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Research Article

Molecular Detection of Endemic Listeriosis and Toxoplasmosis in Shelter Dogs in Istanbul, Turkey

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ABSTRACT

Nowadays it is known that animal sourced infections may have serious threats against human health. In our study, we aimed to determine the molecular positivity of Listeriosis and Toxoplasmosis among shelter dogs in different animal shelters around Istanbul and to describe the role of dogs in the transmission of these zoonoses. Blood samples from 100 dogs were collected and Tag-Man probe based Real Time PCR (qPCR) analyses of the samples were conducted regarding to the high sensitivity and characteristics of this technique and the results were evaluated according to the gender and age of the dogs. According to our results, it is found that 12 dogs (12%) out of 100 are *L. monocytogenes* positive and 19 dogs (19%) are *T. gondii* positive. It is seen that seropositivity among the 0-2 ages group is high in both zoonoses and also according to gender *L. monocytogenes* is high among the females and *T. gondii* is high among the male dogs. We think that these results may be a serious risk for the people living in this city and optimal protective cautions should be taken. We estimate that our study will contribute the data about the prevalence of these zoonoses not only in our country but also all around the world.

Key words: *Listeria monocytogenes*, *Toxoplasma gondii*, dogs, Real Time PCR

INTRODUCTION

Listeriosis and Toxoplasmosis are worldwide infectious zoonoses seen among humans and animals which are especially economically important. It is clearly known that dogs have a potential risk in the transmission of these infections (Dubey, 2008; Schroeder *et al*., 1993; Oni *et al*., 1989). Listeriosis, which is quite common in the nature, is seen as sporadic and endemic infections among domestic animals and sometimes human beings in many countries all over the world. It is caused by facultative intracellular, short, asporous Gram-positive *L. monocytogenes.* It is motile at 22-28°C, but not motile at 37°C. Although the source of the infection is not often known in sporadic cases, epidemia is seen after consuming contaminated cheese, milk or some other animal products. Sometimes a person or an animal can be a porter and it may also be transmitted by infected person or animal's faeces. In such a case, the occurrence of the infection is significant (Bille, 2007; Ooi and Lorber, 2005).

Listeriosis can cause serious health problems, such as meningoencephalitis (meningitis), septicaemia, endo-carditis, conjunctivitis, abortus, arthritis and hepatitis. It might be life threating among some groups of patients including neonatals, pregnants, geriatric populations, under immunosuppressive treatment and with inadequate cellular immune response. Listeria infections are generally diagnosed with serological tests in blood or culture methods in stool samples. Besides serological tests like IFA and ELISA, molecular tests like PCR are highly sensitive and specific (Lorber, 2010; Day and Basavanna, 2015; Liu, 2008). Toxoplasmosis is a zoonotic infection caused by the intracellular protozoan parasite *T. gondii.* It is one of the most common parasitic zoonoses worldwide and the parasite can infect all warm-blooded animals. Human beings are infected by ingestion of oocysts in soil

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or water contaminated with dog or cat faeces, or by ingestion of tissue cysts in undercooked or uncooked meat of infected animals and also by drinking uncooked milk of these animals. Transfusion or organ transplantation from an infected person can also transmit the organism (Garcia and Bruckner, 1997; Tenter, 2000).

Besides cats, dogs have also been considered as a potential risk factor for *T. gondii* infection due to mechanical transmission of oocysts. Although this protozoan does not have an intraepithelial cycle, it must be regarded as a significant case because it is seen more often among children and young people who contact with dogs rather than cats (Lindsay *et al*., 1997; Ali *et al*., 2003; Dubey and Carpenter, 1993). It is difficult to get a clinical diagnosis because most persons are often asymptomatic, do not show any specific symptoms and recover spontaneously. Toxoplasmosis may be seen in different clinical manifestations depending on the duration of infection and the immune system status of people. These manifestations are acute infections, congenital infections, ocular toxoplasmosis, latent infections and reactivations. For diagnosis, some common tests are preferred, such as ELISA (Enzyme Linked Immuno-sorbent Assay), IFA (Indirect Fluorescent Antibody) and PCR (Polymerase Chain Reaction) implications (Bogitsh *et al*., 2013; Gürüz and Özcel, 2007).

In the present study, we aimed to determine the molecular positivity of Listeriosis and Toxoplasmosis among shelter dogs in different animal shelters around Istanbul and to describe the role of dogs in the transmission of these zoonoses.

MATERIALS AND METHODS

In the present study, we went to the dog shelters of Municipality of Istanbul and collected blood samples taken from 100 stray dogs in both genders by a veterinarian. Then, sera of the dogs were stored at -20°C until assayed. TagMan method and Real Time PCR analyses were conducted for the diagnoses of *T. gondii* and *L. monocytogenes* (Espy *et al*., 2006; Edvinsson *et al*., 2006; Le Monnier *et al*., 2011). Regarding to genders and ages of the dogs, correlations between the results were described and the values were commented statistically.

Qiagen Dneasy Blood & Tissue kit was used for the extraction of DNA according to the manufacturer's instructions. For the extraction of DNA, the proteins of the samples were dysed by Proteinase K and the DNAs were connected to Dneasy Mini Spin by the help of tampons. Contaminants were taken away during the centrifuge while the DNAs connected to the Dneasy membrane. After two stages of washing, the rest of the contaminants and enzyme inhibitors were taken away and so the DNAs were extracted.

Amplication was used on the primer design™ genesis® for *Listeria monocytogenes* and *Toxoplasma gondii* Kits (Primerdesing Ltd, Southampton, U.K.) Real-Time PCR has been done in the QIAGEN Rotor Gene Q (QIAGEN GmbH, Hilden, Germany) device. For *T. gondii*, during the application of TagMan probe based Real Time PCR, the primers and probes amplified the specific areas of 529 bp repeat element (RE) gene regions. And for *L. monocytogenes*, it is aimed to evaluate Listeriolysin O (hly A) gene regions.

The checking of positive, negative and internal DNA extraction, and also the preparation of the mixture of pathogen assessment were all done depending on the kit. Into each tube, 15µl of the mixture was placed and 5µl of DNA extracts of the samples was added into each tube. The volume of each tube was 20µl. After the PCR tubes were placed in Rotor Gene Q, the amplification protocol, which is stated in the Real Time PCR kit, was applied according to the software programme (Rotor Gene Q series software) and then the reaction was started. The activation of enzyme took place during the first turn, and the stages of denaturation and collection of data continued for 50 turns. The Amplification protocol was realized for activation of enzyme 2 mins at 95°C for denaturation 10s 95°C for collection of data 60s at 60°C. At the end of each turn, the increase of the fluorescence was identified by Real Time PCR.

After Real Time PCR analysis, the positive results of the samples and the quantitative evaluations, the amplification values and Ct (threshold value cycle) are all calculated and evaluated depending on the data. The measurements of the samples taken in the Green canal were evaluated as positive because inside the kit the probe sets for the agent had been marked with FAM dye. For internal checking, the probes which had been marked with VIC dye were used and with the measurement taken from the Yellow (VIC) canal it was understood that the study went well from the beginning of the isolation. In order to evaluate a sample in the measurement canal as positive, the PCR line must cross the threshold line. The numbers of samples with *T. gondii* and *L. monocytogenes* positive in age groups and genders were compared by using Fisher's exact probability test and Chi square with Yates's correction test. *P* values (two tailed) less than 0.05 were considered significant. Data were analyzed using the software package SPSS for Windows release 21.0.

Permission and ethical approval to conduct the present study were obtained from the Local Ethics Committee in Animal Experiments of Istanbul University.

RESULTS

L. monocytogenes and *T. gondii* are prevalent in our country and all around the world and they are two significant public health problems when the role of dogs on the prevalence is considered. In our study, we found that 12 dogs (12%) were *L. monocytogenes* positive and 19 dogs (19%) were *T. gondii* positive with Real Time PCR. It is an obvious fact that the rates are very significant for public health when the sensitivity of such molecular epidemiological studies is regarded.

In this study of *L. monocytogenes* and *T. gondii* the relations between the dogs' age, gender and positive values are shown in Tables 1 and 2. The isolation of *T. gondii* is higher than the isolation of *L. monocytogenes* and the prevalence of the protozoan is higher especially among the younger dogs. The relations of between the factors like age and gender in the positivity of these infections are evaluated statistically. According to the results, we did not find any statistically significant relation between different age and gender groups of our positive samples (P=0.41).

Table 1: *T. gondii* and *L. monocytogenes*, the relations between the dogs' age and positive values using TagMan method with Real Time PCR

Age Groups (year)	Number of samples	Number of *T. gondii* positive samples	The percentage of *T. gondii* positive samples (%)	p value	Number of *L. monocytogenes* positive samples	The percentage of *L. monocytogenes* positive samples (%)	p value
0-2	53	11	20		8	15	
3-5	31	6	19	0.48	2	6	0.49
≥6	16	1	6		2	12	
Total	100	19	19		12	12	

Table 2: *T. gondii* and *L. monocytogenes*, the relations between the dogs' gender and positive values using TagMan method with Real Time PCR

Gender	Number of samples	Number of *T. gondii* positive samples	The percentage of *T. gondii* positive samples (%)	p value	Number of *L. monocytogenes* positive samples	The percentage of *L. monocytogenes* positive samples (%)	p value
Female	48	7	14		7	14	
Male	52	12	23	0.41	5	9	0.65
Total	100	19	19		12	12	

The molecular methods used for the diagnosis of *L. monocytogenes* and *T. gondii* are limited in our country. For this reason, we preferred to use Real Time PCR technique to monitor the progress of a PCR reaction in real time. Its usage is advantageous in a wide range of applications. The development of fluorescent methods for a closed tube polymerase chain reaction has greatly simplified the process of nucleic acid qualification. We aimed to evaluate the efficacy of a commercially available rapid, sensitive and quantitative Real Time PCR for the molecular detection of *L. monocytogenes* and *T. gondii*. The analyses of TagMan probe based Real Time PCR for *L. monocytogenes and T. gondii* in our study are shown in Figure 1 and 2.

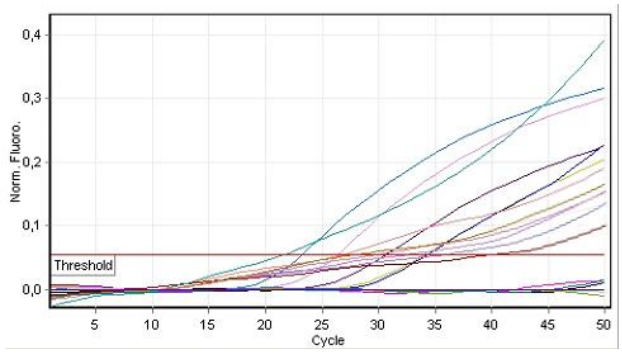

Fig. 1: *T. gondii* positive lines of our samples with Real Time PCR

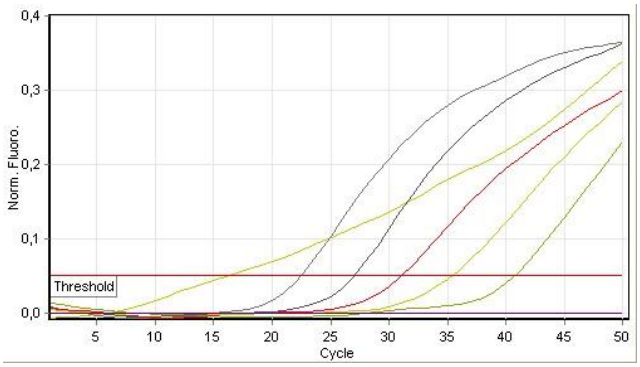

Fig. 2: *L. monocytogenes* positive lines of our samples with Real Time PCR

DISCUSSION

T. gondii, which is an obligate intracellular protozoan, is quite prevalent among dogs. It is essential to understand that it can be transmitted to humans mechanically because the active parasite is found in materials such as tear, rheum, saliva and urine. It may be difficult to diagnose this infection because the infection is subclinical during both intermediate host and definitive host. For this reason, it is required to use reliable and current methods for the diagnoses of this infection (Le Monnier *et al*., 2011; Dubey and Battie, 1998; Lin *et al*., 2000; Silva *et al*., 2002; Salb *et al*., 2008; Yazar *et al*., 2012).

In the studies in several countries, which used different serological methods, it is reported that the seroprevalence of *T. gondii* varies between 7.9% and 76.4% (Ali *et al*., 2003; Tsai *et al*., 2008). In Turkey, the seroprevalence of *T. gondii* is reported to be 15.7% in Kırıkkale and surroundings (Babür *et al*., 1997), 62.06% in Ankara and surroundings (Aslantaş *et al*., 2005), 57.9-97.5% in the eastern cities such as Van, Urfa and Kars (Babür *et al*., 2007; Babür *et al*., 2007; Gıcık *et al*., 2010). In our study, using Real Time PCR, the molecular positivity of Toxoplasmosis is calculated to be 19%. The positivity of this infection is thought to be low because our area is inside the borders of the big city municipality and the selected animals are shelter dogs, so the conditions are hygienic. It is really important to use developed molecular diagnostic methods for the diagnosis of zoonotic toxoplasmosis and describe the molecular epidemiology and potential risks of these infections.

Liu *et al*. found high rates of seroprevalence of *T. gondii* among cats and dogs in the city of Zhenjiang in Eastern China. Sera from 160 dogs and 116 cats were tested for *T. gondii* antibodies using ELISA. The seropositivity by sex and age was also analysed. Overall, 13.1% of the dogs and 20.7% of the cats had antibodies to *T. gondii*. The infection rate in stray dogs (38.7%) was significantly higher than the rate in household dogs (6.9%). The seroprevalence in male dogs was slightly higher than the seroprevalence in female dogs, but not significantly different (P>0.05) (Liu *et al*., 2014). As a result of our investigation, we have a general opinion that the molecular data we obtained may have a role as an

indicator in the environmental prevalence of *T. gondii* among the animals living freely in the nature such as dogs. Besides, the results of the studies in various countries have similar conclusions as our study. Listeriosis is quite prevalent in the nature and it is a zoonotic infection which is seen as a sporadic and enzootic (endemic) infection among domestic animals and sometimes humans in many countries around the world. *L. monocytogenes* can be isolated from faeces of humans and animals or from clinical specimens obtained from normally sterile sites such as cerebrospinal fluid, blood and amniotic fluid. For this reason, the contamination of soil, water and also food with faeces has a significant role for Listeriosis. The importance of this should be considered seriously because dogs might serve a reservoir of *L. monocytogenes* and be a source for Listeriosis (Kocabıyık *et al*., 2005; Broome *et al*., 1991).

Lorber *et al*. have declared that Listeriosis, which is caused by *L. monocytogenes*, is an infection seen in warm and cold climates. In several studies done in Turkey, it is remarkable to see that the rate is higher in cold climates. In our study the rate is lower than the other areas, probably because Istanbul has a warmer climate. Beside this, in other investigations it is reported that the prevalence of Listeriosis is higher among young animals. In our study, we have also found that the rate of the infection is higher among younger dogs and female dogs regarding to gender. In zoonotic studies done several counties, it is reported that the prevalence of Listeriosis is generally higher among younger dogs and females (Lorber, 1997; Low and Donachie, 1997).

There are not many investigations about the relation between dogs and Listeriosis. The studies which have been done so far indicate the importance of dogs for the transmission of the infection to humans and other animals. In the seroprevalence studies, it is stated that the rate in the world is 1.3-20%, but in the investigations made in Turkey the results can be much higher. When the data is investigated, the rate of seropositivity among dogs is 22.3% in the province of Kars, 28-57% in Nevşehir and 26.3% in Erzurum province (Weber *et al*., 1985; Kırbaş *et al*., 2011; Aktaş *et al*., 2010; Gıcık *et al*., 2010).

In Japan, Iida *et al.* investigated the faecal specimens of several kinds of healthy animals, for the isolation of *L. monocytogenes* they used the cold enrichment culture method. The prevalence of *L. monocytogenes* was found to be 1.9% in cattle, 0.6% in pigs, 0.9% in dogs and 6.5% in rats. However, none of *L. monocytogenes* was isolated from chicken or cats (Iida *et al*., 1991). In Nigeria, Oni *et al.* found that out of the serum samples of 100 dogs the prevalence of *L. monocytogenes* is 20%. The investigators used standard tube agglutination test to determine the antibody prevalence to *L. monocytogenes* (Oni *et al*., 1989).

In Turkey, while different results were obtained, the high seropositivity was said to be related to some factors, such as climate, geography, areas of livestock raising, profession and hygienic conditions. Although the positivity with molecular methods is lower according to the other areas of our country, we have the opinion that this might be because Istanbul has a better sanitation, also the samples were collected from animal shelters and there are no exact consensus of the detection of these pathogens from blood via molecular methods like PCR.

As a result, in our investigation we aimed to calculate the prevalence of Listeriosis and Toxoplasmosis, both of which threatens human and animal health, among the shelter dogs in the province of Istanbul. We estimate that our study will contribute the data about the prevalence of these zoonoses not only in our country but also all around the world. Besides, we aim to reach the correct information by using molecular diagnostic methods and to increase the routine usage of these tests in our country. Furthermore, we also think that with the development of Real Time PCR, which is used as a molecular method for the diagnosis of *L. monocytogenes* and *T. gondii*, the usage of it will be beneficial in clinical laboratories. Although we found that the seropositivity of Toxoplasmosis and Listeriosis among shelter dogs in Istanbul is 19% and 12% respectively and lower than the other areas of our country, we have a general opinion that it might be a serious public health risk and necessary precautions must be taken.

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