Polytechnic Journal. 2022. 12(2): 215-224
ISSN: 2313-5727
http://journals.epu.edu.iq/index.php/polytechnic

RESEARCH ARTICLE

Epidemiological Study and Molecular-Based Identification of *Enterobius vermicularis* Among Qushtapa Refugee Camp Children

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Received: 2 April 2021
Accepted: 16 June 2021
Published: March 2023

DOI: 10.25156/ptj.v12n2y2022.pp215-224

ABSTRACT

Background and objectives: Over the past 50 years, international travel, world trade, worldwide wars and conflicts have led to mass movements of people and the eviction of large numbers of people, causing major problems in controlling the border between countries. Children under the age of 18 years old make up 46% of the world's refugees, and these children spend their childhood away from home and may face the psychological and traumatic consequences of war and violence. Every disease has its own mechanisms of affecting humans and different prevention mechanisms as per disease nature and these factors are included in epidemiology of disease while other factors include prevalence of diseases in different population ions, so exactly knowing about disease epidemiology helps governing authorities to prevent the disease.

Methodology: Two hundred and six (206) stool samples were taken from children aged 1 to 12 resident in Qushtapa refugee camp. Samples were collected from beginning of October 2019 to the end of February 2020. A questionnaire was used to collect data on socio-demographic status.

Results: Result reveals an overall prevalence of infection was 33%. Children aged between 6 to 12 have higher prevalence of infection (43.3%) in comparison with those aged less than 6 years (22.5%). Several factors have statistically significant differences in proportion related to enterobiasis which are age (p-value=0.002) and hand washing (p-value=0.039). parent education level (p-value=0.323), family size (p-value=0.424) and nailed finger (p-value=0.57) showed no significant differences of infection. Molecular analysis was carried out using 28S rDNA, isolated from adult female and primers targeting the area of 28S rDNA. Sequence was submitted to Gene bank of NCBI and recorded with an accession number of MZ400682. Analysis of the original sequence using the universal primers of the sample of the study divulged that the parasite belonged to the species *Enterobius vermicularis* (sequence similarity was performed using BLAST from DDBJ and identity percentage is %99).

Conclusion: An overall percentage of infection by pinworm is 33%. Difference in the infection related to several factors. Polymerase chain reaction-based techniques utilizing 28S rDNA sequences can mentioned as investigated to be a faithful tool to diagnose the helminthes species. Identification of the *Entrobius vermicularis* based on the 28Sr DNA sequence analysis represent as a first attempt for the diagnosis of nematode in Iraq.

Keywords: *E. vermicularis*, Enterobiasis, Refugee camps, Qushtapa, 28S rDNA.

INTRODUCTION

Over the past 50 years, international travel, world trade, worldwide wars and conflicts have led to mass movements of people and the eviction of large numbers of people, causing major problems in controlling the border between countries (Doganay and Demiraslan, 2016). Children under the age of 18 years old make up 46% of the world's refugees, and these children spend their childhood away from home and may face the psychological and traumatic consequences of war and violence (Furukawa, *et al*., 2014). The first infectious diseases affecting refugees and other new migrants coming to income countries include parasites (Shetty, 2019). Intestinal parasite infection is one of the most common types of infections. About 3.5 billion people affected by 450 million diseases, most of them children (Tigabu, *et al*,. 2019). In the context of examples, the most common parasitic infections in refugee camps include helminthic infections such as ascariasis, enterobiasis and underlying diseases such as amoebiasis, giardiasis, plantidosis, pruritus (Abdulhaleem, *et al*., 2017). *E. vermicularis* is a nematode that is considered as a harmful parasite that affects approximately one billion people worldwide and causes many health and mental problems in children (Al-Dawoody and Al-Bazzaz, 2020) ; (Al Waaly, 2020). *E. vermicularis* is a small white worm up to 1 cm long that lives in the appendix and adjacent intestine (McConnaughey, 2014). Most *E. vermicularis* infections are asymptomatic or cause some abnormal symptoms such as itching

around the anus causing local skin irritation and secondary bacterial infections. Other symptoms include abdominal discomfort, loss of appetite, weight loss, restlessness, and irritability

(Vermund and Wilson, 2000). Every disease has its own mechanisms of affecting humans and different prevention mechanisms as per disease nature and these factors are included in epidemiology of disease while other factors include prevalence of diseases in different population ions, so exactly knowing about disease epidemiology helps governing authorities to prevent the disease.

MATERIALS AND METHODS

STUDY DESIGN AND STUDY AREA

A prospective study was conducted on Qushtapa refugee camp. It is located at 23 Km Southwest of Erbil province, The Kurdistan Region, Iraq (at the GPS coordinates of 35° 58' 58.626"N and 44° 2' 14.083"E). The area of the camp is 416,268 m². camp constitute of 1744 houses, and approximately 2000 families and number of population individual is 7,719. Displaced individuals are originated from different cities majority is from Qamishli , Diralok and Hassaka. Number of children from age less than one year to 5 years were 1636 among them 796 female and 840 male, On the other hand, the members of the age group 5 to 11 years were 1478 (725 females and 753 males).

SAMPLE COLLECTION

Samples were collected from 206 children (104 females and 102 males with a mean age of 5.98±2.7 years old), aged under one year to 12 years old resident in Qushtapa refugee camp. Samples were collected from beginning of October 2019 until the end of February 2020 and they stored in sterile collection tubes with normal saline and then transferred to the Erbil Health Technical College laboratory for the purpose of further examination. A questionnaire form was used to collect data on socio-demographic status.

MICROSCOPIC EXAMINATION

During the microscopic examination of the samples, various diagnostic methods were used, including iodine wet method (Lee, *et al*., 2001) ; (Mohammadi, *et al*., 2014) and (Al-Jawabreh, *et al*., 2019). The following is an account and description of test procedure: Direct wet mount, one drop of normal saline was added and a small amount of stool about 2-5 grams was spread on a glass, put cover slip, and samples were examined (Parija, *et al*., 2003). Double smears were prepared by this method. The Iodine-Glycerol wet mount of feces was compared with 10% KOH and saline wet mount preparations of feces. On the other hand, scotch tape technique can be used for diagnosing *E. vermicularis*. It was applied to the anal area of infected children with pinworms and their eggs to stick to the cellophane tape (McConnaughey, 2014) ; (Mohammadi, *et al*., 2014). Also, Concentration Method, Formalin-Ethyl acetate sedimentation technique is used in a centrifuge tube add 10 ml of 10% formalin and add 4 gram stool (0.5 teaspoonful), which was strained through two layer of surgical gauze into glass centrifuge tube. Add %10 formalin to fill tube ,then suspension was centrifuged at 500 rpm for 10 minutes, the supernatant decanted and the particulate matter suspended repeatedly until the supernatant was clear. After an additional 10 ml of 10% formalin mixed well by wooden stick, after that add 4 ml ethyl acetate and the suspension shaken vigorously, following centrifugation at about 500 rpm for 10 min, the entire supernatant was poured off (Al-Jawabreh, *et al*., 2019). Two thin films of the sediment were prepared with lugols iodine and without, each of them mounted with cover slip, then examined under light microscope 10X and 40X objective lenses.

MOLECULAR-BASED DIAGNOSIS

DNA EXTRACTION

The genome DNA was obtained from the head of the adult worm (figure 1) with the using of extraction kit (BIONEER, Korea) with some modifications according to the production instructions ; the incubation period in the tissue westernization phase was increased to 3 hours and absolute ethanol was used instead of isopropanol for DNA precipitation as used by Koyee, *et al*., (2016). The samples were immersed in mortars and their contents were transferred to a sterile tube with 200 µl of tissue

insulation and keep it in the incubator for 3 hours. The rehabilitation and quantity for DNA concentration were carried out with NanoDrop (ND-1000, USA). Genomic DNA samples with (A260-A320) / (A280-A320) proportions over 1.7 and the product received more than 0.5 µg

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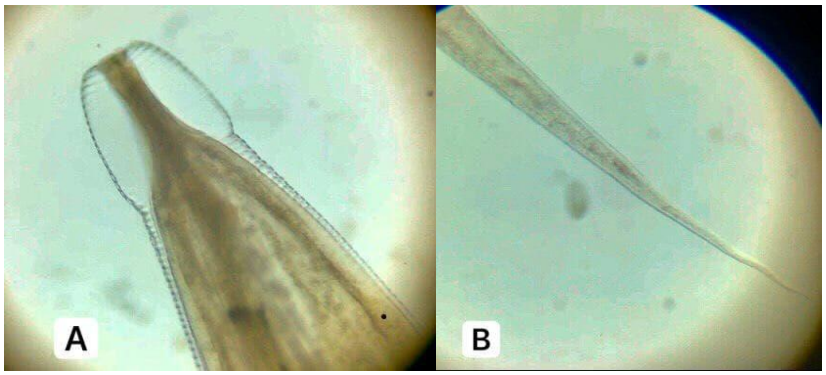

Figure (1): Adult stage of *Enerobius vermicularis*, showing region of DNA extraction from A-head (40X) and B-tail (40X)

DNA AMPLIFICATION AND SEQUENCING

A 28S rDNA region was amplified by Polymerase Chain Reaction (PCR). The primers were universal, forward primer C1 (ACCCGCTGAATTTAAGCAT at position 25), and reverse primer C3 (CTCTTCAGAGTACTTTTCAAC at position 390), they were designed and selected by Mollaret, *et al*. (2000). They were selected to be specific to nematodes. Using MJ Study, Applied Biosystem (AB) thermal cycler, PCR reaction and situation were executed. Fifty µl of reaction mixture prepared in PCR tubes containing 2 µl of DNA template, 25 µl of a (High Pure PCR Template Preparation Kit) master mix, 1 µl per base and 21 µl of double demonized water (ddH_2O). Cycling conditions include initial denaturation for 5 minutes at 94 °C, 35 denaturation cycles at 94 °C for 45 seconds, annealing temperature at 51 ° C for 45 seconds, expansion for 45 seconds at 72 °C and 5 minutes for final expansion at 72 °C. Agarose gel electrophoresis was employed to check the efficiency of PCR reactions. The samples were prepared and run in 2% agarose gel, then stained with ethidium bromide that makes the DNA visible under UV light, with the expected size of the PCR product was 365 bps. In the current research, to identify the order of 28S rDNA nucleotides from the pinworm, the nucleotide sequence analyzer ABI 3130X (SINGAPORE) was also used (sequencing was performed in Turkey). Pinworm PCR fragments were took out from agarose gel and used as a DNA pattern source to amplify special PCR sequences. Two hundred and six children (104 females and 102 males) aged less than one year to 12 years old with a mean age of 5.98±2.7 participated in the study and an overall percentage of infection is 68 (33%) was recorded. Several factors which affecting fluctuation of infection were determined and analyzed during this study among them, age, family size, gender, parent education, hand washing, and finger nailed children as shown in table (1). There is a significant difference among different age groups (p-value= 0.002) as shown in figure 2. Similar result was

recorded by Fan, *et al*., (2019) they recorded an overall prevalence of pinworm infection with 22.4% (88/392) and showed a much higher prevalence in children over 5 years old (32.77%) than in children under 5 year olds (17.95%). whilst disagree with that noticed by Forson, *et al*. (2018). Children aged less than one year to 5 years old had lower prevalence of infection (22.5%) in comparison with those aged over 5 years (43.3%). This difference in infection between age groups may be due to the fact that the concern for personal hygiene is that at the age of ≤5, they are in the care of the parents and under supervision, on the other hand, children less than 3 years of age are fed by one parent and stay more at home than children aged > 5 where they spend more time outdoors. As a result, they are more likely to play with the ground than young children and have more frequent physical contact with their friends; therefore, they are more susceptible to infection with pinworms. Regarding gender, there is a non-significant variation (p-value= 0.843) of the rate of infection between female (33.7%) and male (32.3%). The results of this study are consistent with the results of Chen, *et al*. (2017) ; Fan, *et al*. (2019) and disagree with that mentioned by Hammadi, (2012) from AL-Mahmoudyia area/Baghdad province , (Al-Bayati, 2005) from Kirkuk province, (Al-Daoody and Al-Bazzaz, 2020) in Erbil province. The reason of this result due to their family houses are too small in refugee camp which each house consist only one bedroom that is why all of children sleeping together, the school are mixed, and the large numbers of children were playing every day together and this has easy the spreading of the infection. Education levels of parent showing no any significant relationship to infection by this parasite (p-value = 0.323) and this study confirmed infection by this parasite (27.3%) among children with low parent education level (mother education level) and disagree with that mentioned by Quihui, *et al.*, (2006) ; Forson, *et al.*, (2018) as shown in figure 4. Statistically a significant result (p-value=0.039) was noticed the prevalence lower among children with frequent hand washing (30.1%) and higher in those with infrequent hand washing (48.5%) as shown in figure 3. The habit of washing hands with soap after playing with the soil and before eating is most often associated with pinworm infection. Children who do not wash their hands with soap before eating allow microorganisms, including pinworm eggs attached to their fingernails, to enter their bodies with food. Similar result was recorded by Chen, *et al*. (2017) in Taipei, Taiwan and Muliawati, *et al*., (2020) from that interpreted by Ercumen, *et al*., (2020). Other factors like family size (p-value=0.424) play no any significant role in the variation of the infection rate among children because some large families had personal hygiene in the other hand, same of them small families but did not personal hygiene, and finger nailed children (p-value=0.57) also play no any important role in the variation of the infection rate among children because if they are hands dirt, it is not important which they are kept the nails short or not. these results of the present study, disagree with that of Forson, *et al*., (2018) they mentioned that the family size was a predisposing factor (p-value= 0.031) for parasitic infections (figure 5). In general , the results of the present study (33%) is lower than the study that conducted in the Jaras camp by Bustami, (2010) in Jordon, 448 in total 812 children (55.2%),also Keskin and Ay Bektaş, (2014) from Ankara, Turkey, In Iraq and the Kurdistan region. A low percentage (3.40%) of infection was recorded by Ahmed and Al-Marjan, (2018) among displaced children in Kirkuk province-Iraq. A low percentage 0.35% also was recorded by Hassan and Mero, (2020) among children in various localities of Duhok city and nearby village. 27.13% was recorded by (Al-Daoody and Al-Bazzaz, 2020) in Erbil province; 32.2% was recorded by Turhan, *et al.* (2009) in Turkey.

Table (1): The prevalence of pinworm infection among children in relation to several epidemiological factors.

Characteristics	Details	Positive	p-value*
Age	0-5 years	23 (22.5)	0.002
	6-12 years	45 (43.3)	
Family size	1 to 5 person	29 (30.2)	0.424
	More than 5 person	39 (35.5)	
Gender	Female	35 (33.7)	0.843
	Male	33 (32.3)	
Education level of father	None	18 (32.1)	0.433
	Below secondary	21 (32.8)	
	Secondary	25 (38.5)	
	University or above	4 (19.0)	
Education level of mother	None	15 (27.3)	0.323
	Below secondary	24 (34.3)	
	Secondary	27 (39.1)	
	University or above	2 (16.7)	
Finger nail	Tall	54 (32.2)	0.57
	Short	14 (36.8)	
Washing hand before eating	Frequent	52 (30.1)	0.039
	Infrequent	16 (48.5)	

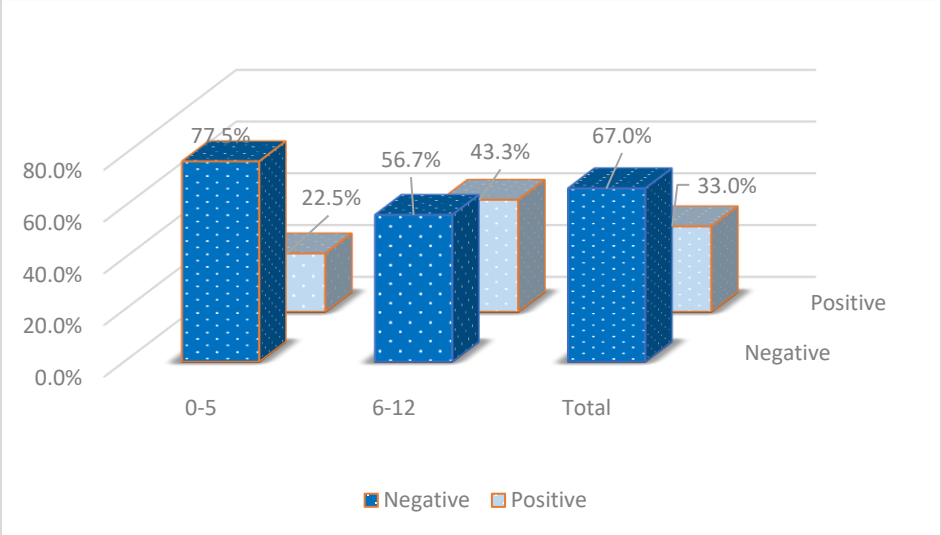

Figure (2): A Chart shows a significant difference by infection among age group.

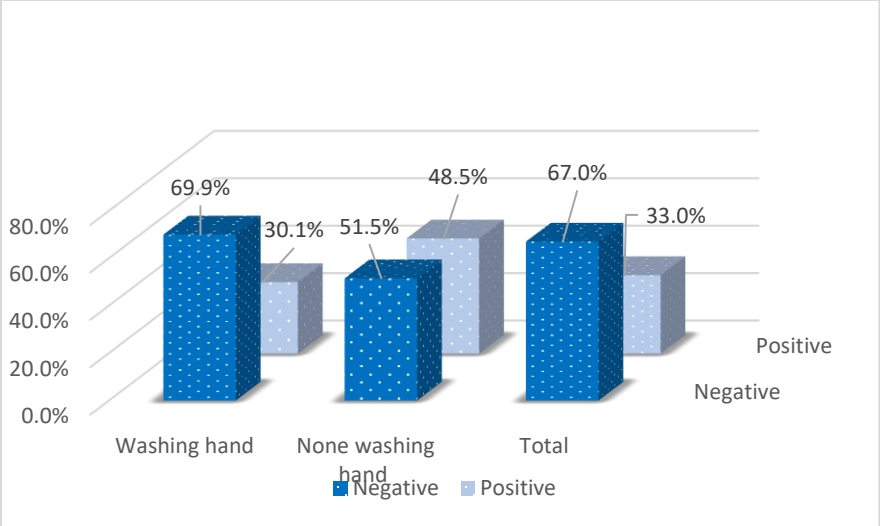

Figure (3): A Chart shows a significant difference by infection among frequent and none frequent hand washing.

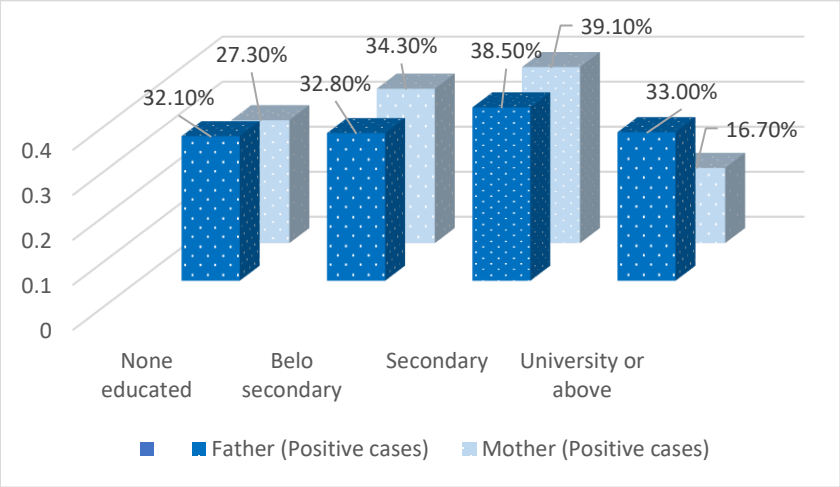

Figure (4): A Chart shows none significant difference by infection regarding to education levels of parent.

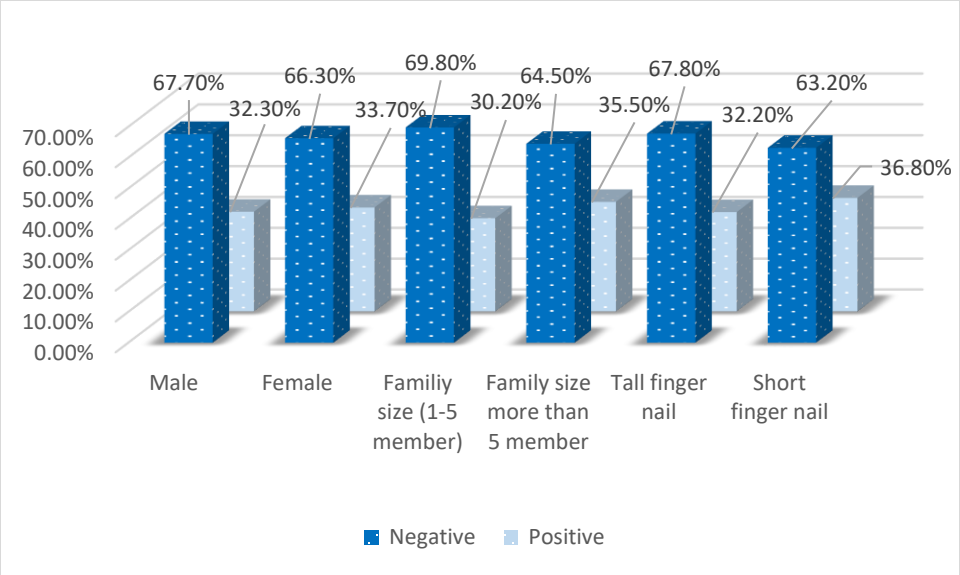

Figure (5): A Chart shows none significant difference by infection depending to several factors.

MOLECULAR STUDY

Polymerase chain reaction (PCR) techniques using 28S rDNA (28S ribosomal RNA gene) sequencing have confirm to be a reliable tool for identifying the helminthes species and their evolution (Verma, *et al*., 2012). 28S rDNA is 28S rRNA gene which was firstly used by Mollaret *et al.*, (2000) to study of phylogenetic analysis of the monogeneans and their affair with Digenea. This sequence is universal and its length is 365bp (Figure 2). In this study, DNA sequence of the pinworms was a 28S rDNA value of 304 bp, the amplified fragment was 365 base pair, while the quality of the sequence analysis was 61 nucleotides missing. PCR were conducted for several isolated adult worm (head of female only) for the result confirmation and the sequencing procedure were re-redoin Turkey country several time until getting to best result and the sequenced graph was placed on BLAST in FASTA format as :

>IRAK_F

CAAATGCGCTACGAGGATCCTTAGTACGGCGAGTGAACAGGGAGAAGCCCAGCGTTGAATT

CTATGATCATTCTATGATCATTTGGAACCTGTAGCGTATAGGTGTGGCCGCTTGATGGTTCTT

TGTATACTCTAAGTCCCCTTGAGTGGGGCTACAGCCCATTGATGGTGCTAGGCCAGTAGGAG

TATCGGGGTTCTGTCAAATTTCGGCTATACTTTGGAGTCGAGTTGCCTGGGATCGCAGCTCA

AAGTGGGTGGTAAACTTCATCTAAGGCTAAATATTACTACGAGACCGATAGCCAACAAGTA

CCGTGAGGGAAAGTTGAAAAGCTTCCTTGAAGAGA

Sequence was submitted to Gene bank of NCBI and recorded with an accession number of MZ400682. and then compared to other stored GenBank *Enterobius* which are recorded previously based on 28S rRNA partial sequence from DNA data bank of Japan (DDBJ). BLAST results (http://ddbj.nig.ac.jp/blast/wabi_blast_2021-0606-0240-00-303-351432) showed that the query sequence was 99% similar to *E. vermicularis* (Figure 3). Pinworm species are genetically identical to other *Enterobius* species

present with the same rDNA sequencing fragment marker from the DNA data bank of Japan (DDBJ). Preliminary sequence analysis using universal primers of working sample coverage prefixes showed that the parasite belongs to the species *E. vermicularis.* Identification of the Entrobius vermicularis based on the 28Sr DNA sequence analysis represent as a fist attempt in the nematode diagnosis in Iraq (communication through Iraqi parasitologist groups).

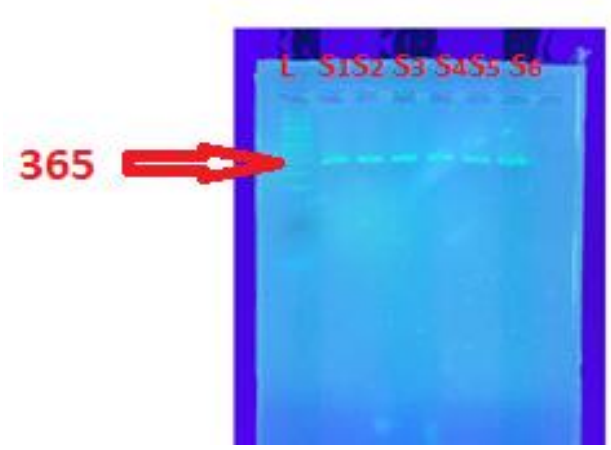

Figure 2: PCR analysis of *E. vermicularis* using 28S rDNA (ladder = 100bp)

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            >LC416069|LC416069.1 Enterobius vermicularis gene for 28S rRNA,
            partial sequence.
           Length = 773
 Score =  663 bits (301), Expect = 0.0
 Identities = 304/305 (99%)
 Strand = Plus / Plus
Query: 160 acggcgagtgaacagggagaagcccagcgttgaattctatgatcattctatgatcatttg 219
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 18  acggcgagtgaacagggagaagcccagcgttgaattctatgatcattctatgatcatttg 77

Query: 220 gaacctgtagcgtataggtgtggccgcttgatggttctttgtatactctaagtcccctttg 279
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 78  gaacctgtagcgtataggtgtggccgcttgatggttctttgtatactctaagtcccctttg 137

Query: 280 agtggggctacagcccattgatggtgctaggccagtaggagtatcggggttctgtcaaat 339
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 138 agtggggctacagcccattgatggtgctaggccagtaggagtatcggggttctgtcaaat 197

Query: 340 ttcggctatactttggagtcgagttgcctgggatcgcagctcaaagtgggtggtaaactt 399
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 198 ttcggctatactttggagtcgagttgcctgggatcgcagctcaaagtgggtggtaaactt 257

Query: 400 catctaaggctaaatattactacgagaccgatagccaacaagtaccgtgagggaaagttg 459
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 258 catctaaggctaaatattactacgagaccgatagccaacaagtaccgtgagggaaagttg 317

Query: 460 aaaag 464
            ||||
Sbjct: 318 caaag 322
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Figure 3: Pairwise alignment of 28S rDNA sequence of *E. vermicularis*. Query is the study or sample sequence and Subject is the GenBank sequence.

CONCLUSION

An overall percentage of infection by pinworm is 33%. Difference in the infection related to several factors. There is a significant variation among age group. Regarding gender, there is a non-significant variation of the rate of infection between female and male. Education levels of parent especially mother education showing no any significant relationship to infection by this parasite. The habit of washing hands with soap after playing with soil and before eating is significantly associated to pinworm infection. Other factors like family size, play no any significant role in the variation of the infection rate among children and finger nailed children also play no any important role in the variation of the infection rate among children. Polymerase chain reaction-based techniques utilizing 28S rDNA sequences can mentioned as investigated to be a faithful tool to diagnose the helminthes species. Identification of the *Entrobius vermicularis* based on the 28Sr DNA sequence analysis represent as a first attempt for the diagnosis of nematode in Iraq.

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