

Survey of *Toxoplasma Gondii* in Livestocks' Meat (Sheep, Goat, Camel), Using Nested PCR Method in Sabzavar District

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Abstract

Toxoplasmosis is a common parasitic zoonosis in the world which led to loss of life and property in the countries annually. *Toxoplasma gondii* is an intracellular protozoan disease. It is estimated that about a third of the world's population are infected with the parasite. Parasites is transmitted in various ways such as contaminated water containing oocytes shed of cats, eating undercooked or raw meat or through congenital infection. This study was conducted in Sabzevar , in which a sample of animals in slaughters in Sabzevar were selected including livestock such as sheep, goats, camels, which were collected and investigated for toxoplasmosis. In this study, DNA of these samples (heart and diaphragm) including 40 sheep, 40 goats, 40 camels were collected and then were examined using B1 marker. The PCR results showed 60% of sheep infected to toxoplasmosis and infection rate in diaphragm and heart samples were 37.5% and 25.5% . Also frequency rate for toxoplasma in goat and camels were obtained 52.5% and 65% and infectivity rate of diaphragm and heart were 35% and 17.5% for goat, 45% and 20% for camels samples. The results showed that infectivity rate of toxoplasmosis in sheep and goats in Sabzavar is more dangerous than other cities. It seems that infection rate was influenced by examination tests, ecological condition, way of growth and feeding and agony of livestock and others factors. Toxoplasmosis were seen more in female sheep. We did not obtain any difference for infectivity in goat and camel gender and related to growing factors. In this study, toxoplasmosis was reported in camels of Iran for first time. The RFLP results showed genotypes of II and III for toxoplasma and it needs to modified studies in this field.

Keyword: *Toxoplasma gondii*, Nested PCR, samples of animals in slaughtered

Introduction

Toxoplasmosis is a common parasitic zoonosis in the world that has been imposed economic and life losses annually to countries (Bahrieni, 2008, Dubey, 2009). The cause of this disease is intracellular parasite called *Toxoplasma gondii*. (Hamzavi, 2007, Dubey, 2009)

It is estimated that about a third of the world's population are infected with the parasite. This parasite is transmitted in various ways such as contaminated water containing oocytes disposed of cats, eat undercooked or raw meat or through congenital infection. (Asgari, 2011) while toxoplasmosis is benign in healthy adults, but clinical development of the parasite in the womb and those with impaired immune systems is severe and common complication of infection in utero include chorioretinitis, encephalomyelitis, hydrocephalus and microcephaly and abortion (Markel, 2001) and (Nov-Brown, 2006). Therefore, infections during pregnancy if the mother is infected for the first time have many problems for the baby in the future and even the risk of miscarriage possible (Moazeni, 2013)

Toxoplasmosis in utero is extremely dangerous disease that unfortunately prevalent in various human societies and in United States it is estimated that each year 5,000 babies are born with toxoplasmosis complications. (Shuhaiber, 2003)

Most important ways of transmission of the parasite to humans in Toxoplasmosis include:

A. eating meat containing tissue cysts

B. eating parasite Oocytes by contaminated soil or water or by contact with contaminated fruits and vegetables (Kendall, 2003)

Since, bradyzoite for the loss of tissue cysts in contaminated meat temperature should be above 67°C and the freezer temperature to reach -12°C, meat and dairy product especially for pregnant women can be a major source of infection. Contamination of meats mostly by eating undercooked meat of sheep, goats and pigs and less often via contaminated beef. (Fallah, 2011. Cook, 2000)

In countries where the culture of consumption of meat and dairy products is in raw or undercooked and there is also high prevalence of Toxoplasma. Also among those who work in slaughterhouses and butcher shops as well as the risk is high. (Boyer, 2005)

In France those who want to eat a lot of uncooked meats (steak of sheep), a significant amount of pollution to Toxoplasmosis and congenital cases have been reported. (Macpherson, 2005)

With a high prevalence of toxoplasmosis serology studies in domestic animals, especially sheep, goats and pigs were demonstrated in a study in 2002 in England to determine the prevalence of parasites in meat and PCR methods showed that 38% of the samples containing tissue cysts were infected with the Toxoplasma (Hurtad, 2001)

In some studies in Iran, the source of pollution prevalence of antibodies against Toxoplasma was in serum of animals, in a study carried out on 12 Iranian provinces to evaluate serum Toxoplasma gondii infection in domestic animals, a total of 13018 samples collected and antibodies against Toxoplasma in 51.8% of samples were observed. (Asgari, 2013)

Some other studies in Iran based on the prevalence of meat infections were done using molecular techniques. In another study at the Shahrekord by Azizi et al. (2014) to assess the prevalence of toxoplasmosis in meat and livestock (sheep, goat) by PCR was observed that infection rates in sheep meat is 37.5% and the infection in goat meat is equal to 22.7%, the highest organ that had been infected include: brain (32%), liver (30%), thigh (28%), tongue (16%) (Kravetz, 2005)

Toxoplasma gondii

Toxoplasma gondii is a mandatory intracellular protozoan which can infect warm-blooded vertebrates including mammals. Human infection with T gondii has a wide range of clinical symptoms depending on the age and immunological conditions of individuals. Cats are the only host that the protozoa that can reproduce both sexually and asexually. Understanding the life cycle of the parasite and to investigate the role of cats in the pathogenesis of disease transmission and prevention methods is necessary (Markell et al, 1995)

Toxoplasmosis is a common disease, with a global scope. The spread of disease varies from one country to another depending on the culture. In cultures that raw meat consumption is recommended, the high prevalence of the disease is seen. According to the cultural and spatial context in the United States of America annual rate of change of serum is 1 to 2%. 10 to 40% of patients with the HIV virus in the United States carriers the tissue that shows hidden infection and genital toxoplasmosis in the 3000 case reported in America (Oksenhendler et al, 1994).

Among the diseases that are killing people with AIDS, it is estimated that almost ten percent of AIDS patients in the United States of America and 30% in Europe die of toxoplasmosis (Hellerbrand et al, 1996)

PCR

In the past, usually chemical methods have been used for the production of nucleotide fragments, but these methods were laborious and require a long time. From 1980 onwards, mainly PCR method has been used in molecular biology laboratories.

In 1971, Khorana and colleagues reported a method to simulate a region of double-stranded DNA using two primers, so that the two ends of 3 which were designed to each other. But the idea of using the property for a reaction cycle repeated was invented 13 years later by Kary Mullis at Cetus.

This technique was introduced in 1984 by Kary Mullis and was used in most of laboratories a for different purposes some of which include:

DNA Cloning to sequencing (Sequencing), phylogenies based on DNA for functional analysis of genes, genetic diagnosis, identification and genetic fingerprinting and tracking and diagnosis of infectious diseases. Mullis for inventing the technique in 1993 was awarded the Nobel Prize. This technique takes its name from one of its key components, DNA polymerase, which is used to provide a large number of copies of a DNA sequence.

Proceed with the PCR process from the initial number of copies of a piece of DNA, which may be one or very few is used as the template and produced in large amounts in the range of several million copies and the final product of PCR generally called Amplicon means that material is reinforced or amplified.

PCR -RFLP

RFLP first time in 1974 as a genetic marker to detect mutations in the virus by Grodzicker and colleagues was used. RFLP use as markers of genetic disease first time (Kon and Dozy, 1974) was used for the analysis of sickle cell disease. Botstein et al 1980 introduced basic theory of this method for mapping disease-related genes in the human.

Backman invented DNA pattern transfer method and probe from gels to nitrocellulose membranes in RFLP (Backman, 1986). He introduced this marker for the first time. Critical applications such as mapping and spatial manipulation of genes controlling the quantitative traits using RFLP in 1983 were expressed by Backman and Soller. This technique represents a powerful multi-gene or genome were analyzed in a number of animal species such as cattle, sheep, goats, horses, pigs and chickens using the marker.

Research questions

How much is *Toxoplasma gondii* infection in sheep meat in Sabzevar?

How much is *Toxoplasma gondii* infection in goat meat in Sabzevar?

How much is *Toxoplasma* infection in camel meat in Sabzevar?

Is the *Toxoplasma gondii* infection in sheep, goats and camels different?

Materials and methods

This is a cross-sectional study. In this study was conducted in the city several examples of animals slaughtered in Sabzevar, including the diaphragm and heart-related livestock (sheep, goats, camels) were collected and the infection toxoplasmosis was investigated. Sampling began in February 2014, and continued until the end of April 2015. All samples were collected from slaughter animals in Sabzevar. In this study, samples of heart and diaphragm in 40 sheep, 40 goats and 40 camels were collected. In addition, 40 samples of minced meat also were collected. Given veterinarian at the slaughterhouse based on a random sample of approximately 50 grams of the heart and diaphragm of each animal with a disposable scalpel isolated and in disposable plastic samples

with a special code printed on every package and every day after sampling, samples were immediately transferred to a -20°C freezer.

Frozen Samples collected from slaughterhouses Sabzevar were transferred to the city of Kerman at -20 C in freezers of Department of Parasitology, Faculty of Medicine Afzalipour.

After observing hygiene such as wearing gloves and disinfect the desktop with 70% alcohol, the samples were removed from the freezer and disposable scalpel blade approximately 5 grams of tissue inside a disposable plates up and then start to grind and crush the meat with a scalpel. This work continued until a completely homogeneous tissue samples obtained. Then homogenized sample is transferred from the plates to pipes Falcon 50 cc and 20 cc of fresh kiwi juice added to it and about 1 h at the laboratory until the tissues are quite loose and crushed. Then, the contents Falcon tubes with shaker, for 10 minutes, stirring until completely mixed. Then mixture was homogenized in 20 ml of saline is added to the total volume of 40 cc. Then, the contents of Falcons again stirred with the help shaker for 10 minutes with a solution of 200 ml pipette contents Falcon tubes were removed and transferred to micro-tubes of 1.5.

In the PCR to determine the diaphragm and heart samples of *Toxoplasma gondii* infection by Nested PCR test marker (gene) B1 were used. Protozoan *Toxoplasma gondii* B1 gene in the genome 35 times (copy) and of the marker in the diagnosis of human and animal toxoplasmosis and various samples of blood, spinal fluid and tissue suspension is used (Burg et al, 1998; Jalal et al, 2004)

To amplification of this gene in samples suspected of being infected with *Toxoplasma gondii* AS1 and AS2 of 2 primers were used in the Nested PCR method.

First corresponding primer sequences according to selected studies were analyzed to identify genes related to specific action. Several articles were evaluated to confirm the primers. Then, primer sequences were sent to Micro gene South Korea for the order. Primer synthesis by the manufacturer for Liufiliz was sent to the Department of Parasitology. Later, according to the protocol described on each primer to each tube, distilled water for injection was added and then dilution of each primer was performed by PMOL and micrograms. Finally primers prepared were stored at -20 ° C.

Results

In this project, 280 samples randomly from slaughter livestock city of Sabzevar, of animals, including sheep, goats and camels were collected over a period of five months. Results expressed in the heart and diaphragm toxoplasmosis infection in sheep, goats and camels in the following table.

Table1: Compare toxoplasmosis infection in heart and diaphragm in sheep, goats and camels in Sabzevar in 2014

Animal	Examined tissue	NO / Per of contaminated samples	Examined tissue	NO / Per of contaminated samples	Examined tissue	NO / Per of contaminated samples
Sheep	Diaphragm muscle	37.5-15	Myocardium	22.5-9	Diaphragm and Heart	60-24
Goat	Diaphragm muscle	35-14	Myocardium	17.5-7	Diaphragm and Heart	52.5-21
Camel	Diaphragm muscle	45-18	Myocardium	20-8	Diaphragm and Heart	65-26
Sheep & Goat	Minced meat	28-7	-	-	-	-
Camel	Minced meat	26.6-4	-	-	-	-

Compare Results molecular about any animal (sheep, goats and camels), and examples of each animal (diaphragm and heart) analyzed by SPSS16, which results in the following tables.

Table 2: Results between pollution per animal (sheep, goats and camels) and where to buy it (the slaughterhouse and wheel of Goods)

Check between two factors	P-Value	Result
Pollution mutton and where to buy it	0.085	-
Pollution ostrich meat and where to buy it	0.217	-
Pollution goat meat and where to buy it	0.052	-
Three animals with the contaminated meat and where to buy it	0.048	+

Table 3: Results between pollution per animal (sheep, goats and camels) and meat

Check between two factors	P-Value	Result
Pollution mutton and Meat	0.012	+
Pollution ostrich meat and Meat	0.011	+
Pollution goat meat and Meat	0.052	-
Three animals with the contaminated meat and Meat	0.001	+

Table 4: Results between infection of Toxoplasma gondii and type of animal (sheep, goats and camels)

Check between two factors	P-Value	Result
Pollution and Toxoplasmosis gondii and type of animal	0.961	-

Toxoplasma gondii infection rate in the diaphragm and heart tissue in animal husbandry (sheep, goats and camels) in Sabzevar to sex are as the following table:

Table 5: The total amount of pollution Toxoplasmosis gondii by PCR in cattle (sheep, goats and camels) by gender

Type of animal	Sex	The number of contaminated	The amount of pollution (in%) according to sex	Examined tissue	The total amount of pollution(in%)
Sheep	Male	9	22.5	Diaphragm and Heart	60
	Female	15	37.5		
Goat	Male	11	27.5	Diaphragm and Heart	52.5
	Female	10	25		
Camel	Male	15	37.5	Diaphragm and Heart	65
	Female	11	27.5		

The statistical comparison between male and female animals was conducted in the infected city as follows:

Table 6: Results between infection of Toxoplasma gondii and type of animal (sheep, goats and camels)

Check between two factors	P-Value	Search result
Contamination of livestock (sheep, goats and camels) And Sex	0.795	-

PCR results on the samples (heart and diaphragm) in three types of cattle, sheep, goats and camels in Sabzevar showed that the infection rate in sheep and goats, 52.5% and 60% is 65% higher.

In the case of B1 gene and PCR products of the marker, some researchers have used the RFLP technique; because the results of this test some preliminary studies show the genotypes of Toxoplasma gondii (Et al, 2009 Michael, 2001. Alfonso)

It should be noted about the demographic strains of the parasite *Toxoplasma gondii* in Europe and North America have shown that the majority of isolates of *Toxoplasma gondii* in three genotypes I, II and III, with that genotype I isolated from congenital toxoplasmosis and ocular Genotype II (the highest percentage of genotype in human toxoplasmosis), and most of congenital toxoplasmosis and immune deficiency (AIDS) and the genotype III have been mostly isolated from infected animals. (Howe, Sibley, 1995)

In this study, RFLP of restriction enzymes *Xho*I was used to test the enzyme markers B1 PCR products related to type II and III infection is related to digestion and the DNA is divided into two or three pieces, but sometimes some genotype in the II, III possible without cutting (digesting) remain.

Also, PCR products were digested genotype I infection without cutting the restriction enzymes *Xho*I remain. In this study, 57 were positive by PCR products which were selected B1 gene amplification and RFLP testing was done about it.

Table 7: RFLP results for the three types of animals (sheep, goats and camels)

Type of livestock	Number of samples	Evaluation of the samples without being digested	
Sheep	19	11 genotypes II and III	8 samples may genotype I or genotypes II and III
Goat	19	7 genotypes II and III	12 samples may genotype I or genotypes II and III
Camel	19	9 genotypes II and III	11 samples may genotype I or genotypes II and III

Discussion and conclusion

In this study the parasite *Toxoplasma gondii* in tissue distribution of sheep, goats, camels by Nested PCR has explored the city of Sabzevar, finally after the process of collecting samples and DNA extraction Nested PCR process on a total of 280 samples including the heart, diaphragm and minced meat was found, examples being infected sheep in a total of 60% reported that 37.5% of contamination in samples of the diaphragm, the heart of the infection was 22.5%. Also, in samples from a total of 52.5% was reported that 35% of toxoplasma infection contamination in the diaphragm, the heart of the infection was 17.5%.

Whole *Toxoplasma* infection was reported in 65% that 45% higher contamination in the diaphragm, the heart of the infection was 20%. Simultaneous infection of the heart and diaphragm in infected sheep, 5 which was 12.5% and pollution at the same time, the heart and diaphragm infected goats, 2 which was equivalent to 5% and also pollution of the heart and the diaphragm is simultaneously infected herd, 8 was equal to 20%.

Further, abundance of parasites in sheep and goat meat minced meat and in minced meat (28%) was 26.6% higher. In the study by Rubens and colleagues in Brazil on 71 samples of tongue and diaphragm goats and RFLP-PCR method was carried out, it was found that the rate of infection of *Toxoplasma gondii* in context, 34% and 66% respectively in the diaphragm muscle. (Rubens, Veronique et al.2007)

In this study, after tests, it was found that there is no significant difference between contaminated meat and gender (significantly higher level of 0.05), that sex does not affect the meat contamination. (P-value=0.795)

In this regard, several studies have been conducted in the country. Asghari et al in a study on 95 sheep meat, 90 pigs, 35 horses and 4 Geese carried out by MAT, infection rates of 29.5% and 18.8% of sheep meat goat meat 40% goose meat samples without contamination. ((Asghari, Sarkari et al.2013)

Further, in study after tests showed that the contamination of meat and meat (muscle and diaphragm) were significantly different and the type of lamb meat on the impact of pollution, also between contamination of camel meat and meat (muscle and diaphragm) there is a significant difference and meat Ostrich meat is the effect of pollution, but no significant difference between infection and goat meat (muscle and diaphragm) does not exist does not affect the type of meat on goat meat contamination.

In a study that was conducted by Moore and Arsalan In Turkey, the prevalence of Toxoplasma were reported in 95.7% in sheep, also in a similar study by Klun et al in Serbia was conducted on sheep meat Toxoplasma infection rate of 84.5% was reported (Klun et al, 2006)

In similar studies in Iran by Ahmadinejad et al in Ahvaz on sheep meat, sheep parasite Toxoplasma gondii infection rate was reported as 72.6, also in another similar study that was conducted by honorable and colleagues in Mazandaran on sheep among sheep parasite prevalence was 35% (Sharif et al, 2007).

Since in this study, all animals that have been studied over 2 years of age have a certain statistical test was used to compare age groups.

In comparing our results with other studies on the contamination of sheep and goats in other countries show that the prevalence of infection in sheep and goats in the region of Khorasan Razavi than some higher and some similar results. In some animal studies to determine the contamination of different organs have been used; for example, in Brazil the parasite Toxoplasma gondii infection percentage of tongue is more than the diaphragm.

It seems that the use of markers (genes) investigated the molecular experiments, tissue study of livestock, livestock raising environment due to air temperature and humidity environment, the frequency of cat and infection of Toxoplasma oocytes environment and other factors contribute to the difference in the trap.

In this study, in the comparison of the results of sheep infected with other results show that the contamination of sheep and goats (especially sheep) was higher in Sabzevar. Unfortunately, in the province, toxoplasmosis infection in animals has not been compared with the results of this research but it seems likely ecological differences among the mountain, the precipitation, the livestock and other factors in the intermediate host infection toxoplasmosis can have a role in this increase in pollution.

Toxoplasma prevalence in this study was seen in female sheep more than males while the prevalence of parasites in goats and camels were more in males. About sheep that have partly been seen in other studies. Perhaps keeping ewes to be fruitful and multiply, and increasing in the environment are more susceptible to infection and it does not apply in the case of goats. Also, the willingness of farmers to keep more male animals in Sabzevar is because of more expensive meat of the male animal.

Ostrich meat contamination on the research conducted for the first time and, unfortunately, no statistics as a source of meat from infected animals within Iran have been mentioned in any reference. It seems that contamination with other animal host of toxoplasmosis is very important in the field.

One of the factors that can accelerate the transmission of toxoplasmosis among livestock farmers for their cattle are fed every way, because the majority of livestock feed such as hay, etc. In order to feed their cattle fodder they use and they are often dumped in the environment that may be crossing over domestic animals such as cats. Also, most of the non-covered animals are kept, which can be a factor near pets such as cats and livestock, and thus facilitate the transfer of parasitic infection.

In this study, the prevalence of *Toxoplasma* in meat stuffing in the frequency of infection in any organ of the diaphragm and heart were lower and this difference was statistically significant, It can be concluded that the presence of parasites and probably in other parts of the body muscles and other meat (meat on sale in stores) relative organs such as the diaphragm, tongue, brain and heart less.

In the case of minced meat in the beef markets, it is remarkable that the cysts would rupture the tissue grinder meat and meat contamination is common.

In a study on 164 products by Fallah et al on meat was performed by RFLP showed the infection of *Toxoplasma* in meat products is 56.6%, which is a form of pollution of ground meat partly related (Fallah et al, 2011). These studies, as well as other studies around the world show that meat can be an important source of *Toxoplasma* parasite.

In this study, RFLP technique was used, PCR products B1 gene was inserted and the results showed that 19 sheep, 11 genotype II and III and 8 sample may genotype I and genotype II and III without digestion were and also goat number 19, number 7 genotype II and III and 12 sample may genotype I and genotype II and III are without digestion.

RFLP testing results also showed that more than 19 cases, 9 genotype II and III and 11 samples may genotype I and genotype II and III are without digestion.

A study by Zia Ali and Keshavarz in northern Iran (Mazandaran and Gilan) on antibody in the serum of infected sheep, goats and chickens live by serology and tissue in mice was carried out. Researchers reported the prevalence of 32.5 sheep and 14.3 in goats. In this study also Multiplex PCR assay for genotyping parasite strains used in this study genotype III and II in sheep and goats were reported. (Zia-Ali et al, 2007). Of course, genotyping of samples contaminated with other possible markers that should be addressed in future studies on this matter.

Finally, according to the results of *Toxoplasma* infection in sheep, goats and camels as one of the main sources in our country, we hope our health plan more attention to different aspects of prevention, diagnosis and treatment of toxoplasmosis.

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