



New prevalence surveillance of *Toxoplasma gondii* among rodents and stray cats by ELISA avidity and nested PCR methods, Northeast of Iran

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Abstract Rodents and stray cats are the sources of many parasitic infections including *T. gondii*, for other animals and human. Toxoplasmosis has a wide range of laboratory factors in its intermediate and definite hosts. Regarding the importance of rodents and stray cats as the hosts that spread the *Toxoplasma gondii*, it is necessary to obtain comprehensive information about these animals in the life cycle of *T. gondii*. The objective was to investigate the new prevalence of toxoplasmosis among target animals in Iran, using GRA6 gene in combination with ELISA avidity. In this study, 286 rodents and 210 stray cats were collected and their heart tissues extracted to obtain DNA, blood samples and IgG Ab of *T. gondii* parasite. We detected the positive tissue samples in our study by the nested-PCR method. Then, we examined *T. gondii* IgG ELISA avidity for assessment of toxoplasmosis among rodents and stray cats. This study, was conducted in January to March 2017, based on the prevalence study. The findings revealed that 246/286 (86.01%) of rodents and 180/210 (85.71%) of stray cats were positive by IgG ELISA avidity methods. moreover, 68 rodents samples and 38 stray cats samples were positive concerning the GRA6 *Toxoplasma* gene; and these positive samples were at intermediate levels for IgG avidity. We concluded that the new prevalence of toxoplasmosis among rodents and stray cats was at high levels,

using the serologic method in Northeast of Iran and the results of quantitative ELISA avidity were as the same as those of the nested-PCR for detecting recent toxoplasmosis in these hosts.

Keywords Toxoplasmosis · New prevalence · Iran · Molecular detection · Serology study

Introduction

T. gondii is an important coccidian with worldwide distribution, which causes a serious disease, toxoplasmosis, in human and animals. The stray and feral cat's definitive hosts can just excrete million oocysts by their feces. Cysts can remain infective for many months in sultry climates and spread in the environment or anything mixed with the feces. Stray cats can be infected by ingesting tissue cyst from intermediate hosts, such as wild rodents (Verma et al. 2018; Wang et al. 2018c).

There are a lot of tests for detecting positive infection with *T. gondii* including ELISA, indirect hem agglutination assay (IHA), ELISA Avidity and PCR for different genes. The LAT, ELISA and ELISA avidity are currently applicable for serological diagnosis of the infection; however the ELISA avidity with 97% sensitivity is a highly user-friendly method to detect toxoplasmosis infection (Hecker et al. 2018; Hou et al. 2018; Oh et al. 2018).

The aim of this study was to investigate the new prevalence of *T. gondii* among rodents and stray cats using ELISA avidity and PCR methods in Northeast of Iran. This investigation was necessary to assess the prevalence of Toxoplasmosis, using both serological and molecular methods.

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Materials and methods

Study area

Golestan province is the main reservoir of the rodents and stray cats as toxoplasmosis circulating agents. The region is located next to the Caspian Sea, and has very sultry and rainy climate. This study was performed in 2017.

Sampling

We collected 286 heart tissues and blood samples of rodents and 210 heart tissues and blood samples of stray cats simultaneously from Golestan forest, where rodents and stray cats live. The samples were collected using intravenous blood sampling with syringe; then the animals were killed to isolate their heart tissues. The samples were transferred to a laboratory to examine nested-PCR, using GRA6 gene for detecting toxoplasmosis. Finally, IgG avidity was assessed in serum samples of the infected hosts, using the ELISA avidity method for evaluation of the infection time. The study was based on the prevalence and randomized abundance analysis. Tissue sample examination, processing and lysis were conducted before DNA extraction and nested-PCR.

Animal species were *Rattus Rattus*, *Rattus norvegicus*, *Mus musculus*, *Rombomys opimus* and *stray cat*.

DNA extraction

Extraction of genomic DNA was conducted using sodium DNG/proteinase K method. The material was purchased from Sinacolon Company and eluted into 50 μ l DDH₂O, according to the manufacturer's instructions. A nested-PCR for *T. gondii* GRA6 gene was applied to confirm probable infection.

We cut 3 g of the sample tissue into small pieces and put them in a 1.5 ml sterile tube. 180 μ l lyse buffer was added for homogenization and 400 μ l DNG solution was also added and homogenized once again; then, 20 μ l proteinase K was added.

We mixed them immediately by shaking for 20 s and incubated at 60 °C for 1 h to lyse the sample. If tissue resists to lysing, the incubation time would increase to 2–3 h. The samples were shaken or inverted in 10–15 min intervals. Then, we added 300 μ l Isopropanol to DNA precipitation. After 5–10 min the tubes were washed by 70% ethanol. Finally, we eluted the DNA by deionized H₂O. The eluted DNA was preserved at – 20 °C using a nested-PCR method for detection of infection. The positive control sample in this study was Tehran strain of *T. gondii*.

The PCR kit was purchased from the Pishgam Company-Iran.

Nested-PCR analysis for *T. gondii* GRA6 gene

Nested-PCR analysis was performed to detect *Toxoplasma* GRA6 gene as a diagnostic gene. The primers were designed according to Pishgam Corporation (Table 1).

Amplification of the GRA6 gene was completed in a 5-min cycle at 95 °C for initial denaturation, followed by 30 one-min cycles at 95 °C, 1 min at 62 °C, and 3 min at 74 °C. The best annealing temperature for PCR 1 was 58 °C and for PCR 2 was 57.5 °C. The segment lengths were 546 bp in PCR1 and 344 bp in PCR2 (Table 2). The reason for choosing this gene was generalization of this molecular marker in various strains of *T. Gondii* and this gene was used in many studies.

Then, we evaluated the serum of the infected rodents and stray cats to detect IgG avidity, using tachyzoite enzyme-linked immune assay method.

Tachyzoite based ELISA avidity test

ELISA avidity test has been used for detection of the *Toxoplasma* IgG in blood serum of the rodents and stray cats, according to the Euro immune ELISA avidity kit with specific conjugation. The positive control in the avidity ELISA test was embedded in the commercial kit. The ELISA avidity procedure was performed using Euro Immune Kit-France, according to manufacturer's instructions.

The formula of ELISA avidity (semi quantitative):

$$\frac{\text{OD patients (serum samples)}}{\text{OD calibrator 2}}$$

The result > 1.1 represents positive toxoplasmosis.

The formula of quantitative ELISA avidity (qIgG):

$$\frac{\text{OD (serum samples) in urea buffer}}{\text{OD (serum samples) in phosphate buffer}}$$

The result $\times 100 = \text{RIA}$ (relative index avidity); the RIA value below 40% is in *low avidity*, between 40 and 60% is in *intermediate avidity* and over 60% is in *high avidity*.

Statistical analysis

In this study, one-sample and two-sample t-tests, correlation table, frequency charts, high-low diagram and SPSS 19 software were used for data analysis. IgGq was nearly equal to the quantitative IgG ELISA avidity.

Table 1 Primer sequences

PCR	Segment length	Primer sequences	Annealing temperature
Primary	546	Forward: 5'-ATTGTGTTTCCGAGCAGGT-3' Reverse: 5'-GCAC CTTCGCTTGTGGTT-3'	58 °C
Secondary	344	Forward: 5'-TTTCCGAGCAGGTGACCT-3' Reverse: 5'-TCGCCGAAGAGTTGACATAG-3'	57.5 °C

q quantitative

Table 2 PCR substrate

Materials	Volume (μl)	Final concentration
Master Mix	7.5	2X
DNA Sample	2	10–100 ng/μl
Work Primer	1	20 Picomol
Distilled water	4.5	–
Total	15.0	–

Results

In this study, 246/286 (86.01%) of rodents and 180/210 (85.71%) of stray cats were positive based on the *Toxoplasma* IgG ELISA avidity method. It was also found that 68 rodent samples and 38 stray cat samples were positive in presence of GRA6 gene. In rodents, 38 samples were *Ratus ratus*, 10 samples were *Ratus norvegicus*, 10 samples were *Mus musculus* and 10 samples were *Rombomys opimus*. These samples were positive in 344 bp location. The primer designing was conducted using BLAST software—NCBI site. Among 131 *Rattus Rattus* samples, 38 were positive (29%), among 45 *Rattus norvegicus* samples, 10 were positives (22%), among 60 *Mus musculus* samples, 10 were positive (16%), among 50 *Rombomys opimus* samples, 10 were positive (20%) and finally among 210 stray cat's samples, 38 were positive (18%) (Fig. 1).

In this survey, we analyzed the prevalence study to show the important correlation between *Toxoplasma* PCR results

(68/286 samples of rodents and 38/210 samples of stray cats) and positivity of *Toxoplasma* IgG ELISA Avidity (246/286 samples of rodents and 180/210 samples of stray cats). However, we found that 246/286 (86.01%) of rodents and 180/210 (85.71%) of stray cats were positive, using IgG ELISA avidity method but just 68/286 of the rodents and 38/210 of the stray cats showed positive band in 344 bp location of GRA6 gene, using the nested PCR method.

Tables 3 and 4 show the *t* test results, and analysis of the infected rodents and stray cats to compare serum levels of semi quantitative IgG and quantitative IgG (IgGq), with 95% confidence interval. Figure 2 shows the frequency histogram tables of the serum level of *Toxoplasma* IgG avidity among the rodents and stray cats. The results were 2–2.5 (toxoplasmosis positive) among 246/286 rodents and 180/210 stray cats, with 95% confidence interval. Figure 3 shows the frequency histogram tables of the serum levels of *Toxoplasma* IgG quantitative avidity among the rodents and stray cats. The results were about 30% with 95% confidence interval, respectively. In this study, we showed that *Toxoplasma* GRA6 gene was detected only in rodents and stray cats with *Toxoplasma* intermediate ELISA avidity (40–60% relative index avidity).

Figure 4 shows the high/low bars of *Toxoplasma* IgG and IgGq (quantitative) positive rodents and stray cats. The results of IgG avidity were in 2–2.5 (semi quantitative ELISA) among these animals with 95% confidence interval. These results were in about 30% among 68/286 rodents and 38/210 stray cats (quantitative ELISA) with 95%

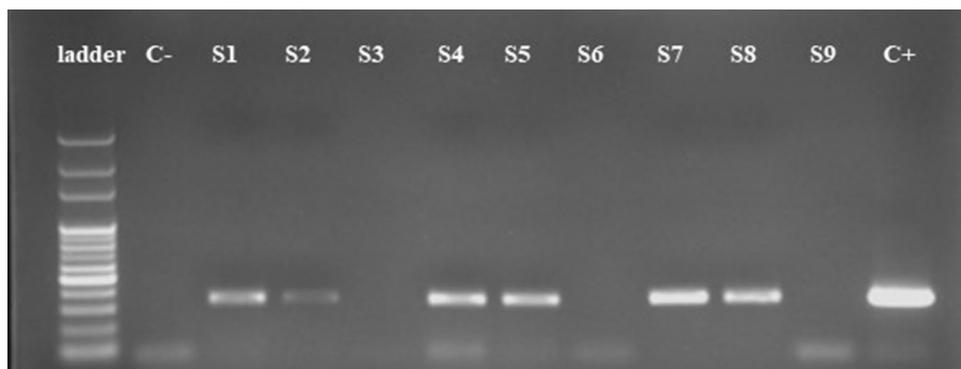
Fig. 1 Nested-PCR result using GRA6 gene

Table 3 One-sample statistics

	N	Mean	SD	SE Mean
IgG rodent	286	2.2067	1.09861	0.06496
IgG stray cat	210	2.3042	1.10651	0.07636
IgGq Rodent	286	31.4301	11.31496	0.66907
IgGq Stray cat	210	31.0095	11.82366	0.81591

q quantitative

Table 4 One-sample t-test

	Test value = 0					
	t	df	Sig. (2-tailed)	Mean difference	95% confidence interval of the difference	
					Lower	Upper
IgG rodent	33.969	285	.000	2.20671	2.0788	2.3346
IgG stray cat	30.177	209	.000	2.30419	2.1537	2.4547
IgGq Rodent	46.976	285	.000	31.43007	30.1131	32.7470
IgGq Stray cat	38.006	209	.000	31.00952	29.4011	32.6180

q quantitative

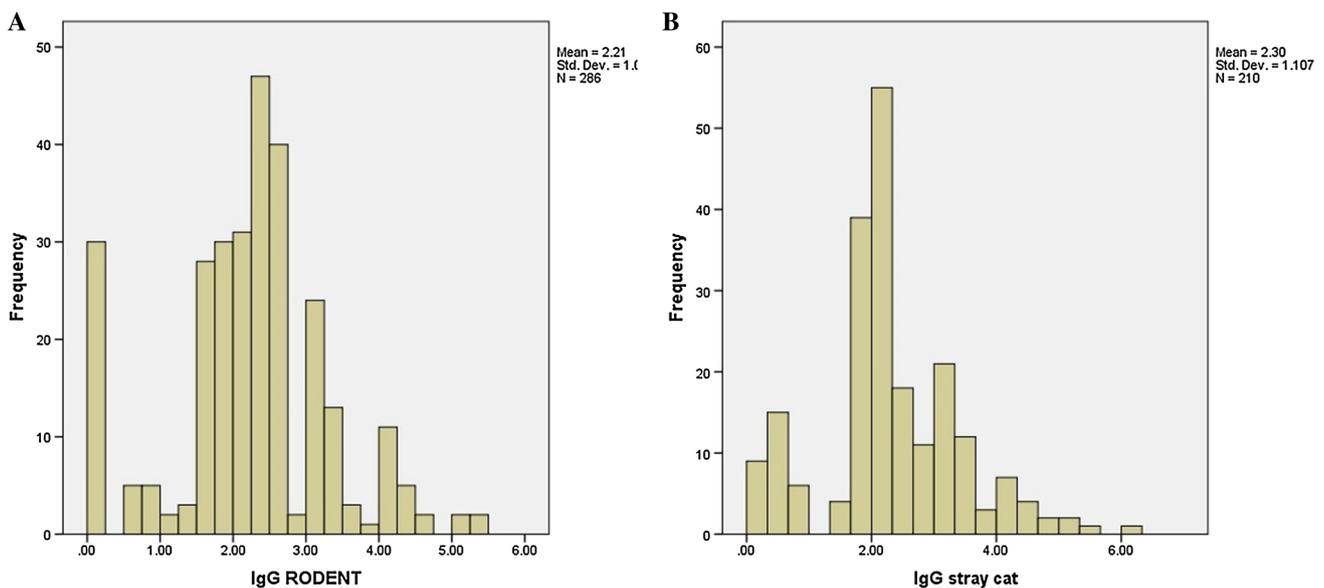


Fig. 2 a and b Histogram table of *Toxoplasma* IgG avidity in rodents and stray cats

confidence interval. Table 5 shows the comprehensive data achieved through analysis of *Toxoplasma* IgG and IgGq rodents and stray cats. According to this table, the mean of *Toxoplasma* IgG was in 2.20 for rodents and 2.30 for stray cats or the mean of *Toxoplasma* IgG q (quantitative) was 31.43 among the rodents and 31.01 among the stray cats. The proved intermediate avidity in these infected hosts and the SD of *Toxoplasma* IgG was 1.099 among the rodents and 1.10 among the stray cats. In addition, the SD of

Toxoplasma IgGq (quantitative) was 11.31 among the rodents and 11.82 among the stray cats. Evaluating the prevalence analysis of the two methods for detecting toxoplasmosis in Northeast of Iran revealed a statistical significance. The results of nested-PCR were just applied for approving the existence of parasite, also semi quantitative ELISA avidity detected the presence of IgG antibody; however quantitative ELISA avidity method was used to evaluate the acuteness of the infection. In this study, we

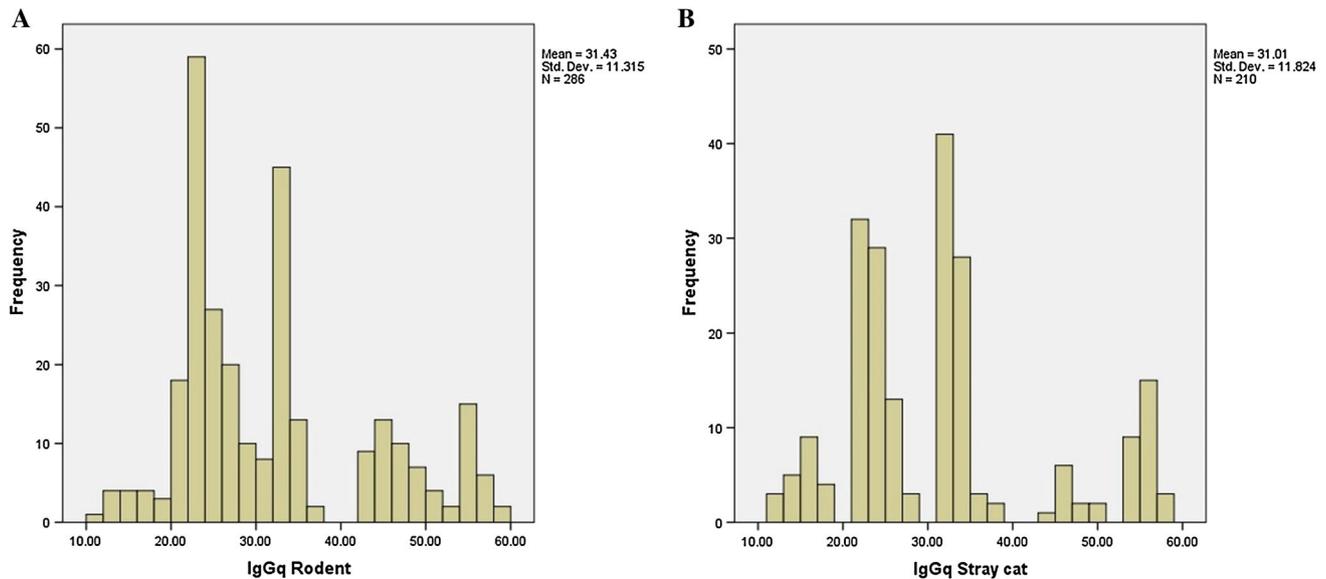


Fig. 3 a and b Histogram table of *Toxoplasma* IgGq avidity in rodents and stray cats

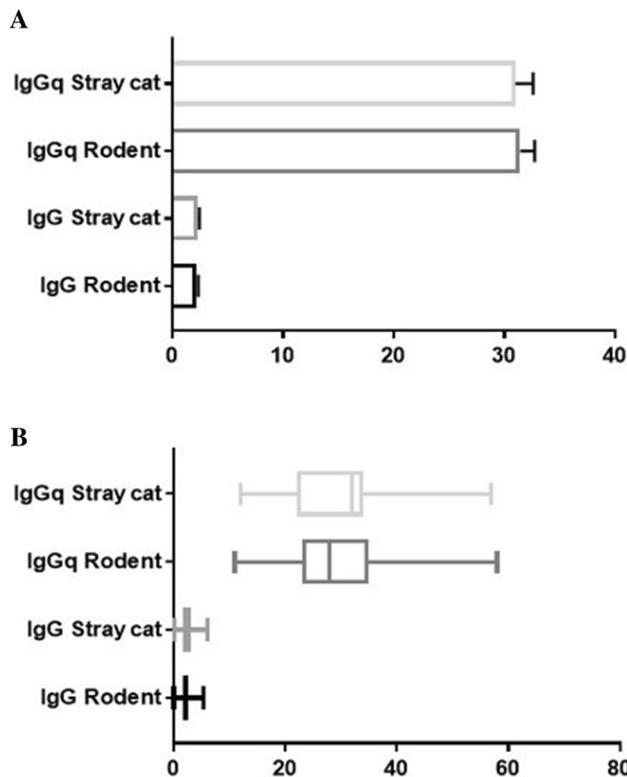


Fig. 4 a and b High low bars of *Toxoplasma* IgG and IgGq avidity in rodents and stray cats. S sample, C control, q quantitative

found that these animals with *Toxoplasma* GRA6 gene had acute infection, based on the quantitative ELISA avidity, but the hosts with chronic infection reported negative for *Toxoplasma* GRA6 gene. It means that GRA6 gene was detected only in quantitative ELISA avidity positive animals.

Discussion

One of the main difficulties of this study was simultaneous sampling of the rodents and stray cats. Golestan province located in Northeast of Iran with a sultry climate. In this study, the serologic and molecular methods have been used simultaneously for the first time to test the serum and tissue samples of the rodents and stray cats in the Golestan province. The results of this study revealed that the prevalence of toxoplasmosis with ELISA avidity procedure was higher than that in the polymerase chain reaction method in Northeast of Iran. Golestan province is the main reservoir of the rodents and stray cats as disseminators of toxoplasmosis. Rodents are the main reservoir host for toxoplasmosis that infects stray cats. Climate characteristic of region is highly suitable for toxoplasmosis. Many studies have been conducted on the occurrence of *T. gondii* among the cats and rodents in Iran. Sharif et al. 2009 have reported prevalence of forty percent *T. gondii* antibodies among the stray cats in Sari, Northern Iran. They conducted *T. gondii* antibodies test with latex agglutination on 100 serum samples collected from stray cats in five urban areas of Sari. Mostafavi et al. detected the differences in *T. gondii* infection between the male and female stray cats. Khademvatan et al. 2013 showed the existence of birds toxoplasmosis in Southwest of Iran (Mostafavi and Jalali Monfared 2012).

In recent years, there were no study to detect rodent and stray cat's toxoplasmosis in Golestan area. The present study showed the important role of the rodents and stray cats in dissemination of toxoplasmosis in sultry areas. It also revealed that the GRA6 gene was a critical marker to

Table 5 Data analyze of *Toxoplasma* IgG and IgG quantitative avidity rodents and stray cats

	IgG rodent	IgG Stray cat	IgGq Rodent	IgGq Stray cat
Number of values	286	210	286	210
Minimum	0.02	0.18	11	12
25% Percentile	1.7	1.775	23	22
Median	2.3	2.1	28	32
75% Percentile	2.625	3.025	35	34
Maximum	5.4	6.1	58	57
Mean	2.207	2.304	31.43	31.01
SD	1.099	1.107	11.31	11.82
SE Mean	0.06496	0.07636	0.6691	0.8159
Lower 95% CI of mean	2.079	2.154	30.11	29.4
Upper 95% CI of mean	2.335	2.455	32.75	32.62
Sum	631.1	483.9	8989	6512

q quantitative

detect the abundance of toxoplasmosis and that the heart tissue was one of the main tissues to follow up GRA6 gene from *Toxoplasma* parasite. Moreover, the rodents and stray cats were important agents of spreading toxoplasmosis in the region. The parasite forms tissue cysts with bradyzoites in various tissues (also in heart tissue), so the detected parasitic DNA (GRA6 gene) comes from bradyzoites more probably than from tachyzoites. The result of the recent studies revealed that toxoplasmosis among the rodents and stray cats of the Golestan province is about 85%. Regarding the climate of the Golestan province, which is theoretically highly suitable for *T. gondii* growth, detection of high rate of seropositive samples is probable. *T. gondii* parasite is responsible for toxoplasmosis as one of the most important diseases that causes ecological problems in rodents and stray cats' environment. Serological test can be applied as an important instrument to diagnose the infection among the herds. This test detects specific anti-*T. gondii* antibodies, and involves techniques such as indirect fluorescence antibody test (IFAT), *toxoplasma* agglutination test (TAT), enzyme-linked immunosorbent assay (ELISA), rapid immune chromatographic test (RIT), and the immunoblot assay. *T. gondii* is an inevitable intracellular coccidian parasite and an important zoonotic pathogen that causes severe diseases in congenitally infected rodents and strays cats and in immune-compromised patients. Rodents and stray cats are the main reservoir for toxoplasmosis in Northeast of Iran. Early detection of the toxoplasmosis is very important to follow up the disease. The intrinsic factors of oocyst dispersal from the initial Drop-off locations of the infected feces may also be a crucial factor contributing to the widespread distribution of oocysts in soil. Antibodies to *T. gondii* was found in the cat,

in 5/28 black rats (18%), and 2/18 brown rats, from Brazil (11%)(Silva et al. 2017; Simon et al. 2017).

In these hosts, the incidence of transmission elevates thereafter to more than 90%, when congenital infection occurs during the late weeks before delivery, which is more likely to be asymptomatic, yet may proceed to neurologic disorders later in childhood or in adolescence. One-third of the mother rodents, who acquire a primary *T. gondii* infection during pregnancy, transmit the infection to their fetuses and disseminate the infection to the environment (Bastien et al. 2018; Brouat et al. 2018; Foronda et al. 2015; Gennari et al. 2015; Mikhail et al. 2017; Normaznah et al. 2015; Wang et al. 2018d).

One of the reasons for using ELISA avidity method in rodents and stray cat's studies was to find out the proper time for the host to be tested for *Toxoplasma* parasites, because finding this time and that the host is at what stage of infection are efficient in the severity of the infection. The Rodents were divided into four groups and stray cats in our study included in the group with the total hosts involved with *Toxoplasma* parasites. According to the results of this study, 246/286 rodents and 180/210 stray cats developed positive antibodies. Moreover, the results revealed that there were 68 positive *T. gondii* GRA6 gene rodents and 38 positive *T. gondii* GRA6 gene cats among the samples. Rodents and cats cannot develop positive antibodies and more probably ELISA test can be positive because of the presence of antibodies against *T. gondii* in the serum of the infected animals. Prevention and treatment of this parasitic infection in animals is not common, even if they are diagnosed in the early acute stage. The preventive measures are only used to avoid infection in domestic animals and human. Toxoplasmosis of rodents and stray cats may not create environmental and health problems for

human, in Northeast of Iran. Despite the efforts of veterinarians to eradicate this disease in northeastern Iran, the infection remains as one of the unresolved problems. In Golestan region, especially in its tourism areas, people are in contact with these animals, and in rare cases, they can lead to severe infections or even epidemics. Early detection of the toxoplasmosis in these animals can be helpful to prevent the spread of infection to human and other hosts. A simple ELISA method used in toxoplasmosis analysis detects presence of the antibodies in these hosts. The molecular method is positively associated with the ELISA avidity method and can contribute to the health of the region (Jung et al. 2017; Must et al. 2017; Pena et al. 2017; Wang et al. 2018a, b, d; Wang et al. 2017).

Prevention of the congenital toxoplasmosis in rodents and stray cats has been based mainly on serological tests for anti-*Toxoplasma* antibodies. Many serological tests, including the ELISA and ELISA avidity methods have been used to detect the antibodies against *T. gondii* (Flegr 2017; Liang et al. 2016; Macaluso et al. 2018; Poulle et al. 2016; Spada et al. 2012; Teimouri et al. 2018). However, infection should be diagnosed at the early acute stages, when treatment is more effective. Therefore, the ELISA avidity method is useful in detection of the congenital toxoplasmosis among the rodents and stray cats and its results will be reliable, if we use the designed ELISA avidity kit from *Toxoplasma* ES antigens (tachyzoite based ELISA avidity) (Ahmadpour et al. 2017; Felin et al. 2017; Naghili et al. 2017; Patel et al. 2017; Pishkari et al. 2017). Toxoplasmosis is not critical disease in Iran but it is necessary to prevent it from spreading animals to humans.

Conclusion

According to the results of this study, the Northeast of Iran is a very important region for following up the toxoplasmosis infection, specifically among the animals such as rodents and stray cats (their main reservoir is Golestan). Moreover, quantitative IgG avidity (qIgG) results were at the intermediate level among the rodents and stray cats with *T. gondii* GRA6 positive gene in the region. We also concluded that the prevalence rate of toxoplasmosis in serology study was higher than that in the molecular detection.

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Author contributions Javid Sadraei contributed on proposal and sampling of this manuscript (25%), Ehsan Shariat Bahadori contributed on analyzing of samples and experimentation (50%) and Abdolhosein Dalimi contributed on data analyze and statistical survey (25%).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

Human and animal rights This study was approved by 52/3595 number on 2015, Ethics committee, Tarbiat Modares University.

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