Isolation, cloning and sequence analysis of the lactate dehydrogenase gene from *Theileria annulata* may lead to design of new antitheilerial drugs

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ABSTRACT: Theileriosis is a serious animal disease that is transmitted by ticks. *Theileria* species are tick-borne obligate intracellular protozoan parasites that cause severe and mild infections in their hosts. Two of them, *Theileria annulata* and *Theileria parva*, cause lymphoproliferative disease with high mortality and morbidity in cattle commonly known as tropical theileriosis and East Coast fever, respectively. Currently available antiparasitic drugs are effective in animals but animals may remain carriers and treatment is most effective in the early stages of the disease. The isolation, cloning and analysis of lactate dehydrogenase from *T. annulata* was the goal of the present study with the ultimate aim of designing new antiparasitic drugs that will hopefully have a wider mode of action in animals. Blood samples were taken from a four year-old Brown Swiss cow showing signs of acute tropical theileriosis and genomic DNA was extracted following the confirmation of the clinical diagnosis. For the first time, in this study, the lactate dehydrogenase sequence was isolated from from a *Theileria* species. Following extraction from genomic DNA by PCR the sequence was cloned into the vector pGEM-T easy. Sequencing of the whole gene from both directions indicated that the open reading frame was interrupted by two introns. Several single nucleotide exchanges, deletions and insertions were also observed in the *T. annulata* lactate dehydrogenase sequence compared to the host. The most remarkable difference between the parasite and host enzyme is a five residue insertion in the active site loop region that might be an attractive target for inhibitors of the enzyme. This study opens a new route to further kinetic and structural studies towards the development of novel inhibitors of *T. annulata* lactate dehydrogenase.

Keywords: *Theileria annulata*; theileriosis; antitheilerials; lactate dehydrogenase; gene cloning

Theileriosis is a serious animal disease that is transmitted by ticks. It is caused by obligate intracellular parasitic protozoa of the genus *Theileria* classified in the phylum of apicomplexan protozoa as *Plasmodium* and *Toxoplasma* (Bishop et al. 2004). *Theileria parva* and *Theileria annulata* are globally the most important species causing East Coast fever and tropical theileriosis, respectively (Bishop et al. 2004).

Tropical theileriosis is an important tick-borne disease of cattle in tropical and sub-tropical countries (Gardner et al. 2005; Mhadhbi et al. 2010). Two hundred and fifty million cattle are estimated to be at risk because of the disease (Bishop et al. 2004). Available control strategies include the application of acaracides for the tick vector, chemotherapy or immunisation with a live attenuated cell-line vaccine for the parasite in endemic areas (Wilkie et al. 1998; Darghouth et al. 1999). The disease resulted in high mortality up to the 1970s due to the lack of effective therapies (Mhadhbi et al. 2010). Parvaquone (McHardy and Morgan, 1985) and buparvaquone (McHardy et al. 1985) have been effectively used since their discovery in the treat-

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ment of *Theileria* infections in cattle in subsequent years without the development of any resistance to these drugs. However, resistance to buparvaquone has been recently reported for the first time in the literature (Mhadhbi et al. 2010) and this has very recently been followed by a new case (Sharifiyazdi et al. 2012). The first study was conducted in a farm located in Tunisia. Four out of seven cattle showing symptoms of acute tropical theileriosis died in spite of repeated buparvaquone injections (2.5 mg/kg). It was reported that buparvaquone and parvaquone have been the only available drugs for the treatment of tropical theileriosis to date and possibly for several more years (Mhadhbi et al. 2010). The second case was reported from Fars Province, southern Iran (Sharifiyazdi et al. 2012). In this study seven out of eight animals were reported to have died despite single (five cases) and double (two cases) injection of buparvaquone (2.5 mg/kg) as a result of the occurrence of resistance to buparvaquone treatment. Resistance was explained by point mutations in the mitochondrial cytochrome *b* gene in the parasite (Sharifiyazdi et al. 2012). This emphasizes the need for the design of new anti-theilerial drugs that will have different modes of action then the currently available drugs. Mining of the genome and the characterization of genes has led to investigations which have focused on essential metabolic enzymes as potential new targets for the design of anti-parasitic drugs (Fernandez-Robledo and Vasta 2010).

Lactate dehydrogenase (LDH) is one of the glycolytic enzymes targeted for the design of such drugs. This enzyme catalyses the interconversion of pyruvate and L-lactate with a concomitant interconversion of NADH and NAD^+ (Holbrook et al. 1975). On the basis of molecular cloning studies, kinetic characterisations and X-ray structure analysis, plasmodial LDH's have been studied in most detail (Dunn et al. 1996; Turgut-Balik et al. 2001a; Chaikuad et al. 2005) among the apicomplexan parasites and have been identified as new enzyme targets for the development of novel antimalarial drugs. Determination of the key residues in the active site of this enzyme from *Plasmodium* showed that this site could be an attractive target for enzyme inhibitors (Dunn et al. 1996; Turgut-Balik et al. 2001b). A series of azole-based compounds were reported to bind within the active site of the enzyme and to preferentially bind *Plasmodium falciparum* LDH vs. the human LDH (Cameron et al. 2004). Comparative analysis of *Theileria* and *Plasmodium* genome sequences for the metabolic potential of parasites indicates that the complete TCA cycle may not be functional in both parasites (Gardner et al. 2005), and studies suggest glycolysis as the main pathway for energy production in *Theileria* (Kiama et al. 1999) as in *Plasmodium*. It is also suggested that *T. parva* schizonts may be similar to the erythrocytic stage of *P. falciparum* that uses glycolysis as the main pathway for the production of its energy (Sherman 1991). In the light of all these studies, it is reasonable to study the same enzyme from a *Theileria* species with the ultimate aim of discovering enzyme inhibitors of the parasite, as drug resistance has now been reported (Mhadhbi et al. 2010; Sharifiyazdi et al. 2012). Because the structural differences between host and parasite LDHs allow discrimination for preferential inhibition of the parasite enzyme (Cameron et al. 2004), LDH was amplified and cloned from a *Theileria* species for the first time in the literature in this study and the sequence was analysed in comparision to the host LDH and some other known LDH sequences.

MATERIAL AND METHODS

All general methods were applied according to Sambrook and Russel (2001) unless otherwise stated.

Bacterial strain, growth media, enzymes and vector

The host bacterial strain used to prepare DNA for cloning and sequencing in the pGEM-T Easy vector (Promega, USA) was *Escherichia coli* JM105 {supE hsdD5 thiD(lac-proAB) D(srl-recA) (306:Tn10) (tet^r) F'[traD36 $proAB^+$ $lacI^q$ lacZDM15 d]}. The *E. coli* JM105 cells were cultured in LB broth. Twenty mg/ml IPTG, 20 mg/ml X-Gal and 100 mg/ml ampicillin were used in media for the selection and growth of transformants where necessary. *Taq* DNA Polymerase was obtained from (Fermentas, #Cat. No: EP0402, Vilnus, Lithuania).

Parasite isolate and genomic DNA

Blood samples were collected in tubes containing the anticoagulant ethylene diamine tetra-acetic acid (EDTA) from a four year-old Brown Swiss cow showing signs of acute tropical theileriosis in Elazig province, Turkey. The clinical diagnosis

was confirmed by observation of *T. annulata* piroplasms and schizonts on Giemsa-stained blood and lymph node biopsy smears (immersion oil objective) at the Laboratory of Parasitology of the Veterinary School of Firat University, Elazig, Turkey. The Wizard genomic DNA purification system (Promega Corporation, #Cat. No: A1120, Madison, USA) was used to prepare DNA according to the manufacturer's instructions.

Amplification of *Theileria annulata* LDH by PCR

The initial sequence of *T. annulata* LDH (*Ta*LDH) was obtained from NCBI with the accession number of XM_948495. This sequence was then used to design two specific oligonucleotide primers for the amplification of the LDH gene from genomic DNA of *T. annulata* Elazig strain. Two oligonucleotide primers were prepared, complementary to the forward and reverse strands of the *Ta*LDH gene. The forward primer was *Ta*3, 5'-CGCGCGGGATCCATGGCAAGAAATAATAAGAGG-3', and reverse primer was *Ta*4, 5'-TTTTCTGCAGTTAGTGATGGTGATGGTGATGTTTAATGAGTGCTTCTAAACG-3'.

The enzyme *Taq* DNA Polymerase was used to amplify the DNA. This polymerase often adds a single deoxyadenosine to the 3'-end of the amplified fragments which makes these products suitable for use for T vector cloning. The reaction mixture contained 5 µl of *Taq* DNA Polymerase buffer, which is supplied with the enzyme, 1.5mM $MgCl_2$, 5 µl of a stock of dNTPs (10 µl of each 10mM dNTPs and 10 µl of H_2O). 50 pmol of each oligonucleotide primer, 1 µl of genomic DNA from *T. annulata* Elazig strain, 1 µl (2.5 units) of *Taq* DNA Polymerase and H_2O to a final volume of 50 µl. DNA was pre-denatured at 95 °C for 5 min prior to amplification. DNA was then denatured at 94 °C for 1 min and 30 s, annealed at 44 °C for 2 min and extended at 72 °C for 2 min for 45 cycles. The reaction ended with a final extension at 72 °C for 10 min. Analysis of PCR products on a 1% agarose gel revealed the presence of a band of the expected size. After confirmation of the product size, PCR was set up using the same conditions again and the DNA band was extracted directly from the agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega Corporation, #Cat. No: A9281, Madison, USA) prior to ligation.

Ligation and transformation

The pGEM-T easy vector system is used for the cloning of PCR products (Promega Corporation, #Cat. No: A1360, Madison, USA). This vector is prepared by cutting with *Eco* RV and adding a 3' terminal thymidine to both ends. The addition of single 3'-T overhangs at the insertion site contributes to the efficiency of ligation of the PCR product into the plasmid. The A-tailing procedure was applied to the PCR product to improve cloning efficiency prior to ligation according to the supplier's instructions. Ligation and transformation were also performed according to the supplier's instructions.

DNA sequencing

Inserts were initially checked by colony PCR using the *Ta*3 and *Ta*4 oligonucleotide primers and obtained bands at the expected size indicated the amplification of the correct DNA locus. Plasmid DNA was then prepared using Wizard Plus SV Minipreps DNA Purification System (Promega Corporation, #Cat. No: A1460, Madison, USA) and submitted for sequencing from both directions. All of the cloning experiments and sequencing were repeated twice independently starting from the genomic DNA step, because of the lack of proofreading activity of *Taq* DNA polymerase.

Database analysis and molecular modelling

LDH sequences of apicomplexans were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). Alignments of sequences at the nucleotide level were performed by using NCBI BLASTn tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The positions of exons and introns were determined by the GSDS utility (http://gsds.cbi.pku.edu.cn/index.php) (Guo et al. 2007). The molecular weight of proteins was calculated using the web based Peptide Properties Calculator (http://www.basic.northwestern.edu/biotools/proteincalc.html). Amino acid sequence alignment was performed manually by using catalytic residues as reference points to set up residue numbering correctly. The Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Chenna et al. 2003) tool was used to align LDH from *T. annulata*, *Bos taurus* and some other apicomplexans and then the obtained alignment file

was downloaded and adapted to MEGA to built the phylogenetic tree (Tamura et al. 2007) using the neighbour-joining method. Modelling studies of *B. taurus* LDH-A and *Ta*LDH was conducted using SWISS-Model automated mode (http://swissmodel.expasy.org/) (Arnold et al. 2006). The nucleotide sequences used in this study are available in GenBank under the following accession numbers: HM176661 for *T. annulata*, BC146210 for *B. taurus* (LDH A), XM_761610 for *T. parva*, DQ198261 for *Plasmodium falciparum*, DQ060151 for *Plasmodium vivax*, AY437808 for *Plasmodium berghei*, BM165756 for *Plasmodium yoelii*, U35118 for *Toxoplasma gondii* (LDH 1), U23207 for *T. gondii* (LDH2), AF274310 for *Cryptosporodium parvum*, AY143389 for *Eimeria tenella* and FJ617009 for *Eimeria acervulina*.

RESULTS

Amplification, cloning and DNA sequencing of the *Theileria annulata* LDH gene

Theileria annulata genomic DNA was amplified using two oligonucleotides a DNA fragment of about 1.6 kb in size was obtained. This product was then purified and inserted into the pGEM-T Easy plasmid vector prior to transformation into *E. coli* JM105 cells. Cloning of the target sequence was pre-checked and confirmed by PCR prior to sequencing (Figure 1). Sequence analysis of the insert demonstrated the presence of the full length

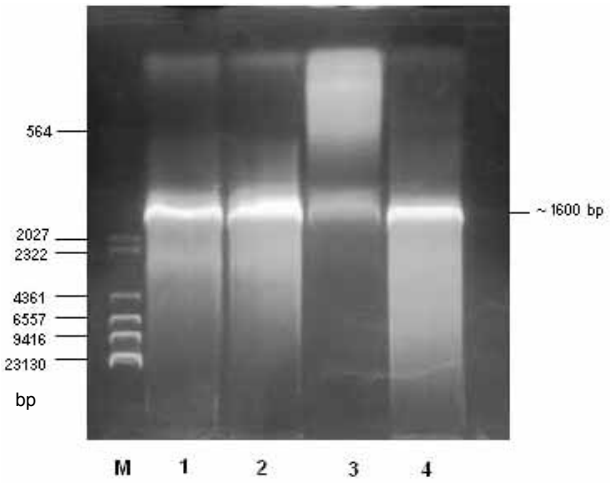

Figure 1. Confirmation of the cloning of *Ta*LDH by PCR. Lines: M = marker, 1,2,4 = positive results, 3 = negative result

*Ta*LDH. This sequence was deposited in GenBank with the accession number of HM176661.

This cloned LDH sequence is the first sequence to be cloned from a *Theileria* species to date, to our knowledge. Cloning and DNA sequencing results indicated that the open reading frame (ORF) of *Ta*LDH consisted of 1591 bp starting with an AUG codon and ending with a TAA codon. The ORF was interrupted by two introns conforming to the GT/AG rule at the splicing junctions. The main finding of this study is the presence of a 15 nucleotide insertion in the substrate specificity loop of the enzyme. The deduced *Ta*LDH protein sequence consisted of 322 amino acids with a calculated molecular weight of 35 220 g/mol.

Multiple amino acid sequence alignment and phylogenetic analysis of *Theileria annulata* LDH with some other known LDHs

The amino acid sequence of *Ta*LDH cloned in this study (HM176661) was aligned with the amino acid sequences of some other species, including *B. taurus* (LDH A: BC146210; LDH B: BC151427), *T. parva* (XM_761610), *P. falciparum* (DQ198261), *T. gondii* (LDH 1: U35118), *E. tenella* (AY143389) and *C. parvum* (AF274310) after removal of the two intron sequences (Table 1). *Ta*LDH showed 88% similarity to *T. parva*, 50% to *P. falciparum*, 47% to *T gondii*, 47% to *E. tenella* and 45% to *C. parvum*, respectively at the amino acid level. This alignment shows that the amino terminal extension observed in mammalian LDHs is lacking in *Ta*LDH. Residues involved in catalysis perfectly matched with the residues and positions of the other known LDHs. Although many characteristic residues are conserved, amino acid differences were observed between host *B. taurus* LDH (*Bt*LDH) and parasite LDH. One hundred and sixty three, 250 and 246 are some important positions (Turgut-Balik et al. 2001b) where amino acid exchanges were observed. Comparison of the *Ta*LDH with *Bt*LDH sequence revealed that *Ta*LDH has deletions at positions 48 and 283 and two insertions. The first insertion was at position 74. The second and most remarkable insertion was a penta peptide or 5 nucleotide insertion, between positions serine 108 and arginine 109 in the active site loop of the enzyme. This five amino acid insertion is present in Apicomplexan parasite LDH sequences, excluding *Cryptosporidium parvum* (Table 1).

Table 1. Alignment of *Ta*LDH amino acid sequence with LDH from *Bos taurus* (LDH A: BC146210; LDH B: BC151427), *Theileria parva* (XM761610), *Plasmodium falciparum* (DQ198261), *Toxoplasma gondii* (LDH 1: U35118), *Eimeria tenella* (AY143389) and *Cryptosporidium parvum* (AF274310). The five residue insertion in apicomplexan parasite LDHs is shown in box

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           1                          2729 32              48   53      61  65     72   76      85
btaurus-A  MATLKDQLIQNLLKEEHVPQNKITIVGVGAVGMACAISILMKDLADEVALVDVMEDKLKGEMMDLQHGSLFL.RTPKIVSGKDYNVTANSRLVI
btaurus-B  ATLKEKLIAPVAEEETTIPNNKITVVGVGQVGMACAISILGKSLTDELALVDVLEDKLKGEMMDLQHGSLFL.QTPKIVADKDYSVTANSKIVV
tannulata  .............MARNNKRKLISLIGSGNIGGIMGYLTQLTELAD.VNFLDIVPNIGAGKSLDIMHANSIQGKAYKCKGTNNYEDISGSDVCI
   tparva  .............MSGIRRKLISLIGSGNIGGIMGYLSQLTELAD.TVFFDIVPNIGAGKSLDIMHANSIQGKAYKCKGTNNYKDIAGSDVCI
    pfalc  ..............MAPKAKIVLVGSGMIGGVMATLIVQKNLGD.VVLFDIVKNMPHGKALDTSHTNVMAYSNCKVSGSNTYDDLAGADVVI
   toxgo1  ...........MAPALVQRRKKVAMIGSGMIGGTMGYLCALRELAD.VVLYDVVKGMPEGKALDDSQATSIADTNVSVTSANQYEKIAGSDVVI
 etenella  ...........MAVFEKVRRPKIALVGSGMIGGTMGFLCSLRELGD.VVLFDVVPNMPAGKALDLCHTAAVADNGVRVQGANSYASLEGADVVI
  cparvum  ..............MIERRKIAVIGSGQIGGNIAYIVGKDNLAD.VVLFDIAEGIPQGKALDITHSMVMFGSTSKVIGTNDYADISGSDVVI

            98           109110  116                        140 143         156    163  168171173
btaurus-A  ITAGARQQEGES.....RLNLVQRNVNIFKFIIPNIVKYSPNCKLLVVSNPVDILTYVAWKISGFPKNRVIGSGCNLDSARFRYLMGERLGVHP
btaurus-B  VTAGVRQQEGES.....RLNLVQRNVNVFKFIIPQIVKYSPACIIIVVSNPVDILTYVTWKLSGLPKHRVIGSGCNLDSARFRYLMAEKLGIHP
tannulata  VTAGLAKAPTKSNEEWNRDDLVGYNSKIIRDVGENIKKYAPEAFVIVITNPMDVMVHLMLKVTGFPKNMVVGMGGLLDSSRMNCYIAEKLGVNP
   tparva  VTAGLAKAPAKSNEEWNRDDLVAFNAKIITEVAENIKKYAPKAFVIVITNPMDVMVHLMLKVTGFSKNMVVGMGGLLDSSRMNCYIAEKLGVNP
    pfalc  VTAGFTKAPGKSDKEWNRDDLLPLNNKIMIEIGGHIKKNCPNAFIIVVTNPVDVMVQLLHQHSGVPKNKIIGLGGVLDTSRLKYYISQKLNVCP
    pknowl VTAGFTKAPGKSDKEWNRDDLLPLNNKIMIEIGGHIKKLCPNAFIIVVTNPVDVMVQLLFEHSGVPKNKIIGLGGVLDTSRLKYYLSQKLNVCP
   toxgo1  VTAGLTKVPGKPDSEWSRNDLLPFNSKIIREIGQNIKKYCPKTFIIVVTNPLDCMVKVMCEASGVPTNMICGMACMLDSGRFRRYVADALSVSP
 etenella  ITAGITKAAGKSDQEWSRKDLLPVNVKILREVGAAIKQFCPHAFVINITNPLDVMVAALREAAGLPAARVCGMAGVLDSARFRRLLADRLGVSP
  cparvum  ITASIPGRPKDD.....RSELLFGNARILDSVAEGVKKYCPNAFVICITNPLDVMVSHFQKVSGLPHNKVCGMAGVLDSSRFRTFIAQHFGVNA

              188    195      204                                  237   243246 250                  270
btaurus-A  LSCHGWILGEHGDSSVPVWSGVNVAGVSLKNLHPELGTDADKEQWKAVHKQVVDSAYEVIKLKGYTSWAIGLSVADLAESIMKNLRRVHPISTM
btaurus-B  SSCHGWILGEHGDSSVAVWSGVNVAGVSLQELNPEMGTDNDSENWKEVHKMVVESAYEVIKLKGYTNWAIGLSVADLIESMLKNLSRIHPVSTM
tannulata  KYVHGSVIGAHGDSMIPLVSRSTVYGIPILQFVEQGYITMEDIKEIEERTVTSAFEILKLYGSGSSYFAPATAAIEMASSYLNDKKCVFPCSCY
   tparva  KYVHGSVIGAHGDSMIPLVSRSTVYGIPILDFVEKGYLTHEDIKEIEERTITSAIEILKLYGSGSSYFAPATAAIEMASAYLNDKKSVFPCSCY
    pfalc  RDVNAHIVGAHGNKMVLLKRYITVGGIPLQEFINNK.LISDAE.LEAIFDRTVNTALEIVNLHASPYVAPAAAIIEMAESYLKDLKKVLICSTL
   toxgo1  RDVQATVIGTHGDCMVPLVRYITVNGYPIQKFIKDGVVTEKQLEEIAEHTKVSGGEIVRFLGQGSAYYAPAASAVAMATSFLNDEKRVIPCSVY
 etenella  RDVQAMVLGVHGDNMVPLSRFATVNGVPLGELARQGWISEAEIREVERQTRAAGGDIVRLLGQGSAYFAPGAAAVAMAEAYLKDQKRVFVCSCY
  cparvum  SDVSANVIGGHGDGMVPVTSSVSVGGVPLSSFIKQGLITQEQIDEIVCHTRIAWKEVADNLKTGTAYFAPAAAAVKMAEAYLKDRKAVVPCSAF

              280       290                  311    318 322
btaurus-A  IKGLYGIKEDVFLSVPCILGQNGISDVVKVTLTHEEEACLKKSADTLWGIQKELQF
btaurus-B  VKGMYGIENEVFLSLPCILNARGLTSVINQKLKDEEVAQLKKSADTLWGIQKDLKDL
tannulata  LEGQYGHR.DIYCGTPAVIGANGVEKVFELKLTPEEQDKYDASIKEIKRLEALIK.
   tparva  LEGQYGHK.EVYCGTPAVIGANGVEKVLELKLTPQEQQKFNDSIKEIRRLESLIK.
    pfalc  LEGQYGHS.DIFGGTPVVLGANGVEQVIELQLNSEEKAKFDEAIAETKRMKALA*...
   toxgo1  CNGEYGLK.DMFIGLPAVIGGAGIERVIELELNEEEKKQFQKSVDDVMALNKAVAALQA.
 etenella  LEGPYGVR.GHCLGVPCVVGAGGVERVIELPLDAREAQLLQASIDEVREMHRQLAAADAAAE
  cparvum  CSNHYGVK.GIYMGVPTIIGKNGVEDILELDLTPLKQKLLGESINEVNTISKVLDNAPAAGA.
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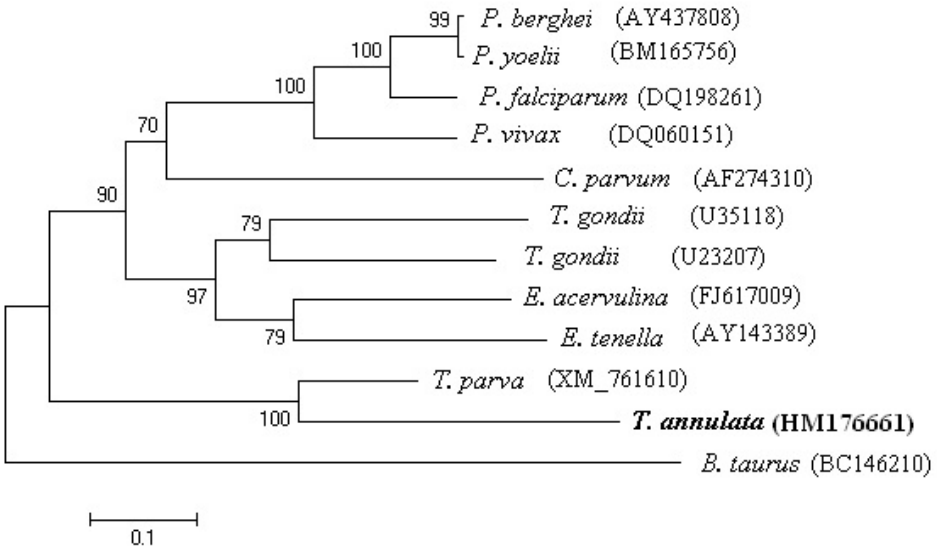

Figure 2. Phylogenetic relationships (neighbour-joining analysis) between *Theileria annulata* LDH identified in this study and some other apicomplexans parasites and *Bos taurus* available in the GenBank database. Numbers above the branch denote the bootstrap support from 1000 replications. The tree was created using the MEGA 4 package. The GenBank accession numbers are in parentheses. The sequence described in this study is in bold. The scale bar represents nucleotide substitutions per position

A phylogenetic tree was also constructed among *Ta*LDH, some apicomplexan LDHs and *Bt*LDH. Analysis of the results showed that *Ta*LDH and *T. parva* LDH are in a different clade than the other LDHs (Figure 2).

Modelling of *T. annulata* and *Bos taurus* LDHs

The deduced *Ta*LDH amino acid sequence obtained after the removal of the two introns from the gene was used to model the 3D structure of *Ta*LDH in

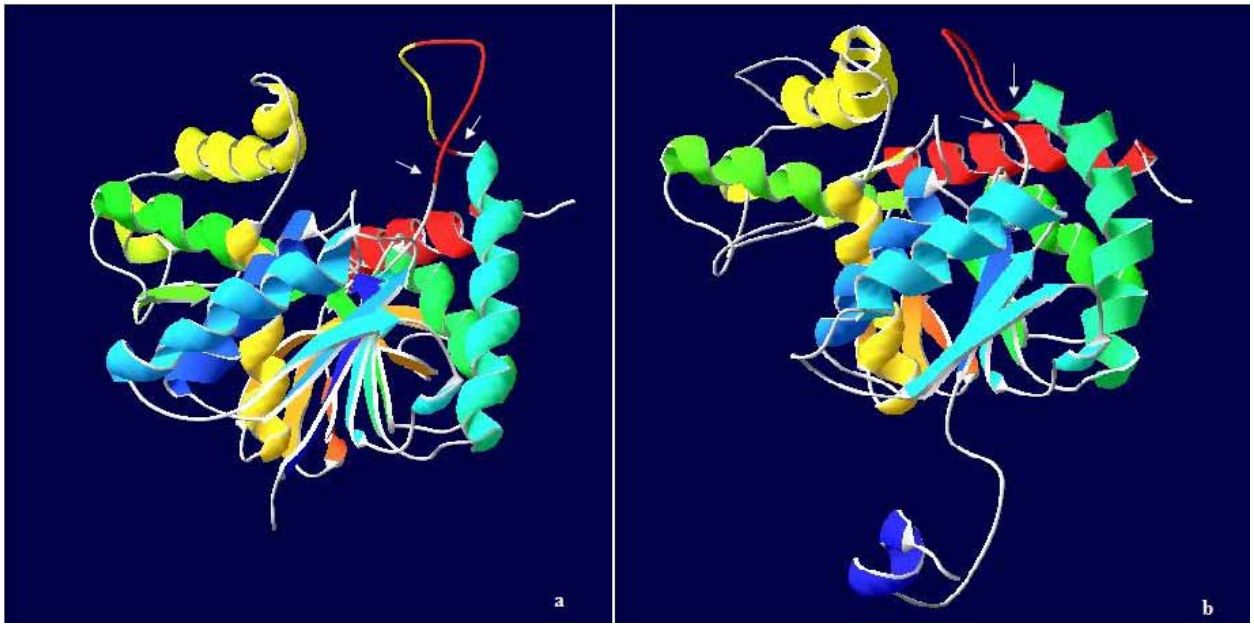

Figure 3. 3D view of *T. annulata* LDH (a) and *B. taurus* LDH (b). Borders of the substrate specificity loop (101–109) shown by white arrows (a and b), five amino acid insertion in the substrate specificity loop of *Ta*LDH shown in yellow (a)

the SWISS-MODEL workspace using the automatic modelling mode (Figure 3). Formation of an extended substrate specificity loop (101–109) caused by the insertion of five amino acids (NEEWN) is clearly observed in *Ta*LDH compared to its counterpart in *B. taurus*.

DISCUSSION

To our knowledge, this study is the first to describe the isolation, cloning and analysis of the LDH gene from a *Theileria* species. The cloning of *Ta*LDH opens up the route to the application of further structural and kinetic studies towards the development of enzyme inhibitors via structure-based drug design studies. The LDH sequence is known for some other apicomplexan parasites such as *Plasmodium* (Turgut-Balik et al. 2004), *Toxoplasma* (Yang and Parmley 1997) and *Eimeria* (Schaap et al. 2004) (Table 1). Characteristic residues responsible for catalytic efficiency of the LDHs (Clarke et al. 1989) are also present in *Ta*LDH. This shows that *T. annulata* LDH was cloned successfully in this study.

Analysis of the genomic sequence of *T. parva* in comparison with *P. falciparum* suggests that *Theileria* parasites do not have a completely functional TCA cycle similarly to plasmodial LDHs and rely heavily on glycolysis for the production of their energy (Kiama et al. 1999; Gardner et al. 2005). Therefore, inhibition of a crucial enzyme, like LDH, in the glycolytic pathway is a rational approach for the development of a novel antitheilerial drug that will have a different mode of action than the existing drugs. Determination of differences between plasmodial LDH and host LDH indicated that both enzymes are distinguishable in their structural and kinetic properties (Vander-Jagt et al. 1981; Royer et al. 1986; Makler and Hinrichs, 1993; Dunn et al. 1996; Turgut-Balik et al. 2001a). Insertion of five amino acids between residues S_{108}-R_{109} in the substrate specificity loop (101–109) is characteristic for the apicomplexan parasite LDHs cloned to date. This insertion is NEEWN in *Theileria*, DKEWN in *Plasmodium*, DS/KEWS in *Toxoplasma* and DQEWS in *Eimeria* (Table 1). This site has been well determined in *Plasmodium* and suggested to be an attractive target for the design of drugs against malaria (Dunn et al. 1996). The crystal structure of *P. falciparum* LDH (Dunn et al. 1996) indicated that this five amino acid insertion creates a distinctive cleft in the surface of the enzyme adjacent to the substrate binding site in contrast to the same region of the host LDH. Similar structures were also observed for *P. vivax* (Chaikuad et al. 2005), *P. berghei* (Winter et al. 2003) and *T. gondii* (Kavanagh et al. 2004) LDHs. Several organic molecules have recently been designed and developed with the aim of blocking LDH from *P. falciparum* (Granchi et al. 2010). Gossypol is a polyphenolic binaphthyl disesquiterpene compound present in cottonseed oil. Its derivatives have been tested against *Pf*LDH and reported to be strong inhibitors of the parasite enzyme (Royer et al. 1986). However, the cytotoxicity of gossypol has stopped any further development with regard to *Pf*LDH inhibition (Granchi et al. 2010). A series of azole-based compounds have also been identified in high throughput enzymatic screening and tested against *Pf*LDH. These compounds inhibited LDH activity at sub-micromolar concentrations *in vitro* and displayed modest antimalarial activity *in vivo* on *P. berghei* in a rodent model (Cameron et al. 2004). The same study also showed that these inhibitors form a network of interactions with residues within the active site of the enzyme. The present study indicates that the active site of *Ta*LDH is quite similar to the same region from *Pf*LDH. Therefore, the application of similar approaches to *Ta*LDH would be expected to produce valuable results as in other apicomplexan LDH studies.

Structure-based drug design studies often proceed through multiple steps before clinical trials (Anderson 2003). The first cycle of the process includes the cloning of the target gene, purification of the protein and determination of its structure by X-ray crystallography, NMR or homology modelling (Anderson 2003). In this study, cloning and sequence analysis in combination with homology modelling of the lactate dehydrogenase gene from *T. annulata* was described with the aim of facilitating structure-based drug design studies. The results show that the active site of *Ta*LDH, extended by five amino acids, is similar to the same sequence from other apicomplexan parasites and may be expected to be similar in character *Plasmodial* LDH's. To the best of our knowledge, this is the first report of the cloning of the LDH gene from a *Theileria* species and provides valuable insight into the structure of LDH from *Theileria* species that will greatly facilitate structure-based drug design studies.

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