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Molecular Identification of *Fasciola gigantica* in Cattle by Using a Conventional PCR in Basrah Governorate

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Abstract

Fascioliasis represents an ignored zoonotic disease that results from liver flukes that belong to the genus *Fasciola* spp. It results in substantial financial damages in the cattle sector. In the current investigation, liver flukes were taken from cattle in two places. The Basrah abattoir and the Aljazeera region. The flukes were found in buffalo and a cow. The nuclear marker genes were used to identify the species and learn about the past of *Fasciola* spp. spread. From a total number of examined Buffalo (11) and Cow (11), the infection with *Fasciola* spp. were in Buffalo and in Cow the percentage of infection was 18.18 % (2/11), 81. 82% (9/11) respectively. The Polymerase chain reaction results showed that all examined samples by PCR were found as *Fasciolagigantica*, with an identity of 100% according to GenBank. In the current study, we isolated *Fasciola* spp. Two worms from sheep and PCR were done, and it showed that it is *F. gig*antica. This indicates that the distribution of the parasite is emerging from the host specificity, which means it might be molecularly done in detail studies in all types of cattle, not only in Basrah city but all over Iraq.

Key Words: Fascioliasis, Basrah Governorate, PCR, Cattle, Liver flukes.

Introduction

Fascioliasis is an overlooked zoonotic illness that results from liver flukes as members of the genus *Fasciola* spp. It results in enormous financial damages to the cattle businesses (1, 2).In addition, cases have been recorded in more than 50 countries, especially in Asia, Africa, and America, making it one of the most frequent cattle infections (3). It is evident that changes in agricultural techniques and climate change have contributed to an increase in the incidence of Fascioliasis during the last 20 years and the improvement of resistance to

anthelmintics(4,5). Two major species of the genus *Fasciola* are *F. gigantica*, native to tropical regions, and *F. hepatica*, endemic to temperate zones (1,2). The coexistence of the two species in the subtropics gives rise to hybrid strains, which are mostly prevalent in Asian countries (9). Lymnaeidae snails serve as intermediate carriers of *Fasciola* spp., facilitating the parasites' entry into the bile ducts and livers of mammals and ruminants (1,2).

The identification of *Fasciola* species was accomplished using PCR-restriction fragment length polymorphism (RFLP) and multiplex polymerase chain reaction (PCR).Combining these methods with DNA polymerase delta (pold) and phosphoenol pyruvate carboxykinase (pepck) allowed for the detection of nuclear DNA. Employing either approach, gene segments from several species, such as *F. hepatica, F. gigantica*, and hybrids, may be identified (6).

Using mitochondrial markers, researchers have investigated the development routes of parasites and the phylogeny of *Fasciola* spp. Flukes in a number of different countries (7-10).The genes for cytochrome C oxidase subunit 1 (cox1) and NADH dehydrogenase subunit 1 (nad1) were successfully exploited to elucidate the evolutionary history of *F. gigantica*, which was originally found in Nigeria and Egypt (9,11).

Extensive research has been conducted on fascioliasis in Basrah, Iraq, and Iraq in general, including a study by (12), which determined that the overall prevalence of fascioliasis infection in buffalo males was 6.3% and buffalo females was 19.1%. This study sought to understand the expansion history of *Fasciola* spp. by identifying liver flukes gathered from cattle in two distinct areas (the Basrah abattoir and the Aljazeera region) using nuclear marker genes. The two species of *Fasciola* share morphological similarities and can be found in different hosts.

Because of the morphological similarities between both species of *Fasciola* and their presence in different hosts, the current work aimed to study the liver flukes gathered from Buffalo and cattle in two distinct regions, Basrah abattoir and Aljazeera region to identify the employing nuclear marker genes and understand the expansion history of *Fasciola* spp.

Materials And Methods

Collection of Samples and Extraction of DNA: There are 11 worms of *Fasciola* spp. isolated from cattle (9 from cow and 2 from Buffalo), respectively, from October 2022 to January 2023 and from two different regions (Basrah slaughtered house and Aljazeera region). Infected livers were dissected to remove liver flukes from the biliary ducts and stored in 70% ethanol as described previously (13). A tiny fluke was used to extract DNA using the following materials:

Genomic DNA Extraction (modified specifically to enable extraction of parasite DNA)

1-A homogenizer was employed to homogenize the parasite sample.

2-Using a vortex, 200 µl of GT Buffer and 20 l of Proteinase K was combined. At 60oC, incubate for a minimum of 10 minutes. Every 3 minutes, invert the tube during incubation. Step two is lysis.

3-The sample was vortexed with 200 µl of GB Buffer for 10 seconds. To ensure clarity,the sample was incubated lysate for a minimum at 70ºC for 10 min. While incubating, turn the tube over every 3 min.

4-The sample lysate was vigorously mixed with 200 µl of 100% ethanol right away.

5-Once the mixture was transferred to the GD Column, including any insoluble precipitate, spin it at 14-16,000 x g for 2 min. After removing the previous 2 ml collection tube with the flow-through, place the GD column in a new 2 ml collection tube.

6-In the GD Column, 400 µl of W1 Buffer was poured. After 30 seconds of 14–16,000 x g centrifugation, the flow-through was discarded.

7-Replacing the GD Column in the 2 ml collection tube was required.

8-Ensure that ethanol was incorporated before adding 600 µl of wash buffer to the GD column. Discard the flow-through after 30 seconds of centrifugation at 14-16,000 x g. The GD Column inside the 2 ml collection tube was required replacement.

9-The sample was centrifuged at 14–16,000 x g for 3 min. for drying the column matrix.

10-The dry GD Column was place in a 1.5 ml microcentrifuge tube.

11-The column matrix's CENTRE was filled with 50 µl of hot elution buffer. Elution was allowed buffer to stand for a minimum of 3 min. to absorb fully.

12-Finally, the purified DNA was centrifuged at 14-16,000 x g for 30 sec.

Agarose gel electrophoresis of DNA: Electrophoresis was used to assess DNA extraction quality and observe PCR product size after PCR. The concentration of the gel varied in accordance with the product classification. The average quality of the DNA on the agarose gel was 0.7%, whereas the standard PCR products was required 1-2%.

The Agarose gel Preparation: Due to the methodology described previously (13), the agarose gel preparation was conducted. To summarize, 1 gram of agarose was dissolved in about 100 ml of a 1X TAE buffer, and then the mixture was brought to a boil in a microwave device. Following the gel's cooling to a temperature range of 45 to 50 °C, Red Safe nucleic acid coloring solution (50 µl) was introduced into the gel cast. It was after that let to rest for around 30 min. until it was totally solidified. The gel plate was positioned into the gel tank and filled with 1X TAE buffer until the whole gel surface was submerged (Table, 1).

Loading the PCR products: Each PCR result was aliquoted with about 5 µl and kept in the relevant hole on the 1% gel. Approximately Safe-Green 100bp Opti-

DNA Marker (5 µl) was added to the initial hole in the gel lines to measure the PCR product's size.

Electrophoresis: After loading the samples and Safe-Green 100bp Opti-DNA Marker, the electrophoresis equipment was set to 90 volts, constant current for 45 min. Lastly, a 320 nm UV light source was used to visualize the PCR products by placing the gel in the UVP system.

Preparing the primers of PCR detection of *Fasciola gigantica*

Following the primer synthesizer company's recommendations, lyophilized primers were dissolved in free ddH2O to a final concentration of µM/µl, forming a stock solution stored at -20 °C. A 10 M/l concentration was prepared by diluting the stock primers, which will serve as the work primer (Table, 2). **Green Master Mix by GoTaq® G2 for conventional PCR**

The GoTaq® G2 Green Master Mix is a 2X concentrated blend of reaction buffer, deoxynucleotides, and premium Taq DNA Polymerase (figure 1) It is ready to be used. It comprises all DNA amplification reagents. An inert green dye and stabilizing agent in the GoTaq® G2 Green Master Mix was allowed samples to be directly transferred to a gel for examination (Table, 3).

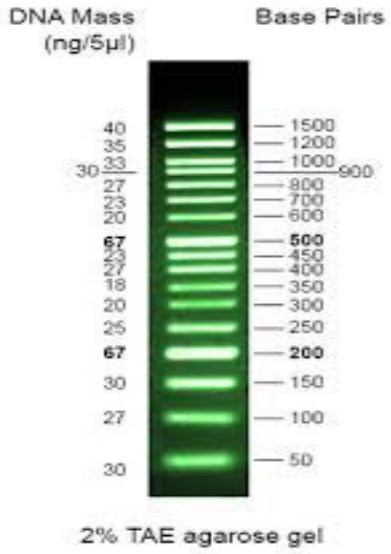

Figure (1): Self- Green 100bp Opti-DNA Marker

Table (1): The conventional PCR solutions Preparation

Components	Concentration	Volume (50 µl)
2X PCR *Taq* Master Mix	1X	25 µl
Forward primer	10 µM/µl	4 µl
Reverse primer	10 µM/µl	4 µl
ddH_2O	-	13 µl
DNA	40 ng	4 µl

Table (2): *Fasciola gigentica* Primer Design

Organism	Target gene	Primer name	5'-3'	PCR Product	Melting Temp (°C)	Reference	Accession number
Fasciola gigantica	cox1-trnT-rrnL	FGF	TGTTATGATTCATTGTTTGTAG	615 bp	63.5	Li et al., 2012	MH621335.1
		FHGR	ATAAGAACCGACCTGGCTCAC		63.5		

Table (3): Conventional PCR Conditions

Phase	Ta(°C)	Time	Cycles
Initial denaturation	94°C	5 min	1X
Denaturation	94°C	30 sec.	35X
Annealing	56°C	30 sec.	
Extension	72°C	1 min	
Final extension	72°C	5 min	1X

Results

From a total number of examined Buffalo (11) and Cow (11), the infected with *Fasciola* spp. were in Buffalo was 2 and in Cow was 9, and the percentage of infection was 18.18% and 81.81%, respectively (Figs. 2-5).

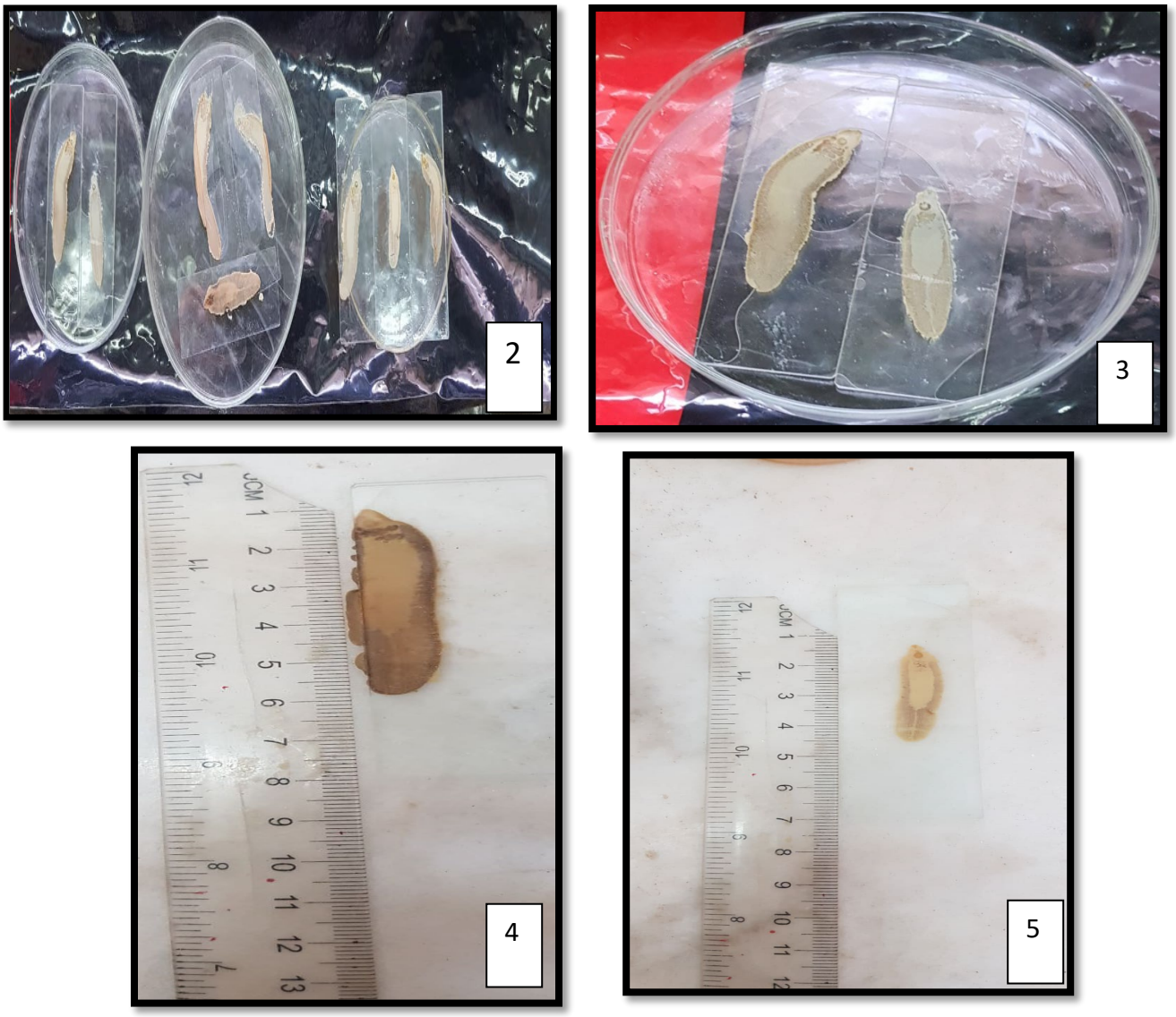

Figure (2-5): Adult worms of *Fasciola gigentica*

The PCR results showed that all examined samples by PCR were found as *Fasciola gigantic*a, with identity 100% according to GenBank. On the other hand, two parasites in slaughtered sheep, and the PCR found that this parasite was *F. gigantica* (Fig. 6).The PCR product has a 615 bp size. Red Safe (Intron, Korea) DNA dye was used on a 1.5% gel. Time: 45 minutes, V: 90. M: A ladder made of DNA

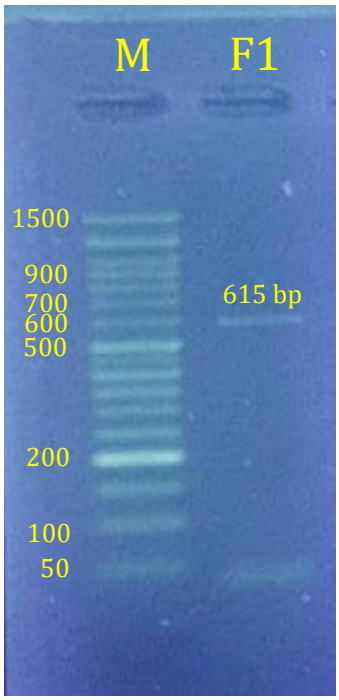

Figure (6): PCR products of the detection of *Fasciola gigantica*

Discussion

Fascioliasis, which primarily relies on the two liver flukes *Fasciola hepatica* (14) and *F. gigantica* (15) to infect humans, domestic animals, and wildlife, is a significant zoonotic infection transmitted through food and water (4, 16). It has been demonstrated that *F. hepatica* has the more cosmopolitan distribution of the two species.

By utilizing molecular methodologies in Kirkuk, Iraq, to classify *Fasciola gigantica* isolates from sheep livers of varying ages and sexes according to molecular analysis of the internal transcribed spacer 1 and sequence analysis to determine the species of parasite. It was feasible to identify the isolated worms as *Fasciola gigantica* with up to one hundred percent similarity to other worldwide isolates (17).

The molecular identification of *Fasciola* spp. is significant since it may aid in illness monitoring, diagnosis, and parasite management (18) The morphological characteristics of adult worms and eggs are used to provide a diagnosis of these two species. Based on clinical, pathological symptoms, immunological and fecal approaches, the difference between the two species of *Fasciola* spp. is invalid (19). These methods were substituted with novel molecular methodologies. Molecular techniques exhibit superior efficiency and sensitivity in comparison to conventional methods due to their examination of the worm genome .All previous studies were dependent on the morphology of the flukes. The common classification was *F. hepatica* in sheep and goats and *F. gigentica* in cows

and buffaloes. However, *F. gigentica* isolated from sheep in Kirkuk. In the current study, we isolated *Fasciola* spp. two worms from sheep and PCR were done, it observed that *F. gigantica*, and this indicates that the distribution of the parasite is emerging from the host specificity, which means it might be done details studies in a molecular way in all types of cattle, not only in Basrah city but in all over of Iraq.

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الكشف الجزيئي عن المتورقة العملاقة في الماشية باستخدام تقنية الـ PCR في محافظة البصرة

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الخـــلاصـــة

داء المتورقات هو مرض تسببه المثقوبة الكبدية المرتبطة بالجنس المتورقة او الفاشيولا, وهو مرض حيواني المنشأ مهمل يؤدي إلى خسائر اقتصادية فادحة في الثروة الحيوانية.في هذه الدراسة ، تم فحص مثقوبات الكبد التي تم جمعها من الماشية في موقعين مختلفين ، مجزرة البصرة ومنطقة الجزيرة وفي حيوانين مختلفين (الجاموس والبقر) للتعرف على الأنواع الفاشيولا المعزولة باستخدام تفاعل البلمرة المتسلسل لفهم مدى انتشار الـ *Fasciola* spp.من إجمالي عدد الجاموس الذي تم فحصه (11) والابقار (11) شخصت الاصابة بـ *Fasciola* sppفي الجاموس (2) والابقار (9) وكانت نسبة الإصابة و 18,18٪ و 81,81٪ على التوالي.أظهرت نتائج الفحص الجزيئي بطريقة الـ PCRتأكيد الاصابة بالنوع *Fasciola gigentica* ، مع تطابق جيني بنسبة 100 ٪ وفقًا لـ GenBank ، وفي دراستنا الحالية قمنا بعزل *Fasciola* spp من الأغنام ونتيجة الـ PCRلها*F. gigentica* وهذا يشير إلى أن انتشار الطفيلي بدأ يخرج من نطاق خصوصية المضيف ، مما يعني أنه يجب إجراء دراسات تفصيلية بالطريقة الجزيئية في جميع أنواع الماشية ليس فقط في مدينة البصرة ولكن في جميع انحاء العراق.

الكلمات المفتاحية: الفاشيولا, مدينة البصرة, تفاعل البلمرة المتسلسل, الماشية, حلزون الكبد.