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ORIGINAL ARTICLE

Seroprevalence and *B1* gene Phylogeny of *Toxoplasma gondii* of Dogs and Cats in Republic of Korea

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Abstract: The outbreak of human toxoplasmosis can be attributed to ingestion of food contaminated with *Toxoplasma gondii*. Toxoplasmosis recently increased in domestic and stray dogs and cats. It prompted studies on the zoonotic infectious diseases transmitted via these animals. Sero- and antigen prevalences of *T. gondii* in dogs and cats were surveyed using ELISA and PCR, and *B1* gene phylogeny was analyzed in this study. Toxoplasmosis antibodies were measured on sera of 403 stray cats, 947 stray dogs, 909 domestic cats, and 2,412 domestic dogs collected at nationwide regions, Korea from 2017 to 2019. In addition, whole blood, feces, and tissue samples were also collected from stray cats (1,392), stray dogs (686), domestic cats (3,040), and domestic dogs (1,974), and *T. gondii*-specific *B1* gene PCR was performed. Antibody prevalence of stray cats, stray dogs, domestic cats, and domestic dogs were 14.1%, 5.6%, 2.3%, and 0.04%, respectively. Antigen prevalence of these animals was 0.5%, 0.2%, 0.1%, and 0.4%, respectively. Stray cats revealed the highest infection rate of toxoplasmosis, followed by stray dogs, domestic cats, and domestic dogs. *B1* gene positives were 5 of stray cats, and identified to high/moderate pathogenic Type I/III group. These findings enforce that preventive hygienic measure should be strengthened at One Health level in dogs and cats, domestic and stray, to minimize human toxoplasmosis infections.

Key words: *Toxoplasma gondii*, seroprevalence, antigen, phylogeny, PCR, Korea

INTRODUCTION

As of 2018, the number of domestic dogs newly registered in Korea is 146,617, which is an increase of 39.8% compared to the previous year [1]. The total number of dogs registered by 2018 is 13,340,477 in Korea [1]. In addition, there were 12,077 rescued or protected abandoned animals in 2018. Among them, dogs accounted for 75.8% and cats for 23.2%. In recent years, the number of companion and stray dogs and cats is on the rise, so it is time to be more alert to the spread of the zoonotic infectious diseases in Korea.

Human toxoplasmosis is an infection caused by the parasite *Toxoplasma gondii* and can occur through ingestion of contaminated meat or exposure to feces from infected animals. Although toxoplasmosis is mostly asymptomatic in humans, it is fatal in immunocompromised people and pregnant women, who may experience birth defects or miscarriage due to infection [2,3]. Toxoplasmosis can occur in all warm-blooded animals, and the feces of the infected cats are a prominent source of transmission [4,5]. Moreover, dogs have recently been identified as positive carrier of *T. gondii* via antigen and antibody tests, and mechanical transmission through dogs has therefore emerged as a growing concern [6-9].

The seroprevalence of *T. gondii* in cats varies greatly among different countries [4,8,10-18]. Even in the same country, the rate of positivity varies considerably depending on where the sample was taken, which test method was used, etc [10,19-21]. In Korea, many researchers have investigated the status of feline toxoplasmosis infection [10,19-24]. However, 1 study regarding the status of infection with toxoplasmosis in dogs has been conducted during 2006-2007 [22]. In this study, we collected the samples from domestic and stray dogs and cats in Korea during 2017-2019, and investigated the infection status of toxoplasmosis of dogs and cats by P30 antibody ELISA and

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B1 gene antigen PCR.

MATERIALS AND METHODS

Sample collection

A total of 7,092 blood, fecal and tissue samples and 4,671 serum samples were collected for antigen detection and serological tests, respectively, from 2017 to 2019 in 9 regions of Korea (Seoul-Gyeonggi-Incheon, Gangwon, Chungbuk, Daejeon-Sejong-Chungnam, Jeonbuk, Gwangju-Jeonnam, Deagu-Gyeongbuk, Busan-Ulsan-Gyeongnam, and Jeju). The protocol for animal experiments was approved by the Institutional Animal Care and Use Committee (IACUC) of the Animal and Plant Quarantine Agency (APQA) (Approval Number 2018-400 and 2019-456). Canine blood samples were collected from domestic dogs (n=1,974), as well as stray dogs (n=686) from the abandoned animal shelter in 9 regions of Korea. Whole blood samples from dogs were collected using syringes with 26-gauge needles and transferred to BD Vacutainer® Heparin blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). Blood and fecal samples were collected from domestic and stray cats in the veterinary clinics. Whole blood samples from cats were transferred to BD Microtainer® Tubes with K_2EDTA (Becton, Dickinson and Company). Feces from cats were obtained manually at animal clinics and shelters of stray cats. From domestic cats, we collected 1,014 of blood samples, and from stray cats, 406 of blood samples and 514 of fecal samples. Cat tissue samples (n=472) were obtained from cats, which were sent to the Department of Disease Diagnosis - Animal and Plant Quarantine Agency for diagnosis. Each cat tissue sample was homogenized, and centrifuged at 13,000 rpm for 1 min, and the supernatants were used for antigen test by PCR. Sera were obtained from whole blood by centrifugation at 3,000×g, 5 min. In addition, sera from domestic (n=438) and stray (n=261) dogs were also separated from blood clot samples by centrifuging at 3,000×g, 5 min. However, the sera could not be obtained from 105 and 3 of whole blood samples from domestic and stray cats, respectively due to small amount and separation problem. Therefore, total number of sera from domestic dogs, domestic cats, stray dogs, and stray cats were 2,412, 909, 947, and 403, respectively. Whole bloods, sera, and feces were stored at 4˚C until testing, and tissues at –20˚C until testing.

ELISA

All sera were tested using a commercial ELISA kit (ID Screen® Toxoplasmosis Indirect Multi-species Kit; IDvet, Grabels, France) to detect antibodies against the P30 protein of *T. gondii*. All sera samples were injected into the corresponding wells. And then the other procedures, such as washing the plates, and adding a multi-species peroxidase conjugate, substrate (3,3′,5,5′-tetramethylbenzidine), and stop solution, were followed by the manufacturer's instruction. For each sample, optical density (OD) values were measured at 450 nm. The positive percentage of the sample (S/P%) was calculated according to the manufacturer's instruction and used to determine the seroprevalence. It is positive if the value of S/P% is more than 70 for dogs and 50 for cats, and negative in both cases if it is less than 40.

PCR and real-time PCR

DNA was extracted from blood and homogenized tissue samples according to the manufacturer's instructions of Maxwell® RSC Whole Blood and Viral TNA Kit (Promega, Madison, Wisconsin, USA), respectively, and Maxwell® RSC Instrument (Promega). The cartridges to be used were placed in the deck tray(s). Each cartridge in the deck tray(s) were placed with well #1 (the largest well in the cartridge) facing away from the elution tubes. All sealing tape and any residual adhesive were removed before placing cartridges in the instrument. Blood samples or tissue lysates were transferred to well #1 of the cartridge. An empty elution tube was placed into the elution tube position for each cartridge in the deck tray. Sixty µl of elution buffer was added to the bottom of each elution tube. And then Maxwell® RSC Instrument was setup and performed. *T. gondii* oocysts were isolated from cat fecal samples using the sugar floatation method. After washing with 1 g of feces in distilled water (D.W.), samples were mixed with sugar solution and suspended for 1 hr. The sugar solution was prepared by dissolving 454 g of sugar in D.W. 355 ml while heating, and adding 6 ml of 37% formaldehyde solution. DNA was extracted from 500 µl of supernatant from the feces sample using QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Stockach, Germany). The extracted DNA was stored at -20˚C until use. DNA was analyzed for the amplification of *B1* gene in *T. gondii* by CFX Connect™ and CFX96™ real-time PCR system (Bio-Rad, Hercules, California, USA) [25,26]. A positive control DNA was extracted by *T. gondii* RH strain tachyzoites cultured in vitro. Further, 2 µl of extracted DNA was added with

Table 1. Primers and annealing conditions of PCR, nested PCR and real-time PCR for *Toxoplasma gondii B1* gene

PCR	Primer	Sequence (5'→3')	Annealing (℃/sec)	Amplicon size (bp)
PCR	T1	GGAACTGCATCCGTTCATGAG	50/30	501
	T2	CAGACGAATCACGGAACTG		
Phylogeny	Primary PCR	Tg1: TGTTCTGTCCTATCGCAACG	48/40	516
		Tg2: ACGGATGCAGTTCCTTTCTG		
	Nested reaction	Tg3: TCTTCCCAGACGTGGATTTC	56/60	
		Tg4: CTCGACAATACGCTGCTTGA		
rt-PCR	TOXO-P	FAM-TCTGTGCAACTTTGGTGTATTCGCAG-TAMRA	50/30	
	TOXO-F	TCCCCTCTGCTGGCGAAAAGT		
	TOXO-R	AGCGTTCGTGGTCAACTATCGATTG		

10 µl of iQTM Supermix (Bio-Rad), 1 µl of mixed primer and probe, and 7 µl of D.W., and PCR was performed at 95℃ for 10 min, 40 cycles of 95℃ for 15 sec and 60℃ for 1 min (Table 1).

Phylogenetic analysis

The *B1* gene amplification of positive samples in real-time PCR was performed by nested PCR assay for phylogenetic analysis (Table 1) [27]. The mixture containing AccuPower® PCR PreMix (Bioneer, Daejeon, Korea) was a total volume of 20 µl containing 2 µl of extracted DNA, 1 µl of each primer, and 16 µl D.W. for the first reaction, followed by 35 cycles of 94℃ for 30 sec, 48℃ for 40 sec and 72℃ 45 sec. The nested reaction was performed in 20 µl of the solution containing 1 µl of the first reaction product, 1 µl of each primer, and 17 µl D.W., followed by 35 cycles of 94℃ for 45 sec, 56℃ for 1 min and 72℃ for 90 sec. The eluted DNA samples were sent for sequencing (Macrogen Inc., Seoul, Korea). Each sequence of amplified gene fragment was analysed by NCBI BLAST software. The aligned dataset of *B1* gene of *T. gondii* was analysed by Molecular Evolution Genetics Analysis (MEGA) version 7.0 software. Phylogenetic tree of aligned sequences was constructed using the neighbor-joining method [28].

Statistical analysis

Seroprevalences and antigen prevalences were calculated from the proportion of positive results of those tested by ELISA and PCR, respectively, and were presented with 95% confidence intervals (CIs). We used Microsoft Excel 2019 software and SPSS (v25.0) to perform descriptive statistical analysis data: the difference in positive rates according to domestic and stray groups in dogs and cats by Student's t-test and Chi-square test. Differences were considered significant at $p < 0.05$.

RESULTS

In Korea, the seroprevalence of *T. gondii* in dogs and cats was 1.6% (95% CI = 1.59-1.61) and 5.9% (95% CI = 5.85-5.94), respectively (Table 2). seroprevalence of stray cats was the highest (14.1%, 95% CI = 13.9-14.3) ($\chi^2 = 14.44$, $df = 1$, $P = 0.0001$), followed by stray dogs (5.6%, 95% CI = 5.55-5.65), domestic cats (2.3%, 95% CI = 2.27-2.33), and domestic dogs (0.04%, 95% CI = 0.038-0.042) (Table 2). Jeju (55.6%) revealed the highest seroprevalence in Korea. Seroprevalence in stray cats in both Daejeon-Chungnam (17.3%) and Seoul-Gyeonggi-Incheon (17.2%) was greater than 10%. In contrast, domestic cats were found only in Seoul-Gyeonggi-Incheon (2.3%) and Gangwon (20.0%). Seroprevalence of *T. gondii* in stray dogs was the highest in Jeonbuk region (9.1%).Seroprevalence greater than 5.0% were Seoul-Gyeonggi-Incheon (8.8%), Jeju (8.1%), Daejeon-Chungnam (7.2%), and Gwangju-Jeonnam (6.7%). The antibody positive reactor of domestic dogs was the only one of 2,412 samples tested in Jeju (Table 2).

In Korea, antigen prevalence of dogs was 0.3% (95% CI = 0.296-0.304) and that of cat was 0.2% (95% CI = 0.198-0.202) (Table 3; Figs. 1, 2). Antigen prevalence of stray cats was the highest (0.5%, 95% CI = 0.49-0.51) ($\chi^2 = 5.95$, $df = 1$, $P = 0.015$), followed by domestic dogs (0.4%, 95% CI = 0.394-0.406), stray dogs (0.2%, 95% CI = 0.19-0.21), and domestic cats (0.1%, 95% CI = 0.098-0.102) (Table 2). In all regions except Daegu-Gyeongbuk, which showed 1.0% antigen-positive rate, dog antigen-positive rate was less than 1.0%. Among domestic dogs, antigen-positive areas were Seoul-Gyeonggi-

Table 2. Antibody prevalence of toxoplasmosis in dogs and cats in Korea during 2017-2019

Region	Positive/Test					
	Dog			Cat		
	Domestic	Stray	Subtotal	Domestic	Stray	Subtotal
Seoul-Gyeonggi-Incheon	0/2,112	15/170	15/2,282	20/876	10/58	30/934
Gangwon	0/91	2/136	2/227	1/5	-	1/5
Daejeon-Chungnam	0/55	6/83	6/138	0/2	23/133	23/135
Chungbuk	-	0/19	0/19	0/1	-	0/1
Jeonbuk	0/16	4/44	4/60	0/1	-	0/1
Daegu-Gyeoungbuk	0/37	1/76	1/113	0/10	4/166	4/176
Gwangju-Jeonnam	0/17	8/119	8/136	-	0/10	0/10
Busan-Ulsan-Gyeongnam	0/51	6/163	6/214	0/12	-	0/12
Jeju	1/33	11/136	12/169	0/2	20/36	20/38
Unknown	-	0/1	0/1	-	-	-
Total	1/2,412	53/947	54/3,359	21/909	57/403	78/1,312
P-value*		< 0.0001			< 0.0001	

*Student's *t*-test. *p*-value compared between domestic and stray groups of dogs and cats.

Table 3. Antigen prevalence of *Toxoplasma gondii* in dogs and cats in Korea

Region	Positive/Test					
	Dog			Cat		
	Domestic	Stray	Subtotal	Domestic	Stray	Subtotal
Seoul-Gyeonggi-Incheon	1/1,250	0/81	1/1,331	3/2,934	2/261	5/3,195
Gangwon	4/331	0/105	4/436	0/9	0/23	0/32
Daejeon-Chungnam	0/86	0/74	0/160	0/17	2/298	2/315
Chungbuk	0/84	0/24	0/108	0/12	-	0/12
Jeonbuk	0/8	0/45	0/53	0/11	0/9	0/20
Daegu-Gyeoungbuk	0/25	1/74	1/99	0/32	0/266	0/298
Gwangju-Jeonnam	0/8	0/81	0/89	0/4	0/48	0/77
Busan-Ulsan-Gyeongnam	1/117	0/108	1/225	0/19	0/51	0/70
Jeju	1/65	0/94	1/159	0/2	0/118	0/120
Unknown	-	-	0/1	-	3/318	3/318
Total	7/1,974	1/686	8/2,660	3/3,040	7/1,392	10/4,432
P-value*		0.291			0.041	

*Student's *t*-test. *p*-value compared between domestic and stray groups of dogs and cats.

Incheon (0.1%), Gangwon (1.2%), Busan-Ulsan-Gyeongnam (0.9%), and Jeju (1.5%). Antigenic positivity of stray dogs was the only 1 in the Daegu-Gyeongbuk region (1.4%). Interestingly, domestic dogs' antigen-positive rate was 0.4% (95% CI = 0.394-0.406;), which was higher than that (0.2%, 95% CI = 0.187-0.213; 1/686) of stray dogs. Domestic cats showed 0.1% (95% CI = 0.098-0.102) antigen-positive rate, which were from Seoul-Gyeonggi-Incheon region, and stray cats showed 0.5% (95% CI = 0.49-0.51) antigen-positive rate. Antigen-positive stray cats were found in Seoul-Gyeonggi-Incheon (0.8%), and Chungcheongnam-do (0.7%, 2/298), and 3 out of 318 stray cats were unknown (0.9%).

All 5 *B1* gene positive samples were obtained from stray cats, which were 18D174 (Stray cat spleen), 4_5SJ11 (Stray cat feces), 19Q130 (Stray cat gut), 18Q89 (Stray cat heart), and 18D174 (Stray cat brain) (Fig. 3). Phylogenetic analysis showed that these samples belong to the same group as the highly pathogenic RH strain (Type I) and the moderately pathogenic VEG strain (Type III) (Fig. 3). Five *B1* gene positive samples belonging to the Type I/III group were not differentiated further (Fig. 3). Genetic difference of *B1* gene between Type I and Type III was only 0.2%. Five *B1* gene positive samples were collected in 2018 and 2019. Samples obtained in 2018 were 18Q89 and 18D174, which were collected in June and July, respectively. Samples taken in 2019 were 4_5SJ11 and 19Q130, which were collected in April and July, respectively.

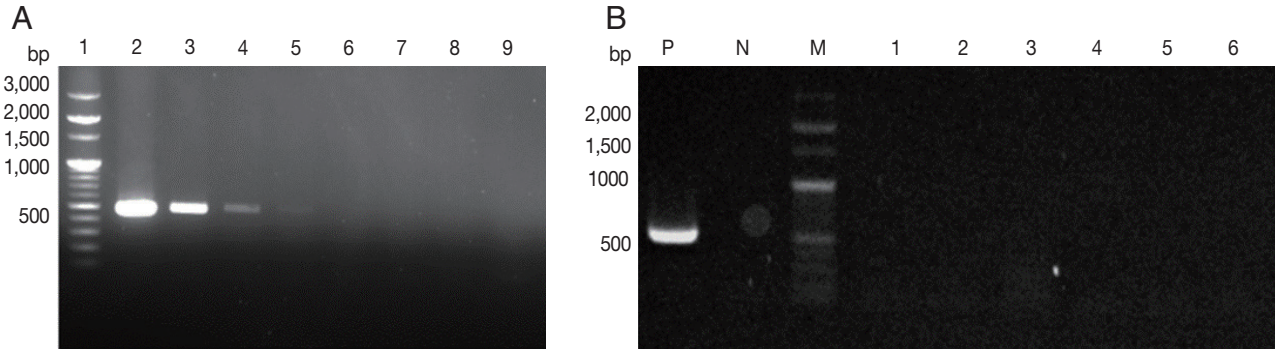
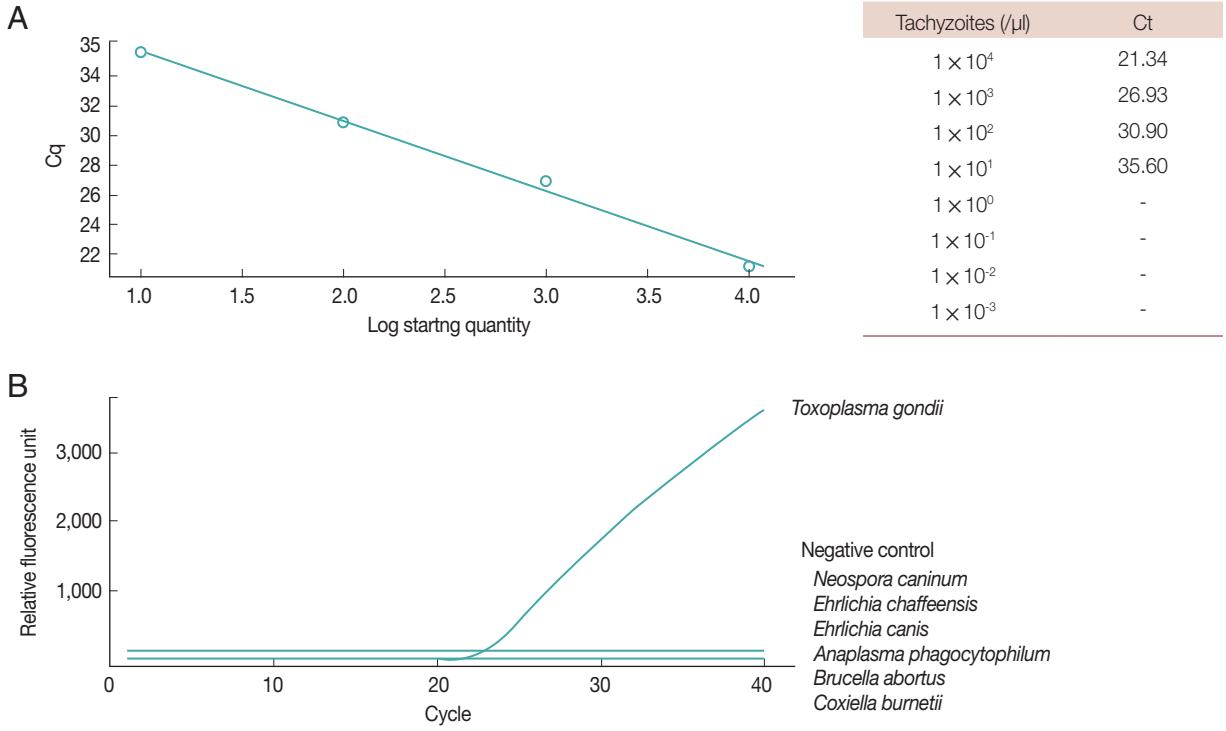


Fig. 1. PCR on *Toxoplasma gondii B1* gene. (A) Sensitivity of *B1* gene PCR. Lane 1, DNA size marker; Lanes 2-9, dilution of *T. gondii* tachyzoites from 10^4 to 10^{-3}. (B) Specificity of *B1* gene PCR. P, positive (*T. gondii*); N, negative; M, DNA size marker. Lane 1, *Neospora caninum*; 2, *Ehrlichia chaffeensis*; 3, *Ehrlichia canis*; 4, *Anaplasma phagocytophilum*; 5, *Brucella abortus*; 6, *Coxiella burnetii*.



Tachyzoites (/μl)	Ct
1×10^4	21.34
1×10^3	26.93
1×10^2	30.90
1×10^1	35.60
1×10^0	-
1×10^{-1}	-
1×10^{-2}	-
1×10^{-3}	-

Fig. 2. Real-time PCR of T*oxoplasma gondii B1* gene. (A) Sensitivity. (B) Specificity.

No changes in genetic lineage between 2018 and 2019 were observed between the positive samples. The positive samples had the highest genetic similarity to the *B1* gene present in the causative agent of Korean rabbit origin (KF038120), aborted sheep fetus from Iran (DQ789361), brain (*Neofelis nebulosa* and *Rattus rattus*) in Thailand (KF425006) (Fig. 3).

DISCUSSION

In this study, we investigated the prevalence of antibodies and antigens in Korean dogs and cats, and the genetic relationship between Korean *Toxoplasma gondii* was compared. In Korea, the seroprevalence of toxoplasmosis in dogs and cats was 1.6 and 6.0%, respectively. Additionally, the antigen prevalence in dogs and cats was 0.3% and 0.2%, respectively. Notably, the seroprevalence of stray cats was 14.1%, the highest among dogs and cats in Korea. Additionally, 5 *T. gondii B1* gene positive samples were found to be in Type I/III group, in which there were both the highly pathogenic RH strain, Type I and the moderately pathogenic VEG strain, Type III (Fig. 3).

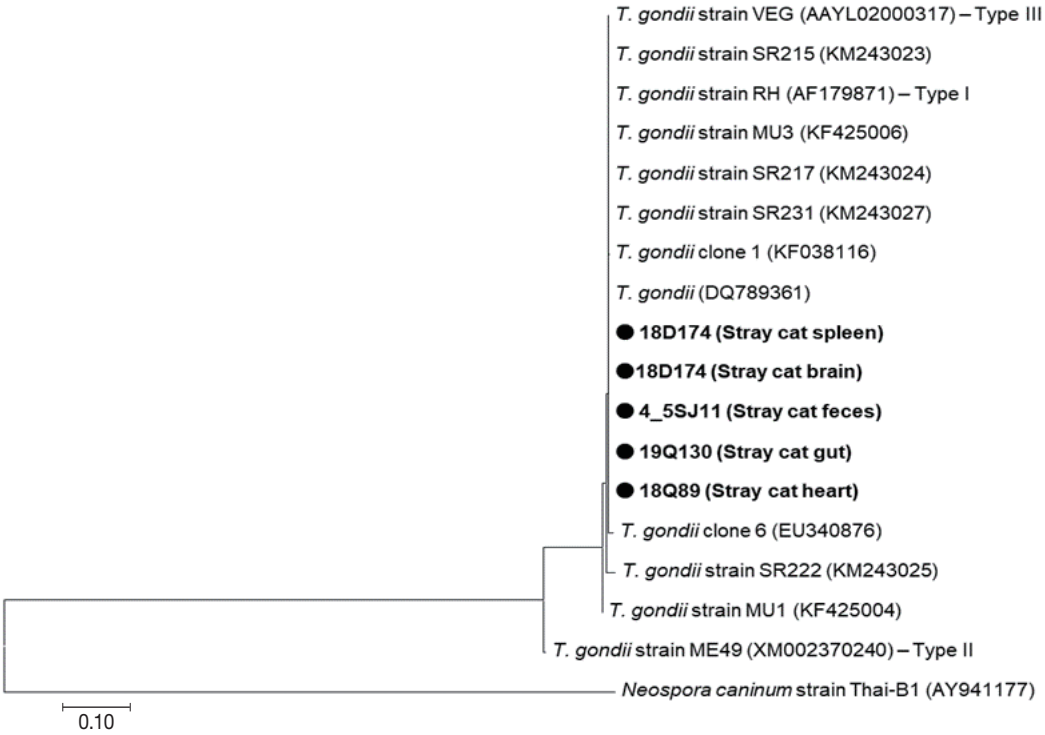


Fig. 3. Phylogenetic tree of *B1* gene of 5 *Toxoplasma gondii* samples from stray cats in Korea. Phylograms were generated by neighbor-joining analysis with 1,000 bootstrapped replicates. GenBank accession number of *T. gondii* is labeled on each line. Sequences of this study are closed circles. Scale bar indicates nucleotide substitution per site.

The seroprevalence in Korean stray cats showed no significant differences by year, and ranged from 8.1% (2008, LAT) to 16.0% (1999, LAT) [10,19-21]. This finding might be attributed to regional differences, as well as differences in the test method used. Although the toxoplasma antibody was not detected in Korean domestic cats in the 2010 survey [21], the seroprevalence of toxoplasmosis in domestic cats in our study was 2.3% (Table 2). Notably, our study is the first to report the presence of toxoplasma antibodies in domestic cats in Korea, emphasizing that additional hygienic practices must be developed to minimize public health concerns in Korea. Cat antibody levels in Thailand were relatively low, ranging from 4.8% to 11.0% [11,29]. Japanese seroprevalence of stray cats was 5.6% to 15.5%, similar to Korean prevalence [8]. Feline antibody levels in Spain, USA, China, Poland, Belgium, and Portugal were 27.2%, 48.0%, 63.2%, 68.1%, 70.2%, and 72.4%, respectively [12,14-18,30], highlighting that toxoplasmosis cat infections are widespread in these countries. The cause of high seroprevalence in these countries seems to be closely related to the ecological habits of stray cats, such as poor hygiene, high temperature, and high humidity of the environment. The seroprevalence of Korean stray and domestic dogs reported in this study was significantly different from the previous survey report, in which the seroprevalence of stray and domestic dogs was reported as 18.5% and 5.1%, respectively [22]. This may be due to the use of a more specific diagnostic method in this study than the LAT test, the sampling areas and ages, and the collecting periods [28]. The seroprevalence of stray dogs in Japan was lower than that of Korea [8], and consequently the environmental contamination of *T. gondii* is likely lower in Japan than in Korea. The seroprevalence of *T. gondii* in dogs in China ranged from 21% to 51.9% and 69.8% in Brazil [9,14,31,32]. Therefore, the environmental toxoplasma contamination of these countries as well as the rate of cat toxoplasmosis infection, is expected to be very high.

Antigen prevalence among stray cats in the present study was 0.5%, which was significantly lower than the values from previous studies, which ranged from 4.7% to 30.6% [10,20,21,23]. The large range in the positive rates reported between studies can be attributed, at least in part, to differences in regions from which the samples were derived. The antigen prevalence in Scotland and China were reported at 19.2% and

52.6%, respectively [14,33]. Therefore, these countries of highly antigen prevalence might be contaminated with *Toxoplasma gondii* in the environment, and then the warm-blood mammals including humans could be infected more easily by environmental and food contaminated *T. gondii*. In China, which showed high antigen-positive rates in cats, the rate of dog antigens was also relatively high, ranging from 3.7% to 8.6% [9,14]. In this study, antigen prevalence of *T. gondii* of domestic and stray dogs and cats in South Korea was very low as much as that of Japan. Based on these results, the infection risk might be low, but the seroprevalence was still high in stray cats and dogs. Therefore, the hygienic handling of pet dogs and cats will be performed steadily to prevent this zoonotic agent.

The phylogenetic analysis of 5 *B1* gene-positive samples from stray cats showed the highest homology with *B1* genes in Indians, American mussels, Thai black rats, and Korean rabbit toxoplasma [34,35]. These strains were all in the same phylogenetic group as the highly pathogenic Type I and moderately pathogenic Type III strain (Type I/III group). Further new targets for phylogenetic analysis were needed to differentiate Type I and Type III clearly. According to a report from China [9], the results of genetic analysis of the dog-derived toxoplasma strains all showed high similarity to the pathogenic Type I strain. On the other hand, in Korea, the result of genetic analysis of two strains reported by Jung et al. [23] was corresponded to the group of low virulent Type II. In this study, all 5 *T. gondii* were grouped as Type I/III strain (Fig. 3). This difference is presumably due to the different regions taken. Based on our findings, we demonstrated that stray cat isolates in Korea are mixed with high pathogenic Type I, moderate pathogenic Type III, or low pathogenic Type II depending on the regions. Genotypes identified in various regions of China – Guangdong, Hunan, Hubei, Anhui, and Guizhou province – have been identified as low virulent Chinese 1 genotypes that differ from Types I, II, and III [14]. Notably, genetic analysis of a larger sample size of isolates in the future studies would likely reveal novel Korean toxoplasma genotypes.

In Korea, the reported seroprevalence of toxoplasmosis in humans varies among different studies, ranging from 0.9% to 17.0% [36-42]. This trend suggests that the seroprevalence of human toxoplasmosis is increasing in Korea, as is the case in the USA [30], where there is a high amount of livestock consumption. In particular, the prevalence of infection in immunodeficiency diseases such as HIV/AIDS patients [43], pregnant women [44], and the elderly [45] increases the necessity of fundamental prevention in animals against toxoplasma, including cats and dogs. As highlighted by the results of our study, measures to prevent infection of stray cats should be strengthened in order to minimize zoonotic toxoplasmosis in Korea. On the other hand, dogs are also highly likely to be infected or mechanically spread from toxoplasmosis from feces contaminated with the environment [6-9]. Therefore, proper management of stray and domestic animals should be prioritized as a factor towards improving overall public health.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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