International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 23s, 2025
https://www.theaspd.com/ijes.php

New Genetic Diversification Of The Type A Pinworm, Enterobius Vermicularis With The Study Of Their Phylogenetic Position And Secondary RNA Structure Analysis

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abstract : *aim of this study was to assess the molecular characterization and genotypes of the pinworm, Enterobius vermicularis isolated from children in the city of Erbil, the capital of the Kurdistan Region of Iraq, given the paucity of existing data on the genetic typing of the parasite among Iraqi children. For this purpose, several worms had been collected from infected children in Erbil City then transported to the laboratory. DNA was extracted from the sample. Sequence amplification had been done using universal primer of Cytochrome Oxidase C Subunit One (COI) gene. Results confirm the occurrences of type A genotypes of the pinworm, Enterobius vermicularis. Six local sequences were isolated and recorded in Gen-Bank under the accession number, PV085602, PV085603, PV085604, PV085605, PV085606 and PV085607. Mitochondrial cytochrome c oxidase subunit 1 gene analysis confirmed that the isolated sequences were belong to the type A pinworm, E. vermicularis based on 100% to 99.33% likeness of fractional groupings with type A. Phylogenetic investigation uncovered that the groupings clustered at the side other type C Chimpanzee related E. vermicularis (under the accession number, AB971673 and AB971669) which varies from human pinworm (type A). Results of the secondary structure analysis of the RNA confirms a distinct genetic variation depending on the number and types of their RNA loops. In conclusion, the present study confirms that the isolated sequences are belong to type A human E. vermicularis and it makes a sister group with type C chimpanzee E. vermicularis. A minor genetic diversification among isolated sequences were noticed. The isolation and investigation of genetically diverse type A pinworm, E. vermicularis from human represent the first investigation in Iraq*
Keywords: Oxyuriasis, RNA folding, Diversity, COI, Erbil

INTRODUCTION

The Study of the mitochondrial cox1 gene in pinworms found from humans and captive chimpanzees showed three different genetic groups called type A, B, and C (Nakano, Okamoto, Ikeda, & Hasegawa, 2006). Traditionally, diagnosis of *E. vermicularis* in vitro has been based on phenotypic strategies that consider their morphological and natural properties. Since these strategies are time expending and in fact exceptionally tiring, molecular strategies based on DNA enhancement strategies are progressively utilized for quick, precise, and particular characterization of parasites. Molecular discovery of human parasitic pathogens gives a comprehensive asset and solid for molecular diagnosis and identification of major human parasitic pathogens. A useful genetic marker that helps identify the organisms is *Cytochrome Oxidase C Subunit One. (COI)* as mentioned by (Al-Taei & Al-Quraishi, 2022; Jain *et al*., 2025). The COI gene quality is regularly utilized in phylogenetic investigations and species separation since of its developmental solidness and presence over an assortment of nematode species (Zhang, Xu, Sun, Dumont, & Han, 2021). Secondary structure is the first step in understanding the more complex tertiary structure. In fact, throughout evolution, members of an RNA family tend to keep their secondary structures the same more than they keep their primary sequences (Yu, 2006). In this report, we focus on predicting RNA secondary structures. Nematodes of the species *Enterobius vermicularis*, commonly called pinworms, are among the

most common nematode disease in the Middle East, Europe and USA (Dogan, 2024; Vanhove *et al.*, 2025). that infect around billion people throughout the word and it is medical intestinal parasite that cause several health disorder among children (Jasim, 2025).

The aim of this study was to assess the molecular characterization and genotypes of the pinworm, *Enterobius vermicularis* isolated from children in the city of Erbil, the capital of the Kurdistan region of Iraq, given the paucity of existing data on the genetic typing of the parasite among Iraqi children

MATERIALS AND METHODS

Description of Study Area.

Erbil City, also called Hawler in Kurdish and historically known as Arbela, is the capital and largest city in the Kurdistan Region of northern Iraq. It is located at the geographic coordinates of 36. 206293 latitudes and 44. 008870 longitudes. In terms of GPS, this translates to 36 degrees 12 minutes 22. 6548 seconds north and 44 degrees 0 minutes 31. 932 seconds east.

Sampling

Worm sampling was done throughout a time ranging from beganing of November 2024 to the end of January 2025 among children attending medical laboratories in different qurters (Figure 1) from Erbil City, Kurdistan Region, Iraq.

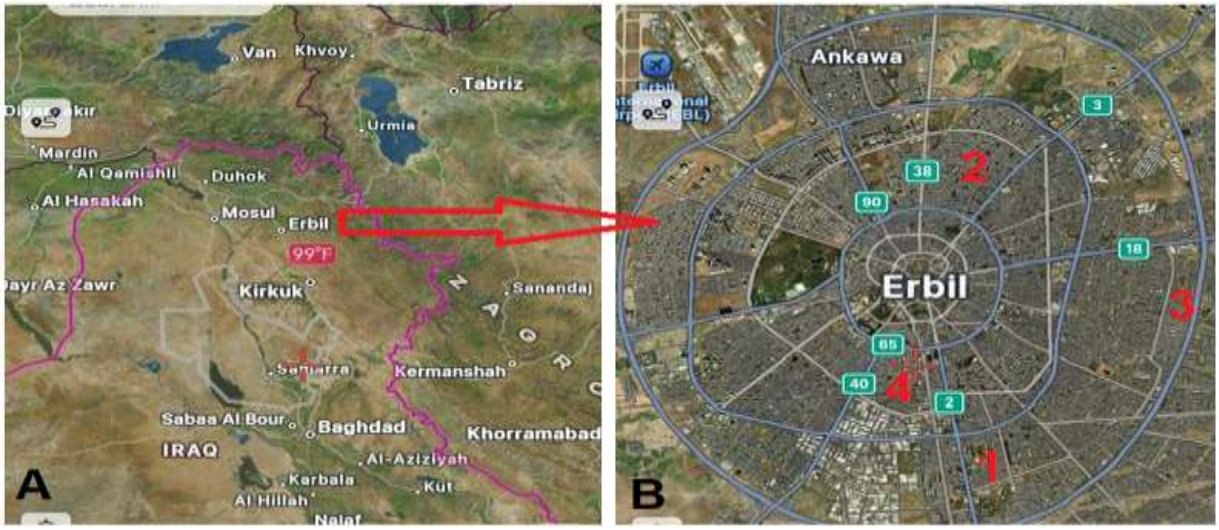

Fig. 1: A-Map of Iraq. B-Map of Erbil City showing the specific quarters within Erbil City where warm sampling was conducted: 1-Farmanbaran, 2- Bakhtyari 3- Zilan City and 4-Zanko Village.

Prepare and Preserve of Samples for Genomic DNA Extraction

In order to prevent the destruction of its genomic DNA and achieve acceptable extraction quality, some of the collected worms were maintained in 95% alcohol and others in sterile injection water. They were then kept frozen at 0 C° in both home and lab refrigerators until they were needed. Ethanol was taken out of the parasites just before extracting the DNA. To eliminate ethanol, the specimens were allowed to air dry.

DNA Extraction

Genomic DNA was isolated from samples and it was extracted by using Beta Bayern Kit for preparing tissue DNA (Beta Bayern GmbH .90453 Bayern, Germany) granting the production's instructions with few modifications.

Quantification and Qualification of Genomic DNA

NanoDrop was used to achieve both the quantity and quality of DNA concentration. (ND- 1000, USA). The results of genome DNA specimens containing more than 0.5 µg and (A260–A320) / (A280– A320) ratio of more than 1.7 qualities was obtained.

Polymerase Chain Reaction (PCR) Amplification

Polymerase chain reaction was done for Cytochrome Oxidase C subunit One (COI) gene. So, each PCR reaction was performed using 50 µl of reaction mixture, which contained 2x Taq DNA Polymerase Master Mix (AMPLIQON A/S Stenhuggervej 22), 10 Picomol (pmol) primers, DNase free water and template DNA (Table 1) by Bioresearch PTC-200 Gradient thermocycler.

Table 1: Pair of primers used in COI gene sequence

Primer code	Sequence 5'-3'	Amplicon size(bp)	PCR Condition
COI-F	5'-GGTCAACAAATCATTAAAGATATTGG-3'	720	95°-5 min; 95°-35 sec, 59°-35 sec, 72°-1 min; 72°-10 min; 4° ∞
COI-R	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'		

Agarose Gel Electrophoresis

Agarose gel electrophoresis is a key part of most standard molecular biology experiments, a technique for separating DNA fragments according to their molecular weight (Linna, 2024; Toranzos, 2024) The method comprises three main steps: agarose gel preparation, DNA fragmentation electrophoresis, as well as DNA fragment visualization. After melting 1.0–1.5% agarose powdered in 1X TBE buffer, the mixture was heated in a microwaves until a pure solution was achieved. The flask was **swirled** so the solutions could **cool** down to about 45 to 37 degrees Celsius. After the agarose gel melted, 5–10 µl of safe dye stain solution was applied and completely mixed. After being poured into the casting tray, the heated agarose solutions were let to harden. Slowly, the combs were removed. The electrophoresis chamber was filled with the gel. sufficient TBE buffer was applied to cover the gel by two to three millimetres5 µl of collected DNA from each sample was combined with 2-3 µl of 6X loading buffer dye and pipetted into different gel wells. Slowly, a DNA ladder (1500–100 base pairs) was pipetted into a different gel well. The same process was used for loading PCR products, but since the AMPLIQON master mix comprised red loading buffer staining, specimens did not need to be mixed with 6X loading solution staining. The power source was linked to the electrode wires, confirming the correct connection between the positive (red) as well as negative (black). The electric source was adjusted between 50 and 100 volts. Once the samples had RNA long enough, the power source was shut off. Gloves were used to remove the gel, which was then visualized under UV light, photographed, and recorded using a UV trans illuminator. DNA segment electrophoresis. Charged molecules can be separated using the electrophoresis process. Since DNA has a negatively charged at neutrality pH, it moves towards the anode once an electric field is introduced across the gel. DNA movement across the gel is based on; DNA molecule size, Agarose concentration, the visualization of DNA fragments using applied current as well as DNA conformational: DNA won't show up on the gel because it isn't naturally colored. Therefore, a DNA-specific dye is applied to the gel prior to electrophoresis. After adding an intercalating dye, such as a safe dye, to agarose gel, the gel is examined under a UV trans illuminator to detect the bands' locations (Aljohani & El-Khatib, 2024).

Sequencing of DNA

The sample of PCR product for the COI partial gene was sequenced by ABI Prism Terminator Sequencing Kit (Applied Biosystem) at Macrogen Molecular Company of Korea. The Finch TV tool software was used to confirm base calls and alter the chromatogram genes.

Sequence Alignment

Isolated sequences had been submitted to NCBI (National Center for Biotechnology Information) in order to obtain on accession number. Sequences were aligned at Basic Local Alignment Search Tool (BLAST)

using, https: // blast. ncbi. nlm. nih. Gov / Blast. cgi to comparing and alignment laboratory or query sequence with the same sequencing fragment marker (COI) to find out more similarity with *E. vermicularis.* The isolated sequences' phylogenetic location was determined by repeated alignment among a number of related pinworm sequences (10 highly query sequence identity 100%). Associated sequences were chosen for this purpose, obtained from NCBI, and then aligned with one another. (adding *Ascaris lumbricoides*, with accession number of BU585703.1 as an out group) utilizing Muscle model within MEGA software v.11 (Program Files\ MEGA11 \ MEGA_64.exe.).

RESULT AND DISCUSSION

In the current study, Cytochrome Oxidase C Subunit One (value of 3000bp) of *E. vermicularis* was analyzed, the primers could yield a band of the expected size of 720bp. The Polymerase chain reaction product was electrophoresed and visualized by 1.5% Agarose gel (Figure 2). Sequencing of PCR products had been done for several samples (six isolated samples) in Korea Country. All the isolated sequences were recorded in the GeneBank from National Center for Biotechnology Information (NCBI) under the following accession number, PV085602, PV085603, PV085604, PV085605, PV085606 and PV085607. BLASTN confirmed that all query sequences (Sample sequences) were aligned significantly with other previous recorded type A genotype sequence (with the accession number of AB971670.1, AB971673.1 and AB971669.1) from the GeneBank with the identical percentage of 100%, 99.83% and 99.33% respectively (Figure 2 and Table 2). (Nakano *et al.*, 2006) in Japan found three different groups of *E. vermicularis*, called types A, B, and C, in both humans and chimpanzees. Their study on how these groups are related showed that all the human samples belonged to type A, but types B and C were found in the chimpanzee.

Morphological identification of all organisms is not dependable especially during genotype analysis. Now, molecular based identification become an alternative tool for solving this problem. Utilizing of the COI gene among the most common gene which was used in the molecular and phylogenetic analysis it inherited maternally and it has little genetic variation over a long period of time (Ahmed, Hraija, & Taher, 2023). Our ability to identify parasites, learn more about their geographic distribution, diversity, and host range, and comprehend their role in disease causation is enhanced by the use of molecular and analytical tools from the fields of population genetics and systematics, which allow the reconstruction of evolutionary relationships between parasites over a wide range of temporal and spatial scales (Lymbery & Thompson, 2012). The molecular traits of the *E. vermicularis* isolates reported in this study serve as the foundation for identifying the genetic variety of human pinworms.

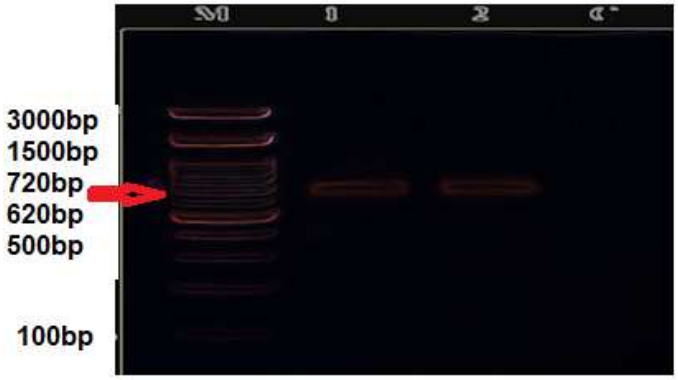

Fig.2: Amplification of partial COI gene from Insect, wells include M; Ladder (3000-100 bp), lane 1-2; sequence bands with the size of 720 bp and C indicate negative control with no band.

Table 2: Sequences of Significant Alignments among Isolated Sequences and GenBank Sequences.

Description	Scientific Name	E value	Per. Ident	Accession

Enterobius vermicularis isolate Saleh-Marjan-1 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	*Enterobius vermicularis*	0.0	100.00%	PV085602.1
Enterobius vermicularis mitochondrial Cox1 gene for cytochrome oxidase subunit 1, partial cds, isolate: YY-22	*Enterobius vermicularis*	0.0	100.00%	AB971670.1
Enterobius vermicularis mitochondrial Cox1 gene for cytochrome oxidase subunit 1, partial cds, isolate: YY-25	*Enterobius vermicularis*	0.0	99.83%	AB971673.1
Enterobius vermicularis mitochondrial Cox1 gene for cytochrome oxidase subunit 1, partial cds, isolate: YY-21	*Enterobius vermicularis*	0.0	99.33%	AB971669.1

The reinstruction of the phylogenetic tree would be utilized to induce developmental relationship among isolated sequences and previous related sequences in the Gen Bank. For this purpose, a total of 9 identical related sequence were downloaded from NCBI (BU585703.1 as outgroup). After sequence alignment, the evolutionary tree had been made (Using Neighbor joining with 100 bootstrap replication). Tree analysis investigated uncovered that the groupings clustered at the side other chimpanzee related type C *E. vermicularis* which had been recorded previously by (*Janthu et al.*, 2022) from Japan under the accession number, AB971673 and AB971669 with significant bootstrap value of 86% and 100% respectively (Figure 3). The result of the present study is disagreeing with that done by (Al-Taei & Al-Quraishi, 2022). They noticed that the pinworm, *E. vermicularis* isolates from local sources in Babylon showed similarity to the *E. vermicularis* human isolates from Iran found in the NCBI-BLAST database.

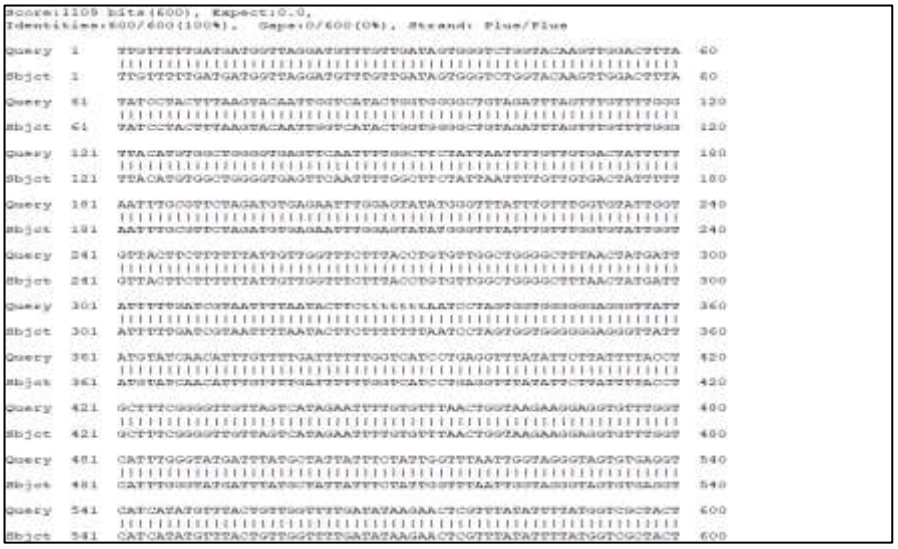

Fig. 2: Result of the Pairwise alignment between sample (query) sequence (PV085602|) and the subject sequence from GeneBank (AB971670.1).

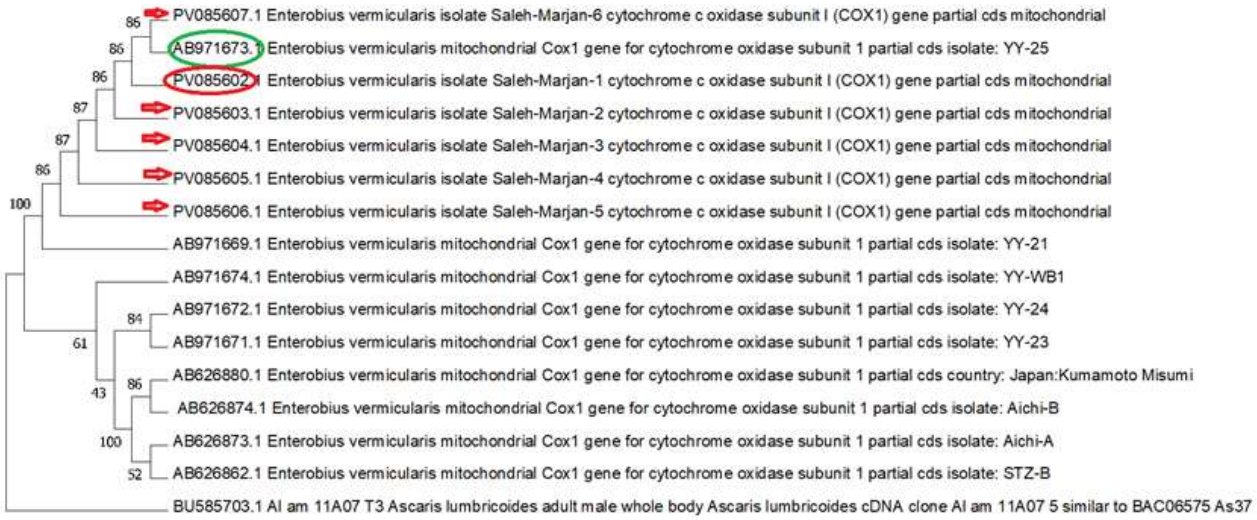

Fig. 3: The phylogenetic position of the isolated sequence(PV085602|) among several recorded human pinworm sequences. Bar, 0.05 substitution per nucleotide position.

In order to investigate the genetic variation among the isolated sequence, the present sequence was compared with the latest subject sequence which was previously recorded by Radi and Al-Omashi (2023) (unpublished) which is belong type A sequence of the same species in Iraq. For this purpose, the subjected sequence (accession number = OR430614) was downloaded from NCBI. Substitution frequency was expected according to Tamura-Nei model between isolated sequence (PV085602) and previous sequence in Iraq (OR430614). Substitution matrix reveals the expected value between 1.82% as minimum value and 30.77% as maximum value and each entry had shown the likelihood of substitution (r) from one base (row) to another base (column) For effortlessness, whole of r values is made = 100, The nucleotide frequencies are A = 20.09%, T/U = 42.82%, C = 10.94%, and G = 26.16%. the percentage of diverse transitional substitutions are appeared in bold and those of transversionsal substitutions are appeared in italics (Table 3).

Table (3): Represent the Maximum Likelihood estimation of the substitution matrix between isolated sequence (PV085602) and previous sequence in Iraq (OR430614).

	A	T/U	C	G
A	-	*7.14*	*1.82*	**15.85**
T/U	*3.35*	-	**7.86**	*4.36*
C	*3.35*	**30.77**	-	*4.36*
G	**12.17**	*7.14*	*1.82*	-

Study of the secondary RNA structure is dependable and confirmation step for the determination of the genetic variation among newly isolated sequences as mentioned by (AL-Marjan, Abdullah, & Kamel, 2024; Sluysmans *et al.*, 2018). For this purpose, the isolated sequence under the accession number PV085602 and the previous recorded sequence in Iraq under the accession number OR430614 of the same species were obtained for this analysis. Maps and the MFE had been drawn and obtained for both sequences, using RNAfold web serverhttp://rna.tbi.univie.ac.at › cgi-bin) as shown in Figure 4, A. Results shows that the minimum free energy for the both sequences was equal to -123.40 kcal/mol and 75.80 kcal/mol respectively. Number and type of loops were calculated for both sequences. Similar type of secondary RNA loops was recorded which include, Hairpin, Internal, External, Multi branched, Bulge and Helical loops

while the number of loops were differ when compare with each other which is equal to 64 and 43 loops for both sequences respectively (Table 4 and Figure 5). Different value of the MFE and the number of the loops confirm the minor genetic variation among isolated sequences compared with the previous isolated local sequence in Iraq. Relative similarity between the map of the secondary structure RNA of both isolated sequence and recent previous sequence confirms that there is a minor genetic polymorphism among the isolated sequences. Previously different studies were confirmed that DNA mutations causing variations in RNA folding (Chowdhury *et al.*, 2015). Therefore, any dissimilarity between the current study and reference studies confirms the genetic changes of the local isolated sequence. The only genetic variation of this parasite that

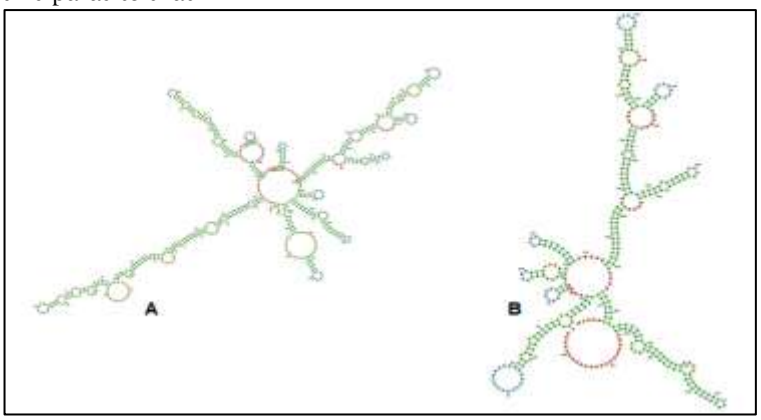

Fig. 4: Schematic representation of the secondary RNA structure of the Cytochrome Oxidase C Subunit One (COI) of the isolated sequence A, PV085602 and previous recorded sequence B, OR430614.

Table 4: Shows number and types of secondary RNA structure loops of both sequences PV085602 and OR430614.

Accession number Isolated sequences	External loops	Internal loops	Bulge Loops	Hairpin Loops	Helices loops	Multi-branched	Total loop
PV085602	1	15	2	9	33	4	64
OR430614	1	10	1	8	19	4	43

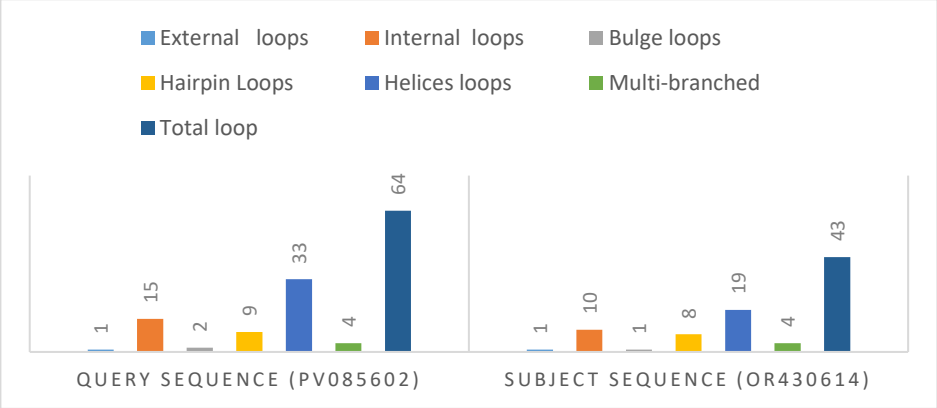

Fig. 5: Histogram shows comparison among the number and types of loops in secondary structure form of COI gene sequence of the isolated pinworm sequence and subject sequence.

has been identified to now is type B in Europe and B1 and B2 subtypes in northwest of Iran (Hagh, Oskouei, Bazmani, Miahipour, & Mirsamadi, 2014). More thorough molecular analyses are needed in order to draw additional findings regarding the genetic diversity of the Iraqi Type A, *E. vermicularis* population.

There was not much information available about the molecular studies of pinworms around the world, especially in Iraq and the Kurdistan region. Several descriptions and articles were gathered and explained related to the genotyping as summarized in Table 5. For more confirmation, a phylogenetic tree was made among previous recorded genotype pinworm in Iraq and isolated sequences. Tree analysis indicates two separate genotype groups, Clade 1 and Clade 2 genotypes (Figure 6). The isolation and investigation of genetically diverse type A pinworm, *E. vermicularis* from human represent the first investigation in Iraq.

Table 5: Shows several recent articles related to the molecular genotyping of the local isolated sequences of the pinworm, *Enterobius vermicularis*

Years	Author/Authors	Study site	GenBank Accession No.	Haplotypes
2018	(Kadhum, Nawar, & Alkhanaq, 2018)	Wasit	HQ646164.1	Type A
2019	Al-Waily, A.G and Alomashi, G.B.	Al-Diwaniyah	MH893634 and MH893635	Type A
2020	(Khazaal, Al-Hadraawy, & Hussein, 2020)	Thi Qar	MN817924-MN817931	Type A
2021	(Khaty, 2022)	Erbil	MZ400682	Type A
2022	(Al-Taei & Al-Quraishi, 2022)	Babylon	MZ505531-MZ505536	Type A

Fig. 6: The phylogenetic position of the isolated sequences among several previously recorded human pinworm sequences in Iraq. Bar, 0.05 substitution per nucleotide position.

Conclusions

This study concludes that the genetic diverse type A *E. vermicularis* is endemic in Iraq. Phylogenetic investigation uncovered that the groupings clustered at the side other Chimpanzee related

type C, *E. vermicularis* (under the accession number, AB971673 and AB971669) which had been recorded previously among Chimpanzee from Japan. Results of the secondary structure analysis of the RNA confirms a distinct genetic variation depending on the number and types of their RNA loops and it could make resistance to various antihelminthes.

Acknowledgements: I would like to extend my thanks to staffs of several Medical Laboratories and Hospital in Erbil City for their kind behaviors and cooperation.

Funding the authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Conflicts of interest: The authors have no competing interests to declare that relevant to the content of this article.

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