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SHORT COMMUNICATION

Comparative Evaluation of Re 529-Bp Sequence and B1 Gene in the Detection of *Toxoplasma gondii* Through PCR in Water Samples of Denizli, Turkey

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Abstract

Purpose While *Toxoplasma gondii* (*T. gondii*) infection is asymptomatic in immunocompetent individuals, it is a life-threatening protozoan in immunocompromised individuals. Its water-borne transmission to humans poses a serious public health concern. Polymerase Chain Reaction (PCR) has a considerable potential for the sensitive and specific detection of *T. gondii* oocysts in waters.

Methods Comparative evaluation of RE 529-bp sequence and B1 gene to detect *T. gondii* tachyzoites and oocysts via PCR in agricultural irrigation water taken from downtown Denizli, Turkey and water samples collected from neighborhood fountains was performed for the first time in Turkish context.

Results Based on real-time PCR targeting the B1 genetic markers and RE 529-bp sequence, *T. gondii* DNA was identified in 6 (16.7%) out of 48 samples collected from agricultural irrigation water. Besides, our PCR analysis did not establish any presence of *T. gondii* in drinking water samples.

Conclusion *T. gondii* showed lower sensitivity in B1-based PCR than in PCR targeting RE 529-bp sequence.

Keywords *Toxoplasma gondii* · 529-bp · B1 gene · Water-borne · Denizli · Turkey

Introduction

Today, most diseases are estimated to stem from insufficiency of safe water and sanitation conditions. Millions of people, more than half of whom are children, die every year owing to water pollution [1, 2]. Parasite oocysts and/or cysts introduced into environment in human and animal feces are transmitted to humans through drinking, utility, and recreation water. Aquatic protozoans such as *Toxoplasma gondii* (*T. gondii*) are responsible for diseases that threaten human health [3]. *T. gondii* is known as a protozoan which infects all types of cells and actively carries out cell invasion [4]. *T. gondii* is transmitted to humans by contamination of oocysts in cat feces into water and food, eating raw or undercooked meat containing bradyzoites, transplacental passage of tachyzoites, organ transplantation, and blood transfusion. Moreover, almost a quarter of the global human population is infected with *T. gondii* [5]. Though it sometimes does not result in any disease in adults, *T. gondii* may induce severe clinical manifestations of ocular disease in immunocompromised individuals, mental retardation in congenitally infected children, and miscarriages in pregnant women. A growing body of research confirms the prevalence of environmental contamination with *T. gondii* oocysts in addition to the increasing importance of toxoplasmosis, and food-borne include transmission by milk and derivadesas well as water-borne pathogens are now assumed to bring about a substantial disease burden [6–8].

PCR, which enables the detection of *T. gondii* in a shorter span of time, is becoming a highly preferred technique for identifying *T. gondii* bradyzoites in water in comparison to

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conventional bioassay tests. Despite the presence of techniques developed for detecting *T. gondii* in clinical samples, modified PCR tests are currently utilized to identify bradyzoites in water samples, probably due to expectedly high concentrations of PCR inhibitor and low *T. gondii* bradyzoites in environmental samples [9–12].

Thus far, no research has been reported on the pollution caused by *T. gondii* in Denizli, which is rich in water resources. This study seeks to investigate the presence of *T. gondii* in agricultural irrigation and drinking water sources, and comparative evaluation of *T. gondii* B1 gene and RE 529-bp sequence has been made for the first time in Turkey.

Materials and Methods

Water Sample Collection & Oocyst Concentration

Located between 37°12'–38°12' N latitude and 28°30'–29°30' D longitude, Denizli is an industrial and tourism city situated in the southwest of Turkey. It is neighbored by Afyon and Burdur to the east, Aydın to the west, Muğla to the south and Uşak to the north. In addition to Gökpınar Dam (also called *Governor Recep Yazıcıoğlu Dam*), which was selected as the water sampling site, Denizli has also other aquatic resources to meet the water need, such as Derindere, Yukarı Santral, and Başkarcı-İsrafil streams. Apart from these resources, many small and large resources feed the networks and reservoires [13, 14].

All water samples were obtained in the period between October 2017 and October 2018. The current study involves collecting water samples from 3 different sampling areas selected from Gökpınar Dam and 4 different neighborhood fountains in downtown Denizli. The sites selected for sample collection included Gökpınar Stream, which feeds the dam, as well as Karakurt and Akhan districts of the dam. The sample fountains, on the other hand, were determined as the ones in the crowded central neighborhoods. The field observations preceding the sample collection phase have revealed that the waste water coming from the neighborhoods in these districts flowed into the dam, and that the dam was contaminated owing to man-made activities. It was also of note that the samples of dam water were turbid, and that there was livestock feeding in the study sites.

Concentration of Water Samples by $Al_2(SO_4)^3$ Flocculation

All water samples collected from different sources in Denizli were purified by $Al_2(SO_4)^3$ flocculation, as described by Karanis and Kimura (2002) [15] and then applied by Kourenti et al. (2003) [16] and Karanis et al. (2006) [17]. 10 L of water from the catchment areas were collected in sterile plastic bottles without chemical additives and immediately transferred to the laboratory for processing. The water in each sample was poured into the dark glass bottle for flocculation. Following the addition of 10 mL of $Al_2(SO_4)^3$ solution, the water samples were left to stand overnight to allow for flock precipitation (pH5.4–5.8).

The water samples were taken from the sources with 10-L disposable plastic drums. 10 mL of aluminum sulfate $Al_2(SO_4)^3$ was added to these samples to achieve pH 5,4–5,8. For precipitation to occur, the samples were kept at room temperature for about 20 to 24 h in dark-room conditions. The supernatant in the upper part of the samples was discarded until 1L remained. It was then centrifuged at 2100 rpm for 10 min at 4 °C. After the upper liquid was discarded, the 50 mL pellet was stored in a refrigerator at 4 °C until used.

Microscopic Detection and Identification of *T. gondii* Oocyst and Tachyzoites

Wet slides in the water pellet smear were utilized for microscopic investigation and analyzed for oocyst and tachyzoites. For giemsa staining, the smears were prepared by taking pea-sized material from water samples with a thin smear on a clean glass slide and stained for 30 min [18].

Purification of Samples (Sucrose Gradient Method)

During the purification process, 0.1 M phosphate-buffered saline (PBS) (pH = 7.2) and sugar solution (200 g (g) glucose, 6.5 g phenol, 320 mL purified water) were prepared. As a result, two solutions (solution A:1/2, solution B:1/4) with different sugar solution/PBS ratio were obtained. First, 15 mL of solution A was placed in a sterile 50 mL polypropylene falcon tube, and 15 mL of solution B was added to obtain a visible phase. A layer was obtained by cautiously placing 10 mL of water sample on this phase. Then, it was made up to 50 mL with distilled water and centrifuged at 2100 × g for 10 min. The supernatant was discarded so that it remained at the bottom of the approximately 2 mL pellet tube, and the pellet was stored in the refrigerator to be employed in DNA extraction [11].

DNA Isolation

DNA isolation from the samples purified by the sucrose gradient method was performed by modifying the QIAamp DNA mini kit (Qiagen) protocol, based on the method suggested by Karanis et al. (2006) [17]. According to this method, lysis buffer was added to the samples and exposed to a freeze–thaw process in liquid nitrogen 15 times. After freezing and thawing processes were carried out for approximately 1 min, *T. gondii* oocyst walls were completely

broken. Then the kit protocol was followed sequentially. The DNA obtained in the last stage was collected in a 50 µL of TE buffer and stored at 4 °C to be used in PCR.

PCR Method

The classic PCR reaction mix was prepared in a final volume of 25 µl. To this end, we used Hot Start TaqDNA polymerase kit (10 × PCR buffer, 5 × Q solution, 25 mM MgCl2, 5 U hotstart taq DNA polymerase) (Qiagen), 25 mM dNTP mix, 10 pmol B1 gene, RE 529-bp sequence specific primers F3 and B3, and 1 µl of DNA (Table 1). Amplification was performed on the Biorad CFX-96 device (BioRad, USA), and positive and negative controls were used for each PCR application. The amplified products were stained with ethidium bromide and loaded on 1.5% agar. The gel electrophoresis was run at 150 V for 30 min and evaluated under UV (GeL Logic 2200-Imaging System (BioRad, USA)).

Results

Though not identified by the standard PCR in the collected drinking water samples, *T. gondii* positivity was detected in 6 (16.7%) of 36 agricultural irrigation water samples. The agarose gel image obtained by PCR is presented in Figs. 1 and 2. When the *T. gondii* B1 gene was analyzed by the standard PCR, only 3 samples collected from the Akhan district of the Governor Recep Yazıcıoğlu Dam turned out to be positive. However, the PCR analysis of the *T. gondii* RE 529-bp sequence revealed that 2 samples from the Akhan district of the dam and 1 sample from the Karakurt district were established to be positive.

Discussion

In this study, PCR tests based on the *T. gondii* B1 gene and the RE 529-bp sequence were investigated for their sensitivity in detecting *T. gondii* oocysts in concentrates of agricultural irrigation and drinking water samples. These genes were particularly chosen for their frequent use and high-copy number targets to identify *T. gondii* in clinical samples [12, 21, 22].

The PCR technique was performed for the genes tested in the study by utilizing B1 primers designed by Fallahi et al. (2014) [19] and the primers of the RE 529-bp fragment detected in the *T. gondii* genome by Homan et al. (2000) [20]. B1 primers are considered the most widely used primer set for PCR detection of *T. gondii* [12, 23, 24]. RE 529-bp

Table 1 Nucleotide sequences of PCR primers (B1gene and RE 529-bp sequence) used in this study for *Toxoplasma gondii* molecular diagnosis

		Amplicon (bp)	Reference
B1 Gene			
Forward	5'-GGA ACT GCA TCC GTT CAT GAG-3'	194 bp	[19]
Reverse	5'-TCT TTA AAG CGT TCG TGG TC-3'	194 bp	[19]
529-bp sequence			
Forward	5'-TGA CTC GGG CCC AGC TGC GT-3'	529 bp	[20]
Reverse	5'-CTC CTC CCT TCG TCC AAG CCT CC-3'	529 bp	[20]

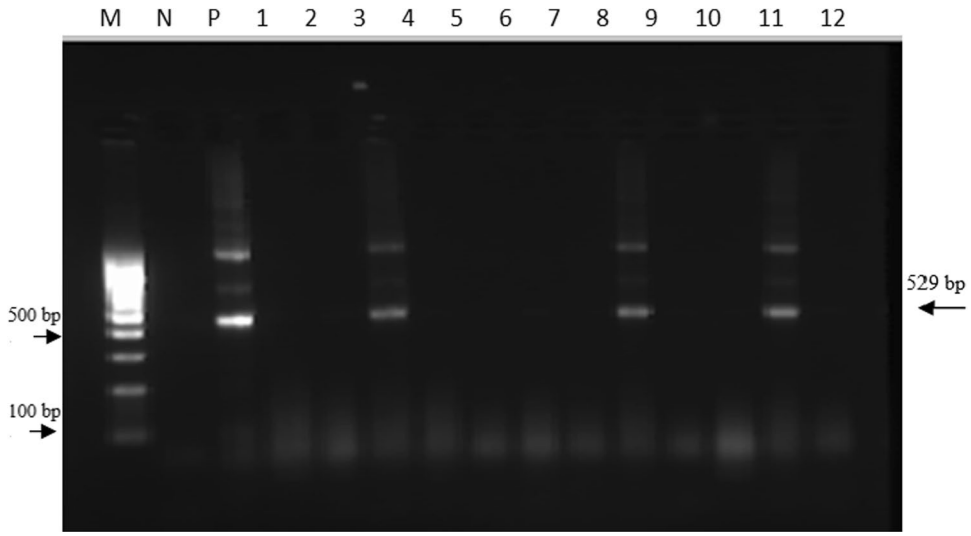

Fig. 1 The image of PCR products of the water samples collected from downtown Denizli, which were analyzed through the standard PCR, on agarose gel; **M:** 100 bp DNA marker; **N:** (distilled water) negative; **P:** *T. gondii* DNA (RE gene region), 3–8–11: water samples analyzed

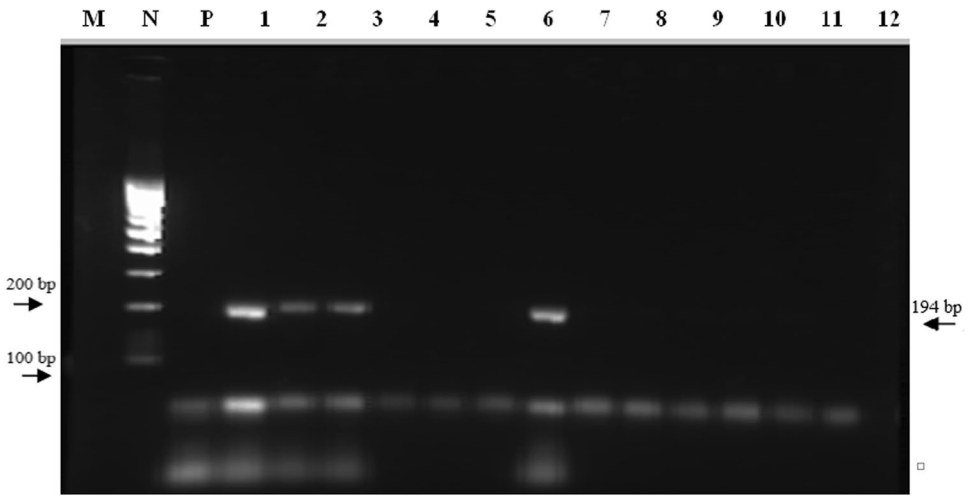

Fig. 2 The image of the PCR products of the water samples collected from downtown Denizli, which was analyzed through the standard PCR, on agarose gel; **M:** 100 bp DNA marker; **N:** (distilled water) negative; **P:** *T. gondii* DNA (B1 gene region), 1–2–6: water samples analyzed

fragment of *T. gondii* are reportedly more sensitive and effective in identifying *T. gondii* [12, 20].

Flávia Minutti et al. (2021) [26] used for serological analysis by indirect fluorescent antibody test (IFAT), modified agglutination test (MAT), and enzyme-linked immunosorbent test (ELISA) to diagnose *Toxoplasma gondii* in free-range chickens. According to molecular analyzes in the study, when qPCR was used as the gold standard, sensitivity (69.4%) in ITS1 nested PCR and specificity were higher in conventional PCR-529 bp (90.7%). However, MAT and ELISA showed similarities in the fit analysis. In addition, the researchers reported that the PCR markers effectively detected parasite DNA. The researchers reported that conventional PCR has similar sensitivity to nested PCR and qPCR and may be a cheaper alternative for diagnosing *T. gondii* infection in chicken tissues. In our study results, conventional PCR is considered to be an effective and less expensive technique for the diagnosis of *T. gondii* infection in water samples.

The findings of the present study confirm higher sensitivity of the RE 529-bp sequence-based PCR experiments in comparison to the B1 gene-based PCR. The analysis of B1 gene of *T. gondii* revealed that only 3 samples were found to be positive from the Akhan region of the Gökpınar Vali Recep Yazıcıoğlu Dam. Besides, according to the PCR result based on the RE 529-bp sequence, 2 samples from the Akhan district and 1 sample from the Karakurt district, where no positive result could be detected with the B1 gene, were established to be positive. That is, the RE 529-bp sequence-based PCR proved more effective in detecting *T. gondii* oocysts than B1 gene-based PCR assays.

The utilization of *T. gondii* oocysts in water samples during PCR detection may be limited due to the non-specificity of some primers and low sensitivity [12]. However, our results suggest that the combined use of the B1 gene and the RE 529-bp sequence can minimize this limitation. Our findings also support the view that the RE 529-bp sequence, originally developed for the analysis of clinical samples, can be utilized effectively in the identification of *T. gondii* oocysts.

The present study could not detect any presence of *T. gondii* in the neighborhood fountains. Demirel et al. (2014) [25] established the presence of *T. gondii* in environmental and drinking water samples in Giresun, Turkey through the standard PCR and the loop-mediated isothermal amplification (LAMP) method. However, they could not find traces of *T. gondii* DNA in any of the drinking water samples.

It was observed during our research that the domestic wastewater and sewage waters of the nearby settlements were mixed with the Governor Recep Yazıcıoğlu Dam without any treatment, and that the dam was contaminated with *T. gondii* as a result. In that regard, the potential risk factors for public health can be prevented by ensuring the control of stray cats and adhering to basic hygiene rules during water use. In addition, priority should be given to the development of diverse methods for the identification of *T. gondii* oocysts in water resources.

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Availability of Data and Materials Data of this study are provided in the paper.

Code Availability Not applicable.

Declarations

Conflict of Interest The authors declare that they have no competing interests.

Ethical Approval Ethics committee approval was not received due to working in water.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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