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Sequencing and Phylogenetic Analysis of Cytochrome b and *TaPIN1* genes of Theileria annulata in Alexandria and Beheira governorates, Egypt

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ABSTRACT

This study was designed to conduct phylogenetic analysis on the cytochrome b and TaPIN1 genes of Theileria annulata and to detect potential associations between mutations in these two genes and resistance to buparvaquone. Positive PCR products for the cytochrome and TaPIN1 genes of two samples were sequenced and phylogenetically analysed. When cytochrome b gene sequences were aligned to the reference isolate (XM949625), the phylogenetic analysis showed the presence of two new T. annulata cyt b gene isolates. These isolates showed 99.9% homology with each other and exhibited high homology with existing Egyptian and global isolates and were deposited in GenBank under accession numbers (PP920503) and (PP920504) for samples from Alexandria and Beheira governorates, respectively. Also, there is one non-synonymous mutation in our two isolates in codon 146 (Alanine146Threonine), which is in the Qo1 region (one of the buparvaquone binding sites). When TaPIN1 gene sequences were aligned to the reference isolate (XM949502), the phylogenetic analysis showed the presence of two new T. annulata TaPIN1 gene isolates. These isolates showed 98.1% homology with each other and showed high homology with global isolates. These two isolates are the first Egyptian Theileria annulata TaPIN1 gene isolates and were deposited in GenBank under accession numbers (PP920505) and (PP920506) for samples from Alexandria and Beheira governorates, respectively. Moreover, phylogenetically, Also, there are two non-synonymous mutations in these two isolates in codon 24 (Isoleucine24Valine) and 52 (Methionine52Threonine), which are related to resistance against buparvaquone. Briefly, this is the first Egyptian study that investigated the TaPIN1 gene of Theileria annulata and the first study in Egypt that investigated both the TaPIN1 and cytochrome b genes in the same samples. This study has introduced evidence of point mutations in the cytochrome b and TaPIN1 genes of T. annulata that might be associated with buparvaquone treatment failure in Egypt.

1. INTRODUCTION

Bovine Theileriosis is one of the most prevalent, endemic, and economically important tick-borne diseases that infects cattle (Jenkins et al., 2016). Bovine tropical Theileriosis is a tick-borne protozoal disease caused by Theileria annulata and is transmitted by ixodes ticks of the genus Hyalomma (Mohammed-Ahmed et al., 2018). It is regarded as one of the tick-borne illnesses that lowers the productivity of native breeds and also kills imported foreign breeds (Ghauri et al., 2019). The most prevalent clinical manifestations of Theileriosis are fever, anorexia, diarrhea, pre-scapular and pre-femoral lymph node enlargement, respiratory distress, jaundice or anemic mucous membrane, and corneal opacity of the eye (Agina et al., 2020).

Parvaquone and buparvaquone (hydroxy naphthoquinone derivatives) were first shown as active medications that primarily targeted Theileria mitochondria's Cytochrome b (Cyt b) (Neelam et al., 2017).

Cytochrome b gene may be employed as a tool to distinguish between various T. annulata genotypes

and as a genetic marker to identify resistant isolates of T. annulata (Mhadhbi et al., 2015). Buparvquone acts primarily as a Qo (Quinone Oxidoreductase) inhibitor by attaching itself to the Qo pockets of cytochrome b protein and effectively inhibiting mitochondrial respiration. However, the drug's fast increasing treatment failures have been linked to single or multiple mutations in the Cytochrome b gene's Qo binding site (Mhadhbi et al., 2015). Treatment failure has also been linked to mutations that occur outside but near the Qo buparvaquone binding region (Mhadhbi et al., 2015; Sharifiyazdi et al., 2012; Walker et al., 1998). T. annulata cytochrome b gene mutations linked to buparvaquone treatment failure have been shown in vivo and in field isolates from animals in Iran, Turkey, Tunisia and Egypt that were unresponsive to treatment with buparvaquone at the recommended dosage (Hacilarlioglu et al., 2012; Mhadhbi et al., 2015; Mhadhbi et al., 2010; Sharifiyazdi et al., 2012; Yousef et al., 2020). The parasite TaPin1 protein can interact with host cell proteins and modulates oncogenic signaling pathways for example, the TaPin1 prolyl isomerase interacts with the host ubiquitin ligase Fbw7, leading to its degradation and subsequent stabilization of c-Jun which promotes host cell transformation (Marsolier et al., 2015). Buparvaquone may function through at least one method, which involves inhibiting the activity of TaPIN1 protein (Marsolier et al., 2019). Mutations in the TaPIN1 gene have been reported in a buparvaquone-resistant parasite that was identified in Tunisia and Sudan (Marsolier et al., 2015; Salim et al., 2019). TaPIN1 inhibitors, such as the anti-Theilerial medication buparvaquone, did not inhibit mutant TaPIN1, according to both in vitro and in vivo experiments (Marsolier et al., 2015). It is found that many parasites also share mutations in both the TaPIN1 and the cytochrome b genes, suggesting that these two genes represent important biomarkers to follow the spread of resistance in Africa, the Middle East and Asia (Salim et al., 2019).

Resistance to buparvaquone was reported in Egypt, especially in Alexandria and Beheira governorates, where there were cases of bovine tropical Theileriosis in which animals did not respond when treated with the recommended dosage of buparvaquone.

The present study was undertaken to conduct phylogenetic analysis of the Cytochrome b and TaPIN1 genes to detect homology of our Theileria annulata isolates with Egyptian and global isolates and to detect potential associations between the resistance against buparvaquone and the occurrence of mutations in the Cytochrome b and TaPIN1 genes of Theileria annulata in cattle under Egyptian circumstances.

2. MATERIALS AND METHODS

2.1. Animals

This study was conducted on 10 cattle from Alexandria and Beheira governorates during the period between (Mai 2023 to June 2024), these cattle were clinically suspected to be infected with Theileriosis and were not responding to buparvaquone treatment at the recommended dose and duration.

2.2. Sampling

One blood sample (4 ml) was collected from each examined animal by jugular vein puncture on vacutainer tubes containing 4 mg EDTA as anticoagulant (1mg/1ml), then stored at -20° C till use in DNA extraction for PCR assay **(Jain, 1993)**.

2.3. Molecular diagnosis

2.3.1. DNA extraction:

Genomic DNA was extracted from 200 µl of the whole blood using QIAamp DNA Mini Kit (**QIAGEN (USA) Catalogue no.51304)** according to the manufacturer's instructions and DNA product samples were stored at −20 ºC until use.

2.3.2. Polymerase chain reaction:

Oligonucleotide primers used in conventional PCR: manufactured by METABION (Germany) are shown in table (1).

Table (1): Oligonucleotide primers sequences:

Gene	Primer sequence	Amplified product	Reference
Cytochrome b	F 5'CAGGGCTTTAACCTACAAATTAAC3' R5'CCCCTCCACTAAGCGTCTTTCGACAC3'	1092 bp	**(Mhadhbi et al., 2015)**
TaPIN1	F 5′-GTCTGTCAAATAGGTAGAAATC-3′ R 5′-GAGAGGAAGTTGAATCAAACAT-3′	526 bp	**(Salim et al., 2019)**

PCR was prepared according to Emerald Amp GT PCR master mix (TAKARA) Code No. RR310Akit. Temperature and time conditions of the two primers during PCR are used according to (Mhadhbi et al., 2015) and (Salim et al., 2019).

2.3.3. Gel electrophoresis:

The amplified DNA samples were electrophoresed on 1.5% agarose gel and stained with ethidium bromide to visualize the amplified DNA fragments under ultraviolet light.

2.4. DNA sequencing and phylogenetic analysis:

PCR products were purified using QIAquick Gel Extraction Kit. **(QIAGEN).**

Bigdye Terminator V3.1 cycle sequencing kit **(PERKIN-ELMER)** was used for the sequence reaction (carried out on two directions using forward and reverse primer set) and then it was purified using Centri-sep spin column. DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan).BLAST® analysis (Basic Local Alignment Search Tool) was carried out to verify the identity of the sequence to GenBank accessions. Phylogenetic analysis was done using maximum likelihood, neighbour joining and maximum parsimony in MEGA 7.

3. RESULT

3.1Results of PCR targeting cytochrome b gene region of the T. annulata, containing the encoding region of the drug-binding sites:

After the amplification of extracted DNA (using primer set targeting cytochrome b gene region of the T. annulata, containing the encoding region of the drug-binding sites). 2 samples were positive for Theileria annulata cytochrome gene. (shown in figure 1). **3.2. Results of PCR targeting TaPIN1 gene containing the encoding region of buparvaquone binding sites:**

DNA products of the 10 samples used to detect (cytochrome b gene) are also used to detect TaPIN1 gene using primer set targeting TaPIN1 gene. The same two samples that were positive for the cytochrome b gene were positive for the TaPIN1 gene. (Shown in figure 2)

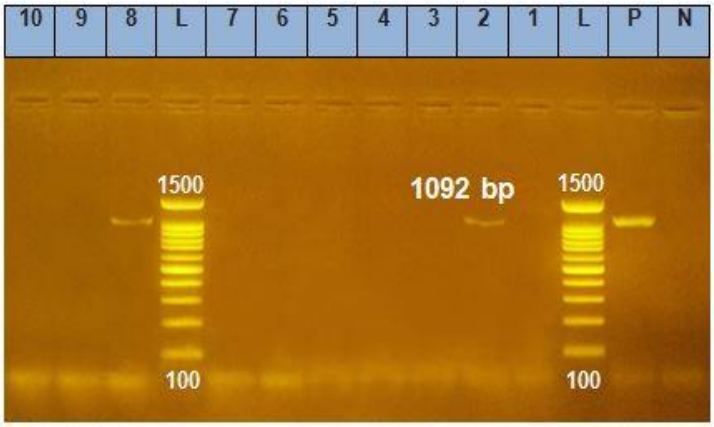

Figure 1: 1.5% ethidium bromide-stained agarose gel electrophoresis for analysis of conventional PCR for detection of T. annulata. by using primer set amplifying cytochrome b gene region of the T. annulata. Lane L: 100 bp ladder marker, Lane P: control positive, Lane N: control negative, Lanes2 and 8 were positive samples for Theileria. annulata showing specific band at 1092 bp and Lanes 1, 3,4, 5, 6, 7, 9 and 10 are negative sample for Theileria. annulata.

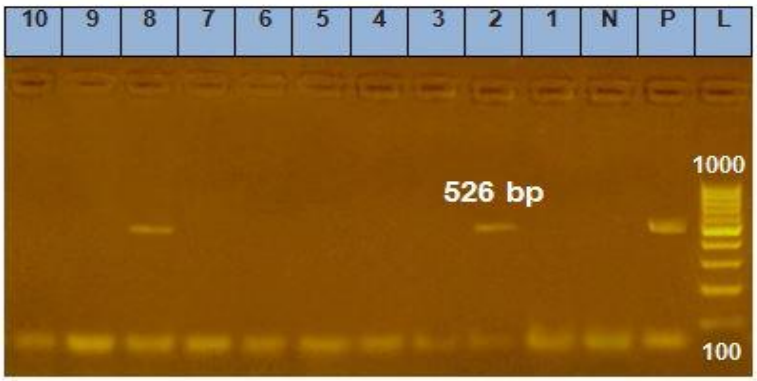

Figure 2: 1.5% ethidium bromide-stained agarose gel electrophoresis for analysis of conventional PCR for detection of T. annulata. by using primer set amplifying TaPIN1 gene. Lane L: 100 bp ladder marker, Lane P: control positive Lane N: control negative, Lanes 2 and 8 positive samples for Theileria. annulata showing specific band at 526 bp and Lanes 1, 3,4, 5, 6, 7, 9 and 10 negative samples for Theileria. annulata.

3.3. Results of sequencing and phylogenetic analysis of Theileria annulata cytochrome b gene:

3.3.1. Results of gene sequencing of cytochrome b gene: Sequencing reaction was applied on 2 PCR products positive for cytochrome b gene.

The obtained sequences were deposited in the GenBank database under accession number (PP920503) for "Theileria annulata SA1 cytochrome b gene isolate" from Alexandria and (PP920504) for "Theileria annulata SA2 cytochrome b gene isolate" from Beheira.

3.3.2. Result of phylogenetic analysis of cytochrome b gene:

3.3.2.1. Sequence distance (Homology) of our isolates with reference isolate:

When our isolates (PP920503 and PP920504) aligned and compared to reference isolate (XM949625), they showed 99.5% and 99.4% homology, respectively.

3.3.2.2. Sequence distance (Homology) of our isolates with each other:

When our isolates (PP920503 and PP920504) are aligned and compared to each other, they showed that they had 99.9 % homology.

3.3.2.3. Sequence distance (Homology) of our isolates with some global isolates:

When our isolate (PP920503) was aligned to the reference nucleotide sequences of Theileria annulata accessed in the GenBank database by using Mega 7 software, it showed that it had 99.8% homology with Theileria annulata isolates (MG787985) and (OP056626) from India, (MK693129) from Turkey and (OR168940) from Pakistan, 99.7% homology with and Indian Theileria annulata isolates (MG787986, MG78798, MN044055), 99.6% homology with Tunisian Theileria annulata isolates (KF732022, KF732024, KF732026, KF732028, KF732029). When our isolate (PP920504) was aligned to the reference nucleotide sequences of Theileria annulata accessed in the GenBank database by using Mega 7 software, it showed that it had 99.7% homology with Theileria annulata isolates (KF732022, KF732024, KF732026, KF732028, KF732029) from, (MG787985) and (MK693129) from India and (OR168940) from Pakistan, 99.6% homology with Indian Theileria annulata isolates (MG787986, MG78798, MN044055).

3.3.2.4. Sequence distance (Homology) of our isolates with Egyptian isolates:

When our isolate (PP920503) was aligned to the reference nucleotide sequences of Theileria annulata accessed in the GenBank database by using Mega 7 software , it showed that it had 99.6 % homology with Egyptian isolates (LC632661, LC632662), 99.5% homology with to Egyptian isolates (LC633288, MK390362, MK390363), 99.4% homology with to Egyptian isolates (LC322660, MK390359), 99.1% homology with Egyptian isolates (LC633286, MK390360, MK390361, MN807050), 98.9% homology with Egyptian isolates (LC633287, MK390364, PP465044) and 97.2% homology with Egyptian isolate (PP465045) and

When our isolate (PP920504) was aligned to the reference nucleotide sequences of Theileria annulata accessed in the GenBank database by using Mega 7 software , it showed that it had 99.7 % homology with Egyptian isolates (LC632661, LC632662), 99.6 % homology with Egyptian isolates (LC633288, MK390362, MK390363), 99.5 % homology with Egyptian isolates (LC322660, MK390359), 99.2 % homology with Egyptian isolates (LC633286, MK390360, MK390361, MN807050), 99.1 % homology with Egyptian isolates (LC633287, MK390364, PP465044) and 97.3 % homology with Egyptian isolate (PP465045).

3.3.2.5. Mutations detected in our isolates of Theileria annulata cytochrome b gene:

Silent and non-synonymous mutations are described in table 2 and 3.

Table (2): Silent mutation of Theileria annulata cytochrome b gene of our obtained sequences in this study (PP920503 and PP920504)

Sample name	GenBank accession No.	Silent mutation GTG/GTA		Silent mutation TCG/TCA		Silent mutation TTA/TTG		Silent mutation TTC/TTT		Silent mutation GTA/GTG	
		Position	Codon	Position	Codon	Position	Codon	Position	Codon	Position	Codon
		138	46	234	78	417	139	429	143	870	290
T. annulata SA1 cyt b gene	**PP920503**	-		+		+		+		+	
T. annulata SA2 cyt b gene	**PP920504**	+		+		+		+		+	

Table (3): Non-synonymous mutations of Theileria annulata cytochrome b gene of our obtained sequences in this study (PP920503 and PP920504).

Isolate name	GenBank accession No.	Non-synonymous mutation GCT/ACT *Alanine146Threonine*	
		Position	**Codon**
		436	146
T. annulata SA1 cyt b gene	**PP920503**	+	
T. annulata SA2 cyt b gene	**PP920504**	+	

Our results revealed that there is one non-synonymous mutation that altered amino acid from Alanine to Threonine at codon 146 (*Alanine146Threonine*) as shown in table (3), which is located in the Qo1 region (one of buparvaquone binding sites; Qo1 (130–148) and Qo2 (244–266)) of our two isolates (PP920503 and PP920504).This point mutation in Qo1 of our T. annulata cytochrome b gene isolates might be associated with buparvaquone treatment failure in Egypt.

3.4. Results of sequencing and phylogenetic analysis of Theileria annulata TaPIN1 gene:

3.4.1. Result of TaPIN1 gene sequencing:

Sequencing reaction was applied on 2 PCR products positive for TaPIN1 gene. The obtained sequences were deposited in the GenBank database under accession number (**PP920505)** for "Theileria annulata SA1 TaPIN1 gene isolate" from Alexandria and (**PP920506)** for "Theileria annulata SA2 TaPIN1 gene isolate" from Beheira.

3.4.2. Result of phylogenetic analysis of TaPIN1 gene:

3.4.2. 1. Sequence distance (Homology) of our isolates with reference isolate:

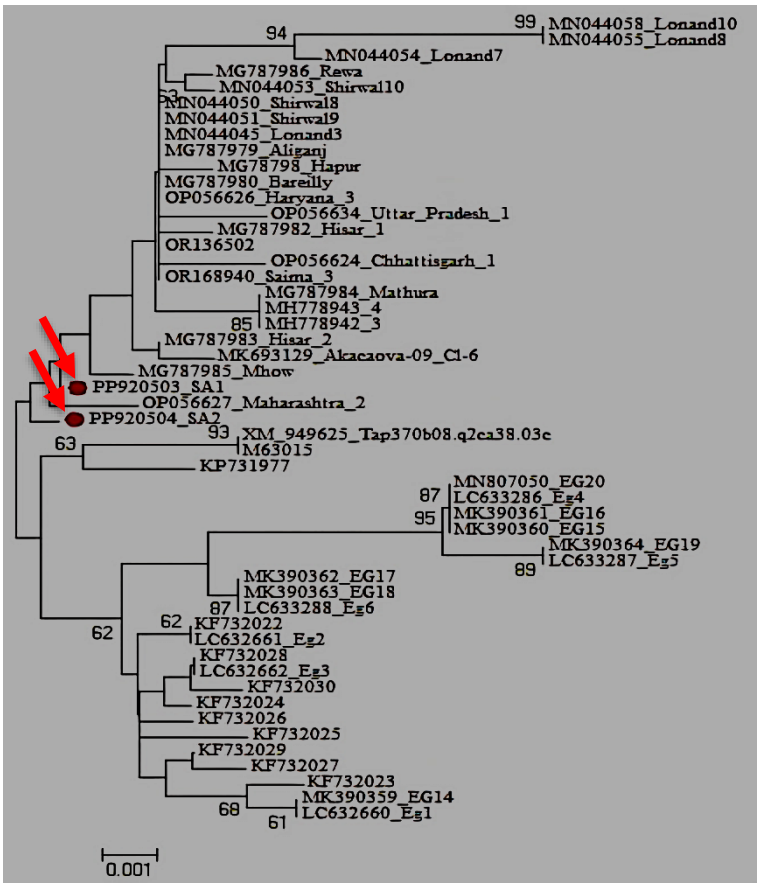

Figure (3): Phylogenetic tree constructed using Neighbor-Joining method illustrates the Genetic relationship of T. annulata isolates (based on cytochrome b gene) obtained in this study and isolates from the GenBank. Our isolates are marked with arrows.

When our isolates (PP920505 and PP920506) aligned and compared to reference isolate (XM949502), they showed 97.5% and 95.7% homology, respectively.

3.4.2.2. Sequence distance (Homology) of our isolates with each other:

When our isolates (**PP920505** and **PP920506**) are aligned and compared to each other, they showed that they had 98.1 % homology.

3.4.2.3. Sequence distance (Homology) of our isolates with some global isolates:

When our isolate (PP920505) was aligned to the reference nucleotide sequences of Theileria annulata accessed in the GenBank database by using Mega 7 software, it showed that it had 98.8% homology with Theileria annulata isolate (OP056644) from India, 98.1% homology with Theileria annulata isolate (OP056643) from India and (MK693121) from Turkey, 97.5% homology with Theileria annulata isolates (MK693122) and (MK693119) from Turkey and (OP056641) and (OP056640) from India, 96-97% homology with Theileria annulata isolates (MK693118) and (MK693120) from Turkey and (OP056639) from India When our isolate (PP920506) was aligned to the reference nucleotide sequences of Theileria annulata accessed in the GenBank database by using Mega 7 software, it showed that it had 96-97% homology with Theileria annulata isolates (MK693121), (MK693119) and (MK693118) from Turkey and (OP056644), (OP056643) and (OP056639) from India and 95-95.9% homology with Theileria annulata isolate (MK693122) from Turkey and (OP056640) from India.

3.4.2.4. Sequence distance (Homology) of our isolates with Egyptian isolates:

There are no Egyptian isolates of Theileria annulata TaPIN1 gene deposited in GenBank

Our isolates are the first isolates of Theileria annulata TaPIN1 gene deposited in GenBank under accession numbers (**PP920505** and **PP920506**)

3.4.2.5. Mutations detected in our isolates of Theileria annulata TaPIN1 gene:

Silent and non-synonymous mutations are described in table 4 and 5

Table (4): Eight Silent mutations of Theileria annulata TaPIN1 gene of our obtained sequences in this study(PP920505and PP920506).

Isolate name		**GenBank accession No.**	**Silent mutation ATT/ATA**		**Silent mutation GTT/GTG**		**Silent mutation CTA/CTG**		**Silent mutation GCT/GCC**	
			Position	**Codon**	**Position**	**Codon**	**Position**	**Codon**	**Position**	**Codon**
			69	23	90	30	108	36	183	61
T. annulata TaPIN1 gene	SA1	**PP920505**	+		+		-		+	
T. annulata TaPIN1 gene	SA2	**PP920506**	+		+		+		-	
Isolate name		**GenBank accession No.**	**Silent mutation GAA/GAG**		**Silent mutation GGG/GGA**		**Silent mutation GAA/GAG**		**Silent mutation GAC/GAT**	
			Position	**Codon**	**Position**	**Codon**	**Position**	**Codon**	**Position**	**Codon**
			243	81	294	98	390	130	396	132
T. annulata TaPIN1 gene	SA1	**PP920505**	+		+		+		+	
T. annulata TaPIN1 gene	SA2	**PP920506**	-		+		-		+	

Our results revealed that there are 4 non-synonymous mutations as shown in table (5), two out of them are located in both our two Theileria annulata TaPIN1 gene isolates (PP920505 and PP920506). These two mutations (Isoleucine24Valine and Methionine52Threonine) are related to buparvaquone resistance. Isoleucine24Valine mutation altered amino acid from isoleucine to valine at codon 24. Methionine52Threonine mutation altered amino acid from methionine to threonine at codon 52. These two mutations and the mutation mentioned before in our cytochrome b gene isolates (PP920503 and PP920504) may be the cause of resistance to buparvaquone.

Table (5): Four non-synonymous mutations of Theileria annulata TaPIN1 gene of our obtained sequences in this study (PP920505 and PP920506).

Isolate name	GenBank accession No.	Isoleucine24Valine ATA/GTA		Histidine34Proline CAC/CCC		Methionine52Threonine ATG/ACG		Glutamate121ysine GAG/AAG	
		Position	Codon	Position	Codon	Position	Codon	Position	Codon
		70	24	101	34	155	52	361	121
T. annulata SA1 TaPIN1 gene	**PP920505**	+		-		+		-	
T. annulata SA2 TaPIN1 gene	**PP920506**	+		+		+		+	

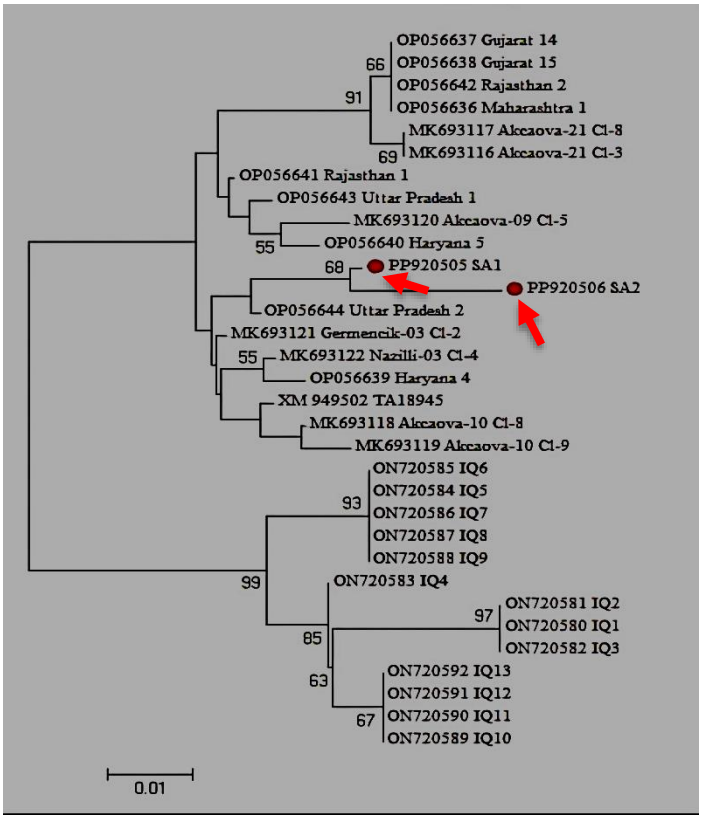

Figure (4): Phylogenetic tree constructed using Neighbor-Joining method illustrates the Genetic relationship of T. annulata isolates (based on TaPIN1gene) obtained in this study and isolates from the GenBank. Our isolates are marked with arrows.

4. DISCUSSION

Treatment of tropical Theileriosis by buparvaquone has a 92% success rate when injected intramuscularly at a dose of 2.5 mg/kg. This medication is effective against both the schizont and piroplasm stages (Gharbi et al., 2015; Hashemi-Fesharki, 1991; McHardy et al., 1985). It has been proposed that buparvaquone and other hydroxynaphtoquinones exert their effect through competitive inhibition of ubiquinone (coenzyme Q) binding to cytochrome b within the mitochondrial cytochrome bc1 complex. This competitive inhibition disrupts the electron transport chain, ultimately leading to the collapse of the membrane potential at the Qo1 (130-148) and Qo2 (244-266) buparvaquone binding sites. Consequently, parasite respiration and pyrimidine biosynthesis are arrested (Hyde, 2007; Mhadhbi et al., 2015). The emergence of drug resistance in protozoan parasites, exemplified by T. annulata, poses a significant challenge in the therapeutic management of protozoan diseases (De Koning, 2017).

Initial reports of T. annulata resistance to buparvaquone on the African continent emerged in Tunisia in 1996 (Darghouth et al., 1996).

For detection of mutations related to drug resistance in the cytochrome b locus of T. annulata, our samples were collected from clinical cases showed treatment failure on the same line of previous studies (Sharifiyazdi et al., 2012) from Iran, (Mhadhbi et al., 2015) from Tunisia and (Chatanga et al., 2019) from Sudan.

Concerning to sequencing and phylogenetic analysis of Theileria annulata cytochrome b gene, our results revealed that sequencing of the 2 positive PCR products of cytochrome b gene results in presence of 2 new isolates of Theileria annulata cytochrome b gene which are deposited in GenBank under accession numbers (PP920503) for "Theileria annulata SA1 cytochrome b gene isolate" from Alexandria and (PP920504) for "Theileria annulata SA2 cytochrome b gene isolate" from Beheira, Our isolates are genetically related with 99.9% homology when aligned and compared with each other and with high homology with many Egyptian and global isolates when compared to reference strain (XM949625).

Our findings revealed that mutations at 6 different codons were found in our T. annulata cytochrome b gene isolates (PP920503 and PP920504) when compared to reference isolate (XM949625). One out of these mutations was non-synonymous observed at codon 146 (*Alanine146Threonine*) that altered amino acid from Alanine to Threonine which resulted in changes in the polypeptide chain of the cytochrome b gene. This mutation is located within Qo1 region [one of buparvaquone binding sites; Qo1 (130–148) and Qo2 (244–266)] of our two isolates (PP920503 and PP920504).

This non-synonymous mutation observed at codon 146 of our T. annulata cytochrome b gene isolates has also been reported exclusively in all resistant isolates of T. annulata in sudan by (Chatanga et al., 2019) who found that only 3 non-synonymous mutations on 50 isolates at codon 146 (50/50), 129 (18/50) and 227 (3/50), but no mutations were present in the Qo2 site in all analysed sequences. This point mutation in Qo1 of our T. annulata cytochrome b gene isolates might be associated with buparvaquone treatment failure in Egypt. And this agreed with (Hacilarlioglu et al., 2012; Hacilarlioglu et al., 2023) who reported that a distinct mutation within the Qo1 drug binding site at position 135 was identified in Turkish isolates of Theileria annulata. This mutation appears to be directly associated with treatment failure for buparvaquone, as it was found exclusively in cattle that did not respond to standard-dose buparvaquone therapy.

Our finding disagreed with (Mhadhbi et al., 2015) who reported in Tunisia that 50% of resistant isolates exhibited mutations within Qo2 region at codon 235 (*Proline253Serine)* or codon 262 (*Leuccine262Serine)* and on contrary to our finding, a study by (Yousef et al., 2020) who reported that two mutations occurred in codons 253 and 262 within Qo2 drug-binding site of all 5 resistant isolates and no mutations in Qo1.

In conclusion of this point, previous studies reported that resistant isolates showed non-synonymous mutations within Qo1, Qo2 or both (buparvaquone binding sites) which leads to inability of buparvaquone to bind to Qo1 or Qo2 pockets.

Not only mutations in buparvaquone binding sites cause drug resistance, but also mutations close to them can cause drug resistance as (Kessl et al., 2004; Walker et al., 1998) who has been proposed that even a single mutation outside the region but near it can lead to mutations that can make antiprotozoal medications like buparvaquone resistant to treatment. Moreover, cytochrome b gene sequences with single or double amino acid mutations that are near the anticipated drug binding site are linked to increased resistance to the antiprotozoal medications' ability to collapse mitochondrial membrane potentials and inhibit respiration, such as atovaquone (Srivastava et al., 1999). Furthermore, studies have shown that these mutations (located near Qo1 or Qo2) induce structural alterations, leading to a reduction in the volume of the drug binding pocket at the target site. This consequently destabilizes the area and disrupts drug binding (Kessl et al., 2005; Walker et al., 1998). For example, mutation at codon 129 serine to proline of the T. annulata cytochrome b gene which also has been reported exclusively in the resistant isolates of T. annulata in Sudan and Tunisia (Ali et al., 2022; Chatanga et al., 2019; Mhadhbi et al., 2015)

The present work has yielded evidence of point mutations in the T. annulata cytochrome b gene that may be linked to treatment failure with buparvaquone in Egypt. We explained this by pointing out that buparvaquone is used extensively in Egypt to treat tropical bovine Theileriosis. To definitively demonstrate T. annulata's resistance to buparvaquone in Egypt, additional experimental research is necessary. Concerning to sequencing and phylogenetic analysis of Theileria annulata TaPIN1 gene, our results revealed that sequencing of the 2 positive PCR products for TaPIN1 gene results in presence of 2 new isolates of Theileria annulata TaPIN1 gene which are deposited in GenBank under accession numbers (PP920505) for "Theileria

annulata SA1 TaPIN1 gene isolate" from Alexandria and (PP920506) for "Theileria annulata SA2 TaPIN1 gene isolate" from Beheira, Our isolates are genetically related with 98.1% homology when aligned and compared with each other. Our isolates are the first Egyptian Theileria annulata TaPIN1 gene isolates accessed in GenBank and have high homology with many global isolates.

Referring to mutations in TaPIN1 gene, when our isolates are compared to reference isolate (XM949502), there are 4 non-synonymous mutations, two out of them (Ile24Val and Met52Thr) are supposed to be related to resistance against buparvaquone.

Ile24Val mutation altered amino acid from isoleucine to valine at codon 24 (isoleucine24valine), this mutation is previously discovered in 3 resistant Theileria annulata TaPIN1 gene isolates from sudan reported by (Salim et al., 2019) who observed several other mutations in resistant isolates, particularly at codons 22, 23, 24 and 26, These positions are unlikely to affect the catalytic activity directly, but they could affect the secretion, processing or targeting of the TaPin1 protein.

Met52Thr mutation altered amino acid from Methionine to Threonine at codon 52 (*Methionine52Threonine*).

Buparvaquone blocks the TaPIN1-induced transformation process of host cells, however a mutation (A53P) at TaPIN1's catalytic loop prevents buparvaquone from blocking PIN1 activity (Marsolier et al., 2015). Subsequent investigations revealed that some, but not all, of the buparvaquone-resistant isolates from Sudan and Tunisia carried the A53P mutation (Marsolier et al., 2015; Salim et al., 2019). While the A53P mutation was not present in our two resistant isolates examined in this study.

Mutation at codon 52 (*Methionine52Threonine*) in our two resistant Theileria annulata TaPIN1 gene isolates was not reported before in previous studies and was discovered for the first time in our isolates. Presence of this mutation directly besides the location of previous mutation at codon 53 in the catalytic loop suggests that this mutation may cause structural changes at catalytic loop portion. So, it is supposed to be related to buparvaquone resistance.

Apicomplexan parasite resistance also results from several various causes, such as overuse of prophylactic drugs, insufficient or ineffective therapeutic treatments for active infections, high genetic and metabolic adaptability of the parasites, and massive proliferation rates that allow certain populations to emerge quickly (Hyde, 2007). So, this suggests that the emergence of these mutations leading to treatment failure may have been facilitated by the overuse or underuse of buparvaquone in the treatment of clinical bovine tropical Theileriosis.

The identification of mutations linked to buparvaquone resistance will be helpful for field drug resistance surveillance. It is also vital to keep in mind that acquired immunity during protracted infection in native breeds in endemic locations may encourage sub-clinically infected carrier cattle, which are crucial in the transmission of mutations linked to buparvaquone resistance to external cattle. Therefore, therapy with a simultaneous combination of tetracycline drugs and buparvaquone, vaccination prophylactic regimens for exogenous animals, and monitoring and assessment of seasonal vector activities may all help to delay or avoid resistance and enhance treatment success (Sharifiyazdi et al., 2012).

5.Conclusion

Presence of non-synonymous mutations at both cytochrome b and TaPIN1 genes of the same samples strongly suggests that resistance against buparvaquone in our samples is attributed to genetic cause.

The phylogenetic analysis of the cytochrome b and TaPIN1 genes of Theileria annulata to investigate the presence or absence of mutations at buparvaquone binding sites (Qo1 and Qo2 regions) can provide genetic evidence about the cause of Theileriosis treatment failure (whether it is attributed to genetic resistance by the parasite or not).

6. REFERENCES

Agina, O., Shaari, M., Isa, N., Ajat, M., Zamri-Saad, M., Hamzah, H. 2020. Clinical Pathology, Immunopathology and Advanced Vaccine Technology in Bovine Theileriosis: A Review. PLoS Pathog. *9*(9): 697.

Ali, Q., Zahid, O., Mhadhbi, M., Jones, B., Darghouth, M. A., Raynes, G., Betson, M. 2022. Genetic characterisation of the Theileria annulata cytochrome b locus and its impact on buparvaquone resistance in bovine. *Int. J. Parasitol. (20)*: 65-75.

Chatanga, E., Mosssad, E., Abdo Abubaker, H., Amin Alnour, S., Katakura, K., Nakao, R., Salim, B. 2019. Evidence of multiple point mutations in Theileria annulata cytochrome b gene incriminated in buparvaquone treatment failure. *Acta Trop. (191)*: 128-132.

Darghouth, M., Miled, L. B., Bouattour, A., Melrose, T., Brown, C., Kilani, M. 1996. A preliminary study on the attenuation of Tunisian schizont-infected cell lines of Theileria annulata. J. *Parasitol. Res. (82)*: 647-655.

De Koning, H. P. 2017. Drug resistance in protozoan parasites. *Emerg Top Life Sci. 1*(6): 627-632.

Gharbi, M., Darghouth, M. A. 2015. Control of tropical Theileriosis (Theileria annulata infection in cattle) in North Africa. *Asian Pac. J. Trop. Dis. 5*(7): 505-510.

Ghauri, H. N., Ijaz, M., Farooqi, S. H., Ali, A., Ghaffar, A., Saleem, S., Ullah, M. R. 2019. A comprehensive review on past, present and future aspects of canine Theileriosis. *Microb pathog. (126)* :116-122.

Hacilarlioglu, S., Bilgic, H., Bakirci, S., Weir, W., Tait, A., Unlu, A., Karagenç, T. 2012. *Are mutations in the cytochrome b gene of Theileria annulata responsible for resistance to the theilericidal drug, Buparvaquone.* Paper presented at the Joint conference on emerging and re-emerging epidemics affecting global health. Orvieto, Italy.

Hacilarlioglu, S., Bilgic, H. B., Bakirci, S., Tait, A., Weir, W., Shiels, B., Karagenc, T. 2023. Selection of genotypes harbouring mutations in the cytochrome b gene of Theileria annulata is associated with resistance to buparvaquone. *PLoS One. 18*(1): e0279925.

Hashemi-Fesharki, R. 1991. Chemotherapeutic value of parvaquone and buparvaquone against Theileria annulata infection of cattle. *Res. J. vet. sci. 50*(2): 204-207.

Hyde, J. E. 2007. Drug-resistant malaria− an insight. *The FEBS journal. 274*(18): 4688-4698.

Jain, N. C. (1993). Essentials of veterinary hematology.

Jenkins, C., Bogema, D. R. 2016. Factors associated with seroconversion to the major piroplasm surface protein of the bovine haemoparasite Theileria orientalis. *Parasit. vectors. (9*): 1-9.

Kessl, J. J., Ha, K. H., Merritt, A. K., Lange, B. B., Hill, P., Meunier, B., Trumpower, B. L. 2005. Cytochrome b mutations that modify the ubiquinol-binding pocket of the cytochrome bc1 complex and confer anti-malarial drug resistance in Saccharomyces cerevisiae. *JBC.280*(17): 17142-17148.

Kessl, J. J., Hill, P., Lange, B. B., Meshnick, S. R., Meunier, B., Trumpower, B. L. 2004. Molecular Basis for Atovaquone Resistance in Pneumocystis jirovecii Modeled in the Cytochrome bc1Complex of Saccharomyces cerevisiae. *JBC. 279*(4): 2817-2824.

Marsolier, J., Perichon, M., DeBarry, J., Villoutreix, B., Chluba, J., Lopez, T., Fritsch, L. 2015. Theileria parasites secrete a prolyl isomerase to maintain host leukocyte transformation. *Nature. 520*(7547): 378-382.

Marsolier, J., Perichon, M., Weitzman, J. B., Medjkane, S. 2019. Secreted parasite Pin1 isomerase stabilizes host PKM2 to reprogram host cell metabolism. *Commun. Biol. 2*(1): 152.

McHardy, N., Wekbsa, L., Hudson, A., Randall, A. 1985. Antitheilerial activity of BW720C (buparvaquone): a comparison with parvaquone. *Res. J. vet. sci. 39*(1): 29-33.

Mhadhbi, M., Chaouch, M., Ajroud, K., Darghouth, M. A., BenAbderrazak, S. 2015. Sequence Polymorphism of Cytochrome b Gene in Theileria annulata Tunisian Isolates and Its Association with Buparvaquone Treatment Failure. *PLoS One. 10*(6): e0129678.

Mhadhbi, M., Naouach, A., Boumiza, A., Chaabani, M. F., BenAbderazzak, S., Darghouth, M. A. 2010. In vivo evidence for the resistance of Theileria annulata to buparvaquone. *Vet. Parasitol. 169*(3-4): 241-247.

Mohammed-Ahmed, G., Hassan, S., El Hussein, A., Salih, D. 2018. Molecular, serological and parasitological survey of Theileria annulata in North Kordofan State, Sudan. *Vet. Parasitol. (13*): 24-29.

Neelam, R. N., Jhambh, R., Virmani, M., Goel, P. 2017. Evaluation of the antioxidants as adjunct therapy in cattle naturally infected with bovine tropical Theileriosis. *Int. J. Curr. Microbiol. App. Sci. 6*(11): 5373-5384.

Salim, B., Chatanga, E., Jannot, G., Mossaad, E., Nakao, R., Weitzman, J. B. 2019. Mutations in the TaPIN1 peptidyl prolyl isomerase gene in Theileria annulata parasites isolated in Sudan. *Int. J. Parasitol. (11)*: 101-105.

Sharifiyazdi, H., Namazi, F., Oryan, A., Shahriari, R., Razavi, M. 2012. Point mutations in the Theileria annulata cytochrome b gene is associated with buparvaquone treatment failure. *Vet. Parasitol. 187*(3-4): 431-435.

Srivastava, I. K., Morrisey, J. M., Darrouzet, E., Daldal, F., Vaidya, A. B. 1999. Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. *Mol. Microbiol. 33*(4): 704-711.

Walker, D. J., Wakefield, A. E., Dohn, M. N., Miller, R. F., Baughman, R. P., Hossler, P. A., Meshnick, S. R. 1998. Sequence polymorphisms in the Pneumocystis carinii cytochrome b gene and their association with atovaquone prophylaxis failure. *J. Infect. Dis. 178*(6): 1767-1775.

Yousef, S. G., El Balkemy, F. A., El Damaty, H. M. 2020. Mutations in Theileria Annulata Cytochrome B Gene Associated with Buparvaquone Resistance in Cattle, Egypt. *Pak. Vet. J. 40*(2).