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RESEARCH ARTICLE

Isolation, Cloning and Sequence Analysis of Enolase Enzyme Encoding Gene from *Theileria annulata* for Assessment of Important Residues of This Enzyme [1]

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[1] *Note: Nucleotide sequence data reported in this paper is available in the GenBank database under GenBank Accession No. HQ646253*

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Summary

Drug resistance against one of the important antitheilerial drugs has been reported for the first time in 2010. For the aim of developing new antitheilerial drugs or vaccines, enolase gene was isolated from the genomic DNA of *Theileria annulata*, cloned for the first time in the literature and analyzed at nucleotide and amino acid levels by using different web based tools. These analyses showed that the gene was consisted of 1365 nucleotides including an intron sequence placed between residues 40-41. Restriction enzyme mapping analysis of the cloned gene showed that, base pair changes in *Ta*ENO Elazig strain caused differences on cutting and non-cutting restriction enzymes compared to the Ankara strain. These differences may help the identification of different strains by restriction mapping and it may be possible to determine the geological distribution of *T. annulata* strains in any region. As the comparison of enolase gene sequences from *T. annulata* and the muscle enolase isoform of the host *Bos taurus* was made, four different insertions in *T. annulata* enolase that do not exist in *B. taurus* enolase was reported as an important discovery of this study. The modeling studies on *T. annulata* enolase gene showed that these insertions constituted loops that do not exist in *B. taurus* enolase, suggesting that these loops could be specific binding sites for enzyme inhibitors.

Keywords: *Theileria annulata, Enolase, Strain identification, Antitheilerial drugs, Structure based drug design, Protein homology modeling*

Theileria annulata'nın Enolaz Enzimini Kodlayan Geninin, Önemli Rezidülerinin Değerlendirilmesi Amacıyla İzolasyonu, Klonlanması ve Dizi Analizinin Yapılması

Özet

Bu yıl ilk kez önemli bir antitheilerial ilaca karşı direnç geliştiği rapor edilmiştir. Yeni antitheilerial ilaçların veya aşıların geliştirilmesi amacıyla *Theileria annulata* genomik DNA'sından enolaz geni izole edilmiş, literatürde ilk kez klonlanmış ve web tabanlı araçlar kullanılarak hem nükleotid hem de amino asit seviyesinde analiz edilmiştir. Bu analizler genin 1365 nükleotidden oluştuğunu ve 40-41 rezidüleri arasında bir intron dizisi içerdiğini göstermiştir. Klonlanan genin restriksiyon enzim haritalama analizinde, *Ta*ENO Elazığ soyundaki baz çifti değişikliklerinin, Ankara soyu ile karşılaştırıldığında, geni kesen ve kesmeyen enzimlerde farklılıklar yarattığı gözlemlenmiştir. Bu farklılıklar, farklı soyların restriksiyon haritalama ile tanımlanmasına yardımcı olabilir ve *T. annulata* soylarının herhangi bir bölgedeki coğrafik dağılımının belirlenmesini mümkün kılabilir. *T. annulata* enolaz geni dizisi ile konak *Bos taurus*'un kas enolaz izoformu karşılaştırıldığında, *T. annulata*'da bulunan 4 farklı insersiyonun *B. taurus* enolazında bulunmadığı belirlenmiş ve bu çalışmanın önemli bir bulgusu olarak rapor edilmiştir. *T. annulata* enolazının modelleme çalışmaları bu insersiyonların *B. taurus*'ta bulunmayan halkalar oluşturduğunu göstermiş ve bu halkaların enzim inhibitörleri için spesifik bağlanma bölgeleri olabileceği önerilmiştir.

Anahtar sözcükler: *Theileria annulata, Enolaz, Soy tanımlama, Antitheilerial ilaçlar, Yapıya dayandırılmış ilaç tasarımı, Protein homoloji modelleme*

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INTRODUCTION

Tropical theileriosis is a serious and widespread disease transmitted by ticks from *Hyalomma* genus and caused by *Theileria annulata* which infects cattle and leads to major economic losses [1]. *Theileria parva* and *Theileria annulata* are the most pathogen species among Theileria species that are factors of theileriosis disease, characterized as lymphoproliferative and causing high morbidity and mortality [2]. Tropical theileriosis factor *T. annulata* is found in tropical and subtropical countries [3] and approximately 250 million animals are living in risky areas [4,5]. Strategies to control this disease can be listed as, acarisides against vector ticks, immunization by live attenuated cell line vaccines, chemotherapy [6,7] and antiprotozoal drugs like halofuginone, parvaquone and buparvaquone [8-10]. Buparvaquone (2-trans (4-t-butylcyclohexyl-methyl)-3-hydroxy-1,4-naphthoquinone) is found to be the most effective antitheilerial drug used against *T. annulata* born cattle theileriosis [4,8,10,11]. However, a resistance of *T. annulata* against buparvaquone was reported first time in 2010 in a study in Tunisia [3]. This situation indicates that, designing new antitheilerial drugs will be very essential in forthcoming years.

Basic metabolic pathways and biochemical features of apicomplexan parasites are potential chemotherapeutic targets for indicating antiparasitic drugs for the treatment of diseases caused by these parasites. For this purpose, the gene encoding enolase enzyme is chosen as a target. Enolase, is a metalloenzyme which catalyzes the inter-conversion of 2-phosphoglycerate (2-PGA) to phosphoenol-pyruvate (PEP) during glycolysis [12]. Since *Theileria* species do not have a proper citric acid cycle and produce their energy mainly via anaerobic respiration, enolase has a crucial importance in the parasites life because of its role in glycolysis.

Aim of this study was isolation, cloning, sequence analysis of *Theileria annulata* enolase encoding gene *(Ta*ENO) and homology modeling for prediction of the 3D structure of the protein. This cloning study will be the first one in literature for *Ta*ENO gene and information presented by it, may lead to a new antitheilerial drug design.

MATERIAL and METHODS

All general methods were applied according to Sambrook et al.[13] unless otherwise stated.

Bacterial Strain, Growth Media, Enzymes and Vector

Escherichia coli JM105 was used as the host bacterial strain to prepare DNA for cloning and sequencing in pGEM-T Easy vector (Promega, USA). The *E. coli* JM105 cells were cultured in 2xYT broth. Long PCR Enzyme Mix was obtained from Fermentas (Lithuania).

Parasite Isolate and Genomic DNA

Blood sample was taken from a 4 years old Brown Swiss cow showing the symptoms of acute tropical theileriosis in Elazig province, Turkey. The samples were kept in a tube containing the anticoagulant ethylene diamine tetra-acetic acid (EDTA). The clinical diagnosis was confirmed by observation of *T. annulata* piroplasms and schizonts on Giemsa-stained blood and lymph node biopsy smears at the Laboratory of Parasitology of the Veterinary School of Firat University, Elazig, Turkey. The Wizard genomic DNA purification system (Promega, USA) was used to prepare DNA according to the manufacturer's instructions.

Amplification of Theileria annulata ENO by PCR

The initial sequence of *Ta*ENO from Ankara strain (accession number XM948248) was obtained from NCBI. Two specific oligonucleotide primers complementary to the forward and reverse strands of the *Ta*ENO gene were designed using this sequence. PCR product was analyzed on a 1% agarose gel and a band of the expected size was observed. After confirmation of the product size, PCR was set up at the same conditions again and DNA band was extracted directly from the PCR product using Promega's Wizard SV Gel and PCR Clean-Up System.

Ligation and Transformation

Ligation and transformation were performed according to the supplier instructions (Promega, USA).

DNA Sequencing

Plasmid DNA was then prepared using Wizard Plus SV Minipreps DNA Purification System (Promega, USA) and submitted for sequencing from both directions twice independently.

Database Analysis and Molecular Modeling

Enolase sequences of apicomplexan parasites, host *B. taurus* and plants were obtained from NCBI. Alignments of sequences at nucleotide level were performed by using ClustalW2 tool. Amino acid sequence alignment was performed manually by using catalytic residues as reference points to set up residue numbering correctly. Clustal W2 tool was used to align ENO from *T. annulata*, *B. taurus,* plants and some other apicomplexans. Modeling studies of *B. taurus* ENO-3 and *Ta*ENO conducted by SWISS-Model [14]. The nucleotide sequences used in this study, including *T. annulata* cloned in this study, with accession numbers: *T. annulata* HQ646253, *Plasmodium falciparum* U00152, *Eimeria tenella* AF353515, *Toxoplasma gondii* AF123457, *Lycopersicum esculentum* X58108, *Arabidopsis thaliana* AY092986 and *B. taurus* NM001034702.

Restriction Enzyme Analysis

*Ta*ENO gene was amplified by PCR again to prepare

template DNA for restriction enzyme analysis. This PCR product was then treated with BamH1 and Acl1 restriction enzymes.

RESULTS

Amplification, Cloning and DNA Sequencing of TaENO Gene

Amplification of *Ta*ENO gene was made by using two oligonucleotides (TaENO1 and TaENO2) and a fragment of about 1.3 kb length was obtained. This product was then purified *(Fig. 1)* following the PCR, inserted into the pGEM-T Easy plasmid vector and transformed into *E. coli* JM105 cells. This cloned sequence of *Ta*ENO was submitted to GenBank with the accession number HQ646253. This was the first time enolase gene was cloned from a *Theileria* species in literature. Sequence analysis of the cloned gene indicated that *Ta*ENO gene was consisted of 1365 base pairs, containing 2 open reading frames (ORF) of 40 bp and 1286bpwhich were divided by an intron sequence of 36bp conforming to the GT/AG rule at the splicing junctions.

Restriction Enzyme Analysis

Restriction enzyme mapping analysis showed that the 37 base pair changes in *Ta*ENO Elazig strain caused differences on cutting and non-cutting restriction enzymes compared to the Ankara strain. Four of these base pair changes generated four new enzyme cutting sites (AflIII, BamHI, BseRI and TstI) and 11 of these base pair changes abolished 11 enzyme cutting sites (AclI, ArsI, BplI, BsmI, BsrDI, DraII, HgaI, PflMI, PpuMI, TspGWI and XbaI) of Elazig strain. EtBr gel electrophoresis results showed clearly that BamH1 restriction enzyme can cut *Ta*ENO gene from Elazig strain while it can not cut *Ta*ENO of Ankara strain and also Acl1 restriction enzyme can not cut *Ta*ENO gene from Elazig strain but can cut *Ta*ENO of Ankara strain.

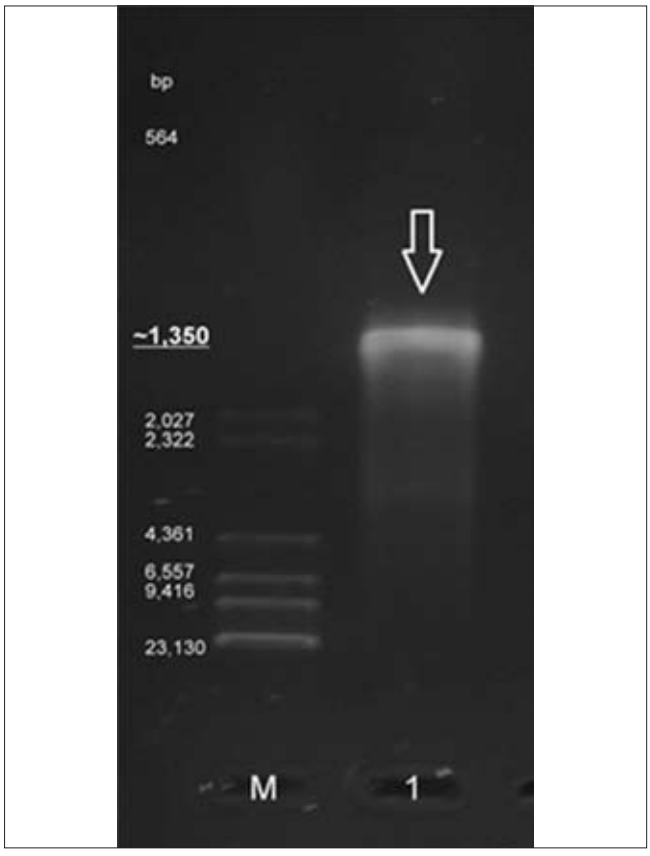

Fig 1. Agarose gel view of amplified and purified *Ta*ENO gene from genomic DNA of *T. annulata*. M: Marker, 1: *Ta*ENO

Şekil 1. *T. annulata* genomik DNA'sından amplifiye edilip saflaştırılmış *Ta*ENO geninin agaroz jel görüntüsü. M: Marker; 1: *Ta*ENO

Multiple Amino Acid Sequence Alignment and Phylogenetic Analysis of TaENO With Some Other Known Enolase Sequences

The amino acid sequence of *Ta*ENO (HQ646253) obtained in this study was first compared with host *B. taurus* muscle form enolase (*Bt*ENO3) (NM001034702). Alignment analysis showed the existence of a penta-peptide, a tripeptide and two dipeptide insertions in *Ta*ENO as $E_{103}W_{104}G_{105}Y_{106}C_{107}$, $T_{147}D_{148}$, $E_{262}K_{263}S_{264}$ and $K_{317}L_{318}$ respectively that do not exist in *Bt*ENO3. The same amino acid alignment comparison was also made with other apicomplexan parasite examples, *T. gondii* ENO1 (AF123457) and ENO2 (AY155668), *P. falciparum* (U00152) and *E. tenella* (AF353515) and a plant example *L. esculentum* (X58108). These comparisons showed that both apicomplexan parasites and plants had similar insertion sites as *Ta*ENO *(Fig. 2)*. Alignement analysis also showed that characteristic residues involved in the catalytic activity of the ENO were conserved [15] in all of the sequences presented in *Fig. 2*. Among these residues, E217 and E174 are involved in the dehydration step; D330, E303 and D252 required for the binding of the substrate; K355 and R384 interacts with the phosphate group and K406 and H383 with the carboxylic group of 2-PGA.

Comparison of T. annulata and Bos taurus ENO's by Homology Modeling and Potential Use of TaENO Gene In Structure Based Drug Design Studies

Structure based drug designing studies are mostly studied on *Plasmodium* sp. among apicomplexan parasites [16]. Because *Plasmodium* species do not have a functional Krebs cycle, as *Thelieria* species, they produce their energy via glycolysis [17,18]. Lactate dehydrogenase enzyme has a crucial role in this parasites life as it catalyzes the reduction of pyruvates to hydroxyls by oxidation of NADH to NAD^+ [16]. It is important to note that, Plasmodial LDH also has a pentapeptide insertion in the active site of the enzyme [19]. Crystallography studies on *Pf*LDH revealed that the pentapeptide insertion in an active site loop between 108. and 109. amino acids which constitutes a cleft on the surface of catalytic ring of the enzyme, but mammalian equivalent LDH does not contain this cleft as it does not have the pentapeptide insertion. From this point of view,

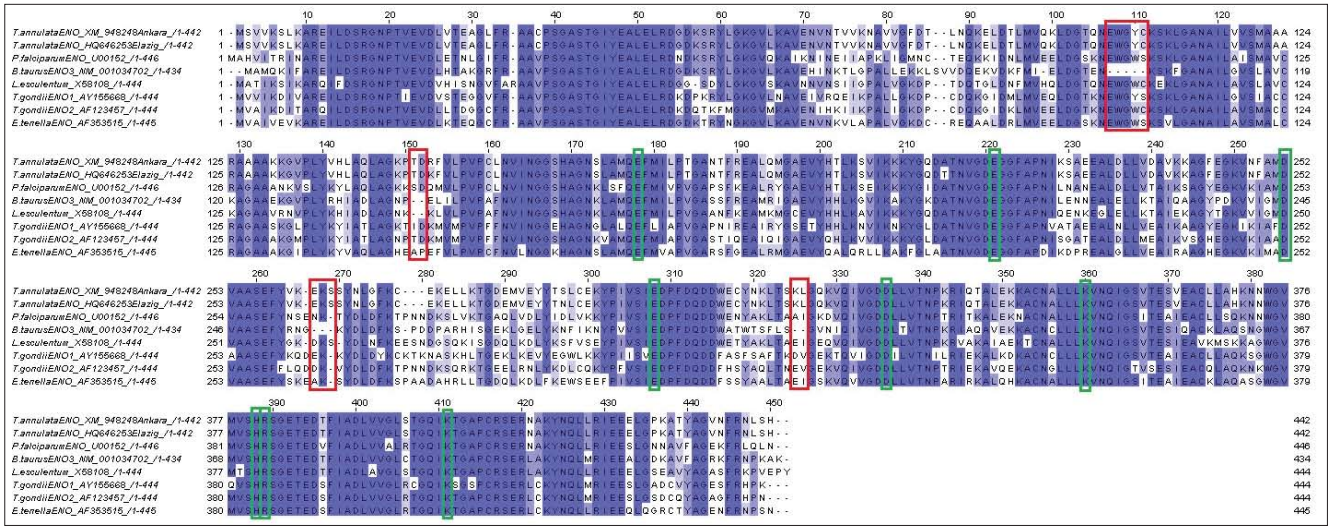

Fig 2. Comparison between amino acid sequences of enolase genes of *Theileria annulata* Ankara and Elazig strains, *Plasmodium falciparum, Bos taurus* ENO3, *Lycopersicum esculentum, Toxoplasma gondii* ENO1 and ENO2 and *Eimeria tenella,* from top to bottom respectively. Sites boxed in red, show the pentapeptide, the tripeptide and two dipeptide insertions. Sites boxed with green show the catalytic residues of enolase

Şekil 2. Yukarıdan aşağı sırayla,*Theileria annulata* Ankara ve Elazığ soyları, *Plasmodium falciparum, Bos taurus* ENO3, *Lycopersicum esculentum, Toxoplasma gondii ENO1 ve ENO2 ve Eimeria tenella*'nın enolaz genlerinin amino asit dizilerinin karşılaştırılması. Kırmızı ile kare içine alınmış bölgeler pentapeptid, tripeptid ve iki dipeptid insersiyonlarını göstermektedir. Yeşil ile kare içine alınan bölgeler enolazın katalitik rezidülerini göstermektedir

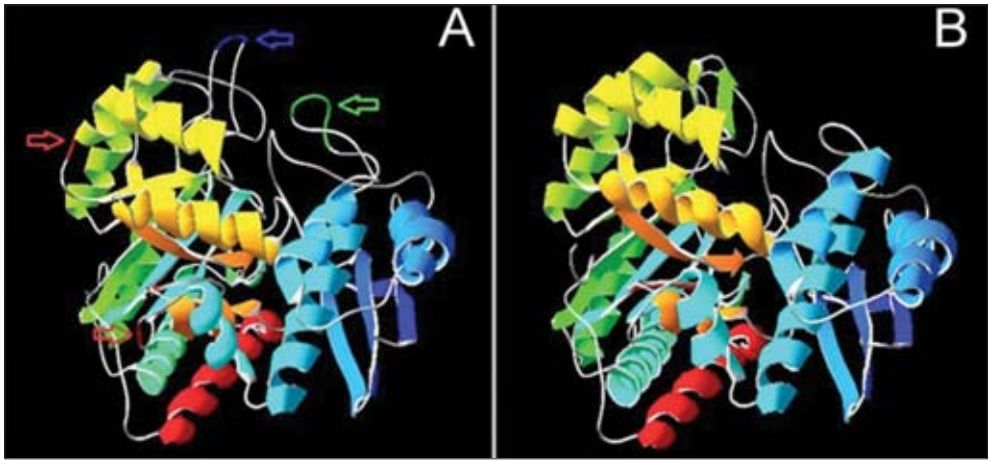

Fig 3. Homology modeling of enolase proteins for prediction of 3D structures of the protein, **A-** Homology model of enolase protein of *T. annulata* Elazig strain in ribbon style. Pentapeptide insertion site is marked with green, tripeptide insertion is marked with blue and dipeptide insertion sites are marked with red, **B-** Homology model of ENO-3 protein of host *B. taurus* in ribbon style

Şekil 3. Enolaz proteinin homoloji modelleme ile 3 boyutlu yapısının tahmini, **A-** Elazığ soyu *T. annulata*'nın enolaz proteininin kurdela modeli ile homoloji modellemesi; pentapeptid insersiyonu yeşil, tripeptid insersiyonu mavi ve dipeptid insersiyonları kırmısı ile işaretlenmiştir, **B-** Konak *B. taurus*'un ENO-3 proteininin kurdela modeli ile homoloji modellemesi

in vitro studies showed that azole based inhibitors can bind to active site of *Pf*LDH protein and stop both the enzyme activity and the parasite development in red blood cells. These compounds were selective against *Pf*LDH than human LDH and crystallography studies showed that the binding regions were also maintained in *P. berghei* LDH forms [16].

Considering these studies on *Plasmodium* species, it is aimed to investigate if it is possible to open a route to design new antitheilerial drugs by the evaluation of the data from isolation and cloning of enolase encoding gene from *T. annulata*. For this purpose, m-RNA sequence of cloned *Ta*ENO gene was compared to host *B. taurus* muscle enolase (ENO-3) using a web based tool to obtain a 3D model of both ENOs.

Amino acid sequence obtained from DNA sequencing of the cloned *Ta*ENO gene in this study was used to model 3D structure of *Ta*ENO by SWISS-MODEL workspace using the automatic modeling mode *(Fig. 3a)*. ENO-3 protein of the host *B. taurus* was also modelled by the same

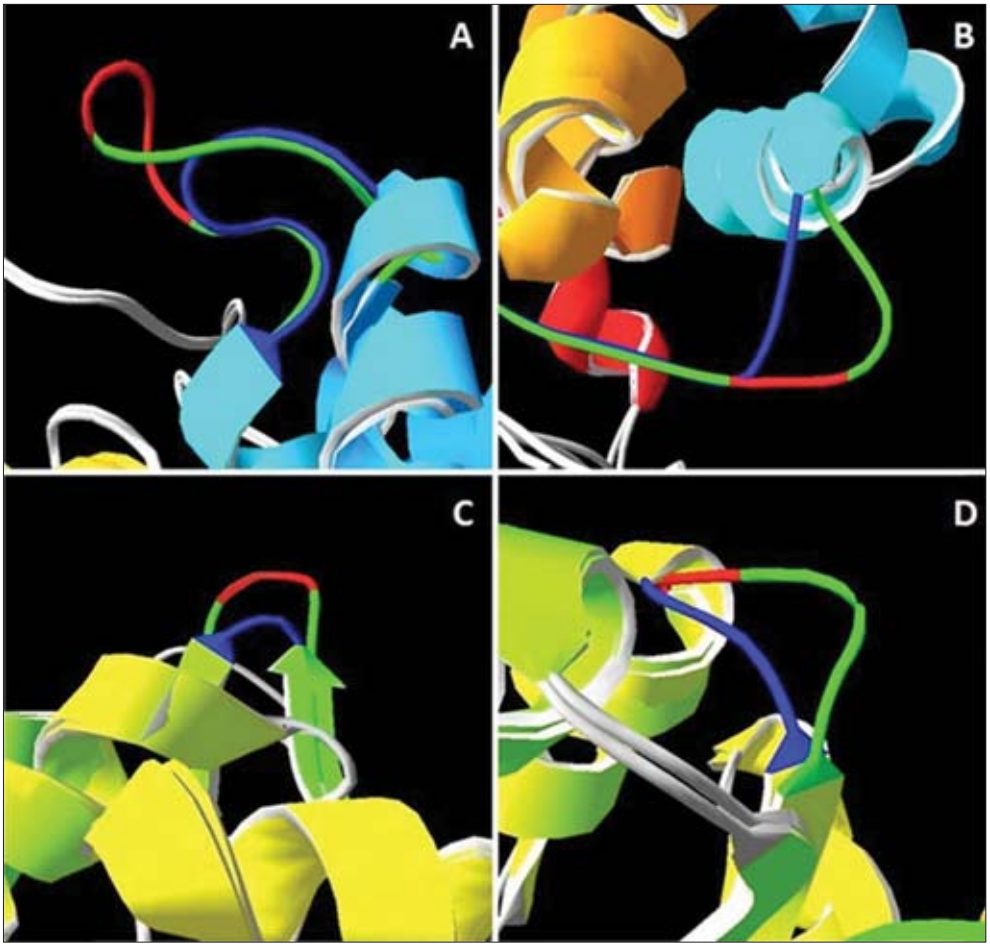

Fig 4. Overlay comparisons of insertion sites of *T. annulata* (green) and *B. taurus* (blue) enolases, **A-** The pentapeptide insertion of $E_{103}W_{104}G_{105}Y_{106}C_{107}$ in *Ta*ENO is shown in red, **B-** A dipeptide insertion ($T_{147}D_{148}$) in *Ta*ENO is shown in red, **C-** The tripeptide insertion ($E_{262}K_{263}S_{264}$) in *Ta*ENO is shown in red, **D-** Another dipeptide insertion ($K_{317}L_{318}$) in *Ta*ENO

Şekil 4. *T. annulata* (yeşil) ve *B. taurus*'un (mavi) enolazlarının insersiyon bölgelerinin (kırmızı) üst üste çakıştırılarak karşılaştırılması, **A-** *Ta*ENO pentapeptid insersiyonu ($E_{103}W_{104}G_{105}Y_{106}C_{107}$), **B-** *Ta*ENO dipeptid insersiyonu ($T_{147}D_{148}$), **C-** *Ta*ENO tripeptid insersiyonu ($E_{262}K_{263}S_{264}$), **D-** Diğer *Ta*ENO dipeptid insersiyonu ($K_{317}L_{318}$)

method using the amino acid sequence from GenBank (NM 001034702) for comparison with *Ta*ENO *(Fig. 3b)*. In detail formation of each loop caused by pentapeptide and dipeptide insertions in *Ta*ENO that do not exist in *Bt*ENO3 were clearly observed when the comparison of overlaid carbon backbone structures of the proteins was made *(Fig. 4)*.

DISCUSSION

Parvaquone [11] and buparvaquone [8] were used as drugs for treatment of tropical theileriosis without facing any resistance against these drugs since 1970's, although the high mortality rate of the disease because of the deficiency of treatment methods. However, a resistance of *T. annulata* against buparvaquone was first reported in Tunisia last year [3]. This situation indicates that designing antitheilerial drugs with different effects is essential. The gene encoding enolase enzyme from *T. annulata* was isolated, to our knowledge, cloned for the first time and analyzed in this study. Results indicated that, *Ta*ENO was consisted of 1365 base pairs including the stop codon and had and intron site of 36 base pairs. The homology modeling studies can give information leading to locate residues that are specific to the parasite and to inactivate the target enzyme of the parasite selectively [17]. As an important finding, four insertion sites, including a pentapeptide ($E_{103}W_{104}G_{105}Y_{106}C_{107}$), a tripeptide ($E_{262}K_{263}S_{264}$)and two di-peptide ($T_{147}D_{148}$and $K_{317}L_{318}$) insertions, were detected by comparison of homology models of *Ta*ENO and the equivalent in host *B. taurus,* muscle enolase (ENO3). It has been suggested that, the loops constituted on enolase by these four insertions of 5, 3 and 2 amino acids, do not exist in host *B. taurus* enolase and can be used as binding regions for specific enzyme inhibitors. Further kinetic, structural and mutagenic analysis of *Ta*ENO in comparison to the host ENO would be of great value towards the drug design studies against *T. annulata.*

Another important data obtained about insertion sites is that the region of the pentapeptide insertion may be an antigenic epitope. Effects on enzyme activity and structure, after removal of a pentapeptide insertion on a surface loop away from the active site by deletion in *P. falciparum* were studied by Vora and colleagues in 2009 [20]. It was reported that this deletion decreases the k_{cat}/K_m values for a 100 times and causes the dimeric form separate to monomers. The serum obtained from mice that were vaccinated by Pfen (*P. falciparum* enolase) and survived the parasite infection, gave a negligible reaction against the protein that carries the deletion, when compared to wild-type enolase. These results show that the insertion site is essential for enolase's proper activity and may constitute a preservative antigenic epitope in parasite's enolase [20]. A region, similar to *Plasmodium*s is also available in *Ta*ENO, which is cloned in this study, and it is suggested that this region may be an antigenic epitope for *Ta*ENO. In addition, virulence has been reported to vary across strains and cloned parasites of *T. annulata* as summarized in Tindih et al.[21]. As differences in ENO sequence of two strains of *T. annulata* from different territories are reported by this study, it may be possible to facilitate determination of virulence of different strains by identification of the strains via restriction enzyme analysis.

In this study isolation, cloning, sequence analysis in combination with homology modeling studies and restriction enzyme analysis were performed for enolase encoding gene from *T. annulata* (Elazig strain). This study is first to describe enolase sequence from this parasite and opens a route to structure based drug design studies after the first report of a drug resistance against a commonly used antitheilerial drug, buparvaquone, and vaccine studies as this study enables to distinguish strains from each other by a simple restriction enzyme analysis that may help to determine the virulence variation between different strains.

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