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Research Article

Development of a Test System Based on Recombinant GM6 Antigen from *Trypanosoma evansi* for the Determination of Surra in Horses

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Abstract | Trypanosomosis, such as Surra, causes significant damage to livestock worldwide. The development of new serological diagnostic methods is highly relevant. The primary objective of this study was to produce a recombinant GM6 antigen derived from *T. evansi* and investigate its immunological properties for the detection and diagnosis of trypanosomosis. To create a recombinant antigen, bioinformatic analysis of the primary structure of *T. evansi* GM6 antigen from several regions was carried out. The protein sequence was reverse translated to the nucleotide sequence, after which the gene was synthesized using solid-phase method. The gene fragment, encoding the GM6 protein, was inserted or cloned into the pET28 expression vector after synthesis. The obtained sequences were checked by sequencing for correspondence to the matrix molecule. For transformation *E. coli* BL21 (DE3) strain was used. To assess the efficacy of the antigen, immunoassay analysis and blot-hybridization techniques were employed. Obtained results showed that 1mM isopropyl ß-D-1-thiogalactopyranoside induction at 2°C for 18 hours was the most optimal condition for expression of recombinant GM6 *T. evansi* antigen. From a 500 ml of growth medium, a total of 3.4 mg of recombinant protein was successfully obtained. A recombinant GM6 antigen of *T. evansi* encoding a 30 kDa polypeptide was developed, which has diagnostically significant sensitivity in the enzyme immunoassay at a dilution of 1:6400 with sera of horses and donkeys infected with trypanosomosis. The proposed test system can be used for diagnosis of trypanosomosis in the early stages of the disease.

Keywords | Serological Detection; Trypanosomosis; Veterinary Medicine; Amino Acid Sequence; Vector System.

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INTRODUCTION

Surra, also known as African animal trypanosomosis, is a significant parasitic disease that affects a wide range of domestic and wild animals in Africa, Asia, and South America. It is caused by the protozoan parasites belonging to the genus *Trypanosoma*, with *Trypanosoma evansi* being the primary causative agent. Surra poses significant economic losses due to reduced productivity, morbidity, and mortality in livestock. Therefore, efforts to control and prevent this disease remain important for the health and well-being of both animals and humans in endemic areas. To date, there are no vaccines against Surra, and the only way to fight it is to isolate infected individuals from the main herd into the quarantine zone. Diagnostic kits based on the use of low-sensitivity tests, such as the precipitation

reaction (PR), agglutination reaction (AR), gelling reaction (GR) do not allow making a reliable and timely diagnosis of trypanosomosis. According to C.A. Rodriguez et al. (2023) enzyme immunoassay (EIA) method is recommended as one of the diagnostic tests of infection determination or confirmation of disease absence after treatment.

According to R. Kumar et al. (2023), it is recommended to use the antigen obtained from trypanosomes lysate for this method. However, K. Sana et al. (2022) demonstrated that standardizing the quality of antigens is a complex task due to the involvement of multiple factors, including the antigen source, purification methods, storage conditions, and assay techniques. The use of this antigen in the test system leads to cross-reactivity and false-positive results. At the same time, recombinant antigens make it possible to increase the specificity of the method, eliminating the need to use animals to obtain antigen. Currently, there exist numerous recommended recombinant antigens for *Trypanosoma* spp. One notable example is the variable surface glycoprotein RoTat1.2. V. Lejon et al. (2005) demonstrated that due to successful expression in insect cells, results are comparable to the native antigen in serological studies aimed at detecting *T. evansi*-infected single-horned camels. Following Tran et al. (2009), glycoprotein 75 (ISG75), present on the surface of trypanosome cells, in a number of 5*104 molecules, is also considered as a potential antigen. Moreover, Goto et al. (2010) showed that trypanosome proteins contain tandem repeats (TR) and induce B-cell response. Some tandem repeats of parasitic proteins of the *Leishmania* and *T. cruzi* species have an immunological dominance.

GM6 is a cytoskeletal protein situated at the connecting site between the membrane microtubules and flagella of the parasite *T. evansi*. This particular protein demonstrates recognition during the early stages of infection when the immune system actively eliminates the parasite. As a result, it represents an excellent choice as an antigen (Honcharenko, 2018). Additionally, GM6 exhibits a high degree of conservation among various trypanosomes, making it a valuable candidate for serving as a potential diagnostic marker in the detection of animal trypanosomosis. The objectives of this study were bioinformatic analysis of the GM6 sequence of the *Trypanosoma evansi* GM6 antigen, obtaining oligonucleotides, cloning them in *E. coli* and obtaining recombinant proteins and testing their immunological properties for the detection of trypanosomosis.

MATERIALS AND METHODS

Synthesis and analysis of *T. evansi* antigen sequences

For search of antigen amino acid sequence, PubMed database and VectorNTI software were used. To determine the consensus sequence, from the NCBI database four molecules of *T. evansi* from different regions were selected: TeGM6 (Indonesia), TeGM6 African (Africa), GM6 (Morocco), TeGM62full (India). The amino acid sequence was back-translated to DNA sequence, and then divided into small pieces, optimized to generate overlapping oligonucleotides, using DNAWorks. Oligonucleotides were synthesized with the phosphonamidite method (Honcharenko, 2018). Reaction columns were dried at 60°C in a vacuum evaporator for 20 minutes. Then oligonucleotides were poured from the column into a 1.5 ml tube. 1 ml of 25% aqueous ammonia was added, then tubes were packed in a pre-cooled steel container and left at room temperature overnight at 800 rpm stirring. The oligonucleotide solution was transferred to new tubes, after centrifuging the tubes at 13200 rpm for 10 minutes. To remove ammonia, the oligonucleotide solution was evaporated in a vacuum evaporator at 40°C for 3 hours until fully dried and then dissolved in 100 µl of TE-1X buffer. Oligonucleotide purity was confirmed using a Bio-Rad vertical 14% PAGE system. Assembling of a trypanosomal gene with 4 terminal restriction sites, including EcoRI and Xhol, was performed under de novo PCR conditions. DNA fragments were separated through horizontal electrophoresis using a 1% agarose gel and then extracted using a mixture of phenol-chloroform and chloroform, with further ethanol precipitation. GM6 gene library was constructed using the pGEM-T Easy vector from Promega, USA. Subsequently, the resulting clones were sequenced using an ABI Prism 3100 system from Applied Biosystems, USA.

pET28 vector usage and transformation

The pET28 expression vector, which includes the T7 promoter, kanamycin resistance gene, and a histidine sequence, was used. The pGEM plasmid carrying the GM6 antigen sequence and the pET28 plasmid were digested with EcoRI and XhoI restriction enzymes obtained from Thermo Fisher Scientific, USA. The resulting fragments were separated by gel electrophoresis using a 0.8% agarose gel. The fragments of interest were then purified using the MinElute PCR purification kit from QIAGEN, USA. Subsequently, the GM6 antigen sequence from *T. evansi* and the pET28 plasmid were ligated together using T4 phage DNA ligase from Thermo Fisher Scientific, USA, at room temperature for 45 minutes. The resulting construct was introduced into electrocompetent *E. coli* strain BL21 (DE3). The transformed cells were plated on lysogeny broth (LB) medium containing 50 µg/ml kanamycin. PCR screening was performed to confirm successful insertion. Positive clones were then subjected to sequencing using BigDye kits on ABI Prism 3100 automatic DNA analysers from Applied Biosystems, USA.

E. coli cultivation and protein expression

E. coli cells were cultivated in LB medium with 50 µg/ml kanamycin at 37°C for 18-24 hours. Once the OD600 reached 0.6, the IPTG (isopropylthio-β-galactoside) inducer obtained from Sigma-Aldrich, USA, was introduced. The inductor was added at various concentrations (0.1 mm, 0.25 mm, 0.5 mm, 1 mm), cultivation was carried out at 25°C and 37°C. Cell disruption was accomplished through sonication (22 kHz for four cycles, 120 seconds each). Subsequently, centrifugation at 10000 g for 60 minutes was carried out to separate the insoluble portion. The resultant pellet was then reconstituted in 10 ml of buffer containing 20 mM HEPES, 500 mM NaCl, and 1 M urea. The reconstituted sample was incubated on an orbital shaker at room temperature for 30 minutes. After another round of centrifugation at 10000 g for 30 minutes, the pellet was resuspended in a metal-affinity chromatography buffer containing 20 mM HEPES, 500 mM NaCl, 20 mM imidazole, 8 M urea, and 10 mM 2-mercaptoethanol. A sonication step was performed at a frequency of 22 kHz for a single cycle lasting 60 seconds. Following a 30-minute incubation on an orbital shaker at room temperature, the suspension was subjected to centrifugation at 6000 rpm for 20 minutes. The resulting supernatant was then utilized for affinity chromatography. The purification of the recombinant protein was carried out using a HisTrap column from Amersham, USA, and elution was achieved by employing a metal-affinity chromatography buffer consisting of 8 M urea and 200 mM imidazole. The relative content of recombinant proteins in the insoluble fraction of the lysates of the producer strain was estimated using the Bradford protein assay, with bovine serum albumin serving as the standard. Electrophoretic separation of proteins was carried out using the Laemmli method in polyacrylamide gels (10% and 12%) under denaturing conditions. *T. evansi trypanosomosis*; H5-H8 – serum of a horse experimentally infected with; H9, H10 – serum; D1-D6 – sera of donkeys suffering naturally from trypanosomosis.

Serum sample collection and analysis

The sample size included serum from 10 horses (4 experimentally infected with *T. evansi;* 4 experimentally infected with *T. equiperdum* and 2 of horses suffering naturally from trypanosomosis) and 5 donkeys confirmed positive for trypanosomosis through parasitological examinations. Additionally, serum from 5 healthy horses and 5 healthy donkeys tested negative served as negative controls. Electrophoresis of the recombinant trypanosomal antigen was conducted on an 11% polyacrylamide gel containing sodium dodecyl sulphate, following the Laemmli method. The antigens were transferred from the gel to a nitrocellulose membrane with a method described by Towbin et al. (1979). For the immunochemical detection of specific antigens, the nitrocellulose membrane was initially incubated overnight at 4°C in a 1% BSA solution. Then it was washed three times with buffered saline and buffered saline with Tween. Subsequently, a 1.5-hour incubation at 37°C was carried out with a diluted solution of anti-6His-tag antibodies. The membrane was incubated at 37°C for 60 minutes with a working dilution of horseradish peroxidase-labelled anti-species antibodies after repeated washing. Additional washing steps were performed prior to the development of the reaction.

Immunoenzymatic assay and specificity testing

The substrate solution for the reaction involved combining 0.01 g of 4-chloro-naphthol with 2 ml of methanol. This blend was then mixed with 18 ml of buffered saline (pH 7.2-7.4) and 0.01 ml of 3% hydrogen peroxide. To determine the specificity of the resulting recombinant antigen, serum from animals affected by trypanosomosis was employed. For immunoenzymatic assay recombinant trypanosomal antigen was immobilized in the wells of a 96-well plate at an optimal concentration of 0.01 mg/ml in 0.05M bicarbonate buffer (pH 9.6) and incubated at 4°C for 12 hours. The wells were blocked with 1% horse serum after washing. Twofold dilutions of positive and negative control sera, starting at 1:100, were added to the wells and incubated at 37°C for 1 hour. Following a wash, an anti-virus conjugate (diluted 1:5000) was added and incubated for 1 hour at 37°C. The reaction was developed using a substrate, and the results were measured with a spectrophotometer.

RESULTS

Bioinformatic analysis of the amino acid sequence

Analysed GM6 molecules contained a consensus region between 43 and 111 amino acids. Three of the four molecules were completely identical. The TeGM Afrikan molecule contained changes in positions 94: alanine for threonine, 101: alanine for valine, 109: alanine for glutamic acid, 110: glutamine for leucine. This region is the most important in the diagnosis of trypanosomosis (Figure 1).

For further work, an amino acid sequence consisting of 262 amino acid residues was selected as shown in Figure 2.

The resulting amino acid sequence was reverse translated to DNA sequence and optimized for *Escherichia coli* BL21 (DE3) strain as shown in Figure 3.

Design of oligonucleotides, synthesis of GM6 gene

After reverse translation overlapping oligonucleotides were generated. As a result, oligonucleotides indicated in Table 1 were obtained.

Table 1: Oligonucleotides used for synthesis of GM6 gene from *T. evansi*

No.	Sequence	Number of nucleotides	Annealing temperature
1	GGAAACCCTGTATAAAATTGAAGATA	26	51.6
2	TATCTTCAATTTTATACAGGGTTTCCGGGCCAATGGT	37	70.5
3	GAATTCAGGAGATATACCATGGAACTG	27	55.5
4	CCAGAAAGCTGCGGCTATCGCTCGCTTTCAGTTTCGCCAGTTCCATGG-TATATCTCCTGA	60	85.6
5	GATAGCCGCAGCTTTCTGGATCCGATGCCGGAAGGCGTGCCGCTGAG-CGAACTGGAACTG	60	91.6
6	CGGCGTTCTTCTTCCATGGTGCTAAATTTTTCATCTTTATCCAGTTC-CAGTTCGCTCAGC	60	82.7
7	CCATGGAAGAAGAACGCCGCAAACTGATTGCGGAAGATCGCGAAGG-CAACGCGGCGCGCA	60	94
8	CGCCTGCGCATGGCTATGTTCGTTCATCGCCGCTTCCAGTTCCGCAATG-CGCGCCGCGTT	60	95.3
9	GCCATGCGCAGGCGGCGGATCGCAGCAGCCAGTTTTGCATTAGCAC-CGGCAAAACCGGCC	60	95.2
10	CAATGGTGCCATCAAAGCATTCCTGCAGGTTGTTATATTCCGCCGGGC-CGGTTTTGCCGG	60	89.9
11	AATGCTTTGATGGCACCATTGGCCCGGAAACCCTGTATAAAATTGAAGA-TAGCCGCGTGA	60	85.5
12	GCACTTCATGCAGCTGCAGGCTTTTTTTCGCGCTTTCTTTCACGCGGC-TATCTTCAATTT	60	85.7
13	TGCAGCTGCATGAAGTGCTGAGCAGCATTAGCTTTAGCAGCCTGGGCG-CGGAAAACATTC	60	88.2
14	CGGTGCGCACCAGGTTGCAGCCATCTTTGCCGTTGCCGCCGCGAATGT-TTTCCGCGCCCA	60	96.8
15	ACCTGGTGCGCACCGATAACAACGGCATTCTGAAAGGCGG-CAGCCCGACCCGCCATAACC	60	92.2
16	TTTCAGTTTGCTGCCAAAGTTCATCACGCCGCCGCCCCAGGTCAGGT-TATGGCGGGTCGG	60	92.2
17	AACTTTGGCAGCAAACTGAAAGCGGGCGGCAGCCACCATCATCATCAT-CATTAAAAGCTT	60	86.1
18	GGCTCGAGAAGCTTTTAATGATGATGATGATGG	33	67.3

The synthesis efficiency was 95%. The thermodynamic properties necessary for PCR reaction are indicated in Table 1. The trypanosomal GM6 gene was assembled from 16 oligonucleotides. The constructed gene was 786 bp long. The DNA fragment was cloned into the pGEM-TEasy multicopy plasmid vector. A library of genes consisting of 19 clones was created. Sequencing of 5 clones showed complete correspondence with the reference sequence.

Expression vector creation

E. coli BL21(DE3) cells were subjected to transformation using a pET28 plasmid with the GM6 *T. evansi* antigen at EcoRI and XhoI restriction sites. Subsequently, the 19 growing colonies were examined for the presence of the inserted sequence via PCR and then sequenced. To confirm the presence of the insert within the plasmid, PCR analysis was performed using a 1% agarose gel, resulting in the expected 786 bp fragment (Figure 4).

Based on the results of sequencing, clones 5-8, 15 and 17-19 fully corresponded to the sequence of reference trypanosomal antigen from the database. Clone 5 was used to optimize the conditions for cultivation and expression.

Expression and purification of GM6 *T. evansi* trypanosomal antigen in *E. coli* BL21 (DE3) strain

Culture temperature and IPTG concentration can significantly impact expression productivity in recombinant protein production. Experiments optimization is essential to determine the most suitable conditions for achieving high expression productivity. The elevated temperature may improve the efficiency of transcription, translation, and protein folding, leading to increased production of the target

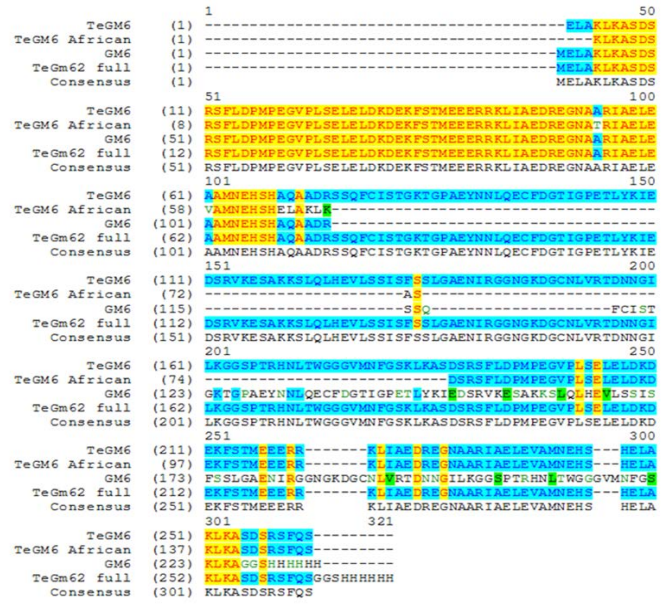

Figure 1: Comparative analysis of trypanosomal antigen amino acid sequences isolated from *T. evansi* across various regions of Kazakhstan

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  1 ELAKLKASDS RSFLDPMPEG VPLSELELDK DEKFSTMEEE RRKLIAEDRE
 51 GNAARIAELE AAMNEHSHAQ AADRSSQFCI STGKTGPAEY NNLQECFDGT
101 IGPETLYKIE DSRVKESAKK SLQLHEVLSS ISFSSLGAEN IRGGNGKDGC
151 NLVRTDNNGI LKGGSPTRHN LTWGGGVMNF GSKLKASDSR SFLDPMPEGV
201 PLSELELDKD EKFSTMEEER RKLIAEDREG NAARIAELEV AMNEHSHELA
251 KLKASDSRSF QS
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Figure 2: The amino acid sequence of GM6 isolated from *T. evansi*

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  1 GAACTGGCGA AACTGAAAGC GAGCGATAGC CGCAGCTTTC TGGATCCGAT GCCGGAAGGC GTGCCGCTGA GCGAACTGGA ACTGGATAAA GATGAAAAAT
101 TTAGCACCAT GGAAGAAGAA CGCCGCAAAC TGATTGCGGA AGATCGCGAA GGCAACGCGG CGCGCATTGC GGAACTGGAA GCGGCGATGA ACGAACATAG
201 CCATGCGCAG GCGCGGATC GCAGCAGCCA GTTTTGCATT AGCACCGGCA AACCGGCGCC GGCGGAATAT AACAACCTGC AGGAATGCTT TGATGGCACC
301 ATTGGCCCGG AAACCCTGTA TAAAATTGAA GATAGCCGCG TGAAAGAAAG CGCGAAAAAA AGCCTGCAGC TGCATGAAGT GCTGAGCAGC ATTAGCTTTA
401 GCAGCCTGGG CGCGGAAAAC ATTCGCGGCG GCAACGGCAA AGATGGCTGC AACCTGGTGC GCACCGATAA CAACGGCATT CTGAAAGGCG GCAGCCGGAC
501 CCGCCATAAC CTGACCTGGG GCGGCGGCGT GATGAACTTT GGCAGCAAAC TGAAAGCGAG CGATAGCCGC AGCTTTCTGG ATCCGATGCC GGAAGGCGTG
601 CCGCTGAGCG AACTGGAACT GGATAAAGAT GAAAAATTTA GCACCATGGA AGAAGAACGC CGCAAACTGA TTGCGGAAGA TCGCGAAGGC AACGCGGCGC
701 GCATTGCGGA ACTGGAAGTG GCGATGAACG AACATAGCCA TGAACTGGCG AAACTGAAAG CGACGCATAG CCGCAGCTTT CAGAGC
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Figure 3: The nucleotide sequence of GM6 trypanosomal antigen gene isolated from *T. evansi*

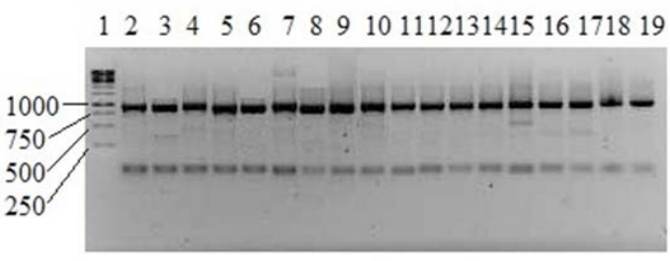

Figure 4: PCR results of *E. coli* clones transformed with pET28/GM6 carrying a trypanosomal antigen insert
Note: 1 – DNA markers; 2-19 – E. coli clones transformed with pET28/GM6.

protein. However, it is important to note that each protein and host organism may have an optimal temperature range for expression. Excessive temperature can result in protein misfolding, aggregation, or degradation. Insufficient IPTG concentration may result in low or no induction of the target gene, leading to low expression productivity. If the inducer concentration is too low, the promoter may not be fully activated, resulting in inadequate transcription and translation of the target gene. Higher IPTG concentrations can lead to stronger induction, but excessive concentrations may have negative effects, such as cellular stress or toxicity. The relative content of the recombinant protein in the soluble fraction of the lysate was determined using different concentrations of the expression inducer, as well as the temperature at which the producer strain was cultivated. The conditions for cultivation of *E. coli* pET28/GM6 strain were optimized. Based on the obtained data, the optimum amount of inductor was 1 mM IPTG and optimal growth temperature was 25°C. The obtained results are presented in Table 2.

Table 2: The relative content of the target protein in the insoluble fraction of the lysate at different cultivation temperatures and expression inducer concentrations

IPTG Concentration (mM)	Temperature (°C)	Relative amount of recombinant protein (%)
0.1	25	50
0.2	25	40
0.5	25	30
1	25	10
0.1	37	8
0.2	37	8
0.5	37	-
1	37	-

The expression of trypanosomal protein was assessed by varying the incubation time with IPTG. The obtained results are presented in Figure 5.

As can be seen from Figure 5, the most productive expression was when the culture was incubated with IPTG for 6 or 18 hours. Long-term incubation allows for sustained exposure to IPTG. This extended duration ensures that the inducer is continuously present in the culture, promoting continuous gene expression throughout the entire growth period. This sustained induction can lead to higher levels of recombinant protein production over time. Longer incubation periods provide more time for cells to grow and reach higher cell densities. Higher cell densities result in increased availability of cellular resources, which are essential for protein synthesis. Consequently, more resources can be allocated to the production of the recombinant protein, leading to higher expression productivity. Prolonged culture incubation allows cells to accumulate higher levels of

the recombinant protein over time. The longer the culture remains active, the more time the cells have to synthesize and accumulate the protein of interest. This can result in higher overall yields of the recombinant protein.

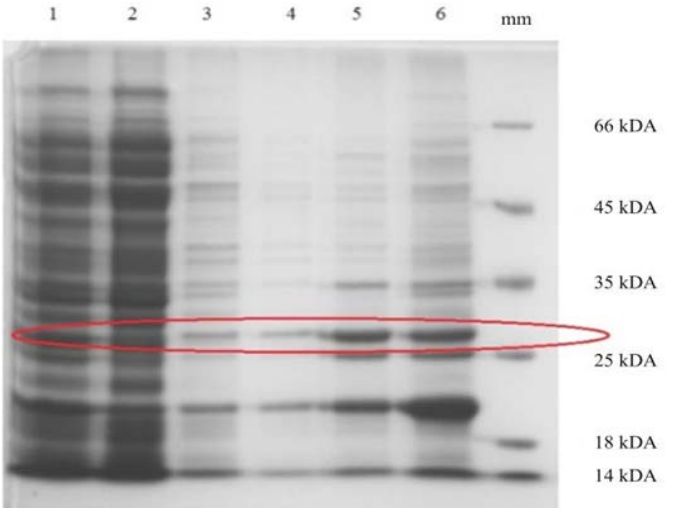

Figure 5: Determination of trypanosomal protein expression depending on time of incubation in IPTG
Note: 1 – cell lysate; 2 – supernatant after centrifugation of cell lysate; 3 – pellet after centrifugation of cell lysate; 4 – pellet of cell lysate without IPTG; 5 – cell lysate pellet after 6 hours' incubation with IPTG; 6 – pellet of cell lysate after 18 hours' incubation with IPTG; mm – molecular weights marker.

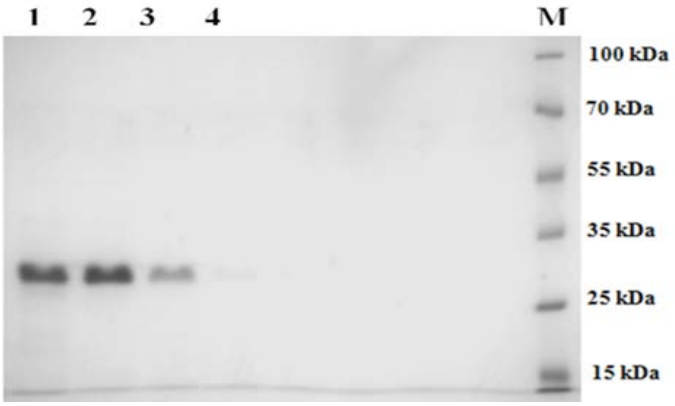

Figure 6: Electrophoregram of purified preparations of trypanosomal antigen GM6
Note: 1-4 – Purified fractions of trypanosomal antigen; M – molecular weights marker.

Figure 6 demonstrates the successful purification of recombinant proteins using metal-chelate chromatography with nickel-sepharose. An elution buffer containing 200 mM imidazole was employed to elute the target proteins. Electrophoretic analysis was performed, and the results indicated the presence of pure preparations of the trypanosomal antigen, with an observed molecular weight of approximately 30 kDa. The electrophoretic analysis was conducted using a 12% polyacrylamide gel (PAGE). From a 500 ml culture of *E. coli* BL21 (DE3)/pET28/GM6, a total of 3.4 mg of recombinant antigen was obtained.

Immunological reactions using the obtained recombinant GM6 antigen

Immunoblotting resulted in a clear band in the region of 30 kDa, corresponding to the recombinant trypanosomal GM6 *T. evansi* antigen (Figure 7).

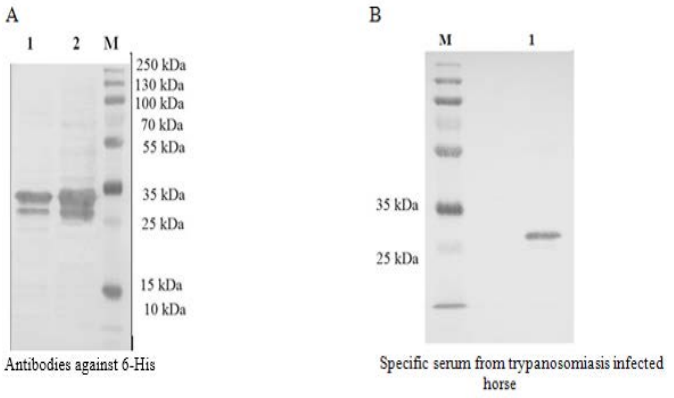

Figure 7: Immunoblotting of recombinant trypanosomal antigen GM6 *T. evansi*
Note: A – antibodies against 6-His; B – serum from trypanosomosis infected horse; 1 – recombinant GM6 protein prior to expression; 2 – recombinant GM6 protein after expression; M – molecular weights marker.

The data obtained show the specific interaction of GM6 *T. evansi* recombinant antigen with serum from *Equus ferus caballus* horse infected with trypanosomosis. Immunochemical and diagnostic characteristics of recombinant trypanosomal antigen GM6 was determined by an indirect enzyme immunoassay. For testing sera of trypanosomosis infected horses *Equus ferus caballus* and donkeys *Equus asinus asinus* were used. Figure 8 presents the results of immunoenzymatic analysis using the recombinant GM6 antigen with sera obtained from horses and donkeys that were infected with trypanosomosis.

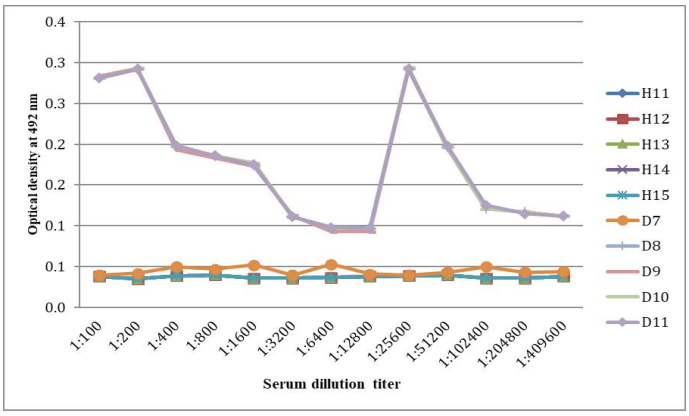

Figure 8: Immunoenzymatic analysis of recombinant trypanosomal antigen with sera from diseased animals
Note: H1-H4 – serum of a horse experimentally infected with T. evansi trypanosomosis; H5-H8 – serum of a horse experimentally infected with T. equiperdum trypanosomosis; H9, H10 – serum of horses suffering naturally from trypanosomosis; D1-D5 – sera

of donkeys suffering naturally from trypanosomosis.

In the case of horses experimentally infected with *T. evansi* (H1-H4), the analysis revealed high optical density values, indicating a strong positive reaction and successful detection of antibodies against the GM6 antigen. This highlights the GM6 antigen's effectiveness in identifying *T. evansi* infections in horses. Similarly, sera from horses experimentally infected with *T. equiperdum* (H5-H8) exhibited elevated optical density values, although slightly lower than those in *T. evansi*-infected horses. This suggests some degree of cross-reactivity between *T. evansi* and *T. equiperdum* antibodies, hinting at the potential broad utility of the GM6 antigen for detecting various Trypanosoma species.

Natural infections in horses (H9, H10) were associated with even higher optical density values compared to the experimentally infected horses, possibly indicating stronger immune responses in natural infections. The positive detection further confirms the GM6 antigen's capability to diagnose natural trypanosome infections. Moving on to donkey sera (D1-D5), these samples also displayed clear positive reactions, with optical density values falling within the diagnostic range, albeit lower than those observed in horse sera. This underscores the GM6 antigen's effectiveness in donkeys, albeit with moderately reduced sensitivity compared to horses. Figure 9, on the other hand, illustrates the analysis results using sera from healthy horses and donkeys.

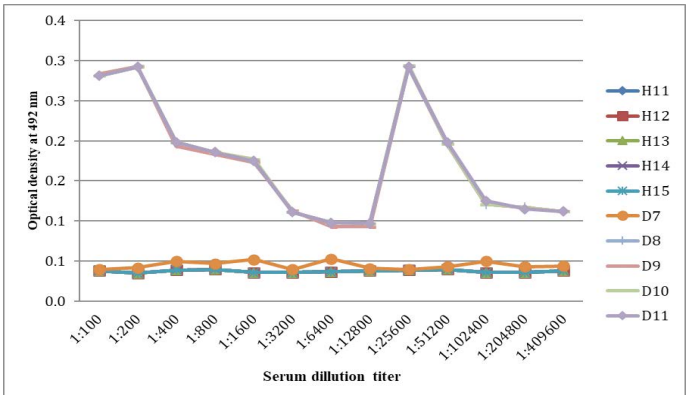

Figure 9: Immunoenzymatic analysis of recombinant trypanosomal antigen with sera from healthy animals
Note: H11-18 – healthy horse serum; D7-15 – healthy donkey serum.

All healthy horse sera (H11-H18) and donkey sera (D7-D15) demonstrated very low optical density values close to the baseline, clearly distinguishing them from the infected samples. This suggests the GM6 antigen's high specificity, with no false positive reactions observed in healthy animals. In summary, the recombinant GM6 antigen has proven to be highly sensitive and specific in detecting Trypanosoma infections in both horses and donkeys. The obtained results demonstrate that serum obtained from horses infected with trypanosomosis exhibits diagnostically significant optical density values at dilutions up to 1:6400. At the same time, naturally infected individuals showed a greater response than artificially infected individuals. The serum of an individual infected with *T. equiperdum* also showed a significant response, indicating the potential of the developed test system for the diagnosis of disease caused by different trypanosome species. Donkey serum also showed significant optical density values, which, however, were naturally slightly lower than for serum obtained from horses. The obtained data indicates a strong positive reaction between the obtained trypanosomal antigen and the antibodies present in the sera. Enzyme immunoassay results demonstrate high diagnostic properties of the recombinant trypanosomal antigen.

DISCUSSION

T. equiperdum and *T. evansi* are the two most commonly found species of trypanosomes. *T. equiperdum* is transmitted through sexual contact and causes Dourine disease in horses, while *T. evansi* causes Surra and is mechanically transmitted by blood-sucking insects like *Tabanus* spp. According to Ferreira et al. (2023) and Desquesnes et al. (2013), there is an urgent requirement for diagnostic tools and mass screenings in the field due to the high prevalence of these diseases. Gizaw et al. (2021) have pointed out that recent outbreaks of trypanosome infections highlight the genuine risk of equine trypanosomosis spreading globally. Therefore, it is crucial to develop accurate and specific diagnostic methods to ensure the safety of international horse trade. The variable presence of trypanosomes in the blood of horses makes direct detection challenging, emphasizing the need for serological approaches. Consequently, the effectiveness of serological methods demonstrated in this study for diagnosing bovine trypanosomosis aligns with the findings of other researchers.

Surra diagnosis currently relies on clinical signs that can be similar to those of other bovine pathogens, often leading to misdiagnosis. Unfortunately, there are no specific diagnostic tools available for identifying *T. evansi* or *T. equiperdum* infections. Furthermore, the treatment approach remains the same regardless of which parasite is detected. Goto et al. (2010) conducted a study demonstrating that repetitive antigens provoke a strong B-cell response. Pillay et al. (2013) emphasized the immunodiagnostic potential of the *T. vivax* GM6 antigen, not only for detecting the corresponding trypanosomes but also for diagnosing trypanosomosis globally. The authors evaluated the *T. evansi* GM6 antigen using diagnostic tools such as ICT and ELISA for equine trypanosomosis, which is not transmitted by

blood-sucking insects like in the case of Dourine disease. ELISA, based on crude antigens, demonstrated high sensitivity and specificity exceeding 90% in detecting trypanosomes. These findings confirm the efficiency and sensitivity of the method described in this study. However, A. Kocher et al. (2015) argue against using ELISA indicators as a diagnostic standard for trypanosomosis.

Pillay et al. (2013) conducted a study indicating that the *T. vivax* GM6 antigen holds promise as a potential candidate for the development of a point-of-care test for diagnosing African animal trypanosomosis in cattle caused by *T. vivax* and *T. congolense*. They expressed and purified the repetitive motif of *T. vivax* (TvGM6) and *T. congolense* (TcoGM6) GM6 antigens, and their immunodiagnostic potential was assessed using indirect ELISA with sera from cattle infected with *T. vivax* or *T. congolense*. In another study, Thuy et al. (2012) highlighted the significant sensitivity of rTeGM6-4r, suggesting its usefulness as an antigen for diagnosing Surra in veterinary screenings. L. Horalskyi et al. (2020) conducted preliminary diagnostic studies using the GM6 antigen from various trypanosome species, including a recombinant β-galactosidase *T. b. gambiense* GM6 fusion protein, which showed high immunodiagnostic sensitivity to serum from cattle infected with *T. brucei* and *T. congolense*. Therefore, the findings from previous studies align with the conclusions drawn in this study regarding the effectiveness of GM6 as a diagnostic tool for trypanosomosis.

Trypanosomes exhibit a notable degree of genetic diversity. Javanshir et al. (2023) have demonstrated genetic distinctions between *T. evansi* strains from different regions of Iran. Considering this, it was essential in this study to analyse the GM6 gene sequences of isolates originating from different regions of Kazakhstan to ensure sufficient conservation of the sequence for detecting infected animals in both regions. Davaasuren et al. (2017) conducted research showing cross-reactivity between *T. equiperdum* GM6 and anti-*T. evansi* GM6 antibodies. They also found a high similarity in the amino acid sequences of GM6 among different trypanosome species, indicating nearly identical antigens. Consequently, GM6-based diagnostic methods not only have potential for diagnosing Surra in equines but also hold utility in epidemiological studies and diagnosing Dourine disease. Verney et al. (2022) identified 12 false positive sera, with five sera from endemic areas of Northern Argentina testing positive for equine infectious anaemia and West Nile virus, potentially leading to cross-reactivity. Cross-reactivity between *T. evansi* and *T. equiperdum* was also observed in their study. These findings suggest that the recombinant antigens obtained can be employed for diagnosing various forms of trypanosomosis caused by different types of trypanosomes.

The findings of the study indicated that reducing the concentration of IPTG led to higher relative yields of the recombinant protein. While it is generally observed that higher concentrations of IPTG can enhance recombinant protein expression, there are instances where lower concentrations can actually result in higher yields. Khani and Bagheri (2020) demonstrated that this phenomenon is particularly evident for complex proteins or those that exert toxic effects on host cells. These proteins can accumulate to harmful levels, negatively affecting the growth and vitality of cells, which ultimately leads to a decline in overall protein production when high IPTG concentrations are induced. On the other hand, lower concentrations of IPTG can alleviate these toxic effects, enabling higher yields of the desired protein. In some expression systems, basal expression levels may already be present even without the addition of an inducer like IPTG (Horalskyi et al., 2022). These basal levels could be sufficient for obtaining the desired amount of recombinant protein. In such cases, the addition of lower concentrations of IPTG can fine-tune the expression levels and promote higher overall yields by avoiding excessive expression and its associated negative consequences. Additionally, Mohammadpour-Aghdam et al. (2021) showed that certain recombinant proteins may exhibit improved stability or proper folding when expressed at lower levels. High IPTG concentrations can induce rapid and excessive protein expression, overwhelming the cellular machinery responsible for protein folding and quality control. In contrast, lower IPTG concentrations can slow down the expression rate, allowing the cells to effectively fold and process the recombinant protein, resulting in higher yields of correctly folded and functional protein.

Looking ahead, the utilization of recombinant antigens offers a stable and reproducible alternative to whole parasite lysate antigens. GM6-based diagnostics hold the promise of affordable and reliable pen-side testing and large-scale surveillance, greatly aiding control efforts. Furthermore, the ability to distinguish between *T. evansi* and *T. equiperdum* infections through species-specific diagnostics enhances treatment precision. Early and accurate diagnosis is instrumental in facilitating timely treatment and the isolation of infected animals, effectively preventing transmission (Popadiuk and Golopura, 2018). Notably, the diagnostic potential of GM6 extends to both horses and donkeys, broadening its utility across affected livestock. Additionally, exploring the antigen's use in vaccine development against trypanosomosis is a promising avenue for future research.

The evaluation of cross-reactivity with other parasite infections is also important, as shared antigens may confound diagnosis. To ensure the consistency and accuracy of diag-

nostic tests, it is crucial to conduct testing on larger sample sizes from various geographical areas. Standardizing protocols and establishing universal cutoff values is essential for widespread adoption, especially in field settings. Additionally, the potential variation in GM6 gene sequences among strains may affect the detection of all variants (Kladnytska et al., 2021).

In this study, the sequences of the *T. evansi* GM6 antigen were analysed, a vector construct was created, and the corresponding recombinant protein was expressed. Its effectiveness as a serological diagnostic tool was confirmed by immunological studies. The obtained results regarding the genetic diversity of *T. evansi*, as well as the effectiveness of diagnostic systems based on GM6 for the determination of a wide range of trypanosomes in cattle, are consistent with the data of other researchers. The obtained data indicate a high potential of the proposed diagnostic method for use in animal husbandry and veterinary medicine.

CONCLUSIONS

In this study, four amino acid sequences of the GM6 antigen from *T. evansi* obtained from different regions of Kazakhstan were analysed. Three sequences turned out to be identical, and the fourth one differed from them by minor amino acid substitutions. Oligonucleotides were designed by reverse translation and synthesized. Recombinant GM6 antigen was successfully obtained by expression in *E. coli*. The results revealed that the most optimal condition for expressing the recombinant GM6 *T. evansi* antigen was achieved through the induction of 1mM IPTG at 25°C for a duration of 18 hours. This specific combination of parameters yielded the maximum relative amount of recombinant protein in the dry residue, which was 50%. The yield of recombinant protein was 3.4 mg from 500 ml of LB medium. This achievement signifies the effectiveness of the established methodology in obtaining a considerable amount of the target protein. The resulting recombinant GM6 antigen of *T. evansi*, with a molecular weight of 30 kDa, has proven to possess diagnostically significant sensitivity in the enzyme immunoassay. The recombinant GM6 antigen has proven to be highly sensitive and specific in detecting trypanosoma infections in both horses and donkeys. The observed optical density patterns align with expected antibody reactions in infected and healthy animals, indicating the GM6 antigen's significant potential for the development of sensitive and accurate diagnostic assays for animal trypanosomiasis. It displays noteworthy optical density values of diagnostic significance, at dilution of 1:6400. Further research is needed to establish cross-reactivity and the prospects of using the proposed diagnostic system for the detection of other types of trypanosomes, as well as their identification. The proposed test system, incorporating this recombinant GM6 antigen, holds promising potential for the accurate diagnosis of trypanosomosis in its early stages.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

NOVELTY STATEMENT

This study introduces an innovative approach for obtaining a recombinant GM6 antigen from Trypanosoma evansi, aimed at enhancing the detection and diagnosis of trypanosomiasis in horses and donkeys.

AUTHORS CONTRIBUTION

All authors contributed equally according to their tasks and approved the final manuscript.

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