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First report of molecular detection and phylogenetic analysis of *Toxoplasma Gondii* in soil, water and vegetables from Chandigarh city, India

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***Toxoplasma gondii*, a zoonotic protozoan parasite, affects up to one-third of the global population. It can be transmitted through consumption of raw or undercooked meat, vertical transmission, or oocysts from contaminated water, soil, or food. However, there are no reports on the molecular prevalence of *T. gondii* in environmental sources like soil, water, and vegetables in India. This study aimed to detect and analyze *T. gondii* in samples from Chandigarh city, India. A total of 100 each soil & water and 500 vegetable samples were collected and analyzed using conventional PCR assay, real-time PCR assay targeting the glycerol-3-phosphate dehydrogenase (B1) gene and real-time LAMP assay targeting both B1 and *Toxoplasma gondii* outer wall protein (TgOWP) genes. Results showed that 15% of water, 9% of soil, and 6.4% of vegetable samples were positive for *T. gondii*. Real-time PCR assay and real-time LAMP assay detected slightly higher positivity rates in water and vegetable samples. Phylogenetic analysis revealed that the *T. gondii* isolates clustered with those from other regions such as Iran, India, Mexico, and those found in cats, pigs, and humans. This study is the first report of *T. gondii* contamination in environmental sources and fresh produce in India. The findings highlight the potential risk of human infection from contaminated water, soil, and vegetables in the region.**

Keywords One health, Real-time LAMP assay, Real-time PCR assay, Soil, *Toxoplasma Gondii*, Vegetables, Water

Toxoplasma gondii (*T. gondii*) is a zoonotic, obligate intracellular protozoan parasite that affects nearly one-third of the global population, including most warm-blooded vertebrates[1]. It can be transmitted through three primary routes: ingestion of raw or undercooked meat from infected animals, vertical transmission from mother to fetus, and ingestion of oocysts via contaminated water, soil, or food[1]. In India, an estimated 56,737 to 176,882 children are at significant risk of congenital toxoplasmosis annually[2].

As a major zoonotic pathogen and a notable cause of food- and waterborne diseases, *T. gondii* has garnered attention from international health organizations such as the World Health Organization, which emphasizes the need for precise epidemiological data on this parasite[1]. Humans and other susceptible animals are exposed to *T. gondii* through water contaminated with oocysts from feline feces[3]. These oocysts are highly resilient to various inactivation processes, including chemical treatments. Recent outbreaks of toxoplasmosis linked to water contaminated with oocysts have highlighted the need for increased awareness of safe water practices and effective remediation strategies. Waterborne outbreaks are more frequently reported in low- and middle-income countries[4]. The NHANES (National Health and Nutrition Examination Survey) survey (1999–2004; 2009–2010) in the USA also found that individuals consuming untreated well or tap water had a significantly higher risk of seropositivity for *T. gondii* compared to those consuming treated water[5].

Soil contamination with *T. gondii* oocysts primarily results from felids, particularly young cats, which excrete millions of oocysts[6]. These oocysts pose a risk to humans, rodents, birds, and other intermediate hosts when soil becomes contaminated[7]. The prevalence of *T. gondii* in soil varies widely, ranging from 0% in the USA to approximately 50% in northeastern France[8].

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While the transmission of *T. gondii* via fresh produce has not been extensively studied in India, the absence of standardized techniques for detecting *T. gondii* DNA in vegetables and other fresh foods has hindered surveillance efforts. To date, only two outbreaks of toxoplasmosis linked to fresh produce have been reported globally[9,10]. Despite advancements in detection methods, the role of oocysts in *T. gondii* epidemiology has been underestimated due to the lack of standardized tests for environmental samples[11]. Further research into the presence of oocysts in various environmental matrices is essential for accurately assessing the risks posed to humans and animals through contaminated produce.

The significance of *T. gondii* as a zoonotic, foodborne, and waterborne pathogen underscores the critical need for comprehensive epidemiological data to identify infection sources, manage the disease, and mitigate its impacts on public health[1]. Unfortunately, only a few countries have conducted regular surveys to track *T. gondii* in humans, and even fewer have investigated its presence in animals or environmental sources[12].

In India, there are currently no reports documenting the prevalence of *T. gondii* in soil, water, or vegetables. However, global studies increasingly recognize environmental transmission as a significant route of human infection[13]. While it was previously believed that contaminated meat was the primary transmission route, the high prevalence of *T. gondii* infection among vegetarians (e.g., Jains in India) suggests that environmental sources are also major contributors[14].

This study aims to detect the prevalence of *T. gondii* in vegetables from open markets and environmental samples, such as water and soil, from the Chandigarh region using molecular techniques. Accurate detection of *T. gondii* in these sources is critical for mitigating the risk of toxoplasmosis in human populations. As the first study in India to investigate the prevalence of *T. gondii* in environmental samples, it represents a significant step toward understanding the parasite's transmission dynamics and informing public health interventions.

Results

Toxoplasma gondii DNA was detected in water collected from tube wells, ponds, tap water collected from government schools, houses, canteens, gurudwaras, public parks, hospitals etc. The parasite was detected in water collected from different areas of Chandigarh which includes Dhanas, Kaimwala (all villages), sector 15, sector 42, sector 45, sector 35, Bapu Dham Colony, Industrial area, sector 12 (Table 1).

Soil samples that tested positive were gathered from various locations throughout the city. The positive samples were collected from urban slums, industrial areas, hospital, public toilet area, kitchen garden, organic farm and public park and all were confirmed to contain *Toxoplasma gondii* using molecular methods (Table 2).

In vegetable samples, thirty-three samples were found positive by real-time LAMP against both the genes for *Toxoplasma gondii*. Coriander, *Coriandrum sativum* ($n=5$) and methi, *Trigonella foenum-graecum* ($n=5$) were found to be highly contaminated with *T. gondii* followed by radish, *Raphanus sativus* ($n=3$), cabbage, *Brassica oleracea* ($n=3$), Beetroot & greens, *Beta vulgaris* ($n=2$), spinach, *Spinacia oleracea* ($n=2$), green chili, *Capsicum annuum* ($n=2$), sprinkle onion, *Allium cepa* ($n=2$), mint, *Mentha* ($n=1$), lettuce, *Lactuca sativa* ($n=1$), swiss chard, *Beta vulgaris* ($n=1$), parsley, *Petroselinum crispum* ($n=1$), arugula, *Eruca sativa* ($n=1$), rape, *Brassica napus* ($n=1$) and tomatoes, *Solanum lycopersicum* ($n=1$). The detailed results of the positive vegetable samples of different types with molecular techniques and from different areas are given in Table 3.

Conventional PCR assay

A total of 100 water samples were analysed out of which 15 (15%) were found positive for *Toxoplasma gondii* by conventional PCR assay. Nine out of 100 samples of soil were found to be positive for *Toxoplasma gondii*. All the samples showed band for *18s* housekeeping gene. Of the 500 vegetable samples, 32 (6.4%) were positive for *T.*

City/country	Water sample collection areas	No. of Toxoplasma gondii positive samples
Chandigarh/India	Dhanas Tubewell, Drinking Water (Public Park), Pond (Cattle bathing area, Near Gurudwara), Drinking Water (H.No. 1614)	3 and 1 (only with Real-Time PCR and LAMP)
	Kaimwala Tubewell (Part 13), Govt. School, Pond 2	3
	Drinking water, Sector 15	1 (only with Real-Time PCR and LAMP)
	Sector 42 Govt. Model School 42B, Drinking water samadhi canteen, Gurudwara drinking water 42B	3
	Sector 45 House No. 2326, 45 C, House no. 2339 drinking water	2
	Model school drinking water, Sector 35 D	1 (only with Real-Time PCR and LAMP)
	Drinking water, Sector 26, Bapu dham colony, Houses	2
	Public park, Industrial Area	1
	Tap water, Hospital, PGIMER	1
Total		18

Table 1. Distribution of water samples positive for *T. Gondii* according to conventional PCR, real-time PCR & real-time LAMP.

City/country	Soil sample collection areas	No. of Toxoplasma gondii positive samples
Chandigarh/India	Urban slums areas/dumping grounds	2
	Hospitals	1
	Industrial area	2
	Public toilet area	1
	Kitchen gardens	1
	Nursery/organic farms	1
	Public parks	1
Total		9

Table 2. Distribution of positive soil samples for DNA of *Toxoplasma gondii* from different areas of Chandigarh city, India.

City/country	Vegetables Sample Collection Areas	Type of Vegetable	Number of Toxoplasma gondii positive samples
Chandigarh/India	Sector 15 (vegetable market (site 1), vendors in local market)	Coriander (*Coriandrum sativum*) leaves site 1 Beetroot (*Beta vulgaris*) Methi *(Trigonella foenum-graecum)* vendor 1 in local market Vendor 2 in local market Vendor 3 in local market	5
	Local vegetable market Nayagaon, local vendor Nayagaon area	Radish (*Raphanus sativus*) leaves Cauliflower (*Brassica oleracea*)	2
	Sector 29 C	Radish (*Raphanus sativus*) leaves vendor 1	1
	Sector 43	Mint (*Mentha*)	1
	Vegetable market, Sector 28	Coriander (*Coriandrum sativum*) leaves	1
	Vegetable market, Sector 33	Coriander (*Coriandrum sativum*) leaves	1 (Only with LAMP)
	Vegetable market, Sector 36	Spinach (*Spinacia oleracea*)	1
	Vegetable wholesale market, Sector 37 C	Lettuce (*Lactuca sativa*)	1
	Kitchen garden 2, Residential colony, PGI	Green chili (*Capsicum annuum*)	1
	Open market, Sarangpur area	Radish (*Raphanus sativus*) Swiss chard (*Beta vulgaris var. cicla*)	2
	Street vendors, open market, Manimajra area	Methi (*Trigonella foenum-graecum*)	1
	Open market, Khudda jassu area	Parsley (*Petroselinum crispum*) Green chili (*Capsicum annuum*) Cabbage (*Brassica oleracea*)	3
	Vegetable market, Sector 43	Cabbage (*Brassica oleracea*) Scallions (*Allium cepa*) Coriander (*Coriandrum sativum*)	4
	Vegetable market, Sector 40	*Methi* (*Trigonella foenum-graecum*) Coriander (*Coriandrum sativum*) Beetgreens (*Beta vulgaris*)	3
	Open markets, Sector 19-sector 20	Tomatoes (*Solanum lycopersicum*) & leaves	1
	Open market, Sector 23- sector 24	Spinach (*Spinacia oleracea*) Scallions (*Allium cepa*)	2
	Vegetable market, Sector 26	Arugula (*Eruca sativa*) Cabbage (*Brassica oleracea*) Rape (*Brassica napus*)	3
Total			33

Table 3. Distribution of positive vegetable samples from different areas of Chandigarh city, India.

gondii. All the positive samples showed a band at 193 bp corresponding to *B1* gene of parasite (Supplementary Fig. 1).

Real- time PCR assay

SYBR Green based Real-Time PCR assay was performed in all the water, soil and vegetables samples for *B1* gene of *Toxoplasma gondii*. Eighteen out of 100 water samples were positive of *T. gondii* (18%). In case of soil samples, out of 100 analysed, 9 were found positive for the parasite. In vegetable samples, thirty-three (33, 6.6%) samples were found positive out of 500 analysed with real-time PCR assay. The samples with C_t value < 30 was considered positive whereas the samples with C_t value > 30 were considered as negative. Melt curve analysis was

also performed in which the samples with fluorescence more than threshold were considered positive and below threshold were considered negative (Supplementary Fig. 2).

Real-time LAMP assay

Real-Time LAMP detected *T. gondii* DNA in 18 samples out of 100 (18%) analysed water samples. Both the *B1* gene targeted LAMP and *TgOWP* gene targeted LAMP was detected same number of positive samples. Water samples collected from tube wells, ponds, tap water collected from government schools, houses, canteens, gurudwaras, public parks, hospitals etc. were found to be contaminated with *T. gondii* oocysts (Supplementary Fig. 3).

Overall, the positivity rate of *Toxoplasma gondii* in water were 15% with conventional PCR assay and 18% with real-time PCR assay & real-time LAMP assay, soil 9% and vegetables 6.4% with conventional PCR assay and 6.6% with real-time PCR assay & real-time LAMP assay (Fig. 1).

Sanger sequencing and phylogenetic analysis

Sanger sequencing was performed for the 12 positive *T. gondii* isolates from water ($n=4$), soil ($n=2$) and vegetable samples ($n=6$) for the *B1* gene. The results for sequencing were obtained in FASTA files which were analysed using FinchTV software. The sequences were submitted to NCBI GenBank database and the accession number obtained were OQ448549, OQ448550, OQ448551, OQ448552. OQ448553. OQ448554 (vegetables samples) OP709775, OP709776 (soil samples) OQ448545, OQ448546, OQ448547, OQ448548 (water samples). MEGA software was used for phylogenetic proximity between the different *T. gondii* isolates ($n=12$) obtained from the study with other non-typing *B1* gene sequences ($n=45$) available on the NCBI database. The phylogenetic analysis revealed that the *T. gondii* isolates from environment and vegetables from our study were clustering with other partial *B1* gene sequences of cat, sheep, pig *T. gondii* isolates from Iran, Mexico, India and also with patient's *T. gondii* isolate from North Indian region. Moreover, the isolates from current study also clustered with *ME49* reference strain and *RH* reference strain of *T. gondii* (Fig. 2). The statistical analysis performed on the positivity rate of water samples using different molecular techniques from different water sources showed significance with p value < 0.0001 and F value 81.00.

Discussion

Toxoplasma gondii (*T. gondii*) is obligate protozoan with wide intermediate host range such as warm-blooded animals including humans, wild mammals etc. and is of significant veterinary importance[15,16]. It affects up to 30% of people worldwide. The infection is often contracted by consuming contaminated water or food with sporulated oocysts from cats infected with *T. gondii*, or by eating raw meat containing bradyzoites or tissue cysts[17,18].

Chandigarh is near the Shivalik Range of the Himalayas (North India) and has an area of around 114 km^2 and is situated on the border with Punjab and Haryana. Climate plays a vital role in the survival of *Toxoplasma gondii* oocysts in the environment. In regions with warm and moist climates, oocysts can survive for extended periods, increasing the risk of infection. Conversely, in colder and drier climates, oocysts may perish faster, leading to a lower prevalence of the parasite.

Hygiene practices can significantly impact the spread of *T. gondii*. Poor sanitation, improper waste disposal, and limited access to clean water increase the likelihood of environmental contamination with oocysts, leading to higher infection rates. At present, the Union Territory of Chandigarh has 23 villages which are divided into the sectoral and non-sectoral villages. There are around a million people residing in Chandigarh, and mostly residing in metropolitan areas. Although they are considered to be non-sectarian villages connected to the city, Kansal and Maloya have less developed infrastructure and socioeconomic status than the city proper and Rajiv

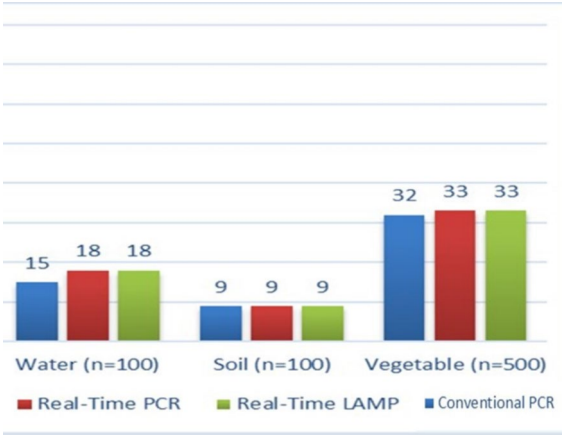

Fig. 1. Detection of *Toxoplasma gondii* DNA in water, soil and vegetable samples using three molecular techniques (Blue color: Conventional PCR, Red Color: Real-Time PCR, Green Color: Real-Time LAMP, n: Total number of samples analyzed for specific category, Numbers above the bars: Total no. of positive samples by technique).

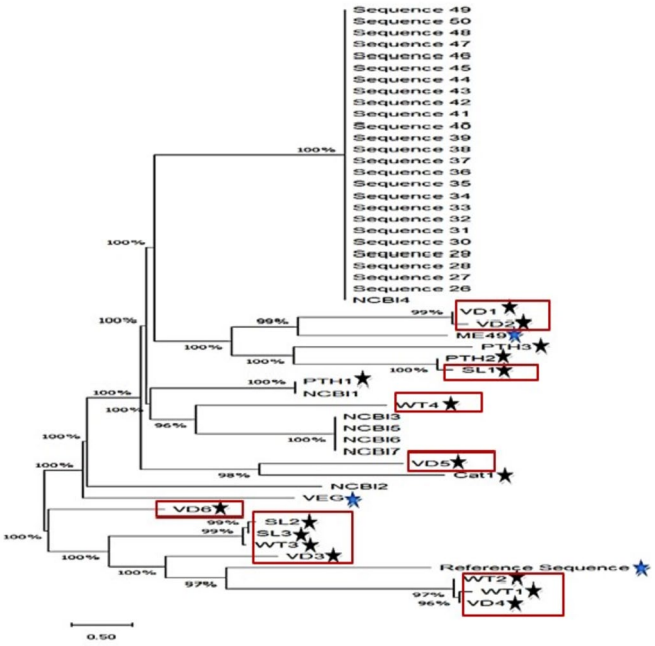

Fig. 2. Molecular phylogenetic analysis of different types of *Toxoplasma gondii*, using the B1 gene region by the Neighbour joining statistical method. The isolates within square box represents the *T. gondii* sequences from the present study (VD1, VD2, SL1, WT4, VD5, VD6, SL2, SL3, WT3, VD3, WT2, WT1, VD4), while the blue star denotes reference sequences (ME49, VEG, Reference sequence (RH)). The unsquared black star marked represent pigs and cats B1 gene sequences from India (PTH3, PTH2, PTH1, Cat1). Other represent similar B1 gene sequences from the GenBank. Percentages (bootstrap values) – Indicates confidence estimate in the branch.

slum colony is located in Sector 38 West. Chandigarh has seen an increase of slum settlements in recent years, particularly on the city's outskirts where low-income families prefer to live owing to the availability of affordable homes[19–21].

Oocysts of *T. gondii* have been identified in water samples across various regions globally such as France, Poland, Germany, Russia, Bulgaria, Scotland, Turkey, Brazil, and Ecuador[7]. The current study represents the detection of *T. gondii* detection in environmental water samples collected from the Chandigarh region using highly sensitive molecular techniques for first time from India according to literature review. In the current study, Conventional PCR for *B1* gene of *T. gondii* detected 15% samples positive for the parasite whereas real-time PCR and real-time LAMP for *B1* and *TgOWP* detected 18% samples positive for *T. gondii*. The findings from current study were similar to other studies such as Pinto-Durate et al., 20,222 which found *T. gondii* in 9.1% of water samples collected from Quindio River basin, Colombia[21]. Hernandez-Cortazar et al., 2017 found 5.4% of the drinking water samples positive for *T. gondii* from an endemic region in Southern Mexico[22]. Adamska. 2018 detected *T. gondii* in 19.4% of water samples collected from natural surface water bodies i.e., lakes, rivers and ponds in Poland[20]. Lass et al., 2022 investigated a total of 214 water samples taken from wastewater treatment plants, slaughterhouse and rivers in China. They detected *T. gondii* type I in 4 samples (1.9%) via real-time PCR and multilocus genotyping[23]. There may be a number of reasons of this the variable incidence (9–58%) of *T. gondii* DNA in water samples across various studies world-wide. Firstly, the source of water in these studies are different and differs from natural environmental sources like rivers, lakes, ponds; to sewage and treated drinking water. These different water sources have diverse environmental, and climatic conditions, as well as range of temperature, pH, and concentration of disinfectants which influence the diverse makeup of host populations. These variable factors which are crucial to the survival of *T. gondii* oocysts cause differences in potential of water sources being source of *T. gondii*.

In the present study, most of the positive samples for *Toxoplasma* were from the village areas and urban slums of Chandigarh city, India. This data would suggest that not all treatments are efficient or that treated water may get polluted, which is especially common in nations with poor water supply infrastructure. As a result, in addition to preventing the contamination of water that has been kept (in tanks, cisterns, and other containers), it is also essential to take into account the efficiency of treatments and handling of treated water for prevention of exposed population from waterborne toxoplasmosis.

This is the first study from India to our knowledge, using molecular techniques to estimate the degree of soil contamination with *T. gondii* at the Chandigarh city, India. It is difficult to detect low oocysts concentration in soil due to lack of techniques with sufficient sensitivity. Molecular methods such as conventional PCR, real-time PCR and real-time LAMP were used in the current study which are highly sensitive and specific and time saving tests. It was seen that 9% of soil samples were positive for *T. gondii* by all the three molecular techniques used. *T.*

gondii DNA was found in soil samples collected from urban slum areas, hospitals, public parks, organic farms, kitchen gardens, industrial area etc. Similar to the findings of our study, Ahmed et al., 2019 conducted a study in soil samples from Baghdad and Kut al-imara cities by targeting *B1* gene of *Toxoplasma gondii*. The results showed highest prevalence in gardens, schools and backyards of homes in Baghdad city (21.42%, 17.4%, 16.66%) and in the Kut al-imara city is (6%, 5.5%, 4%) respectively[24]. Other studies like Pinto-Durate et al. detected 28% positivity rate with nested PCR targeting *B1* gene for *T. gondii* in soil samples collected from Quindio River basin, Colombia[21]. Haghparast-kenari et al., 2020 found *Toxoplasma gondii* DNA in 78.1% of environmental soil samples in Mazandaran Province, Northern Iran by nested PCR of *RE* gene[25].

Soil is one of the environmental sources of *T. gondii* oocysts and these can remain infective for months to years. Understanding the spread of *T. gondii* soil contamination is important for determining the risk factors of toxoplasmosis in intermediate hosts as well as in humans[26]. Routes for soil contamination with *T. gondii* can be many. Cats, including kittens play in the soil residential and educational settings as seen in our study. Cats typically bury their waste in the primary dwelling area, while they occasionally leave smell trails along the outside of their territory[27,28]. Children are considerably more likely to get toxoplasmosis since they may not be aware of adequate hygiene precautions so to stop the spread of *T. gondii*[29].

In the present study, *Toxoplasma gondii* was also detected with molecular methods in green leafy vegetables for the first time in India. Positivity rate for *T. gondii* was found to be 6.4% in vegetable samples with conventional PCR for *B1* whereas real-time PCR and real-time LAMP for *B1* and *TgOWP* detected the parasite in 6.6% of samples and thus prevalence of *T. gondii* in vegetables correlates with other studies. Berrouch et al., 2021 from Marrakech, Morocco revealed an overall contamination of 29.6% with *T. gondii* in leafy green samples by real-time qPCR[30]. In another research conducted by Berrouch et al., the study examined vegetable samples, including carrot, coriander, lettuce, parsley, and radish, and identified a 16.6% contamination rate of vegetables with *T. gondii* using qPCR[31]. Similarly, Lass et al. from China utilized a real-time PCR assay and detected *T. gondii* DNA in 3.6% of the fresh vegetable samples[32].

Vegetable contamination is a direct result of feline activity, which also causes contamination of the surrounding environment, most notably the soil and water. Since they travel freely and can release millions of oocysts of the parasite into the environment through their faeces, stray cats most likely play a significant role in the epidemiology of toxoplasmosis. These animals could wander into regions where fruit and vegetables are being grown and infect them with *Toxoplasma* oocysts in addition to contaminating the water and soil.

In the present study *T. gondii* DNA has been detected in different types of vegetable samples such as coriander *Coriandrum sativum*, fenugreek leaves *Trigonella foenum-graecum*, radish *Raphanus sativus*, cabbage *Brassica oleracea*, beetroot and leaves *Beta vulgaris*, spinach *Spinacia oleracea*, capsicum *Capsicum annuum*, sprinkle onion *Allium cepa*, mint *Mentha*, lettuce *Lactuca sativa*, parsley *Petroselinum crispum*, arugula *Eruca sativa*, rape *Brassica napus and* tomato & leaves *Solanum lycopersicum*. Similar to the types of vegetable found contaminated in present study, Lass et al. detected the presence of *T. gondii* DNA in lettuce, spinach, Chinese cabbage, red cabbage and broccoli and kale samples[32]. Berrouch et al. found maximum contamination of *T. gondii* in parsley, followed by coriander, carrot, lettuce and radish[31]. Furthermore, the surface characteristics may also influence parasitic attachment. Vegetables with rough surfaces are more prone to contamination compared to vegetables with smooth surfaces, such as radish and carrot[33].

In current study, real-time PCR and real-time LAMP detected more positive samples for *Toxoplasma gondii* as compared to the conventional PCR. These results indicate high sensitivity of these techniques, that can detect even low concentration of parasite's genomic DNA in the targeted samples. Similar results were seen by Koloren and Demirel, in which LAMP for *B1* gene detected *Toxoplasma gondii* in 25% of natural water samples from different sources and nested PCR for *18srRNA* detected *T. gondii* in only 11.7% of samples[34]. Additionally, no false positives were reported using molecular methods, as every sample which came positive by atleast two molecular techniques.

The prevalence of *T. gondii* in the Chandigarh region can be attributed to interconnected factors, including poor sanitation practices in slums and villages, which lead to environmental contamination by stray cat feces containing oocysts, and variations in agricultural methods, such as the use of untreated irrigation water and manure from potentially infected animals. Inefficiencies in water treatment and distribution infrastructure further contribute to oocyst persistence in treated water, especially in rural and underserved areas. Additionally, the region's warm and humid climate creates favorable conditions for oocyst survival, increasing the risk of contamination through water, soil, and vegetables.

The isolates from our study showed > 98% sequencing identity with the other *T. gondii B1* gene sequences of cat, sheep, pigs isolates from Iran, India, Mexico and with patient isolate from North India available on the database with accession number LC057646.1, LC057649.1, MK521885.1, MK521884.1, MK521883.1, MK521882.1, MK521881.1, KX270388.1, KX270387.1 etc. Thakur et al. 2019 performed phylogenetic analysis and showed that the positive pig samples for *T. gondii* from Chandigarh region showed clustering with the VEG type III strain which is similar to findings from current study[35]. Low levels of *B1* gene polymorphism did not, however, allow for the differentiation of the clonal lineages of the several isolated strains. To identify the genotypes of *T. gondii* circulating in animal and environmental matrices in India, further research is needed that targets one or more loci of the parasite. Phylogenetic analysis will be crucial for this effort, as it reveals the genetic diversity within the parasite population and helps in understanding its evolution, transmission patterns, and the potential emergence of new strains or lineages[36].

Conclusion

This study provides the first comprehensive assessment of *Toxoplasma gondii* contamination in environmental water, soil, and vegetables in Chandigarh, India, using advanced molecular techniques. The findings highlight significant public health concerns, emphasizing the need for improved sanitation, water treatment, and food

safety measures to mitigate the risk of toxoplasmosis. While the study confirms the presence of *T. gondii* in various environmental sources, further research is required to investigate seasonal variations and the correlation between environmental contamination and human infection rates. Future studies should also explore genetic diversity and emerging strains to enhance surveillance and control strategies. Strengthening public awareness and preventive measures will be crucial in reducing the transmission of *T. gondii* and its associated health risks.

Methods

This prospective cross-sectional study was conducted in Chandigarh, a city in North India, having a predominantly urban population. The present study was ethically approved by the Institutional ethics committee of the Postgraduate Institute of Medical Education and Research, Chandigarh (Approval No: INT/IEC/2021/SPL-960). All the sampling was done strictly as per the guidelines.

Water sampling

Sampling Area and collection: A total of 100 water samples were collected randomly from different drinking and public water sources of Chandigarh region. The study was done from December 2020 December 2022. Sampling was conducted from each phase—Phase I, Phase II, and Phase III—in the Chandigarh region, as well as from villages within and around the area. This approach ensured that water samples were collected from every phase and villages of the city. Water samples were collected from different resources such as from ponds, lakes, tap water from household, public taps located in different schools, government offices and public parks, tube wells etc. (Table 4).

Sample processing

A total of 10 L of water samples was collected from each site in plastic bottles/ containers[37]. All the samples were transported to the department of Medical Parasitology for further processing. The water samples which were having less sediment and collected from the tap water located in schools, public parks, govt. offices, houses etc. were processed via filtration method[38,39]. Millipore water assembly was used with 0.22 µm of filter paper (Millipore). Ten litres of water samples was collected from each site and passed through the filtration assembly (5–6 h). Afterwards, filter paper was dipped in PBS solution (25 µL Phosphate buffer saline + 0.5 µL Tween 20) in 15 mL falcon for overnight. The pellet obtained were transferred into the new 1.5 mL micro centrifuge tube. Processing of dirty and pond water was done using the aluminium sulphate flocculation method[34,40]. Ten liters of homogenized water samples collected from ponds and lakes were treated with 50–100 µL of $Al_2(SO_4)_3$ solution for flocculation. The resulting pellets were washed twice with distilled water before being analysed for the presence of oocysts. These pellets, obtained through filtration and flocculation, underwent DNA extraction using the Qiagen Blood and Tissue Kit Qiagen, Germany (Cat. No. 69504), with modifications to the manufacturer's protocol as described[41]. The modifications included: subjecting samples to 5 freeze (in liquid nitrogen) and thaw cycles (in a water bath at 70 °C), extending the incubation time to 2–3 h for sample lysis using tissue lysis buffer and AL, eluting the DNA in 35–40 µL of elution buffer (AE) to increase the concentration of DNA in the samples. DNA obtained from the samples were stored at -20ºC until used.

Soil sampling

Sampling Area and collection: Sampling sites consist of public parks, urban slum areas, cattle sheds, hospitals, public schools, villages located in the outer boundary of the Chandigarh city, India. A total of 100 samples were collected over the period of two years from December 2020-December 2022 (Fig. 3). Sampling was done from every phase and nearby areas of the city to ensure the random sampling from the area i.e., Phase I, Phase II, Phase III, villages located in and around the Chandigarh region. A total of 20 g of soil was collected in sterile polythene bags from almost 5 cm below the surface layer with the help of a stainless-steel scoop. After collection, it was air dried for 24 h by spreading it on brown paper at room temperature. Air-dried samples were passed

City	Regions	Number of Samples
Chandigarh	Sector 1–30 (urban area)	53
	Sector 31–47 (urban area)	21
	Villages (Kaimwala, Dhanas, Khuda ali sehar, Vikas nagar, Khuda lohra, Maloya, Sarangpur, Khuda jassu)	16
	Near Manimajra	9
	Total	100
	Sampling sources	
	Ponds and lake	5
	Tap water, non-drinking (public taps etc.)	2
	Drinking water (house, temples, gurudwaras, schools, public parks etc.)	72
	Tube well	4
	Other	17
	Total	100

Table 4. Distribution of water sample collection ($n = 100$) from different areas and sources of Chandigarh city, India.

Fig. 3. Distribution of soil sampling area in Chandigarh city.

through a 20 mm mesh sieve to remove the large particles present in the soil that can hinder the DNA extraction process. After sieving, soil samples were stored in sterile uricols at 4 °C for further use[42,43].

DNA extraction

DNA extraction was done using the soil DNA extraction of NucleoSpin Soil Kit (MACHEREY-NAGEL GmbH & Co. KG, Valencienner Str. 11, 52355 Dueren, Germany) and GCC biotech kit (India Pvt. Ltd. Kolkata). Soil DNA extraction was done using these kit with slight modifications in the manufacturer's protocol. The modifications were as follows: (a) 300 mg of soil sample was used instead of the 250 mg specified in the protocol. (b) Among the two lysis buffers provided in the NucleoSpin Soil Kit (SL1 and SL2), SL2 was chosen for DNA extraction due to its superior performance when combined with Enhancer SX. (c) The centrifugation speed was increased to 12,000 rpm throughout all steps to achieve optimal results and high-quality output.

Vegetable sampling

Sample collection

Vegetable samples were collected from different areas of Chandigarh region such as farms (organic and other), street vendors, shops in the market, vegetable market, kitchen gardens etc. The sampling was done for a period of two years i.e., from December 2020 to December 2022. Different types of vegetables were collected such as *Coriandrum sativum* (coriander), *Beta vulgaris* (beetroot greens), *Trigonella foenum-graecum* (methi), *Brassica oleracea* (cabbage), *Raphanus sativus* (radish), *Mentha* (mint), *Spinacia oleracea* (spinach), *Lactuca sativa* (lettuce), *Capsicum annuum* (green chili), *Beta vulgaris var. cicla* (Swiss chard), *Petroselinum crispum* (parsley), *Allium cepa* (scallion), *Eruca sativa* (arugula), *Brassica napus* (rape), *Solanum lycopersicum* (Tomatoes and leaves) etc. Randomized sampling was done from Kaimwala, Dhanas, vegetable markets/shops in sector 26 & 27 and villages of Khuda ali sehar, Vikas nagar, from street vendors in sectors (38, 28,30,31, 32, 33, 34 etc.), Nayagaon, Khuda lohra, Maloya, Sarangpur, Manimajra, Khuda jassu (all villages), areas nearby hospitals, etc. A total of 500 vegetables samples were collected from various areas of the city as detailed above (Supplementary Table 1).

Sample processing

In a zipper bags/ stomacher bag, 50 g of fresh leafy vegetables, and one of each root vegetable (radish, carrot etc.) were placed. Washing solution of about 200 mL which consists of 0.1% Tween 80 in 0.01 M phosphate-buffered saline (PBS; pH 7.4) was added to the bag. The bags were then packed firmly and placed on an orbital shaker (Hoefer, Holliston, MA, USA) for 15 min. The sample bags were then repeatedly manually shakenupside-down. After that wash solution from the bag was transferred to the 50 mL falcons by sieving it through 4-layer gauge filter. Ultracentrifuge was used to centrifuge the wash solution from the bag at 5,000 g for 20 min and extra fluid was dumped. Rewashing the food samples in the zipper bags with 0.01 M PBS solution for 15 min followed by the same centrifugation of the wash solution as previously. The pellets were spun at 1,000 g for 20 min after being transferred to 50 mL tubes in a centrifuge. Finally, micro centrifuge tubes were used to collect pellets[44–46]. DNA was extracted from the pellet obtained using Qiagen Blood and Tissue Kit (Qiagen, Germany) with some modifications in the manufacturer's protocol as described in the processing of water samples.

Molecular analysis in environmental and fresh produce samples

Conventional PCR assay

All the water, soil and vegetables samples were subjected to conventional PCR assay for the housekeeping (*18srRNA*, small subunit rRNA*)* gene and *Toxoplasma gondii* specific glycerol-3-phosphate dehydrogenase, *B1* gene. Reaction mixture was prepared by adding 12.5 µL of master mix (Promega Corporation, USA), 0.8 µL of forward primer, 0.8 µL of reverse primer and variable template DNA (Supplementary Table 2)[47,48].

Real-time PCR assay
Real-Time PCR assay with SYBR Green based chemistry was performed on CFX96 real-time thermal cycler (Bio-Rad laboratories India Pvt.Ltd) machine in all samples for the detection of *Toxoplasma gondii* by targeting *B1* gene. The master mix for the reaction contained, 5µL SYBR Green dye, 0.3µL forward primer & reverse primer respectively (Supplementary Table 2), nuclease free water and template DNA (50 ng/µL of all samples). The reaction was put in opaque real-time PCR strips (0.2 mL). Thermocycling conditions were initial heating at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 0.15 s. and annealing at 60 °C for 0.30 Sects[23,49]. Melt curve analysis (MCA) was also performed from 65 °C to 95 °C at default setting to keep a check for the formation of non-specific products such as primer-dimer etc. Results were analyzed using the Bio-Rad CFX manager software.

Real- time LAMP assay
Real-Time LAMP was performed in all the samples for detection of *T. gondii*. This technique was performed on the Genie III instrument by Optigene Pvt. Ltd. Hyderabad, India. Using real-time LAMP, two genes of *T. gondii* were targeted for the detection i.e., Glycerol-3-phosphate dehydrogenase, *B1* and *Toxoplasma gondii* outer wall protein gene, *TgOWP*. The master mix contained 12.5 µL of Isothermal master mix (Optigene, Hyderabad, Cat. No. AL306), 1 µL of primer mix (40mM BIP, backward inner primer & FIP, forward inner primer, each and 10mM F3 & B3 each), template DNA (2–3 µL) and nuclease free water (Supplementary Table 2)[50].

Sanger sequencing and phylogenetic analysis
The purified PCR products were sent for sanger sequencing at the Central Sophisticated Instrument Cell (CSIC), PGIMER, Chandigarh. For the sequencing, ABI 3730 DNA Analyzer were and sequencing was performed according to the standard protocol[51]. Further, Molecular Evolutionary Genetic Analysis version 11.0.10 (MEGA-X) programme was used to construct neighbour joining tree with the isolates (soil, water and vegetables) obtained from our study and previously published non-typing *B1* gene sequences available on NCBI database[52].

Statistical analysis
One way- Anova was performed for water samples to compares the positivity rate of samples with *Toxoplasma gondii* via different molecular techniques using GraphPad Prism 8.0.2 (263) software (GraphPad Software, Inc.). For soil samples, graph was prepared for positive samples distribution from different sources using Graph Pad Prism software.

Data availability
All the data is submitted to NCBI GenBank Database. Accession numbers are provided in the manuscript for the same.

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Statements & Declarations Authors Information

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Author contributions
D. R.: Data curation, Writing- Original draft preparation, Investigation, Formal analysis, Software, Methodology, Investigation, Visualization P.D.: Validation, Resources, Writing - Review & Editing, Supervision D. S.: Writing - Review & Editing, Formal Analysis, Software C. K. B.: Sample collection R. S.: Funding acquisition, Writing - Review & Editing, Supervision, Project administration, Resources, Validation, Conceptualization.

Declarations

Ethics approval

The present study was ethically approved by the Institutional ethics committee of the Postgraduate Institute of Medical Education and Research, Chandigarh (Approval No: INT/IEC/2021/SPL-960).

Competing interests

The authors declare no competing interests.

Additional information

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