



## DEVELOPMENT AND APPLICATION OF A METHOD FOR GENETIC DETECTION OF *ENTEROBIUS VERMICULARIS* IN SAMPLES OF PATIENTS WITH ENTEROBIASIS

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### ABSTRACT

**Purpose.** The routine diagnosis of enterobiasis consists of a microscopic examination of perianal imprints on a transparent scotch tape for the presence of parasitic eggs. However, this method has low sensitivity when the number of eggs is small. The development of highly sensitive and specific methods, such as PCR, will increase the possibilities of detecting the parasite and will enable its genetic characterization.

**Material/Methods.** The study was conducted from May 2022 to September 2022 and comprised 24 patients infected with *Enterobius vermicularis*. DNA was extracted from parasite eggs/adult worms using PureLink Genomic DNA Mini Kit (INVITROGEN), according to the manufacturer's instructions. Nested *polymerase chain reaction (PCR)* for detection of mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene was applied with two sets of primers.

**Results.** The developed and described method for genetic determination of *Enterobius vermicularis* in samples from infected individuals is suitable and applicable to improve the diagnosis of the disease, as well as for further phylogenetic research of the parasite, which is of great importance for its successful treatment and control.

**Conclusions.** Regardless of existing morphological diagnostic methods, genotyping studies concerning *Enterobius vermicularis* need to be deepened. Molecular-biological analyses increase diagnostic sensitivity, provide valuable knowledge about the geographic distribution and diversity of helminths, and allow the monitoring of therapy.

**Keywords:** *Enterobius vermicularis*, enterobiasis, nested PCR, *cox1* gene,

### INTRODUCTION

Enterobiasis is an intestinal parasitic infection caused by the small nematode *Enterobius vermicularis*, first identified by Karl Linnaeus in 1758. This parasite (also called pinworm) inhabits the lower small and upper large intestines of humans, with females emerging at night actively through the anus and laying their eggs in the perianal folds. Under optimal environmental conditions - oxygen, moisture, and a temperature of 37°C, the eggs mature in 4-6 hours and reach the infective stage [1]. The transmission occurs by the fecal-oral route, by swallowing infective eggs from contaminated food products, various objects, unwashed hands, and also by inhaling dust in which eggs of the parasite have fallen.

The symptoms of the disease caused by *Enterobius vermicularis* are mostly due to the mechano-traumatic effect that the parasites cause when they are fixed in the intestinal mucosa. The pinworms inflict motor and secretory disorders of the digestive tract and toxoallergic effects on the body [2]. A major symptom of the disease is perianal itching, abdominal discomfort, loss of appetite, weight loss, insomnia, and restlessness [3, 4]. Complications such as acute appendicitis, vulvovaginitis, urethritis, and cystitis can occur [5, 6].

Patients with enterobiasis are often reinfected as a result of the large amount of eggs released and their long endurance in the environment. They can remain viable for 2-3 weeks on clothing, bed linens, and other objects [4].

Diagnosis of the disease is mainly microscopic - by helmintoovoscopy of perianal imprints taken with transparent scotch tape (Graham's method) [7]. Adult pinworms can also be detected macroscopically in a fecal sample.

Enterobiasis is a widespread parasitic infection. It occurs most often in children due to their undeveloped hygiene habits [8]. This parasite is common in overcrowded institutions (kindergartens, schools, orphanages, and psy-

chiatry clinics) and among family members of low socioeconomic status, especially those with poor living conditions [9]. In Bulgaria, this is the most common parasitic infection [10, 11].

Despite the wide distribution of *Enterobius vermicularis* worldwide, studies regarding its molecular characterization are limited [12]. There are paleoparasitological studies that detect the presence of *Enterobius vermicularis* in ancient coprolites and compare the sequence similarity between prehistoric and modern helminths [13, 14]. The 5S rRNA intergenic spacer (gene) was successfully used as a PCR target to detect pinworms in Amerindian coprolites [15].

In 2016, Dawood K, et al. developed a PCR technique to determine the 5S rRNA gene of *Enterobius vermicularis* in fecal samples, and the results showed that this gene could be used for the diagnosis of enterobiasis. According to the authors, the applied method is useful for the identification of the parasite and can be used to prove the parasite DNA in samples from infected individuals [16]. In 2020, Khazaal et al. published studies on the diagnosis of enterobiasis by conventional PCR using *coxI*, 12S mtDNA, ITS1, and 18S rRNA genes and determined genetic variation using 18S ribosomal RNA in samples from infected children [17]. In 2006, a phylogenetic study of *Enterobius* was performed in Japan, which identified 3 distinct clusters, designated as type A, B, and C, based on sequencing of the gene encoding mitochondrial subunit 1 of cytochrome c oxidase (*cox1*) in human samples and chimpanzees [18].

However, the existing molecular biological studies regarding *Enterobius* are small in volume and too limited. Therefore, our research aims to develop a method for successful genetic detection of this parasite and to determine its importance and possible application in the diagnosis of enterobiasis.

## MATERIALS AND METHODS

### Study population

The study was conducted from May 2022 to September 2022. The study population comprised 24 patients - 21 children (87.5%) and 3 adults (12.5%). Five of the children were aged between 10-18 years, and 16 were under 10 years of age. Of the individuals enrolled in the study,

12 (50%) were males and 12 (50%) females, aged from three to 41 years (mean age of 11 years).

### Sample collection

From each patient, a perianal swab was collected early in the morning and stored at 4 °C until delivered to the laboratory. Perianal swabs were examined under the light microscope at 100× and 400× magnification. A sample containing at least one *E. vermicularis* egg was considered positive. A total of 24 perianal swabs were tested. Stool samples were also obtained from the examined persons, and in some of them, adult pinworms were detected. Stool samples were further examined by microscopy to rule out the presence of other intestinal parasites.

A questionnaire was used to collect socio-demographic details (e.g. age, gender, residence, and education) of the participants, symptoms suggestive of enterobiasis, the history of other parasitic infections, and their treatment. No personal patient data is disclosed in this study.

### Collection of eggs from a perianal tape impression

Parasite eggs from the perianal tape impression were scraped off the glass slide using a disposable scalpel and transferred into tubes containing aliquots of 3 different solutions (70% ethanol, sterile water, and sterile saline solution) until DNA extraction.

### Collection of adult worms from a stool sample

Adult worms of *E. vermicularis* were obtained from the feces of infected individuals and stored in a saline solution until DNA extraction.

### DNA extraction

DNA was extracted from parasite eggs/adult worms using PureLink Genomic DNA Mini Kit (INVITROGEN), according to the manufacturer's instructions. Thirty µl of proteinase K were added to the samples containing 200 µl ATL Digestion buffer and incubated at 56°C for 24 h. The elution of DNA was in 100 µl Elution buffer. The purified DNA was stored at -20°C.

### PCR

Amplification reactions were carried out according to Piperaki E, et al. (2011) [19]. Nested *polymerase chain reaction (PCR)* for detection of mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene was applied with two sets of primers. The DNA sequences of the primers are shown in Table 1.

**Table 1.** Primer sets used in the study (according to Piperaki E, et al. [19])

Primer set	Primer name	Sequences (5' to 3')	Primer length [Base pair, bp]	Amplicon size [bp]
Outer	Forward - EVM1	TTTTTGGTCATCCTGAGGTTTATATTC	27	390
	Reverse - EVM2	CCACATTATCCAAAATAGGATTAGCC	26	
Inner	Forward - EVIF	TTGGTCATCCTGAGGTTTATATTC	24	379
	Reverse - EVIR	TCCAAAATAGGATTAGCCAACA	22	

Amplifications were performed in a 25- $\mu$ l reaction volume, using AmpliTaq Gold 360 Master Mix (Applied Biosystems by Thermo Fisher Scientific), containing GC Enhancer for optimization of the PCR reaction conditions, 0.2 mM (each) primer and 3  $\mu$ l of template DNA. Thermal cycling was carried out in a model 96G DNA thermal cycler (BIOER Gene Explorer) with the following cycling protocol: – initial denaturation at 94°C for 5 min followed by 45 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, and a final *extension* step at 72°C for 10 min. Three  $\mu$ l of the first PCR reaction were used as a template for the second PCR. Conditions for the second PCR were changed in two directions as follows: 1) the annealing temperature was set at 53°C, and 2) the number of cycles was reduced to 30 [19]. A negative (water) control was included in each round of the nested PCR.

#### Agarose gel electrophoresis

Eight microliters of the products of both the first and the second PCR rounds were separated by electrophoresis in 3% agarose gel containing 0.5  $\mu$ g/ml fluorescent nucleic acid stain (peqGREEN DNA/RNA dye (VWR International GmbH, Germany), visualized on a UV-transilluminator and photographed (SYNGENE gel documentation system (GelVue Model No. GVM20, Synoptics Ltd, UK). A molecular size marker 100 bp (GeneRuler™ 100bp DNA Ladder, Thermo Scientific, Lithuania) was used to determine the product sizes.

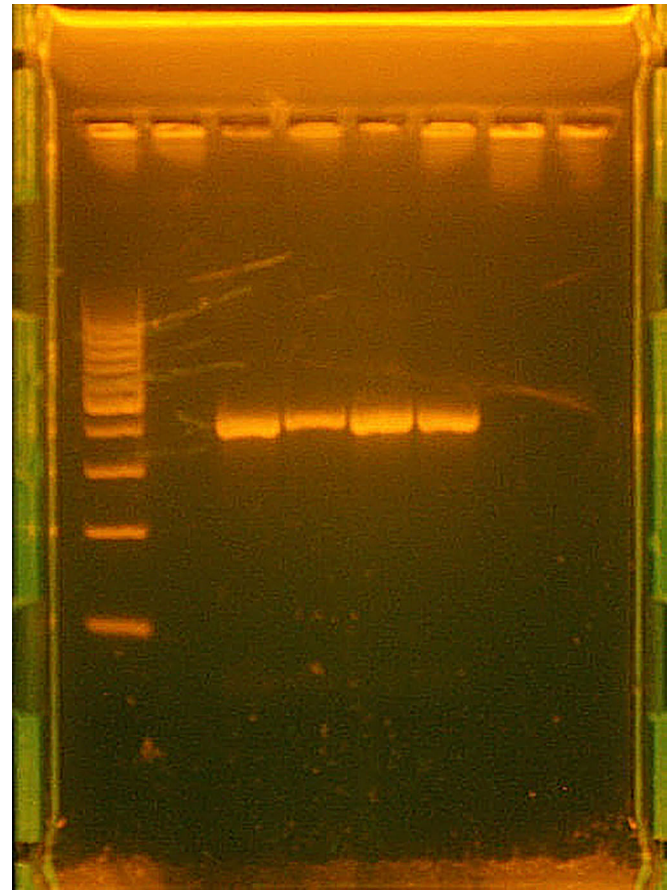
## RESULTS

Nested PCRs were performed using DNAs extracted from stool specimens and *specimens* collected from the skin of the *perianal* area. During the pre-PCR processing, sample preparation and parasite DNA isolation methods were tested. Parasite eggs scraped from the perianal tape impression using a disposable scalpel were collected in 3 different solutions (70% ethanol, sterile water, and sterile saline solution) to investigate the influence of the solution type on DNA isolation. The comparison of the resulting PCR products suggested that the best results were obtained when the parasite eggs were placed in a sterile saline solution. In our study, weak PCR bands were obtained from all DNA samples isolated from eggs collected and preserved in 70% ethanol before DNA extraction. Relatively good results were also obtained when parasite eggs were placed in sterile water before the DNA isolation.

To ensure better destruction of the parasite eggs and adult worms, before DNA extraction, samples were subjected to 3 cycles of freezing at -80°C and thawing at room temperature.

Parasite DNA of sufficient purity and quantity was obtained when, during the collection process, parasite eggs and adult worms were placed in a sterile saline solution (Fig. 1).

**Fig. 1.** Nested PCR results of *Enterobius vermicularis* from the examination of parasite eggs placed in water (3, 4) and in sterile saline (5, 6).



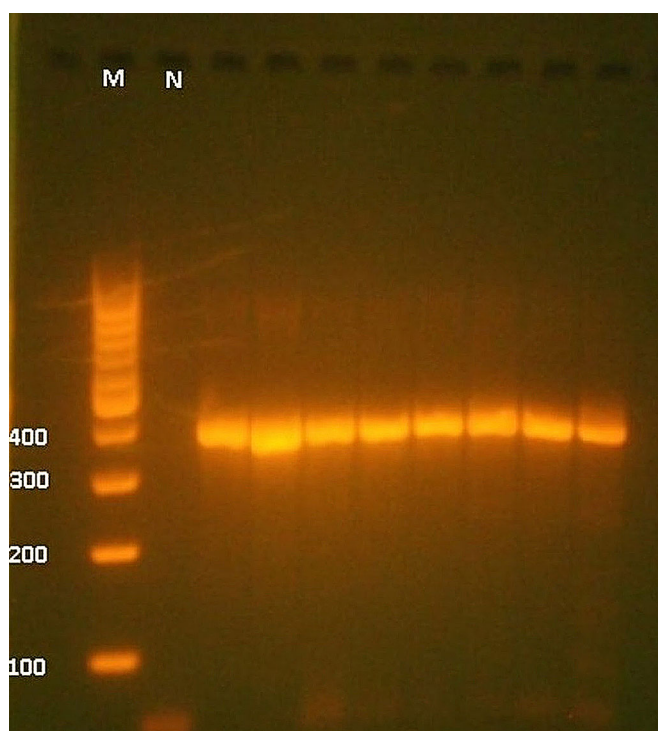
Legend: 1. 100bp DNA Ladder; 2. Negative control; 3, 4, 5, 6 – Patients with enterobiasis

*E. vermicularis*-specific products with the expected length of ~390 bp, amplified with the outer primer pair, were detected in all samples obtained from eggs except one. In the second round of the nested PCR positive signal (amplicon length of 379 bp) was registered from all examined samples (Fig. 2).

PCR products were electrophoresed in 3% agarose gel containing fluorescent nucleic acid stain and visualized under UV light. *E. vermicularis*-specific products of the expected sizes (390 bp and 379 bp with the inner and outer primer pairs, respectively) were successfully amplified from all tested samples isolated from adult forms of the parasite (Fig. 2 - lane 3,4). These results showed that the nested PCR method applies to the genetic detection of both eggs and adult parasites (Fig. 2).

To determine the diagnostic sensitivity of the nested PCR for detection of *E. vermicularis*, DNA was isolated from samples containing different numbers (1 to 5) of eggs

**Fig. 2.** Nested PCR of *Enterobius vermicularis* from Bulgarian persons with enterobiasis (expected product length ~ 379 bp).



Legend: 1. 100bp DNA Ladder; 2. Negative control; 3, 4 - adult forms of *Enterobius vermicularis*; 5, 6 - three eggs of *Enterobius vermicularis*; 7, 8 - two eggs of *Enterobius vermicularis*; 9, 10 - one egg of *Enterobius vermicularis*;

placed in a saline solution. The results showed the presence of characteristic parasite-specific bands in all tested specimens and in DNA samples extracted from one egg as well. This observation confirms the high sensitivity of the PCR technique for the molecular biological analysis of pinworms (Fig. 2 – lanes 5, 6, 7, 8).

## DISCUSSION

*Enterobius vermicularis* is one of the most common parasitic helminths in humans [9]. There are an estimated 200 million people infected with the pathogen worldwide, especially children [4]. Humans can become infected by ingesting contaminated food or water, through direct contact with infected individuals, and rarely, by ingesting airborne eggs [20]. The high contagiousness of enterobiasis favors its widespread distribution. Other factors that determine the high incidence of infection with this parasite are the presence of auto- and frequent re-infections and the existence of asymptomatic carriers of *E. vermicularis*, which are a potential source of transmission and a significant public health problem [21].

The main method for diagnosing enterobiasis is the cellophane tape test method (known as the Scotch tape

method, Graham's scotch tape method). The eggs, if present on the tape, will be visible under a light microscope. However, this method has low sensitivity for weak invasion. Therefore, molecular genetic methods have been intensively developed and applied in recent years.

Both mitochondrial DNA (mtDNA) and internal transcribed spacer (ITS) region are often used in genetic analysis, as it has been found that mitochondrial genes are more suitable for population genetic studies, while the ITS is more useful for parasite identification [22]. The mitochondrial genome of *E. vermicularis* was described and reported in 2009 [23], which is a 14,010-bp circular DNA molecule that encodes 36 genes (12 proteins, 22 tRNAs, and 2 rRNAs). Based on phylogenetic analyses of nucleotide sequences for the 12 protein-coding genes of 25 nematode species, *E. vermicularis* was placed as a sister to the main Ascaridida+Rhabditida group. A unique mtDNA gene order of the parasite, not sharing similarities to any other nematode species, was reported [23].

Phylogenetic studies of *E. vermicularis* based on sequences of mtDNA gene encoding the cytochrome c oxidase subunit 1 (*cox1*) have been performed in samples obtained from chimpanzees and humans, showing the presence of 3 distinct clusters (designated as type A, B, and C) [18]. In 2011, Piperaki et al. performed the first study of *E. vermicularis* genotypes among Greek children by examination of scotch tape samples using primers to amplify a segment of the *cox1* gene of this parasite [19].

In addition to sequence analysis based on the mitochondrial *cox1* gene, nuclear genes coding for the ribosomal RNA (rDNA) of *E. vermicularis* were studied as well [24]. The rDNA has been used extensively as a molecular marker for analyzing phylogenetic relationships and for microbial identification, although the small gene encoding the 5S rRNA in nematodes is highly conserved. Dawood et al. (2016) developed a PCR technique to determine the presence of the *E. vermicularis*-specific 5S rRNA gene in stool samples. This gene was considered a good target for the molecular study of *E. vermicularis* [16]. Data from molecular analyses of rDNA from adult *E. vermicularis* showed that this marker cannot be used to distinguish different parasite isolates but only for diagnostic purposes [24]. The 5S rRNA gene spacer region has been used for systematic, diagnostic, and phylogenetic relationship studies in nematodes based on their size difference and sequence variations [25].

Mitochondrial DNA has several advantages when studying evolutionary interactions in nematodes, especially for distinguishing closely related species [26]. This is due to its high mutation rate and low effective population size, which provides rapid linear sorting between species. A comparative mitochondrial analysis is used to identify cryptic species that cannot be identified by traditional morphological methods [27].

The primers used in our study target the *mitochon-*

drial gene encoding the *cytochrome c oxidase subunit 1* (*cox1*), and the results obtained show that this gene is suitable for genetic identification of the parasite *Enterobius vermicularis*. This analysis was successfully applied to both the parasite adult forms and the eggs released. The high sensitivity of the method allowed the parasite determination even when the examined material contained one egg.

PCR-based identification represents a good possibility for the parasite diagnosis, especially in people without symptoms or when the parasite cannot be demonstrated by microscopic methods. The application of molecular-genetic techniques in the diagnostics process offers new opportunities for conducting subsequent molecular-epidemiological studies of *E. vermicularis*.

## CONCLUSION

Regardless of existing morphological diagnostic methods, genotyping studies concerning *Enterobius vermicularis* need to be deepened. Molecular-biological analyses increase diagnostic sensitivity, provide valuable knowledge about the geographic distribution and diversity of helminths, and allow the monitoring of therapy.

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## REFERENCES:

1. [Clinical parasitology and tropical medicine.] Editors: Petrov P. Kurdova R. Publ. East-West, Sofia. 2016. p. 266. [in Bulgarian]
2. [Parasitology (local and tropical parasitoses).] Editors: Boeva-Bangyozova V. Vutova K. Publ. Arso, Sofia. 2008. p. 404. [in Bulgarian]
3. Vermund SH, Wilson CM. Pinworm (*Enterobius vermicularis*). *Semin Pediatr Infect Dis.* 2000 Oct;11(4):252–256. [Crossref]
4. Burkhart CN, Burkhart CG. Assessment of frequency, transmission, and genitourinary complications of enterobiasis (pinworms). *Int J Dermatol.* 2005 Oct;44(10):837-40. [PubMed]
5. Zahariou A, Karamouti M, Papaioannou P. Enterobius vermicularis in the male urinary tract: a case report. *J Med Case Rep.* 2007 Nov 14;1:137. [PubMed]
6. Panidis S, Paramythiotis D, Panagiotou D, Batsis G, Salonikidis S, Kaloutsis V, et al. Acute appendicitis secondary to Enterobius vermicularis infection in a middle-aged man: a case report. *J Med Case Rep.* 2011 Nov 30;5:559. [PubMed]
7. Garcia LS. Diagnostic Medical Parasitology. 6th Edn. ASM Pres. 15 December 2015: pp.98-99. [Crossref]
8. Cook GC. Enterobius vermicularis infection. *Gut.* 1994 Sep;35(9):1159-62. [PubMed]
9. Al-Daoudy AA, Qadir FM, Tahir A, Mahmood NH, Majeed AF. Risk factors of *Enterobius vermicularis* infection with symptoms among children in Erbil governorate. *Pak-Euro J Med Life Sci.* 2020; 3(2):50-58. [Internet]
10. Harizanov R, Rainova I, Tsvetkova N, Kaftandjiev I, Borisova R, Ivanova A, et al. Prevalence of Intestinal Parasitic Infections among the Bulgarian Population Over a Three Year Period (2015 - 2017). *Helminthologia.* 2020 Jan 25;57(1):12-18. [PubMed]
11. Rainova I, Harizanov R, Tsvetkova N, Borisova R, Kaftandjiev I, Kaneva E, et al. Status of parasitic diseases in Bulgaria in 2018. [in Bulgarian] *General Medicine.* 2020; 22(1):13-18. [Internet]
12. Iñiguez AM, Vicente AC, Araújo A, Ferreira LF, Reinhard KJ. Enterobius vermicularis: specific detection by amplification of an internal region of 5S ribosomal RNA intergenic spacer and trans-splicing leader RNA analysis. *E. vermicularis: specific detection by PCR and SL1 RNA analysis. Exp Parasitol.* 2002 Nov-Dec;102(3-4):218-22. [PubMed]
13. Iñiguez AM, Reinhard KJ, Araújo A, Ferreira LF, Vicente AC. Enterobius vermicularis: ancient DNA from North and South American human coprolites. *Mem Inst Oswaldo Cruz.* 2003; 98 Suppl 1:67-9. [PubMed]
14. Iñiguez AM, Reinhard K, Carvalho Gonçalves ML, Ferreira LF, Araújo A, Paulo Vicente AC. SL1 RNA gene recovery from Enterobius vermicularis ancient DNA in pre-Columbian human coprolites. *Int J Parasitol.* 2006 Nov;36(13):1419-25. [PubMed]
15. Iñiguez AM, Araújo A, Ferreira LF, Vicente AC. Analysis of ancient DNA from coprolites: a perspective with random amplified polymorphic DNA-polymerase chain reaction approach. *Mem Inst Oswaldo Cruz.* 2003; 98 Suppl 1:63-5. [PubMed]
16. Dawood KA, Al-Essami AH, Rathy RM. Identification of 5S rRNA gene of *Enterobius vermicularis*. *AL-Qadisiva Medical Journal.* 2016; 12(21):25-29. [Internet]
17. Khazaal RM, Al-Hadraawy SK, Hussein KR. Molecular identification and phylogenetic analysis of Enterobius vermicularis isolated from children in Thi-Qar City of Iraq. *Int J Pharm Res.* 2020 Oct-Dec;12(4):1919-1930. [Crossref]
18. Nakano T, Okamoto M, Ikeda Y, Hasegawa H. Mitochondrial cytochrome c oxidase subunit 1 gene and nuclear rDNA regions of Enterobius vermicularis parasitic in captive chimpanzees with special reference to its relationship with pinworms in humans. *Parasitol Res.* 2006 Dec;100(1):51-57. [PubMed]
19. Piperaki ET, Spanakos G, Patsantara G, Vassalou E, Vakalis N, Tsakris A. Characterization of Enterobius vermicularis in a human population, employing a molecular-based method from adhesive tape samples. *Mol Cell Probes.* 2011 Apr-Jun;25(2-

3):121-5. [\[PubMed\]](#)

20. Kim BJ, Lee BY, Chung HK, Lee YS, Lee KH, Chung HJ, et al. Egg positive rate of *Enterobius vermicularis* of primary school children in Geoje island. *Korean J Parasitol.* 2003 Mar;41(1):75-7. [\[PubMed\]](#)

21. Al-Daoudy AA. [Prevalence of *Enterobius vermicularis* infection among children of AL-Nusoor kindergarten in Mosul city and the effect of some disinfectant on its eggs viability.] [in Arabic] *Rafidain Journal of Science.* 2005 Aug;16(6):188-201. [\[Internet\]](#)

22. Blouin MS. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus in-

ternal transcribed spacer. *Int J Parasitol.* 2002 May;32(5):527-31. [\[PubMed\]](#)

23. Kang S, Sultana T, Eom KS, Park YC, Soonthornpong N, Nadler SA, et al. The mitochondrial genome sequence of *Enterobius vermicularis* (Nematoda: Oxyurida)—an idiosyncratic gene order and phylogenetic information for chromadorean nematodes. *Gene.* 2009 Jan 15;429(1-2):87-97. [\[PubMed\]](#)

24. Zelck UE, Bialek R, Weiß M. Molecular phylogenetic analysis of *Enterobius vermicularis* and development of an 18S ribosomal DNA-targeted diagnostic PCR. *J Clin Microbiol.* 2011 Apr;49(4):1602-4. [\[PubMed\]](#)

25. Favia G, Bazzocchi C, Cancrini G, Genchi C, Bandi C. Unusual organization of the 5S ribosomal spacer in *Dirofilaria repens*: absence of a canonical spliced leader 1 sequence. *Parasitol Res.* 2000 Jun;86(6):497-9. [\[PubMed\]](#)

26. Hu M, Chilton NB, Gasser RB. The mitochondrial genome of *Strongyloides stercoralis* (Nematoda) - idiosyncratic gene order and evolutionary implications. *Int J Parasitol.* 2003 Oct;33(12):1393-408. [\[PubMed\]](#)

27. Nadler SA, DE León GP. Integrating molecular and morphological approaches for characterizing parasite cryptic species: implications for parasitology. *Parasitology.* 2011 Nov;138(13):1688-709. [\[PubMed\]](#)

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