Genetic characterisation of the *Theileria annulata* cytochrome b locus and its impact on buparvaquone resistance in bovine

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**A R T I C L E   I N F O**

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**A B S T R A C T**

Control of tropical theileriosis, caused by the apicomplexan *Theileria annulata*, depends on the use of a single drug, buparvaquone, the efficacy of which is compromised by the emergence of resistance. The present study was undertaken to improve understanding of the role of mutations conferring buparvaquone resistance in *T. annulata*, and the effects of selection pressures on their emergence and spread. First, we investigated genetic characteristics of the cytochrome b locus associated with buparvaquone resistance in 10 susceptible and 7 resistant *T. annulata* isolates. The 129G (GGC) mutation was found in the Q02 binding pocket and 253S (TCT) and 262S (TCA) mutations were identified within the Q02 binding pocket. Next, we examined field isolates and identified cytochrome b mutations 129G (GGC), 253S (TCT) and 262S (TCA) in 21/75 buffalo-derived and 19/119 cattle-derived *T. annulata* isolates, providing evidence of positive selection pressure. Both hard and soft selective sweeps were identified, with striking differences between isolates. For example, 19 buffalo-derived and 7 cattle-derived isolates contained 129G (GGC) and 253S (TCT) resistance haplotypes at a high frequency, implying the emergence of resistance by a single mutation. Two buffalo-derived and 12 cattle-derived isolates contained equally high frequencies of 129G (GGC), 253S (TCT), 129G (GGC)/253S (TCT) and 262S (TCA) (TCA) resistance haplotypes, implying the emergence of resistance by pre-existing or recurrent mutations. Phylogenetic analysis further revealed that 9 and 21 unique haplotypes in buffalo and cattle-derived isolates were present in a single lineage, suggesting a single origin. We propose that animal migration between farms is an important factor in the spread of buparvaquone resistance in endemic regions of Pakistan. The overall outcomes will be useful in understanding how drug resistance emerges and spreads, and this information will help design strategies to optimise the use and lifespan of the single most drug use to control tropical theileriosis.

**1. Introduction**

Tropical theileriosis, caused by *Theileria annulata* and transmitted by *Hyalomma* ticks, is one of the most important livestock diseases in Asia and North Africa. The disease is highly pathogenic, infecting mononuclear cells of mammalian hosts (Nene et al., 2016). *T. annulata* has a major impact on food production in low- and middle-income countries throughout South Asia, the Middle East and North Africa, where efficient agriculture and livestock production is a priority under the UN Sustainable Development Goals. Tropical theileriosis is less severe in indigenous cattle (20%) than in cross-breed or exotic animals (80%) in Pakistan. The disease become more substantial when programs were launched to increase milk production in this country. Mostly, the disease occurs in subclinical form, leading to significant economic losses. The
cytochrome b catalytic Q atovaquone in 1985). Different studies have shown associations between resistance to parasites of livestock and humans, respectively. These drugs bind to drugs such as buparvaquone. The drug was introduced in the early limited, albeit links between mutations in cytochrome b and the resistance-conferring mutations in the field isolates. Achieving these objectives will lead to clearer understanding of buparvaquone resistance in an important tropical livestock pathogen, with the potential to inform enhanced animal health, improved global food security, and poverty alleviation through reduced production losses.

2. Materials and methods
2.1. Buparvaquone susceptible and resistant T. annulata isolates

We employed four infected cell lines (TA-ank, TA-but, TA-has, TA-mor) produced by the infection of blood mononuclear cells with laboratory-maintained stocks of T. annulata originally isolated in Turkey, India (Katzer et al., 1994), Tunisia and Morocco. The stock was selected to represent buparvaquone susceptible isolates. To extract the gDNA, 50 μl of each isolate was transferred into a fresh tube and centrifuged for 5 min, before removing the supernatant and mixing with 25 μl Direct PCR lysis buffer (Viagen), Proteinase K (Qiagen), and 1M dithiothreitol (DTT). Six known buparvaquone susceptible isolates (674, Battan C, Battan P4, Chargui P5, Jed4, Jed4 P10) were selected from different endemic regions in northeast Tunisia. The Battan C, Battan P4, Jed4 P10 and Jed4 isolates were chosen based on previous studies confirming the susceptibility of these stocks to buparvaquone (Darghouth et al., 1996). The Chergui P5 and 674 isolates were chosen from the animals cured after the first injection of buparvaquone. Seven known buparvaquone-resistant isolates (ST2/13, ST2/19, 739, 881III, 5911, 8307, BC2T) were selected from different endemic regions in northeast Tunisia. Two buparvaquone-resistant isolates (ST2/13, ST2/19) were taken from the previous study by Mhadhbi et al. (2015). The resistant isolates were collected at different times after treatment as follows: isolate ST2/13 was taken 24 h after the first treatment and the ST2/19 isolate was taken 48 h after the third treatment (Mhadhbi et al., 2015). Five stocks (739, 881III, 5911, 8307, BC2T) were initially isolated at various times points from tropical theileriosis clinical cases of treatment failure based on the absence of clinical improvement despite the early initiation of treatment with the conventional dose of 2.5 mg/kg of buparvaquone, and the persistence of a high parasitaemia after at least three doses of buparvaquone. All isolates were collected by lymph node punctures or from whole blood and cultured in a complete RPMI 1640 medium. They had been used at the low passage to avoid in-vitro pressure selection (Mhadhbi et al., 2015). Genomic DNA was extracted using a Promega Wizard DNA Purification kit (Madison, WI, USA) according to the manufacturer’s instructions.

2.2. Theileria annulata field isolates

The study was conducted in nine known tropical theileriosis endemic regions (Lahore, Gujranwala, Chakwal, Qadirabad, Okara, Sahiwal, Hasilabadi, Bahawalpur, Vehari) of the Punjab province of Pakistan. We chose this region because animals are treated sporadically often with generic buparvaquone drugs of unknown quality. Buparvaquone has been widely used for the therapy of tropical theileriosis because they are relatively inexpensive (as per comm of Kiran Afsan).

Field isolates were taken from suspected piroplasm-infected cattle...
and buffalo and presented at veterinary clinics across the endemic regions of Pakistan between 2020 and 2021. Piroplasm-negative cattle blood samples were provided by Dr Tim Connelly, Roslin Institute, University of Edinburgh. The samples were collected by para-veterinary staff under the supervision of local veterinarians following consent from the animal owners. The study was approved by the Institutional Review Board of the Quaid-i-Azam University Islamabad Pakistan (No. #BEC-FBS-QAU/2017). Peripheral blood smears were prepared and stained with 4% Giemsa and examined microscopically to detect piroplasms. Genomic DNA was isolated from positive samples by lysis with GS buffer containing proteinase K as described in the TIANamp Blood DNA Kit (TIANGEN Biotech Co. Ltd, Beijing) and stored at −20 °C. ‘Haemoproteobione’ high-throughput sequencing was performed on piroplasm-positive blood samples to confirm the presence of T. annulata as previously described (Chaudhry et al., 2019). Briefly, 194-piroplasm positive field samples [buffalo (n = 75), cattle (n = 119)] were examined to identify the species of T. annulata involved in the infections.

2.3. PCR amplification, Illumina Mi-Seq run and bioinformatics data handling of cytochrome b locus

A 516 bp fragment of the cytochrome b locus of T. annulata the buparvaquone susceptible and resistant isolates and field isolates was amplified. The primer sets, adapter/barcoded PCR amplification conditions and magnetic bead purification were previously described by Chaudhry et al. (2021) (Supplementary Tables S1 and S2). Ten μl of each barcoded bead purified PCR product were combined to make a pooled library which was subject to agarose gel electrophoresis. Cytochrome b products were excised and purified from the gel using a commercial kit (QiAquick Gel Extraction Kit, Qiagen, Germany), followed by purification of eluted DNA using AMPure XP Magnetic Beads (1X) (Beckman Coulter, Inc.). Purified products were then combined into a single pulsed DNA pool library. The library was quantified with qPCR library quantification kit (KAPA Biosystems, USA) and then run on an Illumina MiSeq Sequencer using a 600-cycle pair-end reagent kit (MiSeq Reagent Kit v2, MS-103-2003) at a concentration of 15 nM with the addition of 15% PhiX Control v3 (Illumina, FC-11-2003) described by Chaudhry et al. (2021).

The Illumina Mi-Seq post-run processing uses the barcoded indices to separate all sequences by sample and generate FASTQ files. The FASTQ files of the buparvaquone susceptible and resistant T. annulata isolates and T. annulata field isolates have been made freely available through the Mendeley database (https://doi.org/10.17632/n6xpdcntvf.1). These files were analysed using Mothur v1.39.5 software (Kozich et al., 2013; Schloss et al., 2009) with modifications in the standard operating procedures of Illumina Mi-Seq in the Command Prompt pipeline described by Chaudhry et al. (2021).

2.4. Allele frequency of buparvaquone resistance-associated SNPs in the cytochrome b locus

The calculation of the relative allele frequencies of T. annulata cytochrome b resistance-associated mutations identified in the buparvaquone susceptible and resistant isolates and field isolates was performed by dividing the number of sequence reads of each isolate by the total number of reads (package ggplot2).

2.5. Genetic models of buparvaquone resistance-associated SNPs in the cytochrome b locus

The sequence reads of the T. annulata cytochrome b locus from each isolate were imported into the FaBox 1.5 tool (birc.au.dk) to collapse the sequences that showed 100% base pair similarity after corrections into a single haplotype (freely available through the Mendeley database https://doi.org/10.17632/n6xpdcntvf.1). The haplotypes of cytochrome b were then aligned using the MUSCLE alignment tool in Geneious v10.2.5 software (Biomatters Ltd, New Zealand) for the analysis of the emergence of buparvaquone resistance-associated mutations in the cytochrome b locus. The haplotypes of the T. annulata cytochrome b locus were selected for neutrality analysis. Tests for selective neutrality were analysed to determine whether the observed frequency distribution of sequence polymorphism in T. annulata cytochrome b locus departed from neutral expectations using the DnaSP 5.10 software package (Librado and Rozas, 2009). The neutrality tests included Tajima’s D (Tajima, 1989) and Fay & Wu’s H (Fay and Wu, 2000) methods.

A split tree of cytochrome b haplotypes was created in the SplitTrees4 software (bio-sof.net) using the neighbour-joining method and the JukesCantor model of substitution for the analysis of the origin of buparvaquone resistance-associated mutations in the cytochrome b locus. The appropriate model of nucleotide substitutions for neighbour-joining analysis was selected by using the jModeltest 12.2.0 program (Posada, 2008). The network tree of T. annulata cytochrome b haplotypes was produced based on the neighbour-joining algorithm built on a sparse network and the epsilon parameter is set to zero default in Network 4.6.1 software (Fluxus Technology Ltd.) for the analysis of the spread of buparvaquone resistance-associated mutations in the cytochrome b locus. All unnecessary median vectors and links were removed with the star contractions. The number of mutations separating adjacent sequence nodes was indicated along connecting branches and the length of the lines connecting the haplotypes is proportional to the number of nucleotide changes.

3. Results

3.1. Buparvaquone resistance-associated SNPs in susceptible and resistant T. annulata isolates

A 516 bp region of the cytochrome b locus of the 10 susceptible (674, Battan C, Battan P4, Chargui P5, Jed4, Jes4P10, TA-ANK, TA-BUT, TA-HAS, TA-MARR) and 7 resistant (739, 8813, 5911, 8307, BC2T, ST2/13, ST2/19) T. annulata isolates were compared. Eleven point mutations in the T. annulata cytochrome b locus were found at codons A133V (GCT/GTT), V135F (GTC/TTC), I136L (ATA/CTA), G138V (GTT/GTG), L139F (TTG/TTT), L140F (TTG/TTT), K141N (AAA/AAC), F143L (CTT/CTC), S129G (GGC) and T129S (TTC) in susceptible isolates (Table 1). Overall, the results provide the genetic link between mutations and drug resistance in T. annulata.

3.2. Buparvaquone resistance-associated SNPs in buffalo- and cattle-derived T. annulata field isolates and their impact on positive selection pressure

A 516 bp fragment of the T. annulata cytochrome b locus was amplified from 75 buffalo-derived and 119 cattle-derived isolates across the endemic regions of Pakistan (Supplementary Table S3). The relative allele frequencies of three mutations S129G (AGG/GGC), P253S (CTT/CCT) and L262S (TTA/TCA) correspond to the buparvaquone binding pocket at codons 129G (GGC) and 253S (TCT) and L262S (TTA/TCA) in resistant isolates compared to P253 (CCT) and L262 (TTA) in susceptible isolates (Table 1). Overall, the results provide the analysis of cytochrome b mutations potentially involved in the development of buparvaquone resistance and the first high throughput genetic link between mutations and drug resistance in T. annulata.
of 1.3–4.3%. 262S (TCA) was not detected in any buffalo-derived T. annulata isolates (Fig. 1, Table 2). In contrast, buparvaquone resistance-associated mutations 129G (GGC), 253S (TCT) and 262S (TCA) were present in 19/119 cattle-derived T. annulata isolates (Fig. 1, Table 2). The 129G (GGC) mutation was present at frequencies of 1.4–100%. 253S (TCT) mutation was present at frequencies between 3.9 and 43%. 262S (TCA) mutation was detected at frequencies of 2.6–13.7%. 129G (GGC)/253S (TCT) was not detected in any cattle-derived T. annulata (Fig. 1, Table 2). Overall, the results indicated the positive selection pressure on the cytochrome b locus of 21 buffalo-derived and 19 cattle-derived T. annulata isolates.

### 3.3. Nature of selective sweeps associated with the emergence of cytochrome b resistance-associated SNPs in buffalo- and cattle-derived T. annulata field isolates

The analysis of 21 buffalo-derived T. annulata revealed that 18 isolates contained the 129G (GGC) resistance haplotype and one isolate (CY110–B) contained the 253S (TCT) resistance haplotype at a high frequency of 100% (Fig. 2A, Table 3), providing evidence of hard selective sweep patterns. In contrast, the CY321–B isolate contained resistance haplotypes of 129G (GGC) at a frequency of 24.5%, 253S (TCT) at a frequency of 59.9% and 129G (GGC)/253S (TCT) at a frequency of 13.8% (Fig. 2A, Table 3). The CY322–B isolate contained resistance haplotypes of 129G (GGC) at a frequency of 24.5%, 253S (TCT) at a frequency of 59.9% and 129G (GGC)/253S (TCT) at a frequency of 13.8% (Fig. 2A, Table 3). The CY321–B isolate contained resistance haplotypes of 129G (GGC) at a frequency of 24.5%, 253S (TCT) at a frequency of 59.9% and 129G (GGC)/253S (TCT) at a frequency of 13.8% (Fig. 2A, Table 3). These two isolates had equally high frequencies of cytochrome b resistance-conferring haplotypes demonstrating evidence of soft selective sweep patterns. Neutrality analysis further revealed a significant departure of neutrality in those 2 isolates providing evidence of a signature of selection at the T. annulata cytochrome b locus (Table 3).

The analysis of 19 cattle-derived T. annulata revealed that 6 (CY212–C, 252–C, 258–C, 6–C, 43–C, 136–C) isolates contained 129G (GGC) resistance haplotypes and one isolate (CY26–C) contained 253S (TCT) resistance haplotype at a high-frequency of 100% (Fig. 2B,
3.4. Phylogeny of cytochrome b resistance-associated SNPs in buffalo- and cattle-derived *T. annulata* field isolates and their impact on the origin and spread of buparvaquone resistance

The analysis of 21 buffalo-derived *T. annulata* revealed 22 unique cytochrome b haplotypes. Six haplotypes encoded 129G (GGC) resistance mutations, 2 haplotypes encoded 253S (TCT) resistance mutations, one haplotype encoded 129G (GGC)/253S (TCT) double resistance mutations and 13 haplotypes encoded S129 (AGC), P253 (CCT) and L262 (TTA) resistance mutations (Fig. 3A, Fig. 4A, Table 3). A split tree analysis showed that 129G (GGC) resistance mutants and 129G (GGC)/253S (TCT) resistance mutants carried 7 haplotypes and 253S (TCT) resistance mutant carried 2 haplotypes were located in a single lineage demonstrating evidence of a single origin. (Fig. 3A). The network analysis showed that the BHAS-129G haplotype was present at a high frequency in eight different isolates collected from the livestock farms in the Okara and Gujranwala regions. The BHA7-129G haplotype was present in three isolates and BHA11-253S haplotype was present in two isolates collected from livestock farms in the Gujranwala region. BHA2-253S haplotype was present in ten different isolates collected from livestock farms in the Okara and Gujranwala regions. BHA7-129G haplotype was present in ten different isolates collected from livestock farms in the Okara and Gujranwala regions. The BHAB1-129G haplotype was present in two isolates collected from livestock farms in the Gujranwala region. BHA2-253S haplotype was present in three isolates and BHA11-253S haplotype was present in two isolates collected from the livestock farms in the Gujranwala region. The BHAB1-129G haplotype was present in two isolates collected from livestock farms in the Gujranwala region. The BHAB1-129G haplotype was present in two isolates collected from livestock farms in the Gujranwala region. The BHAB1-129G haplotype was present in two isolates collected from livestock farms in the Gujranwala region. The BHAB1-129G haplotype was present in two isolates collected from livestock farms in the Gujranwala region.

Table 3, demonstrating evidence of hard selective sweep patterns. In contrast, 9 isolates (CY268-C, 270-C, 272-C, 277-C, 38-C, 39-C, 40-C, 41-C, 42-C) contained 129G (GGC) resistance haplotypes and one isolate (CY150-C) contained 253S (TCT) resistance haplotypes at frequencies ranging between 16 and 84% (Fig. 2B, Table 3). The CY235-C isolate contained resistance haplotypes of 129G (GGC) at a frequency of 34.2% and 262S (TCA) at a frequency of 60.6%. The CY90-C isolate contained resistance haplotypes of 129G (GGC) at a frequency of 34.2% and 262S (TCA) at a frequency of 0.07% (Fig. 2B, Table 3). These 12 isolates had equally high frequencies of cytochrome b resistance-conferring haplotypes demonstrating evidence of soft selective sweep patterns. Neutrality analysis further revealed a significant departure of neutrality in 9 out of 12 isolates providing evidence of a signature of selection at the *T. annulata* cytochrome b locus (Table 3).
indicating that the resistance associated with those haplotypes had not yet spread.

The analysis of 19 cattle-derived *T. annulata* revealed 61 unique cytochrome b haplotypes. 12 haplotypes encoded 129G (GGC) resistance mutations, 6 haplotypes encoded 253S (TCT) resistance mutations, 3 haplotypes encoded 262S (TCA) resistance mutations and 40 haplotypes encoded S129 (AGC), P253 (CCT) and L262 (TTA) susceptible mutations (Fig. 3B and Table 3). A split tree analysis showed that 129G (GGC), 253S (TCT) and 262S (TCA) resistance mutants carried 10, 5 and 2 haplotypes were located in a single lineage (Fig. 3B) demonstrating evidence of a single origin (Fig. 3B). The network tree analysis showed that CHA4-129G haplotype was present at a high frequency in 7 different isolates collected from the livestock farms in the Gujranwala and Qadirabad regions. The CHA9-129G haplotype was present in 9 different isolates collected from the livestock farms in the Bahawalpur, Qadirabad, Vehari and Okara regions. CHA10-129G haplotype was present in 3 isolates, CHA12-129G in 2 isolates, CHA22-129G in 5 isolates at high frequency collected from the livestock farms in the Gujranwala, Qadirabad, Vehari and Okara regions. The analysis further revealed that CHA28-253S haplotype was present in two different isolates collected from the livestock farms in the Gujranwala and Vehari regions. CHA39-253S haplotype was present in three different isolates collected from the livestock farms in the Vehari and Okara regions (Fig. 4B). The data of those 7 haplotypes demonstrate the evidence of a high level of gene flow predicted to occur due to unregulated animal movement. Seven haplotypes (CHA15-129G, CHA16-129G, CHA18-129G, CHA37-129G, CHA41-129G, CHA44-129G, CHA45-129G) were present at a frequency of 100% in 5 isolates collected from the livestock farms in the Gujranwala, Qadirabad, Vehari and Okara regions (Fig. 4B). Four haplotypes (CHA5-253S, CHA24-253S, CHA27-253S, CHA39-253S) were present at a frequency of 100% in single isolates collected from the livestock farms in the Gujranwala and Okara regions (Fig. 4B). CHA5-253S, CHA24-253S, CHA27-253S, CHA39-253S were present at a frequency of 100% in single isolates collected from the livestock farms in the Okara and Lodhran regions (Fig. 4B). These data demonstrated that the resistance conferred by these haplotypes has not yet spread elsewhere.

4. Discussion

The present study was designed to investigate the genetic characteristics of the cytochrome b locus and their impact on buparvaquone resistance in ruminants. The study further describes genetic models to understand the emergence and spread of buparvaquone resistance mutations at the cytochrome b locus of *T. annulata*.

Previous studies using low throughput sequencing methods have shown mutations in the cytochrome b catalytic Q01 and oxidative Q02 binding pockets of *T. annulata* collected from clinical cases of treatment failure in Iran (Sharifiyazdi et al., 2012), Tunisia (Mhadhbi et al., 2015) and Sudan (Chatanga et al., 2019). In the present study, high-throughput deep amplicon sequencing was performed to identify 14 mutations in the cytochrome b catalytic Q01 and oxidative Q02 binding pockets of *T. annulata* collected from clinical cases of treatment failure in the endemic region of Tunisia. Eleven mutations were present at similar frequencies across the buparvaquone susceptible and resistant *T. annulata* isolates. One mutation at codon 129G (GGC) in resistant isolates and S129 (AGC) in susceptible isolates was found in the Q01 binding pocket. Similarly, two mutations at codons 253S (TCT) and 262S (TCA) in resistant isolates and P253 (CCT) and L262 (TTA) in susceptible isolates were found within the Q02 binding pocket. Overall, these data provide a high throughput sequencing analysis of cytochrome b loci involved in the development of buparvaquone resistance and the first genetic link between mutations and buparvaquone drug in *T. annulata*.

The present study used deep amplicon sequencing for the first time to explore the cytochrome b locus of buffalo- and cattle-derived *T. annulata* field isolates collected from the endemic regions of Pakistan. Buparvaquone resistance-associated mutations 129G (GGC), 253S (TCT) and 129G (GGC)/253S (TCT) were identified in 21 buffalo-derived and 129G (GGC), 253S (TCT) and 262S (TCA) were identified in 19 cattle-derived *T. annulata* isolates. This represents the first report of buparvaquone resistance-associated SNPs in *T. annulata* field isolates and their impact on positive selection pressure. A possible explanation for the differences is that were observed in the frequency of buparvaquone-confering mutations with positive selection pressure may be variable drug doses; for example, if the 262S (TCA), 253S (TCT) and 129G (GGC)/253S (TCT)
resistance mutations may be selected at low doses of buparvaquone, while 129G (GGC) may occur at higher doses. Overall, the data provide novel information for screening field samples for the detection of buparvaquone resistance mutations in multiple isolates across different geographical regions. This information could aid in understanding how resistance mutations in multiple isolates across different geographical locations with implications for targeted selective treatment, or use of different drug combinations, including new drugs or the modification of current compounds in resistance mitigation strategies.

Several studies have assessed the selective sweep patterns in pyrimethamine resistance mutations of *P. falciparum* at the dihydrofolate reductase locus from different geographical regions. These emphasise a reduction in polymorphism around the dihydrofolate reductase locus with multiple resistance haplotypes indicative of soft selective sweeps (Hawkins et al., 2008; Shaukat et al., 2019). There is currently no understanding of the nature of selective sweeps associated with the emergence of buparvaquone resistance mutations in *T. annulata*. Both hard and soft selective sweep patterns have been identified in the present study for the buparvaquone resistance mutations in *T. annulata*. A single resistance haplotype at a high frequency was detected in the 19 buffalo-derived and 7 cattle-derived isolates. The selective sweeps on these individual isolates were effectively soft and a genetic footprint of selection was detected by significant departures from the neutrality test. In contrast, multiple resistance haplotypes were detected at high frequencies in the 2 buffalo-derived and 12 cattle-derived isolates. The selective sweeps on these isolates were effectively soft and a genetic footprint of selection was also detected by significant departures from the neutrality test. Overall, the data provide novel information on single and or multiple resistance haplotypes indicative of soft selective sweeps (Hawkins et al., 2008; Shaukat et al., 2019). There is currently no understanding of the nature of selective sweeps associated with the emergence of buparvaquone resistance mutations in *T. annulata*. Both hard and soft selective sweep patterns have been identified in the present study for the buparvaquone resistance mutations in *T. annulata*. A single resistance haplotype at a high frequency was detected in the 19 buffalo-derived and 7 cattle-derived isolates. The selective sweeps on these individual isolates were effectively soft and a genetic footprint of selection was detected by significant departures from the neutrality test. In contrast, multiple resistance haplotypes were detected at high frequencies in the 2 buffalo-derived and 12 cattle-derived isolates. The selective sweeps on these isolates were effectively soft and a genetic footprint of selection was also detected by significant departures from the neutrality test. Overall, the data provide novel information on single and or multiple resistance mutations in this group of parasites that may have implications for targeted selective treatment, or use of different drug combinations, including new drugs or the modification of current compounds in resistance mitigation strategies.

It can be challenging to determine the emergence of resistance

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### Table 3

| Haplotype distribution | Neutrality tests |
|------------------------|-----------------|
| Field isolates         | Fay and Wu H (H) | Tajima (D) |
| Haplotype             |                  |            |
| Total                  |                  |            |
| Buffalo                |                  |            |
| CY110-B                | 3                | 1          | 1.61917   | 1.95807 |
| CY123-B                | 4                | 1          | 1.56652   | 1.95807 |
| CY59-B                 | 2                | 1          | 1.70958   | 2.46226 |
| CY49-B                 | 1                | 1          | N/A       |          |
| CY5-B                  | 1                | 1          | N/A       |          |
| CY48-B                 | 1                | 1          | N/A       |          |
| CY4-B                  | 2                | 1          | 1.55322   | 2.23140 |
| CY60-B                 | 1                | 1          | N/A       |          |
| CY47-B                 | 1                | 1          | N/A       |          |
| CY254-B                | 1                | 1          | N/A       |          |
| CY256-B                | 1                | 1          | N/A       |          |
| CY257-B                | 1                | 1          | N/A       |          |
| CY260-B                | 1                | 1          | N/A       |          |
| CY266-B                | 1                | 1          | N/A       |          |
| CY269-B                | 1                | 1          | N/A       |          |
| CY278-B                | 1                | 1          | N/A       |          |
| CY317-B                | 1                | 1          | N/A       |          |
| CY220-B                | 1                | 1          | N/A       |          |
| CY231-B                | 9                | 4          | 2          | 2         | 1.68770* | 1.75932* |
| CY322-B                | 15               | 9          | 3          | 2         | 1.10119* | 0.88492* |
| CY233-B                | 1                | 1          | N/A       |          |
| Total                  | 22               | 13         | 6          | 2         |           |
| Cattle                 |                  |            |
| CY90-C                 | 20               | 14         | 2          | 2         | 1.02457* | 0.58541* |
| CY136-C                | 6                | 5          | 1          | 2         | 1.26900  | 0.82865 |
| CY150-C                | 6                | 3          | 1          | 2         | 1.39349  | 0.75819 |
| CY6-C                  | 4                | 3          | 1          | N/A       |          |
| CY26-C                 | 5                | 4          | 1          | N/A       |          |
| CY38-C                 | 4                | 1          | 3          | N/A       |          |
| CY39-C                 | 8                | 3          | 5          | 1.54706* | 1.44162 |
| CY40-C                 | 5                | 2          | 3          | 1.74256* | 2.00448* |
| CY41-C                 | 4                | 1          | 3          | 1.69795* | 1.64710 |
| CY42-C                 | 6                | 3          | 3          | 1.62083* | 1.98644 |
| CY43-C                 | 1                | 1          | N/A       |          |
| CY277-C                | 9                | 7          | 1          | 1         | 1.04126  | 0.29438 |
| CY272-C                | 7                | 4          | 3          | 1         | 1.57241* | 1.52030 |
| CY235-C                | 13               | 10         | 1          | 2         | 1.39659  | 0.51934 |
| CY268-C                | 2                | 1          | 1          | 1.67822* | 2.34668 |
| CY212-C                | 2                | 1          | 1          | N/A       |          |
| CY252-C                | 1                | 1          | N/A       |          |
| CY270-C                | 5                | 3          | 1          | 1         | 1.38874* | 1.01660 |
| CY258-C                | 1                | 1          | N/A       |          |
| Total                  | 61               | 40         | 12         | 6         | 3         |

Statistically significant departure from neutrality determined with the use of simulations of the coalescent at p < 0.05*.
Fig. 3. A split tree of 22 buffalo (3A) and 61 cattle-derived (3B) haplotypes of cytochrome b locus was generated in SplitsTrees4 software (Huson and Bryant, 2006). The pie chart circles represent the different haplotypes and the size of each circle is proportional to the number of sequence reads and frequency generated in that haplotype as indicated in the inserted table. The mutations in T. annulata haplotypes are shaded as follows: susceptible haplotypes are shown by grey shading; 129G (GGC) resistant haplotypes are shaded black; 129G (GGC)/253S (TCT) double mutant resistant haplotypes are shown by dots shading; 253S (TCT) resistant haplotypes are shown by white shading and 262S (TCA) resistant haplotypes are shown by black line shading.
Fig. 4. A network tree of 22 buffalo-derived (4A) and 61 cattle-derived (4A) haplotypes of cytochrome b locus was generated in Network 4.6.1 software (Fluxus Technology Ltd). The size of each pie chart circle representing the haplotype was proportional to the number of sequences generated from different isolates. The colours in the pie chart circles replicate the haplotype frequency and their distribution as indicated in the inserted table. The mutation-carrying *T. annulata* haplotypes in different livestock farms are shown in the inserted map. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
mutations with multiple haplotypes, either due to pre-existing mutations before the onset of positive selection or due to recurrent mutations after the onset of positive selection (Chaudhry and Gilleard, 2015). If pre-existing mutations were the only source for the emergence of resistance, similar haplotypes would be present in the isolates (Chaudhry et al., 2016b). For example, in the present study, 2 buffalo-derived isolates (CY321–B and CY322–B) carried four similar resistance haplotypes (BHA2-253S, BHA9-129G, BHA11-253S, BHA13-129G/253S). This striking similarity between the isolates provides evidence for the emergence of resistance by pre-existing mutations. If the recurrent mutations were the only source for the emergence of resistance mutations, different haplotypes would be present in the isolates (Chaudhry et al., 2016b). For example, in the present study, 11 cattle-derived isolates have dramatically carried different haplotypes. This striking difference between the isolates provides evidence for the emergence of resistance by recurrent mutations.

There are several studies demonstrating the evolutionary origin of P. vivax and P. falciparum dihydrofolate reductase resistance mutants (Hawkins et al., 2008; Mita, 2011b; Mita et al., 2007, 2009; Shaukat et al., 2021). A high level of genetic diversity may confer genetic adaptability, enabling the origin of pyrimethamine resistance mutations (Alam et al., 2007; Hong et al., 2016). In the present study, 9 unique buffalo-derived haplotypes of the 129G (GGC), 253S (TCT) resistance mutant and 129G (GGC)/253S (TCT) double resistance mutant were present in a single lineage. Similarly, 21 unique cattle-derived haplotypes of the 129G (GGC), 253S (TCT) and 262S (TCA) resistance mutations were present in a single lineage. These data demonstrate evidence of a single origin of the mutations in the T. annulata isolates examined.

Several studies indicate that there is a large amount of animal movement in the endemic region of Pakistan (Chaudhry et al., 2021), hence migration plays an important role in the spread of benzimidazole resistance mutations in livestock (Ali et al., 2019; Chaudhry et al., 2016a). The present study suggests that animal migration between farms is also important in the spread of buparvaquone resistance in the endemic region. The 129G (GGC), 253S (TCT) and 129G (GGC)/253S (TCT) and 262S (TCA) mutations were present in buffalo- and cattle-derived T. annulata collected from different livestock farms in the endemic regions and their phylogenetic relationship suggests that there is a high level of gene flow; possibly occurring due to unregulated animal movement. Notably, the two resistance haplotypes of the 129G (GGC) mutation predominate in 8 and 10 buffalo-derived T. annulata isolates and the other four resistance haplotypes of 129G (GGC), 253S (TCT) and 129G (GGC)/253S (TCT) mutations were present in 2 and 3 buffalo- and cattle-derived T. annulata isolates respectively. In cattle-derived T. annulata, six resistance haplotypes of the 129G (GGC) mutation predominate between 7 and 10 different isolates and the other two resistance haplotypes of 129G (GGC) and 253S (TCT) mutations were present in 2 and 3 different isolates respectively. Overall, the animal movement has likely contributed to the spread of buparvaquone resistance haplotypes in the endemic region of Pakistan. The spread of resistance mutations may require a degree of reproductive isolation of the hosts and parasites with resistant alleles (Chaudhry et al., 2021).

5. Conclusion

The data obtained in the present study provide the analysis of whether cytochrome b loci are involved in the development of buparvaquone resistance in ruminant livestock under natural field conditions. The results affirm the first link between genetic mutations and buparvaquone drug resistance in T. annulata. The data then obtained in the present study provide information on single and, or, multiple emergences of resistance mutations, albeit it is still unclear how these spread in parasite populations under natural field conditions. Genetic models based on these results could help to inform novel buparvaquone resistance mitigation strategies.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2022.08.004.

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