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DESCRIPTION OF CESTODES INFECTING DOMESTICATED PIGEON (*COLUMBA LIVIA DOMESTICA*) IN EGYPT WITH SPECIAL REFERENCE TO THE MOLECULAR CHARACTERIZATION OF *RAILLIETINA* SPP.

By

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Abstract

This study investigated the prevalence and the specific identity of cestode parasites isolated from the digestive tract of domestic pigeon (*Columba livia domestica*) in Cairo, Egypt. Out of 246 pigeons, 61 (24.79%) were infected by one or more species. Eight helminthes species were recorded; three nematodes; *Ascaridia galli, A. columbae* and *Heterakis gallinarum* and five cestodes; *Raillietina echinobothrida, R. tetragona, R*. sp., *Cotugnia digonopora* and *Choanotaenia infundibulum*. No trematodes were recorded. The morphological observations, using light microscope, were employed for precise identification of cestodes with particular attention to molecular identity of *Raillietina* spp. using two molecular techniques: RAPD-PCR and SSCP-PCR.

Keywords: Prevalence, Pigeon, Cestodes, SSCP-PCR, RAPD-PCR.

Introduction

In Egypt, poultry farming has become one of the most intensive forms of animal husbandry activities (Satish and Priti, 2013). The commonly kept poultry are chickens (*Gallus* spp.), pigeons (*Columba* spp.), ducks (*Anas* spp.), geese (*Anser* spp.) and turkeys (*Meleagris* spp.). Poultry products are considered as one of the most important protein sources (meat and eggs) for man worldwide (FAO, 1997; Branckaert *et al*, 2000). Most of the products are from chickens, but turkeys, pigeons, geese and ducks are important sources as well. Birds in rural production systems are at a constant risk of infections and diseases, especially endoparasites (Catelli *et al*, 1999; Dessie and Ogle, 2001; Eshetu *et al*, 2001).

Helminthes of poultry can reduce weight gain, feed efficiency, and production losses in breeders. It can also lead to significant growth depression and mortality in birds (Ikeme, 1971 and Ruff, 1999). In general, parasitism negatively affects productivity of the local scavenging birds since they either compete for feed or cause distress to the birds (Mungube *et al*, 2008).

Domestic pigeon (*Columba livia domestica*) is a member of order Columbiformes and considered as cosmopolitan of acclimate conditions world-wide (Sari *et al*, 2008). The endo-parasitism is one of the most important forms of disease affecting poultry (Dranzoa *et al*, 1999). The commonest cestode parasites are *Raillietina, choanotaenia, Hymenolepis* and *Davainea* species. *Raillietina* group represents over 200 species (Jadhav and Gore, 2004), with infected ones may cause high rates of morbidity and mortality.

However, the morphological criteria of *Raillietina* spp. in particular, showed a wide range of variations within and between species, difficult to identify by morphology Caira *et al*, 2014). Many molecular techni-ques (RAPD-PCR, RFLP, SSCP & LSSP) clarified knowledge of genus *Raillietina*. Some DNA regions (internal transcribed spacer (ITS) gene, Cytochrome C oxidase (CO1) and nicotinamide adenine dinucleo-tide dehydrogenase subunit 1 (ND1) gene identified helminthes between species (Al Quraishy *et al*, 2019). Molecular techniques with morphological analyses proved useful for helminthic identification (Eom *et al*, 2002; Lavikainen *et al*, 2008).

The present study aimed to explore the intestinal helminth parasite prevalence with particular attention to cestodes. Molecular characterization of *Raillietina* spp. was dete-

rmined using two molecular DNA analysis; RAPD-PCR and SSCP-PCR.

Materials and Methods

The study was performed on 246 domestic pigeons (*Columba livia domestica*) randomly collected from 2015 to 2017. Their intestines were dissected out for examination. They (small intestine and caecum) were opened longitudinally, rinsed with physiological saline (0.85% normal saline) and mucosa was scraped to collect the worms embedded in the mucosal layer. Worms were carefully washed in saline in Petri dishes, examined under a dissecting microscope and prepared for light microscopic examination and molecular investigations.

Light microscopy: Worms were gently relaxed between two slides, fixed in 10% formalin, stained with acetocarmine dye for 30min., differentiated in acidified 70% ethanol followed by ascending series of ethanol and then cleared in clove oil, examined and photographed using light photomicroscope. Parasitic species prevalence was determined as the proportion of the host population infected with a certain parasite seasonally (Thrusfield, 1995).

DNA extraction (CTAB method): Three morphologically *Raillietina* species were (*R. echinobothrida, R. tetragona* and *R.* sp.*)* recovered from pigeon. The tissue samples were homogenized in 250µl of lysis buffer (100mM Tris-HCl, 1.4M NaCl, 20mM EDTA and 10% SDS) then 250µl of CTAB buffer (200mM Tris-HCl, 1.4M NaCl, 20mM EDTA, CTAB powder and 2% B-mercaptoethanol) with 25µl of 20mg/ml proteinase K were added. The homogenates were incubated at 65°C in water bath for 2-3 hours. DNA purification was performed by chloroform-isoamyl alcohol (24:1) and precipitated by isopropanol. Pure DNA pellets were dissolved in 100µl of sterile dH_2O and kept in -20°C until used.

RAPD- PCR technique: Twenty decimer oligonucleotide primers were used to determine those primers that produced reproducible RAPD patterns. Each primer was tested three times. Good and distinct patterns were produced only using 6 arbitrary primers (Tab.1). PCR amplifications were performed (Williams *et al,* 1990), using six selected primers (Tab. 1). The 25µl mixture contained about 1µl of DNA template, 1.5 unit of *Taq.* polymerase, 1µl of 10mM dNTPs, 1µl of 10 pmol primer, and 2.5µl of 10x PCR buffer. Amplifications were done in T-personal thermal cycler (Biometra, Germany), programmed for 45 cycles at 94ºC for 1min, 35ºC for 1min and 72ºC for 1min. An initial denaturation step (3min. at 94ºC) and final extension holding (10min. 72ºC) were included in the first and last cycles, respectively. Reaction products (10µl) were resolved by 1.5% agarose gel electrophoresis in 1X TAE buffer (100mM Tris-HCl, glacial acetic acid and 20mM EDTA). The gel was stained with Ethidium bromide and photographed by digital camera under UV transilluminator.

Table 1: Nucleotide sequences of six arbitrary primers and their (G+C) contents

Primer code	Primer sequence (5`-3`)	MWT	GC%
P8	5`-GAAACACCCC-3`	2966	60
P9	5`-TGTAGCTGGG-3`	3099	60
P10	5`-ACGCGCATGT-3`	3028	60
P12	5`-ACGCGCATGT-3`	3028	60
P13	5`-CACTCTCCTC-3`	2899	60
P33	5`-GCACTAAGAC-3`	2987	50

PCR for specific primers: Specific PCR technique was carried out using two pairs of specific primers to amplify the 12S rRNA and Cytochrome C oxidase genes (Tab. 2). PCR amplifications for Cytochrome C oxidase & 12S rRNA genes respectively using the forward and reverse primers (Dinkel *et al,* 2004). A- Cytochrome C oxidase subunit 1 (*CO1*) gene: Thermocycler conditions were: 5min at 95°C (initial denaturation), 35 cycles of 1min at 95°C, annealing for 1min at 54°C & 1min at 72°C, & 5min at 72°C

(final extension). B- Mitochondrial 12S rRNA gene: PCR program conditions were as follows: 1 cycle at 95˚C for 5min before the 40 cycles of PCR (Hot start PCR) and adding 1 cycle at 72˚C for 10min after 40 cycles. Then 40 cycles (denaturation for 30s at 94˚C, annealing for 1min at 56˚C and elongation for 40s at 72˚C).

Table 2: Nucleotide sequences of the two pairs of specific primers and their (G+C) contents

Primer code		Primer sequence (5`-3`)	Bases number
CO1	JB3 Forward	5`-TTT TTT GGG CAT CCT GAG GTT TAT-3`	24
	JB4.5 reverse	5`-TAA AGA AAG AAC ATA ATG AAA ATG-3`	24
ITS1	EgF Forward	5`-GTC GTA ACA AGG TTT CCG TAG G -3`	22
	EgR Reverse	5`-TAG ATG CGT TCG AAG TGT CG -3`	20

The SSCP assay was performed (Zhu and Gasser, 1998). Four µl of PCR sample was added to 8 µl of loading dye (6X), and mixed well. After denaturation at 94˚C for 10min and subsequent snap cooling on a freeze block (-20˚C), 8µl of each sample were subjected to 10% polyacrylamide gel electrophoresis. After electrophoresis, gel was stained with 0.4% silver nitrate, visualized over Transilluminator plate with ordinary light, and photographed by a digital camera.

Data Analysis: All individual bands were scored as present or absent (1 or 0) for each species. Similarity coefficient (SC) was calculated (Nei and Li, 1979) as follows: SC= 2 Nxy/Nx+Ny. Nxy referred to bands number shared between 2 species (x & y) and Nx & Ny as they were amplified only by species x and y, respectively.

Results

Out of 246 examined samples, total prevalence was 19.5%. In summer (June-Aug), out of 75 samples, 8 (10.6%) were positive, in the fall season (Sept-Nov), 154 of which 40 (25.9%) were positive. In the spring season (March-May), 17 examined samples were cestode-free. But, 9 out of 246 (3.65%) samples were positive with single nematode infection. In summer (June-Aug), 2 out of 75 (2.66%) were positive, in fall season (Sept-Nov), 7 out of 154 (4.54%) were positive with single infection. None was detected in winter and spring. Mixed infections (nematodes and cestodes) were in 4 (2.59%) samples in the fall season.

Collectively, five cestode species were in pigeon's intestines (*R. echinobothrida, R. tetragona, R.* sp., *Cotugnia digonopora* and *Choanotaenia infundibulum*).

1- *Raillietina tetragona* (Molin, 1858): A common tapeworm in the domestic birds. Strobila is 35cm with a small oval scolex (Fig. 1A) of 0.17mm in diameter, Rostellum measures about 0.07x0.06mm, with about 100 hammer-shaped hooks in a single row measured 0.01mm in length. Scolex with four large, oval, and armed suckers measured 0.0x0.05mm in diameter. Suckers armed with 5 to 7 rows of hooks measure less than 0.01mm in length. Neck long measured 0.35x0.02mm, imamture proglottid 0.45x0.2mm. Mature one (Fig. 1B) measured 1.3x0.45mm, testes nearly rounded with 0.04mm diameter ranged from 25 to 28 leading to an oval cirrus pouch, extending beyond ventral longitudinal excretory canal measured 0.27x0.06mm.

Genital pores located unilateral, open anteriorly in segment to middle portion with 0.09mm in diameter, ovary measured 0.2mm in diameter, gravid proglottid (Fig. 1C) measured 0.9x0.6mm, uterus with egg capsules 6 to 9 eggs in each capsule, egg diameter 0.01mm, excretory canal diameter 0.02 mm.

2- *Raillietina echinobothrida:* (Mégnin, 1880): Body long 25cm, scolex spherical in shape and measures 0.27mm in diameter (Fig. 2A). Rostellum disc-shaped, nearly rounded measured 0.1x0.07mm, circularly lined with about 200 mattock-shaped hooks with an extended base and a pointed tip arranged in two distinct rows of 0.011mm in length. Four rounded suckers, rather spaced from one another, each measured 0.09x0.06mm; armed with 6 to 8 rows of

hooklets. Neck region short, unsegmented measured 0.45x0.17mm.

Mature segment (Fig.2B) measured 0.7x0.24mm, with 23 to 26 testes measured 0.04 mm in diameter and cirrus pouch measured 0.06mm in length. Genital pores unilaterally arranged at posterior to middle region of each segment and measured 0.02mm in diameter. Ovary oval and measures 0.04mm in diameter, Gravid one (Fig. 2C) measured 1.2x1mm. Eggs in egg capsule ranged from 4 to 7 with diameter 0.03mm, excretory canal 0.02mm in diameter.

3- *Rallietina* sp.: is a small tapeworm, with a very minute scolex (Fig. 3A), some-how square in shape and measures 0.2 X 0.2 mm in length and width. Rostellum about 0.05x 0.08mm with about 200 hooklets arranged in double rows measured 0.01mm in length. Four large and oval suckers armed with 6 to 8 rows of ho-oklets measured 0.17x0.07mm. Neck very long 1.3x0.1mm, mature proglottid (Fig. 3B) measured 0.65x0.25mm, testes rounded with diameter of 0.02mm with number from 30 to 36. Cirrus pouch oval elongated measured 0.16mm in length, Ovary bilobed measured 0.16mm in diameter.

Genital pores measure 0.06mm in diameter, located unilateral and open anteriorly to the middle portion of each segments. Gravid proglottid (Fig. 3C) measured 1.6x 0.6mm, egg capsules with from 4 to 8 eggs in each, egg diameter 0.01mm, excretory canal 0.03mm in diameter.

4- *Cotugnia digonopora* (Pasquale, 1890): Worm size varied from 7to 12cm in length. Scolex (Fig.4A) large measured 0.6mm in transverse diameter, with four well developed cup-shaped, unarmed suckers. Each measures from 0.1x0.15mm in diameter. Scolex apically with a rostellum, 0.3mm in diameter, armed with double rows of very minute hooks, measured 0.01mm in length and neck measured 0.2mm in width.

Mature segment (Fig. 4B) rectangular in shape with pointed posterior corners measured 2.5x0.5mm, with double set of ge-nital organs and two genital openings (bila-teral) in middle of lateral margins of each segment with diameter of 0.05mm. Testes 37 to 42 in each set, at center of segment measured about 0.0mm in diameter. Cirrus pouch measured 0.17x0.04mm. Ovary bilo- bed measured 0.03mm in width. Gravid proglottid 3.7x1.5mm (Fig. 4C). Each egg capsule with a single egg of 0.03mm diame- ter, excretory canal measured 0.5mm in dia-meter.

5- *Choanotaenia infundibulum* (Bloch, 1779): Worm measured 15-22cm long x up to 3mm wide, scolex (Fig. 5A) triangular to oval in shape, somehow pointed anteriorly and measured 0.27mm in width. Rostellum measured 0.8x0.6mm, armed with 16 slender hooks arranged in one row. Four more or less oval, unarmed suckers measured 0.18x 0.12mm, Neck narrow, short and measured 0.2x0.25-0.3mm.

Immature proglottid measured 0.31x0.23 mm. Mature one (Fig. 5B) more or less bell shaped, broader posteriorly than anteriorly with pointed posterior corners with serrated appearance, measured 1.15x0.7mm in length. Testes nearly rounded with range number from 3 to 40 measured 0.04 mm in diameter. Cirrus pouch elongated, located in anteriorly measured 0.17x0.05 mm, opened in genital pore measured 0.07 mm in diameter and alternates irregularly. Ovary branched measured 0.09mm in diameter with a highly lobed uterus. Gravid srgment (Fig. 5C) large, measured 3x2.5mm. Eggs encapsulated with a single egg in each, rounded measured 0.03 to 0.04mm in diameter. Excretory canal measured 0.04mm in diameter.

Raillietina species molecular characters: Totally, RAPD-PCR analysis used six arbitrary primers on the genome of *R. echinobothrida, R. tetragona,* and *R.* sp. (2 samples of each and one sample of *R.* sp.) showed an a average amplified DNA fragments of 35 bands for *R. echinobothrida*, 33 for *R. tetragona* and 21 bands for *R.* sp. Heterogeneity and homogeneity were among and within species of *Raillietina*.

The RAPD profile obtained with primer

P8 showed that the primer amplified three to five DNA fragments ranged from 600 to 1,200 bp. One dominant band of 800 bp was among the 3 species. It may be considered as a generic molecular marker (Fig. 6A).

P9 primer generated one to seven DNA fragments ranged from 500 bp to 1,200 bp. Two DNA segments of 1,000 and 1,050 bp were common among all species except in one individual. Also, *R. tetragona* samples have four monomorphic bands. The DNA fragment of 500, 600, 1,000 & 1,050 bp present in *R. tetragnona* and *R. echinobothrida* isolates, but absent in *R.* sp. (Fig. 6A).

The primer P10 generated showed eight to twelve DNA bands, some of them were distinct and others were faint. The DNA profile produced various bands ranged from 300 to 1,100 bp. Eight monomorphic DNA bands of 300, 450, 500, 600, 650, 800, 900 and 1,000 bp were dominant among the three species and considered as a generic molecular marker (Fig. 6B). P12 primer generated 3 to 8 DNA fragments ranged from 400 bp to 1,500 bp. DNA fragment of 1,100 bp was in all species samples but faint in *R.* sp. Two fragments of 800 and 1,500 bp were present in all samples except the *R. echinobothrida* sample 2, but DNA fragment of 600 bp was absent only in *R. tetragona* sample 1. Two dominant fragments of 500bp and 1,050bp were prominent in *R. tetragona* samples (Fig. 6C).

Arbitrary primer P13 amplified two to six DNA fragments ranged from 400 to 1,100 bp, some of them were distinct and others were faint. This primer amplified polymerphic bands. Individuals of *R. echinobothrida* showed five monomorphic bands of 400, 500, 600, 1,000 and 1,100 bp. Two DNA bands of 400 and 700 bp were dominant in all samples except one (Fig. 6D).

Primer P33 amplified 1 to 6 polymorphic DNA fragments in range of 200 to 1,200bp for them. Individuals of *R. echinobothrida* and *R. tetragona* showed five monomorphic bands of 200, 300, 500, 800 & 1,200 bp. *R.* sp. showed one band of 600 bp (Fig. 6C).

Primer P12 generated the maximum bands size (1,500bp) among all primers. There were some bands with high intensity and others with faint appearance. Primers amplified different classes, monomorphic, polymorphic and high frequency polymorphic. Primers (P8, P10 & P12) yielded both monomorphic and polymorphic bands, but P9, P13 & P33 generated polymorphic fragments, yielding band patterns with a high degree of divergence among *Raillietina* species that allowed easy distinction between them. The six primers yielded distinguishable band patterns among them and individuals within same species. High similarity coefficient (Tab. 3) was 70% between *R.* sp. & *R. echinobothrida* (R. sp. & R.e1) and between *R.* sp. and *R. tetragona* (R. sp. & R.t2). In one hand, the lowest SC (62%) was found between *R. echinobothrida* and *R. tetragona* (R.e2 & R.t2). The SCs within individuals of the same species were 67% for *R. echinobothrida* (R.e1 & R.e2) and 72% for *R. tetragona* (R.t1 & R.t2).

Table 3: No. of shared bands within 3 species of *Raillietina* with similarity coefficient. (*R.sp: Raillietina* sp. *sample, R.e1&R.e2: R. echinobothrida samples, R.t1&R.t2: R. tetragona samples*)

Species	SB	SC%	GD%
R. sp & R.e1	16	70 %	30 %
R. sp & R.e2	13	50 %	50 %
R. sp & R.t1	15	60 %	40 %
R. sp & R.t2	19	70 %	30 %
R.e1 & R.e2	20	67 %	33 %
R.e1 & R.t1	20	67 %	33 %
R.e1 & R.t2	21	67 %	33 %
R.e2 & R.t1	21	65 %	35 %
R.e2 & R.t2	21	62 %	38 %
R.t1 & R.t2	24	72 %	28 %

SSCP-PCR: *R. echinobothrida* (1 sample), *R. tetragona* (2 samples), and *R.* sp. (1 sample) were subjected to PCR analysis to amplify mitochondrial 12S rRNA and Cytochrome *C* oxidase *(CO1)* genes. DNA samples from them were randomly selected to eliminate individual variation. The amplification of 12S rRNA gene (Fig. 7A) of all isolates produced a fragment of approximately 250 bp. While amplification products of *CO1* (Fig. 7B) were 450 ~ 500 bp fragments in all isolates.

Each PCR product was denatured and loaded in a different lane of a polyacrylamide gel. Although there was no variation in size among the PCR products on agarose gel, three distinct profiles were distinguished among the 3 species in the *CO1* gene in SSCP analysis. *R. tetragona* homozygous individuals have nearly the same pattern except in one band (Fig. 8A, lane 1 & 2), but individuals of *R.* sp. and *R. echinobothrida* presented six and seven bands respectively with different electro-phoretic mobility (Fig. 8A, lane 3 & 4). In 12S rRNA gene, SSCP analysis gave nearly the same profiles among *Raillietina* species but with different electrophoretic mobility for each (Fig. 8B).

Discussion

The whole prevalence of the intestinal helminthes of domesticated pigeon (*C. l. domestica*) was 24.79%. Highest infection rate was in the fall season (33.11%) followed by summer (13.3%), but without infection in winter and spring seasons. This percentage was higher than Eljadar *et al.* (2012) who found prevalence values of 5%, but, relatively low with other studies worldwide. In Iran, prevalence was 84.78% (Radfar *et al*, 2012), in India was 91% (Par-sani *et al*, 2014), and ranged from 46% to 100% in Brazil (Tietz Marques *et al*, 2007), Nigeria (Adang *et al*, 2008; Opara *et al*, 2012; Buba *et al.*, 2018), Iraq (Al-Barwari and Saeed, 2012), Egypt (El-Dakhly *et al*, 2018) and Libya (Alkharigy *et al*, 2018).

In the present study, prevalence was 19.5%, or lower than Chaechi-Nosrati *et al.* (2018) and Laku *et al.* (2018) reported 36.66% & 28% respectively. Also, prevalence of single nematode infection was 3.65%, which different from Borji *et al.* (2012) who reported 21.6%, but nearly similar to cestode 15.3%. Nematode infect-ions % differed from Chaechi-Nosrati *et al.* (2018) who reported 40.5% but similar to Laku *et al.* (2018) who reported 4%.

In the present study, higher prevalence of nematode infections was in summer (2.66%) and fall (4.54%) seasons. This agreed with Moudgil *et al.* (2019) who reported that the higher prevalence was in windy seasons that accompanied by precipitation and also in summer season. This could be attributed to the favorable environmental conditions (high humidity and suitable temperature) for development and prolonged infective parasitic stages survival (Singh *et al*, 2009). Seasonal climatic conditions interfered with feed and water resulted in stress and decreased immunity, leading to more vulnerability to parasitosis (Dhoot *et al*, 2002).

The helminthes prevalence of Egyptian domestic pigeon was relatively low as compared with studies worldwide. This variations in results and percentage in the present study compared to other studies (even higher or lower) and the presence of other reported species of cestodes in other countries may be due to abundance and presence of intermediate hosts (as ants, cockroaches, beetles, earthworms, & some molluscs), host species and its feeding habits, climate factors and geographical factors in Egypt. No trematodes were recorded due to their preferable feeding habits lacking intermediate hosts.

In the present study, for specific identification, the recovered cestode parasites were compared with those from different regions worldwide and they were *R. tetragona* and *Raillietina* sp. with a rounded rostellum and oval suckers, but *R. echin-*

obothrida with small round rostellum and rounded suckers. Also, rostellar hooks of *R. tetragona* were arranged in a single row while those of other 2 species were in 2 rows. Yamaguti (1958); Schmidt (1986) Butboonchoo *et al.* (2016) reported that some morphological criteria confirmed cestodes identifications. The present *R. tetragona* was similar to that of Sawada (1965), Albadalejo (1995) and Butboonchoo *et al.* (2016).

Sawada (1965), Albadalejo (1995), Baker (2008) and Butboonchoo *et al.* (2016) recorded 6 to 12 eggs per egg capsule in *R. tetragona*, that differs from the present study, that recorded 5 to 11 eggs in egg capsule. In the present study, shape and size of *R. echinobothrida* were closely related to that described by (Dehlawi, 2007; Lalchhandama, 2009; Al-Marsomy and Al-Hamadaani, 2016; Ibrahim *et al*, 2018). However, it differed from the des-cription of Al-Marsomy and Al-Hama-daani (2016) in scolex width and roste-llum diameter, they recorded 0.04 & 0.027mm respectively, in the present study, were 0.27 & 0.1mm respectively. The present description of *R. echinobothrida* agreed with the description of Lalchhandama (2009) and Butboonchoo *et al.* (2016). They described an oval to rounded armed suckers, an armed rostellum with two rows of hammer-shaped hooks and unilateral posterior genital. It differed with Lalchhandama (2009) who reported *R. echinobothrida* with a single egg in the egg capsule.

Comparing studies on different species of *Raillietina* described, *Raillietina* sp. of the present study cannot be exactly identified. It differed from *R. tetragona* in the present study in having a double circles of rostellar hooks, a relatively longer neck (1.3 mm in *Raillietina* sp. compared with 0.35 mm in *R. tetragona*), larger number of testes (30-36 *Raillietina* sp. compared with 25-28 in *R. tetragona*), number of eggs per egg capsule (4-8 in *Raillietina* sp. compared with 5-11 in *R. tetragona*) and different shape of scolex (*Raillietina* sp. has a nearly squared scolex compared with an oval one of *R. tetragona*). *Raillietina* sp. also differed with *R. echinobothrida* in scolex shape (*Raillietina* sp. with a nearly squared scolex but rounded one of *R. tetragona*), the length of the neck (1.3mm in *Raillietina* sp. compared with 0.45mm in *R. tetragona*) and number of testes (30-36 in *Raillietina* sp. compared with 23-26 in *R. tetragona*). *Raillietina* sp. in the present study was related to *R. saudiae* that described by Al Quraishy *et al.* (2019). They described a *Raillietina* with 2 rows of hammer-shaped rostellar hooks with range number of 230 to 250 and testes number of 27 to 37, unilateral genital pore and 5 to 6 eggs in egg pouch. They differed in the absence of the neck.

In the present study *Raillietina* sp. may be closely related to *R. zahratis* described by Magzoub *et al.* (1980), as a worm with two rows of hammer-shaped rostellar hooks; unilateral genital pore opening and 3 to 9 eggs in egg pouch. But, it differed in the number of testes (12 to 15 to *R. zahratis* and 30 to 36 in *Raillietina* sp.).

The present *Cotugnia digonopora* was closely similar to *C. magdoubii* of Magzoub *et al.* (1980) in worm size, number of testes and in genital pores arrangement. But, it differed in scolex width, rostellum diameter and suckers and the cirrus pouch length (Magzoub *et al*, 1980). In the present study, *C. digonopora* was similar to that of Meggitt (1926) and Magzoub *et al.* (1980) in having two sets of reproductive organs, cup-shaped muscular suckers, and rostellum with double rows of very small hooks. But, it differs in the number of testes, both reported 100 testes while in the present study there were about 37-42 testes in each set of organs.

In the present study, *Choanotaenia infundibulum* agreed with Lincicome (1939) and Sawada (1970). Baker (2008) reported that *C. infundibulum* was 23cm long; scolex bears an armed rostellum with 16 to 22 hooks arranged in one circle measured 0.02 to 0.03 mm in length. But, Arulmozhi *et al.* (2018) found that *C. infundibulum* measured 15 to

22x 2.5 to 3mm, a small scolex with suckers and rostellum armed with 16-20 slender hooks. Egg had an ovoid shape, measured 0.03x0.045 mm similar to the study *C. infundibulum* described. Intestinal helminthes are frequently identified on the basis of morphological characters, their transmission methods or their pathological effects on their hosts (Lichtenfels *et al,* 1997). These criteria are often insufficient for specific identification. Using molecular techniques for species identification increased knowledge and data for recognition, identification, and phylogenetic relationships of *Raillietina* species.

RAPD-PCR was identified by Gasser (2006) as DNA fingerprinting method easily produced complex and informative genomic fingerprints. In *Raillietina* species (*Raillietina* sp., *R. echinobothrida,* and *R. tetragona*) under investigation, RAPD-PCR data showed that *Raillietina* sp. was closely related to *R. echinobothrida* and *R. tetragona* with 70% similar coefficient. *R. echinobothrida* and *R. tetragona* were 67% related. SSCP technique (12S rRNA and Cytochrome *C* oxidase (*CO1*) genes) proved that the three species were closely related to each other by having nearly identical bands pattern. This agreed with (Littlewood *et al.,* 2008; Butboonchoo and Wongsawad, 2017) who stated that *R. echinobothrida* and *R. tetragona* were more related to each other than *Raillietina* species as their definitive host is domestic chickens but, other species use other birds as final hosts. Ghobashy and Taeleb (2015) used RAPD-PCR to identify *Raillietina* spp. of infected domestic and wild pigeons from Sharkia Governorate found sequence similarities confirmed that *Raillietina* spp. was related to other species of order Cyclophyllidea. Sequence of worm samples showed homology to *R. beveridgei*. Wolf *et al.* (2005) and Jyrwa *et al.* (2014) estimated the validity of ITS2 gene region in distinguishing related *Raillietina* species and recommended that more studies was a must to exploit the ITS2 and other markers for robust molecular characterization of these avian parasites. Ibrahim *et al.* (2018) used molecular analysis of gene (ITS2) to differentiate between two *Raillietina* species (*R. beveridgei* & *R. echinobothridia*) found that ITS2 gene was composed a phylogenetic tree using related sequences available on the GenBank.

Conclusion

RAPD-PCR & SSCP-PCR techniques proved to be the most reliable techniques in identification of species and in detecting intraspecific genetic variations between closely related and similar parasites of same species.

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Explanations of figures

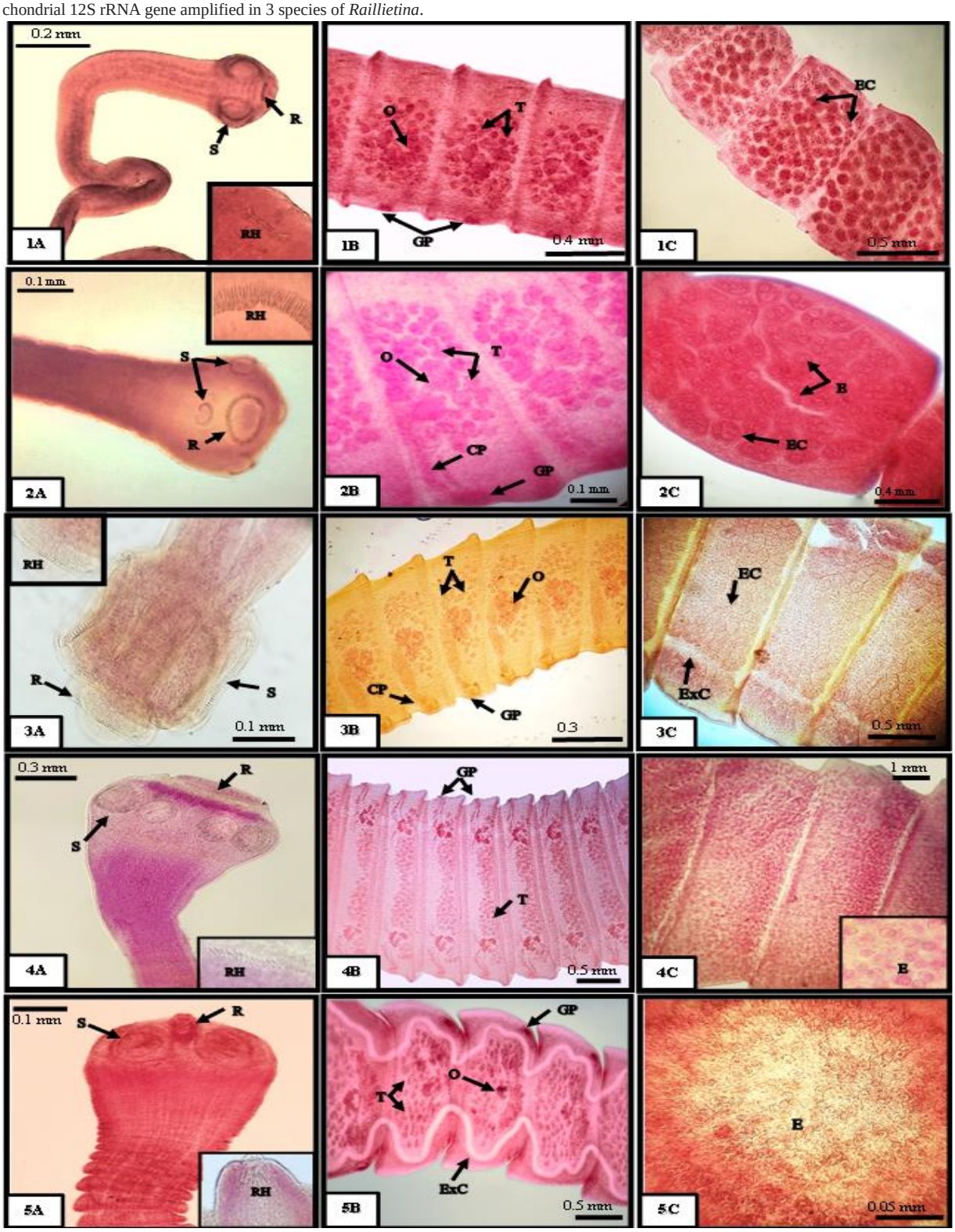
Fig.1: *Raillietina tetragona* recovered from *Columba livia domestica* stained with acetocarmine. A. Scolex with higher magnification of rostellar hooks. (S: Sucker, R: Rostellum RH: Rostellar hooks). B. Mature proglottid. (GP: Genital pore, O: Ovary, T: Testes). C. Gravid proglottid. (E: Egg, EC: Egg capsule)

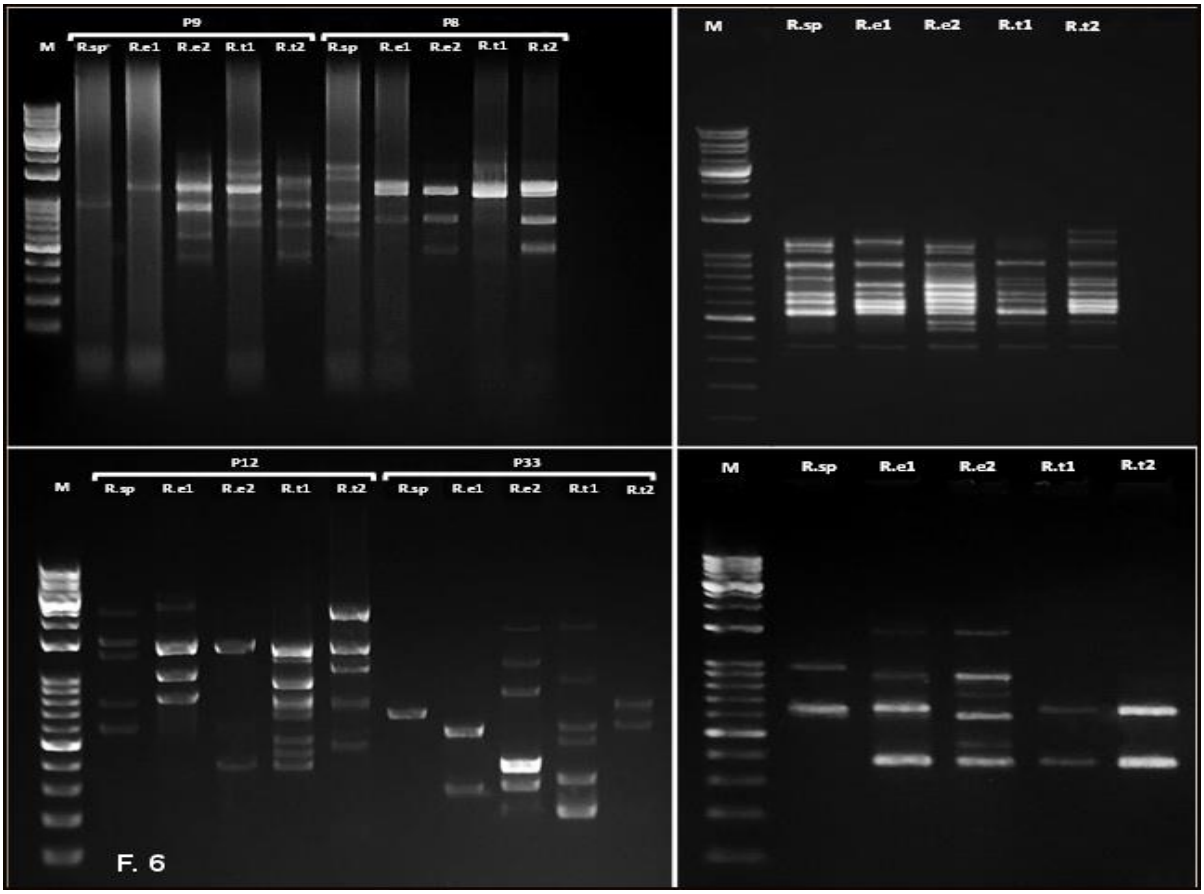
Fig. 2: *Raillietina echinobothrida* recovered from *C. l. domestica* stained with acetocarmine. A. Scolex with higher magnification of rostellar hooks. (S: Sucker, R: Rostellum, RH: Rostellar hooks). B. Mature proglottid. C. Gravid proglottid.

Fig. 3: *Raillietina* sp. recovered from *C. l. domestica* stained with acetocarmine. A. Scolex with higher magnification of rostellar hooks. B. Mature proglottid. C. Gravid proglottid.

Fig. 4: *Cotugnia digonopora* recovered from *C. l. domestica* stained with acetocarmine. A. Scolex with higher magnification of rostellar hooks. B. Mature proglottid. C. Gravid proglottid. (E: Egg)

Fig. 5: *Choanotaenia infundibulum* recovered from *C. l. domestica* stained with acetocarmine, A. Scolex with higher magnification of rotellar hooks. B. Mature proglottid. C. Gravid proglottid.
Fig. 6: Agarose gel electrophoresis of RAPD-PCR of 3 species of *Raillietina*. A. Using primers P8 & P9. B. Using primer P 10. C. Using primers P12 & P33. D. Using primer P13. (R.sp: *Raillietina* sp. sample, R.e1&R.e2: *R. echinobothrida* samples, R.t1&R.t2: *R. tetragona* samples, M: 100 bp DNA ladder)
Fig. 7: Agarose gel electrophoresis of PCR detection of A. Cytochrome C oxidase subunit (CO1) gene. B. Mitochondrial 12sRNA gene in 3 species of *Raillietina*.
Fig. 8: Agarose gel electrophoresis stained with silver nitrate of SSCP-PCR assay of A. Cytochrome C oxidase subunit (CO1) gene. B. Mitochondrial 12S rRNA gene amplified in 3 species of *Raillietina*.

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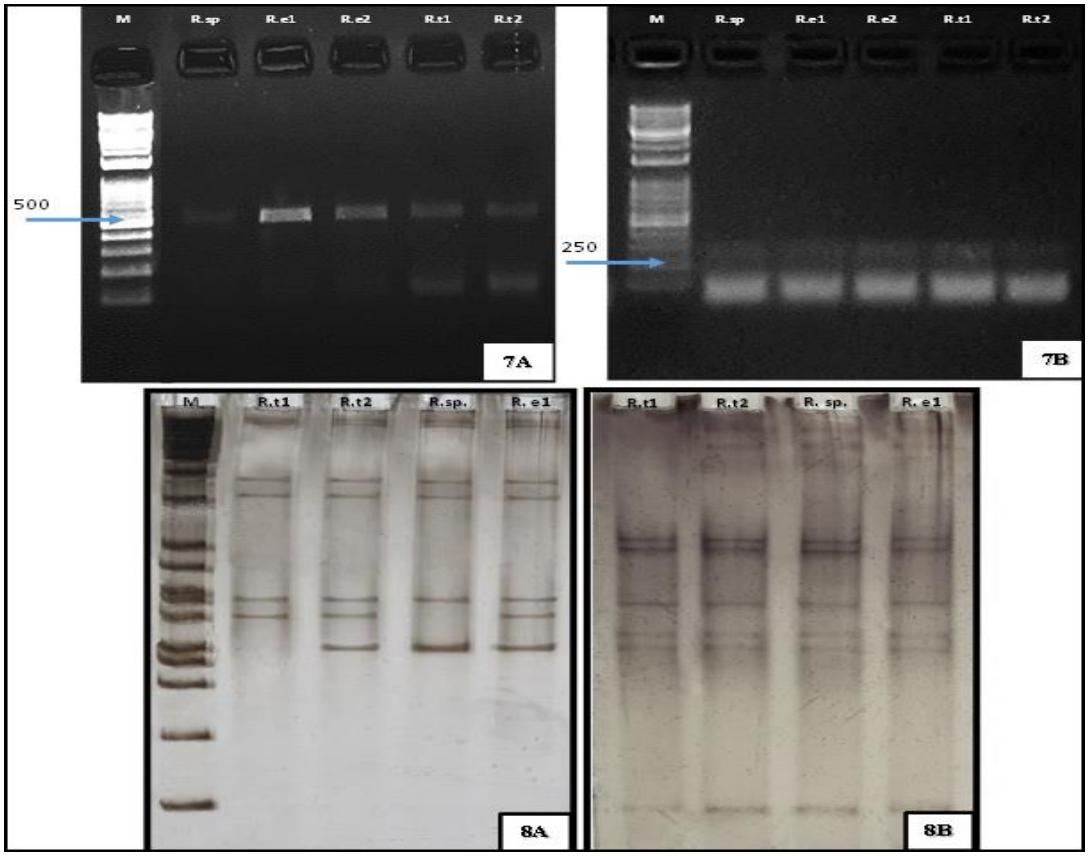
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