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Original Article

Molecular survey of *Toxoplasma gondii* B1 gene in pigs from various localities in Korea

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Abstract

Toxoplasma gondii, a common protozoan parasite, poses significant public health risks due to its potential to cause toxoplasmosis in humans and can be contracted from pigs, which are considered its critical intermediate host. The aim of this study is to evaluate the prevalence of *T. gondii* in slaughtered pigs for human consumption, emphasizing the zoonotic implications and the need for improved biosecurity and monitoring practices in pig farming. A total of 1,526 pig samples (1,051 whole blood samples and 384 lung tissue samples from the local slaughterhouse and 91 aborted fetus samples from local farms) were collected throughout the whole country of Korea in 2020. Among them, 6 (0.4%) were found to be infected with *T. gondii* by nested PCR. When compared by sample type, the prevalence of *T. gondii* was significantly higher in the aborted fetus samples (2.2%, 2/91) than in the blood (0.3%, 3/1,051) and lung tissue samples (0.3%, 1/384). The B1 gene sequence of *T. gondii* was similar (97.9–99.8%) to that of the other *T. gondii* isolates. This study represents the first molecular genotyping survey of *T. gondii* in the lung tissue of fattening pigs and aborted fetuses in Korea. Our findings indicated the importance of adopting preventive measures including the implementation of rigorous farm hygiene protocols and the promotion of public awareness about the risks of consuming undercooked pork. By addressing the gaps in current control strategies and encouraging the One Health approach, this study contributes to the development of more effective strategies to mitigate the transmission of *T. gondii* from pigs to humans, ultimately safeguarding public health.

Keywords: *Toxoplasma gondii*, pig, molecular epidemiology, genotyping, Korea

Introduction

Toxoplasmosis, a zoonosis with a global distribution, is estimated to infect approximately one-third of the world's human population [1]. *Toxoplasma gondii* has consequently emerged as the most significant protozoan foodborne pathogen, which is mostly contracted through meat consumption [2]. A primary mode of human transmission involves the ingestion of raw or undercooked meat from various animals, particularly pigs. Pigs can contract *T. gondii* by consuming food or water contaminated with sporulated oocysts or ingesting cysts in the tissues of infected animals (e.g., rodents, birds, and other pigs) or through congenital transmission [3].

Effective management and monitoring of *T. gondii* infection are important to implement in public health programs. In Korea, surveillance programs for *T. gondii* infection in pigs at slaughter are limited, and the suitability of *Toxoplasma*-infected meat for human consumption remains to be unmonitored. In contrast, the European Food Safety Authority has rec-

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Project administration: Seo MG
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Conflict of interest
The authors declare no conflict of interest related to this study.

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ognized *T. gondii* as a significant biological hazard. It advocates for the inclusion of *T. gondii* in the revised meat inspection regulations, which include not only pigs but also other animals, such as sheep, goats, farmed deer, and farmed wild boar [4].

Numerous seroprevalence studies on *T. gondii* in pigs (1.8–38.3%) were conducted in Korea between 2007 and 2018 [5-8]. Despite this, only a few studies have focused on the molecular surveillance of *T. gondii* within the country's pig population. Given the limited information on the molecular detection of *T. gondii* in pigs designated for human consumption, the present study aims to genetically identify the infectious strains of *T. gondii* as well as gather epidemiological data concerning *T. gondii* in both intensively raised fattening pigs and aborted fetuses throughout Korea's pig farming regions. Through molecular analysis, this study identified and characterized the genotypes of *T. gondii* that are prevalent on these farms. Furthermore, this study presents our efforts to molecularly delineate the strains of *T. gondii* circulating in Korea's domestic pig population.

Materials and Methods

Ethics statement

This study, which was conducted in 2020, was beyond the purview of the Institutional Animal Care and Use Committee (IACUC) at Kyungpook National University (KNU) because the IACUC at KNU only evaluates studies that involve laboratory animals maintained in indoor facilities and does not regulate research involving outdoor animals. Clinically healthy pigs were slaughtered for pig meat at the local slaughterhouse, and the blood and lung tissue samples were collected at that time. The aborted fetus samples were submitted to KNU for diagnostic investigation.

Sample size determination and collection

In 2020, 11,078,032 pigs from a total of 6,078 farms were reared in Korea [9]. The sample size was determined using the following formula with an expected disease prevalence of 10%, accepted absolute error of 5%, and confidence level of 95% with a simple random sampling design [10]:

$$n = \frac{1.96^2 p_{exp}(1 - p_{exp})}{d^2}$$

where n = required sample size, p_{exp} = expected prevalence, and d = desired absolute precision.

Based on the formula, a minimum of 138 samples was required; however, 1,526 pig samples (1,051 whole blood samples and 384 lung tissue samples from the local slaughterhouse from clinically healthy pigs and 91 aborted fetus samples from local farms) were selected throughout the entire country in 2020. The regions were classified as the northern, central, and southern regions and Jeju Island. In the northern region, the samples were collected from Gyeonggi and Gangwon Province. In the central region, collection was performed in Chungcheong Province. The samples in the southern region were collected from Gyeongsang and Jeolla Province. Finally, the remaining samples were collected from Jeju Island. Data on the regions, sample types, seasons, and farm sizes were recorded for subse-

quent analysis.

DNA extraction and PCR detection

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Nested PCR (nPCR) was performed using the AccuPower HotStart PCR Premix Kit (Bioneer, Daejeon, Korea). nPCR was performed to detect *T. gondii* by amplifying the B1 gene with external (Tg1 and Tg2) and internal (Tg3 and Tg4) primers that generated a 531-bp amplicon, as previously described [11]. A sample of *T. gondii* isolated from cats in Korea [12] was used as the positive control, whereas a sample lacking a DNA template was used as the negative control.

DNA cloning

The amplicons from the infected animals were purified using the QIAquick Gel Extraction Kit (Qiagen), ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA), transformed into *Escherichia coli* DH5α-competent cells (Thermo Fisher Scientific, Wilmington, DE, USA), and incubated at 37°C overnight. A plasmid DNA extraction was performed using a plasmid miniprep kit (Qiagen) in accordance with the manufacturer's instructions.

Sequencing and phylogenetic analyses

Recombinant clones were selected and sent to Macrogen, Daejeon, Korea, for nucleotide sequencing. The sequences obtained in this study were aligned and analyzed using the CLUSTAL Omega multiple sequence alignment program Omega (v. 1.2.1 (Bioweb, Ferndale, WA, USA)), and the alignment was corrected using BioEdit (v. 7.2.5 (BioEdit Company, Manchester, UK)). Moreover, a phylogenetic analysis was subsequently performed using MEGA v. 7.0 (Mega software solutions, Madhurawadha, India) based on the maximum likelihood method with the Kimura 2-parameter distance model. The aligned sequences were analyzed using a similarity matrix. The stability of the trees obtained was then estimated via bootstrap analysis with 1,000 replicates.

Statistical analysis

The GraphPad Prism software (v. 5.04; GraphPad, La Jolla, CA, USA) was used for statistical analyses. The differences among the groups were analyzed using a chi-square test. A *P*-value of <0.05 was considered statistically significant. A 95% confidence interval (CI) was calculated for all estimates.

Results

Prevalence

Of the 1,526 pigs, 6 (0.4%; 95% CI, 0.1–0.7) tested positive for the *T. gondii* B1 gene (Table 1). With regard to region, *T. gondii* was the most prevalent on Jeju Island (1.2%; 1/85; 95% CI, 0–3.5), followed by the southern region (0.6%; 3/484; 95% CI, 0–1.3) and central region (0.3%; 2/752; 95% CI, 0–0.6). However, none were detected in the northern region. When compared by sample type, the prevalence of *T. gondii* was significantly higher in the aborted fetus samples (2.2%; 2/91; 95% CI, 0–5.2; $P=0.0179$) than in the blood (0.3%; 3/1,051; 95%

Table 1. Prevalence of *Toxoplasma gondii* in Korean pigs in 2020

Group		No. tested	No. positive (%)	95% CI[a]	χ^2 (df[b]) (*P*-value[c])
Region[d]	Northern	205	0	0	3.086 (3) 0.3785
	Central	752	2 (0.3)	0–0.6	
	Southern	484	3 (0.6)	0–1.3	
	Jeju Island	85	1 (1.2)	0–3.5	
Sample type	Blood	1,051	3 (0.3)	0–0.6	8.051 (2) 0.0179
	Lung tissue	384	1 (0.3)	0–0.8	
	Aborted fetus	91	2 (2.2)	0–5.2	
Season	Spring	650	1 (0.2)	0–0.5	1.949 (3) 0.5831
	Summer	280	2 (0.7)	0–1.7	
	Autumn	246	1 (0.4)	0–1.2	
	Winter	350	1 (0.3)	0–0.8	
Farm size	< 500	252	3 (1.2)	0–2.5	5.271 (2) 0.0717
	500–2,000	562	2 (0.4)	0–0.8	
	> 2,000	712	1 (0.1)	0–0.4	
Total		1,526	6 (0.4)	0.1–0.7	

[a]CI: Confidence interval.
[b]df: degree of freedom.
[c]Differences were considered significant at $P < 0.05$.
[d]Northern region (Gyeonggi and Gangwon Province); Central region (Chungcheong Province); Southern region (Gyeongsang and Jeolla Province).

CI, 0–0.6) and lung tissue samples (0.3%; 1/384; 95% CI, 0–0.8). With regard to season, *T. gondii* was prevalent during the summer (0.7%; 2/280; 95% CI, 0–1.7), followed by the other seasons, such as autumn (0.4%; 1/246; 95% CI, 0–1.2), winter (0.3%; 1/350; 95% CI, 0–0.8), and spring (0.2%; 1/650; 95% CI, 0–0.5). In terms of farm size, *T. gondii* was prevalent on farms with fewer than 500 pigs (1.2%; 2/252; 95% CI, 0–2.5), followed by those with more than 500 but less than 2,000 pigs (0.4%; 2/562; 95% CI, 0–0.8) and with more than 2,000 pigs (0.1%; 1/712; 95% CI, 0–0.4).

Molecular and phylogenetic analyses

The phylogenetic analysis revealed that the B1 gene nucleotide sequences of *T. gondii* identified in this study clustered with previously identified *T. gondii* sequences (Fig. 1). The 6 sequences of the *T. gondii* B1 gene in this study shared a 99.4–100% identity. Moreover, they were 97.9–99.8% identical to the B1 gene sequences of previously reported *T. gondii* isolates. All sequences used in the phylogenetic analysis were submitted to the GenBank database (accession numbers: PP430333–PP430338).

Discussion

In this study, we provided epidemiological and molecular insights into *T. gondii* in slaughtered pigs and aborted fetuses in Korea. Our findings revealed a low prevalence of *T. gondii* infections on swine farms. While the prevalence in pigs decreased, the seroprevalence among Korean residents reportedly increased in recent years [13]; this is mainly attributed to the increased consumption of local or imported pork and other meats susceptible to *T. gondii* infection [2]. Moreover, asymptomatic animals that have *T. gondii* cysts in their

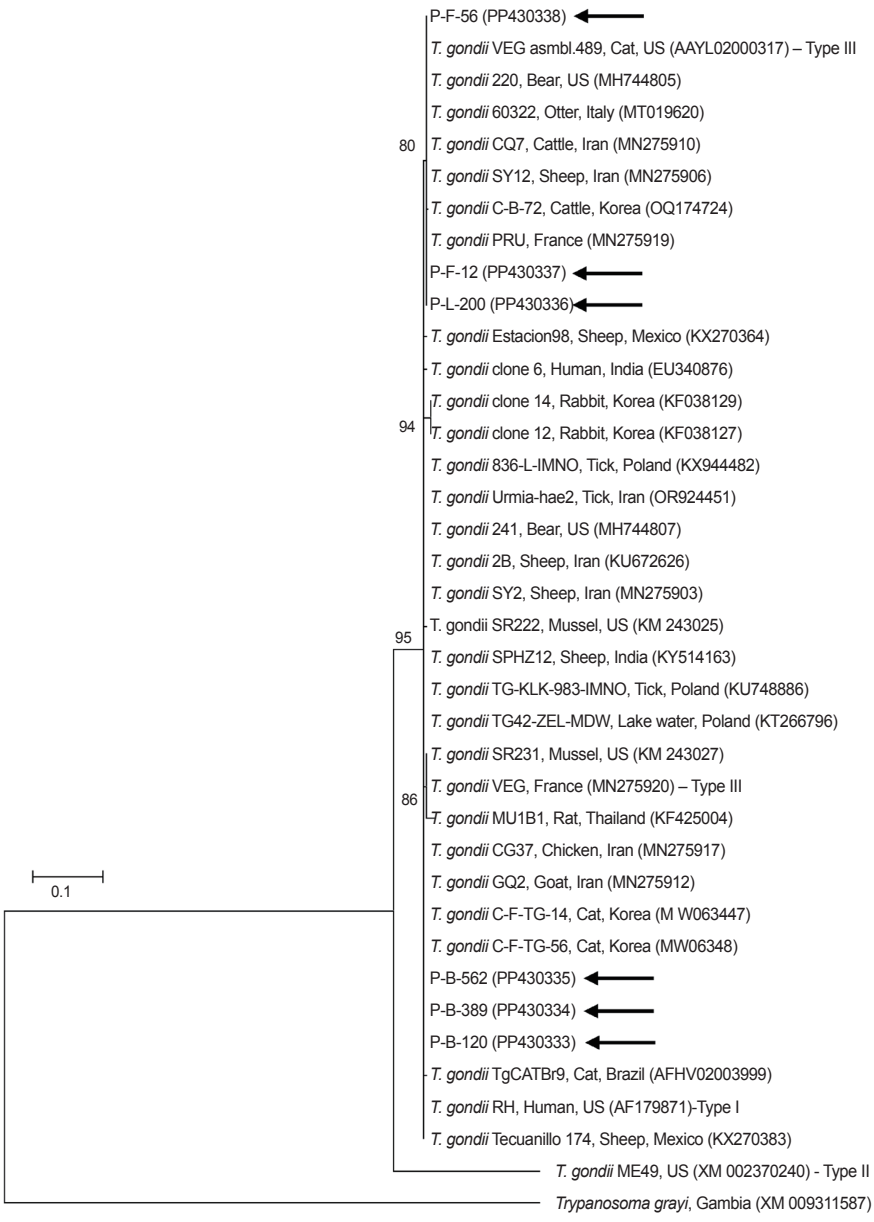

Fig. 1. The phylogenetic tree of *Toxoplasma gondii* on the basis of the B1 gene sequences. The tree was constructed using the maximum likelihood method. The black arrows represent the sequences analyzed in this study. *Trypanosoma grayi* was used as the outgroup. The GenBank accession numbers of other sequences are presented with their names. The numbers on the branches represent the mean bootstrap support based on 1,000 replicates. The scale and scale bar indicate the phylogenetic distance.

muscles may enter the fresh pork market and thus represent a significant foodborne toxoplasmosis risk and public health concern. In Poland, *T. gondii* was found in retail raw meat products including sausages (45.1% of positives), smoked meats (27.4%), ham (8.0%), and minced meat (19.5%) [14]. The results of these studies indicate that raw meat products could be a critical source of *T. gondii* infection for humans. Therefore, the government

should implement health education measures that encourage proper hygiene as well as proper cooking temperatures and methods to deactivate *T. gondii* cysts in meats to prevent human infections caused by contaminated meat [15].

In Korea, studies on *T. gondii* infection in pigs have included various investigations. On Jeju Island in 2002, dead sows (100%, 2/2) and aborted fetuses from the same litter (60%, 3/5) tested positive for *T. gondii* via histopathology and immunohistochemistry, and sows that had aborted or normal sows (41.2%, 7/17) were evaluated using the latex agglutination test [16]. In Gyeonggi Province in 2007, the latex agglutination test (22.8%, 118/516) and PCR test (57.6%, 68/118) were used [7]. In the eastern areas of Gyeongbuk Province in 2008, the prevalence of *T. gondii* infection was evaluated via ELISA (16.8%, 62/368) [5], and in Gyeongnam Province in 2009, ELISA (38.3%, 115/300) and PCR (0%) were used [6]. Finally, in 2018, local (9.1%, 53/583) and imported pork (1.8%, 7/386) from Korean retail meat markets were tested for *T. gondii* using ELISA [8]. These findings suggest that *T. gondii* is widely distributed in pigs from several farms in Korea, reflecting a cumulative probability of exposure to *T. gondii* and the lifelong persistence of antibodies.

With regard to sample type, *T. gondii* showed a significant prevalence in aborted fetuses. Moreover, *T. gondii* infections in pigs are often asymptomatic; however, several instances of clinical disease and mortality were recorded. *Toxoplasma gondii* infection has been linked to reproductive failure in sows, manifesting as abortion, fetal mummification, stillbirth, and neonatal death [17]. This presents significant clinical and economic implications for the agribusiness sector, making it a crucial area for research. Infections in aborted fetuses have also been reported in Korea [16] and Switzerland [17]. A previous study reported elevated abortion rates (up to 44%) and unusually high sow mortality rates (up to 19%) that were primarily attributed to toxoplasmosis in Korea [16]. Moreover, the process of *T. gondii* detection in the lung tissues in this study is consistent with that in studies focusing on piglets and fattening pigs in China [18] as well as aborted fetuses in Korea [16].

With regard to seasonal prevalence, *T. gondii* was more commonly isolated during the summer than during other seasons. This finding is consistent with those of other studies [5,19]. The warmer temperatures and increased humidity that are typical during the summer season create conditions that favor the survival and proliferation of *T. gondii* oocysts in environments including pig farms [19]. Furthermore, the heightened interactions between farm environments and potential intermediate hosts, such as rodents, during the summer months could facilitate the spread of *T. gondii*. These factors, even in predominantly indoor pig farming systems, can lead to higher infection rates during the summer, emphasizing the critical need for stringent biosecurity and hygiene practices throughout the year.

With regard to farm size, small farms have shown a higher prevalence of *T. gondii* than large farms. This finding is consistent with those of other studies [6,20]. Small, family-run farms have been found to have an increased risk of *T. gondii* infections, which is likely due to lower hygienic standards than those found in larger, intensive operations [21]. Therefore, it is important to implement preventive and control measures for swine toxoplasmosis in farms to prevent pigs from coming into contact with sources of *T. gondii* infection, such as cat feces and infected rodents [22]. By adopting appropriate hygiene and preventive strategies, the risk of infection can be further minimized.

In a prior investigation, *Toxoplasma* strains were categorized into 3 genotypes according

to their virulence levels in outbred mice: Type I (highly virulent), Type II (moderately virulent), and Type III (non-virulent) [23]. Type II is identified as the most common genotype in humans [24]. Further research has revealed various *T. gondii* types in pigs. For instance, all 3 genotypes have been distributed in Poland as follows: Type III (49%), Type II (17.3%), Type I (10.2%), and mixed genotypes (23.5%) [14]. Moreover, molecular identification enhances our understanding of the risks associated with meat consumption because the impact of *T. gondii* infection is influenced by host factors and the specific parasite lineages involved, leading to different clinical outcomes for each genotype [25]. In this study, 6 pigs were confirmed to be positive for *T. gondii*, while the analysis of the B1 gene, Type I/III was identified. However, additional phylogenetic studies are necessary to distinguish between Types I and III.

In the present study, we identified a relatively low *T. gondii* infection rate (0.4%) in pigs. This low rate may be due to diminished exposure to common sources of *T. gondii* infection, which includes enhancements in pig farming infrastructure and strong regulations. To our knowledge, this study represents the first molecular genotyping survey of *T. gondii* in the lung tissues of fattening pigs and aborted fetuses in Korea. Toxoplasmosis in pigs can cause substantial economic losses for pig farmers. Thus, it is crucial to educate pig farmers on proper hygiene practices to cultivate healthy and disease-free pigs, thereby mitigating economic losses and preventing foodborne zoonotic diseases. Furthermore, from a public health perspective, the establishment of regulatory measures and control strategies for foodborne toxoplasmosis in Korea is essential.

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