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Morphological and Molecular Diagnosis of Ascarislumbricoides Ova Isolated from Soil in Thi-qar Province, Iraq

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Abstract.

Ascarislumbricoidesis a soil-transmitted helminth (STH)that causes theascariasis disease in human.The present study was conducted for isolation andidentification of Ascarislumbricoides from soil of 5 different regions in Thi-qar province, where 100 samples of soils have been collected during December, January and February 2016. Sedimentation method was used to investigation of Ascaris ova in soil and DNA sequencing method of the ribosomal ITS 1 region was performed for confirming the identification of Ascarislumbricoides.The total contamination percentage of the soil samples withAscarislumbricoideswas 21%, the highest percentage 27.58% in AL-Jibaish district while the lowest percent 17.5% in Nassiriyahdistrictso T- test did find the significant differences. There are no significant differences between months in which was happened the study. All isolates were identified as Ascarislumbricoides by sequences of the ribosomal ITS 1 region and gave 100% similarity with references isolates of Genbank except five isolates (IQ-Soil.No.1, IQ-Soil.No.2, IQ-Soil.No.3, IQ-Soil.No.4 and IQ-Soil.No.5) that show similarity 99.41%, 99.61%, 99.61%, 9941% and 99.41% respectively. The five new isolates recorded in NCBI with accession numbers MK849916.1, MK849917.1, MK849918.1, MK849919.1, and MK849920.1 respectively

Keywords. Sequence Technique, ITS 1, PCR, Ascarislumbricoides, Diagnosis Techniques.

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INTRODUCTION

Intestinal parasitic are being the main health problems in a lot of developed countries [1]. Consumption of vegetables that weren't well washed and digestion is the biggest route of parasitic transmission [(2, 3)].

There are many instructions from several countries to eating vegetables freshly or slightly cooked to preserve the taste and nutrients, but this increases the probability of infection with intestinal parasitic. In some parts of world, the drainage system is applied in the agricultural lands and is considered a familiar base in the transmission of living organisms that causing diseases from soil to the crops leading infection of human [3].

There are billion cases of Ascariasis caused by*Ascarislumbricoides* on aglobal scale [1]. *Ascaris*worm have large size, cylindrical shape, its color tend to whiten and have mouth with three lips. Length of *Ascaris* male 30 cm, and has end curved to forward. Female of Ascaris may be up to 50 cm length with straight end. Ascaris egg is characterized with its high resistance to environmental changes resulting from the presence of thick shell that surrounds it. These eggs also have the greatest ability survive in soil [4].

Morphological-based diagnostic methodsof*Ascarislumbricoides* are depended in infection diagnosis but are low sensitivity and require expertise to achieve.The development of PCR techniques for the detection of parasite species based on specific gene sequences (mitochondrial cytochrome C oxidase subunit 1; cox1, NADH dehydrogenase subunit 1; nad1, the first internally transcribed spacer; ITS1 and the second internally transcribed spacer; ITS2) in different samples have proven highly sensitive than morphological methods[5, 6, 7]. The internal transcribed spacer 1 (ITS1) is located in the ribosomal RNA (rRNA) genes separated between the 3′ end of 18S rDNA gene and the 5′ end of the 5.8S rDNA gene in each transcription unit [8]. Therefore, the PCR techniques are specific tests for detection of *Ascaris*spp, especially by extraction and amplification of DNA from single eggs [9].

The high nutritional value of fruits and vegetables and administrated by people in combination with extent of enduring egg of *Ascaris* to harsh conditions leading to conduct this study to investigation the prevalence of eggs in the farms of different districts of Thi-Qar province by using PCR and sequence technique along with morphological method and discover the causes that helped on its reaching to the regions or its high prevalence rates in specific region than other areas.

MATERIALS AND METHODS

Sample collection

A total of 100soil samples were collected in December, January and February 2016 from agricultural areaswhere vegetables and fruits are grown inthe districts of Nassiriyah, Suq – AL- Shuyukh, AL-Shatra, AL-Rifaai, and AL- Jibaish of Thi-Qar province in a sterile plastic containers and then immediately transferred to the laboratory.

Sedimentation method

Sedimentation method was used to investigation of *Ascaris* ova in soil as follow:

1. A suspension was prepared by adding 1gram of soil to 10 ml of warm water (normal saline) and mixed well, then the suspension was filtered to baker through two layers of wet gauze to remove the large particles.
2. The suspension has been centrifuged at 2500 cycle for one minute.
3. The sediment was taken and repeated its suspension with clean water at the same speed until the supernatant became clear which was removed and kept the deposit.
4. A drop of deposit was placed on slide and a drop of iodine was added to it, then a cover was put on it and examined under microscope[10].

DNA extraction

Genomic DNA was extracted from eggs by using DNA extraction Kit (Bioneer, South Korea). The extraction was done according to company protocol and genomic DNA was eluted in 50 µl of elution buffer. The extracted genomic DNA was checked by electrophoresis, and stored at -20 °C until further use.

Polymerase Chain Reaction

A ~500-bpof the ribosomal internal transcribed spacer (ITS) region was amplified by using primers as ITS1 F (5′-CTTGAACCGGGTAAAAGTCG-3′) and ITS1 R (5′-ATGTGTCTGCAATTCGCACT-3′)[11]. The PCR reaction was prepared by using (Accupower PCR PreMix Kit. Bioneer, South Korea). The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250µM, Tris-HCL (PH 9.0) 10 Mm, KCl 30Mm, MgCl2 1.2Mm, stabilizer and tracking dye). The PCR reaction was prepared according to kit instructions in 20 µl total volume by added 5 µl of purified genomic DNA, 1 µl of 10 pmole of forword primer, 1 µl of 10 pmole of reverse primer and complete the PCR premix tube by deionized PCR water into 20 µl and briefly mixed by Exispin vortex centrifuge (Bioneer, Korea).

The PCR reaction was performed in a thermocycler (MygeneBioneer, South Korea) under the following condition : initial denaturation temperature at 95 °C for 1 min; followed by 30 cycles of 95 °C for 30 s, 59°C for 1min and 72 °C for 1 min and then final extention at 72 °C for 10 min. the PCR products were analyzed by electrophoresis using 1% agarose gel, stained with withethidium bromide and the fragments sizes determined by comparison with a 100 bp DNA ladder and visualized under UV transilluminator.

DNA Sequencing technique

DNA sequencing method was performed for identification of *Ascarislumbricoides* based on the ribosomal ITS 1 region.

The PCR products of the ribosomal ITS 1 region were sent to Macrogen Companyhttp://dna.macrogen.com in South Korea for performed the DNA sequencing after sending the forward and reverse primers and PCR products. The PCR products purification were accomplished by the company for sequencing. *Ascarislumbricoides* nucleotide sequences were identified by Basic Local Alignment search tool (BLAST) followed by National Center for Biotechnology Information (NCBI) "http://www.ncbi.nlm.nih.gov ". Nucleotide sequences was copied and pasted in the "BLAST" after proofreading, then the program was identified the worms with others by comparing their sequence together. The phylogenetic analysis was performed based on Neighbor Distance Phylogenetic tree analysis (Mega version 6).

The statistical analysis

The statistical analysis was performed in this study by usingT-test according to[12].

RESULTS AND DISCUSSION

Prevalence of Ascarislumbricoides

Ascaris ova were detected in 21 out of 100soil samples collected from agricultural areas of five districts of Thi-qar province and appeared with percentage 21% which was in agreement with[13] and disagreement with[14].

Highest percentage of pollution 27.58% in AL-Jibaish district and the lowest percentage 17.5% in district of Nassiriyah so there are significant differences table (1).

The reason may be due to the fact that AL-Jibaish is one of the rural areas that are famous with animal breeding and using non- treated animal fertilizers that contain on such eggs thus increasing their presence in the soil.The femaleof parasiteproduced high number of eggs and the strong and resistant nature of theeggs might have contributed forsurviving in the harsh environment. It is also the eggs can survivein the absence of oxygen, live for 2 years at 5–10°C, and can survivein desiccation for up to 3 weeks[15, 16].

Table 1. Distribution of pollution percentage with ova ofA. lumbricoidesin the districts of Thi-Qar province

Area name	tested samples	contaminated samples	Pollution rate
Nassiriyah district	40	7	17.5%
Suq – AL- Shuyukh	5	1	20%
AL-Shatra district	9	2	22.22%
AL-Rifaai district	17	3	17.64%
AL- Jibaish district	29	8	27.58%
Total	100	21	21%

$T_{Calculated}$ = 11.273 ,d.f = 4 , Sig. = 0.00

The difference of pollution percentage between past and present is due to increased health awareness more than before. There are also attempts to reduce pollution by spraying pesticides and reducing irrigation with sewage [17]. Or may be due to that study took place in winter which represent the least seasons in activation this worm.

Table 2. Distribution of contamination rates of Ascaris egg in various areas of Thi-qargovernorate(December, January, February)

Month	examined samples	contaminated samples	Percentage
December	15	5	33.33%
January	43	11	25.58%
February	42	5	11.90%
Total	100	21	21%

$T_{Calculated}$ = 3.768, d.f = 2, Sig. = 0.064

Pollution with *Ascaris* ova was reported with percentage 33.33%, 25.58% and 11.9% in December, January and February respectively, and there are no significant differences table (table 2).The obtained results in the current studies has shown detectionof*Ascarislumbricoides* ova on the five studied districtshas public health risk to many who have close contact with the soil or ova reach to fruits and vegetables that grown in these areas and consume by people.

PCR amplification of ITS1 and sequence analysis

The extracted DNA from each ova was subjected toPCR for amplifying the ribosomal ITS 1 region. PCR products (bands) were observed on agarose gel electrophoresis under UV transilluminator in comparison with the DNA ladder. All amplicons of the ribosomal ITS 1 region were subjected to sequence and identify.

A homology search of the ribosomal ITS 1 region sequences was performed with BLAST fromNCBI. All isolates were identified as *Ascarislumbricoides* bysequences and gave 100% similarity with references isolates except five isolates (IQ-Soil.No.1, IQ-Soil.No.2, IQ-Soil.No.3, IQ-Soil.No.4and IQ-Soil.No.5) that showed similarity 99.41%, 99.61%, 99.61%, 9941% and 99.41% respectively(table 3).

The isolates IQ-Soil.No.1and IQ-Soil.No.2 were isolated from Nassiriyah district, whereas the isolates IQ-Soil.No.3 was isolated fromAL-Rifaai district and the isolates IQ-Soil.No.4 andIQ-Soil.No.5 were isolated fromAL- Jibaish district.

Table 3. The NCBI-BLAST Homology Sequence identity (%) between local Ascarislumbricoides soil isolates and NCBI-BLAST submitted Ascarislumbricoides isolates

Ascarislumbricoides soil isolates	Genbank Accession number	NCBI-BLAST Homology Sequence identity (%)		
		Identical *Ascarislumbricoides*	Genbank Accession number	Identity (%)
Ascarislumbricoides soil isolate No.1	MK849916.1	*Ascarislumbricoides*	AB110020.1	99.41%
Ascarislumbricoides soil isolate No.2	MK849917.1	*Ascarislumbricoides*	AB571295.1	99.61%
Ascarislumbricoides soil isolate No.3	MK849918.1	*Ascarislumbricoides*	LC422643.1	99.61%
Ascarislumbricoides soil isolate No.4	MK849919.1	*Ascarislumbricoides*	AB110020.1	99.41%
Ascarislumbricoides soil isolate No.5	MK849920.1	*Ascarislumbricoides*	HQ721820.1	99.41%

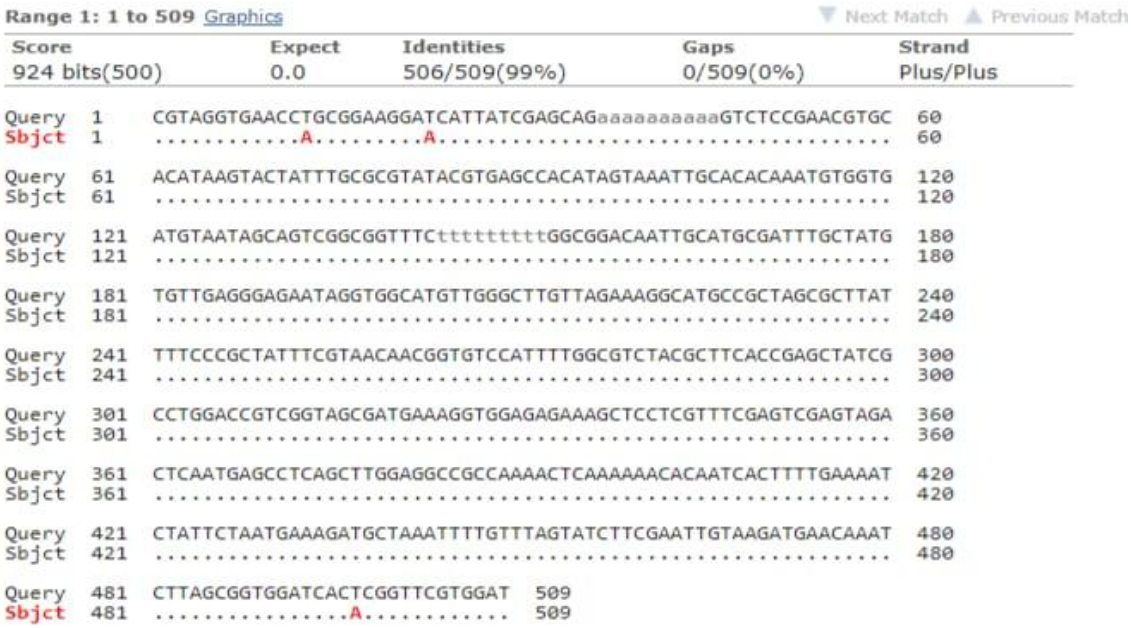
These five isolates were identifiedas new isolates which were different from their referenceisolates in somepositions of the nucleotide sequences. The new isolates werepublished in the National Center for Biotechnology Information (NCBI). Isolate No.1*Ascaris lumbricoides*isolateIQ-Soil.No.1 (Gen Bank: MK849916.1) was closely related(99.41%) to *Ascarislumbricoides* isolate Alj2 (GenBank: AB110020.1) but with three point transversion mutation TT and T instead of AA and A at position 13, 23 and 497 bp respectively (figure 1).

Isolate No.2 *Ascarislumbricoides* isolate IQ-Soil.No.2 (GenBank: MK849917.1) was closely related (99.61%) to *Ascarislumbricoides* isolate ` AscH1 (GenBank: AB571295.1) but with two point transversion mutation A and T instead of T and A at position 9 and 497 bp respectively (figure 2).

Isolate No.3 *Ascarislumbricoides* isolate IQ-Soil.No.3 (GenBank: MK849918.1) was closely related (99.61%) to *Ascarislumbricoides* isolate 144 (GenBank: LC422643.1) but with two point transversion mutation T and T instead of A and A at position 23 and 497 bp respectively (figure 3).

Isolate No.4 *Ascarislumbricoides* isolate IQ-Soil.No.4 (GenBank: MK849919.1) was closely related (99.41%) to *Ascarislumbricoides* isolate Alj2 (GenBank: AB110020.1) but with three point transversion mutation T, A and T instead of A, T and A at position 7, 10 and 23 bp respectively (figure 4).

Isolate No.5 *Ascarislumbricoides* isolate IQ-Soil.No.5 (GenBank: MK849920.1) was closely related (99.41%) to *Ascarislumbricoides* isolate NX(GenBank: HQ721820.1) but with three point transversion mutation T and A instead of A and T at position 23 and 495 bp respectively (figure 5).


Range 1: 1 to 509 Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
924 bits(500)	0.0	506/509(99%)	0/509(0%)	Plus/Plus

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Query  1    CGTAGGTGAACCTGCGGAAGGATCATTATCGAGCAGaaaaaaaaaaGTCTCCGAACGTGC  60
Sbjct  1    ............A.........A.....................................  60

Query  61   ACATAAGTACTATTTGCGCGTATACGTGAGCCACATAGTAAATTGCACACAAATGTGGTG  120
Sbjct  61   ............................................................  120

Query  121  ATGTAATAGCAGTCGGCGGTTTCttttttttttGGCGGACAATTGCATGCGATTTGCTATG  180
Sbjct  121  ............................................................  180

Query  181  TGTTGAGGGAGAATAGGTGGCATGTTGGGCTTGTTAGAAAGGCATGCCGCTAGCGCTTAT  240
Sbjct  181  ............................................................  240

Query  241  TTTCCCGCTATTTCGTAACAACGGTGTCCATTTTGGCGTCTACGCTTCACCGAGCTATCG  300
Sbjct  241  ............................................................  300

Query  301  CCTGGACCGTCGGTAGCGATGAAAGGTGGAGAGAAAGCTCCTCGTTTCGAGTCGAGTAGA  360
Sbjct  301  ............................................................  360

Query  361  CTCAATGAGCCTCAGCTTGGAGGCCGCCAAAACTCAAAAAACACAATCACTTTTGAAAAT  420
Sbjct  361  ............................................................  420

Query  421  CTATTCTAATGAAAGATGCTAAATTTTGTTTAGTATCTTCGAATTGTAAGATGAACAAAT  480
Sbjct  421  ............................................................  480

Query  481  CTTAGCGGTGGATCACTCGGTTCGTGGAT  509
Sbjct  481  .............A............  509
```

Figure 1. Comparisonofthe ribosomal ITS 1 region nucleotide sequencein local Ascarislumbricoides No.1 isolate with NCBI-GenbankAscarislumbricoides isolate Alj2 (GenBank: AB110020.1)

Range 1: 1 to 509 Graphics — Next Match — Previous Match

Score	Expect	Identities	Gaps	Strand
929 bits(503)	0.0	507/509(99%)	0/509(0%)	Plus/Plus

```
Query  1    CGTAGGTGAACCTGCGGAAGGATCATTATCGAGCAGaaaaaaaaaaGTCTCCGAACGTGC  60
Sbjct  1    ........T.....................................................  60

Query  61   ACATAAGTACTATTTGCGCGTATACGTGAGCCACATAGTAAATTGCACACAAATGTGGTG  120
Sbjct  61   ............................................................  120

Query  121  ATGTAATAGCAGTCGGCGGTTTCtttttttttGGCGGACAATTGCATGCGATTTGCTATG  180
Sbjct  121  ............................................................  180

Query  181  TGTTGAGGGAGAATAGGTGGCATGTTGGGCTTGTTAGAAAGGCATGCCGCTAGCGCTTAT  240
Sbjct  181  ............................................................  240

Query  241  TTTCCCGCTATTTCGTAACAACGGTGTCCATTTTGGCGTCTACGCTTCACCGAGCTATCG  300
Sbjct  241  ............................................................  300

Query  301  CCTGGACCGTCGGTAGCGATGAAAGGTGGAGAGAAAGCTCCTCGTTTCGAGTCGAGTAGA  360
Sbjct  301  ............................................................  360

Query  361  CTCAATGAGCCTCAGCTTGGAGGCCGCCAAAACTCAAAAAACACAATCACTTTTGAAAAT  420
Sbjct  361  ............................................................  420

Query  421  CTATTCTAATGAAAGATGCTAAATTTTGTTTAGTATCTTCGAATTGTAAGATGAACAAAT  480
Sbjct  421  ............................................................  480

Query  481  CTTAGCGGTGGATCACTCGGTTCGTGGAT  509
Sbjct  481  .............A............  509
```

Figure 2. Comparison of the ribosomal ITS 1 region nucleotide sequence in LocalAscarislumbricoides No.2 isolate with NCBI-GenbankAscarislumbricoidesisolate AscH1 (GenBank: AB571295.1)

Range 1: 1 to 508 Graphics — Next Match — Previous Match

Score	Expect	Identities	Gaps	Strand
928 bits(502)	0.0	506/508(99%)	0/508(0%)	Plus/Plus

```
Query  1    CGTAGGTGAACCTGCGGAAGGATCATTATCGAGCAGaaaaaaaaaaGTCTCCGAACGTGC  60
Sbjct  1    ....................A........................................  60

Query  61   ACATAAGTACTATTTGCGCGTATACGTGAGCCACATAGTAAATTGCACACAAATGTGGTG  120
Sbjct  61   ............................................................  120

Query  121  ATGTAATAGCAGTCGGCGGTTTCtttttttttGGCGGACAATTGCATGCGATTTGCTATG  180
Sbjct  121  ............................................................  180

Query  181  TGTTGAGGGAGAATAGGTGGCATGTTGGGCTTGTTAGAAAGGCATGCCGCTAGCGCTTAT  240
Sbjct  181  ............................................................  240

Query  241  TTTCCCGCTATTTCGTAACAACGGTGTCCATTTTGGCGTCTACGCTTCACCGAGCTATCG  300
Sbjct  241  ............................................................  300

Query  301  CCTGGACCGTCGGTAGCGATGAAAGGTGGAGAGAAAGCTCCTCGTTTCGAGTCGAGTAGA  360
Sbjct  301  ............................................................  360

Query  361  CTCAATGAGCCTCAGCTTGGAGGCCGCCAAAACTCAAAAAACACAATCACTTTTGAAAAT  420
Sbjct  361  ............................................................  420

Query  421  CTATTCTAATGAAAGATGCTAAATTTTGTTTAGTATCTTCGAATTGTAAGATGAACAAAT  480
Sbjct  421  ............................................................  480

Query  481  CTTAGCGGTGGATCACTCGGTTCGTGGA  508
Sbjct  481  .............A............  508
```

Figure 3. Comparison of the ribosomal ITS 1 region nucleotide sequence in local Ascarislumbricoides No.3 isolate with NCBI-GenbankAscarislumbricoides isolate 144 (GenBank: LC422643.1)

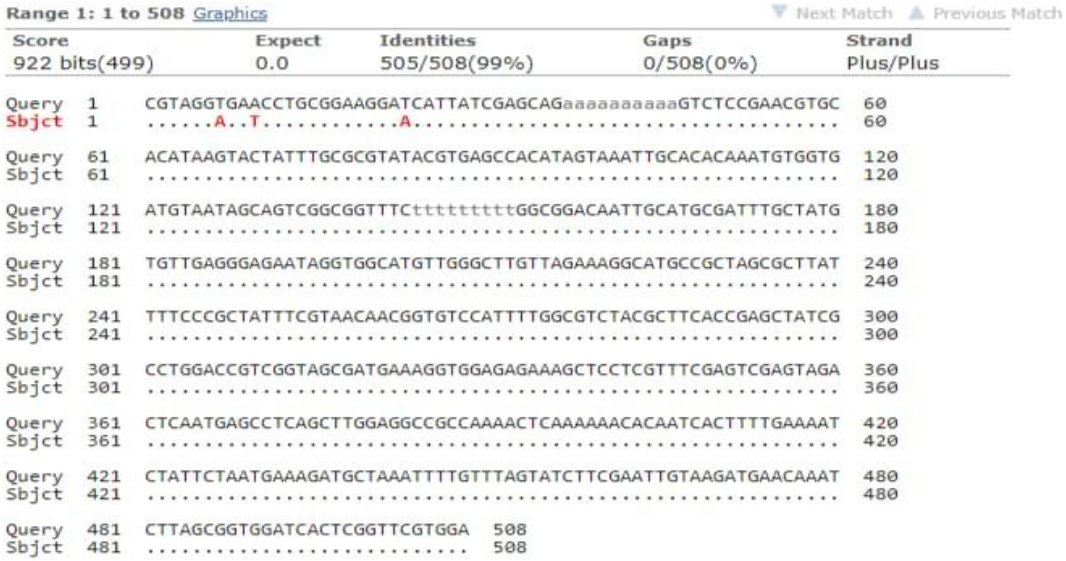

Figure 4. Comparison of the ribosomal ITS 1 region nucleotide sequence in local Ascarislumbricoidesisolate IQ-Soil.No.4 with NCBI-Genbank AscarislumbricoidesisolateAlj2(GenBank: AB110020.1)

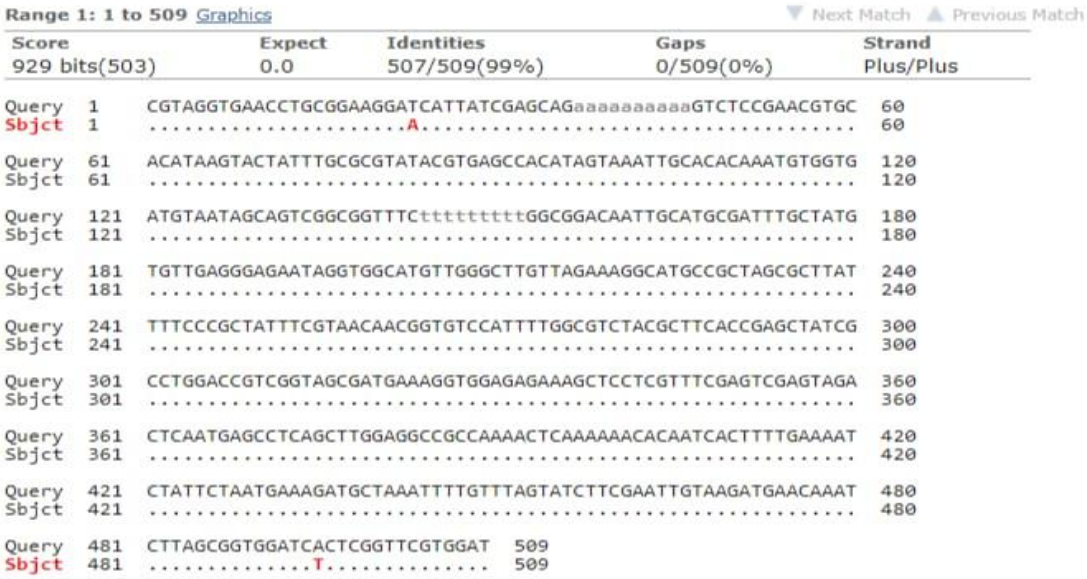

Figure 5. Comparison of the ribosomal ITS 1 region nucleotide sequence in local Ascarislumbricoides No.4 isolate with NCBI-GenbankAscarislumbricoides isolate NX (GenBank: HQ721820.1)

Phylogenetic tree analysis of 5 new local *Ascarislumbricoides* isolates based on the ribosomal ITS 1 region partial sequence refer to similarities with reference isolates that used for genetic analysis study (figure 6). The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version).

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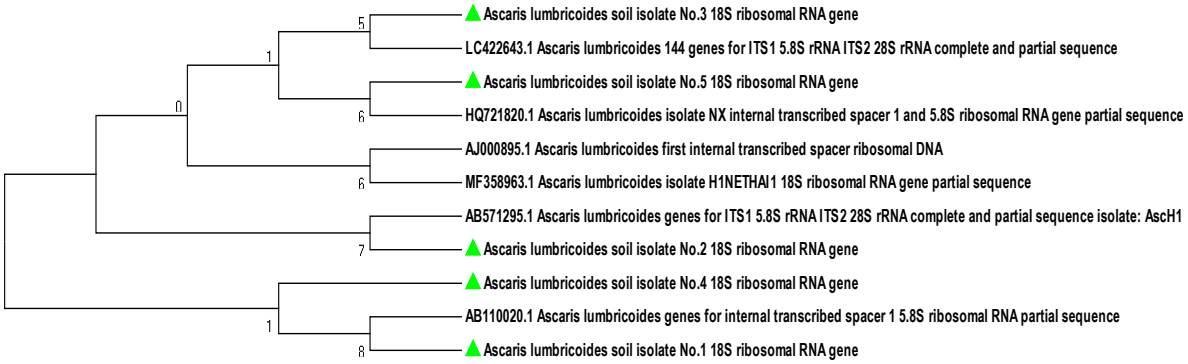

Figure 6. Phylogenetic tree analysis of local Ascarislumbricoides isolates based on the ribosomal ITS 1 region partial sequence

The ribosomal ITS 1 region is usefulness tool as markers for species distinction, where ITS1 evolve much faster than coding regions because substitutions occurring in spacers may be considered neutral[18].The ITS1 region belongs to a family of multiple copy gene provides a several targets for PCRassays to detect mutations may occur by exposing the parasite species to certain harsh environmental factors.

In the present study, it was observed polymorphic PCR ampliconsof our isolates to supportand examine the identities of *Ascaris* samples isolated from soiland showed ITS1 sequence similarity with references isolates in NCBI database. The molecular identification of the isolates by using the ribosomal ITS1 region partial sequenceconfirmed morphological identification of isolates. However, the sequenceanalysis provided additional and more precise informationon the identity of our isolates.

The data of ITS1sequence from *Ascaris*speciesobtain from pigs in Iowa,USA, referred to transmission had occurred from humansto pigs[19].The Sequence of PCR-amplifiedITS1 region of two*Ascaris* egg samples from humans in northern Thailandrevealed a probable hybrid genotype from two human ascariasis cases that have two positions that can be used to distinguish between *Ascarislumbricoides* and *Ascarissuum*[20].

The resultsof thisstudy are beneficial for specialists in Iraq and need to be more aware with hygiene precautions such as washing of hands, fruits, and vegetables before eating andproviding health education to children to prevent infection with ascariasis.

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