

Analysis of the dynamics of *Trypanosoma evansi* strain accumulation during laboratory cultivation

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ABSTRACT

Background: The aim of the study was to characterise the accumulation dynamics, viability, morphology, and antigenic suitability of *Trypanosoma evansi* during experimental infection in mice and cultivation in RPMI- PY medium.

Methods: The study was conducted from January to June 2025 in the parasitology laboratory of the Limited Liability Company "Scientific-Production Enterprise "Antigen" using experimental infection of male mice followed by morphological, culturological, and serological analysis of the pathogen.

Results: The results demonstrated a clear dependence of the infection course and morphological characteristics of the pathogen on the inoculum dose and cultivation conditions. At the lowest inoculum dose, parasitemia in mice reached approximately 4.8×10^6 cells/mL; at the medium inoculum dose, it reached 1.1×10^7 cells/mL; and at the highest inoculum dose, it reached up to 1.3×10^8 cells/mL, accompanied by intensified clinical signs. Morphometric analysis revealed a reduction in cell length from 24.6 μm at the lowest inoculum dose to 20.3 μm at the highest inoculum dose and a reduction in flagellum length from 8.2 to 6.1 μm , along with an increase in the proportion of degenerative forms and kinetoplast displacement. In *in vitro* culture, growth exhibited an S-shaped pattern: from 6.8×10^6 cells/mL on day five to a peak of 1.3×10^7 cells/mL on day nine, followed by a decline in viability to 6 %. Protein analysis showed a concentration of 2.3 mg/ mL with dominant antigenic fractions around 30 and 55 kDa, confirming the diagnostic value of the obtained material.

Conclusion: The results demonstrated dose- dependent changes in parasitemia, morphology, *in vitro* growth, and protein profile of *T. evansi*, highlighting the diagnostic and applied significance of the study.

INTRODUCTION

The study of *Trypanosoma evansi* has practical significance, as this parasite causes surra – a severe disease affecting horses, camels, cattle, and other animals. The infection is accompanied by anaemia, emaciation, reduced productivity, and high mortality, leading to economic losses in animal husbandry. The disease occurs in regions where horse and camel breeding play a significant role in economic activities, and its spread limits agricultural development. The absence of a vaccine, high susceptibility of various animal species, and rapid increase in infection cases exacerbate the epizootiological risk. Additional challenges arise from chronic and latent forms of the disease, which complicate diagnosis and control, highlighting the need for further research on this parasite.

T. evansi strains must be cultivated in a controlled laboratory setting to study the pathogen's biological behaviour, including its growth dynamics, morphological variability, viability, and interactions with host-related factors. To analyse pathogenesis, assess parasite adaptation, and determine the stages at which infection progression may be controlled, stable strain accumulation offers experimental material. Furthermore, the preparation of antigenic material, the enhancement of serological diagnostics, and the testing of possible methods for infection monitoring and control in veterinary practice all depend on cultivated strains

The biological characteristics of *T. evansi* and the extent of its distribution in different regions of the world remain important subjects of analysis in veterinary parasitology. In a review by M. Desquesnes et al. [1], a comprehensive analysis of the global epizootiological situation was conducted, showing widespread presence of the pathogen in countries of Asia, Africa, and South America, as well as isolated cases in Europe and Australia associated with the movement of infected animals. The authors emphasised that the diversity of clinical manifestations across regions made diagnosis difficult and led to delayed detection of the infection. Considering the epizootiological situation in tropical countries, surra remained insufficiently studied, which was highlighted by J. Kim et al. [2], who referred to it as a “neglected disease.” The researchers noted that, unlike in Africa where trypanosomoses were traditionally controlled, in Asia and Latin America the infection received less attention, and detected imported cases in Europe and Australia indicated a potential risk of range expansion. An important addition was data on Central Asia provided by Z. Abay et al. [3] in Kazakhstan, where seroepidemiological studies revealed a high prevalence of infection in camels. These results indicated a threat to traditional camel husbandry and confirmed the need to improve veterinary surveillance systems under specific regional conditions.

Methods for accumulation and cultivation of trypanosomes include the use of both laboratory animals and various culture systems, enabling the acquisition of viable material for research. Isolation and maintenance of strains *in vitro* were described by K. Kamyngkird et al. [4], who established stable conditions for parasite proliferation in nutrient media. The authors showed that growth was sustained for several days and depended on medium composition. Features of axenic culture were examined in R. Kumar et al. [5], demonstrating that induction of reactive oxygen species inhibited division and reduced cell viability. This allowed identification of mechanisms limiting the organism's survival outside the host. Analysis of growth dynamics conducted by S.M. Dzhunisbayeva et al. [6] recorded regular changes in pathogen numbers under laboratory conditions in Kazakhstan, enabling assessment of parasitemia kinetics during experimental infection. Additional insights were provided by G. Castelli et al. [7], who compared different culture media and demonstrated that a modified RPMI- PY medium (RPMI-1640 supplemented with peptone and yeast extract) ensured more stable growth compared to traditional tryptic soy broth-based media.

Accumulated trypanosome strains have been widely used for the development of diagnostic systems, assessment of drug efficacy, and applied veterinary purposes [8]. In the study by M.F. Canever and L.C. Miletti [9], the Pathogen Box® compound library was used, allowing identification of molecules with pronounced antiparasitic activity. The researchers showed that specific compounds inhibited parasite growth, offering prospects for further pharmacological testing. Additional contribution was made by G.T. Nguyen et al. [10], where an antigen obtained through *in vitro* cultivation was applied for enzyme-linked immunosorbent assay (ELISA). The method demonstrated high sensitivity and specificity for detecting infected cattle, enhancing the potential for seroepidemiological control. N. Akhmetsadykov et al. [11] developed a test system based on the recombinant antigen gm6, providing high specificity for detecting infected horses. The authors confirmed that using this tool facilitated diagnosis and improved the

effectiveness of epizootiological monitoring.

Despite available data on pathogen biology and certain cultivation methods, the efficiency of different approaches for accumulating viable *T. evansi* strains under laboratory conditions remains insufficiently studied. The scientific novelty of the study lies in the combined characterisation of *T. evansi* accumulation in an experimental mouse model and its subsequent cultivation in RPMI-PY medium. This work incorporates parasitemia dynamics, dose-related morphometric alterations, viability assessment, *in vitro* growth kinetics, and antigenic evaluation into a single experimental framework, in contrast to research that just concentrate on infection detection or diagnostic application. This method offers a more thorough foundation for choosing appropriate parasite material for pathogenesis research and the creation of serological tests.

The aim of this study was to characterise the accumulation dynamics, viability, morphological changes, and antigenic suitability of the *T. evansi* strain during experimental infection in mice and subsequent cultivation in RPMI-PY medium. To achieve this aim, the following objectives were defined: to perform *in vivo* and *in vitro* cultivation of the parasite; to evaluate the kinetics of its accumulation; and to develop approaches for controlling concentration and viability.

METHODS

The study was conducted as an experimental laboratory investigation with longitudinal observation at the Parasitology Laboratory of the Limited Liability Company (LLC) "Research and Production Enterprise "Antigen" (Almaty, Republic of Kazakhstan). The experiment took place from January to June 2025. This period was selected because of the seasonal availability of fresh biological material in Kazakhstan and the time required to cover the complete *in vivo* and *in vitro* cycle of pathogen accumulation.

The object of the study was a *T. evansi* strain, initially isolated by specialists of the Parasitology Laboratory of the Kazakh Scientific Research Veterinary Institute from a naturally infected dromedary camel (*Camelus dromedarius*), aged 4 years, kept in a private farm in the Turkestan region of Kazakhstan in December 2024, and preserved until the start of the experiment in the specified laboratory. To maintain viability, the material was stored in a Sanyo MDF- U53V cryobank (Japan) at -80 °C with dimethyl sulfoxide (DMSO, Sigma- Aldrich, USA) as a cryoprotectant, ensuring cell stability until the experiment.

For *in vivo* accumulation, male BALB/c mice (*Mus musculus*) were used, commonly applied in parasitological experiments due to their high susceptibility to trypanosomes and stable immune responses. Only males were included to exclude hormonal fluctuations typical of females during the estrous cycle, reducing result variability. Animals were 6–8 weeks old, weighing 18–22 g, corresponding to optimal physiological condition and reproducibility of infection indicators. A total of 30 animals were randomly divided into three groups of 10 (n=10 per group): group A received a standard inoculum of 10⁴ parasites/ mL, group B received a medium inoculum of 10⁵ parasites/ mL, and group C received a high inoculum of 10⁶ parasites/ mL. All 30 animals met the inclusion criteria, and none were excluded during preliminary screening. The exclusion criteria before randomisation were physiological abnormalities, pronounced lethargy, illness, co-infections, or injuries identified during preliminary examination. Animals were maintained under standard vivarium conditions with ad libitum access to food and water. Body weight loss exceeding 1 % of the initial value, marked lethargy, hypothermia, anorexia lasting more than 24 h, or parasitemia $\geq 1 \times 10^8$ cells/ mL were considered predefined humane endpoints rather than exclusion criteria. Animals reaching any of these endpoints were removed from further observation for ethical reasons but were not excluded from the statistical analysis. A modified intention-to-treat approach was applied, whereby all animals initially randomised to the three experimental groups were included in the analysis according to their assigned group. All measurements obtained up to the time of humane removal were retained, while observations after removal were treated as unavailable and were not imputed.

Frozen material was thawed in a 37 °C water bath for 60–90 s, resuspended in warmed PBS-G (phosphate-buffered saline supplemented with % glucose), and washed to remove DMSO by centrifugation at 300 × g for 10 min at 20 °C. Parasite viability was assessed using 0 % trypan blue, and only suspensions with viability of ≥ 9 % were used. The concentration of viable parasites was determined in a Goryaev counting chamber (China; chamber depth, 0.1 mm) at $\times 400$ magnification. Before counting, each suspension was gently mixed to ensure

homogeneous parasite distribution. Two independent chamber counts were performed for each preparation, and the mean value was converted to the number of viable parasites per millilitre. The concentration was corrected according to the proportion of trypan blue-negative cells.

The stock suspension was subsequently diluted with PBS-G containing % heat-inactivated serum according to the equation $C_1V_1 = C_2V_2$ to obtain target concentrations of 1×10^4 , 1×10^5 , and 1×10^6 viable parasites/mL. Each final inoculum was mixed thoroughly and recounted twice in the Goryaev chamber immediately before administration. A preparation was accepted when the duplicate counts differed by no more than 1 % and the mean measured concentration was within ± 1 % of the target value; otherwise, the suspension was adjusted and recounted. The same standardized suspension was used for all animals within the corresponding experimental group. Each mouse received 0.2 mL intraperitoneally, corresponding to actual administered doses of approximately 2×10^3 , 2×10^4 , and 2×10^5 viable parasites per mouse for groups A, B, and C, respectively. Inoculations were performed using 1-mL insulin syringes with 29G needles; brief isoflurane sedation at 1– % for no longer than 2 min was applied when necessary.

Starting from 2 days post-infection, blood was collected daily from the tail into Microvette® capillaries (Sarstedt, Germany) with EDTA, smears were prepared and stained using the Romanowsky-Giemsa method, and counting was performed in a Goryaev chamber; at low parasitemia ($< 10^4$ cells/mL), the buffy-coat method was applied. Clinical monitoring occurred twice daily (activity, body weight, coat condition, signs of anaemia). Peak parasitemia was observed on days 5–9. At $\geq 1 \times 10^7$ cells/mL, cardiac puncture under light isoflurane anaesthesia (1.5– 2. %, O_2 , 2– 3 min) was performed. Blood was collected into EDTA tubes (1.5 mg/mL), cooled, and processed by centrifugation (1,500 g, 10 min, 4 °C) with erythrocyte lysis using ammonium-chloride buffer. Morphometric analysis of trypanosomes measured cell length, cell width, free flagellum length, and described morphotype features, including undulating membrane condition, kinetoplast position, and presence of degenerative forms. Material was allocated for *in vivo* passages, *in vitro* cultures, and antigen preparation; excess material was frozen (-1 °C/min to -80 °C, then stored in cryobank).

For *T. evansi* cultivation, modified RPMI-PY medium was used, consisting of RPMI-1640 (Biowest, France) with 0. % peptone and 0. % yeast extract, supplemented with 1 % fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, USA), 2 mM L-glutamine (Sigma-Aldrich, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Thermo Fisher Scientific, USA) to prevent bacterial contamination. RPMI-PY medium was chosen because RPMI-1640 supplemented with peptone and yeast extract provides additional nutrients that support the short-term growth, motility, and morphological stability of *T. evansi*. Compared with ISCOV or HMI, this medium was more suitable for routine parasite accumulation and antigen preparation under reproducible laboratory conditions. Medium was prepared under sterile conditions, filtered through 0.22 µm membranes, and used freshly. Parasite suspensions post *in vivo* accumulation were washed three times in PBS-G and resuspended in 5 mL fresh medium. Samples were transferred to T-25 culture flasks (Corning, USA) containing 20 mL medium and incubated at 26 °C in a % CO_2 -enriched atmosphere. The % CO_2 -enriched atmosphere was used to maintain the bicarbonate-buffered RPMI-1640 medium at a stable physiological pH during incubation. This condition helped prevent medium alkalization and supported parasite motility, viability, and stable growth during the *in vitro* cultivation of *T. evansi*.

Cell mobility and morphology were monitored daily using an Olympus CKX53 inverted microscope (Japan). Parasite concentration was determined in a Goryaev chamber (China, depth 0.1 mm) at $\times 400$ and expressed as cells/mL; at low concentrations ($< 10^4$ cells/mL), the buffy-coat method was additionally applied. To prevent nutrient depletion and accumulation of toxic metabolites, medium was renewed every 48 h: one-fifth of the culture was transferred to a new sterile flask with 20 mL fresh medium. Cell viability was checked using trypan blue, excluding samples with > 2 % mortality. Cultures were maintained until cell concentration fell below 1×10^3 cells/mL or morphotype degeneration was observed (loss of typical shape, reduced motility, cytoplasmic vacuolization). Upon reaching these criteria, the culture was fixed, and material was used for analysis or frozen with cryoprotectant (9 % FBS +1 % DMSO) for storage.

Soluble somatic antigens were prepared from concentrated *T. evansi* cultures by washing in PBS-G, ultrasonication on ice, and centrifugation; the supernatant fraction was frozen at -80 °C. Protein concentration was measured by the Bradford method using bovine serum albumin for the calibration curve, with optical density read at 595 nm. Protein composition was further

analysed by denaturing SDS- PAGE on a Mini- PROTEAN system (Bio- Rad, USA), allowing identification of protein fractions of different molecular weights. Antigens were used for indirect ELISA: immobilized on 96-well plates, blocked with % albumin, incubated with diluted cattle sera (n=20; 10 positive, 10 negative), and detected using anti-bovine IgG conjugated with peroxidase; TMB served as the substrate, with optical density measured at 450 nm. Each run included positive and negative controls. Positivity threshold was set as the mean of negative samples +3 standard deviations (SD). Sensitivity and specificity were calculated using a contingency table for 20 samples.

All quantitative results were presented as mean $\pm$ standard deviation (M $\pm$ SD). Statistical analyses followed a modified intention-to-treat principle and included all 30 randomised animals in their originally assigned groups. For animals removed after reaching predefined humane endpoints, all available measurements collected before removal were included, without imputation of subsequent missing observations. Differences between two samples were assessed using Student's t- test, while comparisons across more than two groups were performed using one-way ANOVA. Statistical significance was set at $P < .05$.

The study complied with international standards for humane animal handling. All experimental procedures were approved by the local bioethics committee of the Kazakh Scientific Research Veterinary Institute and adhered to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [12]. Additionally, the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines were followed to ensure transparency and reproducibility [13].

RESULTS

Dynamics of parasitemia and clinical manifestations in laboratory animals

T. evansi is a mechanically transmitted hemoflagellate parasite that causes surra in camels, horses, cattle, and other vulnerable hosts. It primarily circulates in the bloodstream of affected animals. It is typically transmitted by biting flies through the transfer of infected blood, unlike a number of other trypanosomes that require cyclical development inside the vector. This biological characteristic helps to explain the parasite's quick spread and the need of keeping viable strains in experimental settings. Since changes in parasitemia, morphology, flagellum length, kinetoplast position, and viability directly reflect the parasite's growth state and adaptation under *in vivo* and *in vitro* conditions, the bloodstream form of *T. evansi* was used as the primary object of observation in the current experiment

All 30 BALB/c mice were included in the modified intention-to-treat analysis according to their original group allocation, with 10 animals in each group. No animal was excluded from the statistical analysis after randomisation. Groups A and B completed the planned observation period, whereas animals in group C reached the predefined humane endpoints and were removed from further observation. Their available data up to the time of humane removal were retained in the analysis, while no values were assigned or imputed for subsequent observation points. The study of parasitemia dynamics allowed tracing patterns of infection development at different inoculum doses and comparing them with clinical changes.

In group A (n=10), which received the lowest inoculum (10^4 cells/ mL), parasites were first detected in the blood only on days 3– 4 post- infection, indicating relatively slow infection development and the need for an adaptation period of the pathogen within the host. Peak parasitemia occurred on days 7– 9, averaging $(4.8 \pm 0.9) \times 10^6$ cells/ mL. Clinical manifestations were mild: mice showed slight lethargy, minor reduction in activity, and mild anaemia. Body weight loss did not exceed 1 % of baseline. The infection in this group had a prolonged course with relatively mild impact on the organism.

In group B (n=10; 10^5 cells/mL), invasion occurred more rapidly, with trypanosomes detected as early as days 2– 3. Peak parasitemia occurred on days 5– 6, reaching approximately $(1.1 \pm 0.2) \times 10^7$ cells/ mL, almost double that of group A, confirming a clear dose- dependent effect. Clinical signs were more pronounced: animals showed apathy, body weight loss of 8– 1 %, pale mucous membranes, and ruffled fur. Despite this, mice survived the observation period, allowing documentation of the full parasitemia cycle. This group exhibited an intermediate course: faster progression than at the lowest dose, with more prominent symptoms, yet not reaching critical severity.

The fastest dynamics were observed in group C (n=10), which received the highest inoculum (10^6 cells/mL). Parasites were first detected as early as day 2, indicating an extremely short adaptation phase. By days 5–6, parasitemia exceeded 1×10^8 cells/mL ($M \pm SD = (1.3 \pm 0.3) \times 10^8$ cells/mL), accompanied by severe lethargy, >1 % body weight loss, marked hypothermia, and progressive anaemia. The observation period for this group was therefore terminated early according to the predefined humane endpoints. This termination was not treated as an exclusion from the analysis, and data collected from all 10 animals up to the endpoint were retained.

Analysis of the results revealed a clear dose-dependent relationship between the initial inoculum dose and the rate of infection development. The low inoculum dose (10^4 cells/mL) resulted in detectable parasitemia only on days 3–4, with peaks on days 7–9 and mild clinical signs. The medium inoculum dose (10^5 cells/mL) led to parasite detection on days 2–3, with maximum levels on days 5–6, accompanied by apathy, ruffled fur, anaemia, and 8–1 % body weight loss. High-dose infection (10^6 cells/mL) resulted in parasite detection by day 2, with a peak on days 5–6, and was associated with severe lethargy, hypothermia, marked anaemia, and >1 % weight loss, necessitating early termination. Clinical observations corresponded with parasitemia data: group A mice maintained stable appetite, active movement, and smooth fur; group B showed reduced activity, decreased food intake, and ruffled fur, indicating metabolic disturbances; group C displayed severe depression, rapid weight loss, hypothermia, and shallow respiration from early days, confirming a severe infection course.

These observations confirmed the key role of inoculum dose in shaping the clinical picture and infection dynamics. The data not only characterised the course of *T. evansi* infection in BALB/c mice but also justified the selection of an optimal model for subsequent applied studies.

Morphological characteristics and viability of the investigated *T. evansi* strain

Morphological examination of the *T. evansi* strain isolated from a camel allowed a detailed description of the parasite's structural features and revealed changes depending on the inoculum dose and infection stage. Light microscopy of blood smears stained using the Romanowsky-Giemsa method showed that the parasites retained the species-specific spindle-shaped morphology, with a slender body, pointed ends, an undulating membrane, and a free flagellum. The cytoplasm stained evenly in blue tones, the nucleus was centrally located and stained deep reddish-violet, and the kinetoplast was clearly visible in the subterminal region. Average cell dimensions ranged from 19–28 μm in length and 1.8–3.2 μm in width, with flagella 6–11 μm long. Analysis of different inoculum groups revealed consistent differences in morphometric parameters and morphological stability (Table 2).

Analysis of the morphometric data demonstrated a clear dose-dependent transformation of *T. evansi* cellular parameters. In group A (10^4 cells/mL), cells maintained the most stable morphology: average length was 24.6 ± 2.1 μm , width was minimal (2.5 ± 0.3 μm), and the flagellum was longest (8.2 ± 1.1 μm). Parasites exhibited elongated bodies, distinct kinetoplasts, and uniform undulating membranes, indicating normal division and high population viability. Only a small fraction (2– %) showed slight vacuolization, which did not affect overall morphotype stability. In group B (10^5 cells/mL), cellular features began to change: average length decreased to 22.8 ± 2.4 μm , flagellum shortened to 7.4 ± 1.0 μm , and width increased to 2.7 ± 0.4 μm . At peak parasitemia (days 5–6), up to 1 % of cells showed morphological deviations, such as shortened flagella, uneven cytoplasmic staining, and minor kinetoplast displacement. These changes indicated the onset of morphofunctional instability, yet the population retained high viability, making this dose suitable for experimental studies: infection developed rapidly enough to study pathogenesis without excessive mortality. In the present experiment, morphofunctional instability was defined by the combined appearance of shortened flagella, uneven cytoplasmic staining, minor kinetoplast displacement, and early vacuolisation, while preserved viability indicated that these alterations had not yet reached a degenerative stage. The most pronounced abnormalities were observed in group C (10^6 cells/mL). Average cell length declined to 20.3 ± 2.9 μm (an 1 % reduction compared to group A), flagellum length decreased to 6.1 ± 1.2 μm (–2 % relative to control), and width increased to 2.9 ± 0.5 μm . Up to 15–2 % of parasites exhibited degenerative changes, including cytoplasmic vacuolization, loss of the undulating membrane, and abnormal kinetoplast positioning. These changes might have been associated with increased parasitemia and intensive parasite replication, which could have created unfavourable conditions for maintaining normal parasite morphology. Consequently, the

population became less stable and the cells functionally compromised.

The observed changes in kinetoplast position have biological significance because the kinetoplast is functionally connected with the basal body and flagellar apparatus. As a result, its displacement could be a sign that normal cell polarity and division processes are being disrupted. These alterations were especially noticeable in the high-dose group in the current investigation, when parasitemia quickly peaked and the percentage of degenerative forms rose. This implied that kinetoplast displacement was linked to the stage of intense parasite proliferation and the shift towards morphofunctional instability rather than being a separate morphological characteristic. Therefore, rather than representing only a change in cell shape, the morphological abnormalities observed at greater inoculum doses might have indicated decreased population stability during peak parasitemia.

In summary, the data demonstrated a clear relationship: the higher the initial inoculum was, the faster the parasites lost their typical elongated shape and functional stability. Biologically, this pattern was associated with increased stress at high parasitemia, accelerated division, and a higher probability of morphogenetic errors. For applied studies, the medium dose was considered optimal because the observed changes were moderate and the cells remained suitable for subsequent *in vitro* cultivation and preparation of serological tests.

Growth characteristics of *T. evansi* cultures *in vitro*

In the experiment, parasites were transferred to a modified RPMI-PY medium, which included RPMI-1640 supplemented with peptone and yeast extract, providing optimal conditions for parasite proliferation. On the first day after transfer, a lag phase was observed, with cell concentration remaining relatively low at $(1.0 \pm 0.2) \times 10^5$ cells/mL. Viability was high (95–9 %), indicating good tolerance of the new conditions. Morphologically, parasites retained elongated bodies, a clear kinetoplast, and an intact undulating membrane. Only occasional cells showed early vacuolization, interpreted as initial adaptive responses to the medium change. By the third day, cell division accelerated sharply. Average concentration increased to $(2.5 \pm 0.4) \times 10^6$ cells/mL, while viability remained high at 92–9 %. Microscopic analysis revealed dense cell clusters with distinct outlines and uniform cytoplasmic staining, marking the onset of the logarithmic growth phase. This phase was critical for material collection, as the population was highly active with minimal morphological changes. On day five, growth reached peak rate: concentration rose to $(6.8 \pm 1.1) \times 10^6$ cells/mL, nearly 27 times the initial level, with viability at 88–9 %. Vacuolization appeared in 5– % of cells but did not compromise overall population stability. Thus, days 3–5 represented the period of maximal growth and were considered optimal for experimental purposes.

By day seven, growth slowed; average concentration was $(1.1 \pm 0.2) \times 10^7$ cells/mL, viability 85–8 %. Morphological changes appeared, including shortened flagella and uneven cytoplasmic staining in some cells, indicating early morphofunctional instability due to metabolite accumulation and nutrient depletion. On day nine, cell numbers peaked at $(1.3 \pm 0.3) \times 10^7$ cells/mL, but viability dropped to 70–7 %, and up to 2 % of cells displayed degenerative changes such as cytoplasmic vacuolization, loss of the undulating membrane, and altered cell size. These signs reflected the culture reaching a stationary limit, making further maintenance without subculture impossible. By day eleven, concentration fell to $(0.9 \pm 0.2) \times 10^7$ cells/mL, viability dropped to 60–6 %, and morphological degradation became dominant, limiting use for applied purposes. Regular subculturing every 48 h maintained high viability and morphological stability, which was essential for antigen preparation.

The results demonstrated that *T. evansi* growth *in vitro* followed a characteristic S-shaped curve. The lag phase was short (1–2 days), followed by exponential growth (days 3–5), during which cell concentration increased several tens of times. The stationary plateau occurred on days 7–9, with a decline by day 11. This pattern was consistent with classical unicellular culture models and confirmed that, under the experimental conditions, RPMI-PY medium supported stable populations for a limited period. Viability analysis was particularly important. Despite increasing cell numbers, viability began to decrease from day five, reflecting rising stress. By day nine, a 20–2 % decline corresponded with pronounced morphological defects, highlighting a limited temporal window for applied research. Morphological assessment confirmed these findings: early culture phases retained “classic” forms (elongated body, clear kinetoplast, developed flagellum), but increased culture density led to flagellum shortening, cytoplasmic vacuolization,

and gradual loss of the undulating membrane. By the final phase, up to a quarter of the population was degenerated, sharply reducing material utility for biochemical and serological tests.

Overall, the optimal period for high-quality material was days 3–7 post-subculture, when cells combined high density, maximal activity, and morphological stability. Extended culture without subculturing led to rapid resource depletion and morphological degradation, making such cultures unsuitable for applied purposes.

Biochemical properties of antigenic material and serological test results

Biochemical analysis of antigenic material obtained from axenic cultures of *T. evansi* revealed stable protein content and a characteristic spectrum of protein fractions. The average protein concentration was 2.3 ± 0.4 mg/mL, confirming sufficient material for subsequent serological studies. Electrophoretic separation showed bands in the 25–75 kDa range, with the most prominent signals at approximately 30 and 55 kDa, corresponding to several dominant antigenic proteins. This profile indicated the presence of both surface glycoproteins and intracellular metabolic enzymes, providing a broad range of potential immunogenic determinants. The material was homogeneous and showed no signs of degradation, demonstrating the adequacy of the preparation conditions.

For serological testing, twenty bovine serum samples were used: ten from animals with a confirmed diagnosis of surra and ten negative controls. ELISA results demonstrated clear separation between the two groups. The mean optical density (OD) of positive samples was 1.21 ± 0.14 , with a range of 0.95–1.38, whereas negative samples remained low at 0.18 ± 0.05 , ranging from 0.10 to 0.27. The difference between groups was pronounced and statistically significant.

The cut-off threshold was set at 0.30, allowing complete separation of positive samples from most negatives. The calculated sensitivity was 10 % within the analysed sample set ($n=20$). Specificity reached 9 %, with one negative sample showing a value near the borderline, possibly due to cross-reactivity with other hemoparasites. Overall, the method demonstrated high reliability, and the signal-to-noise ratio ensured clear distinction between infected and uninfected animals.

Table 4 summarises the data, showing mean optical density values and ranges. Positive samples formed a compact group with closely aligned values, reflecting a stable immune response. Negative samples remained well below the cut-off, excluding the possibility of random fluctuations. These results confirmed the suitability of the prepared antigenic material for use in serological assays.

Analysis of the obtained data showed that the electrophoretic protein profile included both highly immunogenic surface glycoproteins and cytoplasmic components, which explained the high sensitivity of the test. The 30 and 55 kDa fractions contributed most significantly, as they generated the primary signal when interacting with serum antibodies. The presence of these components ensured a broad recognition spectrum and reliable detection of positive cases. ELISA results demonstrated that using *T. evansi* antigens from axenic cultures allowed clear discrimination between positive and negative samples. The optical density of positive samples was more than six times higher than that of negatives, minimising the likelihood of diagnostic errors. In situations where the sensitivity of traditional microscopy is limited by parasitemia levels, the serological test provided reliable identification of infected animals. The single recorded false-positive result did not significantly affect the overall assessment of the method. It is important to note that, with a larger number of samples, the proportion of such reactions might vary; however, even in the current dataset, specificity remained high and met diagnostic requirements.

Overall, the data confirmed that antigenic material derived from *T. evansi* cultures possessed stable biochemical properties and provided high diagnostic value in serological testing. The well-defined protein spectrum and reproducibility of the ELISA results supported the use of this approach as a reliable tool for diagnosing surra in livestock

Tables

Group (dose)	Time of parasite detection (days)	Peak (days)	Mean peak level (cells/mL)	Main clinical signs
A (n=10; 10 ⁴ cells/mL)	3–4	7–9	(4.8±0.9)×10 ⁶	Mild lethargy, <1 % weight loss, mild anaemia
B (n=10; 10 ⁵ cells/mL)	2–3	5–6	(1.1±0.2)×10 ⁷	Apathy, 8–1 % weight loss, anaemia, ruffled fur
C (n=10; 10 ⁶ cells/mL)	2	5–6	(1.3±0.3)×10 ⁸	Severe lethargy, >1 % weight loss, hypothermia, severe anaemia

Source: compiled by the authors.

Table 1: The table presents the time of initial parasite detection, the period and mean level of peak parasitemia, and the principal clinical manifestations observed in groups receiving inoculum doses of 10⁴, 10⁵, and 10⁶ cells/mL. Values for peak parasitemia are expressed as mean ± standard deviation (M ± SD; n = 10 per group).

Group (Dose)	Cell Length, μm	Width, μm	Flagellum Length, μm	Morphotype Features
A (n=10; 10 ⁴ cells/mL)	24.6±2.1	2.5±0.3	8.2±1.1	Typical elongated forms, distinct kinetoplast
B (n=10; 10 ⁵ cells/mL)	22.8±2.4	2.7±0.4	7.4±1.0	Shortened flagellum, partial cytoplasmic vacuolization
C (n=10; 10 ⁶ cells/mL)	20.3±2.9	2.9±0.5	6.1±1.2	High proportion of degenerative forms, displaced kinetoplast

Table 2: The table compares mean cell length, width, flagellum length, and dominant morphotype features in mice infected with inoculum doses of 10⁴, 10⁵, and 10⁶ cells/mL. Values are presented as mean ± standard deviation (M ± SD; n = 10 per group), demonstrating progressive cell shortening, reduced flagellum length, and increased morphological degeneration at higher inoculum doses.

Observation day	Cell concentration (cells/mL)	Viability (%)	Morphological features
1	(1.0±0.2)×10 ⁵	95–97	Adaptation phase, elongated forms
3	(2.5±0.4)×10 ⁶	92–94	Active division, clear membrane
5	(6.8±1.1)×10 ⁶	88–90	Vacuolization in 5– % of cells
7	(1.1±0.2)×10 ⁷	85–87	Flagellum shortening in some cells
9	(1.3±0.3)×10 ⁷	70–75	Degenerative forms in 20–2 %
11	(0.9±0.2)×10 ⁷	60–65	Morphological degradation

Table 3: The table presents changes in cell concentration, viability, and dominant morphological features from day 1 to day 11, demonstrating an initial adaptation phase, subsequent active proliferation, peak growth on day 9, and progressive degeneration during the stationary and decline phases. Values for cell concentration are expressed as mean ± standard deviation (M ± SD).

Serum group	n	Optical density	Range	Diagnostic interpretation
Positive	10	1.21±0.14	0.95–1.38	Clearly positive
Negative	10	0.18±0.05	0.10–0.27	Below cut-off
Cut-off	–	0.30	–	–

Note: M±SD — mean ± standard deviation.

Table 4: The table presents mean optical density values, observed ranges, and diagnostic interpretations for each serum group, demonstrating clear separation between positive and negative samples relative to the established cut-off value. Values are expressed as mean ± standard deviation (M ± SD).

DISCUSSION

The conducted study allowed tracking the dynamics of parasitemia and clinical manifestations in mice administered varying inoculum doses of *T. evansi*, as well as identifying characteristic

morphological changes in parasites depending on the inoculum dose. The results confirmed that high inoculum doses caused pronounced cellular degeneration and severe clinical conditions in the hosts, whereas the medium inoculum dose produced a stable course suitable for experimental modelling. *In vitro*, the cultures could be maintained for a limited period, enabling the preparation of antigenic material. Biochemical and serological analyses demonstrated the presence of immunogenic proteins and high diagnostic efficacy of the obtained antigen in indirect ELISA.

Parasitemia dynamics in mice showed a clear dose-dependent pattern: at 10^4 cells/mL, the peak occurred on days 7–9 with levels of $(4.8 \pm 0.9) \times 10^6$ cells/mL; at 10^5 cells/mL, the peak was on days 5–6 with $(1.1 \pm 0.2) \times 10^7$ cells/mL; and at 10^6 cells/mL, maximum parasitemia was reached during the same period at $(1.3 \pm 0.3) \times 10^8$ cells/mL. Clinical signs ranged from mild anaemia and moderate lethargy to severe lethargy, hypothermia, and over 1 % body weight loss, indicating a direct relationship between clinical severity and inoculum dose. These observations are consistent with the findings of A.M. Sadek [14], who reported that increasing the inoculum dose in laboratory animals led to earlier parasite appearance and accelerated development of parasitemia. In the present study, the peak at 10^6 cells/mL occurred on days 5–6, whereas at 10^4 cells/mL it was recorded only on days 7–9. Morphological changes in highly virulent infections have been described by D.H. Sawitri and R. Damayanti [15], including flagellum shortening and pronounced degenerative forms. Similar phenomena were observed in Group C of this experiment, where cell length decreased to 20.3 ± 2.9 μm , and up to 2 % of trypanosomes exhibited signs of morphological degeneration. Disruption of brain oxidative status and behavioural alterations in mice were reported by M.A. Dkhil et al. [16], confirming the role of *T. evansi* in systemic disorders. These results corresponded with the clinical manifestations in Group C, where animals displayed marked lethargy, body weight loss exceeding 1 %, and severe anaemia. Comparative analysis of diagnostic methods by A. Sadek et al. [17] demonstrated the superiority of serological approaches over microscopy at low parasitemia. A similar effect was observed in this study, where ELISA reliably distinguished positive and negative samples even at parasite concentrations below 10^4 cells/mL.

The results indicated that increasing the inoculum dose was accompanied by a reduction in cell and flagellum length, as well as an increase in the proportion of degenerative forms, reflecting the morphofunctional instability of the parasite. The most pronounced changes were observed at an inoculum dose of 10^6 cells/mL, where the average cell length decreased to 20.3 ± 2.9 μm and the flagellum shortened to 6.1 ± 1.2 μm . It has been established that after γ -irradiation, *T. evansi* underwent molecular and morphological alterations, as shown by R.T. Kangethe et al. [18], which corresponded with the observed morphotype degradation at high inoculum doses. This observation underscores the role of stress factors in accelerating morphofunctional instability.

During antiparasitic therapy, S.M. Barghash [19] reported the formation of morphological anomalies and a decrease in the proportion of functionally active cells, which corresponded to the changes observed at the high inoculum dose. However, a key distinction in this study was that such transformations occurred naturally, without chemical intervention, solely due to intensive replication. Experiments by G. Guayaquil et al. [20] demonstrated that genetic diversity and structural features of variable surface glycoproteins determined the resilience of *T. evansi* populations, aligning with the morphotype changes observed at high inoculum doses. These findings indicate that morphological instability may be influenced not only by the inoculum dose but also by antigenic structural characteristics. In addition, M.G. Sawerus et al. [21] showed that phytotherapeutic compounds induced significant morphological shifts and reduced parasite viability, corresponding to the degenerative forms observed at the highest inoculum dose. In contrast, in the present study, these changes occurred naturally during rapid proliferation rather than due to treatment.

The growth of *T. evansi* in RPMI-PY medium followed a typical S-shaped curve: an exponential phase was observed on days 3–5 with concentrations reaching $(6.8 \pm 1.1) \times 10^6$ cells/mL; a plateau was reached on days 7–9 with a maximum of $(1.3 \pm 0.3) \times 10^7$ cells/mL; and after day 11, culture degradation began, with viability dropping to 60–6 %. The most favourable period for material collection was days 3–7, when cells maintained morphological stability and high activity. C.S.D. Vieira et al. [22] reported that maintaining glucose metabolism supported stable growth of *T. cruzi* in culture, which aligns with the observed dependence of *T. evansi* viability on the nutrient medium. A.I. Ward et al. [23] established that long-term persistence of *T. cruzi* was achieved through the formation of tissue foci; in contrast, *T. evansi* growth *in vitro* ceased after

nine days due to medium depletion. According to P.C. Dumoulin and B.A. Burleigh [24], the high metabolic flexibility of *T. cruzi* amastigotes allowed adaptation to stress conditions. The present findings indicated that *T. evansi* was less resilient and began to degrade as early as day seven. N. Martinez-Peinado et al. [25] demonstrated that metabolic modifiers effectively suppressed *T. cruzi* growth in culture, corresponding with the observed sensitivity of *T. evansi* to environmental changes and accumulation of toxic metabolites.

The ELISA results demonstrated a clear distinction between positive and negative samples: in infected cattle, optical density reached 1.2 ± 0.14 , whereas control samples did not exceed 0.18 ± 0.05 , confirming the high diagnostic value of the prepared antigen. The method's sensitivity was 10 %, with a specificity of 9 %. In the study by W.D. Bhutia et al. [26], the use of protease and kinase inhibitors effectively reduced *T. evansi* viability *in vitro* and improved the accuracy of therapeutic assessment. These findings correspond with the current results, where the antigen material exhibited high specificity in serological testing. J.M. Bustamante et al. [27] reported that a modified treatment regimen could completely eliminate both active and latent trypanosome forms in mice, aligning with the high diagnostic capability of ELISA, which allowed detection of both overt and subclinical infections. According to W.G. Aregawi et al. [28], diminazene and isometamidium treatments were effective in controlling surra in laboratory animals, confirming the practical relevance of serological tests for monitoring therapy. A. Majeau et al. [29] identified conserved *T. cruzi* antigens for the development of universal diagnostic systems, supporting the present findings in which the detected protein fractions enabled reliable identification of infected animals.

The average protein concentration of the antigen was 2.3 ± 0.4 mg/mL, providing an appropriate working concentration for subsequent serological procedures. Electrophoresis revealed a protein spectrum ranging from 25– 75 kDa, with dominant bands around 30 and 55 kDa, indicating the presence of immunodominant components suitable for standardising antigen preparations. C. Reck et al. [30] demonstrated that using buffered *T. evansi* antigen allowed reliable detection of antibodies in horses, supporting the diagnostic potential of these dominant proteins. K. Benfodil et al. [21] reported that combining serological methods with molecular tests increased the sensitivity of trypanosomosis diagnosis, reinforcing the need for antigen standardisation. J.R. Durán et al. [32] showed that the cultivation conditions of *T. cruzi* directly influenced the formation of morphological and antigenic structures, highlighting the critical role of stable protein fractions for reproducibility in serological tests. M.A. Vanegas et al. [33] proposed a system for isolating and maintaining *T. cruzi* cultures to obtain stable antigenic material, which aligns with the present findings and confirms the value of standardised protein fractions for diagnostic development.

Overall, the data demonstrated that the morphological characteristics, *in vitro* growth patterns, and protein profile of *T. evansi* depended on the inoculum dose and culture conditions. A high inoculum dose led to increased cell degradation, whereas a limited cultivation period preserved stable forms. Immunodominant proteins were identified with diagnostic relevance, suitable for serological applications. Collectively, these results emphasise the importance of standardising cultivation methods and controlling antigen composition to improve the reliability of diagnostic assays.

The integrated description of *T. evansi* behaviour across two related stages — experimental infection in mice and subsequent *in vitro* cultivation in RPMI-PY medium — is the study's primary contribution. The findings demonstrated that while RPMI-PY cultivation made it possible to produce antigenic material appropriate for serological testing, the medium inoculum offered a balanced model characterized by sufficiently rapid parasite multiplication, preserved viability, and moderate morphological alterations. Therefore, the work's uniqueness is in connecting infection kinetics, morphometric stability, culture viability, and diagnostic usefulness in a single experimental scheme rather than being restricted to a single methodological component.

The study revealed that the development of parasitemia in BALB/c mice was strongly dependent on the inoculum dose. At the lowest inoculum dose, parasites appeared in the bloodstream on days 3–4, reaching a maximum of approximately 4.8×10^6 cells/mL by days 7–9, accompanied by mild anaemia and body weight loss of less than 1 %. At the medium inoculum dose, peak parasitemia reached 1.1×10^7 cells/mL on days 5–6, with clinical signs including pronounced anaemia, apathy, and weight loss of 8–1 %. The highest inoculum dose resulted in a sharp increase in parasitemia to 1.3×10^8 cells/mL, leading to severe disease characterised by weight loss exceeding 1 %, hypothermia, and lethargy. These findings confirmed a direct relationship

between the severity and progression of the disease and the initial inoculum dose. Morphological characteristics of *T. evansi* also varied according to the inoculum dose. At the lowest inoculum dose, cells were elongated, with a mean length of 24.6 ± 2.1 μm and a flagellum length of 8.2 ± 1.1 μm , with the kinetoplast clearly and stably positioned. At the medium inoculum dose, cell length decreased to 22.8 ± 2.4 μm and flagellum length to 7.4 ± 1.0 μm , with partial vacuolisation observed in the population. The highest inoculum dose resulted in further reductions in cell length to 20.3 ± 2.9 μm and flagellum length to 6.1 ± 1.2 μm , an increased proportion of degenerative forms, and displacement of the kinetoplast. These results demonstrated that increasing the inoculum dose directly affected parasite structural integrity.

In vitro growth of *T. evansi* in RPMI-PY medium followed an S-shaped curve. During the initial adaptation phase (day 1), cell density was around 1.0×10^5 cells/mL with viability up to 9 %. Exponential growth occurred on days 3–5, reaching 6.8×10^6 cells/mL with 88–9 % viability. By day 7, cell concentration increased to 1.1×10^7 cells/mL with viability of 85–8 %, peaking at 1.3×10^7 cells/mL on day 9 with a viability decrease to 70–7 %. After day 11, cell counts dropped to 0.9×10^7 cells/mL, viability fell to 60–6 %, and the proportion of degenerative forms increased to approximately one-quarter of the population. These data indicated that the optimal window for harvesting stable antigenic material was between days 3–7. Protein analysis showed an average concentration of 2.3 ± 0.4 mg/mL, sufficient for serological assays. Electrophoresis revealed protein bands in the 25–75 kDa range, with dominant signals at approximately 30 and 55 kDa. ELISA testing demonstrated clear separation of samples: positives had an optical density of 1.21 ± 0.14 , negatives 0.18 ± 0.05 , with a cut-off threshold of 0.30. These results confirmed the diagnostic value, reproducibility, and suitability of the antigenic material for epizootiological monitoring of trypanosomosis.

A limitation of the study was that experiments were conducted exclusively in BALB/c mice, limiting the extrapolation of results to other animal species. Future research should include a broader range of models and different animal lines to confirm the generalisability of the findings.

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CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

Data Availability Statement

Data availability: Request from corresponding author.

AUTHOR CONTRIBUTIONS

All authors contributed to all aspects of this research, approved the final manuscript and agreed to be accountable for this work.

AI Use Statement. Grammarly was used solely for language editing, including improvements to grammar, syntax, and clarity. The authors carefully reviewed and approved all suggested changes and take full responsibility for the accuracy, integrity, and final content of the manuscript, including all data presented. No generative AI was used to create scientific content, data, figures, or other visual materials.

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