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Detection and genotyping of *Toxoplasma gondii* oocysts in environmental samples in Gaza, Palestine: A combined parasitological and molecular approach

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ABSTRACT

Objective: This study evaluated the prevalence and genotypes of *Toxoplasma* (*T.*) *gondii* oocysts, the infectious environmental stage, in soil and water samples collected from Gaza, Palestine.

Methods: A total of 290 environmental samples (200 soil, 90 water) from diverse sites in Gaza, including Wadi Gaza, residential areas, markets, wells, and the main sewage plant. Samples were analyzed using polymerase chain reaction (PCR) and Sheather's flotation, with genotyping via Mse I endonuclease.

Results: Out of 290 environmental samples, 22 (7.6%) were positive by PCR and 31 (10.7%) were positive by Sheather's flotation. The prevalence of *T. gondii* significantly varied by location, with residential yards showing the highest contamination and public squares and gardens the lowest. Among soil samples, 26 (13.0%) tested positive by Sheather's method, while PCR detected contamination in 18 (9.0%). Water had lower contamination rates, with 4 (4.4%) testing positive by PCR and 5 (5.6%) by Sheather's. All positive water samples were from Wadi Gaza. Genotyping confirmed all four tested samples belonged to *T. gondii* Type I.

Conclusions: The study demonstrated a statistically significant relationship between oocyst contamination prevalence and the source location of the samples. It concluded that *T. gondii* contamination is present in the soil and water in the study area, with soil showing higher contamination levels than water.

KEYWORDS: *Toxoplasma gondii*; Oocysts; Genotyping; Soil; Water; Gaza

1. Introduction

Toxoplasma (*T.*) *gondii* is an obligate intracellular protozoan infecting all warm-blooded animals. It reproduces sexually in the feline gut, producing oocysts that sporulate in the environment, where they remain infectious and highly resistant for years[1]. Ecological factors like humidity, temperature, soil and water matrices, and land cover influence the survival of *T. gondii* oocysts, which can remain infectious in soil in soil, water and food environments[2]. After ingestion by intermediate hosts, sporulated oocysts release sporozoites that invade the intestinal epithelium, develop into tachyzoites, and rapidly replicate asexually within host cells[1]. During infection, inflammation and tissue damage can cause clinical symptoms. As immunity develops, parasite replication slows, and tachyzoites convert into bradyzoites, forming tissue cysts[3]. By forming tissue cysts,

Significance

Toxoplasma (*T.*) *gondii* oocysts are recognized as environmental contaminants with significant public health implications, yet data from Gaza, Palestine, remain scant. This study provides the first combined parasitological and molecular evidence of *T. gondii* prevalence and genotypes in Gaza's soil and water, demonstrating a statistically significant association between contamination rates and sample source location. The findings reveal that *T. gondii* contamination occurs in both soil and water, with soil exhibiting higher contamination levels, emphasizing the need for targeted interventions to mitigate toxoplasmosis transmission in the region.

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T. gondii establishes chronic infection. Humans mostly acquire infection through ingestion of oocysts from contaminated sources or by consuming raw or undercooked meat containing tissue cysts[4]. Recent research reinforces waterborne transmission as a major contributor to toxoplasmosis outbreaks. A molecular survey conducted in Chandigarh, India, identified *T. gondii* oocysts in 15%-18% of public water samples, revealing persistent contamination risks[5]. Likewise, a significant 2018 outbreak in Santa Maria, Brazil, was traced to municipal drinking water, with bioassay confirming oocyst presence[6]. Heavy rainfall and agricultural runoff drive the spread of *T. gondii* by transporting oocysts from contaminated land into water bodies. This increases contamination in freshwater and coastal environments, raising the risk of waterborne toxoplasmosis outbreaks in humans and animals[7]. Toxoplasmosis is one of the most prevalent zoonoses worldwide. This is supported by a 2019 systematic review, after analyzing data from over 648 010 individuals, calculated a global pooled seroprevalence of 25.7% for *T. gondii* infection[8]. It is noteworthy that the seroprevalence of *T. gondii* is particularly high in regions where raw or undercooked meat is commonly consumed[9]. Tropical areas in Latin America and Africa show high *T. gondii* seroprevalence due to large cat populations and climates that favor oocyst survival. Warm, humid conditions increase environmental contamination and transmission risk, with seroprevalence rates over 50% in Latin America and up to 80% in sub-Saharan Africa, indicating a significant public health challenge in these regions[10,11]. On the other hand, approximately 15% of U.S. women aged 25 to 44 are infected with *T. gondii*, with 400 to 4 000 recorded annual cases of congenital toxoplasmosis[12]. In addition, a study on 24 food-borne parasites ranked *T. gondii* fourth in economic and trade impact[13]. Sporadic waterborne toxoplasmosis incidents have occurred globally, including the initial epidemic in Panama (1979) and a major outbreak in British Columbia (1995), where over 100 individuals contracted the disease from contaminated water[14,15]. While in Brazil, one of the largest recorded outbreaks occurred in 2001 in Santa Isabel do Ivaí, Paraná State, where contamination of the municipal water supply network resulted in illness in more than 400 persons[16]. According to a systematic review of human toxoplasmosis outbreaks from 1967 to 2018, 12 (35.3%) of the 34 published outbreaks occurred in Brazil and involved 776 of the 1 721 affected persons, highlighting the particularly high burden in this country. Several of these Brazilian outbreaks were associated with exposure to *Toxoplasma gondii* oocysts, especially through contaminated drinking water, fruits and vegetables irrigated or processed with contaminated water[17]. Numerous studies have assessed *T. gondii* seroprevalence among pregnant and aborted women in Palestine. Al-Hindi and Lubbad found that 17.9% of 312 women with abortions in Gaza tested positive for IgG, and 12.8% for IgM[18]. In Hebron, a seroprevalence of 27.9% was reported among 204 pregnant women, with rural women showing higher rates than urban women[19]. Similarly, Al-Jarousha found a 30.9% seropositivity rate among 255 pregnant women in Gaza[20]. Detecting *T. gondii* prevalence in the environment is challenging, as just one oocyst ingested from contaminated water, soil, fruits, or vegetables can cause significant health issues in humans and animals[2]. The study aimed to detect *T. gondii* oocysts in environmental samples (soil and water) in Gaza, Palestine, using conventional and molecular techniques, and identify the genotypes of Toxo-DNA isolates.

2. Methods

2.1. Study area and sample collection

A total of 200 soil samples were randomly collected from marketplaces, garbage areas, Wadi-Gaza, public squares, gardens, and private yards, with 40 samples taken from each location. The samples were collected from May to August 2019, as detailed in Table 1.

Wadi Gaza is a water stream originating in the Hebron mountains, flowing through Gaza for 8 kilometers before emptying into the Mediterranean Sea. Soil samples from Wadi Gaza were collected between the Salah El Deen bridge and 1 km east (Supplementary Figure 1)[21]. Additional samples were taken from Feras Market, Gaza's largest and busiest vegetable market. Additional soil samples were collected near waste bins in busy city areas and from yards of houses selected after surveying neighborhoods with high domestic and stray cat populations. Lastly, soil samples were collected from public gardens and areas with high cat populations after monitoring. Water samples were taken from Wadi Gaza (sewage and rainwater mix), a wastewater treatment facility west of Gaza, and wells used for irrigation and household purposes across the city and suburbs.

2.2. Detection of T. gondii oocysts

A total of 290 soil and water samples were collected and processed from the previously described locations. The soil sample preparation was based on the protocols outlined by Lass *et al*.[22], with slight methodological adjustments. Each soil specimen, weighing 300 g, was collected at a depth of 2-5 cm beneath the surface and subsequently air-dried at room temperature for two days. Thereafter, 40 g of each dried sample were mixed with 250 mL of distilled water and sieved through a 75 μm mesh. The filtrate was transferred into a 250 mL graduated cylinder and allowed to sediment overnight. The following day, 4 mL of the supernatant was gently aspirated and combined with the 8 mL of sediment from the cylinder's base. This 12 mL suspension was then centrifuged at 2 500 rpm for 10 min. Post-centrifugation, 2 mL of the supernatant was discarded, and the remaining mixture was prepared for microscopic examination using the flotation technique with Sheather's solution. A total of 90 water samples (30 from each of the three previously specified locations; 500 mL per sample)were collected for analysis. Each was filtered using a Büchner funnel equipped with a 0.45 μm nylon membrane filter. The membranes were rinsed with saline solution and examined microscopically using Sheather's flotation technique under light microscopy. Samples that tested contamination were transferred to Eppendorf tubes and stored at –20 °C for subsequent molecular analysis by polymerase chain reaction (PCR).

Table 1. Distribution of samples by locality.

Locality	Samples from soil	Samples from water
Wadi-Gaza	40	30
Trash dumpster premises	40	/
House yards	40	/
Public squares and gardens	40	/
Markets	40	/
Sewage treatment plant	/	30
Wells	/	30
Total	200	90

2.3. Molecular identification of *T. gondii*

2.3.1. DNA extraction

DNA extraction was performed at the laboratories of the Faculty of Health Sciences, Islamic University of Gaza, Palestine. Furthermore, Genomic DNA was extracted from the resuspended pellets using the Wizard Genomic DNA Extraction Kit A1120 (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions, and the extracted DNA was stored at –20 °C until use for PCR analysis.

2.3.2. Primer design and PCR conditions

PCR amplification was carried out using the primer set TOXO-F (5'-GGA ACT GCA TCC GTT CAT GAG-3') and TOXO-R (5'-TCT TTA AAG GGT TGG TGG TC-3'), as described by Lass *et al*., targeting a 194 bp fragment of the 35-copy B1 gene. Positive samples were further confirmed using an additional primer set, TOXO-F (5'-AGG GGA GGG TGA GGA TGA-3') and TOXO-R (5'-TGG TCT CGT CTG GAT CGC AT-3'), designed to amplify a 200-300-fold repetitive element (GenBank AF146527). PCR reactions were prepared using AccuPower PCR PreMix tubes (Bioneer Corporation-Hylabs), and amplification was performed on a Biometra thermal cycler (Germany). The thermal cycling protocol included an initial denaturation at 95 °C for 5 min, followed by 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 90 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min[22]. Distilled water served as a negative control, whereas a reference *T. gondii* DNA sample, supplied by the Health Sciences Department at the Islamic University of Gaza, was used as a positive control, as previously described by Dardona *et al*.[23].

2.4. Genotyping

Genotyping of four PCR-positive samples was performed using the method described by Norouzi *et al*.[24]. In this approach, the Mse I restriction endonuclease was utilized to digest the PCR products, targeting specific cleavage sites within the GRA6 gene to differentiate parasite genotypes based on fragment sizes. Specifically, digestion produced fragments of 168 bp and 544 bp for type I, 75 bp and 623 bp for type II, and 97 bp and 544 bp for type III. Following enzyme addition, the digestion mixture was incubated at 37 °C for 4 h and subsequently subjected to gel electrophoresis for analysis[24].

2.5. Statistical analysis

Data analysis was performed using the SPSS software package version 15.0. *Chi*-square tests were applied to assess the association between *T. gondii* oocyst contamination and two variables: the type of environmental sample (soil *vs*. water) and the sampling month. A *P* value of less than 0.05 was considered indicative of statistical significance in evaluating these associations.

2.6. Ethical statement

This study did not involve human participants or vertebrate animals. Sampling of enviromental materials was conducted in accordance with local environmental and public health regulations.

3. Results

The findings of the present study showed a notable prevalence of *T. gondii* oocysts in certain environmental samples examined. Definitely, PCR detected contamination in 22 (7.6%) out of 290 tested water and soil samples, while Sheather's solution identified contamination in 31 (10.7%) samples. PCR specifically found 18 contaminated soil samples and 4 contaminated water samples, whereas Sheather's solution detected 26 contaminated soil samples and 5 contaminated water samples (Table 2). The study determined that water samples collected from Wadi Gaza were the sole source of oocyst-contaminated water, with PCR and Sheather's solution detecting 4 and 5 contaminated samples, respectively. Conversely, *T. gondii* oocysts were not detected in samples obtained from wells or the sewage treatment facility. However, contamination was observed in samples from all sources of soil samples studied. The highest contamination rate was detected in samples collected from residential house yards, where Sheather's solution detected 9 samples and PCR detected 7 samples with *T. gondii* oocysts. Following this, Feras Market and Wadi Gaza showed intermediate levels of contamination, with 5 samples detected by Sheather's solution and 5 by PCR in Feras Market, and 5 samples detected by Sheather's solution and 3 by PCR in Wadi Gaza. Soil samples collected from garbage dumpster areas showed 4 samples contaminated according to Sheather's solution and 2 samples according to PCR. Public gardens and squares exhibited the lowest contamination rate, with 3 samples contaminated by Sheather's solution and 1 sample by PCR. Table 2 indicates a significant variation in the occurrence of *T. gondii* oocysts among different environmental samples as assessed by both PCR and Sheather's solution, with *P* values of 0.01 and 0.04, respectively, both below the significance threshold of 0.05.

Regarding the timing of sample collection, the study observed varying contamination rates across months, with the highest numbers of contaminated samples found in November and December. Conversely, July and August showed the lowest contamination rates. These findings are shown in Table 3.

Table 2. The prevalence of *T. gondii* oocysts in the environmental samples, based on the source of sample using both PCR and Sheather's techniques (n=290).

Type	Sample	Positive (Sheather's)	Positive (PCR)
Wadi –Gaza (Running water)	30	5	4
Sewage treatment plant (Sewage water)	30	0	0
Wells (Drinking and irrigations)	30	0	0
Wadi – Gaza (Soil)	40	5	3
Trash cans (Soil)	40	4	2
House yards (Soil)	40	9	7
Public gardens (Soil)	40	3	1
Feras market (Soil)	40	5	5
Total	290	31 (10.7)	22 (7.6)
χ^2		14.86	16.79
P		0.04*	0.01*

Table 3. Monthly prevalence of *T. gondii* oocyst in the environmental samples in Gaza city.

Month	Soil	Water	Positive (Sheather's)	Positive (PCR)
July	28	15	3	1
August	41	26	2	2
September	12	8	4	2
October	50	7	7	4
November	42	18	8	6
December	27	16	7	7
Total	200	90	31 (10.7 %)	22 (7.6 %)
χ^2			8.60	9.05
P			0.13	0.12

The correlation between sample contamination with oocysts, assessed using both Sheather's technique and PCR, and the months of sample collection was not statistically significant, with *P* values of 0.13 and 0.12, respectively, as presented in Table 3. Both values exceeded the 0.05 threshold for significance.

Additionally, Figure 1 illustrates the PCR results, revealing that 31 samples tested positive by Sheather's method, while PCR targeting the *B1* gene confirmed *T. gondii* oocyst contamination in only 22 samples. Regarding genotyping, the addition of Mse I endonuclease to four PCR-positive samples produced bands at 544 and 168 bp in all samples, as shown in Figure 2. This pattern indicates that the four samples belong to Toxo-type I.

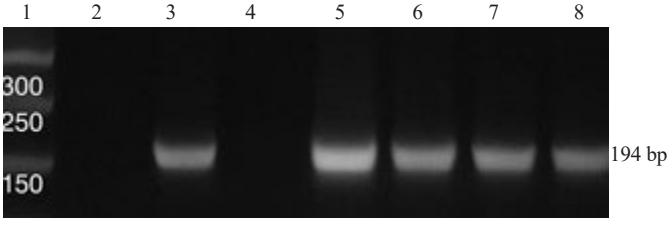


Figure 1. Agarose gel electrophoresis of PCR products. Detection of *T. gondii* oocysts in artificially contaminated water and soil samples. The amplification products of the *B1* gene were separated on a 2% agarose gel. Line 1 corresponds to the molecular weight marker (Marker 50 bp, Bioline, Italy). Line 2 represents the negative control, while line 3 is the positive control. Line 4 is the no-template control (negative control). Positive samples contaminated with *T. gondii* oocysts are shown in lines 5, 6, 7, and 8.

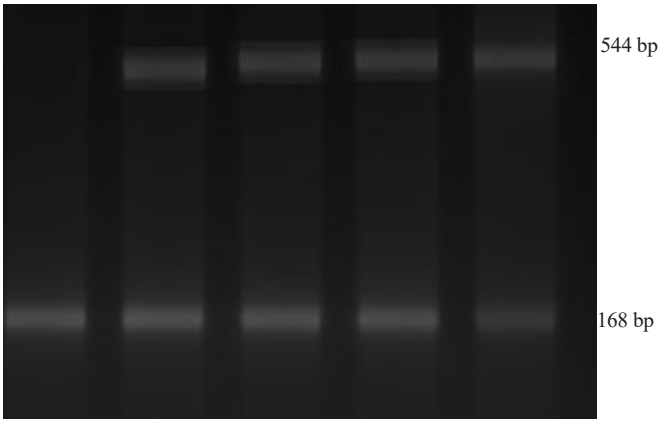


Figure 2. PCR-RFLP pattern of the GRA6 gene cut with Mse I endonuclease (168 bp and 544 bp bands), corresponding to the pattern of type I *T. gondii* on a 2.0 % agarose gel.

4. Discussion

The main goal of the study was to detect *T. gondii* oocysts in soil and water samples in Gaza. Soil contact is a major transmission route for *T. gondii* infections, emphasizing soil as a critical environmental factor in human infection[25]. Research from Europe indicated that 17% of *T. gondii* infections stem from exposure to contaminated soil, based on surveys of affected women. This highlights the significant risk posed by contaminated soil due to the long-lasting infectivity of oocysts, which can persist for more than a year[26]. Definitely, soil constitutes a crucial environmental reservoir for *T. gondii* infection[27]. This assertion is supported by our study's findings, which revealed the occurrence of oocysts across all sampled soil sites. Oocysts, exclusively excreted in cat feces, were prevalent at all sampling sites where cats were present, leading to the highest contamination rates observed in Wadi-Gaza and residential yards. Stray cats are ubiquitous in Gaza, particularly in Wadi-Gaza and surrounding agricultural areas, where abundant food sources and suitable habitats are available. Some of these cats have been identified as carriers of *T. gondii*, consistent with findings by Al-Hindi *et al*. who detected *T. gondii* oocysts in fecal samples from stray cats in Khanyounis Governorate, located south of Gaza City[28]. Although the prevalence was relatively low (5.4%), this finding confirms that stray cats in the region can shed *T. gondii* oocysts, contributing to environmental contamination. This association is underscored by cats' established role in oocyst dissemination. Our study findings align with research from Poland, which detected oocysts in 18 (17.8%) out of 101 soil samples using PCR[22]. Similarly, a study in China reported contamination in 12.7% soil samples[29]. The study revealed contamination of the surface water stream, Wadi Gaza, with *T. gondii* oocysts. This stream, comprising rainwater and sewage, flows from east to west through Gaza into the Mediterranean Sea. The existence of cats near the stream is implicated in its contamination, facilitated by the availability of food sources due to improper waste disposal practices along the stream. Additionally, local and migratory birds, rats, and decomposing animal carcasses contribute to the nutritional resources available to cats in this area. Other environmental factors influencing oocyst spread in surface water sources include rainfall, wind, soil, and mechanical vectors such as insects, flies, and earthworms[2]. Water samples from other locations showed no oocyst contamination, likely due to the absence of infected cats and other factors known to facilitate oocyst transmission. Wells and the water treatment plant were effectively secured against cat presence and other potential oocyst-spreading factors. A concurrent study in the same area, focusing on parasitic contamination in kitchen water by AL-Hindi *et al*., corroborates these findings, reporting no *T. gondii* oocysts but detecting cryptosporidium instead[30]. It is well-established that household water in the region is predominantly drawn from local wells. Unlike cryptosporidium, *T. gondii* is not considered a major concern as an aquatic pathogen[2]. However, given recent

toxoplasmosis outbreaks among humans, the present study underscored the importance of monitoring oocyst contamination in environmental samples. Similar findings were reported in a study from France, where oocysts were detected in 7.7% of environmental water samples[31]. The French study's author concluded that rainwater washing contaminated soil into water sources was the primary cause of water contamination. This explanation aligns with the findings of the current study. Rainwater mixed with soil and sewage likely contributed to the oocyst contamination in Wadi Gaza, a major surface water stream flowing into the Mediterranean Sea. To validate this phenomenon, water samples were collected from Wadi Gaza approximately one kilometer before it reaches the Mediterranean. This strategic sampling point allowed the researchers to assess the impact of soil and other environmental factors on water contamination. Additionally, the study's results are consistent with findings from a Brazilian study, which reported that 7.7% of environmental water samples from surface water streams, mainly used for drinking, were contaminated with oocysts[32]. This research, along with previous studies, confirms the occurrence of oocyst contamination in surface water sources, highlighting a significant contamination rate, particularly for water used in irrigation. The current investigation is among the few that have focused on detecting oocyst contamination or even parasites in the Gaza environment. Dardona *et al*. also reported the presence of *T. gondii* oocysts in raw leafy vegetables commonly consumed in Gaza. Their findings suggest that soil or water contamination, along with various environmental factors, significantly contribute to the spread of oocysts to vegetables[23]. In the Gaza region, Hilles *et al*. documented the contamination of the Gaza shoreline with certain parasites but did not identify *T. gondii* among them[33]. The slight variations in results across different studies conducted in various cities and countries could be attributed to several factors. These include the differing examination methods used, the varying environmental conditions that facilitate oocyst transmission, and the differences in local cat population densities near the sampling sites. Even before the *T. gondii* life cycle was elucidated in 1970, there was ongoing debate about the epidemiological significance of *T. gondii* infection, specifically whether it primarily occurred through the ingestion of oocysts or tissue cysts[34]. Although the relationship between the contamination of soil and water samples and the month in which the samples were collected was not found to be statistically significant, it is noteworthy that the highest contamination rates occurred in November and December, while the lowest were observed in July and August. This can be explained by the increased rainfall during the winter months, which raises the water level in the valley's stream, accelerating the flow and transporting potential sources of contamination. Additionally, the elevated stream levels lead to flooding, which spreads water onto the agricultural lands flanking the valley, thereby increasing contamination rates. The likelihood of environmental factors such as rainwater and wind contributing to the transmission and dissemination of Toxoplasma oocysts also rises during the winter[2]. The initial observation made upon detection was that both vegetarians and non-vegetarians are susceptible to *T. gondii* infections, indicating that meat consumption is not the sole source of these infections[35]. The level of *T. gondii* infection in cats is closely linked to the infection rates in birds, rodents, and other cat food sources[36]. Infected cats excrete millions of oocysts into the soil after ingesting a single tissue cyst or bradyzoite, and these sporulated oocysts can remain infectious in the soil for up to 18 months, regardless of temperature fluctuations. While light microscopy is sufficient for detecting oocysts in heavily contaminated samples, the molecular method is preferable due to its high sensitivity and speed. PCR amplification of the *B1* gene offers a more efficient approach for detecting *T. gondii*[2]. This study utilized a PCR-RFLP assay targeting the GRA6 gene to genotype four DNA isolates. Notably, this is the first study in the region to determine the genotyping of *T. gondii* isolates. The study's findings indicated that all analyzed oocyst DNA isolates belonged to Toxo-Type I. Genotyping of the parasite is crucial in understanding disease manifestation, as evidenced by various mouse model trials. It is well-documented that genotype I strains are highly virulent, whereas other types are significantly less harmful[37]. It is significant to note that *T. gondii* DNA isolates are categorized based on their virulence in outbred mice. Nevertheless, initial phylogenetic studies of *T. gondii* strains revealed less complexity than anticipated[38]. One of the most critical aspects of genotyping is its use in tracking epidemics[39]. Significant geographical variations exist in the distribution of *T. gondii* genotypes. For instance, studies in Spain and Portugal have reported the presence of types I and III[40,41]. Conversely, genotyping studies in Crete and Cyprus have identified type III in both regions[42]. To round out the findings, French research revealed that only four of 86 *T. gondii* isolates belonged to genotype I. On the other hand, genotype I was found to be extremely dominant in a study of isolated strains from the cerebrospinal fluid of eight patients with acquired immunodeficiency in the United States, where the majority were infected with *T. gondii* genotype I[43,44]. Additionally, a study conducted in Brazil on *T. gondii* isolates from water supply samples, using the SAG2 locus for genotyping, demonstrated that genotype I is highly pathogenic[16].

5. Conclusions

The findings highlight the widespread presence of *T. gondii* oocysts in the environment, particularly in residential soils, underscoring a significant public health risk in Gaza. The exclusive contamination of water from Wadi Gaza further indicates potential exposure pathways that require urgent attention. To mitigate the risk of toxoplasmosis transmission, it is imperative to implement regular environmental monitoring, improve sanitation and waste management practices, and raise public awareness about contamination sources. Future policies

should prioritize integrated water and soil quality management and adopt molecular surveillance techniques to enhance early detection and prevention efforts.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

Dardona Z played the key role in data collection, composing the article, identifying relevant sources, writing, providing photographic documentation, and revising the text through linguistic and editorial editing. Al Hindi A, Hafidi M and Boumezzough A contributed to the research review, offered support in the writing process, funding and project administration. Sharif F contributed to PCR methodology and data analysis. Boussaa S supervised the entire project, performed a comprehensive review, managed communication with the journal, and provided significant assistance in writing, data collection, and editing. All authors read and approved the final manuscript.

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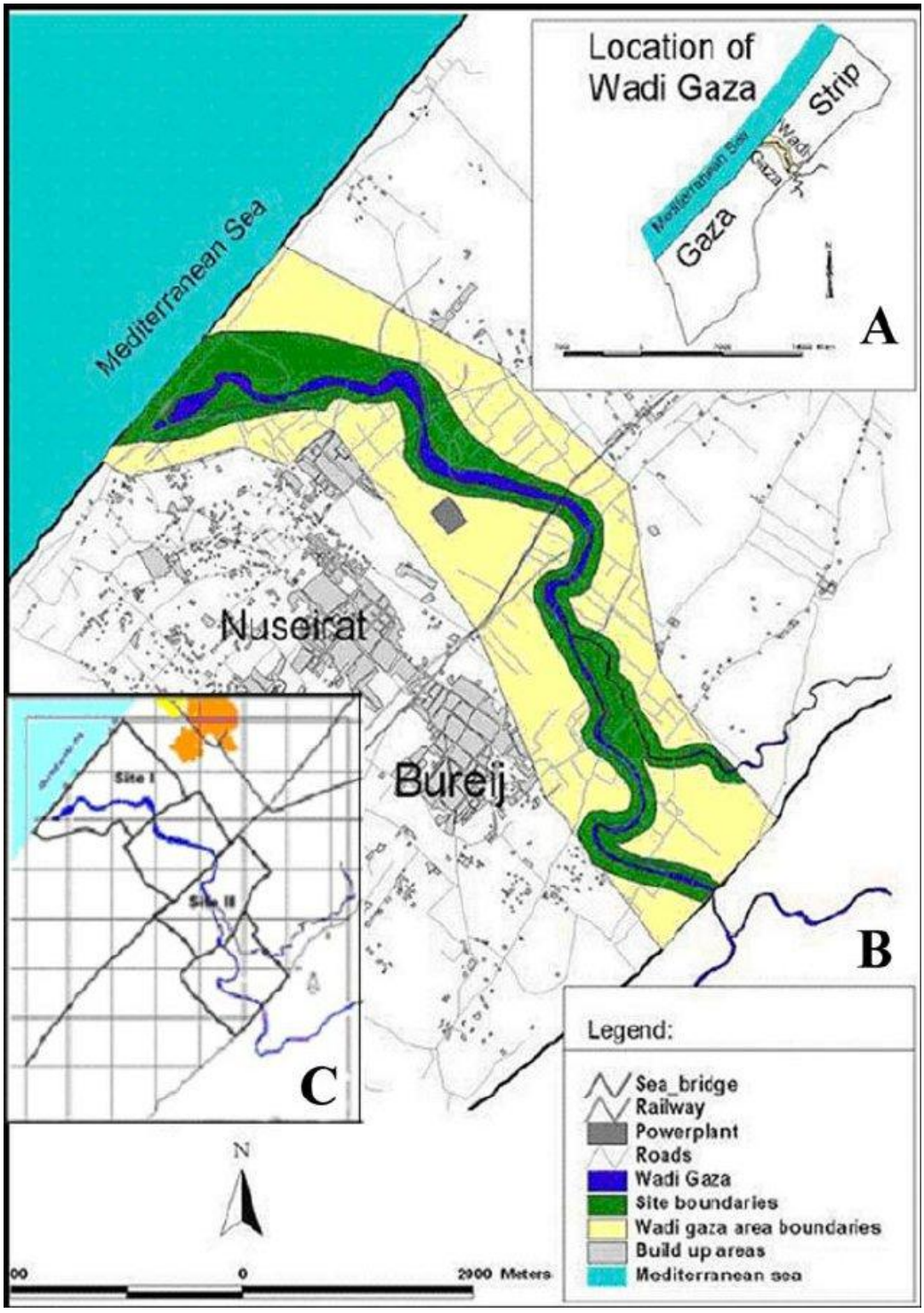
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Supplementary Figure 1. The location of Wadi Gaza[21].