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Microscopical and Molecular Diagnosis of Gastrointestinal Nematodes Infecting Small Ruminants in Menofia Governorate

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ABSTRACT

Gastrointestinal nematode is the most common problem affecting the health and productivity of small ruminants. The current study aimed to identify nematodes infecting small ruminants microscopically and characterizes them molecularly by using RAPD-PCR. The study was carried out on 45 slaughtered small ruminants (19 sheep and 26 goats) for microscopical and molecular diagnosis of recovered nematodes. The gastrointestinal tracts of slaughtered animals were examined by sieving method for nematode recovery. The recovered nematodes were washed, cleared, mounted and identified. RAPD-PCR was carried out on 10 samples (adults and larvae) by using 3 primers. The results revealed that the infection rate of nematodes was higher in sheep than goats. The recovered nematodes were *Haemonchus contortus, Trichostrongylus axei, Cooperia* sp., *Ostertagia ostertagi* and *Trichuris ovis*. The infection rate of different nematodes was recorded. Comparison between *Trichuris ovis* from sheep and goats revealed genetic polymorphism and genetic variability in DNA amplification using three primers. Overall, RAPD-PCR indicated genetic polymorphism and genetic variability between different nematodes and within the same species.

Key words: Nematodes, RAPD-PCR, genetic polymorphism, genetic variability, DNA amplification

INTRODUCTION

Gastrointestinal nematodes are the most numerous, complex and variable between helminthes of domesticated animals. On earth, the nematodes are second only to arthropods with regard to their numbers and complexity of life cycles. Both adult and larval stages of nematodes can produce significant pathology in domesticated animals[1]. Gastrointestinal nematodes majorly concerned about productivity, health of small ruminants, industry and threaten sustainability. However, more common and of major economic impact are sub clinical infections, which may cause significantly reduced performance of infected animals as are without obvious symptoms[2]. Traditional diagnostic methods for nematodes need faecal culture and coproscopic detection of eggs or larvae. Advanced molecular diagnostic techniques are more reliable and efficient methods than traditional techniques[3]. The utilization of molecular biology

techniques brought new approaches for diagnosis of nematodes. Methods to extract DNA for different forms of parasites in fecal samples of animals have allowed the use of molecular tools for the diagnosis of several organisms[4]. With the application of the Polymerase Chain Reaction (PCR) it was possible to extend these studies. The PCR technique has the potential to find out DNA of different organisms, detect the pathogens in secretions and tissues and differentiate morphological similar helminthes. *Haemonchus contortus* and *Haemonchus placei* have been differentiated genetically based on PCR method[5]. When the target sequence is unknown, RAPD-PCR is useful[6]. This technique can be distinguished from the other PCR techniques by using a single very short arbitrary oligonucleotide, generally with 10 bases[7]. This is in contrast to others that require information concerning the target DNA for designing specific primers. It is a rapid technique requiring small amount of DNA and has been widely used allowing accomplishment of studies of genetic analysis in diverse species. Studies with nematodes of plants and humans demonstrate its great efficiency in the differentiation of profiles of amplification and capability to distinguish polymorphisms between organisms[8]. So this study aimed for microscopic identification of nematodes infecting small ruminants and characterizes them molecularly by using RAPD-PCR.

MATERIALS AND METHODS

Worm recovery: Standard methods were used to recover worms from the gastrointestinal tract of 45 slaughtered small ruminants (19 sheep and 26 goats). As soon as possible after removing the alimentary tract from the body cavity, the abomasal/duodenal junction was ligatured. Separate the abomasums, small intestine and large intestine. The abomasum was opened; its contents washed into a bucket under running water and made the total volume up to 2-4 L, filtered through the sieve with an aperture of 250 µm capable of retaining the larvae. A duplicate of 200 mL was transferred to a labeled plastic container and preserved in 10% formalin. The 20 mL of the sub-sample was taken onto a Petri dish and 2-3 mL of iodine solution for coloration to facilitate easy identification and examination of worms under stereomicroscope. Small and large intestines were treated like the abomasum according to Urquhart *et al.*[1].

Identification of collected nematodes: The collected worms were washed with normal saline, fixed in hot glycerin alcohol, cleared in Lactophenol and mounted in glycerol jelly. Morphological description and identification of the worms were done on mounted specimens according to the keys illustrated by Urquhart *et al.*[1] and MAFF[9].

Samples preparation and extraction of DNA: Ten Samples were tested with RAPD PCR, between them 7 samples were adult worms and 3 samples were larvae. After that adult worms were recovered from GIT, washed several times with PBS and then preserved in eppendorf tubes that stored at -20°C till further use. The larvae and *Toxocara vitullorum* were used from positive preserved identified samples in the lab. Extraction of DNA by QIAamp DNA Mini Kit was performed according to manufacturer´s instructions. DNA quality was checked by electrophoresis on 1% agarose gel.

RAPD-PCR: Three random primers (primer 1. 5´-TCGCGAATTC-3´; primer 2. 5´-AACGCGCAAC-3´; primer 3. 5´-AAACGGTTGGGTGAG-3´) were used to amplify the targeted DNA. PCR was performed in 25 µL of a mixture containing 0.5 µL of the extracted DNA template, 50 pmol of each primer and 22.5 mL of 2x PCR Master mix Solution (iNtRON Biotechnology Inc., Korea). The reactions were performed under the following conditions: 1 min at 94°C, 45 cycles (30 sec of denaturation at 94°C, 20 sec of annealing at 55°C, 60 sec of extension at 72°C) and 5 min of final extension at 72°C in a G-STORM PCR system. Negative controls were included in all runs[10].

Screening of amplified fragments: The PCR products were subjected to electrophoresis in 1% agarose gel and then visualized under an ultraviolet (UV) light after staining with ethidium bromide.

RESULTS

Prevalence of recovered nematodes: The recovered nematodes were *Haemonchus contortus, Trichuris ovis*, *Trichostrongylus axei, Ostertagia ostertagi* and *Cooperia curticei*, their measurements were recorded in Table 1 and 2. The morphology of these worms was shown in Fig. 1-5.

The results in Table 3 showed the infection rate of the different recovered worms. The 11 out of 19 examined sheep were infected with nematodes with an infection rate of 57.89%. While, 12 out of 26 examined goats were infected with nematodes with an infection rate of 46.15%. Infection rate of sheep (57.89%) with nematodes was higher than that of goats (46.15%). Concerning to *Haemonchus contortus*, 9 out 19 sheep and 10 out 26 goats were infected with *Haemonchus*

Table 1: Measurements of recovered worms males and females in mm

		Total length	Breadth	Esophageal length	Vulvar flap		Tail length	Spicules
					Breadth	Length		
Haemonchus contortus	Male	10-16 (13)	0.20-0.30 (0.25)	1.00-1.15 (1.075)				0.32-0.34 (0.33)
	Female	13-25 (19)	0.25-0.55 (0.40)	1.00-1.30 (1.15)	0.09-0.22 (0.15)	0.15-0.55 (0.35)	0.40-0.70 (0.55)	
Ostertagia ostertagi	Male	7.00-9.50 (8.25)	0.08-0.12 (0.10)	0.35-0.45 (0.40)				0.20-0.27 (0.235)
	Female	9.00-10.00 (9.50)	0.10-0.14 (0.12)	0.45-0.55 0.50	0.06-0.09 (0.075)	0.15-0.19 (0.17)	0.25-0.45 (0.30)	
Cooperia curticei	Female	5.00-8.00 (6.50)	0.09-0.11 (0.10)					
Trichostrongylus axei	Female	6.00-8.00 (7.00)	0.08-0.10 (0.09)					

Table 2: Measurements of *Trichuris ovis* male and female in mm

	Total length	Length of thin part	Length of thick part	Breadth of thin part	Breadth of thick part	Spicule
Male	34.50-37.50 (36.00)	25.00-27.00 (26.00)	9.50-10.50 (10.00)	0.05-0.08 (0.65)	0.25-0.29 (0.27)	4.00-6.00 (5.00)
Female	35.00-42.00 (38.75)	24.50-30.50 (27.50)	10.50-12.00 (11.25)	0.05-0.10 (0.075)	0.50-0.70 (0.60)	

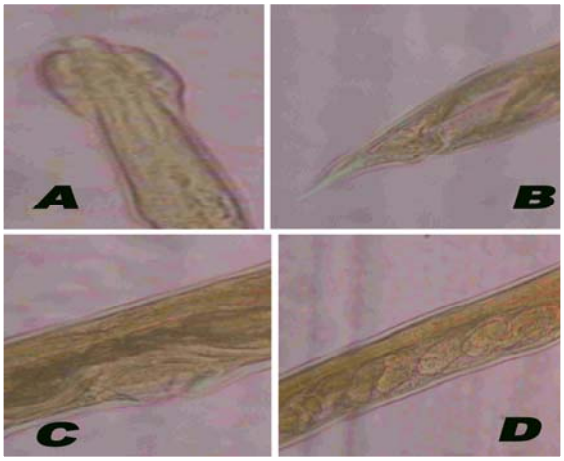

Fig. 1: *Cooperia curticei* X_{10} A-Anterior end showing cephalic vesicle X_{10}, B-Posterior end of female X_{10}, C-Vulvar region of female X_{10}, D-Uterus containing eggs X_{10}

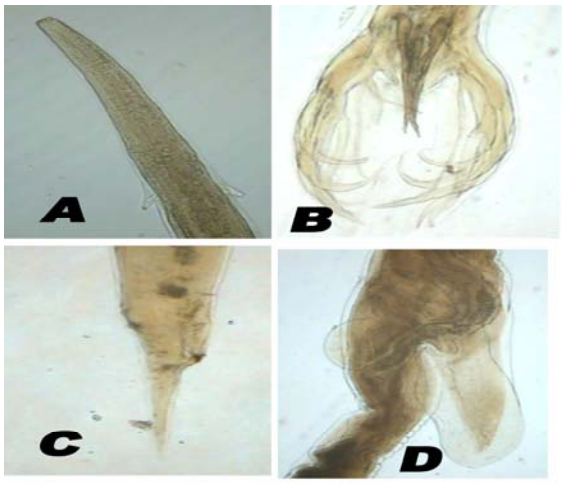

Fig. 2: *Haemonchus contours*, A-Anterior end X_{10}, B-Male posterior end X_4, C-Female posterior end X_4, D-Female vulvar flap X_4

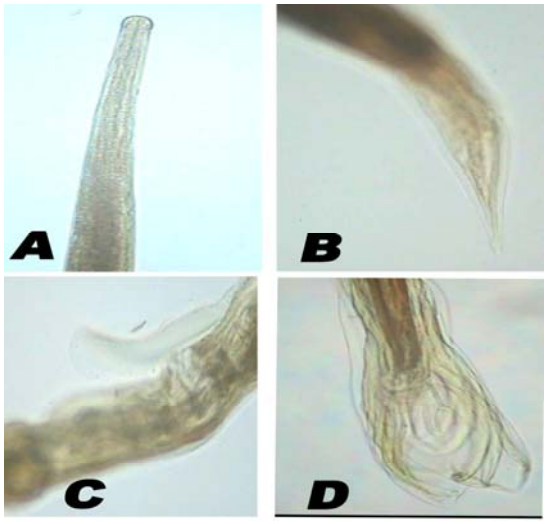

Fig. 3: *Ostertagia ostertagi*, A-Anterior end X_{10}, B-Female posterior end X_4, C-Female vulvar flapX_4, D-Male posterior end X_4

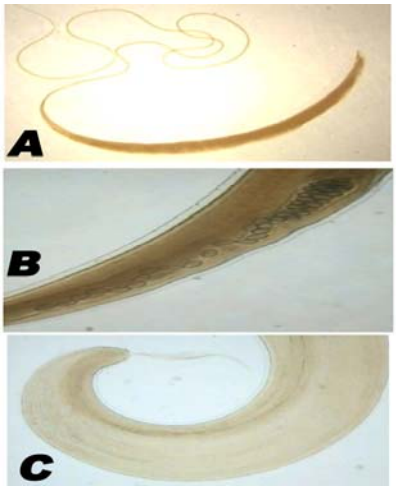

Fig. 4: *Trichuris ovis*, A-Whole worm X_2, B-Vulvar region X_4, C-Male posterior end

Table 3: The infection rate of the different recovered nematodes

	Sheep		Goats	
	Number examined 19		Number examined 26	
Species	Number infected	Percentage of infection	Number infected	Percentage of infection
Haemonchus contortus	5	26.31	8	30.76
Trichuris ovis	0	0	2	7.69
Ostertagia ostertagi	1	5.26	0	0
Mixed (*Haemonchus contortus, Trichostrongylus axei* and *Cooperia* sp.)	1	5.26	0	0
Mixed (*Haemonchus contortus, Trichuris ovis*)	3	15.78	2	7.69
Mixed (*Haemonchus contortus, Ostertagia ostertagi*)	1	5.26	0	0
Total infection	11	57.89	12	46.15

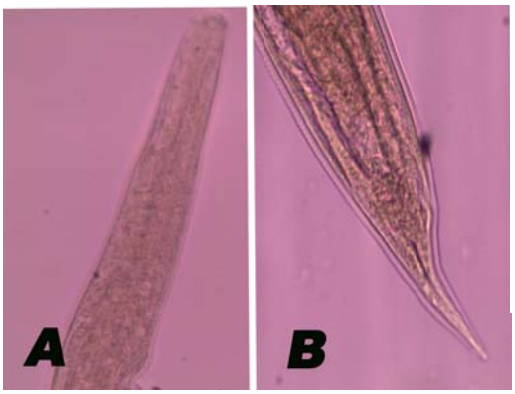

Fig. 5: *Trichostrongylus axei* X_{10} (A) Anterior end showing excretory notch, (B) Female posterior end

contortus with an infection rate of 47.36 and 38.46% respectively. Infection rate of *Haemonchus contortus* in sheep (47.36%) was higher than goats (38.46%). Regarding to *Trichuris ovis,* 3 out 19 sheep and 4 out 26 goats were infected with *Trichuris ovis* with an infection rate of 15.78 and 15.38%, respectively. Infection rate of *Trichuris ovis* in sheep (15.78%) was higher than goats (15.38%).

The infection rate of *Trichostrongylus axei, Cooperia curticei* and *Ostertagia ostertagi* was 5.26% in sheep and without any infections in goats. Neither *Trichostrongylus axei* nor *Cooperia curticei* and *Ostertagia ostertagi* were recorded in goats. Mixed infections with different nematodes were recorded in sheep and goats as recorded in Table 3.

RAPD-PCR analysis: PCR conditions of 10 nematodes samples were checked with 3 different primers. The genetic profiles of 10 nematodes samples (7 worms and 3 larvae) from different ruminants were compared.

RAPD-PCR analysis using Primer 1 yielded only one fragment (1300 bp) with only *Trichuris ovis* from goats (Fig. 6). While, the RAPD-PCR analysis using primer 2 showed in Fig. 6, yielded

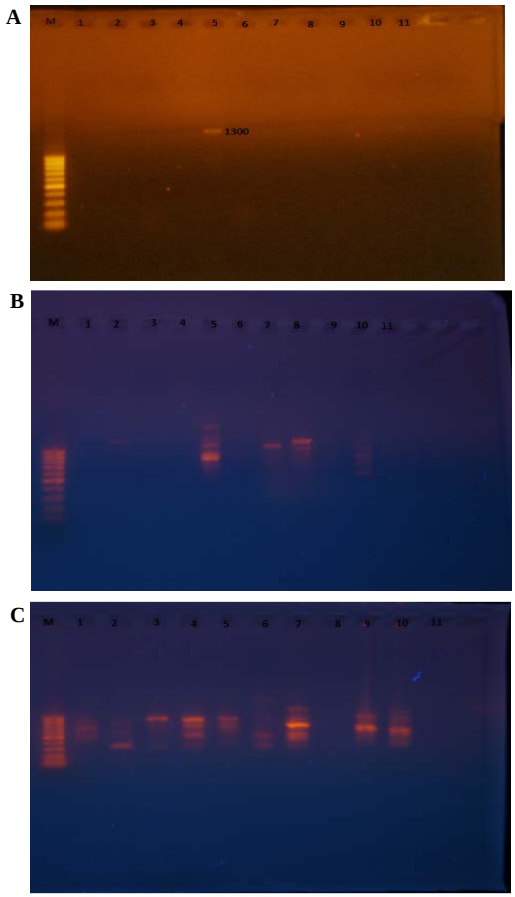

Fig. 6: DNA amplification fragments obtained by RAPDs-PCR using A-primer 1, B-primer 2, C-primer 3, Analysed by electrophoresis in 1% Agarose gel, M-DNA marker; 1-ovine *Haemonchus contortus*, 2-*Toxocara vitullorum* from buffaloes; 3-ovine *Ostertagia osteraagi*; 4-ovine *Trichuris ovis*; 5-caprine *Trichuris ovis*; 6-*Toxocara vitullorum* from cattle; 7-ovine *Cooperia curticei*; 8-ovine *Strongyloides papillosus* larvae; 9- ovine *Nematodirus filliollis* larvae; 10-ovine *Haemonchus contortus* larvae; 11-Negative control

5 fragments (900, 1000, 1100, 1200 and 1400 bp) with ovine *Trichuris ovis* and without any fragments with caprine *Trichuris ovis*. Primer 2 gave 3 fragments (900, 1100 and 1200 bp) with ovine *Cooperia curticei* and 4 fragments (800, 900, 1000 and 1100 bp) with ovine *Nematodirus filliollis* larvae.

Primer 3 gave 3 fragments (700, 800 and 900 bp) with ovine *Haemonchus contortus*, one fragment (1000 bp) with ovine *Ostertagia ostertagi*, 3 fragments (700, 900 and 1000 bp) with ovine *Trichuris ovis* and only 2 fragments (900 and 1000 bp) with caprine *Trichuris ovis*. But, primer 3 gave 5 fragments (500, 600, 800, 900 and 1100 bp) with ovine *Cooperia curticei*, 2 fragments (800, 1000 bp) with ovine *Nematodirus filliollis* larvae and 4 fragments (700, 800, 900, 1000 and 1100 bp) with ovine *Haemonchus contortus* larvae.

There were no fragments recorded with the RAPD-PCR analysis using 3 tested Primers with ovine *Strongyloides papillosus* larvae as showed in Fig. 6. Overall, the RAPD-PCR analysis indicated polymorphism using 3 tested primers as illustrated in Fig. 6.

DISCUSSION

The present study aimed to throw more light on the different diagnostic methods of nematodes infecting small ruminants including microscopic and molecular diagnosis (RAPD-PCR). Examination of slaughtered sheep and goats was carried on 19 sheep and 26 goats. According to this study sheep and goats were infected with different gastrointestinal nematodes with an infection rate of 57.89 and 46.15%, respectively. The highest recorded nematode was *Haemonchus contortus*.

Concerning to the morphological examinations and measurements of recovered worms (*Haemonchus contortus, Ostertagia ostertagi, Cooperia curticei, Trichostrongylus axei* and *Trichuris ovis*) that collected from gastrointestinal tracts of slaughtered sheep and goats were in agreement with MAFF[9] and Soulsby[11].

Regarding to RAPD-PCR, PCR conditions of 10 nematodes samples were checked with 3 different primers. The genetic profiles of 10 nematodes samples (7 worms and 3 larvae) from different ruminants were compared. The RAPD-PCR analysis indicated genetic polymorphism and genetic variability by using 3 primers. RAPD-PCR analysis using Primer 1 yielded only one fragment (1300 bp) with only *Trichuris ovis* from goats. RAPD-PCR analysis using primer 1 yielded no fragments with other samples including *Haemonchus contortus*. These results disagreed with Rabouam *et al.*[12] which recorded that RAPD-PCR analysis using this primer yielded 8 fragments with *Haemonchus contortus* and this may due to genetic polymorphism with genetic variability in *Haemonchus contortus*.

There were no fragments recorded with RAPD-PCR analysis using the three checked primers with ovine *Strongyloides papillosus* larvae. Comparison between *Trichuris ovis* from sheep and goats revealed genetic polymorphism with genetic variability observed in DNA amplification with the three tested primers. This explanation agreed with Guclu *et al.*[13] which reported that comparisons between the morphologically similar species showed lower levels of sequence divergence than those between different spp. Molecular techniques provide more specific tool than traditionally employed in epidemiological studies[14]. RAPD-PCR technique is able to explain the genetic relationship between *Helminthes* spp. infecting small ruminants.

CONCLUSIONS

This study showed that the infection rate of nematodes was higher in sheep than goats. RAPD-PCR technique is able to explain the genetic relationship between *Helminthes* spp. infecting small ruminants. RAPD-PCR indicated genetic polymorphism and genetic variability between different nematodes infecting small ruminants and within the same species.

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