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Molecular characterization of *Fasciola* flukes using mitochondrial 28S rRNA gene in Naimi Saudi sheep

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ABSTRACT

Fasciolosis is a parasitic disease of medical and economic importance. This retrospective study was conducted on 110 *Fasciola* flukes collected from livers of 14 infected Naimi sheep slaughtered at Riyadh abattoir in Saudi Arabia during winter season of 2016. Collected specimens were analyzed for their species identification on the basis of partial sequences of mitochondrial 28S rRNA gene. Results have shown the presence of both *Fasciola hepatica* (*F. hepatica*) and *Fasciola gigantica* (*F. gigantica*) species. Where *Fasciola hepatica* was predominate (80%). Both intra-species and interspecies genetic distance was studied and results showed that the intraspecific variability among individuals of both species i.e., *F. hepatica* and *F. gigantica*, ranging between 0 and 1% while the interspecific diversity between *F. hepatica* and *F. gigantica* was only 1%. In conclusion, mitochondrial 28S rRNA gene is a proved as a good marker in identifying *Fasciola* of different species. Where, the *F. hepatica* and *F. gigantica* are present in sheep breed in Riyadh region, Saudi Arabia.

1. Introduction

Fascioliosis, caused by *Fasciola* spp., is one of the common parasitic diseases between humans and animals that cause serious health problems and great economic losses worldwide (Mehlhorn, 2008). Genus *Fasciola* (Platyhelmintha: Digenea) are considered of the common liver flukes of a wide range of animals (Mas-Coma et al., 2005). Moreover, *Fasciola* spp. has played an important role in weight loss and decrease of meat production in livestock animals as well as fertility reduction of animals that produced severe negative economic impact in endemic regions (Mas-Coma et al., 1999).

In the last decades, the prevalence of animal fasciolosis has been carried out in different parts of KSA (Khanjari et al., 2014). *Fasciola* infection to sheep in some parts of KSA is recorded as a very serious problem, where the consumer shifts from local to imported sheep in Riyadh markets (Degheidy and Al-Malki, 1998).

The species of *Fasciola hepatica* and *Fasciola gigantica*, present different geographical distributions, which overlap in many regions, the former species is reported mainly in Europe, Americas, and Oceania, while the latter species is found in Africa and Asia, and in which the differentiation of both species is usually difficult because of the many similarities in their morphological characteristics (Marcilla et al., 2002).

In humans, the differential diagnosis between *F. hepatica* and *F. gigantica* infection is very important because of their different transmission and epidemiological characteristics (Marcilla et al., 2002). As is known, the classification of the *Fasciola* species can't be achieved by clinical, pathological or immunological methods. Also in human infections, clinical, parasitological, and serological findings do not distinguish between these species (Esteban et al., 1998).

As regards to the health and economic importance of Fascioliosis in KSA, various studies were developed to identify *Fasciola* types. Of which, morphological characteristics of adult worms and eggs is used but this method of identification is affected by different factors including parasite age and good sample fixation (Mas-Coma et al., 2005). Due to the presence of many similarities in morphological characteristics, differentiation between *Fasciola* species using classical morphological taxonomy become difficult (Mas-Coma and Bargues, 1997; Itagaki and Tsutsumi, 1998). *F. hepatica* and *F. gigantica* are reproducing sexually through fertilization by mating with other individuals or by self-fertilization (Massoud et al., 2012).

As a result of difficulties in differentiating between two species of *Fasciola* morphologically, molecular methods using a

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polymerase chain reaction (PCR) based on the DNA sequences of several nuclear genes and gene spacers (Shoriki et al., 2016; Yuan et al., 2016). Also, mitochondrial genes such as mitochondrial 28S rRNA gene have been previously developed for precise identification, genotyping, intera-specific and inter-specific variations, and phylogenetic studies of these parasites (Conceição et al., 2004; Huang et al., 2004; Kleiman et al., 2007; Prasad et al., 2008; Nguyen et al., 2009; Chamuah et al., 2016).

In this study, partial sequences of mitochondrial 28S rRNA gene was used as a marker for molecular barcoding and retyping of *Fasciola* collected from Saudi Naimi sheep to determine the diversity prevalence of *Fasciola* species among sheep in Riyadh region of Saudi Arabia.

2. Materials and methods

2.1. Sample collection

Every slaughtered sheep was carefully examined and the rate of liver helminthic infection was recorded on prepared sheets. The livers of total 326 slaughtered sheep were examined where 110 *fasciola* specimens were collected, washed in physiological saline, inspected to recognize morphometric criteria according to standard taxonomic keys described to identify to the species level (Yamaguti, 1958; Sahba et al., 1972). *Fasciola* specimens were fixed in 80% ethanol and stored at −80 °C until used for molecular identification.

2.2. DNA extraction and polymerase chain reaction amplification

After removing the samples from −80 °C, small part from each ethanol-preserved *Fasciola* specimens was cut, washed in distilled water before DNA isolation, allowed to dry and crushed in sterile 1.5 ml micro-centrifuge tubes. Whole genomic DNA was extracted from individual specimens using QIAGEN DNeasy® Blood and Tissue Kit (Catalogue # 69504), according to the manufacturer's instructions.

The polymerase chain reaction (PCR) was used to amplify the barcoding region of the partial mitochondrial 28S rRNA gene from each specimen using the primer (AJ439738.1) Forward sequence (5′-ACGTGATTACCCGCTGAACT-3′) and (AJ439738.1) Reverse sequence (5′-CTGAGAAAGTGCACTGACAAG-3′) (Marcilla et al., 2002).

Polymerase chain reaction was performed in 20 μl reaction volume, comprising of 4.0 μl of Solis BioDyne 5x FIREPol® Master Mix (Reagents: FIREPol® DNA polymerase, 5x Reaction Buffer B (0.4 M Tris–HCl, 0.1 M (NH4)2SO4, 0.1% w/v Tween-20), 12.5 mM MgCl2 (1x PCR solution – 2.5 mM MgCl2) and 2 mM dNTPs of each (1x PCR solution – 200 μM dATP, 200 μM dCTP, 200 μM dGTP and 200 μM dTTP)), 0.6 μl of each primer, 2.0 μl of DNA template and finally 12.8 μl of nuclease free water. Polymerase chain reactions were carried out in an Applied Biosystems® Veriti® Thermal Cycler and the thermal cycling conditions involved were: an initial denaturation of 15 min at 95 °C, followed by 35 cycles of 45 s at 95 °C as denaturation step, an annealing step for 45 s at 51 °C, and an extension for 45 s at 72 °C. This was followed by a final extension of 10 min at 72 °C. Agarose gel electrophoresis was performed to confirm amplification and 1.5% agarose gel was used to separate the PCR products.

2.3. DNA sequencing and bioinformatics analysis

The PCR products were sequenced using the same primers as were used in the PCR with the sequencing kit Big Dye terminator V3.1 (Applera, Foster City, CA, USA). Results were analyzed using an ABI 3700 DNA Analyzer (Applied Biosystem, Foster City, CA, USA).

Sequences were trimmed to approximately 565 bp and were then aligned with their respective reference species present in Gene Bank using BioEdit Sequence Alignment Editor version 7.2.5 (Hall, 1999), and with ClustalW Multiple Alignment program with the maximum number of 1000 iterations (Thompson et al., 1994).

2.4. Biostatistical analysis

Phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (−807.0585) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 6 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 523 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

3. Results

One hundred ten trematodes of *Fasciola* spp. were collected during abattoir inspection from livers of 14 naturally infected Naimi sheep imported from China and slaughtered at an abattoir in Riyadh, Saudi Arabia out of a total of 326 investigated sheep.

All collected specimens were identified based on partial nucleotide sequences of mitochondrial 28S rRNA gene. A 600 bp fragment of mitochondrial 28S rRNA gene (Fig. 1) from each collected

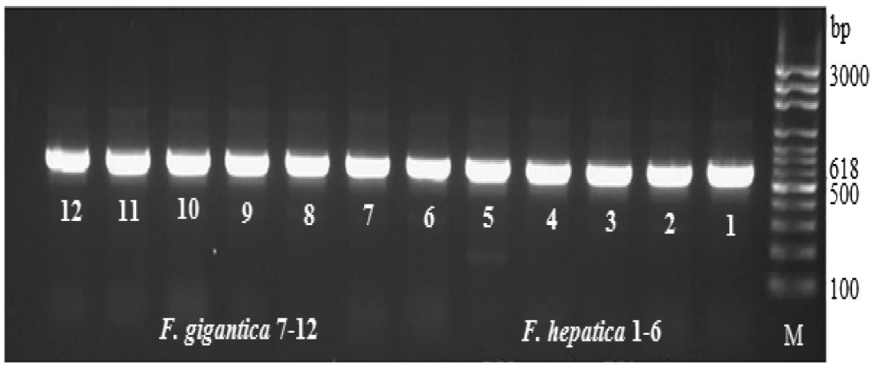

Fig. 1. PCR products after amplification of partial mitochondrial 28S rRNA gene. Lane M: DNA marker (100 bp), Lanes: 1–12 represent different samples of Fasciola species.

Fig. 2. Alignment of the partial sequence of mitochondrial 28S rRNA gene of *Fasciola gigantica* specimens (1–6) with the reference, *Fasciola gigantica* isolates (AB674551.1). Dots represent sequence similarities.

specimen was sequenced. The obtained sequences were identified using online BLAST tool. According to blasting results two species were identified as *Fasciola hepatica* and *Fasciola gigantica*. Results of the present study showed that from a total of 326 Naimi sheep slaughtered at an abattoir northern Riyadh governorate one hundred ten livers out of total sheep were infected, of which 21 (19%) were identified as *Fasciola gigantica* and 88 (80%) were identified as *Fasciola hepatica*, while one sample (1%) didn't give any result.

Ninety-nine percent similarities were recorded in the present investigation in comparison with the available data of reference *Fasciola* spp. registered in GenBank database (Figs. 2 and 3) as six individuals of each species were picked up randomly and demonstrated in included figures. Where the sequence under accession number HM369231.1 was used as a reference for *F. hepatica* species, while the sequence under the accession number AB674551.1 was used as a reference for *F. gigantica* species collected and identified in this study.

Intra-species and interspecies genetic distances were analyzed using BioEdit Sequence Alignment version 7.2.5. Where sequences were aligned and selected sequences are demonstrated in Tables 1

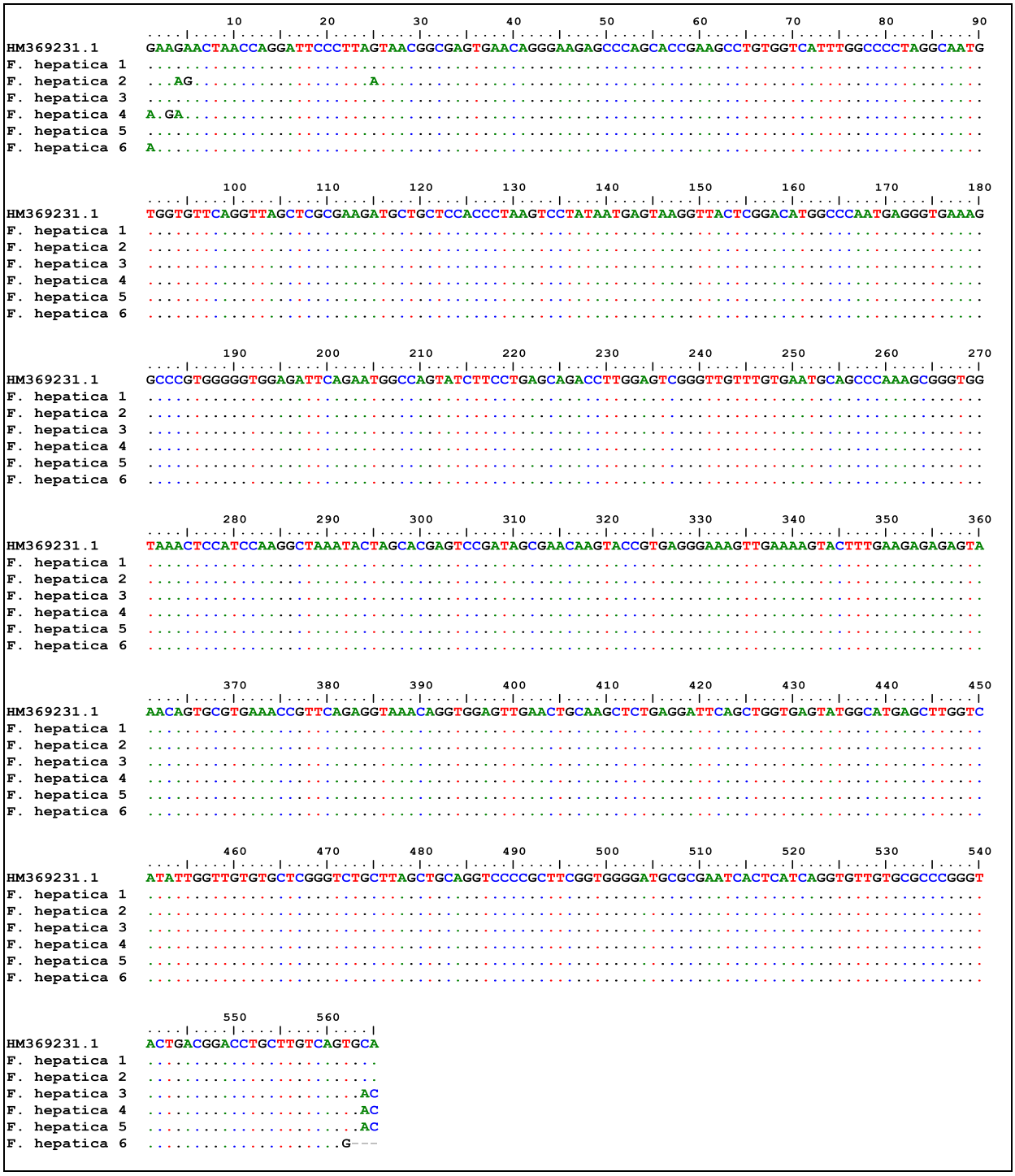

Fig. 3. Alignment of the partial sequence of mitochondrial 28S rRNA gene of *Fasciola hepatica* specimens (1–6) with *Fasciola hepatica* isolate 3.3 (HM369231.1). Dots represent sequence similarities.

Table 1
Percentage of difference of specimens with reference species: *Fasciola gigantica* gene for 28S rRNA, partial sequence, isolate (AB674551.1).

Sp	3	6	7	9	10	13	15	21	22	23	24	26	30	37	76	77	84	95	98	102	103
Ref	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%

and 2. The least genetic distance was recorded as intraspecific variability among individuals of both species i.e., *F. hepatica* and *F. gigantica*, ranging between 0 and 1% while the interspecific diversity between *F. hepatica* and *F. gigantica* was only 1%. Phylogenetic tree supported the classification done in the present study. Two clades were observed; the first clade includes specimens of

Table 2
Percentage of difference of partial sequence of 28S rRNA gene, of *Fasciola* specimens with the reference species, *Fasciola hepatica* isolate 3.3 (HM369231.1).

Specimen No.	1	2	4	5	8	11	12	14	16	17	18	19	20	25	27	28	29	31	32	33	34	35
Reference	1%	1%	1%	1%	1%	1%	0%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	0%	0%	1%
Specimen No.	36	38	39	40	41	42	43	44	45	46	47	48	49	51	52	53	54	55	56	57	58	59
Reference	0%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	0%
Specimen No.	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	78	79	80	81	82	83
Reference	1%	1%	1%	1%	1%	1%	0%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	0%	1%	1%	1%
Specimen No.	85	86	87	88	89	90	91	92	93	94	96	97	99	100	101	104	105	106	107	108	109	110
Reference	0%	1%	0%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	1%	0%

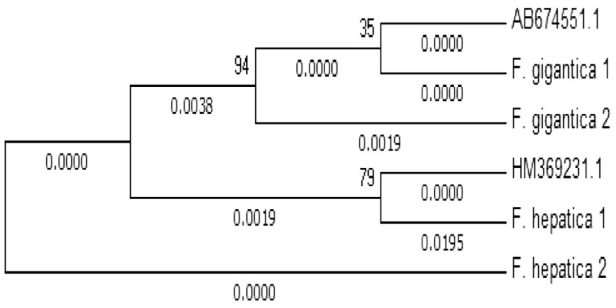

Fig. 4. Phylogenetic tree using the maximum likelihood method (ML) with the Tamura and Nei model with the bootstrap values of 1000 replicates. (AB674551.1) represent *Fasciola gigantica* reference and (HM369231.1) represents *Fasciola hepatica* reference.

F. gigantica species with their used reference. While the second clade contains individuals of *F. hepatica* with their used reference (Fig. 4) only two sequences from each species were selected randomly to construct the phylogenetic tree. Also phylogenetic tree showed degrees of intra and interspecific variability between individuals of the same species and among species of the same genera.

4. Discussion

Molecular methods in comparison with other diagnostic methods for *Fasciola* parasites have been more accuracy investigated by Marcilla et al. (2002). Moreover differentiation between species of *Fasciola* according to life cycle and species-specific intermediate host are also documented by Mas-Coma et al. (2005).

Many studies based on both nuclear and mitochondrial sequences have revealed that *Fasciola hepatica*, is the commonest and most widespread species in temperate regions while *F. gigantica* is found in tropical countries of Africa (Agatsuma et al., 2000; Ali et al., 2008). Pure and mixed forms of *Fasciola* adults in human and ruminants, particularly in Asian countries like Iran (Ashrafi et al., 2006), China (Huang et al., 2004; Lin et al., 2007; Peng et al., 2009) Korea (Itagaki et al., 2005a), Japan (Itagaki et al., 2005b; Terasaki et al., 1998) and Vietnam (Itagaki et al., 2009; Le et al., 2008) have already been investigated.

The percentage of *F. hepatica* (80%) noted among the slaughtered Naimi sheep in the present study gave an idea about the adaptation of this fluke to the local environment in Riyadh. Occurrence of this digenea, which may have been introduced into this country from Europe through imported infected animals (Mas-Coma et al., 2005; Lotfy et al., 2002), was supported by a previous report suggesting the role of *Radix natalensis* (the principal snail host of *F. gigantica*) as a potential intermediate host for *F. hepatica* (Dar et al., 2010). The other fasciolid, *F. gigantica* present in 19% in the analyzed samples of the present work, was also considered as a local species of *Fasciola* found in Egypt (Farag, 1998), it was also recorded that Fascioliosis the causative agents, *Fasciola hepatica* and *Fasciola gigantica*, present geographical distributions, which overlap in many regions of Africa and Asia, and in which the differentiation of both species is usually difficult because of the many similarities in their morphological characteristics. The differential diagnosis between *F. hepatica* and *F. gigantica* infection is very important because of their different transmission and epidemiological characteristics (Amor et al., 2011). However, Teofanova et al. (2011) have proposed existence of 2 lineages for mitochondrial 28S rRNA gene in *F. hepatica* in Eastern Europe based on the polymorphism at 105th nucleotide in mitochondrial 28S rRNA gene in F. *hepatica*. Moreover, in *F. gigantica* are proposed on the existence of variable 284th lineages nucleotide of the mitochondrial 28S rRNA gene. Similarly, ninety *Fasciola* samples from three different geographical regions (Khorasan, Fars and East Azerbaijan provinces) and two different hosts (sheep and cattle) were identified as either *F. hepatica* or *F. gigantica* by PCR-RFLP (Marcilla et al., 2002; Huang et al., 2004; Karimi, 2008; Rokni et al., 2010).

Concerning imported animals, fasciolosis has been increasingly reported in KSA, the infection rate among imported sheep was 15.1% higher than of local one (4.9%) (Youssuf et al., 2013). Degheidy and Al-Malki (1998) investigated the prevalence of fasciolosis in imported slaughtered cattle from Al-Taif, KSA with a total of (16.3%) livers were condemned due to fasciolosis and abscessation affections. Of the slaughtered cattle, a significantly ($p \leq 0.05$) higher prevalence of fasciolosis was recorded (8.6%) than abscessation (1.1). Fasciolosis with a percentage of 52.06 in cattle was considered the main cause of liver condemnation and was responsible for total liver condemnation. The economic importance of such infections in terms of lost meat and offal were also estimated with a great negative impact on meat industry annually.

In the present study both intra and inter specific variations were studied, where interspecific variations range between 0 and 1% while variations between studied species was 1%. Similar intraspecific variations were obtained by Galavani and Gholizadeh (2016) in their genetic characterization to *fasciola* spp (*F. hepatica* and *F. gigantic*) isolates from Iran. While interspecific variation reach 3%.

5. Conclusion

The results of the present study concluded that the variability between two species of Fasciola was ranged from 0 to 1% for intraspecific and 1% for interspecific diversity, and this was

confirmed by phylogenetic tree results. So, there is a need to lower the incidence of Fasciolosis in Saudi Arabia, identification of Fasciola species based on the sequences of mitochondrial 28S rRNA gene can be useful in a further trail to formulate appropriate control strategies and decrease the economic loss due to Fascioliosis among local sheep.

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