



Detection of Molecular Markers of Drug-Resistance in *Trypanosoma evansi* From Camels in Egypt

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Abstract

Trypanosoma evansi is the parasite responsible for causing Surra, a significant veterinary disease worldwide. The development of drug resistance is a major obstacle in controlling trypanosomiasis. Typically, drug resistance occurs due to changes in the drug transporters of parasites. However, the prevalence of drug-resistant trypanosomes in Egypt's endemic regions is not well understood. In this study, we analyzed the TbAT1 and AQP2 genes from six *T. evansi* strains collected from camels across various locations in Egypt. PCR was utilized to amplify 164 bp TBR1/2 primers, followed by the amplification of 1600 bp and 1416 bp regions corresponding to the adenosine transporter P2 gene and the aquaglyceroporin transporter gene, respectively. Sequencing and BLAST analysis were conducted to compare the sequences with those in GenBank, and phylogenetic analysis was performed on all strains related to the TBR1/2, AQP2, and AT1 genes. All six DNA samples tested positive for the TBR1/2 primers. Three strains from South Sinai, Matrouh, and Halaib did not have both the AQP2 and TbAT1 genes, while the other three strains were positive for both genes. The AQP2 gene showed no variations at the nucleotide or amino acid level in any of the strains. However, the AT1 gene sequences had substitutions at different sites, which could contribute to drug resistance. The Behera strain did not show any variations at the nucleotide or amino acid level. Our findings revealed the presence of the TbAT1 gene in three out of six *T. evansi* strains, with some mutations. Additionally, three local strains lacked both the AQP2 and TbAT1 genes, potentially linked to drug resistance in *T. evansi*. This study provides molecular evidence of drug resistance in *T. evansi* in Egypt, which could be valuable for the treatment and control of Surra in the country.

Keywords: Aquaglyceroporins, Adenosine transporter, Drug Resistance, African Trypanosoma.

Introduction

Chemotherapy is crucial in fighting against *T. evansi*. These parasites and other protozoan species cannot produce purines from scratch for their metabolic pathways and the biosynthesis of nucleic acid [1]. Instead, extracellular parasite protozoa have developed ways to obtain essential purines from their hosts. They have different nucleoside transporters

with unique substrate specificities, different from mammals. This provides potential targets for controlling Trypanosomatidae members through chemotherapeutic methods. [2]

Drug resistance is a major challenge in treating and controlling trypanosome diseases [3]. Research on drug resistance has been ongoing since its discovery in 1908, especially after finding cross-

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resistance between different trypanocides [4]. As known, changes to the drug transporter of the parasite usually cause resistance, with several transporters responsible for drug uptake. [5]The P2 transporter, encoded by the TbAT1 gene, can transport drugs like melaminophenyl arsenical [6] – and diamidine to African trypanosomes [7,8,9,10]. Any disruption in the P2 transporter can lead to drug resistance [11,12] as shown by studies detecting drug-resistant parasites with defects in P2-mediated transport due to various mechanisms like point mutations and TbAT1 gene deletion [13,5,9] The gene TevAT1, which is similar to the gene found in *T. evansi*, has been sequenced and cloned. It has a 99.7% similarity with the TbAT1 gene of *T. brucei*. Researchers have identified the subtelomeric region of chromosome 5 as the genomic location of TbAT1. They found that TbAT1 was missing in the *T. brucei* gambiense STIB 386 mutant that is resistant to melarsomine hydrochloride. However, TbAT1 was present in the melarsomine hydrochloride-resistant *T. brucei brucei* STIB 247 and the diminazine-resistant *T. equiperdum* lines. Further analysis showed that the expression of TbAT1 RNA was undetectable in the *T. brucei brucei* STIB 247 and *T. equiperdum* diminazine-resistant strains. [14]. Moreover, eight *T. evansi* isolates that were unable to amplify the TevAT1 gene may be resistant to naganol, suramin, isometamidium, and quinapyramine constitute the most widely prescribed drug groups in the Philippines [15]. Another two transporters involved in drug transmission are a high-affinity pentamidine transporter (HAPT1) and a low-affinity pentamidine transporter (LAPT1). Aquaglyceroporins (AQPs) are crucial proteins present in all organisms that help maintain osmotic balance and facilitate the uptake of glycerol and urea. A study found that AQP2 encodes the high-affinity pentamidine transporter (HAPT1) [16], which is necessary for sensitivity to pentamidine and melaminophenyl arsenicals [17]. Mutations or changes in the expression of AQP2, a drug transporter, can lead to drug resistance thus, Knocking out the AQP2-specific gene was shown to cause cross-resistance to melarsoprol and pentamidine in trypanosomes [9]. Furthermore, AQP2 increases the sensitivity of trypanosomes to these drugs, and its loss of function can determine if cross-resistance is innate or acquired. There were no observed differentiation defects in cells lacking AQP2 during the life cycle, and drug sensitivity remained consistent throughout different stages. Likewise, a significant decrease in sensitivity to melarsoprol and pentamidine has been detected in trypanosomes lacking AQP2 compared to wild-type organisms. However, reintroducing AQP2 expression restored sensitivity to these drugs.

Since there is limited information available on drug resistance of *Trypanosoma* in Egypt, our study, aims to elucidate the mechanism of resistance in different *T. evansi* strains by molecular detection of mutations in the AT1 and AQP2 genes, in six isolates of *T. evansi* representing our country. Our findings provide the first insight into the mechanism of drug resistance in *Trypanosoma* spp. in Egypt, which is valuable for its treatment and control.

Material and Methods

Specimen collection and molecular identification

Six EDTA blood samples were collected from naturally infected camels in different localities of Egypt. Two samples from Cairo and four from Behera, Matrouh, Sinai and Halaib. Cairo2; kindly supplied by Dr. Tahani S. Behour. DNA extraction was performed at the laboratory of the Parasitology Department, Faculty of Veterinary Medicine, Alexandria University. DNA was extracted using the GeneJet Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions. Agarose gel electrophoresis was conducted to confirm the intensity of the DNA product.

The first PCR was conducted to amplify a 164 bp subgenus-specific sequence for trypanozoon using the TBR1/2 primer targeting mini-chromosome satellite DNA. The sequences for the TBR1/2 primer can be found in Table 1. The PCR mixture, with a total volume of 25 μ L, included 12.5 μ L of Dream Taq Green master mix (Thermo Scientific), 1.5 μ L of DNA template, 1 μ L of each primer (10 pmol/ μ L), and nuclease-free water to complete the volume. The amplification process was carried out in a 3Prime thermal cycler (TECHNE, UK) with an initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds, and extension at 72 °C for 30 seconds. A final extension step was performed at 72 °C for 7 minutes. Subsequently, 10 microliters of the PCR products were loaded onto a 1% agarose gel stained with ethidium bromide (0.5 μ g/mL) and electrophoresed using a 100 bp DNA ladder from Jena Bioscience (105 ng/ μ g/L) as a size marker. The results were visualized using the gel documentation system UVP PhotoDoc-itTM Imaging System (Analytikjena, USA)

Six DNA samples that tested positive for TBR1/2 from 6 different strains were amplified using specific primers (AQP2 and TBAT1) Table (1), to detect a 1416 bp segment of the Aquaglyceroporin 2 transporter gene and a 1600 bp segment encoding the adenosine transporter P2. The PCR mixture was prepared in a 25 μ L total volume, including 12.5 μ L of Dream Taq Green master mix (Thermo Scientific),

5 µL of DNA template, 1 µL of each forward and reverse primer for each gene (10 pmol /µL), and 5.5 µL of nuclease-free water. The amplification process was carried out in a ³Prime thermal cycler (TECHNE, UK) with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 62 °C for 45 s, and extension at 72 °C for 1.5 min. The final extension step was at 72 °C for 10 min. PCR was utilized to amplify 164 bp TBR1/2 primers, followed by the amplification of 1600 bp and 1416 bp regions corresponding to the adenosine transporter P2 gene and the aquaglyceroporin transporter gene, respectively.

Sequencing and phylogenetic analysis

The PCR products were purified and then sequenced in both forward and reverse directions using an Applied Biosystems 3130 automated DNA sequencer (ABI, 3130, USA) with the Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA, Cat. No. 4336817). Six samples from different regions two from Cairo, Beherah, Halaib, Sina, and Matrouh) were sequenced following the manufacturer's instructions. Contigs for each paired sequence were created using BioEdit software. A BLAST® analysis was conducted to confirm sequence identity with GenBank accessions, with matches between 98 and 100% and the lowest E value being considered.

A comparison was made between the sequences from this study and sequences from a database using the CLUSTALW multiple sequence alignment program in MEGA11. The Gblocks server was used to remove poorly aligned positions and divergent regions in the DNA alignment, making it better suited for phylogenetic analysis.

The evolutionary history was inferred by using the maximum likelihood method and the Tamura 3-parameter model [18]. The bootstrap consensus tree inferred from 100 replicates [4] was constructed to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 56% of bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model and then selecting the topology with the superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.8436)). This analysis involved 14 nucleotide sequences, and evolutionary analyses were conducted in MEGA11.[19]. A colored phylogenetic tree was constructed with the iTOL server.

Results

DNA extracted from six different regions tested positive for the TBR1/2 primer. The PCR products showed multiple bands due to the repetitive nature of the target gene. Multiple sequencing alignment for all six *T. evansi* strains with other isolates in Genbank is shown in Fig.1. Additionally, the two isolates of Cairo and Behera isolate were positive for both the AQP2 and TeAT1 genes, with specific bands at 1416 and 1600 bp, respectively. On the other hand, the Sinai, Matrouh, and Halaib isolates tested negative for both genes. Alignment and sequencing analysis were conducted, and the results can be found in Additional files 1, 2, 3, and 4. The percentage identity of each gene is illustrated in (Fig. 2,3,4)

TBR gene

The BLAST analysis of the consensus sequences confirmed that the representative sample was a *T. evansi* isolate with a 99% identity match. The results showed similarities to various *T. evansi* strains such as the *T. evansi* strain Gedaref 3 minisatellite sequence, the *T. evansi* strain Kassala minisatellite sequence, the *T. evansi* strain Gedaref 2 minisatellite sequence, the *T. evansi* strain New Halfa 1 minisatellite sequence, the *T. evansi* strain Ad Damer 2 minisatellite sequence, the *T. evansi* strain Ad Damer 1 minisatellite sequence, the *T. evansi* strain Sidoon minisatellite sequence and the *T. evansi* strain Port Sudan minisatellite sequence. Fig.2 and Table 2.

A phylogenetic tree constructed using the maximum likelihood method (Fig. 2) revealed that the five *T. evansi* isolates from different areas (Sina, Matrouh, Halaib, Cairo1, and Behira) were highly similar to each other (bootstrap 100), while samples from Cairo2 seemed to constitute an outgroup. All samples were grouped in a cluster with an MF 142291 *T. evansi* strain Sidoon minisatellite sequence, an MF 142293 *T. evansi* strain Ad Damer 2 minisatellite sequence, an MF 142297 *T. evansi* strain Gedaref 2 minisatellite sequence, an MF 142290 *T. evansi* strain Port Sudan minisatellite sequence, an MF 142298 *T. evansi* strain Kassala minisatellite sequence, an MF 142299 *T. evansi* strain Gedaref 3 minisatellite sequence, an MF 142292 *T. evansi* strain Ad Damer 1 minisatellite sequence, and an MF 142294 *T. evansi* strain New Halfa 1 minisatellite sequence.

AQP2 gene, AT1 gene

The alignment analysis of the AQP2 gene did not show any variations in nucleotides or amino acids. Similarly, the Cairo 1 isolate of the AT1 gene also did not exhibit any polymorphisms at the nucleotide or amino acid level. However, the Cairo 2 isolate had nucleotide substitutions at various locations, and

surprisingly, the Behera isolate had both nucleotide substitutions and insertions. These changes led to the addition of new amino acids and the replacement of existing ones. Table (3)

The AQP2 phylogenetic tree showed that three isolates (Behera, Cairo 1, and Cairo 2) were very similar to each other and grouped with XM-822804-*T. brucei brucei* TREU927 (bootstrap 98), (Fig. 3). The TeAT1 gene phylogenetic tree indicated that Cairo 2 was closely related to LC467218 *T. evansi* S4 TevAT1 and clustered with the Behera isolate and *T. brucei gambiense* DAL972 (accession numbers XM011773670 and FN554966). Cairo 1 was in a separate subgroup with 17 other isolates. (Fig. 4)

Discussion

Currently, drug resistance in *Trypanosoma* is linked to a decrease in drug intake by transporters due to mutations. The results confirmed that all strains were *T. evansi*. The AQP2 gene was present in three isolates (Cairo 1, Cairo 2, and Behera) without any genetic variations when sequenced. However, the AT1 gene was also found in these three isolates with some mutations, suggesting that these isolates may be resistant to drugs.

Loss of either the TbAQP2 or TbAT1 gene leads to a loss of susceptibility to melaminophenyl arsenical (MPA) and pentamidine. The B48 strain, which lacks both genes, is more resistant to pentamidine and MPA compared to other strains that only lack one of the two genes. This highlights the importance of these genes for cross-resistance [16]. Point mutations or deletion linked to the P2 transporter cause resistance [20,21] also showed that both P2 and HAPT1 carrier proteins need to be deleted in order to achieve large levels of melaminophenyl arsenical resistance. It was discovered that these two resistance mechanisms functioned independently yet tightly in tandem during experiments.

Similarly, field isolates of *T. b. brucei* and *T. b. rhodesiense* that are resistant to diminazene have similar AT1 point mutations. Moreover, mutant *T. b. gambiense* arises from different mutations in TbAQP2, resulting in either loss of AQP2 or loss of the wild-type TbAQP2 allele and subsequent chimeric TbAQP2. The mutant *T. b. gambiense* is 3- to 5-fold less sensitive to melarsoprol and 40- to 50-fold less sensitive to pentamidine compared to reference isolates, indicating a significant correlation between mutant *T. b. gambiense* and decreased sensitivity to these drugs [17]. On the other hand, genetic engineering was used to knock out the AT1 gene in *T. evansi* through RNA interference, resulting in a 10-fold decrease in TevAT1 mRNA. The induced parasites showed 5.5-fold greater

propagation at an in vitro Berenil concentration compared to the IC50 of the wild-type strain. [22]

According to the alignment and phylogenetic results, the analysis investigated the population diversity of *T. evansi* in the six governorates and revealed five closely related clusters and one outgroup. Interestingly, our samples from these areas were clustered with the MF 142291 *T. evansi* strain Sidoon minisatellite sequence, the MF 142293 *T. evansi* strain Ad Damer 2 minisatellite sequence, the MF 142297 *T. evansi* strain Gedaref 2 minisatellite sequence, the MF 142290 *T. evansi* strain Port Sudan minisatellite sequence, the MF 142298 *T. evansi* strain Kassala minisatellite sequence, the MF 142299 *T. evansi* strain Gedaref 3 minisatellite sequence, the MF 142292 *T. evansi* strain Ad Damer 1 minisatellite sequence, and the MF 142294 *T. evansi* strain New Halfa 1 minisatellite sequence. These findings confirm that all the strains are *T. evansi* with an overall low degree of genetic variability.

Even then, we need to know more about the link between the observed mutations and the drug transport activity of the gene product, which will require functional analysis and expression of all detected chimeras and mutants. Only if we can gain sufficient insights into all the structural determinants of drug transport by AT1 and AQP2 may we be able to base treatment decisions on their sequence analysis.

Conclusions

Five of the six *T. evansi* strains that were being studied, which came from various locations around our nation, had a tight relationship with one another. An out group was produced by the strain Cairo 2. The genes for AQP2 and AT1 were missing from the strains Sinai, Matrouh, and Halaib. Variants encoding these genes include Cairo1, Cairo2, and Behera without any polymorphism variation or genetic reassortment in the AQP 2 gene. Conversely, several substitutions and insertions at various locations in the AT1 gene have resulted in changes that make it more susceptible to trypanocide resistance. To combat medication resistance in Egyptian *T. evansi* strains, more follow-up research is necessary to find new genetic markers

Abbreviations

AQP2: Aquaglyceroporine 2; AT1: Adenosine transporter 1; TBR: *Trypanosoma brucei* repeat

Supplementary information

Additional file 1: Nucleotide alignment between the query sequence and database sequences of AQP2 sequences. (Notepad file).

Additional file 2: Amino acid alignment between the query sequence and database sequences of AQP2 sequences. (Word file).

Additional file 3: Amino acid alignment between the query sequence and database sequences of AT1 sequences. (Notepad file).

Additional file 4: Nucleotide alignment between the query sequence and database sequences of AT1 sequences. (Notepad file).

Acknowledgments

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Declarations

Ethical approval

Ethical approval was obtained from the ethical committee of the Faculty of Veterinary Medicine, Alexandria University, Egypt (Approval No. AU 1308072024 119).

Consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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TABLE 1. Primer sequences for the target genes

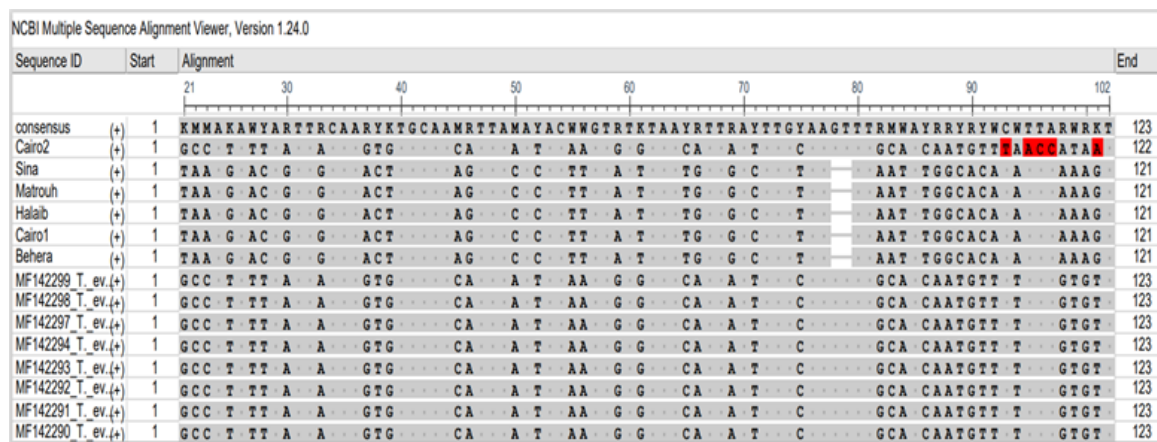
Primer name	Primer sequence	Amplicon length	Accession no.
TBR-1	5'-GAATATTAACAATGCGCAG-3' 5'-CCATTTATTAGCTTTGTTGC-3'	164 bp	[22]
AQP2	5'-AAGAAGGCTGAACTCCACTTG-3' 5'-CTTCGGGAGAAACAAACCTC-3'	1416 bp	[17]
TbAT1	5'-GAAATCCCCGCTTTTTCTCAC-3' 5'-ATGTGCTGAGCCTTTTCCTT-3'	1600 bp	[17]

TABLE 2. BLAST results of six nucleotide sequences with percentages of identity to *Trypanosoma evansi*

Sequence	Accession number	e-value	Identity %	Reference
<i>Trypanosoma evansi</i> strain Gedaref 3 minisatellite	MF142299.1	2e-59	99%	[23]
<i>Trypanosoma evansi</i> strain Kassala minisatellite sequence	MF142298.1	9e-58	99%	[23]
<i>Trypanosoma evansi</i> strain Gedaref 2 minisatellite sequence	MF142297.1	9e-58	99%	[23]
<i>Trypanosoma evansi</i> strain New Halfa 1 minisatellite sequence	MF142294.1	9e-58	99%	[23]
<i>Trypanosoma evansi</i> strain Ad Damer 2 minisatellite sequence	MF142293.1	9e-58	99%	[23]
<i>Trypanosoma evansi</i> strain Ad Damer 1 minisatellite sequence	MF142292.1	9e-58	99%	[23]
<i>Trypanosoma evansi</i> strain Sidoon minisatellite sequence	MF142291.1	9e-58	99%	[23]
<i>Trypanosoma evansi</i> strain Port Sudan minisatellite sequence	MF142290.1	9e-58	99%	[23]

TABLE 3. Substitution and insertion mutations of the AT1 gene and their positions

Isolate	Nucleotides	Amino acids
Cairo 1	No change	No change
Cairo 2	Mismatch G>A at position 521 Mismatch G>T at position 619 Mismatch A>C at position 641 Mismatch A>T at position 665 Mismatch T>G at position 723 Mismatch T>G at position 740 Mismatch A>C at position 933	Ins "F" at position 340
Behera	Ins" GTGT" at position 406 Ins" GC" at position 512 Ins" AA" at position 614 Ins "CCAAC" at position 626 Ins "TAATAATT" at position 650 Ins" TTTGGC" at position 695 Mismatch A>T at position 648 Mismatch A>G at position 694 Mismatch "TGCT>CTTC" at position 410 Mismatch C>G at position 703 Mismatch A>G at position 759 Mismatch TA>AG at position 790 Mismatch C>G at position 804 Mismatch G>A at position 849 Mismatch A>G at position 851 Mismatch A>C at position 854	Ins "W" at position 271 Mis "KK > RT" at positions 272 and 273 ins "F" at position 340

**Fig.1. Multiple sequence alignment (MSA) of *T. evansi* (the present study) with other *T. evansi* isolates present in databases**

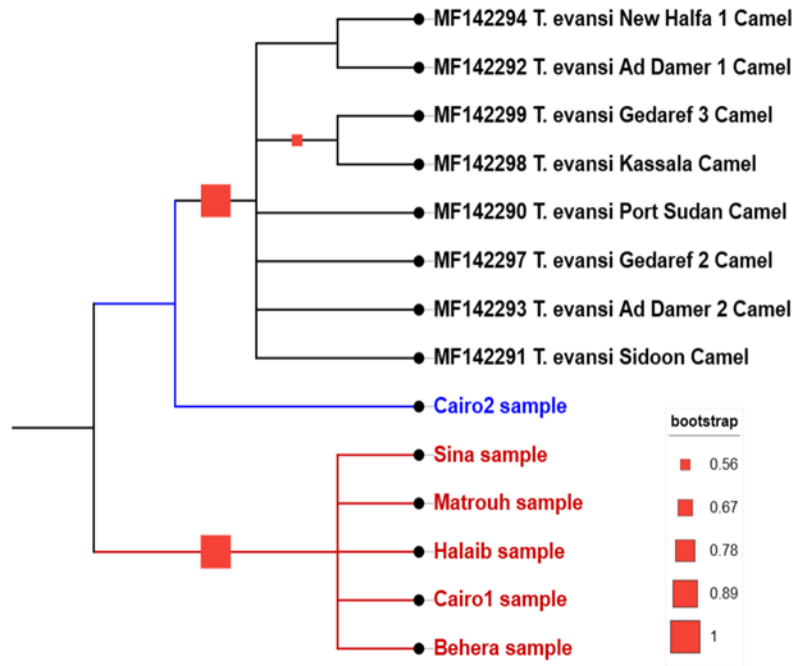


Fig. 2. The relatedness of the six strains under study according to the TBR gene and their relatedness with other isolates in the GenBank. The dendrogram was constructed by the maximum likelihood method. The distance of similarities was based on the molecular basis of the evolution of minisatellite sequence regions in the genomic DNA

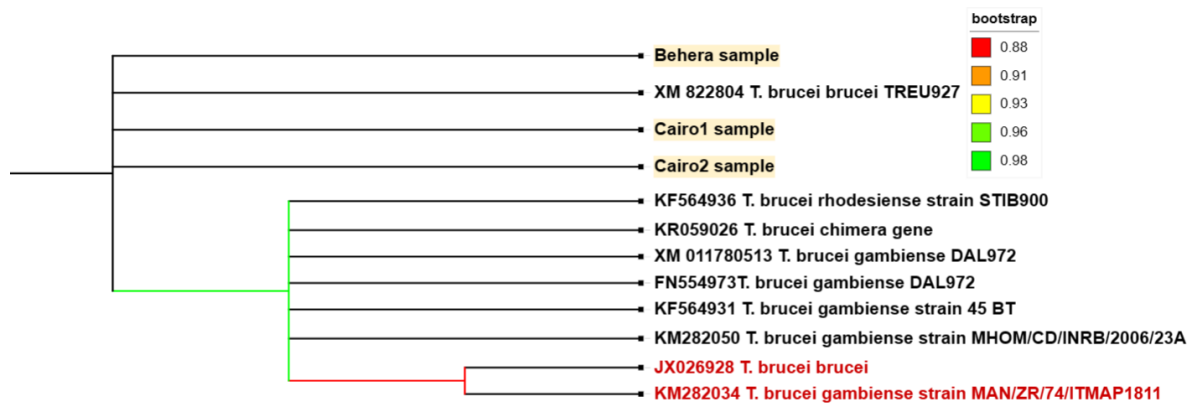


Fig. 3. Relationships among the Cairo1, Cairo 2, and Behera strains according to the AQP2 gene and their relatedness with other isolates in the gene bank. The dendrogram was constructed by the maximum likelihood method. The distance of similarities was based on the molecular basis of the evolution of minisatellite sequence regions in the genomic DNA

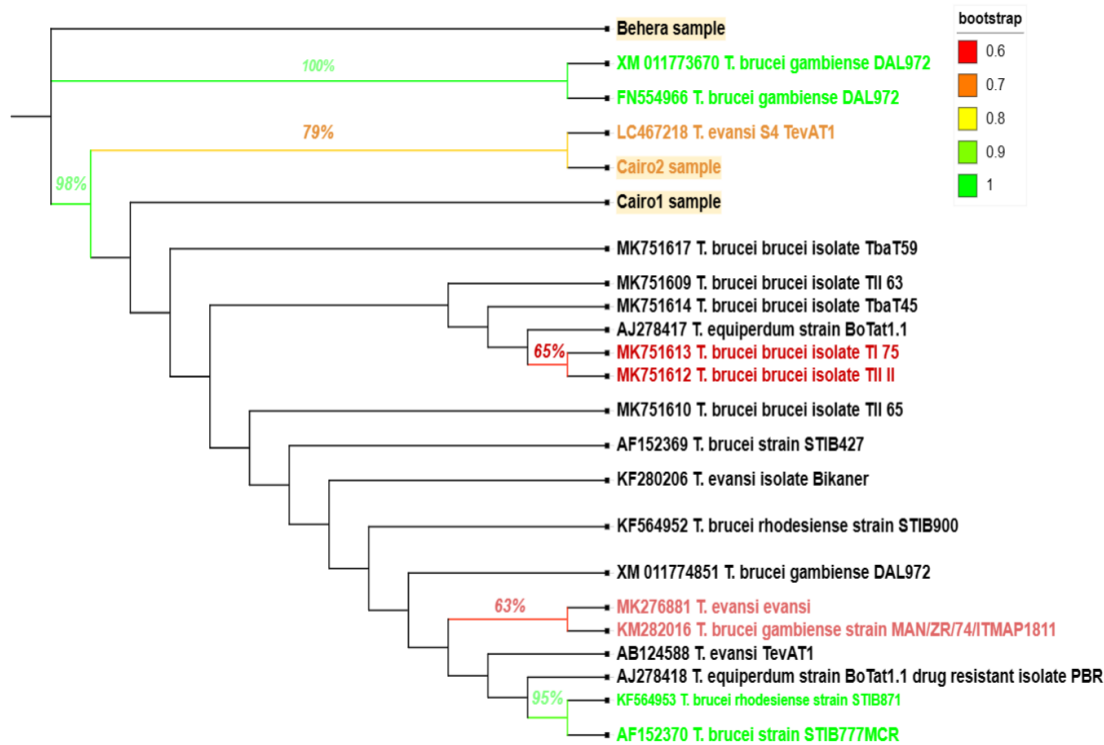


Fig. 4. The relatedness among the Cairo1, Cairo 2, and Behera strains according to the AT1 gene and their relatedness with other isolates in the GenBank. The dendrogram was constructed by the maximum likelihood method. The distance of similarities was based on the molecular basis of the evolution of minisatellite sequence regions in the genomic DNA

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الكشف عن العلامات الجزيئية لمقاومة الأدوية في طفيل التريبانوسوما ايفنسى من الإبل في مصر

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الملخص

المتقبية ايفانسي هي العامل المسبب لمرض السرة، وهو أحد أهم الأمراض البيطرية ذات الأهمية الاقتصادية في العالم. يمثل ظهور مقاومة للأدوية عائقًا كبيرًا أمام السيطرة العلاجية الكيميائية على داء المتقبيات. عادة ما تنتج مقاومة الأدوية عن تغيرات في ناقلات الأدوية للطفيليات. وفي مصر، لا يزال انتشار داء المتقبيات المقاوم للأدوية في المناطق الموبوءة غير مفهوم جيدًا. قمنا بتسلسل جينات *TbAT1* و *AQP2* من ست سلالات حقلية من التريبانوسوما ايفنسى تم جمعها من الجمال في مناطق مختلفة والتي تمثل دولة مصر. تم إجراء اختبار البلمرة المتسلسل لرصد جين *TBR1* / *TBR1* بمقدار 164 بيز بير. تم استخدام ست عينات الحمض النووي ايجابية لجين *TBR1/2* للكشف عن وجود جين ال *AT1* بحجم 1600 بيز بير والتي تشفر الجين *P2* الناقل للأدينوزين وجين *AQP2* بحجم 1416 بيز بير. تم إجراء تحليل التسلسل لتحديد هوية التسلسل للتسلسلات التي تم الوصول إليها من بنك الجينات والتحليل الوراثي لجميع السلالات المرتبطة بجينات *TBR1/2* و *AQP2* و *AT1*. أكدت النتائج ان جميع السلالات كانت ايجابية لوجود جين *TBR1 / 2* مما يؤكد ان جميعها تخص متقبية ايفانسي. كما اوضحت النتائج ايضا ان ثلاث سلالات تمثل جنوب سيناء ومطروح وحلايب تفتقر إلى جينات *AQP2* و *TbAT1*. وكانت السلالات الثلاث الأخرى وهى القاهرة 1 والبحيرة والقاهرة 2 ايجابية بالنسبة لجينات *AQP2* و *TbAT1* وأظهرت نطاقات محددة عند 1416 و1600 بيز بير على التوالي. كشفت نتائج محاذاة الجين *AQP2* عن عدم وجود تعدد الأشكال على مستوى النوكليوتيدات أو الأحماض الأمينية في أي من السلالات التي تم اختبارها. كشفت محاذاة تسلسل الجينات *AT1* عن بعض البدائل في مواقع مختلفة، مما قد يكون سبباً لمقاومة الأدوية المسببة للطفرة. لم تظهر سلالة البحيرة أي تعدد أشكال على مستوى النوكليوتيدات أو الأحماض الأمينية.

الكلمات الدالة: اكوجليسروبورين، ناقل ادينوزين، مقاومة الادوية، التريبانوسوما الافريقية.