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Geno-typing and Comparison of Conventional and Molecular Diagnostic Techniques for Detection of Blastocystis on health centers in Kerman, Iran

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Running Title: Geno-typing and Comparison of Conventional and Molecular Diagnostic Techniques for Detection of Blastocystis

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SUMMARY

Background: Blastocystis hominis is a common intestinal protozoan with a prevalence reported variously from different countries. Considering the relatively high prevalence of this condition and the emphasis on its pathogenesis, this study determined Geno-typing and Comparison of Conventional and Molecular Diagnostic Techniques for Detection of Blastocystis on health centers in Kerman, Iran

Methods: A total of 210 single samples of stool were collected from patients presenting to healthcare centers in Kerman. The samples were transferred to laboratory in the same day and examined for diagnosis of infection with blastocystis hominis using direct method, concentrating method (formalin-ether), Trichrome staining method (trichrome), and Real-time PCR molecular method.

Results: The frequency of blastocystis hominis using direct method and Trichrome staining method showed that 30 cases under study (%14.3) were positive including 17 females and 13 males. In the molecular method, 66 cases (%31.42) were positive for infection with blastocystis hominis including 30 (%45.5) men and 36 (%54.5) women. In this study, the maximum and minimum frequencies pertained to genotype (subtype) 3 (%56.06) and genotype 2 (%1.51) with no cases found for genotype 6. Also, mixed infection with genotypes 3 and 4 (%42.88), genotypes 4 and 5 (%14.28), genotypes 3 and 5 (%14.28), genotypes 1, 2 and 3 (%14.28), genotypes 3, 4, and 7 (%14.28) were reported.

Conclusion: Hence, promoting the knowledge about personal improving environmental sanitation, and supplying healthful drinking water can prove effective in decreasing infection with blastocystis hominis.

Keywords: Blastocystis hominis, direct method, trichrome staining method, molecular method.

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*** Highlights (for review)**

*Outbreaks of human Blastocystis associated with the Public Health status, often occur in Kerman city.

*Our findings also showed with no cases found for genotype 6. Also, mixed infection with genotypes 3 and 4 (%42.88), genotypes 4 and 5 (%14.28), genotypes 3 and 5 (%14.28), genotypes 1, 2 and 3 (%14.28), genotypes 3, 4, and 7 (%14.28) were reported.

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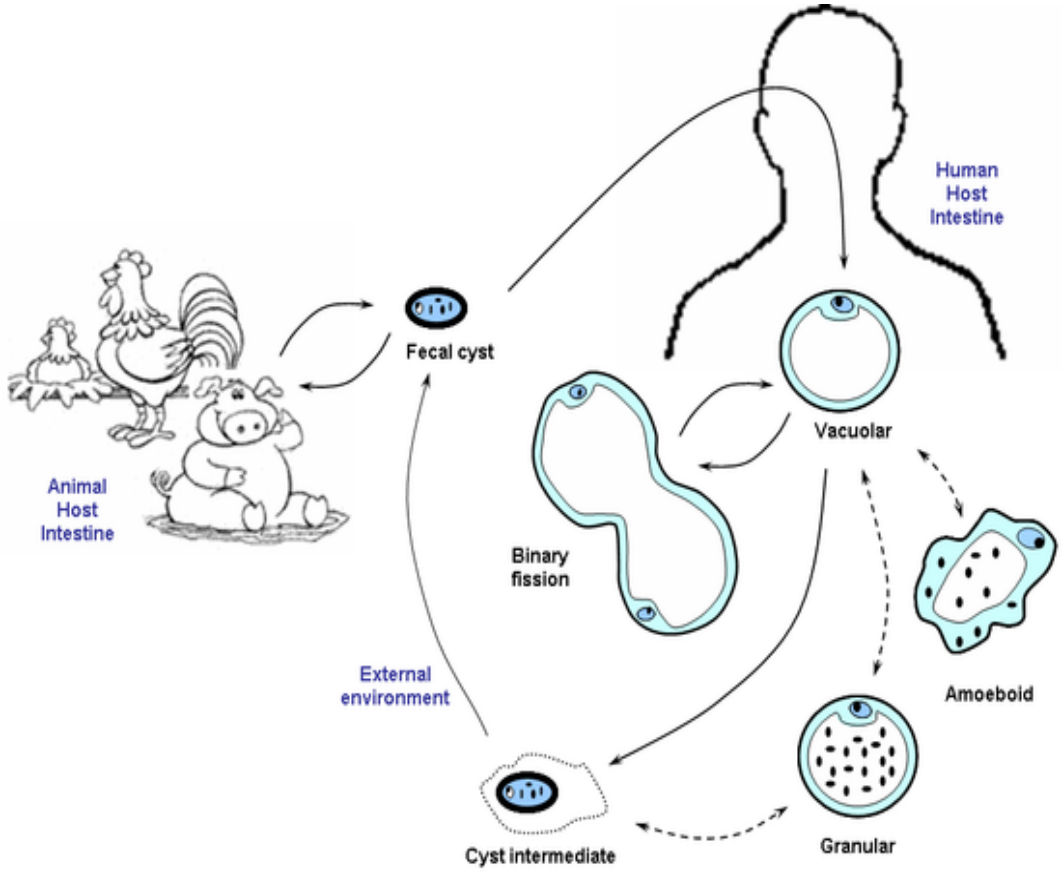
*Improving of environmental sanitation and supplying healthful drinking water can prove effective in decreasing infection with Blastocystis hominis.



Graphical abstract

INTRODUCTION

The prevalence of enteric parasitic disorders is directly correlated with the level of hygiene and socio-economic status of each region. Its prevalence is also not the same in different parts of each country and varies depending on climatic conditions, public health, and dietary habits. Factors such as gender, age, and education level may play a determining role in some cases of parasitic infections like *Enterobius vermicularis*. Its low prevalence in various age groups is justifiable on the basis of pattern of parasite transmission (1). Blastocystis is a kind of protozoan and zoonotic parasite found in the large intestine of man and many other vertebrates (2). It was identified in human defecate since one century ago, but it was rendered as non-pathogenic factor for a long time. However, in recent years, many studies revealed many unknown ambiguities about it and its significance was manifested for Parasitologist (3). The cyst or infectious factor of this parasite is resistant against water and is readily transferred through water or contaminated food to various hosts which are not very specific (4). The prevalence of this parasite varies around the world between %0.8 and 61.8 while this rate varies in different countries and even in different regions of each country. Generally, its prevalence is higher in developing countries, especially in tropical regions. The factors influencing its increased prevalence include poor hygiene and close proximity with animals. Contrary to some other gastrointestinal parasites, some studies indicated that its prevalence is higher in adults with an increased percentage of prevalence in some seasons of the year (5). Many studies have reported its pathogenic nature and attributed symptoms like acute and chronic diarrhea, abdominal pain and flatulence, constipation, nausea, irritable bowel syndrome, gastroenteritis, joints pain, and skin rashes to this infection (6). This parasite with various developmental courses and different multiplication methods such as dyadic (duplicate) division, proliferation, endodyogeny, sporogony, schizogony, and blastomia and also various morphological forms like vacuolated, granulated, ameboid and cystic forms with 2-200 nm diameters is rendered as a notorious and mysterious unique parasite. Unfortunately, these traits have reduced specificity and sensitivity in microscopic examinations (7). This study Geno-typing and Comparison of Conventional and Molecular Diagnostic Techniques for Detection of Blastocystis on health centers in Kerman, Iran.

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METHODS

Collection of Samples

A total of 210 stool samples were collected from clients presenting to the healthcare facilities in Kerman. Demographic information of the clients such as age, gender, occupation, education level, and place of residence was recorded at the time of stool sampling. The samples were examined with formalin-ether method, Trichrome staining method, and molecular method to compare sensitivity and specificity of the three diagnostic methods while the genotypes of blastocystis hominis were determined in Kerman for the first time.

Diagnostic Methods

1. Direct (Formalin- ether) Method

Using a wooden applicator, some stool (0.5 g) was taken from various parts of the sample.
2. The obtained sample was placed in a tube containing 7 mL of %10 formalin.
3. The four-layered gauze was placed in a funnel and the funnel was placed in a centrifuge tube.
4. The stool suspension was filtered through the gauze.
5. Five mL of ether were added to the test tube and shaken well after fastening the tube cork.
6. The resulting suspension was centrifuged for 1 min at 2000 rpm. After centrifugation, four layers were formed in the tube. The first layer is ether and fat, the second is stool particles, the third is formalin, and the fourth layer is precipitated materials or deposits.
7. After removing the tube cork gently, the layer of fat formed on the upper part of the tube was separated and the overlaying fluid was discarded.

8. The deposit at the bottom of the tube was mixed well and one drop of this fluid was placed on a microscope slide and examined under microscope with 10x-40x object piece magnification (8). Although this method does not enjoy sufficient sensitivity for identifying blastocystis, the stool sample was examined with this method to increase the chance of demonstration of this parasite in the stool sample.

2. Trichrome Staining

In using the fresh samples of stool, it should be placed in Bowen fixing solution immediately after preparation of extension. If the sample is dried in any stages of extension, it will be of no use. The time required for this stage was estimated to be 15 min. Then, the extension was placed in %70 alcohol for 1 min. After 15 min, it was placed in a tray containing Trichrome for 30 s and then it was placed in acid-alcohol solution for 30 s. Subsequently, it was placed in %96 and %100 alcohol solution for 1 min, respectively and finally, it was placed in xylol clearing agent for 5 min. Ultimately, the prepared smear was examined for various forms of the parasite under light microscope with 1000x magnification (oily lens) (9). This method possesses high accuracy and sensitivity. The positive samples for blastocystis were stained with trichrome staining to show the different forms of the parasite.

3. Molecular (REAL- Time PCR) Method

A. DNA Extraction

DNA extraction was performed using Accuprep Genomic DNA Extraction Kit (Bioneer) with LOTNO K3032 on the basis of the manufacturer's instructions. The Real-Time PCR molecular method was carried out with general primers given in Table 1 using SYBR Green with Line-Genek fabricated by the BioER Company, China.

Table1. General primers used for determining blastocystis hominis with Real-Time PCR.

	SEQ	MW		%GC	TM	H_2O
		calculated	measured			
SBF	5´- AGTAGTCATACGCTCGTCTCAAA-3´	7.007.6	7.013.2	43.5	61.1	300
SBR	5´- TCTTCGTTACCCGTTACTGC-3´	6.010	6.013.1	50	58.4	300

Table2. The temporal and thermal schedule of Real-Time PCR reaction.

Stage	Temperature (ºC)	Time	Number of cycles
Primary denaturation	95	15min	1
Secondary denaturation	95	20 s	35
Annealing amplification	60	40 s	

Finally, the fluorescent color was measured at the green canal at 60ºC. Then, the melting curve was plotted at each temperature from 60 to 95ºC for 10 s (Fig. 1 and Fig. 2).

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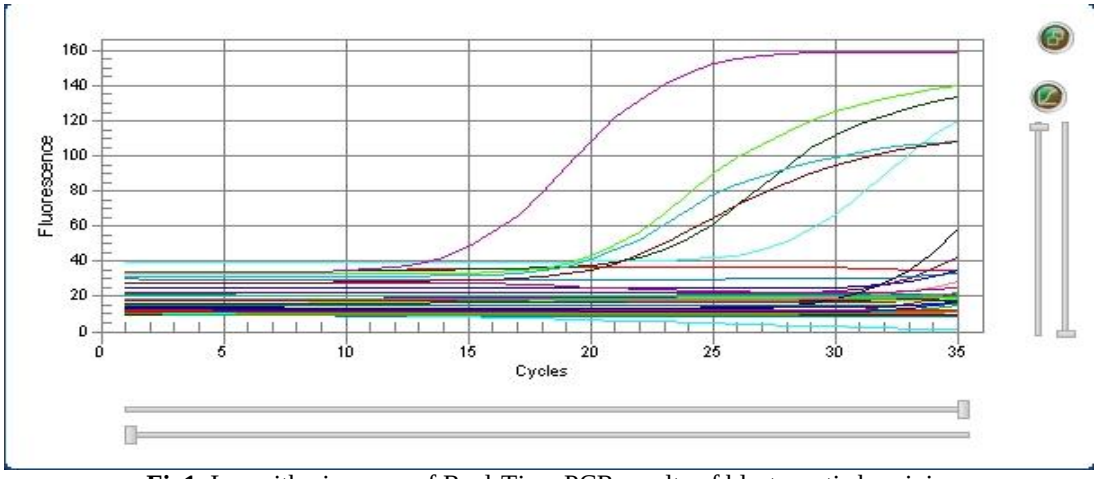

Fig1. Logarithmic curve of Real-Time PCR results of blastocystis hominis.

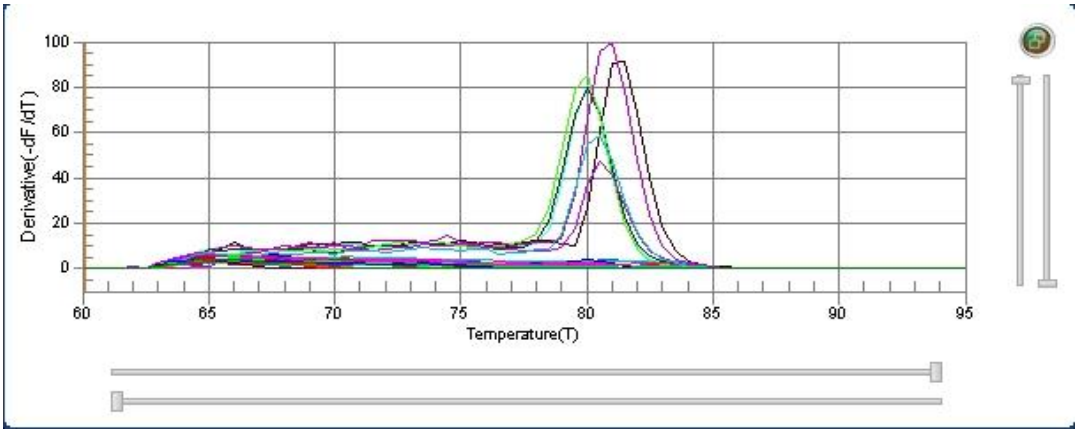

Fig2. Melting curve of positive results of blastocystis hominis

B. Genotyping Test

This test was performed using seven pairs of specific blastocystis hominis primers (10)with lyophilization master mix (Bioneer, Accu power PCR Premix, South Korea) under the following thermal protocol.

Table3. The temporal-thermal PCR protocol for all primers.

Step	Temperature	Time	Cycle
Predenaturation	95°c	10 Min	
Denaturation	95°c	20 Sec	
Annealing	55°c	30 Sec	**35**
Extension	72°c	40 Sec	

The specific bonds of 351bp, 704bp, 526bp, 487bp, 317bp, 338bp, and 650bp were determined, respectively, according to this protocol and then underwent electrophoresis.

C. Data Analysis

The gleaned data were analyzed with SPSS20 using Chi-square test. Normality of data distribution was compared among the three groups using ANOVA.

D. Ethics

Ethics The study was reviewed and approved by The Research and Ethical Review Committee of the School of Medicine; protocol identification number: Code: IR.MEDICINE.REC.1395.298.

RESAULTS

A total of 210 clients presenting to healthcare facilities in Kerman, Iran, participated in this study. Ninety-four (%%44.8) subjects were male with a mean age of 32.43±8.46 years and 116 were female with a mean age of 22.76±7.68 years. Also, 30 samples were positive using direct method and Trichrome staining method including 17 females and 13 males. However, 66 samples (30 males and 36 females) were positive using molecular method. The rate of prevalence of blastocystis is not the same in all parts of Iran and varies according to epidemiological,

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geographical, hygienic, sanitary, and cultural factors. Estimation of frequency percentages of infection with blastocystis hominis using direct method, Trichrome staining method, and molecular method in terms of age revealed a significant difference between age and frequency of blastocystis hominis using direct method and Trichrome staining method with most infected subjects being in the 0-10 years age group (%26.7) (P= 0.011). Nevertheless, no significant difference was observed between age and frequency of blastocystis hominis using the molecular method (P=0.185). In the study by Qolami et al. (2017), the highest prevalence of blastocystis was observed in the 40-50 years age group with %4.8 while the least frequency was seen in the -20 years age group with %0.4(11). The difference may be attributed to the point that there is increased exposure to the parasite with increasing age leading to increased infection with it. Determination of frequency percentages of infection with blastocystis hominis using direct method, Trichrome staining method, and molecular method in terms of occupation demonstrated no significant difference between occupation and frequency of blastocystis hominis with most infected subjects being house-keepers while the least percentage belonged to university students and employees(P=0.423, 0.139). The results of study conducted in Sari, northern Iran (2014) indicated that the rate of infection was greater in free jobs (%7.8) and farmers and animal husbandmen (shepherds) (%3.5) compared to other occupations (11). Estimation of frequency percentages of infection with blastocystis hominis using direct method, Trichrome staining method, and molecular method in terms of education level showed no significant difference between education level and frequency of blastocystis hominis with most infected subjects holding a sub-diploma degree while the least infection rate belonged to BS/BA+ degree (P=0.525, 0.124).Yet, the results of the study by Mahami Oskooyee et al. (2009) who investigated the prevalence of blastocystis hominis in Tabriz, northwest of Iran, showed a higher infection rate among those with lower levels of education (12). Also, the results of the study by Hazrati Tappeh et al. (2011) suggested that the rate of infection with gastrointestinal parasites decreased with increasing levels of education in families(13). Similar results were obtained in Sari, northern Iran, (2004) indicating a decreasing rate of infection with increasing level of education so that most cases of positive results (%8.3) held a primary school degree while the least rate of positive results (%0.4) belonged to those who had academic education (11). Additionally, a study in Nigeria showed that high prevalence rate of infection with parasites can be attributed to poor sanitary situations, economical poverty, and poor hygienic behaviors (14). Consequently, it may be concluded that the high rate of infection in subjects with lower levels of education may be due to lack of knowledge and awareness or low level of hygienic and sanitary conditions. Furthermore, determination of frequency percentages of infection with blastocystis hominis using direct method, Trichrome staining method, and molecular method in terms of gender revealed no significant difference between gender and frequency of blastocystis hominis using direct method, Trichrome staining method, and molecular method (P= 0.891, 0.865). In this regard, the results of a study in northern Iran indicated a significant correlation between prevalence of blastocystis hominis and gender so that the rate of prevalence of infection in females (%11.4) was greater than that of males (%2.8) (Qolami et al., 2017)(11). Furthermore, estimation of frequency percentages of infection with blastocystis hominis using direct method, Trichrome staining method, and molecular method in terms of place of residence revealed a significant difference between place of residence and frequency of blastocystis hominis with the three methods with most infected subjects living in the southern parts of Kerman while the least frequency belonged to those living in the western parts of Kerman. The frequency of blastocystis hominis genotypes among the 66 samples under study were as the following: genotype (subtype) 3 (%56.06), genotype 4 (%10.6), genotype Mix (%10.6) genotype 5 (%9.09), genotype 1 (%7.57), genotype 7 (%4.57), genotype 2 (%1.51), and genotype 6 (%0.0).

Table4. Frequency of genotypes in the samples under study.

Genotypes	Frequency	%
Genotype 1	5	7.57
Genotype 2	1	1.51
Genotype 3	37	56.06
Genotype 4	7	10.6
Genotype 5	6	9.09
Genotype 6	0	0
Genotype 7	3	4.57
Genotype Mix	7	10.6

As displayed in Table 5, the frequency of mixed genotypes in patients with positive results of infection with blastocystis hominis is a follows:

Table5. Frequency of mixed genotypes in the samples under study.

Mixed genotypes	Frequency	%
Genotypes 3 & 5	1	14.28
Genotypes 3 & 4	3	42.88
Genotypes 4 & 5	1	14.28
Genotypes 1, 2, & 3	1	14.28
Genotypes 3, 4 & 7	1	14.28

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DISCUSSION

The study by Khushnud et al. (2014) on investigating the prevalence and genotype variety of blastocystis hominis in clients presenting to healthcare facilities of Baghmalek town, the identified subtypes included ST_7, ST_5, ST_4, and ST_3 with ST_4 (%40.9) having the highest prevalence (15). Also, in an investigation of the subtypes in Hamedan, Iran, by Sardarian et al. (2012) the prevalence of ST_1, ST_3, and ST_2 were %56.1, %22, and %7.3, respectively with infection rate of mixed $ST_{1,3}$ being %14.6 (16). The highest frequencies belonged to ST_2, ST_3, and ST_1, respectively (16). A study was carried out in Tehran on 100 isolates of blastocystis with or without gastrointestinal symptoms with specific primers of subtypes using PCR. They identified four subtypes from the isolates which were ST_1, ST_5, and ST_2, respectively (17). Baadparva et al. (2012) reported the frequencies of ST3 (%56.7), ST5 (%20), ST_2 and (%13.3), respectively. Also, they reported the mixed infection with $ST_{2,3}$ (%6) and $ST_{3,5}$ (%3.3) (18). An investigation of subtypes in patients with gastrointestinal infections and hives in Malaysia, Singapore, and America, ST_3 was identified as the dominant subtype (2). Moreover, ST_3 was reported as the dominant subtype in immunodeficiency and cancer patients (19). There was also a report on identifying the subtypes in China reporting ST_3 as the dominant subtype followed by ST_1 ranking second (7). ST_4 is the second most common subtype in England, Europe, and North America. Various subtypes like ST_4, ST_2, and ST_1 were reported from Italy in addition to ST_3 (20). The most frequent subtypes reported in Sweden in patients with gastroenteritis and irritable bowel syndrome were ST_2, ST_1, and ST_3, respectively (21). In another study in Colombia, %100 of the patients with diarrhea were infected with ST_1 simultaneously while ST_1 was identified in all individuals without any clinical signs and symptoms (22).

CONCLUSION

Our findings indicated that the rate of infection with blastocystis hominis parasite was %31.42 in Kerman. This rate reveals that this parasite has a high prevalence in this region and, hence, any ignorance toward it may impose many problems for the patients. Considering these results, it was concluded that most patients infected with this parasite were aged 0-10 years, were female, had sub-diploma education, were unemployed, and lived in the poor suburbs. There was a statistically significant correlation between the rate of prevalence of blastocystis hominis parasite and place of residence; however, there was no significant correlation between "occupation, education, and gender" and "the parasite prevalence. The correlation coefficient was significant for age with both direct method and trichrome staining method; yet, it was not significant using the molecular method. In this study, the highest and lowest frequencies pertained to genotype 3 (%56.06) and genotype 2 (%1.51), respectively and no case was found for genotype 6. Also, there were mixed genotype infections of $ST_{3,4}$ (%42.88), $ST_{4,5}$ (%14.28), $ST_{3,5}$ (%14.28), $ST_{1,2,3}$ (%14.28) and $ST_{3,4,7}$ (%14.28). Additionally, our results demonstrated that awareness of and knowledge about this parasite and the application of suitable diagnostic methods in labs and healthcare centers can improve the early and prompt diagnosis of the infection predisposing to the promoted level of healthcare services and community health. We found that the direct method and Real-Time PCR technique were obviously different in diagnosing the positive cases. Consequently, the use of molecular methods is highly recommended in investigating the frequency of this parasite. It is also advised to apply structural modifications in the diagnostic trend of this parasite both in diagnostic methods and in comprehensive programs developed for staff education. Considering the development of molecular diagnostic methods and the confirmation of the existence of a correlation between specific species of infective factors like blastocystis hominis with high virulence and also given the low number of studies in this field in Iran, it is recommended that more future studies be conducted on identifying the potentially pathogenic strains of this protozoan. Physicians and clinicians should also pay more meticulous attention to the quality of laboratory and clinical diagnostic techniques used in identifying this parasite. In the cases that long-term clinical signs and symptoms are manifested, the use of therapeutic procedures is recommended. Also, some complementary studies ought to be completed to identify the factors effective in pathogenesis of the parasite in various parts of Kerman Province. Finally, it is recommended that this study be replicated on various groups including diabetics, HIV patients, and residents of retirement homes, kindergartens, and schools to obtain a better understanding of the distribution of this parasite in the community.

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