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RESEARCH ARTICLE

Mutations in *Theileria Annulata Cytochrome B* Gene Associated with Buparvaquone Resistance in Cattle, Egypt

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A B S T R A C T

Buparvaquone is now the principle medicinal drug utilized against tropical theileriosis. The current study was conducted to determine the potential association between the resistance against buparvaquone and the occurrence of mutations in the *Cytochrome b* (*Cyto b)* gene of *Theileria annulata* (*T. annulata*) in cattle under Egyptian conditions. We sequenced the *Cyto b* gene of *T. annulata* of 6 blood samples collected from 1 responding and 5 non-responding cattle to buparvaquone treatment. The analysis revealed that two mutations occurred in codons 253 and 262 within the quinol oxidation (Qo2) drug-binding site of the five resistant isolates. Two mutations in the Qo1 site were found together in the buparvaquone sensitive isolate only. Our findings suggest that the failure of buparvaquone therapy could be attributed to the presence of single or double point mutations at the Qo2 site of *T. annulata Cyto b* gene.

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INTRODUCTION

Buparvaquone is now the principle medicinal drug utilized against tropical theileriosis. In the 1970s, parvaquone and buparvaquone (hydroxy naphthoquinone derivatives) were introduced as active drugs acting mainly on *Cytochrome b* (*Cyto b*) of *Theileria* mitochondria. Since then, buparvaquone has been the global choice drug for the treatment of theileriosis (Neelam *et al.*, 2017). Hydroxy naphthoquinone is a competitive inhibitor of energy metabolism and mitochondrial respiration. It blocks the Q-cycle of the inner mitochondrial membrane at the level of cytochrome bc1complex (Staines *et al.*, 2018). This leads to the collapse of inner mitochondrial membrane potential and subsequent death of the parasite. Atovaquone is a hydroxy naphthoquinone that had been developed in the 1990s and had wide spectrum activity against apicomplexan parasites (Staines *et al.*, 2018). It is mainly a Qo inhibitor that binds to the Qo pocket within the *Cyto b* gene and stops the mitochondrial respiration effectively. Nevertheless, rapidly grown treatment failures associated with this drug frequently recorded due to single or double mutations in the Qo binding site of the *Cyto b* gene (Goodman *et al.*, 2017). In this vein, the resistance of *Theileria* parasites to buparvaquone becomes established in the field. This study aims to determine the mutations in the *Cyto b* gene of *T. annulata* isolated from clinical cases of cattle under Egyptian conditions that have or haven't responded to the treatment with buparvaquone.

MATERIALS AND METHODS

During summer 2019, ear vein-puncture smears from 6 crossbred cattle aged 2-5 years showed clinical signs of acute theileriosis in El-Sharkia Governorate Egypt were examined. Parasitemia was expressed as the proportion of infected erythrocytes (1000 erythrocytes) using an ordinary microscope after examination at a magnification of 1000 x according to Neelam *et al.* (2017). Additionally, 10 mL of blood was collected from the jugular vein on EDTA vacuumed tubes for the detection of hematocrit value using the auto hematology analyzer (Rayto, RT-7200, Germany) and for DNA extraction. Buparvaquone (25 mg/10 kg BW) was administered immediately after the initial onset of the illness in all cases and repeated after 48h. The iron supply (10mL) was given twice a week per animal (Saravanan *et al.*, 2017). This study was issued by the Institutional Animal Care and Use Committee of the Zagazig University (Ref. No: ZU-IACUC/2/F/25/2019).

PCR amplification of *Cyto b* gene: DNA extraction was applied using a QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturers` instructions. PCR amplification was performed using Cyto F (5'CAGGGCTTTAACCTACAAATTAAC3') and Cyto R (5' CCCCTCCACTAAGCGTCTTTCGACAC 3') primers to amplify 1092 bp of *Cyto b* gene as described elsewhere (Mhadhibi *et al.*, 2015).

DNA sequencing and analysis: Sequencing was performed using a ready reaction Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Full-length *Cyto b* gene sequences of all isolates (n=6) were compared and aligned with the reference sequence of Ankara strain (*T. annulata*, accession No. XM949625). Sequence identities and divergences were computed using MegAlign software (DNA STARR Lasergene® version 7.2, USA). A phylogenetic tree was created (Tamura *et al.*, 2013), while comparative alignment of the *Cyto b* amino acid sequences was performed using the Clustal W Multiple alignments inside BioEdit software. A hierarchical clustering supported by a heat map was created using the mutation in Qo regions (Qo1 and Qo2) scored as binary data (presence=1, absence=0) using a Metabo Analyst online server (Chong and Xia, 2018).

Nucleotide sequence accession numbers: The *Cyto b* nucleotide sequences of *T. annulata* isolates generated in this study deposited into the GenBank database with accession numbers MK390359- MK390364.

Statistical analysis: Fisher exact test (two-tailed) conducted to determine the significance of the association between the occurrence of point mutations in the *Cyto b* gene and the resistance against buparvaquone using GraphPad Prism software (v.5). For validation, we compared the 6 studied sequences with other previously published data (3 Iranian isolates (Sharifiyazdi *et al.*, 2012) and 9 Tunisian clones (Mhadhbi *et al.*, 2015).

RESULTS AND DISCUSSION

Cattle in the present study showed typical symptoms of acute tropical theileriosis. These findings are consistent with Saravanan *et al.* (2017). *Theileria annulata* intraerythrocytic stages detected in the stained blood smears with a mean percentage of parasitemia 6%. The mean hematocrit value was 25.14±5.9, which is in agreement with Mhadhbi *et al.* (2010). Cattle showed no evidence of improvement either clinically or upon the parasitological examination 48 hours after two doses of buparvaquone. The mean temperature values still more than 40^{o}C, mean parasitemia elevated to be 23%. Meanwhile, the hematocrit values declined to 21.26±6.26. Signs of anemia greatly exaggerated, complete rumen cessation and recumbence of cattle occurred on the 4th day of treatment. Three animals died on the 5th day of treatment, meanwhile, the other two cows died one day later indicating the treatment failure as mentioned by Mhadhbi *et al.* (2010). Meanwhile, the responded cow to buparvaquone therapy showed complete clinical recovery within a week and a decline in parasitemia to 0.3 that agreed with Neelam *et al.* (2017).

Mutations in nucleotide sequences of the studied isolates: Alignment of nucleotide and amino acid sequences of *Cyto b* revealed specific mutations at 17 positions, 11 out of them were non-synonymous resulting in alterations of the translated protein. Analysis of amino acid sequences within Qo1 (130-148) and Qo2 (244-266) regions revealed the presence of two non-synonymous mutations including, *phenylalanine143leucine* and *alanine146threonine*, which are located in the Qo1 of the sensitive isolate only (accession No. MK390359). There were no observed mutations in the Qo2 site of the sensitive isolate.

In respect to the buparvaquone resistant isolates, two mutations were identified at 253 and 262 codons and were expressed together by MK390364. The *Proline253Serine* mutation was detected in MK390362 and MK390363, whereas the *Leucine262Serine* mutation was observed in MK390361 and MK390362 isolates. These findings are in line with Mhadhbi *et al.* (2015) who found that the same mutation at codon 143 in a Qo1 site of their sensitive clone (KF732023) and only (3/6; 50%) of resistant clones exhibited mutations at Qo2 and were either *Proline253Serine* or *Leuccine262Serine* whereas, all the studied clones showed *Alanine146Threonine* mutation in Qo1 region. These results are almost in line with that reported by Sharifiyazdi *et al.* (2012) who found the *Proline253Serine* mutation to be associated with a resistant isolate (JQ308839), whereas the sensitive isolate (JQ308837) showed no mutations at this site.

On contrary to our findings, a recent study by Chatanga *et al.* (2019) found that only 3 non-synonymous mutations on 50 isolates at codon 146 (50/50), 129 (18/50) and 227 (3/50), while no mutations were present in the Qo2 site in all studied sequences. The possible explanation is that the samples used in its study were collected from animals treated with buparvaquone regardless of whether or not these animals responded to the therapy.

Other non-synonymous mutations near the Qo1 region at codon 129 and near Qo2 site as *Valine227Methionine* and *Serine347Leucine* mutations were expressed in MK390360, MK390361and MK390364 sequences of resistant isolates. All reported mutations herein indicate that the more error-prone nature of this gene as suggested by Goodman *et al.* (2017) who explained that the *Cyto b* gene is encoded by multiple copies on the mitochondrial genome and the mitochondrial polymerase enzyme has poor proofreading character.

A non-significant association was detected between drug resistance and mutation occurrence on both Qo1 and Qo2 regions or in the Qo1 region only (P-value >0.05 for both). On the other side, this relationship was significant with mutation occurrence in the Qo2 site (P-value = 0.02) as shown in Table 1. These results suggest that the failure of buparvaquone therapy might be due to the presence of single or double point mutations at the Qo2 site which agreed with Mhadhbi *et al.* (2015) who reported that mutations in the Qo1 region did not relate to drug resistance as the resistant clones not exhibited specific Qo1 mutation. Such resistance may be due to irresponsible and misuse of buparvaquone in the treatment of bovine theileriosis in Egypt, Along with its absolute use by veterinarians not only against theileriosis but also against blood parasites as a whole.

Table 1: Frequency of mutation in various target sites of the *Cytochrome b* gene and the significance of its association with buparvaquone resistance

Isolate type	Mutation in both Qo1[a] and Qo2[b] sites		Mutation in Qo1 site		Mutation in Qo2 site	
	Absent	Present	Absent	Present	Absent	Present
Sensitive (S) (n=5)	1	4[c]	1	4[c]	5	0
Resistant (R) (n=13)	1	12	7	6	4	9[d]
P value	0.49 (NS)		0.28 (NS)		0.029 (*)	

[a]Quinol oxidation 1; [b]Quinol oxidation 2, NS: non-significant; The analysis was done using 6 Egyptian isolates [MK390359-MK390364], 3 Iranian isolates [JQ308837 (S), JQ308838 (R) and JQ308839 (R)] and 9 Tunisian clones [KF732022 (S), KF732023 (S), KF732029 (S), KF732024 (R), KF732025 (R), KF732026 (R), KF732027 (R), KF732028 (R) and KF732030 (R)]. *Significance was determined at a cutoff value ≤0.05; [c]Included MK390359 Egyptian isolate; [d]Included MK390360 - MK390364 Egyptian isolates.

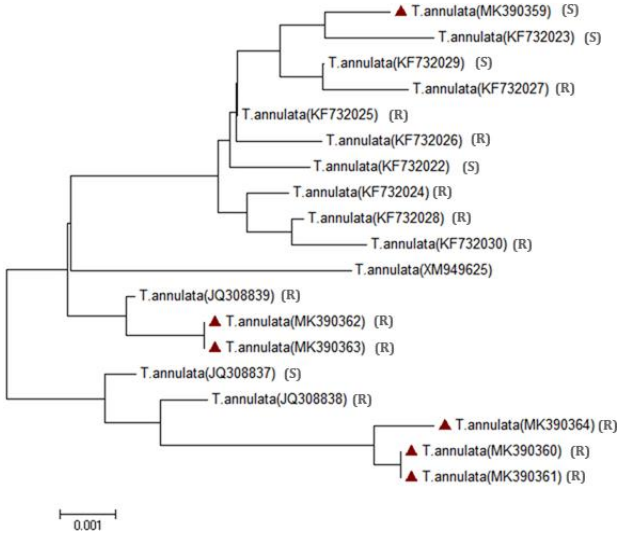

Fig. 1: Phylogenetic tree of *T. annulata* based on the *Cytochrome b* nucleotide sequences of the retrieved reference strains from GeneBank and 6 Egyptian isolates (marked with red triangle beside) using the Neighbor-joining. (N-J) method and 1000 bootstrap replicate.

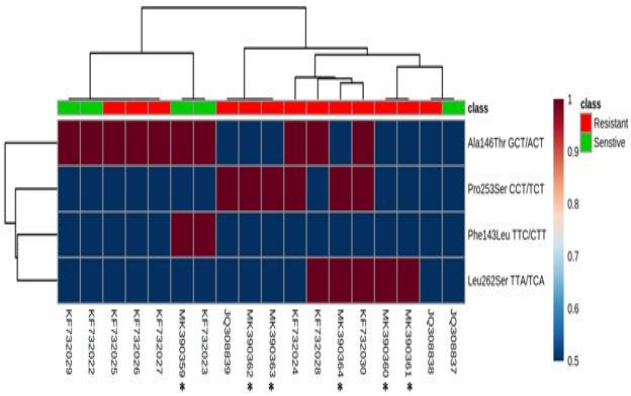

Fig. 2: Hierarchical cluster showing the relationship between *Theileria annulata* strains (n=18) including the study isolates (marked with strikes beside), the heat map built based on the presence and absence of the respective mutations at the site of buparvaquone action.

Phylogenetic analysis and the heatmap: The studied sensitive isolate (MK390359) were closely related to KF732023 and KF732029 Tunisian sensitive clones as it is shown in Fig. 1, meanwhile the studied resistant isolates (MK390360- MK390364) were closely related to Iranian resistant ones. The hierarchical cluster of *T. annulata* strains presented in Fig. 2 showed a separate cluster pattern of resistant isolates (10/13; 77%), while that pattern was not evident for the sensitive isolates, which could be explained by their small numbers (n=5). This strengthens the idea that the *Cyto b* gene can be used as a genetic marker to distinguish resistant *T. annulata* isolates as concluded by Mhadhbi *et al.* (2015). One limitation of our study is the sample size because of the lack of available clinical cases which failed to treatment and limited funding. Thus, we suggest that future studies consider larger sample size. We also encourage field researchers to investigate buparvaquone resistance in other bovine species, particularly water buffaloes.

Conclusions: Qo2 specified mutations are involved in buparvaquone resistance because of their exclusive association with the resistant isolates of *T. annulata*. To the best of our knowledge, this is the first report on sequencing and phylogenetic analysis of *Cyto b* gene in unresponsiveness field cases of tropical theileriosis to buparvaquone therapy in Egypt.

Authors contribution: SGY, FAE and HME contributed to the proposal, revised and approved the manuscript; SGY collected the samples needed for the experiment and analyze the data; SGY and HME carried out the experimental procedures.

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