MOLECULAR CHARACTERIZATION OF *FASCIOLA* SPP. ISOLATED FROM THE GALLBLADDER OF INFECTED CATTLE IN DUHOK PROVINCE, KURDISTAN REGION/ IRAQ

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Abstract:

***Fascioliasis* caused by the liver flukes of the genus *Fasciola* is considered as the most significant Trematodes infection of ruminants in both temperate and tropical countries. In the present study fifty adult *Fasciola* flukes (Platyhelminthes: Trematoda: Digenia) were collected from the bile ducts and gallbladder of infected bovine hosts (cattle) slaughtered at Duhok abattoir, Kurdistan region, Iraq. Genomic DNA extraction was performed using a Genomic DNA Extraction kit. ITS-1 and ITS-2 ribosomal DNA sequences have been used to characterize these liver flukes as a specific marker. The PCR products were separated by electrophoresis in 1.5% agarose gel, visualized by staining with ethidium bromide, and photographed. ITS-2 marker for *F. hepatica* was amplified successfully and the length of produced band for ITS-2 was 330 bp. The present study is the first trail for molecular characterization of *F. hepatica* in cattle in Duhok, Kurdistan Region, Iraq using the ITS-2 rDNA as a reliable genetic marker.**

Key word: *Fasciola hepatica; Cattle; ITS-2 ribosomal DNA; Duhok.*

Introduction

Fascioliasis is an important zoonotic parasitic disease caused by the liver fluke of genus *Fasciola*, mainly *F. hepatica* and *F. gigantica* (Mas-Coma *et al*., 2005 and Zhou *et al*., 2008). It represent a veterinary health problem to herbivorous animals like sheep, cattle, goats and water buffalos causing great economic loses in various countries worldwide, and leading to mortality of animals, increasing the cost of diagnosis, and treatment of condemned liver, in addition to the reduction of milk, meat production and fertility disorder (Keiser and. Utzinger, 2007; Caprino *et al*., 2007, and Chen and Mott, 2010).

The distribution of both species of *Fasciola* overlap in many areas, *F. hepatica* has a worldwide distribution occurring mainly in temperate zones like Europe and Middle East, while *F. gigantica* occur mainly in Africa and Asia, and both species are present in tropical and subtropical areas of Africa and Asia (Mas-Coma *et al*., 2009)

According to the records of WHO (1995) at least 830,000 people were infected with *F. hepatica* or *F. gigantica* in Nile delta of Egypt. The disease has been recognized as food-born zoonotic disease affecting about 2.4- 17 million people worldwide (Mas-Coma, 2004)

Traditionally the two species of *Fasciola* can be differentiated depending on morphological characters, which are time consuming and unreliable method, such as the ratio of body length to width, the presence of cephalic cone, the size of both suckers (Brown and Nova, 1997 and Rokni *et al*., 2010).

The improvement in molecular assay technology for identifying *Fasciola* species, such as randomly amplified polymorphic DNA analysis (RAPD), has overcome these limitations (Vaughan *et al.*, 1997). However, the nuclear ribosomal DNA is practically useful for molecular studies because it is highly repeated and contains variable regions flanked by more conserved region (Ramadan *et al.*, 2010). The majority of studies on *Fasciola* species in Iraq were focused on the development, the prevalence, and the epidemiological and immunological aspects. These studies included the effect of different temperatures on the development of intra-Mollusca stages of *F. gigantica*, the prevalence of liver fluke among slaughtered animals in Al-Najaf abattoir, epidemiological and immunological study for *F. gigantica* among cattle in Babylon province and epidemiological study on *F. hepatica* in children and animals at Babylon City (Hillis and Dixon 1991; Al-Habbib and Al-Zako , 1981; Khalil , 2011 ; Al-Delemi , 2005 and Abdalnabi, 2012).

Therefore, proper identification of the species isolated from animals or humans is crucial (Rokni *et al.*, 2010). For more precise identification of *Fasciola* species, isolated from animals or humans several molecular methods have been used (Chaichanasak *et al*., 2012, and Ichikawa and Itagaki, 2012), among these, the

sequencing of the first and second internal transcribed spacers (ITS-1 and ITS-2) for rDNA, and mtDNA provided reliable genetic markers for species level identification (Lin Ai *et al*., 2011 and Ayoub *et al.,* 2015). ITS-2 sequence is located between the 5.8 S and 28 S coding region of rDNA having few inter-specific nucleotides and is highly conserved. Therefore, it is useful, for genetic characterization and identification of both species of *Fasciola*.

Since no such work has been performed in Duhok province, for this reason the present study is attempted to characterize and identify the species of *Fasciola* isolated from infected cattle slaughtered at Duhok abattoir using PCR technique.

Methods

Fifty adult *Fasciola* flukes were collected during March and April 2015 from the infected liver of bovine hosts (cattle) slaughtered at Duhok abattoir /Kurdistan region/Iraq. The infected livers were brought to the laboratory in boxes containing crushed ice, the flukes were isolated washed three times with buffer solution, kept in clean screwed containers with 90% ethanol and stored in a refrigerator at 4°C until used.

Genomic DNA extraction and purification were performed using a Genomic DNA Extraction kit (provided by Jena Bioscience GmbH/ Germany) based on the guidelines. The DNA quality was checked on a DNA Nanodrop (2000, Thermo, U.S.A)

The DNA was amplified through polymerase chain reaction (PCR) four pairs of primers were used each two from the known sequences of *F. hepatica* and *F. gigantica*. The sequences of primers were obtained from NCBI data base (Table 1).

Table 1: Primers pairs used for amplification of *Fasciola* ITS markers with Gene bank accession Numbers.

Primer	Sequence	Gene Bank Accession No.
FG-ITS -1	**F**: GCG ACC TGA AAA TCT ACT CTT ACA CAA GCG **R**: GAC GTA CGT ATG GTC AAA GAC CAG GTT	**EF612472**
FG- ITS -2	**F**: GCT TAT AAA CTA TCA CGA CGC CCC AC **R**: GAA GAC AGA CCA CGA AGG GTA CCG TC	**EF612484**
FH-ITS -1	**F:** CTA CTC TCA CAC AAG CGA TAC ACG **R:** GTA CGT ATG GTC AAA GAC CAG GG	**EF612469**
FH-ITS -2	**F:** GCT TAT AAA CTA TCA CGA CGC CC **R:** GAA GAC AGA CCA CGA AGG G	**EF612481**

The PCR Profile for FG ITS-1 and FH ITS-1 was as follows; one cycle of initial denaturation at 95 ˚C for 2 min; then 30 cycles of denaturation at 95˚C for 30 sec, annealing at 55˚C for 30 sec, and extension at 74˚C for 1.5 min; and followed by one cycle of final extension at 72˚C for 7 min. PCR optimization for FG ITS-2 and FH ITS-2 was done accordingly: one cycle of initial denaturation at 95 ˚C for 2 min; then 30 cycles of denaturation at 94˚C for 30 sec, annealing at 45˚C for 30 sec, and extension at 72˚Cfor 1.5 min; and followed by one cycle of final extension at 72˚C for 7 min. PCR products were analysed by agarose gel electrophoresis in 1X TBE buffer at 100 V for 120 min in gel composed of 1.5% agarose. 100bp ladder DNA Marker was run with PCR products for sizing of the bands. Gels were stained with ethidium bromide solution (concentration 0.5 µg/ml) for 30 min, then visualized with a UV transilluminater and photographed using digital camera.

Results and Discussion

The FG-ITS -1, FG- ITS -2 markers for *F. gigantica* and FH-ITS -1 marker for *F. hepatica* were not amplified, whereas, the FH- ITS-2 marker for *F. hepatica* was amplified successfully and produced a band of 330 bp (Figures 1 and 2).

Figure 1: Agarose gel (1.5%) of PCR products obtained with species-specific primers.

Lane (M) indicates 100-bp DNA marker.
Lane (1): Primer FG-ITS -1
Lane (2): Primer FG- ITS -2

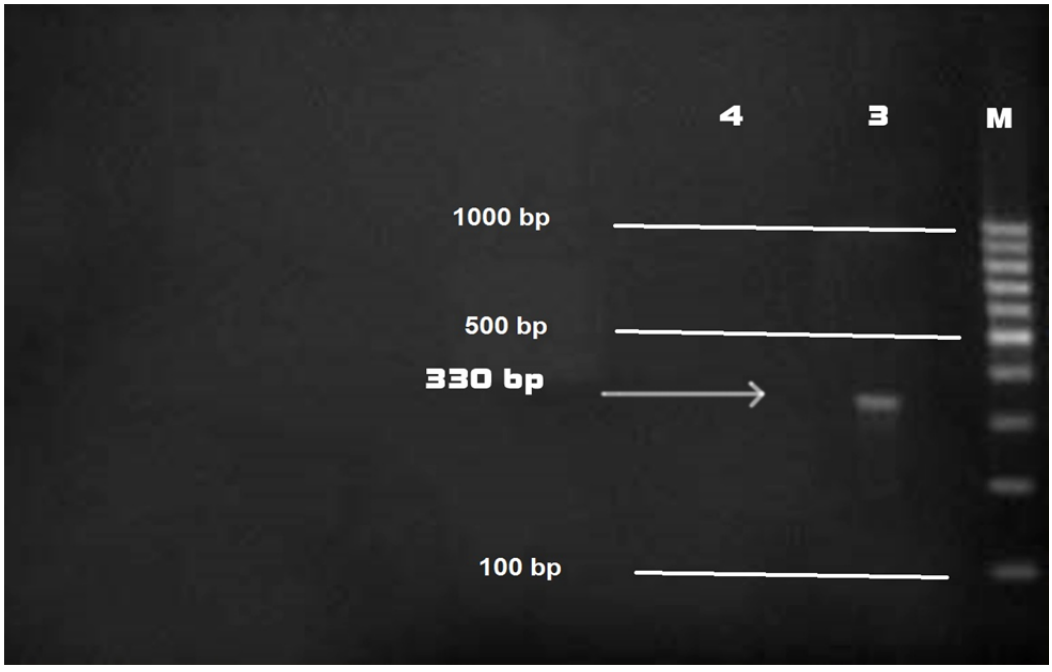

Figure 2: Agarose gel (1.5%) of PCR products obtained with species-specific primers.
Lane (M) indicates 100-bp DNA marker.
Lane (3): amplified product of FH ITS-2 (330 bp).
Lane (4): FH-ITS -1

The 330 bp band confirms that the studied species is *F. hepatica.* A study of this kind was conducted for the first time in Kurdistan with the major purpose of identifying the existing *Fasciola* species in Duhok province on the basis of Molecular techniques. This was performed by using sequences of ITS-1 and ITS-2 of DNA for *Fasciola spp.* The presence of ITS-1 indicate that the studied *Fasciola species* is *F. hapitica* .The sequences of ITS-1 and ITS-2 DNA are present between the 18S, 5.8S, and 28S coding regions. These markers have been successfully used for diagnosis (Kostadinova *et al.*, 2013). The ITS-2 sequences have been used more frequently for molecular identification of flukes as compared to any other marker (Huang *et al*. 2004). It has highly repeatable and conserved sequences and is therefore, particularly useful in molecular studies (Prasad *et al*., 2008).

Recent studies on species identification have been extensively conducted in different parts of the world using ITS-1 and ITS-2 sequences of flukes from Japan, Korea, Spain, India and Turkey were characterized to differentiate between *F. hepatica* and *F. gigantica* (Hashimoto *et al.*, 1997; Itagaki and Tsutsumi, 1998; Agatsuma *et al.*, 2000; Semyenova *et al.*, 2005; Alasaad *et al.*, 2007; Prasad *et al.*, 2009 and Erensoy *et al.*, 2009). It is not possible to differentiate between the two species on the basis of clinical, pathological, or immunological findings and morphologically their eggs are very similar (Lotfy and Hillyer, 2003). The specific differentiation of species can only be made by either a morphological study of adult flukes or by molecular tools (Ashrafi *et al.*, 2006 and Periago, 2008). Intermediate characters can create misunderstanding, especially in areas, where both species prevail and can interbreed giving hybrids (Lotfy and Hillyer, 2003). Therefore, molecular techniques based on genomics are very valuable for species identification, epidemiological and diagnostic tools as well as for research on genetic variation of the parasitic organism (Mas-Coma *et al*., 2005).

Previous molecular systematic studies of Platyhelminthes showed that the sequences of the internal transcribed spacers (ITS-1 and ITS-2) of ribosomal DNA provide reliable genetic markers for characterization of species (Mas-Coma *et al.*, 2009).

Conclusion

The result of this study revealed that all *Fasciola* samples examined represent a single species, which was *F. hepatica*. IST-2 sequence provided a reliable genetic marker for the differentiation of *F. hepatica* from *F. gigantica*.

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دیارکرنا کرمێن میلاکێ ب کارئینانا ئیك ژڤان ریگین بایولوجی (PCR) ل باریزگەها دهوکێ / کوردستان عیراق

پوختە:

وەکی یا دیار توشبوون ب کرمێن میلاکی Fasciola ئیك ژ ترسناکترین نەخوشێن مشەخور اوێت توشی کیانەوەرا دبن و هەروەسا دبیت ئەگەرێ ژ ناڤجوونا گیانەوەرین گیاخور ل جهێن فێنك و استوائی. و ل ڤێ ڤەکولینێ دا دی رابین ب کومکرنا 50 سامپل ژ کرمێن میلاکێ Fasciola flukes ژ زەراڤێ و میلاکا چێلێن تووشبووی و ئەڤیت هاتین سەرژێ کرن ل گوشکەها دهوکێ. پاشی ژ هاتە درئیخستن مادە (DNA) ب کارئینانا ریکین هەرسکرنا ئەنزیما. و دی رابین ب کارئینان چار جووتین (باندا) کەل تشتیت گەل دا (ITS-1 and ITS-2 rDNA) و رابوون بکرنا هندك کارلێکێن (البلمرە) دیڤ ئیك دا PCR وپاشی مەبرن سامپلێت خو سەر ئەگەروزی. دیاربوون ئەنجامێ مە کو باندێت ITS-2 تایبەتێ ژ جورێ *Fasciola hepatica* ئەنجام درکەت ب قەبارەێ 330 چووتا ژ باندین نایترۆجینێ. تێتە گووتن کو ڤەکولینا نوکە ئیکمین ڤەکەلینە ژ ڤێ جوری تێت کرن ل پاریزگەها دهوکێ – کوردستان عیراقێ و ب کارئینان ئیك ژ نیشانێن رەسەنن (DNA).

التوصيف الجزئيي لانواع الديدان الكبدية المعزولة من كيس المرارة للابقار في محافظة دهوك / كوردستان العراق

الخلاصة:

تعتبر الاصابة بديدان الكبد Fasciola من اخطر الامراض الطفيلية التي تصيب الحيوانات وهي تسبب خسائر كبيرة للمجترات في المناطق الدافئة والاستوائية. في هذه الدراسة تم جمع 50 عينة من الديدان الكبدية Fasciola Flukes من كيس االمرارة للابقار المصابة والتي ذبحت في مجزرة دهوك. تم عزل مادة الدنا المجيني باستخدام طريقة هضم الانزيم ، ثم استخدم اربعة ازواج من البادئات ضمن التتابعات

(ITS-1 and ITS-2 rDNA) ونفذت تفاعلات البلمرة المتسلسل PCR ومن ثم ترحيل العينات على هلام الاكاروز. اظهرت النتائج بان البادئ ITS-2 الخاص بالنوع *Fasciola hepatica* قد انتج حزمة بحجم 330 زوجا من القواعد النتروجينية. تعد الدراسة الحالية اول دراسة من نوعها تنجز في محافظ دهوك بكوردستان العراق وباستخدام احدى المؤشرات الرصينة للدنا المجيني.