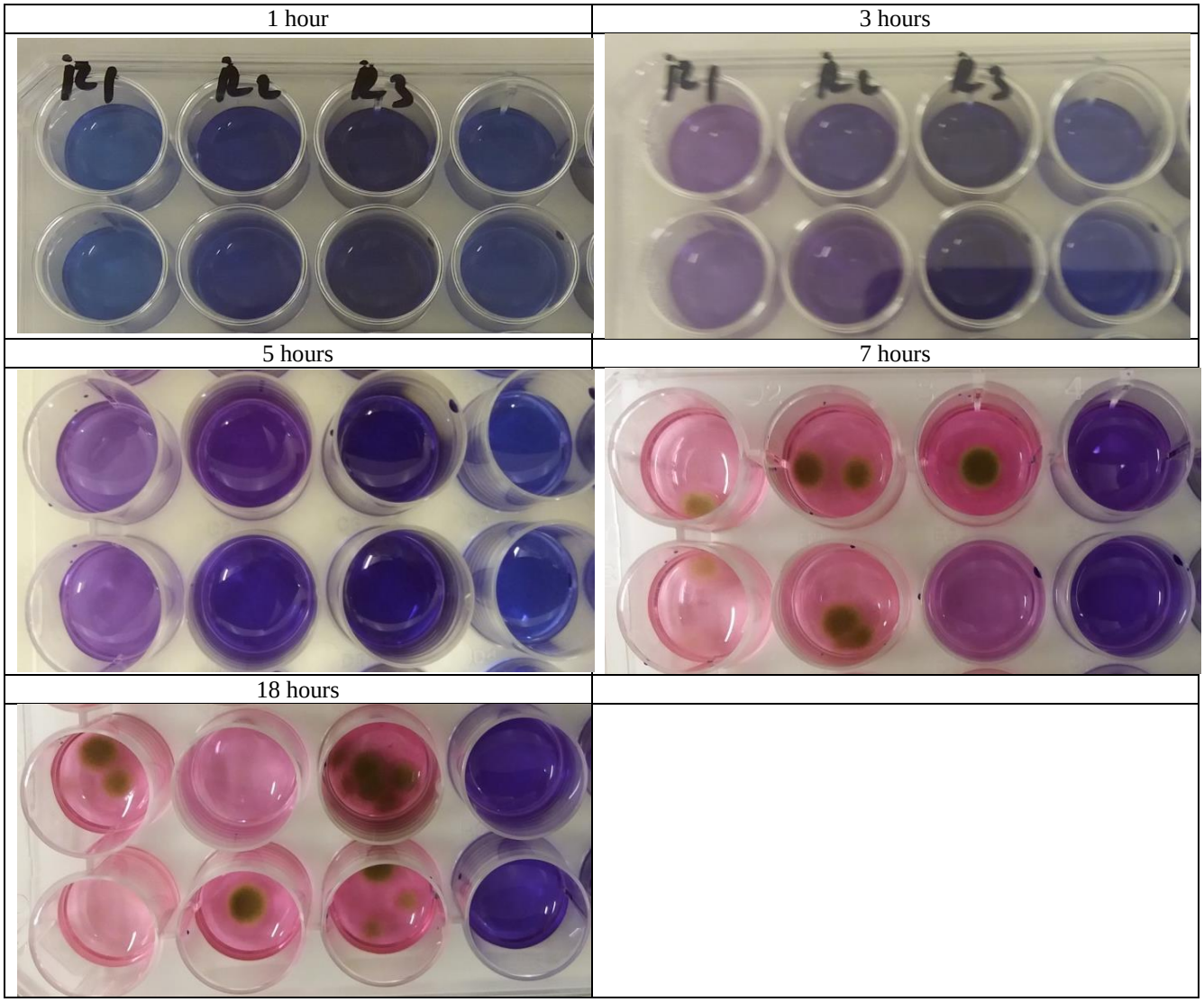

Figure 8. A plate to see the colorimetric changes of Resazurin (2.4 mM) in each reading time after incubation with pentamidine (80 ng/mL)

Drug sensitivity assays

Based on the optimized Resazurin assay for cell density, Resazurin concentration, and incubation period (Figure 5), Resazurin was deployed for the sensitivity of *T. theileri* epimastigote to pentamidine. Accordingly, Resazurin solution at a concentration of 2.4 mM was used to assess the response of *T. theileri* epimastigotes to different concentrations (1-80 ng/mL) of pentamidine (Figure 9). Therefore, there was a negative correlation (-0.8826) between the reduction in fluorescence signal and a significant increase in drug concentration ($p < 0.05$), as described in Figure 9and Table 4.

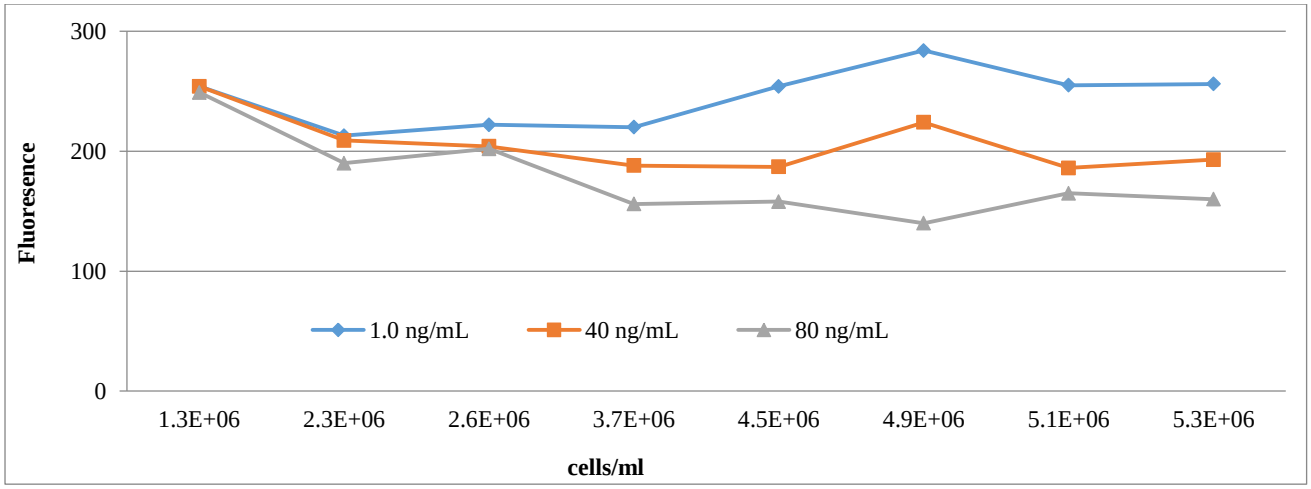

Figure 9. The reduction in fluorescence of epimastigotes of *Trypanosoma theileri* due to the pentamidine at different concentrations using 2.4 mM Resazurin solution

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Table 4. Signals from Resazurin (2.4 mM) after addition of three different concentrations of pentamidine

	Cells/mL with its respective fluorescence								r value	P value
Pentamidine	1.3×10^6	2.3×10^6	2.6×10^6	3.7×10^6	4.5×10^6	4.9×10^6	5.1×10^6	5.3×10^6		
1ng/ml	254	213	222	220	254	284	255	256	0.533	0.1740
40ng/ml	254	209	204	188	187	224	186	193	-0.649	0.0817
80ng/ml	249	190	202	156	158	140	165	160	-0.8826	0.0037

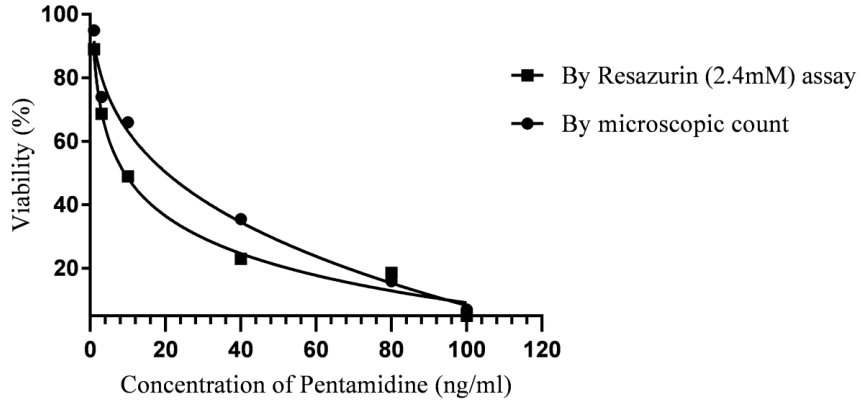

Figure 10. Viability of epimastigote after exposure to Pentamidine (1-100ng/mL) using Resazurin and microscopic counting

The role of Resazurin colorimetric assay was compared by calculating percentages of anti-epimastigotes (% AE) activity of pentamidine(1 to 100 ng/mL). For this purpose, a microscopic count was done and calculated based on Equation 1as described in materials and methods. As indicated in Figure 10, the Resazurin assay and microscopic count were compared. As a result, there was a significant reduction in the percentage of viability of the epimastigotes as the pentamidine concentration increased after 48 hours of contact time. IC_{50} values of 9.25ng/mL and 16.29 ng/mL were determined using Resazurin ($r = -0.957$; $p < 0.05$) and manual counting ($r = 0.90$, $p < 0.05$), respectively.

Experimental infection of *Trypanosoma theileri*

Before inoculating a calf with *T. theileri*, blood samples were taken to check whether there was a natural infection. No *T. theileri* was confirmed either in Giemsa stained slides or by culturing PBMC and buffy coat in RPMI 1640 and HMI 9 medium. The calf was inoculated intravenously through a jugular vein with a density of 4.5×10^6/mL to 7.3×10^6/mL in 5-8ml. Moreover, the same amount and concentration were administered orally. Experimental infections were performed three times.

PCR confirmation of *Trypanosoma theileri*

WithDNeasy®Blood and Tissue Kit (Qiagen, Hilden, Germany) a very low concentration of DNA could be achieved. As a result, a higher DNA concentration (16.5-274 µg/m) could be extracted using the phenol-chloroform method. Gradient PCR amplification using *T. theileri* specific PCR (Tth625-PCR) revealed the 465 bp amplification product (Figure 11). In addition, the full-length 18S ribosomal DNA sequence of *T. theileri* DNA was detected at 730 bp (Figure 12). They were stained with ethidium bromide in 1% agarose gel. A DNA ladder (100 bp) was used on the left side of the gel. The PCR amplification for the third primer (for annealing the Internal Transcribed Spacer (ITS) of the sequence of the ribosomal gene) was not included due to the poor image quality.

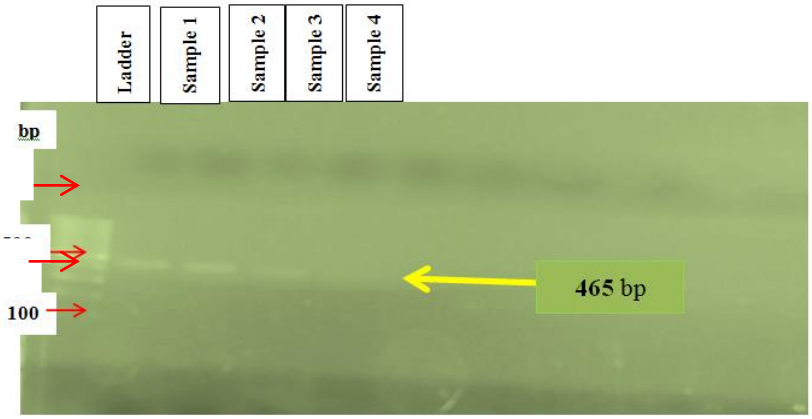

Figure 11. Detection of PCR of *Trypanosoma theileri* DNA from the cultured epimastigote using species-specific Tth625-PCR primers. The products were separated on a 1% agarose mini-gel

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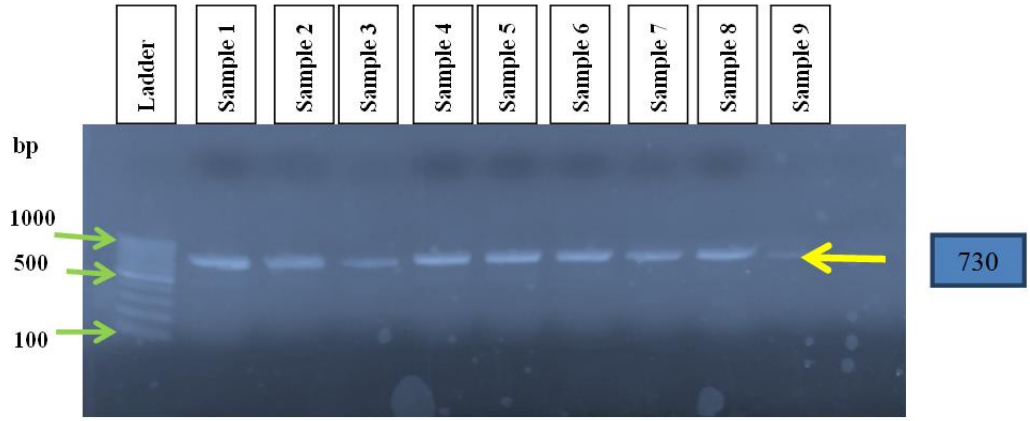

Figure 12. Detection of PCR of *T. theileri* of 18S ribosomal DNA from the cultured epimastigote using 18STnF2 and 18STnR3. The products were separated on a 1% agarose mini-gel

In silico analysis

Multiple Sequence Alignment of the sequences of commonly used trypanocidal drug targets (Figure 13) with *T. theileri* nucleotide found in the NCBI database was run. Multiple Sequence Alignment was performed at both the genomic (DNA) and protein levels. The Basic Local Alignment Search Tool (BLAST) and Clustal W program (from ExPASy bioinformatics resource portal) was run to demonstrate homology between *T. theileri* genome/ proteome and /or drug target sequences. Based on the determined protein/nucleotide sequence, phylogenetic trees were constructed to find out the possibility of evolutionary relationships. At the genomic (DNA) level, no significant similarity was found. However, to a lesser extent, a similarity of 294/392 (75%) was observed between *T.grayi* cathepsin L-like protein (CATL) gene (NCBI Gene bank accession No: XM_009318006.1) and HQ664735.1 of *T. theileri* isolate Tthb19 clone 3 cathepsin L-like protein (CATL) gene. The Cluster W alignment of these two similar genes from both species of *Trypanosome* was is described in Figure 14.

On the other hand, MSA among anti-trypanosome drug targets excluding *T. theileri* showed that there was above 99.85% identity among accession number of XM_824336.1(100%), J02771.1(99%), and AF042286 (99%). These listed accession numbers all indicated the Ornithine decarboxylase gene in *T.brucei*. In addition, the phylogenetic tree showed that these were similarities with accession Numbers: DQ887563.1 and XM_009318006.1, which both described the topoisomerase gene from *T.congolense* and the CATL gene from *T. grayi*, respectively (Figure 15).

CLUSTAL format alignment by MAFFT FFT-NS-i (v7.215)

```
XM_009318006.1  AACGGCGGCTTGATGGACGACGCCTTCACATGGATCATCCAGGACCACAACGGCACGGTG
HQ664735.1      AATGGCGGCTTGATGGACGACGCCTTCCAGTGGCTCGTGGATTCGAACAAGGGCAAGGTG
                **.************************  .*** **.*  *     **** **** ****

XM_009318006.1  GACACAGAGGCCAGCTACCCCTACGTCTCGGGCGCGGGCTACTCCCCGAAGTGCAGGACA
HQ664735.1      TACACGGAGAACAGCTATCCCTACGTCTCTGGCTCCGGTCAAACGCCGGCGTGCTCGACA
                 ****.***. ******.*********** *** * **..*  * ***. ****  ****

XM_009318006.1  GCTAGCCACGAGTTCGGCGCAGCCATCAGCGGCTACAATGACCTGCCGAATGATGAGGAC
HQ664735.1      AGTGAACATGAGGTTGGTGCGACAATCACCGGCTTTGTGGACTTGCCAAAAGATGAGGAC
                . *.. **.*** *.**.**..* **** ***** ..  ***.****.** *********

XM_009318006.1  AAGATGGCCGCGTGGCTGGCTGTCCACGGCCCCATTGCCATCGCCGTCGACGCCACCAGC
HQ664735.1      AAGATGGCGGCATGGCTTGCTACCAATGGCCCCATTGCTATCGCTGTCGACGCCAACAGC
                ******** **.***** ***..* *.**********.*****.********** ****

XM_009318006.1  TTCCAGTTCTACATGGGTGGCGTCCTGACGAACTGCATCTCTGAGCAGCTCGACCACGGG
HQ664735.1      TTTCTGTCGTACGTAAGTGGTGTTTTGACGAACTGTGAATCGGACCAGTTGAACCACGGT
                **.* **. ***.*..****.**..**********..  ** ** ***.* .*******

XM_009318006.1  GTGCTTCTTGTGGGCTACGACGACAGCAACAGCCCGCCGTACTGGATCATCAAGAACTCG
HQ664735.1      GTGCTTCTTGTCGGCTACGACGACAGCAGCAATCCACCGTACTGGATCATCAAGAAC---
                ********** ***************.**..**.********************
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Figure 13. Cluster W alignment of *Trypanosoma theileri* (HQ664735.1) and *T.grayi* (XM_009318006.1) for the cathepsin L-like protein gene

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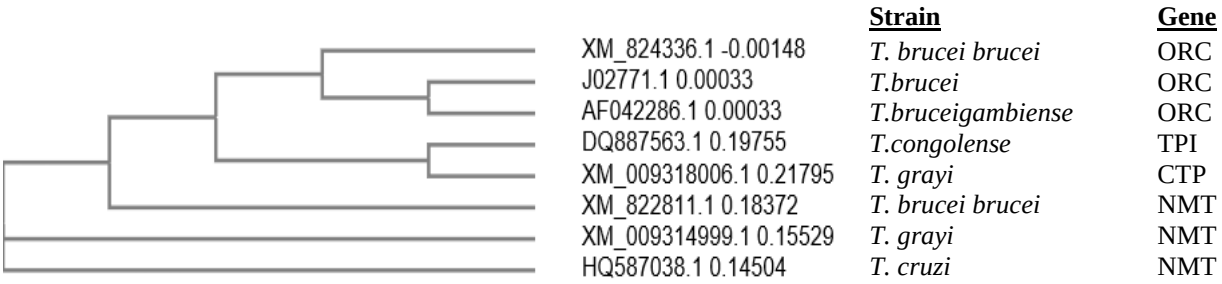

Figure 14. Multiple sequence alignments phylogenetic tree of the genome (DNA) of trypanosome showing relationships between common drug targets. ORC: Ornithine decarboxylase, TPI: Topoisomerase, CTP: Cathepsin L-like protein partial mRNA, NMT: N-myristoyl transferase

Table 5. Percentage of identity and divergence between common anti-trypanosomal drug targets identity

Divergence		1	2	3	4	5	6	7	8	
	1: XM_824336.1	100	99.93	99.85	44.01	41.48	40.87	44.4	42.34	1
	2: J02771.1	99.93	100	99.93	42.89	41.22	41.2	43.46	42.2	2
	3: AF042286.1	99.85	99.93	100	43.07	41.22	41.25	43.42	42.08	3
	4: DQ887563.1	44.01	42.89	43.07	100	58.45	43.5	44.17	43.38	4
	5: XM_009318006.1	41.48	41.22	41.22	58.45	100	41.52	43.44	40	5
	6: XM_822811.1	40.87	41.2	41.25	43.5	41.52	100	63.21	65.15	6
	7: XM_009314999.1	44.4	43.46	43.42	44.17	43.44	63.21	100	68.7	7
	8**:** HQ587038.1	42.34	42.2	42.08	43.38	40	65.15	68.7	100	8

```
XM_824336.1     TCTGTGAATTGTCTTGTAGCACAAACGGAGAAATCTATGGACATTGTCGTGAACGATGAC
J02771.1        TCTGTGAATTGTCTTGTAGCACAAACGGAGAAATCTATGGACATTGTCGTGAACGATGAC
AF042286.1      TCTGTGAATTGTCTTGTAGCACAAACGGAGAAATCTATGGACATTGTCGTGAACGATGAC
                ************************************************************

XM_824336.1     TTGAGTTGTCGCTTTCTTGAAGGGTTTAATACGAGGGATGCCCTCTGTAAAAAGATCAGT
J02771.1        TTGAGTTGTCGCTTTCTTGAAGGGTTTAATACGAGGGATGCCCTCTGTAAAAAGATCAGT
AF042286.1      TTGAGTTGTCGCTTTCTTGAAGGGTTTAATACGAGGGATGCCCTCTGTAAAAAGATCAGT
                ************************************************************

XM_824336.1     ATGAATACGTGTGACGAAGGTGATCCGTTTTTTGTTGCCGATCTCGGGGACATTGTAAGG
J02771.1        ATGAATACGTGTGACGAAGGTGATCCGTTTTTTGTTGCCGATCTCGGGGACATTGTAAGG
AF042286.1      ATGAATACGTGTGACGAAGGTGATCCGTTTTTTGTTGCCGATCTCGGGGACATTGTAAGG
                ************************************************************

XM_824336.1     AAGCACGAAACATGGAAAAAATGCCTTCCCCGCGTCACGCCGTTTTACGCGGTCAAATGC
J02771.1        AAGCACGAAACATGGAAAAAATGCCTTCCCCGCGTCACGCCGTTTTACGCGGTCAAATGC
AF042286.1      AAGCACGAAACATGGAAAAAATGCCTTCCCCGCGTCACGCCGTTTTACGCGGTCAAATGC
                ************************************************************

XM_824336.1     AACGATGACTGGCGCGTACTTGGAACGCTGGCGGCTCTCGGCACGGGATTTGATTGTGCT
J02771.1        AACGATGACTGGCGCGTACTTGGAACGCTGGCGGCTCTCGGCACGGGATTTGATTGTGCT
AF042286.1      AACGATGACTGGCGCGTACTTGGAACGCTGGCGGCTCTCGGCACGGGATTTGATTGTGCT
                ************************************************************

XM_824336.1     AGCAACACTGAGATACAACGTGTGAGAGGCATTGGTGTGCCACCGGAAAAAATAATATAT
J02771.1        AGCAACACTGAGATACAACGTGTGAGAGGCATTGGTGTGCCACCGGAAAAAATAATATAT
AF042286.1      AGCAACACTGAGATACAACGTGTGAGAGGCATTGGTGTGCCACCGGAAAAAATAATATAT
                ************************************************************

XM_824336.1     GCGAACCCTTGTAAACAAAATTCACACATACGGTACGCGCGTGATAGCGGCGTTGATGTC
J02771.1        GCGAACCCTTGTAAACAAATTTCACACATACGGTACGCGCGTGATAGCGGCGTTGATGTC
AF042286.1      GCGAACCCTTGTAAACAAATTTCACACATACGGTACGCGCGTGATAGCGGCGTTGATGTC
                ******************* ****************************************

XM_824336.1     ATGACATTTGATTGCGTGGATGAACTGGAAAAGGTCGCTAAAACGCATCCAAAGGCAAAG
J02771.1        ATGACATTTGATTGCGTGGATGAACTGGAAAAGGTCGCTAAAACGCATCCAAAGGCAAAG
AF042286.1      ATGACATTTGATTGCGTGGATGAACTGGAAAAGGTCGCTAAAACGCATCCAAAGGCAAAG
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XM_824336.1     ATGGTATTAAGAATTTCTACGGATGATTCGTTGGCTCGATGCCGTCTCAGTGTGAAGTTT
J02771.1        ATGGTATTAAGAATTTCTACGGATGATTCGTTGGCTCGATGCCGTCTCAGTGTGAAGTTT
AF042286.1      ATGGTATTAAGAATTTCTACGGATGATTCGTTGGCTCGATGCCGTCTCAGTGTAAAGTTT
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XM_824336.1     GGTGCAAAGGTGGAAGACTGTAGGTTTATCTTGGAGCAGGCAAAGAAACTGAATATCGAC
J02771.1        GGTGCAAAGGTGGAAGACTGTAGGTTTATCTTGGAGCAGGCAAAGAAACTGAATATCGAC
AF042286.1      GGTGCAAAGGTGGAAGACTGTAGGTTTATCTTGGAGCAGGCAAAGAAACTGAATATCGAC
                ************************************************************

XM_824336.1     GTCACTGGTGTGAGTTTTCACGTGGGAAGCGGATCTACAGATGCCTCTACCTTCGCTCAA
J02771.1        GTCACTGGTGTGAGTTTTCACGTGGGAAGCGGATCTACAGATGCCTCTACCTTCGCTCAA
AF042286.1      GTCACTGGTGTGAGTTTTCACGTGGGAAGCGGATCTACAGATGCCTCTACCTTCGCTCAA
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XM_824336.1     GCCATATCTGACTCCCGTTTCGTTTTCGACATGGGTACTGAGCTTGGGTTCAATATGCAC
J02771.1        GCCATATCTGACTCCCGTTTCGTTTTCGACATGGGTACTGAGCTTGGGTTCAATATGCAC
AF042286.1      GCCATATCTGACTCCCGTTTCGTTTTCGACATGGGTACTGAGCTTGGGTTCAATATGCAC
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J02771.1        ATTCTTGATATCGGTGGTGGGTTTCCAGGGACGAGGGATGCACCACTTAAATTTGAAGAG
AF042286.1      ATTCTTGATATCGGTGGTGGGTTTCCAGGGACGAGGGATGCACCACTTAAATTTGAAGAG
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XM_824336.1     ATTGCTGGTGTCATCAACAATGCGCTGGAAAAACATTTTCCACCTGACCTCAAGCTTACC
J02771.1        ATTGCTGGTGTCATCAACAATGCGCTGGAAAAACATTTTCCACCTGACCTCAAGCTTACC
AF042286.1      ATTGCTGGTGTCATCAACAATGCGCTGGAAAAACATTTTCCACCTGACCTCAAGCTTACC
                ************************************************************

XM_824336.1     ATTGTTGCCGAGCCGGGAAGGTACTACGTTGCTTCAGCTTTCACACTTGCCGTAAATGTT
J02771.1        ATTGTTGCCGAGCCGGGAAGGTACTACGTTGCTTCAGCTTTCACACTTGCCGTAAATGTT
AF042286.1      ATTGTTGCCGAGCCGGGAAGGTACTACGTTGCTTCAGCTTTCACACTTGCCGTAAATGTT
                ************************************************************

XM_824336.1     ATTGCCAAGAAGGTGACACCAGGGGTTCAGACCGACGTCGGTGCCCATGCTGAATCAAAC
J02771.1        ATTGCCAAGAAGGTGACACCAGGGGTTCAGACCGACGTCGGTGCCCATGCTGAATCAAAC
AF042286.1      ATTGCCAAGAAGGTGACACCAGGGGTTCAGACCGACGTCGGTGCCCATGCTGAATCAAAC
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XM_824336.1     GCACAGAGTTTTATGTATTATGTGAATGATGGCGTGTATGGTTCATTTAATTGCATCCTG
J02771.1        GCACAGAGTTTTATGTATTATGTGAATGATGGCGTGTATGGTTCATTTAATTGCATCCTG
AF042286.1      GCACAGAGTTTTATGTATTATGTGAATGATGGCGTGTATGGTTCATTTAATTGCATCCTG
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XM_824336.1     TATGACCACGCAGTCGTCAGGCCTTTGCCCCAGAGGGAGCCAATCCCCAATGAAAAGCTC
J02771.1        TATGACCACGCAGTCGTCAGGCCTTTGCCCCAGAGGGAGCCAATCCCCAATGAAAAGCTC
AF042286.1      TATGACCACGCAGTCGTCAGGCCTTTGCCCCAGAGGGAGCCAATCCCCAATGAAAAGCTC
                ************************************************************

XM_824336.1     TATCCCTCAAGTGTATGGGGTCCCACATGTGATGGTCTTGATCAGATAGTTGAACGATAC
J02771.1        TATCCCTCAAGTGTATGGGGTCCCACATGTGATGGTCTTGATCAGATAGTTGAACGATAC
AF042286.1      TATCCCTCAAGTGTATGGGGTCCCACATGTGATGGTCTTGATCAGATAGTTGAACGATAC
                ************************************************************

XM_824336.1     TATCTTCCCGAGATGCAAGTGGGGGGAATGGCTGCTCTTTGAGGATATGGGTGCCTACACG
J02771.1        TATCTTCCCGAGATGCAAGTGGGGGGAATGGCTGCTCTTTGAGGATATGGGTGCCTACACG
AF042286.1      TATCTTCCCGAGATGCAAGTGGGGGGAATGGCTGCTCTTTGAGGATATGGGTGCCTACACG
                ************************************************************

XM_824336.1     GTCGTAGGAACTTCTTCCTTTAATGGATTCCAGAGTCCGACTATTTACTATGTAGTCTCC
J02771.1        GTCGTAGGAACTTCTTCCTTTAATGGATTCCAGAGTCCGACTATTTACTATGTAGTCTCC
AF042286.1      GTCGTAGGAACTTCTTCCTTTAATGGATTCCAGAGTCCGACTATTTACTATGTAGTCTCC
                ************************************************************

XM_824336.1     GGGCTACCAGACCATGTTGTCCGGGAGTTGAAAAGTCAAAAATCATAA------------
J02771.1        GGGCTACCAGACCATGTTGTCCGGGAGTTGAAAAGTCAAAAATCATAAATGGAAGCGAAG
AF042286.1      GGGCTACCAGACCATGTTGTCCGGGAGTTGAAAAGTCAAAAATCATAAATGGAAGCGAAG
                ********************************************
```

Figure 15. Cluster W alignment between the three homologous sequences of anti-trypanosome drug targets

Furthermore, apart from the genomic level (DNA), MSA was conducted at the protein level. After the BLAST and Cluster W analysis of protein sequences of drug targets with *T. theileri* proteome, which was found in NCBI databases, these drug targets showed a 12/17 (71%) homology with hypothetical *T. theileri* proteins (TM35) and pyruvate kinase (Figures 17 and 18).

On the other hand, among the drug targets themselves, the highest identity was detected in accession number ofXP_829429.1 (445/445(100%)), which indicates ornithine decarboxylase protein (Figure 19). Furthermore, there was homology with AAD02222.1 and AAA30219.1, which encode the same protein from *T.grayi* and *T.brucei*, respectively. The MSA and the phylogenetic trees witnessed the homology of these sequences.

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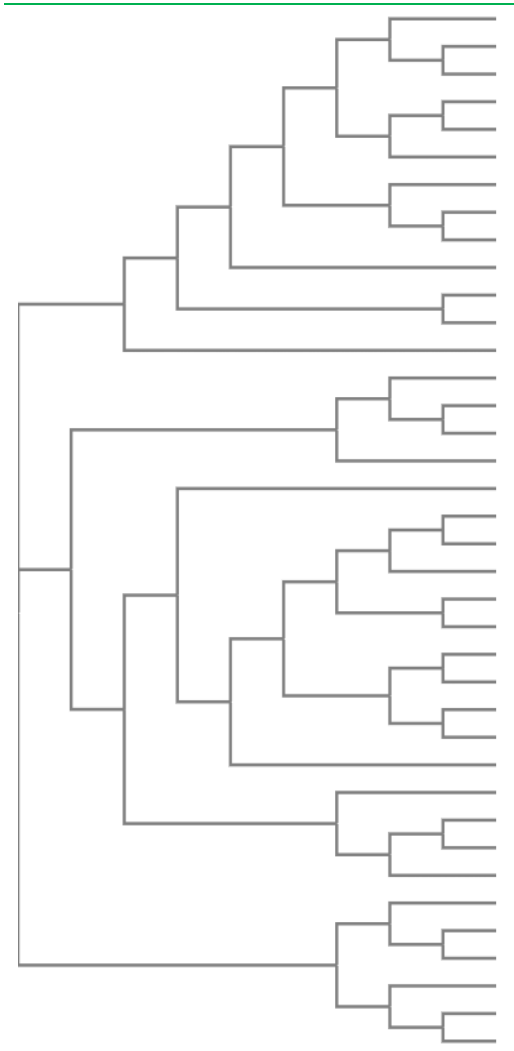

	Target protein	Species/strain
XP_829429.1 0.00049	Ornithine decarboxylase	TB
AAD02222.1 -0.00118	Ornithine decarboxylase	TG
AAA30219.1 0.00118	Ornithine decarboxylase	TB
ORC93851.1 0.37181	**Dihydroxy-3-ketomethyldioxygenase**	**TT**
ORC93850.1 0.37127	Hypothetical protein TM35_000017270	TT
ORC93848.1 0.37177	**Hypothetical protein TM35_000017250**	**TT**
XP_827904.1 0.18659	N-myristoyl transferase	TB
XP_009313274.1 0.1557	N-myristoyl transferase	TG
AEP82716.1 0.14652	N-myristoyl transferase partial	TC
ORC93856.1 0.40966	Hypothetical Protein TM35_000017330	TT
ORC93859.1 0.41854	Hot spot (RHS) protein, partial	TT
ORC93840.1 0.42146	Hypothetical protein TM35_000017170	TT
ORC93853.1 0.41573	Hypothetical protein TM35_000017310	TT
ABI96917.1 0.39109	**Topoisomerase**	**TC**
ORC93854.1 0.36738	**Hypothetical protein TM35_000017310**	**TT**
ORC93843.1 0.31262	Hypothetical protein TM35_000017200	TT
ORC93842.1 0.41766	Coatomer alpha subunit	TT
ORC93849.1 0.3919	Hypothetical protein TM35_000017260	TT
XP_009309990.1 0	Pyruvate kinase 2	TG
KEG11772.1 0	Pyruvate kinase 2	TG
ORC92589.1 0.05295	**Pyruvate kinase 2**	**TT**
ESL09806.1 0.05353	Pyruvate kinase 2	TR
3QV9_B 0.05068	Pyruvate Kinase(Tcpyk)	TC
CCC94421.1 0.05016	Pyruvate kinase 1	TC
CCC52316.1 0.03772	Pyruvate kinase 1, partial	TV
EAN78782.1 0	Pyruvate kinase 1	TB
XP_827894.1 0	Pyruvate kinase 1	TB
ORC93858.1 0.38152	Hot spot (RHS) protein, partial	TT
ORC93855.1 0.4241	Hypothetical protein TM35_000017320	TT
XP_009316281.1 0.3720	Cathepsin L-like protein	TG
ORC93844.1 0.37795	Proteasome beta 7 subunit	TT
ORC93841.1 0.40854	Alkylated DNA repair protein	TT
CAA44870.1 0.37945	Trypanothione reductase	TB
ORC93857.1 0.2975	**Hypothetical Protein TM35_000017340**	**TT**
ORC93847.1 0.31333	RNA-binding protein	TT
ORC93852.1 0.40894	Hypothetical protein TM35_000017300	TT
ORC93845.1 0.36971	Phosphatidylinositol 3-kinase tor	TT
ORC93846.1 0.38247	Cell differentiation protein	TT

Figure 16. Phylogenetic tree following MSA of the drug targets and *Trypanosoma theileri.* TB: *T. brucei*, TG: *T. grayi*, TT: *T. theileri* , TC: *T. cruzi*, TR: *T. rangeli*, TV: *T. vivax*, TC: *T.congolense*

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```
XP_009309990.1  GPSTQSVEALKGLMKSGMSVARMNFSHGSHE—YHQATINNVRTAAAELGLHIGIALDTK
3720
KEG117721       GPSTQSVEALKGLMKSGMSVARMNFSHGSHE—YHQATINNVRTAAAELGLHIGIALDTK
3720
ORC925891       GPSTQSVEALKGLMKSGMSVARMNFSHGSHE—YHQTTINNVRTAAAELGMHIGIALDTK
3720
CCC944211       GPSTQSVEALKGLMKSGMSVARMNFSHGSYE—YHQTTINNVRAAAAELGLHIGIALDTK
3720
CCC523161       GPSTQSVEALKGLMKSGMSVARMNFSHGSHE—YHQTTIKNVRQAAAELGLHIGIALDTK
3720
ESL098061       GPSTQSIEALRSLIKSGMSVARMNFSHGSHE—YHQTTINNVRAASAELGVHIGIALDTK
3720
3QV9_B          GPSTQSVEALKGLIRSGMSVARMNFSHGSHE—YHQTTINNLRAAATELGAHIGLALDTK
3720

XP_0093099901   GPEIRTGLFVGGEAVL-NPGDTVFVTTDPAFE----------KKGTKEKFYVDYPRLAT
3780
KEG117721       GPEIRTGLFVGGEAVL-NPGDTVFVTTDPAFE----------KKGTKEKFYVDYPRLAT
3780
ORC925891       GPEIRTGLFVGGEAIL-MTGDTVLVTTDPAFE----------KTGTKEKFYIDYPRLAT
3780

XP_0093099901   ----FHRLTDRKGCNLPGCDVDLPAVSAKDREDLKFGVEQGVDIIFASFIRTAEQVQEVR
3850
KEG117721       ----FHRLTDRKGCNLPGCDVDLPAVSAKDREDLKFGVEQGVDIIFASFIRTAEQVQEVR
3850
ORC925891       ----GHRLTDRKGCNLPGCEVDLPAVSAKDREDLKFGVEQGVDMIFASFIRTAEQVREVR
3850
CCC944211       ----HHRLTDRKGINLPGCEVDLPAVSEKDRKDLQFGVEQGVDMIFASFIRTADQVREVR
3850
CCC523161       ----HHRLTDRKGCNLPGCDVELPAVSEKDRKDLIFGVEQGVDMIFASFIRTAEQVREVR
3850
ESL098061       ----AHYLTDRKGCNLPGCEVDLPAVSEKDREDLKFGVEQGVDMIFASFIRTAEQVREVR
3850
3QV9_B          ----AHFLTDRKGCNLPGCEVDLPAVSEKDREDLKFGVEQGIDMVFASFIRTAEQVQEVR
3850
EAN787821       ----HHRLTDRRGINLPGCEVDLPAVSEKDRKDLEFGVAQGVDMIFASFIRTAEQVREVR
3850
XP_8278941      ----HHRLTDRRGINLPGCEVDLPAVSEKDRKDLEFGVAQGVDMIFASFIRTAEQVREVR
3850

KEG117721       AQMMLISKCNVAGK------------------PVICATQMLESMTANPRP---TRAEVSD
3970
ORC925891       AQMTLISKCNVAGK------------------PVICATQMLESMTTNPRP---TRAEVSD
3970
CCC944211       AQMCIISKCNVAGK------------------PVICATQMLESMTTNPRP---TRAEVTD
3970
CCC523161       AQMCIISKCNVAGK------------------PVICATQMLESMTTNPRP---TRAEVSD
3970
ESL098061       AQMILISKCNVAGK------------------PVICATQMLESMTTNPRP---TRAEVSD
3970
3QV9_B          AQMILISKCNVAGK------------------PVICATQMLESMTTNPRP---TRAEVSD
3970
EAN787821       AQMCIISKCNVVGK------------------PVICATQMLESMTSNPRP---TRAEVSD
3970
XP_8278941      AQMCIISKCNVVGK------------------PVICATQMLESMTSNPRP---TRAEVSD
3970
```


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```
XP_0093099901   VANAVFNG-----------ADCVMLSGETAKGKYPNEVVRYMARICVEAQSATNQ-----
4010
KEG117721       VANAVFNG-----------ADCVMLSGETAKGKYPNEVVRYMARICVEAQSATNQ-----
4010
ORC925891       VANAVFNG-----------ADCVMLSGETAKGHYPNEVVQYMARICVVAQSATNQ-----
4010
CCC944211       VANAVFNG-----------ADCVMLSGETAKGKYPNEVVQYMVRICIEAQSATHD-----
4010

ESL098061       VANAVFNG-----------ADCVMLSGETAKGKYPSEVVQYMARICVEAQSATNQ-----
4030
3QV9_B          VANAVFNG-----------ADCVMLSGETAKGKYPNEVVQYMARICLEAQSATNQ-----
4030
EAN787821       VANAVLNG-----------ADCVMLSGETAKGKYPNEVVQYMARICVEAQSATHD-----
4030
XP_8278941      VANAVLNG-----------ADCVMLSGETAKGKYPNEVVQYMARICVEAQSATHD-----
4030

XP_0093099901   SNSGRSARLTSKYRPDCPIICVTTRMRTCRQ---------------------LNVTRSVE
4150
KEG117721       SNSGRSARLTSKYRPDCPIICVTTRMRTCRQ---------------------LNVTRSVE
4150
ORC925891       SNSGRSARLASKYRPNCPIICATTRMRTCRQ---------------------LNITQSVE
4150
CCC944211       SNTGRSARLISKYRPNCPIICATTRLLTCRQ---------------------LNVTRSVE
4150

AAD022221       GPTCDGLDQIVERYYLPEMQVGEWLLFEDMGAYTVVGTS---SFNGFQSPTIYYVVSGLP
4210
AAA302191       GPTCDGLDQIVERYYLPEMQVGEWLLFEDMGAYTVVGTS---SFNGFQSPTIYYVVSGLP
4210
XP_8294291      GPTCDGLDQIVERYYLPEMQVGEWLLFEDMGAYTVVGTS---SFNGFQSPTIYYVVSGLP
4210

XP_0093099901   SVFYDAERCGADEDKENRVQLG--VESAKKKGYVVPG-------DIVVAVHADHKVKGYP
4210
KEG117721       SVFYDAERCGADEDKENRVQLG--VESAKKKGYVVPG-------DIVVAVHADHKVKGYP
4210
ORC925891       SVFYDAERYGPDDDKENRVQLG--VEFAKKKGYVVPG-------DVMVVVHADHKVKGYP
4210
CCC944211       SVYYDVDAHGEDNDREKRVQLG--VDWAKTKGYVSAG-------DVMVIVHADHSVKGYP
4210
```

Figure 17. Alignment of drug targets with *Trypanosoma theileri* (XP_0093099901) protein found in the NCBI database

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```
AAD022221       -------EIQRVRGIGVP-PEKIIYANPCKQISHIR---------------------- 550
AAA302191       -------EIQRVRGIGVP-PEKIIYANPCKQISHIR----------------------
XP_8294291      -------EIQRVRGIGVP-PEKIIYANPCKQNSHIR----------------------
ESL098061       -------YLTDRKGCNLPGCEVDLPAVSEKDREDLK-----------------------
3QV9_B          -------FLTDRKGCNLPGCEVDLPAVSEKDREDLK-----------------------
XP_0093099901   -------RLTDRKGCNLPGCDVDLPAVSAKDREDLK-----------------------
KEG117721       -------RLTDRKGCNLPGCDVDLPAVSAKDREDLK-----------------------
CCC523161       -------RLTDRKGCNLPGCDVELPAVSEKDRKDLI-----------------------
EAN787821       -------RLTDRRGINLPGCEVDLPAVSEKDRKDLE-----------------------
XP_8278941      -------RLTDRRGINLPGCEVDLPAVSEKDRKDLE-----------------------

AAD022221       --YARDSGVDVMTFDCVDELEKVAKTHPKAKMVLR-ISTDDSLARCRLSVKFGAKVEDCR 610
AAA302191       --YARDSGVDVMTFDCVDELEKVAKTHPKAKMVLR-ISTDDSLARCRLSVKFGAKVEDCR
XP_8294291      --YARDSGVDVMTFDCVDELEKVAKTHPKAKMVLR-ISTDDSLARCRLSVKFGAKVEDCR
ESL098061       --FGVEQGVDMIFASFIRTAEQVREVRAALGEKGKDILVISKIENHQGVQNIDAIIEASD
3QV9_B          --FGVEQGIDMVFASFIRTAEQVQEVREALGEKGKDILIISKIENHQGVQNIDGIIEASD
XP_0093099901   --FGVEQGVDIIFASFIRTAEQVQEVRAALGEKGKDTLVISKIENHQGVQNIDGIIAVSD
KEG117721       --FGVEQGVDIIFASFIRTAEQVQEVRAALGEKGKDTLVISKIENHQGVQNIDGIIAVSD
CCC523161       --FGVEQGVDMIFASFIRTAEQVREVRAVLGEKGKDTMIISKIENHQGVQNIDAIIEASD
EAN787821       --FGVAQGVDMIFASFIRTAEQVREVRAALGEKGKDILIISKIENHQGVQNIDSIIEASN
XP_8278941      --FGVAQGVDMIFASFIRTAEQVREVRAALGEKGKDILIISKIENHQGVQNIDSIIEASN
CCC944211       --FGVEQGVDMIFASFIRTADQVREVRAALGEKGKDTLIISKIENHQGVQNIDAIIEASD
XP_0093132741   --ENHLEPRIICEINFLCVHKQLREKRMAPILIQEVTRRVNLLNIWQAIYTAGALLPTP-
AEP827161       --EXYLEPRKICEINFLCVHKLLRAKRLAPILIKEVTRRVHLMNIWQAVYTAGRLLPTP-

AAD022221       F--ILEQAKKLNIDVTGVSFHVGSGSTDASTFAQ--------------AISDSRFVFDMG 670
AAA302191       F--ILEQAKKLNIDVTGVSFHVGSGSTDASTFAQ--------------AISDSRFVFDMG
XP_8294291      F--ILEQAKKLNIDVTGVSFHVGSGSTDASTFAQ--------------AISDSRFVFDMG
ESL098061       G--IMVARGDLGVEIAAEKVVVAQMILISKCNVAG-----------KPVICATQMLESMT
3QV9_B          G--IMVARGDLGVEIPAEKVVVAQMILISKCNVAG-----------KPVICATQMLESMT
XP_0093099901   G--IMVARGDLGVEIPAEKVVVAQMMLISKCNVAG-----------KPVICATQMLESMT
KEG117721       G--IMVARGDLGVEIPAEKVVVAQMMLISKCNVAG-----------KPVICATQMLESMT
CCC523161       G--IMVARGDLGVEIPAEKVVVAQMCIISKCNVAG-----------KPVICATQMLESMT
EAN787821       G--IMVARGDLGVEIPAEKVCVAQMCIISKCNVVG-----------KPVICATQMLESMT
XP_8278941      G--IMVARGDLGVEIPAEKVCVAQMCIISKCNVVG-----------KPVICATQMLESMT
CCC944211       G--IMVARGDLGVEIPAEKVVVAQMCIISKCNVAG-----------KPVICATQMLESMT
XP_0093132741   ----FTSGRYFHRSLNPEKLVAIAFSRIPPQYQKF-----------QNPMSMLKRFYQVP
AEP827161       ----FATADYYHRSLNPEKLVAVGFSXIPQQYQKF-----------QNPLSMIKRFYELP
XP_8279041      ----FAKGHYFHRSLNSQKLVDVKFSGIPPHYKRF-----------QNPVAVMERLYRLP

AAD022221       KKVTPGVQTDVGAHAESNAQSFMYYVNDGVYGSFNCILYDHAVVRPLPQ----------910
AAA302191       KKVTPGVQTDVGAHAESNAQSFMYYVNDGVYGSFNCILYDHAVVRPLPQ----------
XP_8294291      KKVTPGVQTDVGAHAESNAQSFMYYVNDGVYGSFNCILYDHAVVRPLPQ----------
ESL098061       SPEDAVCSSAVNSVYEVRAKVLLVLSNSGRSARLASKYRPNCPIVCATT-----------
3QV9_B          SPEEAVCSSAVNSVYEVRAKALLVLSNSGRSARLASKYRPDCPIICATT-----------
XP_0093099901   SPEEAVCCSAVNSVYEVRAKVLLVLSNSGRSARLTSKYRPDCPIICVTT-----------
KEG117721       SPEEAVCCSAVNSVYEVRAKVLLVLSNSGRSARLTSKYRPDCPIICVTT-----------

EAN787821       CPEEAVCSSAVASAFEVQAKAMLVLSNTGRSARLISKYRPNCPIICVTT----------- 910
XP_8278941      CPEEAVCSSAVASAFEVQAKAMLVLSNTGRSARLISKYRPNCPIICVTT-----------

AAD022221       REPIPNEKLYPSSVWGPTCDGLDQIVERYYLPEMQVGE-------------WLLFEDMGA
AAA302191       REPIPNEKLYPSSVWGPTCDGLDQIVERYYLPEMQVGE-------------WLLFEDMGA 970
XP_8294291      REPIPNEKLYPSSVWGPTCDGLDQIVERYYLPEMQVGE-------------WLLFEDMGA
ESL098061       RMRTCRQLTITRSVEPVFYDAERYGDDESKEKRVQLG---------------VEWAKKRG
3QV9_B          RMRTCRQLTITRSVDAVFYDAERYGEDENKEKRVQLG---------------VDCAKKKG
XP_0093099901   RMRTCRQLNVTRSVESVFYDAERCGADEDKENRVQLG---------------VESAKKKG
KEG117721       RMRTCRQLNVTRSVESVFYDAERCGADEDKENRVQLG---------------VESAKKKG
```

Figure 18. Multiple sequence alignmentsamong the drug targets excluding *Trypanosoma theileri* protein

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Table 6. Percent identity and divergence of Cluster W alignment of drug targets

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
1: XP_829429.1	100	99.78	99.76	18.72	17.82	17.82	17.52	15.81	17.22	16.92	17.03	17.03	14.58	14.5	13.33	10.98	18.22	1
2: AAD02222.1	99.78	100	100	18.72	17.82	17.82	17.52	15.81	17.22	16.92	17.03	17.03	14.88	14.5	13.33	10.98	18.22	2
3: AAA30219.1	99.76	100	100	19.23	16.5	16.5	16.18	13.47	16.18	15.53	16.79	16.79	15.19	14.41	13.53	10.98	18.6	3
4: ABI96917.1	18.72	18.72	19.23	100	24.4	24.4	25.81	23.55	24.8	24.19	23.36	23.36	20.66	15.37	14.78	10.68	18.86	4
5: XP_009309990.1	17.82	17.82	16.5	24.4	100	100	82.97	88.18	86.37	86.17	80.34	80.34	22.2	16.77	18.81	16.81	18.37	5
6: KEG11772.1	17.82	17.82	16.5	24.4	100	100	82.97	88.18	86.37	86.17	80.34	80.34	22.2	16.77	18.81	16.81	18.37	6
7: CCC94421.1	17.52	17.52	16.18	25.81	82.97	82.97	100	91.21	82.16	82.36	86.89	86.89	23.17	17.07	18.51	16.38	19.59	7
8: CCC52316.1	15.81	15.81	13.47	23.55	88.18	88.18	91.21	100	84.55	85.15	87.91	87.91	23.59	17.14	19.25	16.74	17.2	8
9: ESL09806.1	17.22	17.22	16.18	24.8	86.37	86.37	82.16	84.55	100	89.58	80.34	80.34	22.44	17.38	20	17.67	15.92	9
10: 3QV9_B	16.92	16.92	15.53	24.19	86.17	86.17	82.36	85.15	89.58	100	80.63	80.63	21.71	17.07	18.81	17.24	16.33	10
11: EAN78782.1	17.03	17.03	16.79	23.36	80.34	80.34	86.89	87.91	80.34	80.63	100	100	23.16	17.86	17.77	14.97	18.27	11
12: XP_827894.1	17.03	17.03	16.79	23.36	80.34	80.34	86.89	87.91	80.34	80.63	100	100	23.16	17.86	17.77	14.97	18.27	12
13: CAA44870.1	14.58	14.88	15.19	20.66	22.20	22.20	23.17	23.59	22.44	21.71	23.16	23.16	100	14.33	13.65	14.07	19.83	13
14: XP_827904.1	14.5	14.5	14.41	15.37	16.77	16.77	17.07	17.14	17.38	17.07	17.86	17.86	14.33	100	59.64	63.52	13.58	14
15: XP_009313274.1	13.33	13.33	13.53	14.78	18.81	18.81	18.51	19.25	20	18.81	17.77	17.77	13.65	59.64	100	69.45	11.93	15
16: AEP82716.1	10.98	10.98	10.98	10.68	16.81	16.81	16.38	16.74	17.67	17.24	14.97	14.97	14.07	63.52	69.45	100	12.28	`6
17: XP_009316281.1	18.22	18.22	18.6	18.86	18.37	18.37	19.59	17.2	15.92	16.33	18.27	18.27	19.83	13.58	11.93	12.28	100	17

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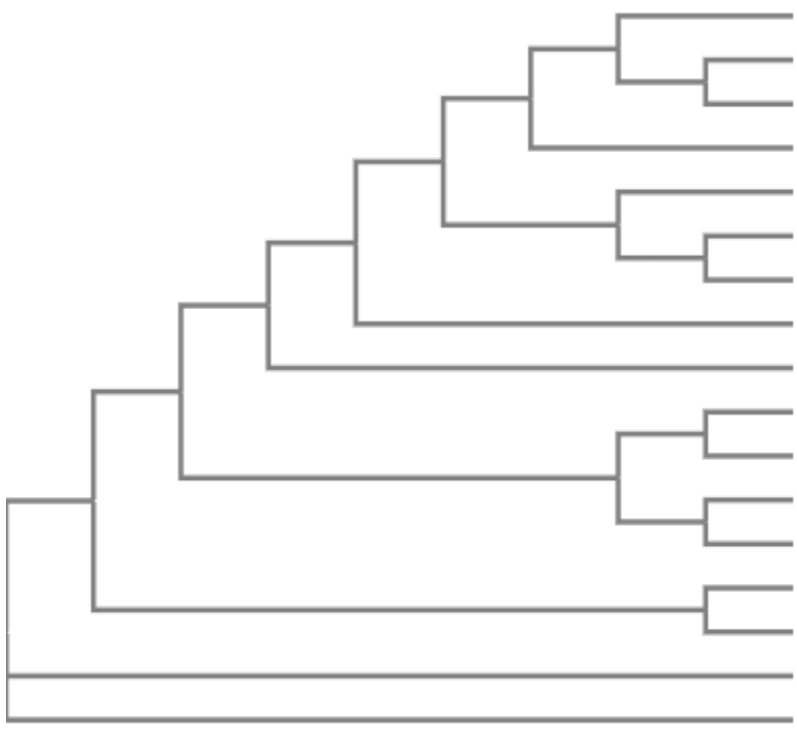

	Target protein	Species/strain	
XP_829429.1 0	Ornithine decarboxylase		T B
AAD02222.1 -0.00239	Ornithine decarboxylase		TG
AAA30219.1 0.00239	Ornithine decarboxylase		TB
XP_009316281.1 0.40926	Cathepsin L-like protein		TG
XP_827904.1 0.19547	N-myristoyl transferase		TB
XP_009313274.1 0.14334	N-myristoyl transferase		TG
AEP82716.1 0.16212	N-myristoyl transferase partial		TC
ABI96917.1 0.38256	Topoisomerase		TC
CAA44870.1 0.39386	Trypanothione reductase		TB
XP_009309990.1 0	Pyruvate kinase 2		TG
KEG11772.1 0	Pyruvate kinase 2		TG
ESL09806.1 0.05239	Pyruvate kinase 2		TR
3QV9_B 0.05182	Pyruvate Kinase(Tcpyk)		TC
EAN78782.1 0	Pyruvate kinase 1		TB
XP_827894.1 0	Pyruvate kinase 1		TB
CCC94421.1 0.05135	Pyruvate kinase 1		TC
CCC52316.1 0.03653	Pyruvate kinase 1, partial		TV

TB: *T.brucei*, TG: T. grayi, TT: *T. theileri* , TC: *T.cruzi*, TR: *T. rangeli*, TV: *T.vivax*, TC: *T.congolense*

Figure 18. Phylogenetic tree following multiple sequence alignments showing relationships among common trypanocidal drug targets

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DISCUSSION

Growth media and condition

Trypanosoma theileri is one of the least studied trypanosomes although it is prevalent worldwide. This distribution may be due to the pathogenic nature of the parasite, which, compared to pathogenic ones, cannot cause a remarkable loss of production and productivity in livestock. However, nowadays researchers are focusing on *T. theileri* since it has been used as a setup and new tool for trypanosomatid-based delivery (Mott et al., 2011) for the treatment of pathogenic trypanosome and other hemoparasites affecting livestock and humans. Nevertheless, due to its low parasitemic nature, it is rarely detected by direct smear microscopy. In view of this, Verloo et al. (2000) developed a kit called KIVI as an excellent tool for isolating *T. Theileri* which they found successful.

There are a number of media that must be cultured for both epimastigote and trypanosome's blood flow stages. For the blood flow stages of *T. theileri* (Trypomastigotes), RPMI medium 1640 with 10% FCS, supplemented with murine spleen cells as a feeder layer, was used (Verloo et al., 2000). In addition, it could be cultured in tissue culture fluid NCTC-109 (Splitter and Soulsby, 1967), 50% HMI-9 medium (Hirumi and Hirumi, 1989) supplemented with 20% FCS, 10% Serum, and 50% MDBK-conditioned media (Mott et al., 2011).

In the present study, it was possible to grow *T. theileri* epimastigotes in SDM 79 with 10 % FCS at 26^{o}C without CO_2betweenfour media tested (Figure 2). Under similar growth conditions, Wink (1979) cultured *T. Theileri* epimastigotes at 25°C with 10 % FCS, but with different growth media, Glossina Cell culture Medium (GCM). Moreover, in the second growth condition (at 37°C with 5% CO_2) there was slight growth of *T. theileri* epimastigotes in RPMI 1640 (Figure 3), which was confirmed by Verloo et al. (2000), who could culture *T. theileri* with RPMI 1640. However, the growth of *T. theileri* epimastigote in RPMI 1640 at 26^{o}C with CO_2 was less than the time cultivated at 26^{o}C without CO_2 (Figure 2) in the present study. This could be due to the reluctance to use PMI conditioning since they used a feeder layer of murine spleen cells. In addition, Verloo et al. (2000) cultured the blood flow stage that was directly isolated from the blood.

Growth pattern and doubling time

The culture of *T. theileri* in eight different tissue culture flasks showed maximum growth on the sixth day during the eight experiments, except for the seventh experiment on the seventh day. On the other hand, as can be seen in Figure 3, the log phase starts clearly from the third day. This indicated that the logarithmic phase of growth was started from the third to the sixth day and from the third to the seventh day for the seventh experiment. The maximum number of cells that could be grown among the eight flasks was estimated to be 1.7×10^7 cells/mL (Experiment 2). Hence, the logarithmic phase extended from 3.2×10^4cells/mL, which was on the third day of the experiment. Second, to the highest 1.3×10^7cells/mL from the same flask on the sixth day except for Experiment seven. The doubling time in the logarithmic growth phase was estimated to range from 13.43 to 19.0 hours with an average estimate of 17.43 hours (0.73 day) in eight experiments. The doubling time of the present study is slightly higher than the doubling time of *T. theileri* reported by Wink (1979) with a doubling time of 10-14 hours. However, the growth pattern of *T. theileri* is2.5 times higher than the doubling time of *T.b.brucei*as described by Sykes and Avery (2009) and Melissa et al. (2009).

The doubling time could indicate that *T. theileri* grows more slowly than other similar reports. The slow growth rate could be due to different media types and growing conditions. It is in a sense that, if the parasite has got a favorable growth environment, they may have the opportunity to divide within a short period. Furthermore, the slower growth of *T. theileri* compared to the growth of other pathogenic Trypanosomesmay be linked to the factors that could make the parasite a pathogen. This means that the longer replication time and the lower parasitemic features can naturally cause the least amount of anemia. The serious losses from pathogenic trypanosomes are principally due to anemia. Furthermore, the lower detection level in the blood can limit the distribution of the parasite to different visceral organs and haemopoietic tissues, such as the spleen. Moreover, virulent trypanosomes have a shorter incubation period than pathogen ones (Magona et al., 2008). The longer the doubling time which was needed in the present study, can be related to this point. The same applies to the study by Böse et al. (1987), who reported that after identifying the stages of infection of *T. theileri* in the gut and feces of tabanids, it could be determined that the minimum prepatent period of around four days in infected cattle despite no apparent signs of disease were detected.

Resazurin assay

The epimastigote (5.2×10^5/mL to 8.5×10^6/mL) was grown in a microtiter plate for three different concentrations of Resazurin. Initially, there was a lower fluorescence signal throughout the three Resazurin concentrations. However, it increased significantly from 1.2×10^6 cells/ml and reached the highest fluorescence signal detection at 3.5×10^6 cells/mL following the seventh hour of incubation with Resazurin. It indicated that there was a positively correlated ($r = 0.7517$-0.9252; $p < 0.05$) fluorescence signal with an increase in cell density and Resazurin concentrations.

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On the other hand, there was a positive correlation ($r = 0.8297$; $p < 0.05$) among fluorescence signals as a result of 2.4 mM Resazurin than the other two concentrations with an increase in incubation time (Figure 8) until 7 hours using an optimal 1.2 $\times 10^6$ cells/mL of cell density. The fluorescence decreased dramatically after the seventh hour of incubation with Resazurin (Figure 7). It is in agreement with a study by Tana et al. (2012) who reported that Resazurin was reduced linearly after three to four hours of incubation with 25 mg/mL (20 µL/well) Resazurin though it was done on *T. brucei*. They also reported that the maximum fluorescence was recorded in the range of 2-5 $\times 10^6$ cell/mL which was almost similar to the optimal cell density found for higher fluorescence (1.2-3.5$\times$ 10^6 cells/mL) with 2.4Mm Resazurin. There was a very high and linear correlation ($r = 0.9876$)observed in the fluorescence signal of the parasite in the range of 1.3 $\times$ 10^6 to 5.3 $\times$ 10^6 cells/ml upon incubating with 2.4 mM Resazurin for 7 hours at 26°C. It is slightly higher than the incubation time used for fluorescence assay for *T. cruzi* which was 5 hours as reported by Miriam et al. (2006). Similarly, according to Tana et al. (2012), there was a linear reduction of Resazurin after a 3-4 hours period of contact time since there might be a saturation of the fluorescence at such high parasite densities. A report by Miriam et al. (2006) also showed the highest concentrations of Resazurin (3 mM) among the Resazurin concentrations they tested (0.5 to 3 mM), showed the highest Resazurin reduction. The same is true from our experiment since the highest Resazurin concentration (2.4 mM) showed a similar pattern. It may be due to the fact that whenever we use the lowest concentration of Resazurin, the Resazurin (blue) can be converted to a more fluorescent resorufin(pink) immediately within a short time. Then, if it was allowed for a long incubation time, the resorufin which is the fluorescent one will be converted to a non-fluorescent stage finally. It shows an imbalance between the highest numbers of cells with the lowest concentration of Resazurin used. Due to this, maybe the highest concentration showed a significant correlation in the magnitude of fluorescent signal with an increase in cell density and incubation time.

Drug sensitivity

The Resazurin assay enables the measurement of parasite viability as an indicator of the ability to recover from compound effects (Nare et al., 2010; Tana et al., 2012). By using the optimization conditions we established for cell density and Resazurin concentration, the Resazurin-lead assay was applied to assess the susceptibility of *T. theileri* epimastigotes to Pentamidine (Sigma, 1-100 ng/mL). As a consequence, a negatively correlated ($r = -0.8826$) and statistically significant ($p < 0.05$) difference were observed between the reduction in fluorescence signal and an increase in pentamidine concentration predominantly in the highest drug concentration. A study was done on *T.b.brucei* by Tana et al. (2012) also indicated a similar response of the parasite following 72 hours of incubation with Pentamidine which result in a dramatic reduction of Resazurin signal. The viability percentage was determined by comparing both microscopic counting which is anti-epimastigotes (%AE) and the resazurin assay with Pentamidine (1-100 ng/mL). IC_{50} values of 9.25 ng/mL and 16.29 ng/mL were found by using Resazurin ($r = -0.957$, $p < 0.05$) and microscopic counting ($r = 0.90$, $p < 0.05$) respectively. These two tests showed roughly similar outcomes though Resazurin assay was more preferable since it has relatively lower IC_{50} (Figure 11). Furthermore, it allowed screening a large sample size as far as microscopic counting is time-consuming. It was in agreement with the report by Miriam et al. (2006) and Sykes and Avery (2009) mentioning that Resazurin was preferable to test the viability of *T.b. brucei* for Pentamidine and Suramin. The IC_{50} value was higher than the reports by Sykes and Avery (2009) and Tana et al. (2012), who reported 5ng/mL and 40 nM, respectively for *T.brueci.*

Experimental infection of calves

During the experimental infection of the present study, the identification of parasites both in Giemsa stained slides and through culturing the PBMC and the buffy coat in RPMI 1640 and HMI 9 medium was unsuccessful. However, a similar method of experimental infection was followed by Mott et al. (2011). The problems could be, first, the parasite could die in transit prior to inoculation since the farm was a few kilometers away from the laboratory where the parasite was cultured. Secondly, it might be due to the time it took for replication to happen. According to the doubling time calculated during the in vitro culture, they needed 13.43 to 19 hours. Third, based on the amount of the parasite, the calf was inoculated between five and nine ml. Finally, maybe another co-founding factor has not yet been realized. Nevertheless, a similar study was done by Mott et al. (2011) after experimental infection. However, they extracted DNA directly from whole blood samples than growing the parasite after identification from buffy coat and/or PBMC since it's rarely detected due to the low level of parasitemia. However, the focus of the present study was on the culturing of the blood-streaming form in mass after isolating from the blood for further proteomic studies; to compare the epimastigote and trypomastigotes proteomics.

PCR confirmation of *Trypanosoma theileri*

Trypanosoma theileri could be differentiated from other trypanosomes through a species-specific primer (Tth625) as described by Rodrigues et al. (2003), PCR-amplified spliced-leader transcript, 18S ribosomal DNA, and internal transcribed spacer of ribosomal genes (ITS gene, Geysen et al., 2003). In the present study, the results obtained by PCR

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and agarose gel electrophoresis based on Species-specific primers confirmed a 465 bp amplification product which was comparable with 450 bp DNA fragment (Tth625 fragment) by Rodrigues et al. (2003)and 472 bp by Lee et al. (2013). For the second amplification for the 18S rDNA sequence of *T. theileri*, a band with a size of730 bp was detected, which was almost expected to be 722 bp, as reported by Geysen et al. (2003).

In silico analysis

The BLAST and Clustal W alignment were done both at nucleotide (DNA) and protein level with target sequences of common anti-trypanosome drugs. Following a BLAST and Cluster W alignment using these common drug targets, there was only one slight homogeneity (75%) gene (CATL gene) which was found in both *T.grayi* (XM_009318006.1) and HQ664735.1 of *T. theileri* isolate in common. On the other hand, among the drug targets only, excluding *T. theileri* genome; they had a very high identity especially among XM_824336.1 (100%), J02771.1 (99%), and AF042286 (99%) which entirely indicated Ornithine decarboxylase gene in*T.brucei.* The phylogenetic tree and ClusterW alignment also showed these relationships. Lee et al. (2013)have got the homology of their isolate (TWTth1) from Taiwan with isolates by Rodrigues et al. (2003), Gene Bank Accession No: AF537201 (99.5%) and AF537202 (98.8%) from Brazil. They did the comparison based on *T. theileri* DNA. In addition, based on the full-length 18S rDNA sequence amplicon of their isolate, they found 100% identity with AB007814.1. Based on ITS sequences, Lee et al.(2013)reported that there was a similarity amongAB007814 (100%), AY773707 (97.3%), and AY773708 (98.0%) which are Japanese and Brazilian isolates.

At the protein level, there was almost no similarity of these drug targets with the proteins found in the NCBI database except a slight similarity with hypothetical *T. theileri* proteins (TM35). However, among the drug targets, a prominent homology was detected among XP_829429.1(100%), AAD02222.1, and AAA30219.1 which indicated ornithine decarboxylase protein as was obtained after analyzing at DNA level in the current study. The MSA and the phylogenetic trees witnessed the homology of these sequences.

Accordingly, BLAST was used in the current study and aligned only with partial and hypothetical sequences of the *T. theileri.* However, there were homologies of sequences from anti-trypanosome drug targets specifically for Ornithine decarboxylase from *T.brucei*. Similarly, Rodrigues et al. (2003) reported that *T. theileri* and '*T. brucei* clade' trypanosomes shared artiodactyl host species with overlapping distributions and commonly bovids carrying mixed trypanosome infection in the field.

CONCLUSIONS

In the present study, *T. theileri* was successfully cultured *in vitro* in SDM 79 at 26^{o}C. The growth pattern, viability, and response to pentamidine were assessed by Resazurin assay. *T. theileri* parasite took a longer time to double the population, compared to other trypanosomes. Moreover, the Resazurin assay using pentamidine was deployed as the reference drug to confirm the effectiveness of this assay technique. Consequently, it could be possible to use such a sensitive and inexpensive assay for high-throughput screening of anti-trypanosome compounds. On the other hand, after extraction of the DNA by the PhenolChloroform protocol from cultured *T. theileri*, the parasite was confirmed using PCR amplification by species-specific primers.

Furthermore, the BLAST and MSA were performed with common anti-trypanosome target sequences. Subsequently, in contrast to the anti-trypanosome targets of pathogenic trypanosomes, significant similarity at both the DNA and protein level with *T. theileri* was detected. However, some similarities to hypothetical. *theileri* proteins (TM35) were observed. Both at DNA and protein level, significant homologyamongXP_829429.1, AAD02222.1, and AAA30219.1 were detected which all referred to ornithine decarboxylase protein in *T.brucei*. The lack of homology in *T. theileri* might be due to the lack of a complete genome sequence. Finally, whole-genome and transcriptome analyses using *T. theileri* can reveal the phylogenetic relationship between *T. theileri* and other pathogenic trypanosomes, which can be used as a tool in the development of new therapeutic drugs for the treatment of the pathogenic trypanosome.

Based on the above conclusions, the following recommendations are put forward. The present study provides baseline data for the next research on the parasite. In the case of experimental infection, it is better to infect the test animal as soon as possible, while the parasite could soon die if transported with PBS. Long and continuous blood sampling should be performed after experimental infection. In the present study, only two consecutive weeks were checked after each infection. There is a chance that the parasite may not be immediately naturally isolated due to a low level of parasitemia. Hence, most of the isolation of *T. theileri* from natural infection was not purposively confirmed. Rather, it was found in an unexpected time. It can be isolated while researchers pursue other goals. For example, the total leukocyte and differential count, and PCV are examined during the macrophage culture while the Bovine Leukemia Virus(BLV) is examined from lymphocyte cultures of cows infected with it. It is better to use a reader with higher fluorescence to get a higher magnitude of the fluorescence signal. Better to use more than two drugs for treatment so that the drug sensitivity assay is representative and comparable.

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DECLARATIONS

Authors' contribution

Tewodros Fentahun contributed to data collection, lab activities, and a write-up of the manuscript. Jan Paeshuyse was involved in data analysis. Both authors confirmed the final revised manuscript.

Competing interests

The authors have no conflicts of interest.

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Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

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