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Original Article

Serological and Molecular Evaluation of *Toxoplasma gondii* in Patients with Colorectal Cancer

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Abstract

Background: *Toxoplasma gondii* is an obligate intracellular protozoan parasite that causes severe disease in immunocompromised individuals. The relationship between this parasite and cancer is complex, as the infection may either promote cancer progression or exert antitumor effects. Colorectal cancer (CRC) is one of the most common cancers. This case–control study aimed to evaluate the serological and molecular prevalence of *T. gondii* infection in patients with colorectal cancer.

Methods: The study was conducted at Mashhad teaching hospitals, Khoran Razavi Province, Iran from Sep 2021 to Mar 2023, involving 156 participants. This included 78 people with CRC and 78 people without cancer, whose biopsy samples were subjected to pathological evaluations to detect cancer. Serological tests for anti-*Toxoplasma* IgG and IgM antibodies were performed using ELISA. Molecular tests utilized PCR analysis of biopsy samples to detect *T. gondii* DNA.

Results: 52.6% of CRC patients tested positive for anti-*Toxoplasma* IgG, compared with 46.2% in the control group. However, this difference was not statistically significant (P=0.423). Only one patient in the CRC group tested positive for IgM, indicating a recent infection. Molecular analysis did not detect any *T. gondii* DNA in the tumoral and normal tissues. Environmental risk factors associated with IgG positivity included living in rural areas, exposure to cats, and consumption of spring water.

Conclusion: Our study found no significant association between CRC and *T. gondii* infection. Though limitations such as small sample size and lack of Immunohistochemistry (IHC) warrant further investigation.

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Introduction

Toxoplasma is an obligate intracellular parasite belonging to Apicomplexa. This parasite can infect almost all warm-blooded animals and humans, making it one of the most common parasites worldwide. *T. gondii* is widely recognized as one of the most significant foodborne and waterborne parasites, with major implications for both veterinary and medical fields (1). This disease is a worldwide health problem for which various new treatments have been studied (2-4).

The primary concern of this infection is generally related to the fetus and immunocompromised individuals; it rarely causes disease in immunocompetent individuals (5). *T. gondii* can regulate innate immune cells by attacking myeloid cells and usually produces a robust Th1 immune response (6, 7).

Moreover, *T. gondii* is considered an agent with anti-tumor effects through mechanisms such as inducing hypoxia, inhibiting tumor angiogenesis, and promoting Th1 responses (8, 9). Immune and cellular mechanisms that result from Toxoplasma infection play an important role in enhancing Th1 cell polarization, which is essential for effective antitumor immunity (10).

The effects of *Toxoplasma* have been evaluated as a potential treatment for a wide range of cancers, including melanoma, breast, ovarian, lung, prostate, and pancreatic cancers. This promising research offers hope for the future of cancer treatment (10). On the other hand, infection or exposure to *T. gondii* is significantly associated with an increased risk of hematological malignancies and brain tumors, specifically glioma and meningioma (11). The antitumor or carcinogenic effects of Toxoplasma may depend on different conditions, including different responses of the immune system and the involved tissue. However, its specific role in CRC remains unclear and requires targeted investigations distinct from other cancer types.

Colorectal cancer (CRC) is one of the most common neoplasms in humans, and the contribution of different environmental risk factors has been widely investigated. Given the diverse reports about the effect of *Toxoplasma* on cancers, more studies are needed to clarify intricacies of its role in CRC (12). Our study investigated the serological and molecular aspects of *Toxoplasma* in patients with colorectal cancer (CRC).

We aimed to evaluate the relationship between seroprevalence as well as the presence of *Toxoplasma* DNA in colorectal tissue biopsy and CRC to provide a preliminary investigation of co-infection with the parasite and cancer.

Materials and Methods

In this case–control study conducted in Mashhad teaching hospitals Khoran Razavi Province, Iran from Sep 2021 to Mar 2023. Moreover, 156 participants were included, consisting of 78 individuals with CRC and 78 individuals without CRC, all over the age of 20. Patients were excluded from the study if they declined consent, had insufficient biopsy tissue for molecular analyses, or lacked adequate serum samples for serological testing. Our control group included patients who underwent biopsy due to chronic abdominal pain, chronic diarrhea, and unexplained iron deficiency anemia, with histology confirming the absence of colorectal cancer. Because we needed patients who had a biopsy sample but were negative for CRC, patients with other diseases such as inflammatory bowel syndromes and malignancies other than CRC were not excluded from our study. All participants underwent clinical evaluations, biopsy, and serological and molecular tests. Additionally, demographic information such as age, gender, occupation, place of residence, contact with animals (especially cats), and water sources was collected using a structured questionnaire.

Informed consent was obtained from all individuals participating in this project, and the text of the consent forms and ethical standards for conducting this project were approved by the Ethics Committee of Mashhad University of Medical Sciences with code IR.MUMS.MEDICAL.REC.1401.451. This work was supported by Mashhad University of Medical Sciences, Grant Number: 4001871

Serological Tests

Blood samples were collected, and serum was separated for anti-toxoplasma IgG and IgM tests using an ELISA kit (Pishtaz Teb Diagnostics). The absorbance was measured using a microplate reader (Epoch, BioTek Instruments, Inc., Highland Park, Winooski, USA) at 450 nm. Positive and negative samples were determined by calculating the index for each sample by dividing the sample OD by the Cut-off value, according to the kit protocol. Patients with positive pathology reports were included in the test group, while patients without cancer were included in the control group.

Molecular Tests (PCR)

While real-time PCR is known for its higher sensitivity, conventional PCR was chosen for this study due to its specific aims. Conventional PCR is sufficient for detecting higher levels of *Toxoplasma* DNA, which was adequate for our primary objective of determining the presence or absence of the parasite's DNA in tumor tissue (13).

Biopsy samples were analyzed to detect the presence of *T. gondii* DNA. First, tissue samples were cut into small pieces and homogenized using a homogenizer. After that, the sample was placed in a 58 °C water bath overnight with a pre-lyse solution and proteinase K, and DNA was extracted using an extraction kit (Parstous tissue Genomic DNA Extraction Kit, Parstous company, Mashhad, Iran). Nanodrop (yielding a mean 260/280 ratio of 1.85) and gel electrophoresis confirmed the accuracy of the extraction.

Our study aimed to detect the overall presence of *T. gondii* DNA, regardless of the life stage. Therefore, we focused on the REP and B1 genes, which are more sensitive for general *Toxoplasma* detection (13). Subsequently, a two-reaction PCR test was conducted for each sample using specific primers, ToxoREP and ToxoB1 (Table 1). A negative control consisting of distilled water and a positive control containing *Toxoplasma* DNA were included in each PCR run. The PCR amplification was performed using a thermal cycler (ASTEC PC-818S thermal cycler) with an initial denaturation at 95 °C for 4 min, followed by 35 cycles of amplification (denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 45 sec) with a final extension at 72 °C for 10 min. PCR products were analyzed using gel electrophoresis.

Table 1: Sequence of primers used for the two REP and B1 genes in this study

REP	**Forward**	**CGCTGCAGGGAGGAAGACGAAAGTTG**
	Reverse	CGCTGCAGACACAGTGCATCTGGATT
B1	Forward	AAATGTGGGAATGAAAGAGACGCTAATGT
	Reverse	GCGACCAATCTGCGAATACACCAAAGT

Statistical analysis

The data were analyzed using SPSS software ver. 23 (IBM Corp., Armonk, NY, USA). Quantitative variables were analyzed using the t-test, and qualitative variables were analyzed using the chi-square test. Logistic regression models were employed to assess the relationship between *Toxoplasma* positivity and environmental factors. A P-value of less than 0.05 was considered statistically significant.

Results

52.6% of the patients with CRC tested positive for anti-*Toxoplasma* IgG, compared to 46.2% in the control group. However, the difference in seroprevalence rates between the two groups was not statistically significant (*P*=0.423). Furthermore, only one patient in the CRC group tested positive for anti-*Toxoplasma* IgM, indicating a recent infection, while no positive samples were found in the control group.

In molecular analysis of tumor biopsies, no Toxoplasma DNA sequences were detected among the 156 samples evaluated, indicating the absence of the parasite in tumor and normal tissues (Fig. 1).

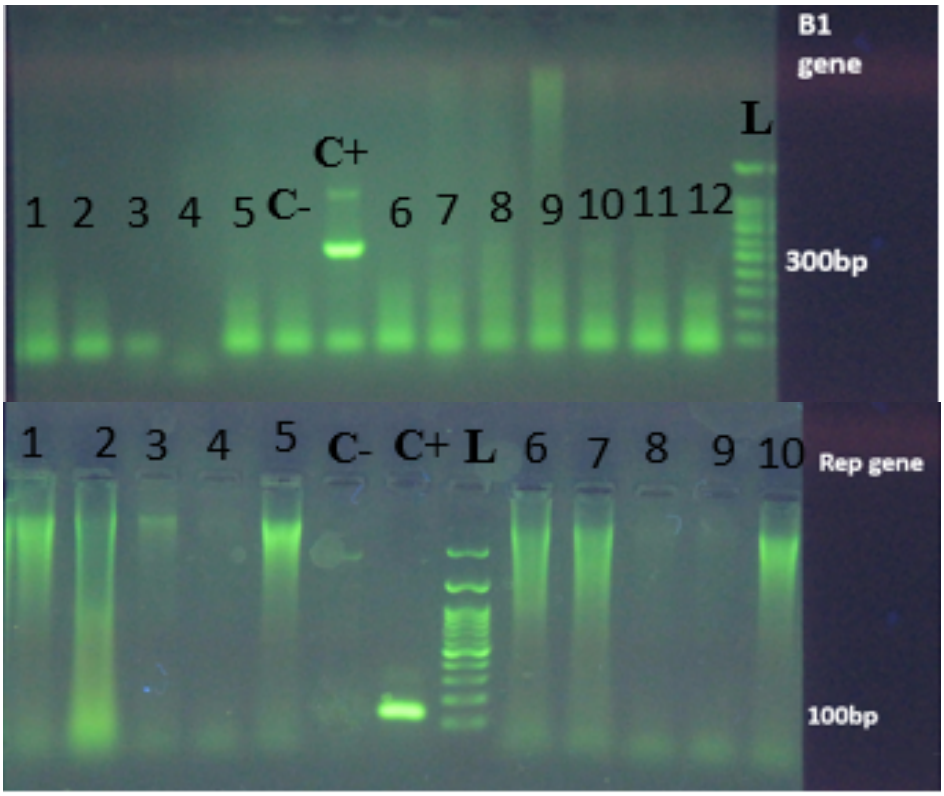


Fig. 1: Agarose gel electrophoresis of PCR products

The PCR electrophoresis gel displays the DNA ladder (L), positive control (C+), and negative control (C−). The corresponding samples are numbered 1 to 12 for the B1 gene and 1 to 10 for the Rep gene. A distinct band is clearly visible at C+, confirming that the PCR reaction worked properly. In contrast, the complete absence of bands in all numbered sample lanes indicates that no *T. gondii* DNA was present in the tested specimens.

Three important factors were associated with *Toxoplasma* IgG positivity: rural residence (*P*=0.001), exposure to cats (*P*<0.001), and consumption of spring water (*P*=0.001). These findings indicate that exposure to environmental risk factors, regardless of cancer status, plays an important role in the transmission of *Toxoplasma*. However, no direct causal link to CRC was established. IgG seropositivity did not differ significantly between CRC patients and controls, either overall (P = 0.423) or within any subgroup defined by sex, cat exposure, drinking water source or place of residence (Table 2).

The average age of cancer patients was 59.87 ± 11.57 yr, significantly higher than that of the control group (48.28 ±17.32, $P<0.001$). No significant difference was observed in gender distribution between groups ($P=0.521$). This age disparity may influence seroprevalence comparisons and should be controlled for in future analyses.

Table 2: Association between socioeconomic/environmental risk factors and anti-T. gondii IgG seropositivity among patients with colorectal cancer (CRC) and without cancer

Risk factor	CRC patients N(%)		Without cancer N(%)		*P* value
	IgG+	IgG−	IgG+	IgG−	
Overall	42 (53.8)	36 (46.2)	36 (46.2)	42 (53.8)	0.423
Sex					
Male	21 (45.7)	25 (54.3)	15 (39.5)	23 (60.5)	0.663
Female	21 (65.6)	11 (34.4)	21 (52.5)	19 (47.5)	0.338
Cat contact					
Yes	16 (69.6)	7 (30.4)	15 (75.0)	5 (25.0)	1.000
No	26 (47.3)	29 (52.7)	21 (36.2)	37 (63.8)	0.257
Drinking water source					
Spring water	15 (75.0)	5 (25.0)	11 (73.3)	4 (26.7)	0.711
Well water	4 (50.0)	4 (50.0)	4 (80.0)	1 (20.0)	0.565
Tap water	23 (44.9)	27 (55.1)	21 (36.2)	37 (63.8)	0.430
Residential area					
Rural	17 (53.1)	15 (46.9)	15 (83.3)	3 (16.7)	0.216
Urban	23 (50.0)	23 (50.0)	21 (35.0)	39 (65.0)	0.164

Values are n (%), where % is the proportion of that group within the row category (IgG+ and IgG− percentages therefore sum to 100% within each group).

P values: Fisher's exact test comparing IgG seropositivity between CRC patients and controls within each row category

Discussion

The relationship between *T. gondii* and cancer has emerged as a significant area of research, contributing to the broader discussion on the impact of infectious agents in the pathogenesis of this cancer. The ability of *T. gondii* to induce immune reactions against tumors is a fascinating object of investigation. *Toxoplasma* infection and its antigens increase the antitumor response against cancers such as melanoma, breast, ovarian, pancreatic, and lung cancer by stimulating the immune system and inducing the production of interferon gamma and interleukin 12 and activating $CD8^+$ cells (14-28). On the other hand, *T. gondii* infection/exposure is linked to an elevated risk of developing brain tumors, particularly gliomas and meningiomas (11). Additionally, according to another study, toxoplasmosis could be linked to an increased risk of hematological cancers (12).

Based on these findings, we aimed to evaluate *Toxoplasma* status in patients with CRC and to investigate the relationship between *Toxoplasma* antibody status and the detection of its genomic sequence in CRC cancer biopsies. CRC is a malignancy that affects the colon or rectum and is one of the most common types of cancer in the world, contributing to a remarkable number of deaths (29). Moreover, risk factors such as age over 50 yr, family history, genetic syndromes, and lifestyle factors including smoking, obesity, and physical inactivity, are recognized as predisposing factors for CRC (30).

Our study used PCR to detect *T. gondii* DNA. Numerous real-time PCR tests using different repetitive PCR targets for enhanced detection of *T. gondii* have been conducted, including the B1 gene, which contains 35 copies in the parasite's genome (31-33). Nonetheless, REP-529 gained prominence when a small amount of *T. gondii* DNA was detected in a sample, as this target is present in greater abundance than the *B1* gene (31,34). REP-529 demonstrated greater sensitivity than *B1* in detecting a single parasite in the sample, whereas *B1* had a limit of detection of 100 parasites (32- 36). We also used primers for these two genes for molecular evaluations. The failure to detect DNA may be due to limited assay sensitivity, sampling constraints, or actual absence, which future studies could clarify using real-time PCR or Immunohistochemistry (IHC) for localization.

Another gene that has been used in many articles is *BAG1*. The *BAG1* gene encodes a bradyzoite-specific antigen, a small heat-shock protein (HSP30) (37).

Other genes, such as *P30* and ribosomal primers, could detect 1 pg of *T. gondii* genomic DNA. However, due to their lower sensitivity compared to the *B1* gene, they were not used in our study (38).

In this study, we used conventional PCR for molecular investigation. Real-time PCR is more sensitive than conventional PCR. Real-time PCR was compared with conventional PCR for the SAG1 gene assay of *T. gondii*; the former was able to detect as few as 1 tachyzoite/reaction. Such high sensitivity provides an excellent way for very early detection of *T. gondii* in infected specimens. On the other hand, conventional PCR showed much lower sensitivity, detecting only about 100 tachyzoites. This lower sensitivity meant it could not detect the parasite in blood samples and showed positive results only in the spleen and lungs, significantly later than real-time PCR (39). The use of conventional PCR can be suitable for examining the presence of *T. gondii* DNA in tissue samples such as those evaluated in our study. Future studies could employ IHC to investigate the parasite's localization within tumor tissue, potentially providing insights into the spatial relationship between *Toxoplasma* and CRC cells.

Anvari and colleagues, based on systematic review and meta-analysis findings from 57 studies, reported a pooled prevalence of *T. gondii* infection at 30.8% in cancer patients worldwide. Interestingly, the highest prevalence was observed in 81% of patients who have breast cancer, though strong associations between infection rates and several factors, including type of cancer, sex, age, and geographic region, were also disclosed (40).

In a study which investigated the relationship between *T. gondii* and CRC, 77.42% of patients tested positive for *Toxoplasma* IgG antibodies, compared with 20.54% in the control group (41). In our research, although anti-IgG levels against *Toxoplasma* were higher in the CRC group than in the control group, no significant difference was observed between the two groups. This non-significance may be influenced by our small sample size and the age disparity between groups, which future studies could address by using larger cohorts and multivariate analyses controlling for confounders such as age.

Moreover, our evaluation revealed that three factors, residing in rural areas, contact with cats, and consumption of spring water, are associated with the transmission of *Toxoplasma*, consistent with previous studies (1).

A case-control study of 287 colorectal cancer patients and 287 matched healthy controls in eastern China, reported a significantly higher overall T. gondii seroprevalence in CRC patients (16.7%) compared to controls (9.1%, P=0.006), driven predominantly by IgG positivity (16.0% vs. 9.1%, P=0.007), while IgM rates did not differ significantly (2.8% vs. 1.1%, P=0.13). Multivariable logistic regression identified rural residence (OR 2.83) and chemotherapy treatment (OR 2.16) as independent risk factors for infection. In contrast, our study in an Iranian population found a

higher overall seroprevalence (52.6% in CRC vs. 46.2% in controls) but failed to demonstrate a statistically significant association between *T. gondii* infection and CRC ($P = 0.423$). Additionally, while Yu et al. reported significant risk factors including rural residence and chemotherapy, our study identified rural residence, cat exposure, and spring water consumption as environmental risk factors, though none were specifically associated with CRC. The discrepancy between these findings may be attributed to differences in sample size (287 vs. 78 per group), geographic and dietary variations between eastern China and Iran, and the absence of molecular detection of *T. gondii* DNA in our CRC tissue biopsies, which was not evaluated in the Yu et al. study (42).

Finally, the mechanisms by which *Toxoplasma* might influence cancer are complex and not fully understood. The interaction may be indirect or mediated through other immune factors, making direct correlations difficult to establish. Variability in the patient population, including genetics, lifestyle, and environmental exposures, may influence the prevalence of *Toxoplasma* and the development of colorectal cancer in ways that are not immediately obvious. Our study confirmed the association between *T. gondii* IgG positivity and rural residence, cat exposure, and spring water consumption. Future studies could investigate how these risk factors might indirectly influence CRC development. For instance, research could explore the interplay between *Toxoplasma* infection and other established CRC risk factors, such as diet, lifestyle, or genetic predisposition, in individuals with these risk factors. Additionally, incorporating detailed clinical data on CRC (e.g., tumor type, stage, size) could help design more targeted studies, as this information was not collected here but would enhance understanding of parasite-tumor dynamics.

Our research is strong because it combines serological and molecular approaches, providing a thorough assessment of *Toxoplasma* infection in patients with CRC. However, the study is limited by a relatively small sample size, which restricts generalizability and statistical power, and by its focus on a single geographic region (Mashhad, Iran), warranting caution in extrapolating to broader populations. The absence of IHC or alternative techniques to localize T. gondii within tumor tissues limits insights into parasite-tumor interactions. Additionally, the lack of longitudinal follow-up precludes assessment of *T. gondii's* influence on CRC progression or outcomes. The significant age disparity between groups was not fully adjusted for, potentially biasing seroprevalence comparisons. The study also did not include detailed clinical data on CRC, such as tumor type, stage, or size, which could inform subgroup analyses.

Future research should validate these findings in larger, multi-center studies to increase statistical power and representativeness. Incorporating IHC for parasite localization, real-time PCR for enhanced sensitivity, longitudinal designs to track outcomes, and adjustments for confounders such as age would substantiate the conclusions. Collecting comprehensive CRC clinical data (type, stage, size) would also aid in designing more robust investigations.

Conclusion

There was no significant association between *T. gondii* infection and CRC, although these results are preliminary due to methodological limitations, confounding factors such as age, and small sample size. Further research is needed to investigate the impact of chronic infections on cancer development and any potential links between *T. gondii* and cancers.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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