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Genotyping of *Enterobius Vermicularis* in Patients of Northern Baghdad

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Abstract

Background: *Enterobius vermicularis* is a common intestinal parasite with a global distribution. Accurate molecular identification is essential for understanding its genetic variation and epidemiology.

Aim: This study aimed to detect and genetically characterize *Enterobius vermicularis* in stool samples using molecular techniques targeting the cox1 gene.

Methods: A total of 85 stool samples were collected from individuals suspected of *E. vermicularis* infection. DNA was extracted and purified, and concentrations were measured using a Nanodrop device. PCR was employed to amplify the cytochrome c oxidase subunit 1 (cox1) gene. Amplified products (407 bp) were visualized using gel electrophoresis. Selected PCR products were sequenced via the Sanger method using the ABI-310 Genetic Analyzer (Macrogen, Korea). Sequences were aligned and compared with entries in the GenBank database (NCBI) to confirm species identity and analyze genetic variation.

Results: *E. vermicularis* DNA was confirmed in 35 samples. DNA concentrations ranged from 9.6 to 19.5 ng/µl. Sequence alignment revealed that all positive samples belonged to the *E. vermicularis* species. Samples 28 and 31 showed 100% identity with the Japanese strain (accession number NC_056632.1), while sample 12 showed the lowest identity at 88%. Genetic variation included both transition (purine-to-purine or pyrimidine-to-pyrimidine substitutions) and transversion mutations (purine-to-pyrimidine or vice versa).

Conclusion: PCR and sequencing of the cox1 gene effectively identified *E. vermicularis* in stool samples and revealed notable genetic diversity among isolates. These findings underscore the importance of molecular methods in parasitological diagnostics and epidemiological studies.

Keywords: Enterobius vermicularis, PCR, Genotyping, cox1 gene, Molecular identification.

1. Introduction

Enterobius vermicularis is a parasitic nematode that inhabits the human intestine and is primarily transmitted through ingestion of eggs found in contaminated water, food, dust, or surfaces *Enterobiasis*, an infection caused by this parasite, is widespread and affects millions of people worldwide, especially in communities with limited socioeconomic resources [1]. *E. vermicularis* is the only nematode in the family *Oxyceridae* that can cause infection in humans. The disease is characterized by direct transmission from infected individuals to susceptible persons without the need for an intermediate host [2].

Enterobius vermicularis typically completes its life cycle in approximately 2 to 3 months [3]. Female worms reach maturity within about one month, while males perish shortly after mating. Pregnant females move to the colon, laying around 2,000 eggs daily in the perianal region [4]. E. vermicularis may also be ectopically present, that is, apart from residing within the large intestine, it occupies places such as the appendix, uterus, kidneys, urinary tract, eyes, and reproductive organs of females and may even be found under the subcutaneous tissues [5].

Clinical presentation ranges from asymptomatic cases to characteristic symptoms such as anal pruritus, abdominal discomfort, sleep disturbance, restlessness, irritability, and nocturnal enuresis. In some cases, scratching the perianal area may lead to secondary bacterial infection [6]. This may also lead to misdiagnosis

since the commonest tests, such as the adhesive tape method and direct smear, are sensitive but not very specific; it has been associated with some complications like acute appendicitis [7,8].

Polymerase Chain Reaction (PCR) is another of the many tools that will be highly efficient in the molecular detection of parasites, and difficulty lies in obtaining either eggs or adult worms for such analysis [9]. Problems arise from the quality of the stool sample used for DNA extraction and identity of other inhibitors that can interfere with PCR [10-12]. Effective methods employed for isolation and purification of DNA are thus very important when PCR is to be used in identifying *E. vermicularis* [13, 14].

Molecular diagnostic techniques offer high precision in parasite detection [11]. These tools contribute to understanding transmission pathways and distinguishing persistent from recurrent infections [12]. Additionally, molecular methods can differentiate between pathogenic and non-pathogenic strains, analyze genetic diversity, and identify virulence factors critical for treatment strategies [11]. DNA extracted from *E. vermicularis* eggs has been successfully amplified and sequenced, aiding in the study of genetic variability, phylogeography, and host adaptation [6]. Modern genetic studies have also confirmed the presence of this pinworm in ancient human feces from the New World, highlighting its long-standing relationship with human hosts [13].

The aim of this study is to detect and genetically characterize *Enterobius vermicularis* in suspected cases using PCR amplification and sequencing of the cox1 gene.

2.Materials and methods

2.1 Stool samples collection

Fecal specimens were collected from children and adolescents aged 1 to 18 years from both genders over several locations spread across northern Baghdad Governorate and within its districts and sub-districts. Sampling was done at random from schools, health clinics, as well as from kindergartens. The samples were collected in sterile plastic containers, preferably in the morning to ensure that the samples were as fresh as possible. Information on the participants' age, sex, place of residence, and clinical symptoms such as perianal pruritus or abdominal discomfort was collected through a structured questionnaire. Immediately upon receipt, the samples were examined for the presence of adult pinworms or eggs using a microscope at 40x and 100x magnification within two hours. Any visible worms detected during the examination were preserved in 70% ethanol at −20°C for subsequent analysis, following a modified protocol adapted from [15].

2.2 Extraction DNA

Genomic DNA was isolated from Enterobius vermicularis adult worms and eggs previously preserved in 70% ethanol at −20°C. The concentration and purity of the extracted DNA were evaluated through agarose gel electrophoresis and Nanodrop spectrophotometry. The isolated DNA was stored at −20 °C until subsequent PCR analysis.

2.3 Polymerase chain reaction (PCR) and sequencing

in this study, the primers are designed to amplify *Enterobius vermicularis* using the cox1 gene from mitochondrial cytochrome c oxidase subunit 1. Sequence data were obtained from NCBI GenBank (accession number AP017684.1), and the design of the primers was done using Primer3Plus software. Primers were then made in Baghdad, Iraq, by Nabu Company. The cox1-targeted 407-bp fragment was amplified using these following primers: forward primer: 5'-TGTGTTGGCTGGGGCTTTA-3' and reverse primer: 5'-GCTGCACAACTAAACGTCCC-3'. Nonessential PCR reactions used i-Taq PCR PreMix.

Each reaction mixture volume of 25 µl consisted of 1.5 µl of DNA template, 1µl of forward primer, 1µl of reverse primer, 5 µl of i-Taq PCR PreMix, and 16.5 µl of distill water. The mixture was shortly vortexed for 3 min at 3000 rpm in an Exispin vortex centrifuge and subsequently subjected to thermal cycler conditions. The PCR cycle conditions were as follows: initial denaturation at 95°C for 5 min; denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 2 min for 35 cycles; final extension step at 72°C for 5 min and hold for 5 mins at 4°C. The PCR products were analyzed on 1.5% agarose gel. For gel preparation, the

agarose was heated in an Erlenmeyer flask in water at 100 °C in a water bath, cooled to 50 °C, mixed with 3 µl ethidium bromide for staining, and poured into a casting tray for gelation for 15 min.

Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray. The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer.10µl of PCR product were added in to each comb well and 3µl of]100bp Ladder) in first well. Then electric current was performed at 100 volt and 80 AM for 1hour. PCR products were visualized by using UV Transilluminator [16]. PCR products and COX1 gene sequencing were sent by Macrogen Korea Sequence analysis was performed against nucleotide databases using the National Center for Biotechnology Information]NCBI) Basic Local Alignment Search Tool (Bio-ID) to identify the sample and submit it to GenBank (ID Sequences relevant to the sample(s) were obtained from the NCBI Nucleotide Database (www.ncbi.nlm.gov/nucleotide) and included using BioID software [17].

2.4 Genetic tree analysis

Gene6 software was used to conduct genetic analysis of the Cox1 gene sequences in pinworm isolates. Alignment was performed using the Neighbor-Joining method. The genetic tree was drawn using Gene6 software, and evolutionary distances were calculated using the Jukes-Cantor model.

3. Results

DNA was extracted from all 35 *Enterobius vermicularis* samples. Clear bands appeared on a 1.5% agarose gel after electrophoresis for one hour at 70 volts, thus confirming DNA presence in every sample . Purity of the extracted DNA ranged from 1.801 to 1.987, while the concentrations ranged from 9.6 ng/ml to 19.5 ng/ml. Sample 26 had the least concentration at 9.6 ng/ml, while sample 15 showed the maximum concentration at 19.5 ng/ml. The cox1 gene was successfully amplified from all DNA samples using PCR with specific primers, producing a fragment of 407 base pairs. The bands were clearly visible on the 1.5% agarose gel and matched the size when compared with a 1000 bp DNA ladder (Figures1).

Genomic DNA extraction was performed on all specimens of *Enterobius vermicularis*, and upon visualizing them on a 1.5% agarose gel under electrophoresis for one hour at 70 volts, clear bands indicating the presence of DNA were observed in all samples (Figure 1). The extracted sample has been found to have varying extents of purity, such as 1.801 to 1.987, while the concentration ranged from 9.6 ng/ml to 19.5 ng/ml, with 9.6 ng/ml found in number 26 and 19.5 ng/ml in sample 15. *Enterobius vermicularis* cox1 gene was amplified from the twenty-five extracted DNA samples utilizing the specific forward and reverse primers through transmission with PCR, where the copious product was expected to be 407 bp, as verified by visible bands upon 1.5% agarose gel electrophoresis against a 1000 bp DNA ladder (Figures 2 and 3).

Analysis confirmed that all 35 samples belonged to *Enterobius vermicularis*. By comparing the sequences to those in global databases, the molecular analysis identified genetic variations in several samples, suggesting the presence of novel strains unique to Iraq. Alignment with NCBI-registered sequences indicated that samples 28 and 31 exhibited 100% similarity to Enterobius vermicularis sequences from Japan (accession number NC_056632.1), while sample 12 showed the lowest similarity at 88%. the results were divided into four groups according to the sampling regions (The first group is Al-Tarmia, the second is Al-Tajji, the third is Al-Mushahidah, and the fourth group includes the Al-Sha’la and Al-Kadhimiya areasAs shown in the figure 4,5,6,7,8,9,10,11.

4. Discussion

Recent progress in helminth genotyping has generated significant insights into their geographic spread. Molecular techniques applied to genetics and population studies facilitate the exploration of evolutionary connections between parasites and their hosts, providing valuable understanding of their distribution, genetic variation, and factors contributing to parasitic infections (18,19,20). As seen from Sequence analysis results of samples collected from five districts in northern Baghdad, the nucleotide sequences were found to be 99%

identical to those of *Enterobius vermicularis* from Japan, as mentioned in GenBank of NCBI with accession ID NC_056632.1 except a few samples from Al-Kadhimiya that displayed 95% identity. The alignment results presented in figures 1-11 resulted in the identification of nucleotide variations including the substitution of G with A, C with T, and A with G. These findings line with a study in Wasit Governorate that managed to isolate and identify *Enterobius vermicularis* from fecal samples of children. By employing molecular methods focused on the cytochrome c oxidase I (cox1) gene, the study elucidated the evolutionary relationships of local strains and their connections to global isolates. It further highlighted the cox1 gene's utility in detecting genetic variability among parasitic worms, establishing it as an effective diagnostic marker [21]. These findings are consistent with research demonstrating that the cox1 gene effectively supports molecular diagnosis, genotype identification, and phylogenetic analysis [22]. The cox1 gene was selected for detecting parasitic worms due to its higher genetic variability compared to other genomic regions, making it particularly valuable for population genetics research [23,24]. Its elevated mutation rate further enhances its suitability for analyzing genetic patterns in *Enterobius vermicularis* [25]. Studies have shown that mitochondrial DNA has several advantages when studying evolutionary interactions in parasitic worms, especially for distinguishing between closely related species. This is due to the high mutation rate and low effective population size that provides rapid linear sorting between species. Comparative mitochondrial analysis is also used to identify cryptic species that cannot be identified by traditional morphological methods [26,24]. One of the samples, number 17, collected from the Taji region, was registered in the US NCBI GenBank under accession number PQ634874 ID: PQ634874 This sample was also aligned and compared with E. vermicularis sequences, with a match rate of 100%. The genetic relationship of this sample with the closest samples was studied using the Mega-x program, as shown in Figure [12]. Studies have also shown that the phylogenetic tree is important in understanding the genetic relationships between species. This tree requires molecular information about the organism, i.e. the sequence of nitrogenous bases from DNA, ribonucleic acid (RNA), or protein sequence. Phylogenetic analysis can be performed using alignment-based tools. Alignment-based tools are often used to build the phylogenetic tree, a computer program to build the genetic relationship of evolution between organisms [26]. The figure [13] shows the protein profiling study resulting from the translation of the nitrogenous base sequences in the COX1 gene for samples of the parasitic worm *E. vermicularis*. The appendix also shows the protein forms resulting from the translation of the nitrogenous base sequences. It is noted that there is a difference in the forms of the proteins resulting from the gene under study. This difference is due to the presence of mutations in the gene under study, which caused a change in the gene expression of these sequences, depending on the regions from which the samples were collected.

6. Conclusions

The PCR technique targeting the cox1 gene demonstrated high effectiveness in detecting *Enterobius vermicularis* in the analyzed samples. All positive samples were confirmed to belong to the same species, although they exhibited varying degrees of genetic similarity. Notably, samples 28 and 31 showed complete (100%) similarity with strains previously registered in Japan, while sample 12 showed the lowest similarity at 88%, suggesting possible genetic divergence. The detected mutations included both transition and transversion types, reflecting genetic variation among the local isolates. These findings reveal clear genetic diversity within the studied *E. vermicularis* population, which may be influenced by evolutionary processes or geographic distribution.

7.Acknowledgments:

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Ethical Approval and Study Type

This cross-sectional molecular study was conducted on stool samples collected from individuals with suspected Enterobius vermicularis infection. The study was coordinated with the Department of Biology, College of Education for Women, University of Tikrit, Tikrit, Iraq, and the General Hospital of Tarmiya. Official permission was obtained as documented by the facilitation letter number 3/7/1263 dated 24 January 2023, issued by the University of Tikrit. Informed consent was obtained from all participants or their legal guardians prior to sample collection.

Study Limitations

This study was limited by the relatively small number of PCR-confirmed positive samples and the inability to conduct follow-up clinical assessments. Additionally, genetic sequencing was performed only on a subset of samples, which may limit the generalizability of the molecular diversity findings.

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Conflict of Interest

The authors declare that there are no conflicts of interest related to this study.

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Figure (1) Electrophoresis of PCR products on 1.5% agarose gel at 70 V for 1.5 h for the cox1 gene in *E. vermicularis* for 35 samples

Nitrogenous base sequence alignment in BLAST

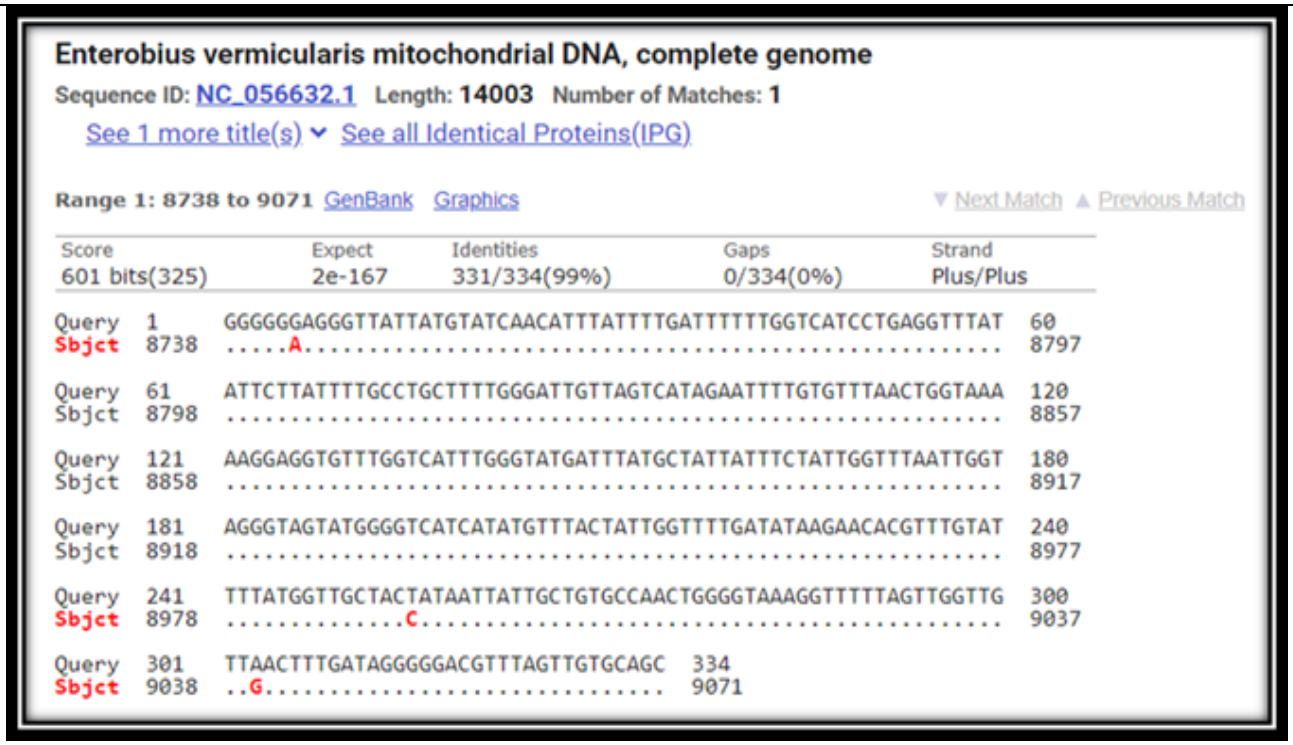

Figure (2) **Nitrogenous base sequence alignment in BLAST** (Al-Tarmia)

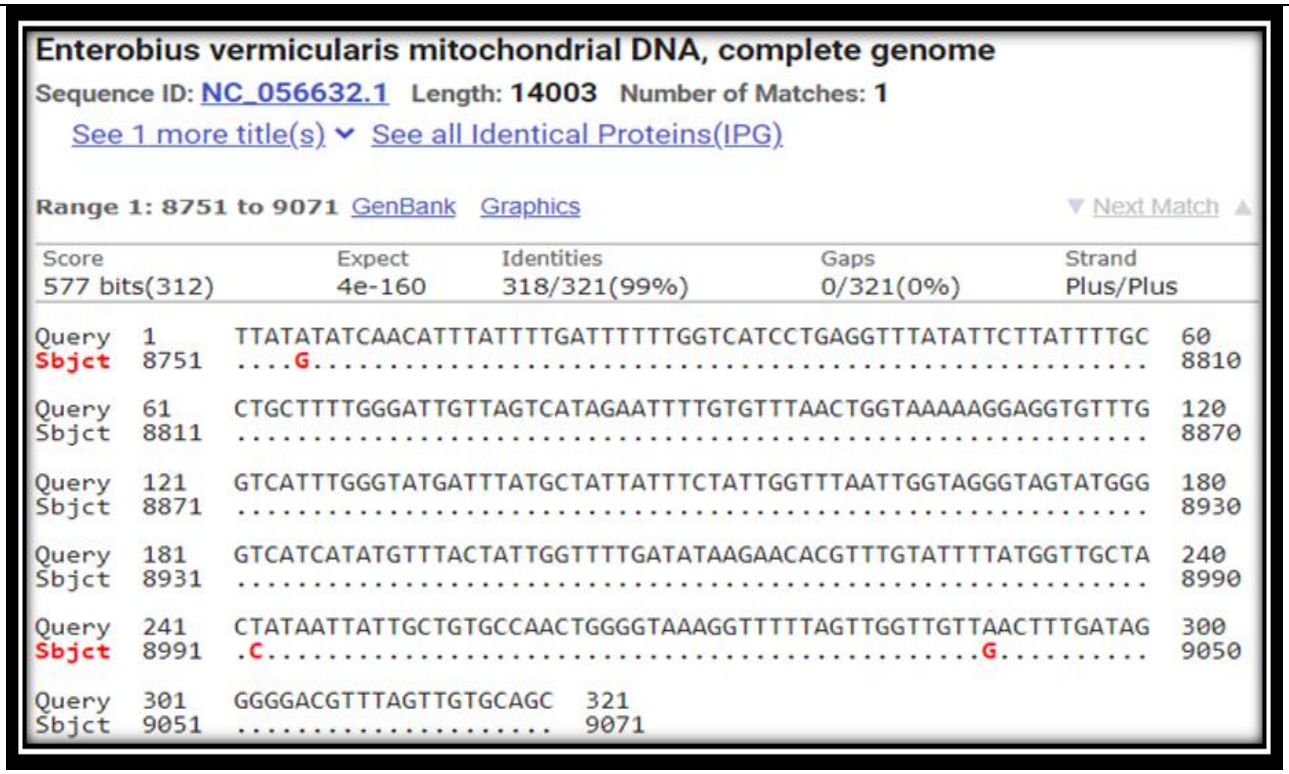

Figure (3) **Nitrogenous base sequence alignment in BLAST** (Al-Tajji)

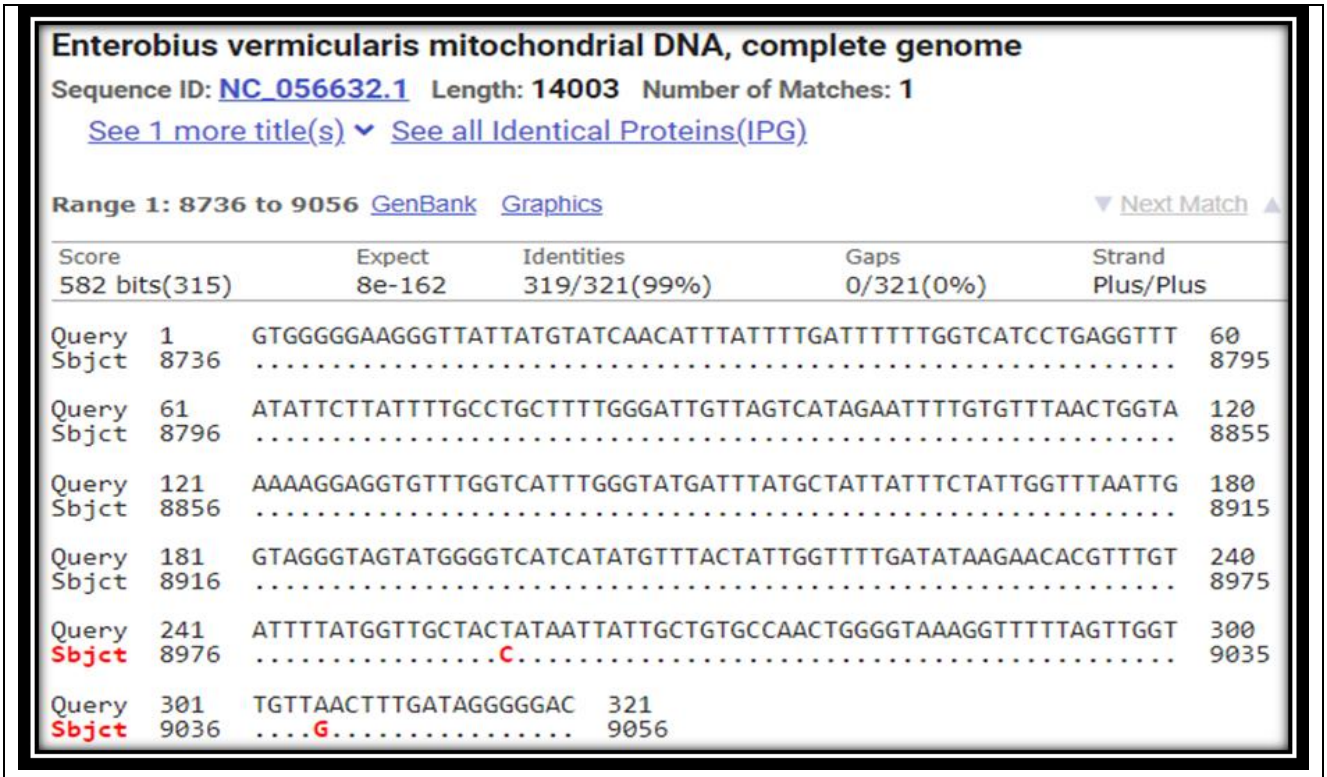

Figure (4) **Nitrogenous base sequence alignment in BLAST** (Al-Mushahidah)

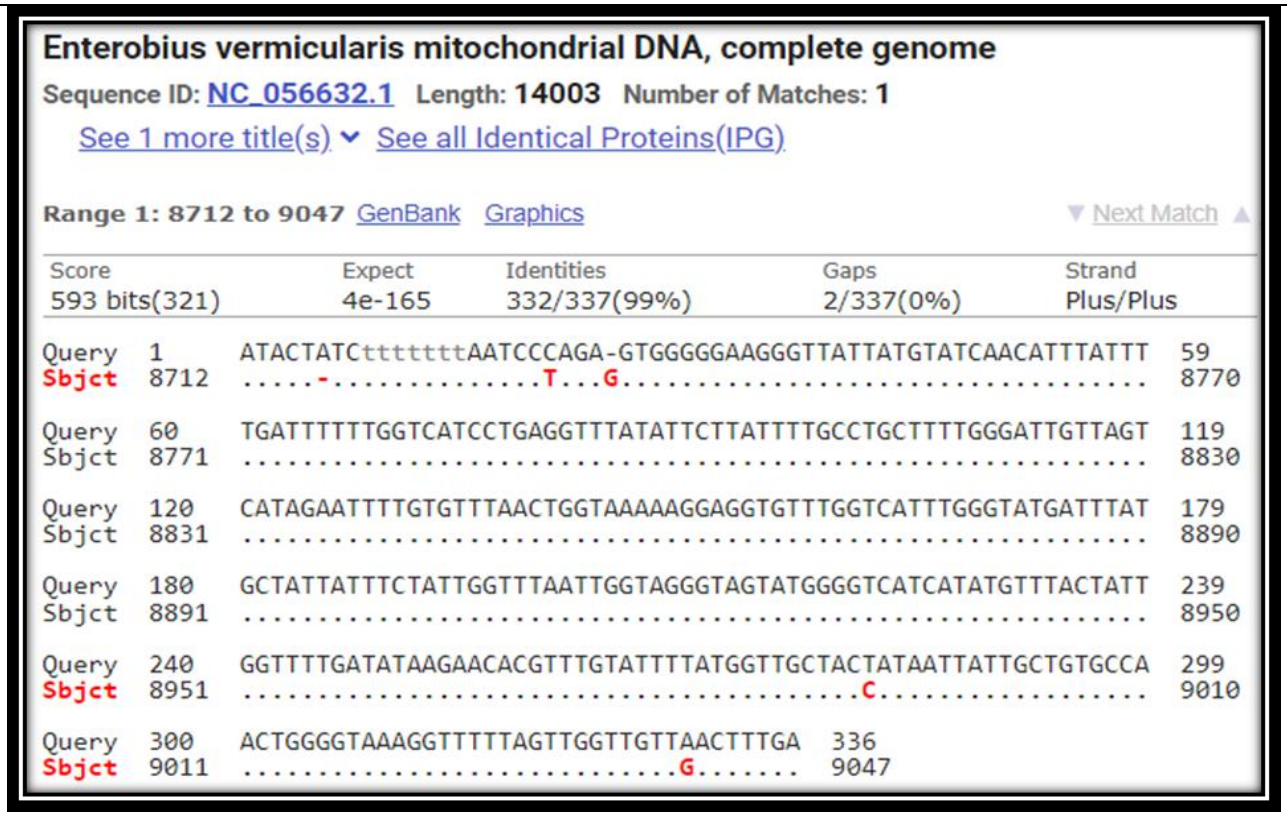

Figure (5) **Nitrogenous base sequence alignment in BLAST** (Al-Sha'la).

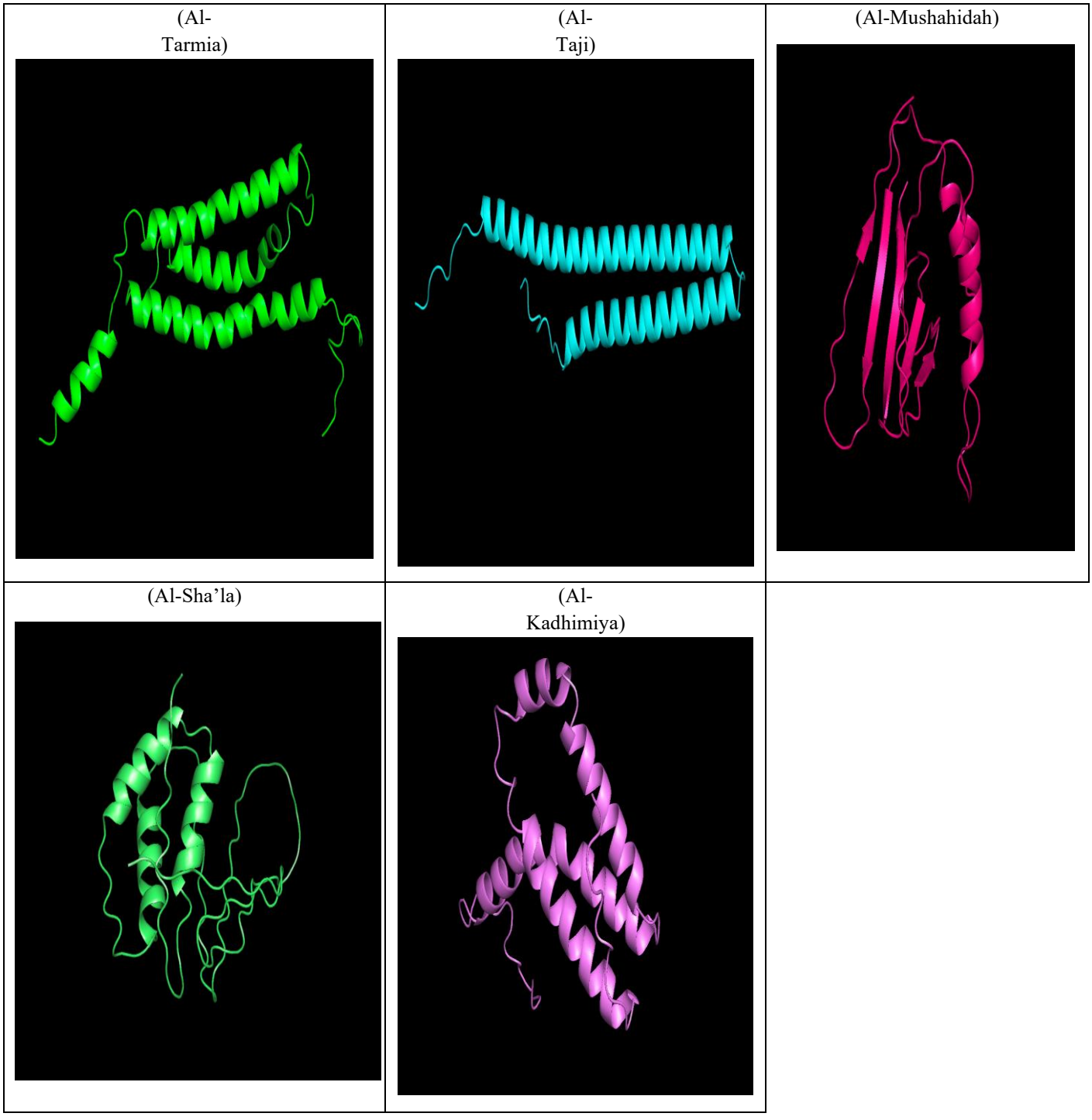

Figure (6) it also shows the protein forms resulting from the translation of the nitrogenous base sequences COX1 gene from samples of the parasitic worm *E. vermicularis*. Variations in the protein forms resulting from the gene under study are noted. This variation is attributed to the presence of mutations in the gene, which led to changes in gene expression for these sequences in the regions from which the samples were collected.