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Phylogenetic Analysis of *Toxoplasma gondii* Type II and Type III by PCR-RFLP Plus Sequencing on Wild-Rats of Golestan Forest, Iran

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Abstract

Introduction: *Toxoplasma* is a genus of intracellular parasites, which was observed in the many types of mammalians. *Toxoplasma* protozoa that were detected from many types of rats have the equal phenotypic characteristics but have different clinical symptoms and genotypes. Mostly, domestic animals and rodents are the hosts in the reservoirs in the environment. The objective of this article was to phylogenetic identification of toxoplasmosis in wild-rats of Golestan forest using secretory antigens type 1 gene.

Materials and Methods: In this surveillance, the researchers gathered 286 wild rodents from Golestan forest and earned DNA from brain and heart tissues to prove SAG1 gene from these hosts. They split these rodents into four groups and then analyzed the positive samples by the Polymerase chain reaction basis.

Results: In this surveillance, they discovered 24% of tissues of the wild-rats were infected for these protozoa. Over 55.8% of tissues belonged to *Ratus ratus*, 17.6% tissues belonged to *Ratus norvegicus*, 14.7% of tissues belonged to *Mus musculus*, 11.7% of tissues belonged to *Rombumys opimus*, also with attention of phylogenetic analysis, 50 infected rats belonged to genotype III and 18 infected rats belonged to genotype II.

Conclusion and Discussion: Conclusion showed that different types of wild rats disseminated this type of infection; also SAG1 molecule was a very important molecule to detect toxoplasmosis in rats of Golestan area.

Keywords: Toxoplasmosis; PCR-RFLP; SAG1; Golestan forest; Rats

Introduction

Toxoplasma gondi is an intracellular extra-cytoplasmic protozoa lives next to many types of animals in Iran [1-5].

The main reservoirs of toxoplasmosis are rats in Iran. Soft tissues and muscles of rats are infected by this parasite. The SAG1 gene is an important biomarker that was extracted from these infected organs and indicated toxoplasmosis. SAG1 gene is one of the important molecules that earned from tachyzoite of all *Toxoplasma gondi* genotypes [2,6-9].

Toxoplasmosis cycle is defined as the transmission of infection from rats to cats and human. This route is very simple and important way to contamination of human environment [10-12].

Two main genotypes of *Toxoplasma protozoa* exist in Iran, type II and type III. The purpose of this study, therefore, was to phylogenetic analysis of *Toxoplasma* in wild rats from Golestan forest for the presence of SAG1 in their brain and heart tissues to identify as toxoplasmosis [4,13-16].

Materials and Methods

The procedure of this detection was polymerase chain reaction based plus sequencing (phylogenetic tree) to complete the investigation. Different locations of Golestan forest have different climate. The dominant condition of this area is maximum humidity (Figure 1).

Figure 1: Iran plane from Golestan forest.

Sampling

The researchers gathered 286 wild rodents from Golestan forest. In order to study the infection status of rodents, organs (the brain and heart) of these animals were removed. These rodents were separated

into four types. To analyze infection status, the brains and hearts of the rodents were collected in 95% ethanol and 4°C frizzier until using.

DNA extraction

They used three methods to extraction of DNA, consists: 1-DNG method that used isopropanol, ethanol and lysis buffer, 2 Viogen kit and 3-phenol chloroform proteinase k method. In these methods we extracted much purified DNA from tissues of that wild-rats. Extracted DNA preserved on 4°C frizzier until using.

Polymerase chain reaction analysis for *T. gondii* SAG1 gene

Polymerase chain reaction analysis was done to detect SAG1 as a *Toxoplasma* marker. The primers of SAG1 gene were blasted from NCBI website and designed by Pishgam Company.

5´- GCTGTAACATTGAGCTCCTTGASTTCCTG-3´forward

5´- CCGGAACAGTACTGATTGTTGTCTTCTG-3´reverse

SAG1 amplification was performed in the following conditions in an Eppendorf thermal cycler. Then we used PCR-RFLP method to genotype this parasite from these wild rodents (Table 1).

S. no	Materials	Quantity
1	Water, nuclease free	17 µl
2	10 × Fast Digest green buffer	2 µl
3	DNA (sample)	10 µl
4	Fast Digest Enzyme	1 µl
	Total Volume	30 µl

Table 1: Polymerase chain reaction-RFLP substrates.

The statistical study was done with SPSS 18 software.

Results

In this study, 68 tissues of rats were found to be positive for SAG1: Over 38 (55.8%) tissues belonged to *Ratus ratus*, 12 (17.6%) tissues belonged to *Ratus norvegicus*, 10 (14.7%) tissues belonged to *Mus musculus*, 8 (11.7%) tissues belonged to *Rombumys opimus*, also with attention of phylogenetic analysis, 50 (73.5%) infected rats belonged to genotype III and 18 (26.5%) infected rats belonged to genotype II (Importance of phylogenetic tree).

These results in Figure 2 showed that positive samples in 1180 Bp area using SAG1 gene. 29% of *Rattus rattus*, 26% of *Rattus norvegicus*, 16% of *Mus musculus* and finally 16% of *Rombomys opimus* were positive. The samples were gathered from brain and heart tissues from these rodents. In this study, we found that 68/286 brain tissues and 63/286 heart tissues were positive. The brain tissue is the most suitable organ to invade *Toxoplasma* parasites (Figures 3 and 4).

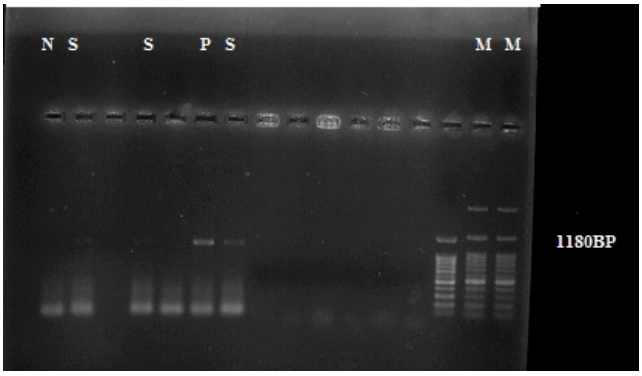

Figure 2: Amplified SAG1 gene. P: positive control; M: size marker; S: positive sample; and N: negative control.

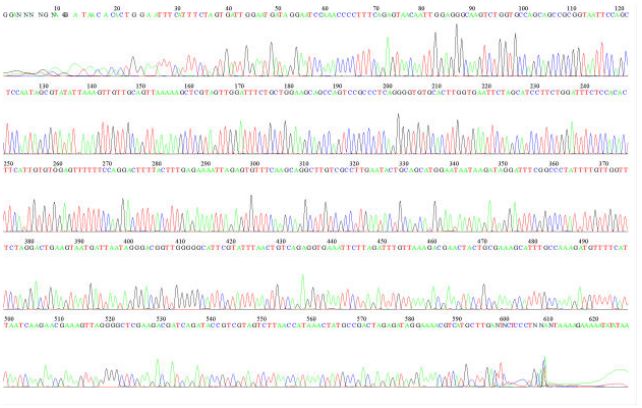

Figure 3: The sequence analysis of SAG1 gene of *Toxoplasma gondii*.

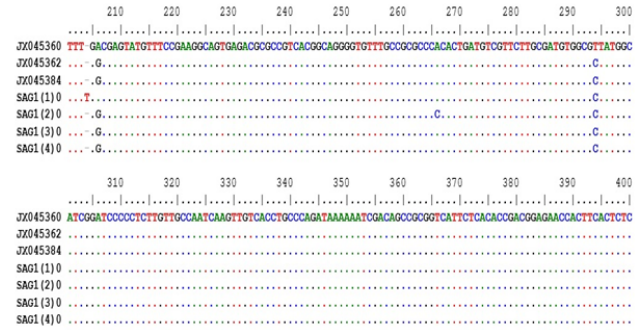

Figure 4: Multi alignment results of SAG1 gene.

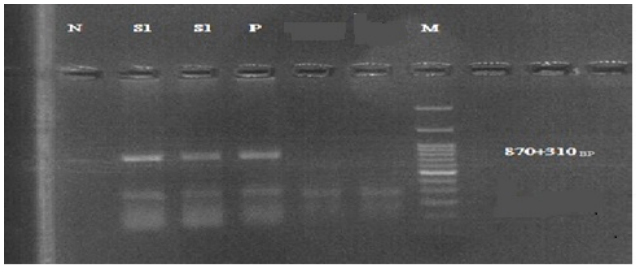

Figure 5: PCR-RFLP result using SAG1 gene of *Toxoplasma gondii*.

In the Figure 5, 870+310 Bp bands showed genotype III of *Toxoplasma gondii* in presence of SAG1 gene (P=positive control, N=negative control, S=sample, M=size marker).

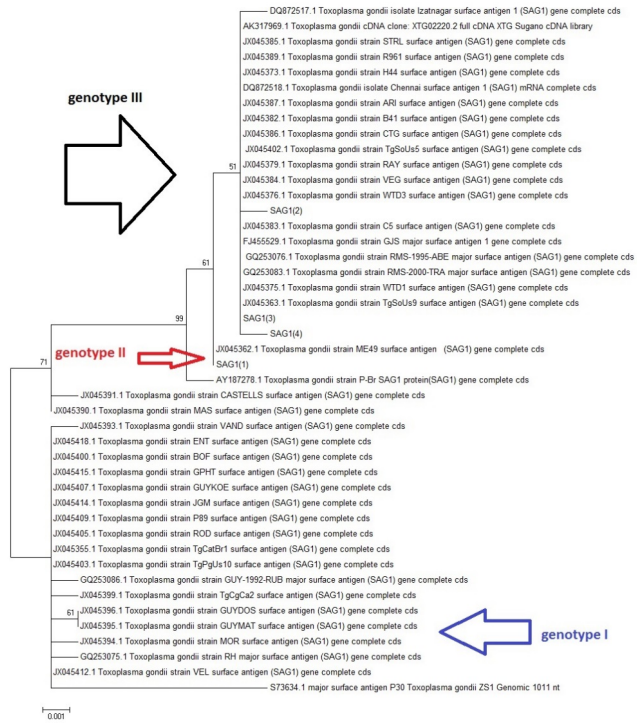

Figure 6: Phylogenetic tree of *Toxoplasma gondii* SAG1 gene.

>SAG1(1)

CAATGTGCACCTGTAGGAAGCTGTAGTCACTGCTGATTCTCGCTGTTCTCGG
CAAGGGCTGACGACCGGAGTACAGTTTTTGTGGGCAGAGCCGCTGTGCAGC
TTTCCGTTCTTCTCGGTTGTGTCACATGTGTCATTGTCGTGTAAACACACGG
TTGTATGTCGGTTTCGCTGCACCACTTCATTATTTCTTCTGGTTTTTTTGACG
AGTATGTTTCCGAAGGCAGTGAGACGCGCCGTCACGGCAGGGGTGTTTGCC
GCGCCCACACTGATGTCGTTCTTGCGATGTGGCGCTATGGCATCGGATCCC
CCTCTTGTTGCCAATCAAGTTGTCACCTGCCCAGATAAAAAATCGACAGCCG
CGGTCATTCTCACACCGACGGAGAACCACTTCACTCTCAAGTGCCCTAAAAC
AGCGCTCACAGAGCCTCCCACTCTTGCGTACTCACCCAACAGGCAAATCTGC
CCAGCGGGTACTACAAGTAGCTGTACATCAAAGGCTGTAACATTGAGCTCCT
TGATTCCTGAAGCAGAAGATAGCTGGTGGACGGGGGATTCTGCTAGTCTCG
ACACGGCAGGCATCAAACTCACAGTTCCAATCGAGAAGTTCCCCGTGACAA
CGCAGACGTTTGTGGTCGGTTGCATCAAGGGAGACGACGCACAGAGTTGTA
TGGTCACAGTGACAGTACAAGCCAGAGCCTCATCGGTCGTCAATAATGTCG
CAAGGTGCTCCTACGGTGCAGACAGCACTCTTGGTCCTGTCAAGTTGTCTGC
GGAAGGACCCACTACAATGACCCTCGTGTGCGGGAAAGATGGAGTCAAAGT
TCCTCAAGACAACAATCAGTACTGTTCCGGGACGACGCTGACTGGTTGCAA
CGAGAAATCGTTCAAAGATATTTTGCCAAAATTAAGTGAGAACCCGTGGCAG
GGTAACGCTTCGAGTGATAAGGGTGCCACGCTAACGATCAACAAGGAAGCA
TTTCCAGCCGAGTCAAAAAGCGTCATTATTGGATGCACAGGGGGATCGCCT
GAGAAGCATCACTGTACCGTGCAACTGGAGTTTGCCGGGGCTGCAGGGTCA
GCAAAATCGTCTGCGGGAACAGCCAGTCACGTTTCCATTTTCGCCATGGTGA
CCGGACTTATTGGCTCTATCGCAGCTTGTGTCGCGTGAGTGATTACCGTTG

>SAG1(2)

CAATGTGCACCTGTAGGAAGCTGTAGTCACTGCTGATTCTCGCTGTTCTCGG
CAAGGGCTGACGACCGGAGTACAGTTTTTGTGGGCAGAGCCGCTGTGCAGC
TTTCCGTTGTTCTCGGTTGTTTCACATGTGTCATTGTCGTGTAAACACACGG
TTGTATGTCGGTTTCGCTGCACCACTTCATTATTTCTTCTGGTTTTTTGGCGA
GTATGTTTCCGAAGGCAGTGAGACGCGCCGTCACGGCAGGGGTGTTTGCCG
CGCCCCCACTGATGTCGTTCTTGCGATGTGGCGCTATGGCATCGGATCCCC
CTCTTGTTGCCAATCAAGTTGTCACCTGCCCAGATAAAAAATCGACAGCCGC
GGTCATTCTCACACCGACGGAGAACCACTTCACTCTCAAGTGCCCTAAAACA
GCGCTCACAGAGCCTCCCACTCTTGCGTACTCACCCAACAGGCAAATCTGCC
CAGCGGGTACTACAAGTAGCTGTACATCAAAGGCTGTAACATTGAGCTCCTT
GATTCCTGAAGCAGAAGATAGCTGGTGGACGGGGGATTCTGCTAGTCTCGA
CACGGCAGGCATCAAACTCACAGTTCCAATCGAGAAGTTCCCCGTGACAAC
GCAGACGTTTGTGGTCGGTTGCATCAAGGGAGACGACGCACAGAGTTGTAT
GGTCACAGTGACAGTACAAGCCAGAGCCTCATCGGTCGTCAATAATGTCGC
AAGGTGCTCCTACGGTGCAAACAGCACTCTTGGTCCTGTCAAGTTGTCTGCG
GAAGGACCCACTACAATGACCCTCGTGTGCGGGAAAGATGGAGTCAAAGTT
CCTCAAGACAACAATCAGTACTGTTCCGGGACGACGCTGACTGGTTGCAAC
GAGAAATCGTTCAAAGATATTTTGCCAAAATTAAGTGAGAACCCGTGGCAG
GGTAACGCTTCGAGTGATAATGGTGCCACGCTAACGATCAACAAGGAAGCA
TTTCCAGCCGAGTCAAAAAGCGTCATTATTGGATGCACAGGGGGATCGCCT
GAGAAGCATCACTGTACCGTGCAACTGGAGTTTGCCGGGGCTGCAGGGTCA
GCAAAATCGTCTGCGGGAACAGCCAGTCACGTTTCCATTTTCGCCATGGTGA
CCGGACTTATTGGCTCTATCGCAGCTTGTGTCGCGTGAGTGATTACCGTTG

Figure 7: Two samples of sequencing results using SAG1 gene.

In Table 2 and Figure 6, we showed that 50 (73.5%) samples were in genotype III and 18 (26.5%) samples were in genotype II; hence, the dominant genotype from this area was III (Figure 7).

	Brain infection rate	Heart infection rate	Total genotype II	Total genotype III
Rattus rattus	13%	11%	3%	10%
Rattus norvegicus	4%	3%	1%	2%
Mus musculus	3.40%	3.10%	1%	2%
opimus	2%	1%	0.60%	2%

Table 2: Total abundance rate of toxoplasmosis in rodents of Golestan forest.

Discussion

With a focus on living wild rodents, cats and also their presence in large number in wild areas, calculating data about *Toxoplasma* in the

population of rodents and cats in wild areas is critical for prevention programs [17-19].

The aim of this study was to carry out a phylogenetic study of toxoplasmosis infection in wild rodents of Golestan forest using SAG1 gene. Toxoplasmosis is a zoonotic infection between rodents and humans. All of them have been presented with toxoplasmosis, which is one of the major zoonotic infection diseases in Iran. The climate condition of Golestan forest is very suitable to spread toxoplasmosis in that wild area.

The significant difference between these two similar studies may be due to the difference in sampled population. By contrast, a significant difference was observed in *Toxoplasma* infection between male and female cats in Sari [20-24].

Most of the studies did not report significant differences in *Toxoplasma* infection between two sexes and the effect of sex on *Toxoplasma* exposure remains to be elucidated. A number of genotyping studies have been done on *T. gondii* from different hosts in various countries [4,6-8,11,18,25-27]. All of these studies had used molecular methods [18]. These and other research studies showed the importance of wild rodents in spreading of toxoplasmosis in humid wild areas. Sharif and colleagues reported 40% prevalence for *Toxoplasma* antibodies in cats from Sari, northern Iran [28]. They used latex agglutination assay to detect anti *Toxoplasma* antibodies in 100 serum samples from stray cats in five urban areas of Sari. The city is located near Golestan forest in northeastern Iran, with humid climate favorable for *Toxoplasma* development. But *Toxoplasma* infection of stray cats in Sari is lower than that of feral cats in Golestan forest [28]. In this study, they found that about 23% brain tissues and 22% heart tissues from rodents were positive for toxoplasmosis in Golestan forest. In recent years, there are no studies about rodent toxoplasmosis in Golestan forest. In this surveillance, they detected that the SAG1 molecule was very simple and reliable gene to detect toxoplasmosis in rats of Iran, especially in northeast of this country. Also we found that two main genotypes were existed in northeast of Iran, type II and type III. In this study we showed that 74% infected rats had genotype III *Toxoplasma* whereas 26% of infected rats had genotype II. These variations of phylogenetic study altered in different areas of Iran [24-29].

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