JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2014. Vol. **8**(5), p. 3995-3999

Genetic Classification and Differentiation of *Enterobius vermicularis* Based on Mitochondrial Cytochrome C Oxidase (*cox1*) in Northwest of Iran

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(Received: 17 May 2014; accepted: 05 July 2014)

***Enterobius vermicularis* is one of the most prevalent parasitic helminths, particularly in children. According to the studies, three different genotypes (A, B and C) were revealed from human and chimpanzee. This study conducted to investigate the existence and distribution of different *E. vermicularis* genotypes based on mitochondrial cytochrome c oxidase subunit 1 (*cox1*) in northwest of Iran. 45 positive scotch tape samples of *E. vermicularis* were collected from various areas of Tabriz. After DNA extraction, the targeted DNA region was amplified for the mitochondrial cytochrome c oxidase 1(*cox1*) gene by nested PCR method. All amplicons were sequenced and then analyzed by specific phylogenetic software. Results of the present study showed that, *Enterobius vermicularis* have two subtypes including B1 and B2 in northwest of Iran by Cox1 gene sequencing method. In conclusion, B1 and B2 subtypes of *Enterobius vermicularis* in human are the predominant genotype of this nematode in northwest of Iran. Regarding to the prevalence and public health importance of the disease and role of the parasite genotyping in prevention, control and treatment, further studies are needed to determine genotypes of *E. vermicularis* in other regions.**

Keywords: *Enterobius vermicularis*, Cytochrome c oxidase 1 (*cox1*), Genetic classification, Iran.

Enterobius vermicularis (pinworm or threadworm), is an intestinal nematode with the cosmopolitan distribution that causes Enterobiasis in human[1]. Graham method (scotch test) is the specific method for detection of the disease because the worm migrates and release ova in perianal. These parasites may causes itching and sleep disturbances, while most infections are asymptomatic[1,2]. According to the studies, the prevalence of *E. vermicularis* in children reported as 39% in Thailand[3], 37% Sweden[4], 29% Denmark[5] and 7.3 – 39.9% in Iran[6-9]. Person-to-person transmission of this infection commonly found among family members and institutionalized populations[10]. Currently, molecular investigations are being widely used for studying genetic diversity of parasites. These studies can provide appreciated insight into the geographic distributions, characterize their host variety and allows to monitoring of possible genetic restructuring[11, 12]. Also, phylogenetic analysis is a declaration about the evolutionary relationship between a set of homologous characters of one or several organisms[12]. Molecular studies on the

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characterization of *E. vermicularis* are limited. Nakano et al in Japan reported 3 different clusters of *E. vermicularis* (designated as type A, B and C) from human and chimpanzee. This phylogenetic study revealed that, all human samples were compromise of A type, while B and C type were detected in chimpanzees samples[13]. In the similar genotypic analysis studies, Ferrero *et al.*,[5] and Piperaki *et al.*,[14] from Denmark and Greece recognized three major clusters of pinworms. On the contrary to Nakano results[13], all human samples in these studies were clustered in type B. Regarding to relatively high prevalence (39.9%) of enterobiasis in northwest of Iran, which have temperate climates and due to lack of available data about molecular studies on *E. vermicularis* in Iran, present study conducted for the first time to investigate the existence and distribution of different *E. vermicularis* genotypes based on mitochondrial cytochrome c oxidase subunit 1 (*cox1*) by direct sequencing method in Iran.

MATERIALS AND METHODS

Sample collection

In this study, 45 positive scotch tape samples of *E. vermicularis* were collected from various areas of Tabriz, northwest of Iran. The slides were washed using ova elution KIT (Pak gene Yakhteh Co., Cat No. PGSO 10100) and the ova were sediment and stored at 4°C until DNA extraction.

DNA extraction

Approximately one hundred ova were used to DNA extraction by using Pak gene Yakhteh KIT (Cat No. PGEX 4030). All extracted DNA stored at -20°C.

Nested PCR method

The targeted DNA regions were amplified for the mitochondrial cytochrome c oxidase 1 (*cox1*) gene by nested PCR method using the outer primes EVM1 (5-TTTTTGGTCATCCTGAGG TTTATA TTC-3), EVM2 (5-CCATCCAAAATAG GATTAGCCAACA-3) and inner primers EVIF (5-TTGGTCATCCTGAGGTTTATATTC-3), EVIR (5-TCCAAAATAGGATTAGCCAACA-3) (14). The product size for outer and inner amplifications was 390 bp and 379 bp respectively. Thermal cycler condition which used for the first PCR was: 6 min initial denaturation at 94°C followed by 45 cycles at 94°C for 1 min, 58.2°C for 1 min, 72°C for 1 min, and a final extension for 10 min at 72°C. The second PCR was done with the same condition, but the annealing temperature was set at 50.1°C and the number of cycles was reduced to 30. Electrophoresis was performed on 2% agarose gel containing 10 µl / dl safe stain (CinnaGen Co., Iran).

Nucleotide sequence and Phylogenetic analyses

Cytochrome oxidase 1 gene from 45 isolates was sequenced by applying to related company and using Genetic Analyzer 3130 ABI. All sequences were aligned using ClustalW. Sequence similarity was carried out using the Basic Local Alignment Search Tool (BLAST). Multiple sequence alignment and then phylogenetic analysis was performed by using MEGA 4.0 software. Different statistical methods were undertaken for phylogenetic analysis of the aligned sequences in the algorithm of this software. Finally, the phylogenetic tree was constructed by the Maximum Composite Likelihood method.

RESULTS

In this study, 45 positive *E. vermicularis* scotch tape samples were successfully amplified (Figure 1) and then analyzed. Regarding to genotypic analysis of the sequences, all the samples were clustered in type B and there were no any A and C types. Also in the present study, *E. vermicularis* type B includes 2 subtypes by phylogenic analysis (Figure 2). Twenty and twenty five of our samples were subtype B1 and subtype B2, respectively. One of each B1 and B2 subtypes submitted and registered in GenBank under following accession numbers KJ780776 and KJ780777. In this investigation, all sequences that identified B1 subtype were different from other sequences in GenBank; of course it has one nucleotide variation with an isolate of Germany (JQ411508) and Denmark (JQ411498). B2 sequences were closely similar to isolates of Denmark (JQ411485) and Greece (HQ317440). Reference genotypes of *Enterobius vermicularis* with accession numbers that previously registered in GenBank and genotypes obtained from this study are shown in table 1.

Table 1. Enterobius vermicularis genotypes obtained from the present investigation and reported of other molecular studies from different countries

*E.vermicularis*genotype	Host	Accession number	Reference
EvF1-EvF3	Human	-	Present study
EvF4 (B1)	Human	KJ780776	Present study
EvF5-EvF20	Human	-	Present study
EvF21-EvF26	Human	-	Present study
EvF27 (B2)	Human	KJ780777	Present study
EvF28-EvF45	Human	-	Present study
A	Human	AB221470-72	[13]
A	Chimpanzee	AB221473-74	[13]
B	Chimpanzee	AB221467-69	[13]
B	Human	HQ317429-40	[14]
B	Human	HQ395270-71	[14]
B	Human	JQ411483-509	[5]
C	Chimpanzee	AB221457-66	[13]

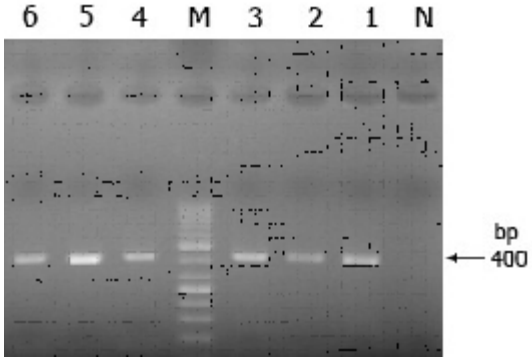

Fig. 1. Agarose gel electrophoresis of 6 PCR products of human *E. vermicularis*. Lanes 1-3 and 4- 6: positive human isolates, N: negative control M: 50 bp DNA size markers.

DISCUSSION

According to the results of the present study, *Enterobius vermicularis* have two subtypes including B1 and B2 in northwest of Iran by *cox1* gene sequencing method. The *cox1* gene has been reported to display high variability than the other regions and proposed to phylogenetic studies[15]. Our study showed that, all (n=45) *E. vermicularis* scotch test samples from humans in northwest of Iran were clustered as type B. In the similar studies which conducted in Greece, Germany and Denmark; all samples of *E. vermicularis* were B genotype and eleven haplotype[14]. On the contrary to this work, Nakano *et al.*, (2006) isolated type A sequence from human in Japan, whereas all captive chimpanzee samples clustered in type B and C. In this study, 18 haplotypes in the nucleotide sequences were reported[13]. In the present investigation, we have not any A and C types based on sequences analyses that it may be due to unavailability of chimpanzee to human in this region. To the best of our knowledge, many animals are also parasitized by *E. vermicularis*, but this disease is transmissible to humans only by chimpanzee[16]. A and B types have been revealed to exist in both human and chimpanzees but infection with type C occurs in chimpanzee and erenow; there is not any study to approve C type of *E. vermicularis* in human samples. Result of our study indicates new information on the variety of *E. vermicularis* genotypes in human from Iran. The phylogenic tree of *E. vermicularis* in this study showed that all sequences lied in two haplotype. This finding suggests that all of our study populations may be infected by a common source. This work was undertaken on human adhesive tape samples to finding *E. vermicularis* eggs. Due to the low amount of DNA and may be containing PCR inhibitors on the adhesive tape samples, it was necessary to use an appropriate method as like as nested PCR, for the amplification of this parasite DNA[13,15]. Mitochondrial gene sequencing, which used in the present study, is more preferable method for direct genotyping of *E. vermicularis* from clinical samples. This technique can be use for molecular and epidemiological studies to explain the genetic variability for this interesting parasite.

Fig. 2. Phylogenic tree based on *cox1* gene sequences of *E. vermicularis* isolates. The tree was constructed by using the Maximum Composite Likelihood model with 500 bootstrapping in MEGA4 package. The numbers above the branches indicate the percentage of bootstrap. Ascaris suum was used as outgroup

In conclusion, B1 and B2 subtypes of *Enterobius vermicularis* in human are the predominant genotype of this nematode in northwest of Iran. Regarding to the prevalence and public health importance of the disease and role of the parasite genotyping in prevention, control and treatment, further studies are needed to determine genotypes of *E. vermicularis* in other regions.

ACKNOWLEDGMENTS

This work was supported fully by Tabriz Infectious and Tropical Diseases Research Center (Grant no: 91-14), Tabriz University of Medical Sciences, Tabriz, Iran. This is a report of a database from thesis of Vahid Rahnamaye Hayati Hagh entitled "Genotyping of Enterobius vermicularis from the infected human samples in Tabriz city using mitochondrial cytochrome oxidase 1(cox1) gene by PCR-Sequencing method" registered in Tabriz University of Medical Sciences. The authors declare that there is no conflict of interest.

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