



# Molecular detection and genotype identification of *Acanthamoeba* species from bronchoalveolar lavage of patients with pulmonary symptoms suspected of cancer

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**Abstract** *Acanthamoeba* spp. are the most common free-living amoeba worldwide, inducing life-threatening diseases such as Granulomatous Amoebic Encephalitis, pulmonary infection, and amoebic keratitis. This study aimed to identify the FLA and *Acanthamoeba* genotypes in patients with pulmonary symptoms suspected of cancer in Kashan's hospitals, Kashan, Iran. This cross-sectional study was conducted on 97 bronchoalveolar lavage samples of patients with respiratory symptoms suspected of lung cancer, who were admitted to the Shahid Beheshti Hospital of Kashan from 2019 to 2020. The samples were cultured onto 1.5% non-nutrient agar enriched with killed *Escherichia coli* and examined for the presence of FLA. Following amoeba isolation and DNA extraction, *Acanthamoeba* spp. were determined by Polymerase Chain Reaction using JDP1 and JDP2 primers, which amplified a 490 bp fragment from the 18 S rDNA gene. Eighteen *Acanthamoeba* isolates were sequenced, and the genotypes were identified. The prevalence of FLA and *Acanthamoeba* and the relationship between symptoms and demographic variables were analyzed with SPSS Software

version 16. The prevalence rates of FLA and *Acanthamoeba* in the BAL samples was 86.6% and 73.2%, respectively. All *Acanthamoeba* isolates belonged to the T4 genotype. The most symptoms among *Acanthamoeba*-positive patients were dyspnea and cough; however, their difference was not statistically significant. The findings indicated the high prevalence of FLA and *Acanthamoeba* in BAL in the population suspected of cancer in Kashan. Since the T4 genotype is a pathogenic genotype of *Acanthamoeba*, training health and improving sanitation levels would help to prevent infection.

**Keywords** *Acanthamoeba* · Free-living amoeba · Bronchoalveolar lavage · Genotype · Cancer patient

## Introduction

Free-living amoeba (FLA), including *Acanthamoeba* Spp., are amphizoic protozoa isolated from environmental sources such as dust, soil, water, bottled mineral water, air-conditioning units, and swimming pools (Visvesvra et al. 2007).

Among these species, the *Acanthamoeba* spp. are the most common FLA worldwide (Retana et al. 2015). Previous studies have indicated that various environmental sources, ventilation ducts, and dental and dialysis units play a critical role in transmitting this protozoan to humans.

According to previous reports, the dust contamination rates of *Acanthamoeba* in the hospital wards of Kashan and Tehran were 52.5 and 52.9%, respectively (Golestani et al. 2018; Lasjerdi et al. 2011).

*Acanthamoeba* exists in two trophozoite and cyst forms. The cyst is formed under adverse environmental conditions and causes the survival of this protozoan in nature (Visvesvra et al. 2007; Edrisiian et al. 2008).

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Moreover, various bacterial agents such as *Legionella pneumophila*, *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Chlamydomphila pneumoniae*, *Bacillus cereus*, *Staphylococcus aureus*, and *Vibrio cholera* can be carried out via *Acanthamoeba* and induce microbial infection; hence, *Acanthamoeba* can also act as a bacterial reservoir (Khan 2006).

This parasite can cause Granulomatous Amoebic Encephalitis (GAE) in immunocompromised patients, which may lead to death (Bloch and Schuster 2005; Kalra et al. 2020). Moreover, pulmonary involvement and disseminated infections have been reported in transplant patients (Martínez-Girón et al. 2008; Duarte et al. 2006). The amoeba has also been detected in the lavage, lung tissues, human nasal cavities, throat, pharyngeal swabs, and the purulent discharge of the ear (Khan 2006; Siddiqu and Khan 2012).

Keratitis is an important sequel of *Acanthamoeba* infection in immunocompetent individuals wearing eye contact lenses (Khan 2006; Haddad et al. 2019). Until now, 22 genotypes of *Acanthamoeba* (T1-T22) have been detected using molecular analyses (Behera et al. 2016; Fuerst and Booton 2020). Among these genotypes, the T4 genotype is the most common type of parasite isolated from amoebic keratitis (AK) and AGE diseases in humans.

Bronchoalveolar lavage (BAL) is a special sample used to diagnose a variety of respiratory infections in patients with pneumonia (Meyer 2007). However, little is known about *Acanthamoeba* in respiratory samples and its correlations with clinical symptoms. Accordingly, the objectives of the present study were as follows: (1) Determining the prevalence of FLA and *Acanthamoeba*, (2) Identifying *Acanthamoeba* genotypes, (3) Detecting the morphological feature of the protozoa, and (4) Examining the relationship between *Acanthamoeba* and symptoms and the demographic features of individuals with pulmonary symptoms suspected of cancer.

## Materials and methods

### Ethics statement

This study was approved by the Ethics Committee of the Kashan University of Medical Sciences, Kashan, Iran (Code: IR.KAUMS.MEDNT.REC.1398.056).

### Sampling area

Kashan is a city in the Northwest of Isfahan Province, Kashan, Iran. Kashan has an arid climate with hot summers and mild winters. The average temperature in this

city is 19 °C, with the maximum temperature of 50 °C (Modarres and Silva 2007; Tabari and Hosseinzadeh 2011).

### Sample collections

This cross-sectional study included ninety-seven patients with respiratory symptoms suspected of lung cancer, who were admitted to the Shahid Beheshti Hospital, Kashan, Iran, during 2019–2020. The participants' age ranged from 18 to 92 years. All demographic questionnaires were filled out, and informed consent was obtained from all participants.

### Culture and morphological identification

The samples were cultivated on 1.5% Non-Nutrient Agar (NNA), Bacto Agar (DIFCO, USA) enriched with a layer of the suspension of autoclaved *Escherichia coli*, and the plates were then incubated with some modifications at 32 °C for two months, as previously described (Visvesvra et al. 2007; Memari et al. 2015). Then, one colony from the positive plates were transferred to the new culture plates by a scraper. For the better growth of Amoeba, the culture medium should be wet; therefore, 4 ml sterile normal saline and 200 µl autoclaved *E. coli* were added to the plate, incubated at 32 °C, and examined by an inverted microscope daily. The plates were incubated at 4° C for 2–3 days to transform trophozoite to cyst and then incubated at 30–32 ° C for 1–2 weeks for transforming cyst to trophozoite (Zanella et al. 2012). After the mass cultivation of amoeba, the agar surface was scraped with the blade, and the plate was washed three times with normal saline and centrifugation (400 g, 5 min). Then, the pellet was stored at –20 °C for DNA extraction and molecular analyses.

To determine the morphological features of the amoeba, a wet mount slide was prepared and examined by light microscopy. Furthermore, the sample was stained by 20% Giemsa for 20 min and examined with 100X. Morphological identification was based on the shape and size of the cysts, according to the criteria set by Pussard and Pons (1977). The size of twenty cysts was measured to establish the average size.

### DNA extraction

The DNA was extracted from the BAL samples by a commercially available kit (DNP kit, Sinaclon, Tehran, Iran) according to the manufacturer's instructions, and it was

then stored at -20 °C. Moreover, DNA was isolated from 10<sup>6</sup> trophozoites or cysts of the harvested culture.

### PCR and genotyping

Polymerase Chain Reaction (PCR) was conducted using a pair of *Acanthamoeba*-specific primers JDP1 (5'GGCCCA GATCGTTTACCGTGAA-3') and JDP2 (5'TCTCACAAG CTGCTAGGGAGTCA-3'), which specifically amplify a 490 bp fragment from 18 S rDNA genes (Schroeder et al. 2001). Positive and negative controls were used in each

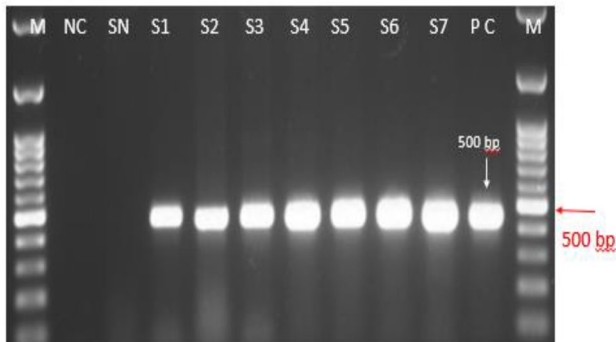
reaction. The annealing of PCR was 61.2 °C for 45 s. The PCR products were electrophoresed and visualized under the UV transillumination. The PCR products of eighteen *Acanthamoeba* isolates were sequenced and deposited in the GenBank database.

### Statistical analysis

Data were analyzed with the SPSS software version 16 (SPSS Inc., Chicago, IL) using the chi-squared and Fisher's exact tests.

### Results

Out of 97 BAL samples examined cytologically, nine samples (9.3%) were diagnosed with lung cancer, and 88 samples were negative. The patients' mean age was  $60.5 \pm 16.6$  years. Among the 97 BALs, 84 (86.6%) were positive for FLA using the culture method, and 71 (73.2%) were confirmed as *Acanthamoeba* spp. using the PCR test (Fig. 1). Out of nine positive cancers patients, six samples were *Acanthamoeba* positive and three ones were *Acanthamoeba* negative by PCR method. Tables 1 present the distribution *Acanthamoeba* spp. in the BAL samples suspected of cancer regarding the patients' demographic characteristics. Among the patients infected with *Acanthamoeba*, 85% were above 65 years ( $p = 0.025$ ) (Table 1). Furthermore,



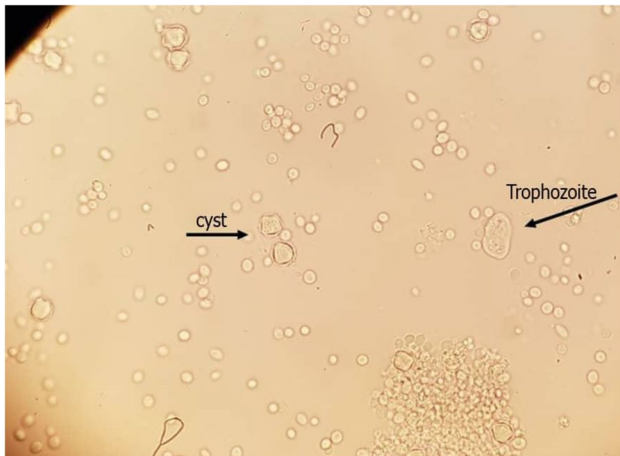
**Fig. 1** Gel electrophoresis of the 500 bp PCR product of *Acanthamoeba* spp. using JDP1 and JDP2 primers, isolated from BAL culture of suspected of cancer in Kashan hospital, Iran. (NC: negative control, S1–S7 positive *Acanthamoeba* samples; marker 100 bp and PC: Positive control)

**Table 1** Distribution of *Acanthamoeba* in BAL samples suspected of cancer according to demographic characteristics, Kashan, Iran

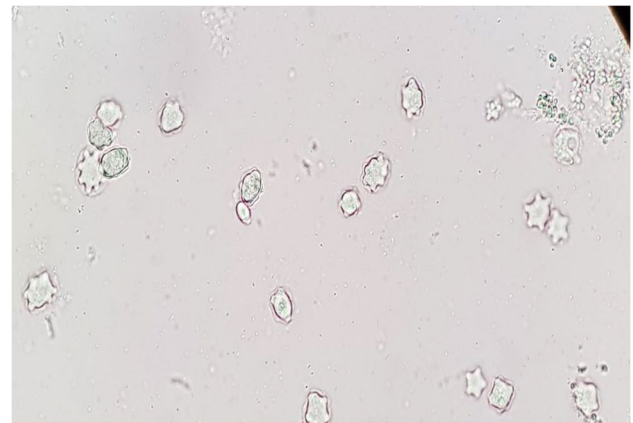
Risk factors	Variables	N	<i>Acanthamoeba</i> positive cases N (%)	<i>Acanthamoeba</i> Negative Cases N (%)	Free- living amoeba N (%)	P value	Cancer positive
Sex	Male	58	45(77.6)	9(15.5)	4(6.9)	0.136	8
	Female	39	26(66.7)	5(12.8)	8(20.5)		1
Age	<50	23	19(82.6)	2(8.7)	2(8.7)	0.025	3
	51–60	34	18(52.9)	8(23.5)	8(23.5)		2
	>65	40	34(85)	4(10)	2(5)		4
Education level	Less than diploma	82	62(75.6)	13(15.9)	7(8.5)	0.035	6
	Diploma and above	15	9(60)	1(6.7)	5(33.3)		3
Job	Housekeeper	30	21(70)	3(10)	6(20)	0.567	1
	Employee	4	2(50)	0(0)	2(50)		0
	Labor	13	10(76.9)	2(15.4)	1(7.7)		2
	Farmer	9	6(66.7)	2(22.2)	1(11.1)		1
	Retired	10	8(80)	1(10)	1(10)		1
	Educational	4	3(75)	1(25)	0(0)		3
	Other	27	21(77.8)	5(18.5)	1(3.7)		1
Diabetes	Yes	10	9(90)	0(0)	1(10)	0.485	2
	No	87	62(71.3)	14(16.1)	11(12.6)		7
Smoking	Yes	15	11(73.3)	2(13.3)	2(13.3)	1	5
	No	82	60(73.2)	12(14.6)	10(12.2)		4

**Table 2** Distribution of *Acanthamoeba* in BAL samples suspected of cancer according to symptoms

Symptom				<i>Acanthamoeba</i>		FLA	P value
				Positive cases (N)	Negative cases (N)		
Dyspnea	Yes	66		48(72.7)	10(15.2)	8(12.1)	1
	No	31		23(74.2)	4(12.9)	4(12.9)	
Cough	Yes	49		36(73.5)	7(14.3)	6(12.2)	0.998
	No	48		35(72.9)	7(14.6)	6(12.5)	
Tumor Mass	Yes	12		12(100)	0(0)	0(0)	0.119
	No	85		59(69.4)	14(16.5)	12(14.1)	
Fever	Yes	3		1(33.3)	2(66.7)	0(0)	0.093
	No	94		70(74.5)	12(12.8)	12(12.8)	
Sputum	Yes	13		9(69.2)	1(7.7)	3(23.1)	0.437
	No	84		62(73.8)	13(15.5)	9(10.7)	
Pneumonia	Yes	6		4 (66.7)	1(16.7)	1(16.7)	0.816
	No	91		67(73.6)	13(14.3)	11(12.1)	



**Fig. 2** *Acanthamoeba* trophozoite and cyst isolated from BAL samples of suspected of cancer patients, Kashan, Iran (400X)

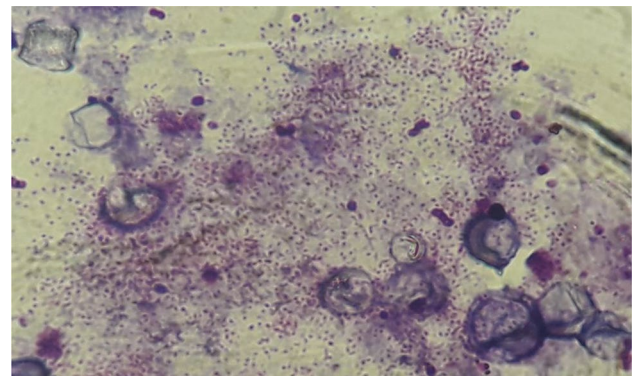


**Fig. 3** Star and polygonal shape cysts of *Acanthamoeba* isolated from BAL samples of suspected of cancer patients, Kashan, Iran (400X)

the prevalence rates of *Acanthamoeba* among patients with elementary education and those with diploma were 75.6 and 60%, respectively ( $p=0.035$ ).

The frequencies of pulmonary symptoms such as dyspnea, cough, tumor mass, fever, sputum, and pneumonia in the *Acanthamoeba*-positive patients were not significant (Table 2).

The selection of eighteen *Acanthamoeba* strains for genotyping was based on pathological positive lung cancer or observing a 500 bp sharp band by PCR. Out of 18 sequenced *Acanthamoeba* samples, four ones were belong to cancer patients and positive *Acanthamoeba* and the others had sharp band of *Acanthamoeba* by PCR. All *Acanthamoeba* spp. isolates (100%) belonged to the



**Fig. 4** *Acanthamoeba* cysts stained with Giemsa isolated from BAL samples of suspected of cancer patients, Kashan, Iran (1000X)



**Table 3** Genotype, Accession number, similarity, morphology, and size of 18 *Acanthamoeba* isolated from BAL samples suspected of cancer in Kashan hospital

No.	Isolate no	Genotype	Similarity %	Accession no.	Shape of cyst	Size of cyst $\mu\text{m}$
1	RT-BAL1	T4	99.5	LC522143.1	–	–
2	RT-BAL2	T4	98.7	LC522142.1	Wrinkled, Polygonal	16.5
3	RT-BAL15	T4	93.8	LC522144.1	Wrinkled Polygonal	9.3
4	RT-BAL16	T4	89.36	LC581474.1	–	–
5	RT-BAL18	T4	99.5	LC522141.1	Wrinkled, Polygonal	
6	RT-BAL35	T4	97.25	LC581471.1	–	–
7	RT-BAL38	T4	99.18	LC581472.1	–	–
8	RT-BAL20	T4	99.27	LC581473.1	Wrinkled, Polygonal	9.3
9	RT-BAL25	T4	99.3	LC598382.1	Round, smooth	8.2
10	RT-BAL32	T4	99.76	LC598383.1	Wrinkled, Polygonal	10.8
11	RT-BAL29	T4	98.88	LC598384.1	Wrinkled, Polygonal	9.6
12	RT-BAL45	T4	100	LC598498.1	Wrinkled, Polygonal	11.3
13	RT-BAL55	T4	99.77	LC604809.1	Wrinkled, Polygonal	9.5
14	RT-BAL66	T4	99.77	LC604810.1	Wrinkled, Polygonal	10.3
15	RT-BAL74	T4	100	LC604811.1	Wrinkled, Polygonal	13.8
16	RT-BAL61	T4	99.77	LC604812.1	Round, smooth	8.4
17	RT-BAL41	T4	98.65	LC604813.1	Wrinkled, Polygonal	10.8
18	RT-BAL80	T4	98.36	LC598792.1	Wrinkled, Polygonal	10

T4 genotype. Most *Acanthamoeba* cysts were diagnosed with double-wall cysts, endocyst polygonal in the size of 9.5–11.5 $\mu\text{m}$  (Figs. 2, 3, 4) (Table 3).

## Discussion

The present research was the first research on the *Acanthamoeba* genotypes in a population with respiratory symptoms suspected of cancer, who were admitted to Kashan's hospitals in Iran. The findings indicated that, out of 97 BAL samples, 86.6% were positive for FLA, and *Acanthamoeba* was confirmed in 73.2% of the cases. The prevalence of FLA in the oral cavity of cancer patients in Kashan was reported to be 89% (Taghipour et al. 2021).

Golestani et al. (2018) reported that the rates of *Acanthamoeba* in the dust, soil, and water samples of the Beheshti Hospital in Kashan were 52.5, 62.5, and 50%, respectively. Furthermore, the contamination rates of rural drinking water of Kashan to FLA and *Acanthamoeba* were 35.2% and 11.7%, respectively (Mostafaei et al. 2019). In a study conducted in Iran, *Acanthamoeba* infection was found to be 100% and 98.4% in the BAL samples of immunocompromised patients with respiratory disorders using culture and molecular methods, respectively (Eslamirad et al. 2020).

According to Lanocha et al. (2009), *Acanthamoeba* was isolated from 3.13% of the BAL samples of patients with immunodeficiency, and the T4 genotypes were identified. Nineteen cases of *Acanthamoeba* spp. were reported in immunodeficiency patients with pneumonia in Poland,

the U.S.A, Australia, India, Japan, and France (Kot et al. 2021). In a study in Peru, *Acanthamoeba* spp. and the T4 and T15 genotypes were reported from the nasal swap samples of healthy individuals (Cabello Vilchez et al. 2014).

However, the prevalence rates in the present study are higher than those reported by other researchers (Lanocha et al. 2009; Kot et al. 2021) and lower than Eslamirad et al.'s reported rate (2020). The high prevalence of FLA and *Acanthamoeba* in this study may be due to modifications in culturing the BAL samples, which agrees with Taghipour et al.'s (2021) findings. Accordingly, the high rate of FLA and *Acanthamoeba* in the BAL samples in this study may probably occur due to the climatic conditions of the Kashan desert region, including dry climate, low rainfall, seasonal storms, and dust. Moreover, the high contamination rate of drinking water and dust in Kashan may also contribute to this high prevalence rate (Golestani et al. 2018; Mostafaei et al. 2019).

Since *Acanthamoeba* is the most common free-living amoeba, ubiquitously distributed in nature and everywhere (Siddiqui and Khan 2012), it can cause a high infection rate in patients without lung cancer. The detection of the *Acanthamoeba* in the BAL samples may not actually be the cause of pathology, and it may only be colonization (Khurana et al. 2015). Golestani (2018) reported that the rate of *Acanthamoeba* in the dust of the Beheshti Hospital in Kashan was 52.5% (Golestani et al. 2018).

We also found that the rate of *Acanthamoeba* infection was higher in diabetic patients (90%); however, the

difference was not significant. Diabetes has been reported as one of the risk factors for *Acanthamoeba* infection (Shimmura-Tomita et al. 2018).

Since *Acanthamoeba* is ubiquitously distributed in nature, it may be a risk factor for the transmission of the parasite to health officials and immunocompetent (Niyiyati et al. 2009; Kot et al. 2021).

The high prevalence of positive *Acanthamoeba* in negative lung cancer may be due to pulmonary diseases such as chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis (CF), which cause parenchymal lesions and the colonization of *Acanthamoeba* in the lung (Vanspauwen et al. 2012; Colson et al. 2011).

In this study 18 *Acanthamoeba* genotypes were identified in the BAL specimens of pulmonary patients for the first time and deposited in the GeneBank database. Moreover, their similarity with the recorded isolates in the GeneBank was 98–100%. Other genotypes such as T1, T4, T5, T10, T11, T12, and T15 have been reported from clinical specimens worldwide (Kot et al. 2021; Cabello Vilchez et al. 2014; Walochnik et al. 2008; Booton et al. 2005; Memari et al. 2016).

Most of the *Acanthamoeba* genotypes in the dust of the Beheshti Hospital and the drinking water of Kashan were T4 (Golestani et al. 2018; Mostafaei et al. 2019). This finding was in a similar with the findings of the present study, as the T4 genotype was detected in all BAL samples suspected of cancer and had 98% similarity with the T4 genotype of the dust samples.

T4 is the predominant genotype in GAE and pulmonary *Acanthamoebiasis*. Since the T4 genotype is a potential pathogen in patients with immunocompromising conditions, differential diagnosis of *Acanthamoeba* should be more considered in these patients (Bloch and Schuster 2005; Shin and Im 2004; Kalra et al. 2020). Accordingly, physicians and health professionals should be more attentive to this lethal infection and diagnostic options, especially in suspected encephalitis.

The results of the present study indicated the higher rates of *Acanthamoeba* infection in patients suspected of cancer, who aged above 66 years (85%,  $p=0.025$ ). Furthermore, the prevalence of *Acanthamoeba* was higher in the individuals with elementary education than in others; however, the difference was statistically significant ( $p=0.035$ ). Kot et al.'s (2021) study showed that most patients with *Acanthamoeba* pneumonia had weight loss and respiratory failure, which is consistent with the findings of the present study.

Some case reports have been published on *Acanthamoeba* pneumonia with immunodeficient status (Shin and Im 2004). The death of cancer patients from unidentified encephalitis has been reported in several studies in Iran (Edrisiian et al. 2008).

The results of the present study indicated that pulmonary symptoms such as dyspnea, cough, fever, sputum, and pneumonia in the population with *Acanthamoeba* positive suspected of cancer were not significant.

Because this protozoon can act as a reservoir of a variety of bacterial agents such as *Legionella pneumophila*, *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Chlamydomphila pneumoniae*, and *Staphylococcus aureus* (Dey et al. 2020; Medie et al. 2011; Siddiqui and Khan 2012), it is recommended to conduct a microbial study on these patients. However, pulmonary diseases such as asthma may cause these symptoms.

## Conclusion

In general, the present findings indicated that higher frequencies of FLA and *Acanthamoeba* in BAL samples with pneumonia symptoms suspected of cancer compared to other previous studies. The successful cultivation of FLA and the high prevalence of *Acanthamoeba* in this study are due to modifications in culturing the BAL samples. The high rate of *Acanthamoeba* in the BAL samples may be due to parasite colonization.

Since the T4 is a pathogenic genotype, pulmonary symptoms may occur due to this parasite. Accordingly, health education and sanitation is recommended to prevent this infection.

**Authors' contributions:** Authors contributed to the study conception and design: (SR), sampling and data collection: (MS), (TT), Methodology: (SR), (TT), analysis: (GAM), (SR), (TT) and (MD), writing the draft of manuscript: (SR) and editing of the manuscript: (MD), (HH) and (ZE). All authors revised the initial manuscript and approved its final version.

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**Data availability** The data that support the findings of this study are available from the corresponding author, (Sima Rasti), upon reasonable request.

## Declarations

**Conflicts of interest** The authors declare that there are no conflicts of interest.

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