Occurrence of zoonotic fascioliasis in donkeys in Egypt with emphasis on PCR-RFLP of 28S rRNA gene

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SUMMARY

Fascioliasis is an important parasitic zoonosis worldwide and has a veterinary problem in herbivorous animals. This study aimed to investigate the occurrence and molecular identification of Fasciola spp. in donkeys in two localities in Egypt (Giza Zoo, Giza Province and Zagazig region, Sharkia Province). One hundred and thirty one fecal samples were collected from donkeys slaughtered at Giza Zoo and also from animals at Zagazig region. Feces were examined by simple sedimentation concentration technique. The overall prevalence rate of fascioliasis was 17.6% (23 out of 131). The infection rate was 32.1% (17 out of 53) in donkeys at Giza Zoo and 7.7% (6 out of 78) at Zagazig region. A postmortem examination was carried out for animals at Giza Zoo. The recovered worms were morphotyped as Fasciola gigantica. As a first molecular typing of fascioliasis of donkeys in Egypt, the 28S rRNA gene of adult worm specimens from infected ten donkeys at Giza Zoo was amplified and digested with the restriction enzyme Ava II using PCR-RFLP. The RFLP patterns of all specimens revealed two fragments of 322 and 269 bp characteristic for Fasciola gigantica. No Fasciola hepatica or intermediate forms could be detected. This study drew an attention for the control of fascioliasis in donkeys to avoid public health hazard.

Keywords: Occurrence, Fasciola gigantica, Donkeys, 28S rRNA gene, PCR-RFLP, Egypt.

RÉSUMÉ

Fasciolose chez les ânes en Égypte : typage par PCR-RFLP du gène de l’ARNr 28S

La fasciolose est une zoonose parasitaire de prévalence mondiale et un problème de santé animale chez les animaux herbivores. Cette étude vise à déterminer la présence et identifier de manière moléculaire Fasciola spp. chez les ânes dans deux localités en Égypte (Zoo de Giza, dans la province de Gizeh et la région Zagazig, province Sharkia). Ont trent et un âne de la région ZagaZig. Un examen post-mortem a été effectué sur les animaux du Zoo de Giza. Les vers ont été récupérés après concentration par sédimentation simple et amplifiés par l’enzyme de restriction Ava II et amplification par PCR-RFLP. Les profils RFLP de tous les âne de la région Zagazig. Un examen post-mortem a été effectué sur les animaux du Zoo de Giza. Les vers ont été récupérés après concentration par sédimentation simple et amplification par PCR-RFLP. Les profils RFLP de tous les âne de la région Zagazig. Un examen post-mortem a été effectué sur les animaux du Zoo de Giza. Les vers ont été récupérés après concentration par sédimentation simple et amplification par PCR-RFLP. Les profils RFLP de tous les échantillons ont révélé deux fragments de 322 et 269 pb caractéristique pour Fasciola gigantica. Aucun cas de F. hepatica ou formes intermédiaires n’a été détecté. Cette étude montre l’importance du contrôle de la fasciolose chez les ânes afin d’éviter les risques de zoonose.

Mots clefs : épidémiologie, fasciolose, Fasciola gigantica, âne, ARNr 28S, PCR-RFLP, Égypte.

Introduction

Fascioliasis is one of the most prevalent helminthic infections of ruminants, equines and humans with a global geographical distribution. It is caused by the digenetic trematodes, Fasciola gigantica and Fasciola hepatica [27]. Fasciola gigantica is found in tropical regions of Africa and Asia, while F. hepatica has a cosmopolitan distribution, mainly in temperate regions. Thus, the two fasciolid species overlap in large regions [21].

Fascioliasis in livestock has been recognized as a veterinary problem on a world wide scale. In Egypt, the disease is highly enzootic affecting cattle, sheep, donkeys, goats and buffaloes [2, 8, 10, 24, 25].

Severe negative economic impact has been estimated in ruminants due to significant morbidity, mortality, reduction in host fecundity, decrease in milk and wool production [4, 12]. In addition, human fascioliasis constitutes a significant health problem, as the estimated number of people infected with fascioliasis in Egypt is 830,000 cases [13], while population at risk is considered to be 27 million [32]. The Nile Delta considered one of the endemic areas in the world for human fascioliasis [6, 28].

Equines still receive more interest in many countries as draft animals, source of leather and other related products. Also, donkeys have a prominent position in the agricultural systems of many developing countries. The epidemiology, pathogenicity and immunology of fascioliasis in equids in general, and in donkeys in particular have not been fully determined. In Egypt, information on equine fasciolosis in donkeys is sparse [11, 12] Traditional methods of identification of Fasciola spp. have relied on morphological features of adults and eggs. The problem has even been increased by the existence of morphologically intermediate forms between the two fasciolid species and genetic hybrids of both in overlapping areas, endemic for animals [15] and humans in Africa [26]. So the molecular approach for accurate identification is more decisive [18, 23]. Thereby, this study aimed to determine occurrence of Fasciola spp.
in donkeys at two localities in Egypt (Giza Zoo and Zagazig region), and also to identify the flukes to the species level using PCR-RFLP of amplified 28S rRNA gene for the first time.

**Material and Methods**

**SAMPLING AND FECAL EXAMINATION**

One hundred and thirty one fecal samples (53 from donkeys slaughtered at Giza Zoo and 78 from live animals at Zagazig region) were collected from the rectum in labeled plastic bags during March 2012 to September, 2013; and examined for *Fasciola* spp. eggs using a simple sedimentation concentration technique [19].

**PARASITE COLLECTION**

Worms were collected from livers of slaughtered animals at the Abattoir of the Zoo, Giza province. In brief, bile ducts were searched thoroughly for presence of fasciolid worms. Detected flukes were removed and washed extensively in phosphate buffer saline (PBS). Portions of specimens were fixed in formalin 10% and stained with acetic acid alum carmine stain according to the technique of KRUSE and PRITCHARD [17], while other portions were subsequently fixed in 70% ethanol and stored at -20°C until used for molecular characterization.

**GENOMIC DNA EXTRACTION**

The genomic DNA was isolated from apical zones of liver flukes, obtained from infected ten donkeys slaughtered at Giza Zoo according to Chelex®-based DNA extraction method [5]. Mature worms were mechanically disrupted in 200 µl of Chelex® 5% under liquid nitrogen and then incubated at 56°C for one hour; and at 95°C for 30 minutes in a water bath. Centrifugation of the mixture was done at 13,000 xg for seven minutes. The DNA supernatants were collected and stored at -20°C.

**PCR AMPLIFICATION OF 28S rRNA GENE**

To amplify the 28S rRNA gene of the specimens, the oligonucleotide primers 28F1 (5’ACGTGATTACCGCTGGAATC’3) and 28R600 (5’CTGAGAAAGTGCACTGACAAG’3) were designed [20]. The nucleotide sets were synthesized in Metabion international AG, Martinsried, Deutschland. Each PCR reaction mixture (25 µl) contained 1 µl of template DNA, 12.5 µl of Dream Taq Green PCR Master Mix (2X) (ThermoScientific, Fermentas), 0.25 µl of each primer (25 pmol) and 11 µl of molecular water. The amplification of reaction mixture was performed in a thermal cycler with a minor modification of PCR conditions [20]. Briefly, an initial denaturation of samples was at 94°C for 4 min, followed by 30 cycles as a denaturation at 94°C for 1 min; then annealing at 55°C for 1 min for 30 cycles and extension for 1 min at 72°C for 30 cycles. The final extension step was at 72°C for 10 min. The PCR products (10 µl) were analyzed by electrophoresis on 1.5% agarose gel stained with ethidium bromide. A negative control was included in a PCR reaction. A Gene Ruler 100 bp DNA Marker (Fermentas) was included on the gel for base-pair comparisons.

**PCR-RFLP OF 28S rRNA GENE**

A specific restriction enzyme Ava II (Jena Bioscience GmbH, Jena, Germany) was selected to digest the amplified 28S rRNA gene of *Fasciola* spp. to identify the recovered worms to a species level [20]. Ten microliters of PCR products were completely digested with 3 µl Ava II and 2.5 µl buffer at 37°C. Analysis of digested PCR products was carried out by ethidium bromide staining. Undigested PCR product (without addition of Ava II) was used as a control. A GeneRuler 50 bp DNA Marker (Fermentas) was included on the gel. The length of restriction fragments was predicted by Gene Runner software v. 3.05 (Hastings Software Inc, 1994).

**Results**

As shown in Table I, twenty three donkeys were infected with fascioliasis with an overall prevalence rate of 17.6%. The infection rate was 7.7% (6 out of 78) at Zagazig region, while it was 32.1% (17 out of 53) at the Zoo, Giza Province. Most of infected donkeys suffered from emaciation, had pale mucous membranes and leaf-like flukes were present in grossly enlarged and thickened bile ducts, particularly in the ventral lobe of the liver.

Regarding the morphology, the recovered eggs from feces were oval, golden yellow in color, operculated and measured 105-147 µm in length (aver. 126 µm) and 72-90 µm in maximum width (aver. 81 µm). The mean egg length/width ratio was 1.6 (Figure 1, A). Morphologically, adult worms showed the characteristics of *F. gigantica*; where the worms were grayish, dorsoventrally flattened, with narrow shoulders, parallel sides and differed in size from 30-48 mm

<table>
<thead>
<tr>
<th>Localities</th>
<th>No. Examined</th>
<th>No. Infected</th>
<th>% Positive</th>
</tr>
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<tbody>
<tr>
<td>Giza ZOO</td>
<td>53</td>
<td>17</td>
<td>32.1</td>
</tr>
<tr>
<td>Zagazig region</td>
<td>78</td>
<td>6</td>
<td>7.7</td>
</tr>
<tr>
<td>Total</td>
<td>131</td>
<td>23</td>
<td>17.6</td>
</tr>
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**Table I**: Occurrence of fascioliasis in examined donkeys at two localities in Egypt.
FASCIOLIASIS IN DONKEYS AND PCR-RFLP OF 28S rRNA GENE

(aver. 39 mm) in length and 10 mm-17 mm (aver. 13.5 mm) as maximum breadth. The cone length ranged from 4-5 mm (aver. 4.5 mm), maximum cone width was 6-8 mm (aver. 7.0 mm), shoulder width was 9-12 mm (aver. 10.5 mm) and the postacetabular length was 36-44 mm (aver. 40 mm) (Figure 1, B&C).

With respect to PCR-RFLP, the amplified 28S rRNA gene of all specimens yielded PCR products of 618 bp which was identical for Fasciola spp. (Figure 2). After digestion of amplicons with the restriction enzyme Ava II, the RFLP profile revealed two fragments of 322 and 269 bp characteristic for F. gigantica (Figure 3).

Discussion

The infection with fascioliasis in domestic ruminants, equines and more recently in humans is a well-recognized problem worldwide. Studies reported by MAS-COMA et al. [22] in Bolivia and by HARIDY et al. [12] in Egypt showed that next to domestic ruminants, donkeys are the second important final reservoirs of F. hepatica and F. gigantica and play a zoonotic significance in human infection [33].

Concerning the overall prevalence of fascioliasis in examined donkeys (17.6%) as shown in Table I, nearly similar percentages of 17.05% in Giza [11] and 14.1% in Kafr El-Sheikh [14]. On the contrary, lower percentages of fascioliasis were cited in different Provinces in Egypt: 0.37% in Assuit [16]; 3.03% in Gharbia [12]; 6.7% in Al-Fayoum [25]; and 3.08% in Giza [1]. However, 44.4% of examined working donkeys in Ethiopia were proved to be positive for fascioliasis [9]. The variations in these results may be attributed to the age of the examined donkeys which coincides with the finding of GETACHEW et al. [9], who stated that the infection intensity of fascioliasis was significantly higher in donkeys...
8 years old and above. In addition, the higher prevalence rate of fascioliasis in donkeys in this study may be accounted for grazing of these donkeys in heavily contaminated areas with encysted metacercariae. The infected donkeys shed viable Fasciola eggs and have capability to infect lymnaeid snails, and the produced metacercariae are infective for other definitive hosts. This referred to the greatest role played by donkeys in the epidemiology of both livestock and human fascioliasis [30]. The higher infection rate of fascioliasis was noticed in donkeys slaughtered at Giza Zoo than that in animals at Zagazig region may be attributed to aged donkeys at the Zoo.

Dealing with morphological and morphometric identification of Fasciola spp. detected in the present study by microscopical examination, only F. gigantica was identified from infected animals (Figure 1). Adult worms showed characteristic features resembling that of the early described for this parasite [7, 29]. However, identification of the species depending on morphological and morphometrical criteria is not decisive due to overlap in the values of most measurements [18, 23]. Eggs showed similar characteristics of Fasciola species eggs, although egg measurements were lower than that described for both F. gigantica and F. hepatica recovered from cattle in Egypt [31].

Molecular genetic techniques based on the differences in nucleotides, a PCR-RFLP technique was developed mainly to differentiate between F. hepatica and F. gigantica [20]. In the present study, the amplified 28S rRNA genes of the specimens collected from donkeys at Giza Zoo were digested with the restriction enzyme Ava II. The RFLP patterns of all specimens revealed two fragments of 322 and 269 bp characteristic for F. gigantica (Figure 3). The present result was concordant with the finding of MARCILLA et al. [20], who distinguished between both fasciolid species obtained from South America, Europe and Africa based on a 618-bp-long sequence of the 28S rRNA gene. These results confirmed the morphological findings and support the early studies which found that F. gigantica is the commonest fasciolid fluke affects donkeys in Egypt [3, 14]; and contradict with finding of AHMED et al. [1], who isolated F. hepatica from 3.08% from necropsied donkeys at Moshtohor and Giza Zoo.

Conclusion

Donkeys are considered as important reservoirs for F. gigantica in Egypt. Thereby, preventive and control measures of fascioliasis in donkeys are crucial to avoid public health hazard. This study clarified that PCR-RFLP assay is a useful alternative tool to distinguish between the two fasciolid worms.

References