Philippine Journal of Science
152 (5): 1989-1998, October 2023
ISSN 0031 - 7683
Date Received: 29 Jun 2023

Molecular Detection and Prevalence of *Toxoplasma gondii* in Ready-to-eat Vegetables and Oysters in Central Luzon, Philippines

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Toxoplasmosis, a parasitic infection caused by *T. gondii*, may be considered as a neglected disease in the Philippines. Consumption of raw or undercooked cyst-containing meat and accidental ingestion of oocysts excreted in the environment are two of the main routes in acquiring the infection. In the Philippines, there is no comprehensive or updated information on the prevalence of *T. gondii* among human populations and as a food-borne pathogen. We detected *T. gondii* DNA in ready-to-eat (RTE) vegetables and oysters sold in markets in Central Luzon, Philippines using molecular method. Six (10%) out of the 60 collected vegetable samples tested positive through nested PCR amplification of *B1* gene. As for the oyster samples, four (9.09%) out of the 44 collected pooled samples were positive for *T. gondii.* Phylogenetic analyses revealed all the DNA sequences retrieved from positive samples clustered with *T. gondii* with a virulent Type 1 genotype (accession no: KX270388). Further studies should be done to identify the exact genotypic profiles of the *T. gondii* detected in the vegetable and oyster samples to infer pathogenicity and possible sources of contamination. Our findings suggest possible transmission patterns of *T. gondii* oocysts as a potential health threat to consumers.

Keywords: B1 gene, food consumption, nested PCR, oocyst, toxoplasmosis

INTRODUCTION

Toxoplasma gondii is a single-celled cyst forming protozoan and is usually described as one of the most successful parasites due to its ubiquitous distribution, wide range of host infection, and high prevalence rates around the world (Delgado *et al.* 2022). It is considered one of the medically significant apicomplexan parasites as the causative agent of toxoplasmosis (Pappas *et al.* 2009) that has infected an estimated 30% of the world's population, causing severe congenital infections, neurological and ocular diseases (Dubey *et al.* 2020; Maubon *et al.* 2008; Montoya and Liesenfeld 2004). However, most cases in healthy adults are mainly asymptomatic (Kawashima *et al.* 2000). Humans can acquire toxoplasmosis either by the consumption of raw or undercooked meat containing *T. gondii* tissue cysts or by the ingestion of oocysts present in soil, water, and contaminated food such as produce (Marín-García *et al.* 2022; Plaza *et al.* 2020; Ybañez *et*

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al. 2019; Jones and Dubey 2012).

Toxoplasmosis is considered to be the second most common cause of foodborne illness in the United States; however, toxoplasmosis is not typically treated as a major foodborne pathogen by people in general since it is traditionally associated with domestic and wild cats as hosts, rather than being associated with food (Hoffmann *et al.* 2012). The environmentally robust stage of *T. gondii* is the oocyst, which plays an important role in the epidemiology of this zoonotic parasite (Dumètre *et al.* 2013; Dubey 2004). Currently, it is unclear for the majority of populations with endemic infection of *T. gondii* whether oocyst ingestion or tissue cyst ingestion holds more significance in the transmission of the disease among humans (Shapiro *et al.* 2019). However, in Brazil, there have been several instances of oocyst-induced outbreaks, with water and produce being recognized as the main sources of exposure. From the 25 recorded outbreaks over the past 50 years, 72% of cases were found to be linked to oocysts through food, soil, or water as the primary sources, whereas 24% were linked to the ingestion of tissue cysts from undercooked or raw meat. Additionally, 4% were associated with the consumption of tachyzoites through unpasteurized milk (Ferreira Neto *et al.* 2018).

While there is a lack of local studies regarding the presence of *T. gondii* in environmental matrices, the presence of *T. gondii* oocysts in soil (Egorov *et al*. 2018; Jones *et al.* 2009) and aquatic environments (Galvani *et al.* 2019; Adamska 2018; Ajonina *et al*. 2018) of different countries have been observed. The definitive hosts, where the sexual stages exclusively occur, are limited to members of the Felidae family, which includes cats (Zulpo *et al.* 2018). Based on the available literature, studies on toxoplasmosis in the Philippines mostly aim at determining the prevalence of *T. gondii* in terrestrial animals such as pigs (Ybañez *et al.* 2019), rats (Salibay and Claveria 2005), cats (Ybañez *et al.* 2019; Chua *et al.* 2014; Advincula *et al.* 2010), dogs (Guy and Penuliar 2016), and marine mammals like cetaceans (Obusan *et al.* 2015, 2019; de Guzman *et al.* 2020), with limited studies on humans (Kawashima *et al.* 2000; Ybañez *et al.* 2019; Cross *et al.* 1977; Salibay *et al.* 2008). Also, there has not been any attention given to the potential presence of oocysts in the food chain, knowledge on which is valuable in understanding reservoirs, infection routes, and risks of *T. gondii* transmission in the country.

In the present study, *T. gondii* was detected in ready-to-eat (RTE) vegetables and oyster samples in Central Luzon, Philippines through nested polymerase chain reaction (PCR) targeting the *B1* gene. The findings of the study could help in assessing the likelihood of acquiring *T. gondii* infection by eating food at risk of contamination by oocysts.

MATERIALS AND METHODS

Sampling

Convenience sampling was performed in four provinces of Central Luzon, Philippines (Figure 1). The following vegetables were collected from local supermarkets in provinces of Bulacan, Pampanga, Tarlac, and Nueva Ecija: lettuce (*Lactuca sativa*) (n = 12), cabbage (*Brassica oleracea* L. var. *capitata*) (n = 12), carrot (*Daucus carota* subsp. *sativus*) (n = 12), cauliflower (*Brassica oleracea* var. *botrytis*) (n = 12), and mung bean (*Vigna radiata*) sprout (n = 12). On the other hand, oysters (*Magallana bilineata*) (n = 44) were collected from the markets in the provinces of Bulacan and Pampanga (Figure 2). Sampling of vegetables and oysters was done from July–November 2022. The samples were placed in sterile plastic bags, stored in a cooler, kept under 10 °C, and transported to the Microbial Ecology of Aquatic and Terrestrial Systems Laboratory in the Institute of Biology, College of Science, University of the Philippines Diliman. Oocysts recovery was done 6–8 h after collection.

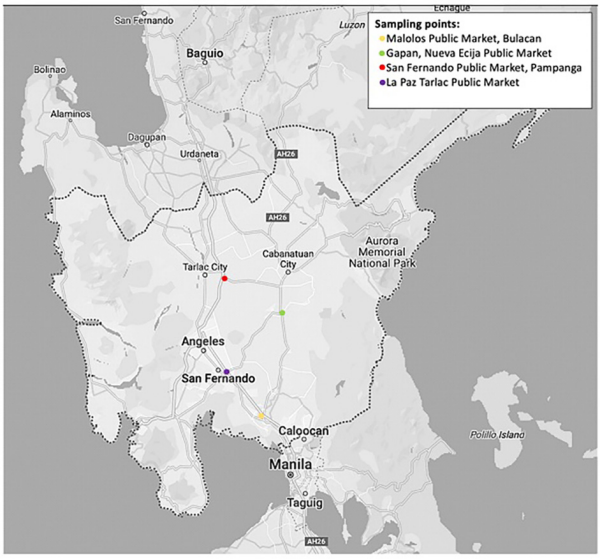

Figure 1. Map of Central Luzon, Philippines highlighting the four municipalities where vegetable samples were collected (source: Google Maps, www.google.com/maps).

T. gondii Oocysts Recovery

Vegetable samples. Following the protocol of Marchioro *et al.* (2016), 50 g of each vegetable sample was manually washed with 100 mL of 1% Tween 80 for 1 min. The resulting liquid was filtered through a cellulose ester membrane (47 mm diameter, 0.2 m pore size; Millipore®) with the aid of a negative pressure vacuum pump (10–15 mmHg). The filters were washed with phosphate buffer of pH 7.8 with the addition of 0.01% Tween 80, and the rinsates were centrifuged for 10 min at 1,050×g. The pellet

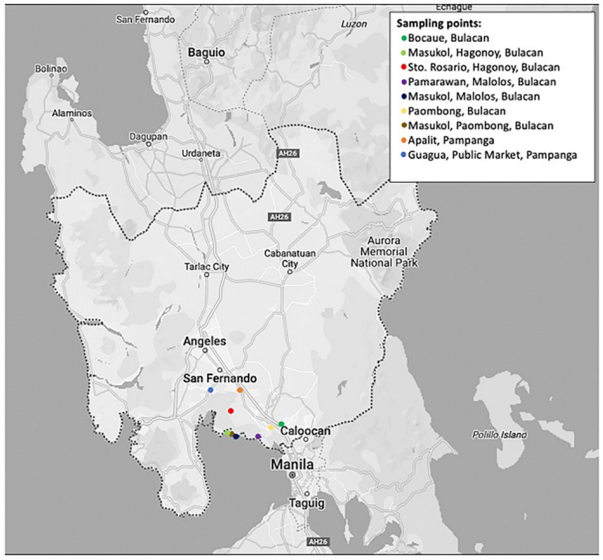

Figure 2. Oyster collection sites in the Province of Bulacan and Pampanga, Philippines. (source: Google Maps www.google.com/maps).

obtained was suspended in 200 L of distilled water (Sroka *et al.* 2006) and immediately kept at –20 °C for further use.

Oyster samples. A total of 440 live oysters were collected from Bulacan and Pampanga. Samples were grouped into 44 pools, with 10 individuals per pool. The oysters' hemolymph, gills, and digestive glands were homogenized with the addition of sucrose solution. Samples were then placed in a stomacher (8 strokes/s for 10 min) and thereafter sieved through a double layer of gauze and centrifuged at 1,050×g for 10 min. The pellets were neutralized by adding 1.2% sodium bicarbonate and were centrifuged again for another 10 min. The supernatant was discarded and the resulting pellets were subjected to genomic DNA extraction (Kim and Shapiro 2021; Cong *et al.* 2017).

Molecular Analyses

DNA extraction. Prior to each DNA extraction procedure, the material obtained from the samples was prepared using five freeze-thaw cycles (using liquid nitrogen for 5 min and a water bath at 80 °C for 5 min) to destroy the walls of the oocysts and improve the efficiency of DNA extraction (Cong *et al.* 2017). Afterward, DNA extraction was performed using DNA extraction kits (DNeasy® PowerWater® Kit, Qiagen for vegetable samples, and Wizard® Genomic DNA Purification Kit, Promega for oyster samples) following the manufacturer's protocol. The resulting DNA was stored at –20 °C (Lass *et al.* 2019). A nanodrop spectrophotometer was used to determine the quality of the collected DNA.

Detection of *T. gondii* through nested polymerase chain reaction (PCR). *T. gondii* glycerol-3-phosphate dehydrogenase or the *B1* gene was amplified through nested PCR (Yai *et al*. 2003). The primers Toxo 1 (5' AGC GTC TCT CTT CAA GCA GCG TA 3') and Toxo 2 (5' TCC GCA GCG ACT TCT ATC TCT GT 3') (IDT, Integrated DNA Technologies, Inc.) were used to amplify a 300-bp DNA fragment in *T. gondii* genome. Each reaction tube contained 5 µL Taq DNA Pol 2.0 Master Mix (Lot No.5200300, GoTaq Green Master Mix, Promega), 0.5 µL forward and 0.5 µL reverse primers, and 3.0 µL nucleotide-free water at a final volume of 10 µL. Thermal cycling was set to: initial denaturation for 5 min at 94 °C, followed by 30 cycles of denaturation for 20 s at 94 °C, annealing for 20 s at 55 °C, extension for 20 s at 72 °C, and final extension for 5 min at 72 °C. For the nested reaction, the primers Toxo 3 (5' TGG GAA TGA AAG AGA CGC TAA TGT G 3') and Toxo 4 (5' TTA AAG CGT TCG TGG TCA ACT ATC G 3') (IDT, Integrated DNA Technologies, Inc.) were used to amplify the 155-bp fragment. Briefly, each reaction tube contained 5 µL master mix, 0.5 µL forward and reverse primers, and 3.5 µL nuclease-free water for a total volume of 10 uL. Thermal cycling was set at the following conditions: initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation for 20 s at 94 °C, annealing for 20 s at 55 °C, extension for 20 s at 72 °C, and final extension for 5 min at 72 °C (Cong *et al.* 2017). Each run comprised a no template control and *T. gondii* CTG strain (Su, The University of Tennessee, Knoxville) as a positive control.

The expected amplicons (155-bp) were analyzed by agarose gel electrophoresis in 1.5% (w/v) agarose in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA at pH 8.0) with 15% (v/v) 5000X SYBR® Safe DNA Gel Stain (Life Technologies, CA, USA) using Bio-Print ST4 (Vilber Lourmat, Marne-La Vallée, France) and Vision-Capt v. 16.08 (Vilber-Lourmat). The molecular weights of the products were checked using VC 100bp Plus DNA Ladder (Vivantis, CA, USA).

Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics version 20 software. Crosstab-Pearson chi-square test was used to calculate the association between the presence of the target *T. gondii* gene in the samples and variables such as type of vegetable samples (leafy greens, cruciferous, root, and legume) and the source of oysters (Bulacan and Pampanga market).

Phylogenetic Analyses

Nucleotide sequences retrieved from Macrogen (Korea) were assembled and analyzed using MEGAX software (Stecher *et al.* 2020). The phylogenetic tree was

constructed using the maximum likelihood method and Jukes-Cantor (JC) model with 1000 bootstraps replication and *Neospora caninum* AY941177.1 as an outgroup. Distance-based analyses and trees were obtained by applying neighbor-join and BioNJ algorithms. A discrete gamma distribution was used to model evolutionary rate differences among sites (Jukes and Cantor 1969).

RESULTS

Detection and Prevalence of *T. gondii* in Vegetables and Oysters

A total of 60 vegetable samples from the four provinces of Central Luzon were analyzed for the detection of *T. gondii* DNA through nested PCR targeting the *B1* gene. The DNA of the parasite was detected in six (10%) vegetable samples: two mung bean sprout samples and one carrot, cabbage, cauliflower, and lettuce sample. Three of the positive samples came from Tarlac, two were from Bulacan, and one from Nueva Ecija. As for the oysters collected from Bulacan and Pampanga, four (9.09%) out of 44 samples were positive for *T. gondii*. The chi-square test shows that there is no significant relationship between the sampling sites and the presence of *T. gondii* in vegetable samples ($p = 0.392$) and oyster samples ($p = 0.426$). Phylogenetic analyses revealed that retrieved sequences from DNA extracted from vegetable and oyster samples clustered with *T. gondii* isolate with a Type 1 genotype obtained from infected sheep from a hyperendemic region in the Pacific coast of Mexico (Figure 3; Appendix I).

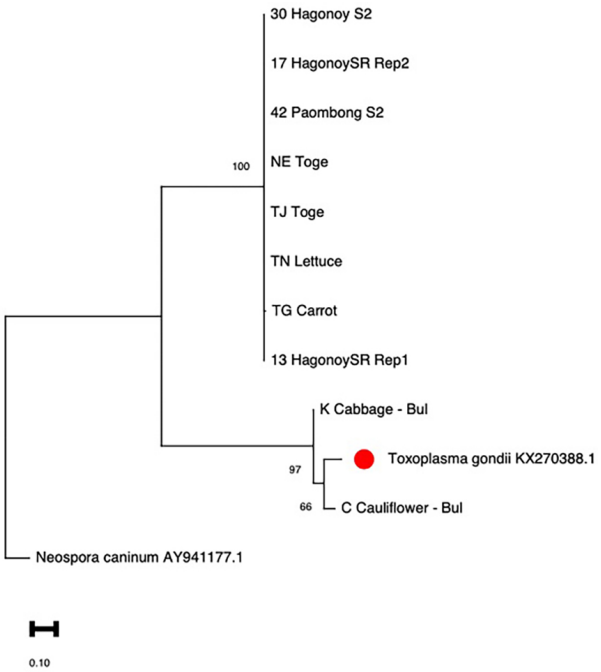

Figure 3. Maximum likelihood tree of the glycerol-3-phosphate dehydrogenase (*B1*) gene nucleotide sequences of positive samples that clustered with *T. gondii* isolate accession number KX270388 (red) with *Neospora caninum* AY941177.1 as outgroup. The values on each node represent bootstrap supports from applying neighbor-join and BioNJ algorithms, respectively. Bootstrap values greater than 95 were significant. The scale bar represents one nucleotide substitution for every 10 nucleotides.

DISCUSSION

The findings confirmed the presence of *T. gondii* in the food chain and suggest a potential risk to human health through consumption. This is of particular concern as toxoplasmosis is recognized to be the second leading cause of death resulting from foodborne illness in the United States (CDC n/d; Hoffman *et al.* 2012). Globally, the parasite has infected approximately 30% of the population, with higher rates of infection reported in South America (Pappas *et al.* 2009). Oocyst-borne infections are more prevalent in Brazil, the largest country in South America, due to the high levels of oocyst contamination in the environment, the presence of multiple *T. gondii* genotypes and a diverse feline population in peri-urban, rural, and forested locations, as well as a lack of control over stray domestic cats in urban and peri-urban regions (Shapiro *et al.* 2019). The understanding of this route of transmission, *i.e*. the ingestion of oocysts through the consumption of fresh produce and seafood, is not yet fully recognized due in part to the lack of standard detection methods for this parasitic stage (Dubey 1996; Elmore *et al*. 2010; Hohweyer *et al.* 2016; Shapiro *et al.* 2019). Hence, this study in the Philippines provides evidence that this mode of transmission among humans is possible. Based on phylogenetic analysis, the genotype of the detected *T. gondii* is inferred as Type I, which is highly virulent to mice as it can lead to the death of an infected one within a two-week period (Martínez-Flores *et al.* 2017; Dubey *et al.* 1981). Saraf *et al.* (2017) provided a comprehensive summary of the typical methodologies employed to assess *T. gondii* virulence in laboratory mice and it appeared that Type 1 are highly virulent strains, Type II are intermediate virulent strains, and Type III are non-virulent strains.

Furthermore, phylogenetic analyses illustrated that *T. gondii* sequences retrieved from vegetable and oyster samples clustered with *T. gondii* isolate with a Type 1 genotype (Figure 3), which was obtained from infected sheep (Martínez-Flores *et al.* 2017). It is important to identify the genotype of the parasite because it determines the degree of virulence to the host, although the disease

development is influenced by factors such as the host's type, genetic makeup, and – critically – its immune condition (Sanchez and Besteiro 2021). Shwab *et al.* (2016) found out that the genotypes prevalent in Europe and North America (archetypal Types II and III strains), North Africa (Types II, III, and 12), and Asia (Chinese Type I) are non-lethal to mice at low infection dose, whereas a large proportion of *T. gondii* strains identified in South America (Types I, II, and III) are highly virulent and lethal to mice. Thus, it may be suggested that the consumption of oysters and RTE vegetables contaminated with *T. gondii* with Type I genotype could potentially lead to symptomatic foodborne toxoplasmosis. The virulence displayed by these genotypes in mice may not directly correlate with their effects on humans. For example, strains with a Type II genotype are regarded as having intermediate virulence in mice, potentially resulting in minor infections among those in good health. These same strains can result in serious congenital infections and acute toxoplasmic encephalitis in individuals with AIDS. On the other hand, Type I strains have been linked to a few limited groups of cases involving human congenital toxoplasmosis and opportunistic infections in individuals with AIDS, which suggests that Type I strains might have a higher propensity to induce illness in susceptible hosts (Khan *et al.* 2005, 2009).

T. gondii oocysts contamination of fresh produce usually occurs at primary production sites in farms – specifically, water contaminated with the feces of felids used in irrigation, washing, or processing (Shapiro *et al.* 2019; Lass *et al.* 2019). As definitive hosts are primarily responsible for the excretion of *T. gondii* oocysts, there is a potential for these animals to reach areas where vegetables are cultivated and contaminate them with the parasite's oocysts (Lass *et al.* 2019). In 2014, Bahia-Oliveira and colleagues observed that fresh produce can become contaminated with *T. gondii* oocysts during primary production on farms. These sources of contamination, both direct and indirect, are especially common in low and middle-income countries where hygiene, sanitation, and water quality may not meet optimal standards (Shapiro *et al.* 2019; Dixon 2016). Nonetheless, crops have been discovered to be contaminated with the parasite (Bahia-Oliveira *et al.* 2017; Shafa-ul-Haq *et al.* 2014). Water in irrigation systems, rivers, lakes, beaches, and coasts, as well as wastewater and groundwater, can be contaminated with environmentally resistant oocysts (López Ureña *et al*. 2022). Additionally, areas with hot, humid climates and lower elevations often exhibit the highest infection rates since oocysts thrive particularly well in such environmental conditions (CDC n/d). The sites of sampling collection in Central Luzon undergo a tropical climate marked by well-defined wet and dry periods. This condition significantly contributes to establishing the region as an agricultural center (Hernandez 2022). However, it also amplifies the potential for parasite contamination, for example, of *T. gondii.*

The present study confirmed the presence of *T. gondii* DNA in different types of vegetable samples – including lettuce, cabbage, cauliflower, carrot, and mung bean sprout. Based on the results, leafy greens were more heavily contaminated compared to root vegetables. Leafy greens possess dense foliage, broad leaves, and a large surface area, which increases the likelihood of extensive contact with contaminated soil and water, resulting in higher contamination rates compared to root vegetables. Additionally, the surface structure plays a role in parasitic attachment, making vegetables with rough surfaces more susceptible to contamination, whereas smooth-surfaced vegetables like radishes and carrots are less prone to such risks (Berrouch *et al.* 2020). The level of contamination of the collected samples was 10%, which is lower than what has been previously reported in other Asian countries (Lass *et al.* 2019; Shafa-ul-Haq *et al.* 2014; Ajmal *et al.* 2013). However, it may be noted that we have a relatively small sample size, which suggests an increased likelihood of identifying a higher prevalence of the parasite with increased samples. Detection of the oocysts in vegetable samples could be correlated to the interconnectivity of freshwater bodies and vegetable farming through irrigation water runoff from agricultural lands, urban areas, and animal waste. It is also possible to consider that the way vegetables are managed post-harvest impacts both their contamination level and the potential risk to the general public (Lass *et al.* 2019). Additionally, vegetables can also be contaminated if proper hygiene practices are not followed. On the other hand, control measures such as scrubbing or washing vegetables in running water and ensuring the separation of fruits and vegetables from raw foods like meat, poultry, and shellfish should be observed to decrease the risk of acquiring *T. gondii* (CDC n/d).

Shellfish are known as a potential source of *T gondii,* and eating raw shellfish is one of the major causes of gastroenteritis in the world as they can transmit enteric viruses to humans (Marquis *et al.* 2019). One of the popular shellfish, oysters, is considered a delicacy, particularly in coastal areas in the Philippines. Oysters are recognized as ecosystem engineers due to their filter-feeding behavior, which has an impact on the quality and turbidity of estuarine water. They also provide habitat for other species and are a valuable source of food for humans (Marquis *et al.* 2019; Rick *et al.* 2016; Dumbauld *et al.* 2009). Their cosmopolitan distribution, sessile lifestyle, and high efficiency of filtration make them capable of bioaccumulation, which allows them to be efficient sentinels for monitoring the health of estuarine ecosystems (Silva *et al.* 2020; Staggs *et al.* 2015; Palos

Ladeiro *et al.* 2014). *T. gondii* oocysts can survive in oysters, retaining its infectivity that may serve as a source of *T. gondii* infection in marine mammals and possibly humans (Coupe *et al.* 2019; Monteiro *et al.* 2019; Miller *et al.* 2008). It has been reported that eating raw shellfish is a significant risk of *T. gondii* infection in humans (Jones *et al.* 2009). The herein reported prevalence (9.09%) is comparable with those reported elsewhere: oyster samples in Italy (3.3 and 6.60%) (Putignani *et al.* 2011; Esmerini *et al.* 2010); in eastern oysters in the Scarborough River (14.3%) and Bagaduce River (4.3%) in the United States (Marquis *et al.* 2015); Brazilian oysters in Bahia, Brazil (8.1%) (Ribeiro *et al.* 2015); cultured oysters in Pará, Brazil (5.8%) (Monteiro *et al.* 2019), market-sold oysters in China (2.61%) (Cong *et al.* 2017), and farmed oysters in Brazil (2.5%) (Gyawali and Hewitt 2020).

To the best knowledge of the authors, there are no local studies yet on *T. gondii* prevalence in freshwater bodies, but as an emerging marine pathogen, *T. gondii* has been detected in cetaceans stranded in Philippine waters as part of a nationwide marine mammal health surveillance program (Obusan *et al.* 2015, 2019; de Guzman *et al.* 2020). Our findings suggest the contamination of the marine environment by *T. gondii* oocysts where these oysters are being cultivated and provide evidence for another negative effect of human activities such as the transfer of biological pollutants from land to water through land-based effluents and surface runoffs. Additionally, the presence of *T. gondii* in these seafoods is not only a risk to animals that consume them but also serves as a significant threat to humans. Nonetheless, the most sustainable approach for reducing the risk of *T. gondii* exposure through the consumption of seafood should focus on reducing *T. gondii* contamination at its source, as well as mitigating the flow of contaminated runoff to water bodies (Shapiro *et al.* 2019).

Foodborne toxoplasmosis remains to be neglected because of the difficulty in establishing the connection between infection and food consumption and also due to the lack of validated sensitive and specific detection methods (Shapiro *et al.* 2019; Hohweyer *et al.* 2016). As in vegetables, the association between shellfish consumption and *T. gondii* infection often goes unrecognized since there are no documented specific cases of toxoplasmosis in humans caused by the consumption of raw shellfish or contact with seawater (Putignani *et al.* 2011; Robertson 2007). Moreover, the methods for detecting *T. gondii* in seafood remain inconsistent, which hinders direct comparison of prevalence and distribution among studies (Shapiro *et al.* 2019). Precise detection of *T. gondii* oocysts in environmental samples is challenging, even though the nested PCR technique is commonly used and is sensitive for detecting this protozoon (Silva *et al.* 2020). Thus, differentiating between viable and non-viable oocysts is also challenging, unless viability assays like cell culture and bioassays are employed. In this case, viable oocysts can develop and exhibit characteristic stages of the parasite's life cycle, whereas non-viable oocysts cannot (Khan *et al.* 2017; Chatterton *et al.* 2002). The *B1* gene was used in the present study because it is widely used for the detection of *T. gondii* in shellfish specifically using the nested PCR (Gyawali and Hewitt 2020; Silva *et al.* 2020; DeMone *et al.* 2020). The *B1 gene* exhibits a high degree of specificity towards *T. gondii* since it is repeated 35 times in the genome of the parasite. Therefore, it serves as a preferred target for amplification in detecting the presence of the parasite (Sağlam *et al.* 2021; Sardarian *et al.* 2018).

CONCLUSION

The study investigated the presence of *T. gondii* oocysts DNA in select RTE vegetables and market-sold oysters collected from the provinces of Central Luzon, Philippines using nested PCR targeting the *B1* gene. The parasite was PCR positive in six (10%) out of the 60 vegetable samples, specifically in two mung bean sprout samples and one carrot, cabbage, cauliflower, and lettuce samples. Among oyster samples, four (9.09%) out of the 44 collected samples were positive for *T. gondii*. Phylogenetic analysis revealed that the DNA sequences of the detected *T. gondii* form a clade with *T. gondii* Type 1 genotype. Contamination of these food products suggests that environmental matrices such as soil and water together with improper food handling and consumption pose a potential risk of *T. gondii* infection among consumers. We recommend the conduct of a more intensive screening and analysis of other environmental matrices such as soil and water in order to determine the possible extent of routes of transmission of *T. gondii* in the study sites. Moreover, it is essential to examine the genetic variation of the parasite through genotypic analysis to gain a better understanding of the epidemiology and transmission of toxoplasmosis.

ACKNOWLEDGMENTS

This study was funded by the Natural Sciences Research Institute, College of Science University of the Philippines, Diliman, Quezon City, Philippines (Project Code BIO-22-1-03).

DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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