

Regional Differences in the Amino Acid Sequences of *Fasciola hepatica* Cathepsin L2 From a Turkish Isolate

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ABSTRACT

Objective: This study describes cloning and sequence analysis of the gene coding for the cathepsin L2 gene from a *F. hepatica* isolate from Turkey.

Methods: The adult form of *F. hepatica* was collected from the infected cattle liver and cDNA was obtained following total RNA isolation. After amplification of the gene by PCR, ligation and transformation was performed. Finally, DNA sequencing and phylogenetic analysis were performed.

Results: The gene consists of an open reading frame of 981 nucleotides corresponding for 327 amino acids. There are five amino acid positions where Fırat *F. hepatica* cathepsin L2 differs from the *F. hepatica* cathepsin L2 Dublin strain. This sequence was the first cathepsin L2 gene from a Turkey isolate and the second cathepsin L2 sequence entered into the database. A comparative amino acid sequence analysis indicated that there is about 99% homology with the *F. hepatica* cathepsin L2 reported in the literature, 98% homology with the *F. hepatica* cathepsin L-like protease, and 85% homology with the *F. hepatica* cathepsin L1 isolated in the Elazığ region at the nucleotide level.

Conclusion: The amino acid differences between sequences could be evaluated for further immunization and diagnosis studies.

Key Words: *Fasciola hepatica*, cathepsin L2, nucleotide, amino acid sequence

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Introduction

Fasciola hepatica is a liver trematode, which is a member of the family Fasciolidae under the subclass Digenea, and causes fasciolosis in humans and animals. While fasciolosis is more widespread in countries breeding cattle and sheep, it is a common problem in both developed and developing countries (1, 2). Although fasciolosis was traditionally regarded as a world-wide disease of livestock, the disease has recently emerged as an important human zoonosis in South America, Egypt, Iran, and Vietnam (3). Its medical importance has also increased in Turkey compared to the previous years (4, 5).

Although the history, clinical symptoms and laboratory findings may suggest fasciolosis in a patient, these only support the diagnosis. Instead, coprologic methods, serologic tests, radiologic imaging techniques, and molecular biology techniques are used in diagnosis (6-9). Demonstration of *Fasciola hepatica* or its eggs provides a definite diagnosis. However, examination of feces is frequently inadequate for diagnosis. This is because eggs are not detected in the stool during the acute phase of infection and ectopic settling (6, 10). For this reason, serologic methods are frequently used in the diagnosis of particularly acute fasciolosis. ELISA, IHA, counter-electrophoresis, and Western blot are the preferred tests. As cross reactions are

rare in these tests, E/S antigens (excretion-secretion) or partially purified parasite products are used. Cysteine proteinases, which are the most frequently found E/S antigens, are secreted by both the adult and young forms, and are quite antigenic in animals and humans. For this reason, they are valuable in diagnosis (8). Purified and recombinant antigens have begun to be used in recent years (11-13).

Cathepsin L1 and L2, are predominant in adult *F. hepatica* gut contents and excretion/ secretion products (14). The adult stage of *F. hepatica* secretes several cysteine proteases and the cathepsin L-like proteinases are proposed to be involved in activities such as invasion of tissues, feeding, immune evasion, or egg shell formation (14). Cathepsin L1 and L2 proteins are potential molecules to be used in diagnostic methods (8, 11, 12) and in immunization studies for protection purpose (15-19) as they are specific to *F. hepatica*.

Genetic variations are widespread in parasite populations. The understanding of the parasite life cycle, transmission, study of population biology, epidemiology, as well as genetic variation in parasite populations is important for the control and effective prevention of parasitic diseases in humans and animals. Thus, the accurate analysis of genetic variation in parasites has important implications (20). Additionally, in order to eliminate or minimize the impact of these parasites and

to be used in diagnostic methods and immunization studies, it is also important to understand the genetic diversity of the liver fluke populations and the relationship between parasite and host at regional bases (21). It is shown that there are regional differences in Cat L1 of *F. hepatica* (22) and these differences in amino acid residues appear to affect immunogenicity of the protein involved. Cornelissen and collaborators (23) proved with synthetic peptides that the difference in only three amino acids results in major differences in immunodiagnostic potential. These differences in the amino acid residues may also affect the serological diagnostic tests to show the same specificity and sensitivity at every region. Therefore, we suggest that local studies are of importance in the selection of antigens for serological diagnostic tests.

This study was carried out to determine regional differences in the amino acid sequences of *Fasciola hepatica* secreted cathepsin L2 in an isolate from Turkey.

Material and Methods

Parasite

The adult forms of *Fasciola hepatica* were collected from the infected cattle liver. The parasites were washed 5 or 6 times with physiologic saline and transferred into the phosphate buffered saline (PBS). They were stored in PBS at 37°C until a total RNA isolation process.

Vector, Bacterial Strain and Growth Media

pGEM-T easy vector system (Promega, U.K.) was used for cloning of the PCR product. The host bacterial strain used for transformation was *E. coli* JM109 for cloning. Ampicillin was used in LB agar for the selection and growth of transformants where necessary. Bacterial cells were grown on minimal agar and made competent using CaCl₂ solution (24).

Isolation of Total RNA

All steps of total RNA isolation were performed under sterilised conditions. 3 adult *F. hepatica* stored in PBS as live parasites were weighed and washed with PBS a few times. These were then cut into small pieces using a blade and put into the centrifuge tubes. 1ml of TRI reagent (Sigma Chemical Co. St. Louis, MO, ABD) was added to each 50-100 mg of the parasite weight and centrifuged at 13.500 rpm, for 10 minutes at 4°C. The upper phase was transferred into a clean tube. 0.2 ml of chloroform was added onto this sample per ml of TRI reagent used in the previous steps. It was then mixed gently for 15 seconds prior to centrifugation at 13.500 rpm, for 15 minutes at 4°C. The upper phase that includes RNA was transferred into a clean tube. 0.5 ml isopropanol was added per ml of TRI reagent used in the previous steps, incubated at room temperature for 10 minutes and centrifuged at 13.500 rpm for 10 minutes at 4°C. The supernatant was removed and 1 ml of 70% cold ethanol was added onto the pellet per ml of TRI reagent used in the previous steps and centrifuged immediately at 13.500 rpm, for 5 minutes at 4°C. Supernatant was removed following the centrifugation. RNA was air dried and dissolved in 100 µl of dH₂O containing 1 µl RNasin (Promega Co, Madison, WI, ABD). RNA was stored at -80°C until needed.

Obtaining cDNA by Reverse Transcription

Two specific oligonucleotide primers complementary to the forward and reverse strands of the *F. hepatica* cathepsin L2 gene were designed using the gene sequence of *F. hepatica* cathepsin L2 (Genbank Accession No: U62289) for the amplification of the gene by RT-PCR. These oligonucleotide primers were called P1 (GGCTCGAGATGCGGTGCTTCGTA) and P2 (GGGTCGACTCACGGAAATCGTGCC) and synthesized by The Midland Certified Reagent Co, Texas, ABD.

P1 and P2 oligonucleotide primers were diluted to 20 pmol prior to the reverse transcription process. 2.5 µl of each primer and 5µl of RNA were transferred into an Eppendorf tube and incubated at 70°C for 5 minutes. This was then cooled to the room temperature and mixed with 1 µl M-MLVRT (Moloney-Murine Leukemia Virus Reverse Transcriptase, Promega Co Madison, WI, ABD), 5µl Reverse Transcriptase Buffer (Promega Co Madison, WI, ABD), 0.7 µl RNasin (Promega Co Madison, WI, ABD), and 4µl of dNTP mixture (10 µl of each 10 mM dNTPs and 10 µl of H₂O) (Promega Co Madison, WI, ABD). This mixture was incubated at 37°C for 1 hour to obtain the cDNA.

Amplification of the *F. hepatica* Cathepsin L2 Gene by PCR

The enzyme ExTaq DNA Polymerase from Takara was used to amplify the DNA. This polymerase often adds a single deoxyadenosine, in a template independent fashion, to the 3'-end of the amplified fragments, which allows these products to be used for T vector cloning (Promega, U. K.). The reaction mixture contained, 5 µl of buffer containing MgCl₂ (supplied with the enzyme), 4 µl of dNTPs (stock at 2.5 mM), 2 µl of each oligonucleotide primers (stock 20 pmol), 5µl of cDNA from *F. hepatica*, 1 µl of Ex Taq DNA Polymerase (stock 5U/µl) and dH₂O to a final volume of 50 µl. DNA was first incubated at 95°C for 2 minutes and then denatured at 94°C for 1 minute, annealed at 44°C for 2 minutes and extended at 72°C for 2 minutes for 35 cycles. This was followed by a 10 minutes final extension at 72°C.

Analysis of PCR product on a 1% agarose gel revealed the presence of a band at about the right size. This band was extracted from the gel using Promega's Wizard SV Gel and PCR Clean-Up System (25).

Ligation and Transformation

Both ligation and transformation were performed according to the suppliers instructions (Promega, U. K.).

DNA Sequencing

Inserts were initially checked by colony PCR using the P1 and P2 oligonucleotide primers and obtained expected size PCR bands, indicating that there might be positives. Plasmid DNA was then prepared using Wizard Plus SV Minipreps DNA Purification System (Promega U.K.) and submitted for sequencing from both directions.

Phylogenetic Analysis

Sequence data were analysed using Genetix-Mac, and EMBL/GenBank database searches were performed with the FASTA program. The alignment of sequences was carried out using the CLUSTAL W program (26) available over the World Wide Web (<http://www.ddbj.nig.ac.jp/E-mail/homology.html>).

Results

Image of the PCR-amplified *F. hepatica* cathepsin L2 gene in 1% ethidium bromide-stained gel is given in Figure 1. After cloning of this 981 bp product into the pGEM-T vector, the correct sequence was confirmed by sequencing.

Figure 2 shows the cDNA sequence of cathepsin L2 acquired by sequence analysis of the recombinant vector pGEM-T (GenBank Accession No: EF611824). Once the sequence was confirmed by sequencing, the gene was then called Firat *F. hepatica* cathepsin L2.

Figure 3 presents a comparative amino acid sequence analysis between *Fasciola hepatica* secreted cathepsin L2 that cloned in the present study and some other members of the family Fasciolidae, and figure 4 shows a family tree of Cathepsin L2.

Discussion

Nucleotide and amino acid sequence of cathepsin L2 gene was detected in our study at cDNA and recombinant vector levels. The nucleotide sequence of cathepsin L2 detected in our study is the second *F. hepatica* cathepsin L2 nucleotide sequence reported in the literature, and shows 99% homology with the *F. hepatica* cathepsin L2 reported by Dowd and collaborates (27) (GenBank Accession No: U62289). The open reading frame of *F. hepatica* cathepsin L2 gene cloned in this study (Firat *F. hepatica* cathepsin L2) consists of 981 base pairs corresponding to 327 amino acids. Amino acid difference was observed at 5 positions compared to the Dublin *F. hepatica* cathepsin L2 (Figure 5).

Firat *F. hepatica* cathepsin L2 was also detected to have 98% homology with the *F. hepatica* cathepsin L-like protease (GenBank Accession No: Z22764) reported by Heussler and Dobbelaere (28) and 85% homology with the *F. hepatica* cathepsin L1 (GenBank Accession No: AY573569) isolated in Elazig region (29).

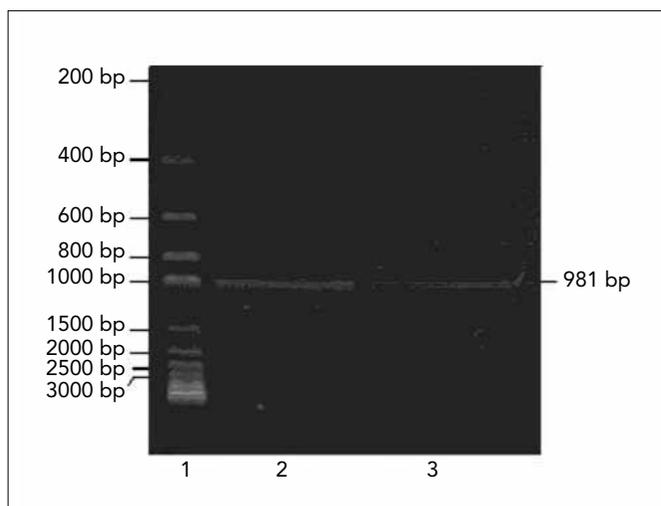


Figure 1. Image of the PCR-amplified *F. hepatica* cathepsin L2 gene in 1% ethidium bromide-stained gel. Line 1: Hyper-Ladder I Marker; Line 2 and 3: Cathepsin L2

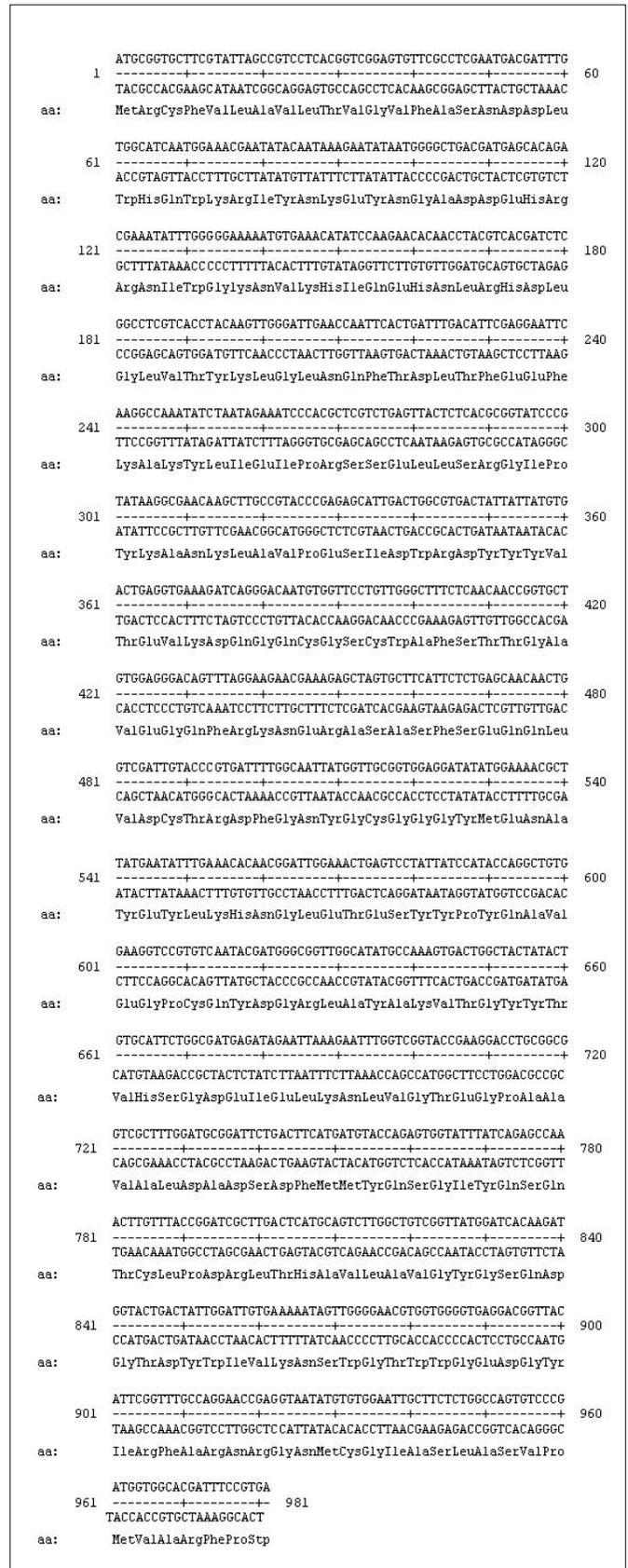


Figure 2. The nucleotide and amino acid sequence of *Fasciola hepatica* secreted cathepsin L2 (FheCL2). Gene Bank Accession No: EF611824

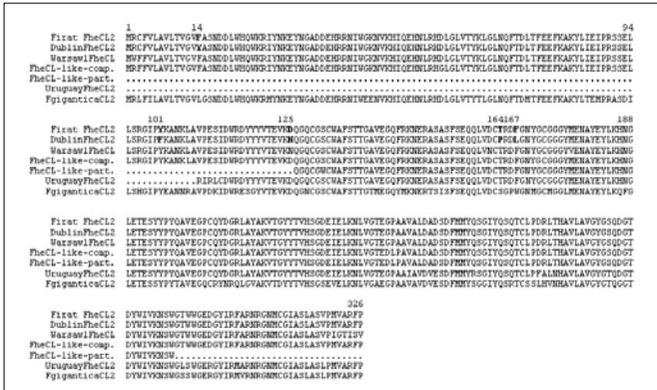


Figure 3. The comparative amino acid sequences of *Fasciola hepatica* secreted cathepsin L2 and other members of the family Fasciolidae. Firat FheCL2: *Fasciola hepatica* secreted cathepsin L2 (Strain Firat) (GenBank Accession No: EF611824), Dublin FheCL2: *Fasciola hepatica* secreted cathepsin L2 (Strain Dublin) (GenBank Accession No: U62289), Warsaw1FheCL: *Fasciola hepatica* isolate Warsaw 1 cathepsin L mRNA (GenBank Accession No: EF407948), FheCL-like-comp.: *Fasciola hepatica* cathepsin L-like protease mRNA complete cds. (GenBank Accession No: Z22765), FheCL-like-part.: *Fasciola hepatica* cathepsin L-like protease mRNA partial cds. (GenBank Accession No: Z22764), UruguayFheCL2: *Fasciola hepatica* cathepsin L2 mRNA partial cds. (GenBank Accession No: DQ533985), FgiganticaCL2: *Fasciola gigantica* cathepsin L2 (GenBank Accession No: AF510856). The amino acid differences between Firat FheCL2 and Dublin FheCL2 are shown in bold

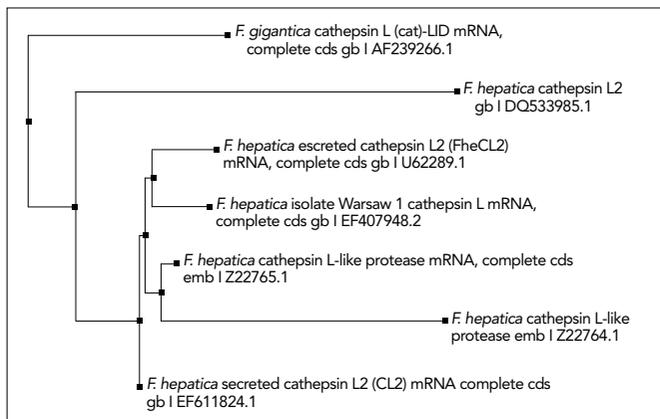


Figure 4. Family tree of cathepsin L2. Firat FheCL2: *Fasciola hepatica* secreted cathepsin L2 (Strain Firat) (GenBank Accession No: EF611824), DublinFheCL2: *Fasciola hepatica* secreted cathepsin L2 (Strain Dublin) (GenBank Accession No: U62289), Warsaw1FheCL: *Fasciola hepatica* isolate Warsaw 1 cathepsin L mRNA (GenBank Accession No: EF407948), FheCL-like-comp.: *Fasciola hepatica* cathepsin L-like protease mRNA complete cds. (GenBank Accession No: Z22765), FheCL-like-part.: *Fasciola hepatica* cathepsin L-like protease mRNA partial cds. (GenBank Accession No: Z22764), Uruguay FheCL2: *Fasciola hepatica* cathepsin L2 mRNA partial cds. (GenBank Accession No: DQ533985), FgiganticaCL2: *Fasciola gigantica* cathepsin L2 (GenBank Accession No: AF510856)

Parasite populations showed frequent genetic variations. It is documented that there are regional differences in CatL1 of *F. hepatica* (22). However, there is no adequate information for cathepsin L2. The effective control of parasitic diseases requires the understanding of the parasite's life cycle, transmission, epidemiology as well as genetic variation in *Fasciola* spp. populations. Therefore, the accurate analysis of genetic variation in parasites has important implications (20).

It has been reported that studies with cathepsin L1 in fasciolosis diagnosis revealed no cross reactions, and that the specificity and sensitivity was high in the ELISA method where it is used as an antigen (8). However, in the literature, there are no adequate studies on the place of cathepsin L2 in the diagnosis of fasciolosis. We believe it can be suggested that the recognition of regional differences in cathepsin L2 of the liver fluke populations is important in selection as an antigen for use in diagnostic methods.

Another significant issue related to fasciolosis is the loss of yield, particularly in stock animals, and the economic losses due to animal deaths. It is emphasized in the studies on the prevalence of fasciolosis and the economic loss rates that it may lead to high economic losses in the countries where it is common, and also in our country; and that an urgent and efficient protection strategy should be developed (30). However, there are no adequate numbers of immunization studies yet. There is no commercial vaccine available against fasciolosis. However, studies on developing such a vaccine are pending. Various molecules such as cathepsin L1, cathepsin L2, leucine amino-peptidase (LAP), glutathione S-Transferase (GST), hemoglobin, fatty acid-binding protein (FABP), and paromyosin are tested as vaccines against *F. hepatica* (15-19).

Testing of cathepsin L1, as an E/S product cysteine protease of *Fasciola hepatica*, as a vaccine on its own and together with cathepsin L2 gave promising results (31). A protection rate of 33-69% was reported in various studies. Furthermore, egg shedding was also reported to be reduced by 40-71% (15, 16). These results, displaying a scene far from complete protection, led to new searches, and brought the DNA vaccines into consideration (17, 19). In a study where 50 µg single vaccine with cDNA coding the cathepsin L1 gene of *F. hepatica* was used, the protectivity of the vaccine was reported to be 74% in female rats, and 100% in male rats (19). However, there is no study on cathepsin L2 cDNA vaccine yet. Further studies are needed on DNA vaccines, which is a quite new area of research. Using cathepsin L2 cDNA obtained in the present study could increase present knowledge in the design of new DNA vaccines against *F. hepatica*. However, it is shown that there are regional differences in the CatL1 of *F. hepatica* (22). Importantly, these differences in amino acid residues appear to affect immunogenicity of the protein involved. Cornelissen and collaborators (23) proved with synthetic amino acid antigens that a difference in only three amino acids results in major differences in the immunodiagnostic potential. Therefore, consideration of the regional differences in cathepsin L2 of the liver fluke populations in selection of the optimal immunogenic protein or the DNA fragment for study of vaccination may be necessary.

As a result, the cDNA coding the *F. hepatica* cathepsin L2 gene was cloned and transformed to the competent JM109

E. coli cell in this study. The nucleotide sequence and amino acid pattern of the *F. hepatica* cathepsin L2 was determined and presented as the second such sequence in the database. The importance of the five amino acids difference in Firat *F. hepatica* cathepsin L2 compared to the Dublin *F. hepatica* cathepsin L2 sequence could be exploited for immunization and diagnostic studies. These studies will be possible with the vector containing the cloned cDNA, or the recombinant cathepsin L2 protein to be acquired in the further stages of the study.

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Conflict of Interest

No conflict of interest was declared by the authors.

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