Microscopical and molecular diagnosis of gastrointestinal nematodes infecting small ruminants in Menofia governorate

Ahmed Elkhatam¹, Mahmoud R. AbouLaila², Nasr Elbahy³, Tamer M. Roshdy⁴

¹,³ Department of Parasitology, Faculty of Veterinary Medicine, University of Sadat City, 32897, Menoufia, Egypt
² Department of Parasitology, Faculty of Veterinary Medicine, Damanhour University, Egypt.
⁴Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Menoufia, Egypt.
elkhtama@yahoo.com

Abstract: Gastrointestinal nematodes are the most common problems affecting the health and productivity of small ruminants. 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.

Keywords: Nematodes, RAPD-PCR, Genetic polymorphism
1. Introduction:
Gastrointestinal nematodes are the most numerous, complex and variable between helminths of domesticated animals. On planet 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 [11]. Gastrointestinal nematodes are of major concern to the productivity, health of small ruminants, and their 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 without obvious symptoms [10]. Traditional diagnostic methods for nematodes require laborious laboratory extraction, culture and microscopic examination of eggs or larvae from faecal samples. Advances in molecular technology offer the potential for more efficient and reliable methods [4]. The utilization of molecular biology techniques brought new approaches for diagnosis of nematodes. Methods to extract DNA of different forms of parasites in faecal samples of animals have allowed the use of molecular tools for the diagnosis of several organisms [8]. With the application of the polymerase chain reaction (PCR) it was possible to extend these studies. The PCR technique has been used to detect DNA of diverse organisms, facilitating the biological diagnosis of the parasites in tissues and secretions. Another important application of the PCR is the differentiation of helminth species that are morphologically indistinct. Through this technique were able to genetically differentiate *Haemonchus contortus* from *Haemonchus placei* [14]. When the target sequence is unknown, RAPD-PCR is useful [13]. This technique can be distinguished from the other PCR techniques by using a single very short arbitrary oligonucleotide, generally with 10 bases [1]. This is in contrast to the others that require information regarding the target DNA for the specific drawing of primers. It is a fast methodology requiring small amounts 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 [3]. So this study aimed to microscopic identification of nematodes infecting small ruminants and characterizes them molecularly by using RAPD-PCR.

2. Methodology:

2.1 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 liters, filtered through the sieve with an aperture of 250 μm capable of retaining the larvae. A duplicate of 200ml was transferred to a labeled plastic container and preserved in 10 % formalin. 20 ml of the sub-sample was taken onto a Petri dish, and 2-3ml 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 [11].

2.2 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 [5,11].

2.3 Samples Preparation and Extraction of DNA
Ten Samples were tested with RAPD PCR, between them 7 samples (adult worms) and 3 samples (larvae). After adult worms were recovered from GIT, washed several times with PBS and then preserved in epindurrf tubes that stored at -20°C till further use. The larvae and *Toxocara vitulorum* were used from positive preserved identified samples in our 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.

2.4 RAPD-PCR
Three random primers (primer 1. 5’- TCGCGAATTCC- 3’; primer 2. 5’-AACCGCGCAAC- 3’; primer 3. 5’- AAACGGTTGGGTGAG- 3’) were used to amplify the targeted DNA. PCR was performed in 25 ul of a mixture containing 0.5 ul 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 s of denaturation at 94 °C, 20 s of annealing at 55 °C, 60 s 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 [6].

2.5 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.

3. Results:

3.1 Prevalence of recovered nematodes
The recovered nematodes were Haemonchus contortus, Trichuris ovis, Trichostrongylus axei, Ostertagia ostertagi and Cooperia curticei, their measurements were recorded in tables (1, 2). The morphology of these worms was shown in figures (1, 2, 3, 4, and 5). The results in table (3) showed the infection rate of the different recovered worms. 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 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).

| Table 1. Measurements of recovered worms males and females in mm |
|---------------------------------------------------------------|
|                  | Male     | Female    | Male     | Female   |
|                  | Total length | breadth | Esophageal length | Vulvar flap length | Tail length | spicules breadth |
| Haemonchus contortus | 10-16 (13) | 0.20-0.30 | 1.00-1.15 (1.075) | 0.32-0.34 | 0.15-0.27 (0.165) |
| Ostertagia ostertagi | 7.00-9.5 (8.25) | 0.08-0.12 | 0.35-0.45 (0.40) | 0.20-0.27 (0.235) |
|                  | 9.00-10.00 (9.50) | 0.10-0.14 | 0.45-0.55 (0.50) | 0.06-0.15-0.25 (0.075-0.17-0.30) |
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  | 25.00-27.00         | 9.50-10.50           | 0.05-0.08            | 0.25-0.29             | 4.00-6.00 |
|          | (36.00)      | (26.00)             | (10.00)              | (0.65)               | (0.27)                |         |
| **Female** | 35.00-42.00 | 24.50-30.50         | 10.50-12.00          | 0.05-0.10            | 0.50-0.70             |         |
|          | (38.75)      | (27.50)             | (11.25)              | (0.075)              | (0.60)                |         |

Table 3. The infection rate of the different recovered nematodes

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

**Figure 1.** *Cooperia curticei* X10 A–anterior end showing cephalic vesicle X10; B–posterior end of female X10; C–vulvar region of female X10; D–uterus containing eggs X10

**Figure 2.** *Haemonchus contortus*, A–anterior end X10; B–male posterior end X4; C–female posterior end X4; D–female vulvar flap X4
3.2 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 (Figure 6). While, The RAPD-PCR analysis using primer 2 showed in (Figure 6), yielded 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 filliovus 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 any fragments recorded with The RAPD-PCR analysis using 3 tested Primers with ovine *Strongyloides papillosus* larvae as showed in (Figure 6). Overall, The RAPD-PCR analysis indicated polymorphism using 3 tested primers as illustrated in Figure 6.

![Figure 6](image)

**Figure 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 vitulorum* from buffaloes; 3-ovine *Ostertagia ostertagi*; 4-ovine *Trichuris ovis*; 5-caprine *Trichuris ovis*; 6- *Toxocara vitulorum* from cattle; 7- ovine *Cooperia curticei*; 8-ovine *Strongyloides papillosus* larvae; 9- ovine *Nematodirus filliollis* larvae; 10- ovine *Haemonchus contortus* larvae; 11- Negative control

4. **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. Sheep and goats were infected with different gastrointestinal nematodes with an infection rate of 57.89% and 46.15% in sheep and goats 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 [9,5].

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 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 [7] that 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 any fragments recorded with The 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 [2] 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 [12].

In conclusion, RAPD-PCR technique is able to explain the genetic relationship between helminths spp. infecting small ruminants.

5. 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 helminths spp. infecting small ruminants. RAPD-PCR indicated genetic polymorphism and genetic variability between different nematodes infecting small ruminants and within the same species.

6. References

[1] Ferreira, M.E. and Gratapaglia, D. (1996): Introdução ao Uso de Marcadores Moleculares em Análise Genética, Embrapa, Brasília 220 pp.

[2] Guclu, F.; Saldem, R.; Guler, L. (2004). Differential identification of cattle *Sarcocystis* spp. By random amplified Polymorphic DNA -Polymerase chain reaction (RAPD-PCR). Revue Méd. Vét., 155( 8-9):440-444.

[3] Jobet, E.; Bougnoux, M.; Morands, S.; Rivault ,C.; Cloarec, A. Hugot, J.P. ( 1998). Use of random amplified polymorphic DNA (RAPD) for generating specific DNA probes for oxyuroid species (Nematoda). Parasite 5: 47-50.

[4] Learmount, J.; Conyers, C.; Hird, H.; Morgan, C.; Craig, B.; von Samson-Himmelstjerna,G.; Taylor, M. (2009). Development and validation of real-time PCR methods for diagnosis of *Teladorsagia circumcincta* and *Haemonchus contortus* in sheep. Vet. Parasitol., 166(3-4):268-274.

[5] MAFF (1986). Manual of Veterinary Parasitological Laboratory Techniques. Ministry of Agriculture, Fisheries and Food. Reference Book No. 418. Her Majesty’s Stationary Office, London, UK.

[6] Nashwa, I.; Lobna, M.S.; Maha, M.A. and Nabila, A. (2010). Molecular Genetic Approach by using the RAPD-PCR Technique for Detection of Genetic Variability in Non-Human Isolates of *Fasciola*. Amer. Sci. J., 6(9):773-779

[7] Rabouam, C.; Comes, A.; BretAgnolle, V.; Humbert, J.; Periquet, G. and Bigot, Y. (1999). Features of DNA fragments obtained by random amplified polymorphic DNA (RAPD) assays. Mol. Ecol., 8(3):493-503.

[8] Schneider, T.; Heise, M. and Epe, C. (1999). Genus-specific PCR for the differentiation of eggs or larvae from gastrointestinal nematodes of ruminantes. Parasitol. Res., 85: 895-898.

[9] Soulsby, E.J.L. (1986): Heminths, Arthropods and protozoa of domesticated animals. 7th ed. Baillier, Tidal and Cassel, London.

[10] Suarez, V.H. and Busetti, M.R. (1995): The epidemiology of helminth infection of growing sheep in Argentina’s Western Pampas, *Int. J. Parasitol.*, 25: 489–94.

[11] Urquhart, G.M.; Armour, P.; Duncan, J.L., Dunn, A.M. and Jennings, F.W. (1996): Veterinary Parasitology. ELBS. Longman. England 2nd ed.
[12] Verhasselt P., Voet, M. and Volckert, G. (1992). DNA sequencing by a subcloning-walking strategy using a specific and semi-random primer in the Polymerase Chain Reaction. Int J Parasitol., 28: 1053-1060.

[13] Williams, J.G.K.; Kubelik, A.R.; Rafalski, J.A. and Tinger, S. (1990): DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18: 6531-6535.

[14] Zarlenga, D.S.; Stringfellow, F.; Nobary, M. and Lichtefels, J.R. (1994). Cloning and characterization of ribosomal RNA genes from three species of Haemonchus (Nematoda: Trichostrongyloidea) and identification of PCR primers for rapid differentiation. Exp. Parasitol. 78: 28-36.

7. Acknowledgements:
This study was conducted in Department of Parasitology, Faculty of Veterinary Medicine, University of Sadat City. Authors are grateful to abattoirs veterinarians at Menoufia and University of Sadat City for support during this study.