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Genotyping Of Toxoplasma Gondii Strains In Patients With Hepatitis B And C Demonstrated A Predominant Distribution Of The Type II Genotype

Nawadir H. Jasim[1] , Ali B. M. Al-Waaly [2]
[1,2]Department of Biology, College of Sciences, University of Al-Qadisiyah, Al-Qadisiyah 58001, Iraq

ABSTRACT
Background: Toxoplasma gondii is a globally prevalent intracellular parasite causing toxoplasmosis, with serious health and economic impacts. It infects various warm-blooded hosts and can affect multiple organs, including the liver. Its pathogenicity is linked to genetic diversity, primarily among genotypes I, II, and III. The GRA6 gene, due to its high polymorphism, is a reliable molecular marker for distinguishing these genotypes and assessing strain variation more effectively than the commonly used B1 gene.
Methods: A total of 100 samples from chronic hepatitis patients were screened for Toxoplasma gondii using ELISA; 62 were positive. B1 gene PCR confirmed infection in selected samples, and 10 B1-positive samples (5 HBV, 5 HCV) were tested for the GRA6 gene using nested PCR. DNA was extracted with a commercial kit, and amplification used specific primers. Genotyping was performed via PCR-RFLP targeting the GRA6 gene, using the MseI enzyme to distinguish T. gondii strains based on fragment sizes.
Results: This study used nested-PCR and RFLP targeting the GRA6 gene to genotype Toxoplasma gondii in hepatitis B and C patients in Thi-Qar, Iraq. Of 100 ELISA-screened samples, 20 tested positive for the B1 gene by PCR, and 5 from each group (HBV, HCV) were GRA6-positive. RFLP analysis using MseI differentiated genotypes I, II, and III. Among chronic liver diseases patients, genotype II was dominated.
Conclusions: Our recent study genotyped Toxoplasma gondii in HBV and HCV patients using nested-PCR and RFLP targeting the GRA6 gene. Genotypes II predominated in Hepatitis patients. These findings align with regional data showing genotype II's prevalence in immunocompromised individuals, highlighting the importance of molecular genotyping for understanding strain distribution and its potential impact on disease outcomes.
KEYWORDS: Toxoplasma gondii; PCR-RFLP; GRA6 gene; Nested-PCR and Genotyping

INTRODUCTION

Toxoplasma gondii, a single-cell obligate intracellular apicomplexan protozoan parasite that can infiltrate and multiply inside all nucleated cell types of warm-blooded mammals, is the causative agent of the most common zoonotic disease, toxoplasmosis. One-third of the world's population is infected with T. gondii, which has a sero-prevalence of 10% to above 90% [1]. It causes potentially dangerous diseases in both humans and animals, which has a significant negative impact on global public health and the economy. The asexual tachyzoite, the active and lytic form of the parasite that causes life-threatening infections, is found in a wide range of intermediate hosts, including humans, sheep, pigs, rodents, and birds, according to reports. The parasite's slow-growing tissue cyst type can primarily develop cysts in the tissues of the brain and muscles. Definitive hosts belong to the Felidae family [2]. A T. gondii infection can range in clinical manifestation from asymptomatic to extremely sick. The host's liver, heart, eyes, lymph nodes, and central nervous system may all be affected by the parasite. Several liver histological aberrations such as granuloma, hepatitis, necrosis, and hepatomegaly are associated with the parasite. Meanwhile, an epidemiological investigation found a link between T. gondii infection and liver cirrhosis [3]. Global studies have shown extensive genetic and phenotypic diversity of T. gondii among which, three main clonal lines (types I–III) have been described which harbor phenotypic variations in virulence and laboratory mouse lethality [4]. Parasitic-DNA may not be sufficient to get the desired effect. By the PCR-RFLP method using the Tru1I (MseI) restriction enzyme, the GRA6 gene (dense granule protein gene) can distinguish three T. gondii genotypes, and this gene is a single copy gene and has higher polymorphism rate compared with other markers [5]. Since it discriminates between the three genetic types and some atypical genotypes of the parasite, the GRA6 gene, which is a highly polymorphic locus, is frequently considered one of the best markers for evaluating the genetic variability of T. gondii. Isolates with the GRA6 gene, therefore, exhibit more unique features when compared to isolates with the B1 gene

[6,7]. The current work aimed to molecularly

MATERIALS AND METHODS

Sample collection

A total of 100 samples were collected from patients suffering from chronic liver diseases (Hepatitis B and Hepatitis C) during the period from January 2024 to December 2024. Initially, these samples were screened for Toxoplasma gondii infection using the ELISA technique. 62 of 100 were sero-positive for ELISA test. Subsequently, ELISA-positive samples were all subjected to confirmatory testing by detecting the B1 gene using the PCR technique (Data not shown). Afterward, 10 samples (5 samples from Hepatitis B and 5 samples for Hepatitis C) of B1 gene-positive samples were subjected to confirmatory testing by detecting the GRA6 gene using the nested PCR technique. Informed consent was obtained from all individual participants included in the study.

DNA extraction

Genomic DNA was extracted from the positive samples using a commercial kit (gSYNC™ DNA Extraction Kit) provided by (Geneaid Biotech LTD, Korea) and following the manufacturer's instructions. The purity of the extracted DNA was then assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA). Extracted DNA was kept at -20 C for further genetic analysis.

Nested-PCR amplification targeted GRA6 gene

A 10 positive samples of B1 gene were randomly chosen and then subjected to further molecular analysis through nested polymerase chain reaction (nPCR) targeting the GRA6 gene. Two pairs of primers were employed in this procedure: GRA6-FO1 (5′-GGCAAACAAAACGAAGTG-3′) and GRA6-RE1 (5′ CGACTACAAGACATAGAGTG-3′) for the first round of amplification, followed by GRA6-F1x (5′ GTAGCGTGCTTGTTGGCGAC-3′) and GRA6-R1x (5′-TACAAGACATAGAGTGCCCC-3′) for the second round [8–11]. The initial PCR reaction was carried out in a total volume of 25 µL, consisting of 10 µL of 2X PCR master mix (Promega Corporation, USA), 1 µL of each primer (forward and reverse), 5 µL of DNA template, and 8 µL of nuclease-free water. The second round of amplification was performed using the same total volume and reagent concentrations; however, the template used was 5 µL of the first-round PCR product. The thermal cycling conditions for both rounds included an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing 55 °C for 30 seconds and extension at 72°C for 1 minute, and a final extension at 72°C for 6 minutes. The annealing temperature was 55°C for the first round and 58°C for the second round. Amplified products were then analyzed using 2% agarose gel electrophoresis to visualize the nPCR results

PCR-RFLP for strain detection

To genetically characterize Toxoplasma gondii strains, PCR-restriction fragment length polymorphism (PCR-RFLP) analysis targeting the GRA6 gene was performed using the MseI restriction enzyme (New England Biolabs, UK). This technique uses particular nested PCR (nPCR) amplicon digestion patterns to differentiate Toxoplasma gondii genotypes. Type I strains produce fragments of 168 and 544 base pairs, type II strains produce fragments of 75 and 623 base pairs, and type III strains produce fragments of 97 and 544 base pairs. The MseI enzyme cuts the polymerase-amplified fragments of GRA6 genes into distinct sizes. Based on the instructions from the manufacturer, digestion was carried out by incubating the nPCR products with the restriction enzyme MseI for four hours at 37°C. After the digestion, the enzyme-treated fragments were electrophoretically separated in 2% agarose gels. This step allowed the verification of the digestion by the enzyme and the identification of the allelic profiles of the Toxoplasma gondii strains.

RESULTS

For the GRA6 marker on chromosome X, we employed RFLP-nPCR to give a highly sensitive genotyping of T. gondii. The variation in this gene was identified by the GRA6 nPCR marker. Using MseI to digest the amplified GRA6 product allowed for the differentiation of genotypes I, II, and III. As a result, using different RFLP patterns, the GRA6 marker can clearly identify all three genotypes. Blood samples from patients with hepatitis (B and C) disease were used in this investigation. Blood was available from the Digestive System Hospital in Thi-Qar province, Iraq. The samples came from 71% men and 29% women, ages ranging from 20 to 75 years old.

Out of 100 suspected cases, 62 blood samples tested positive on the ELISA test (data not shown), the results obtained from the ELISA-positive samples were further confirmed through the detection of the toxoplasma gondii B1 gene using polymerase chain reaction (PCR). The analysis revealed that amplification of the B1 gene was observed in 10 out of 34 samples (29%) obtained from patients infected with hepatitis B virus (HBV). Similarly, among patients infected with hepatitis C virus (HCV), 10 out of

28 samples (36%) tested positive for the B1 gene (data not shown). In a subsequent step, the samples that tested positive for the Toxoplasma gondii B1 gene were re-examined to confirm the presence of the GRA6 gene using nested polymerase chain reaction (nested-PCR). Positive results were identified by the appearance of specific DNA bands corresponding to Toxoplasma gondii at an expected size of 791 bp through nested PCR amplification, and after transferring PCR products to a 2% agarose gel, amplified fragments were detected under UV light. The analysis showed that 5 out of the 10 samples tested positive for the Toxoplasma gondii GRA6 gene among both groups of patients (HBV and HCV, respectively), after using the nested PCR method by amplifying the GRA6 gene target.

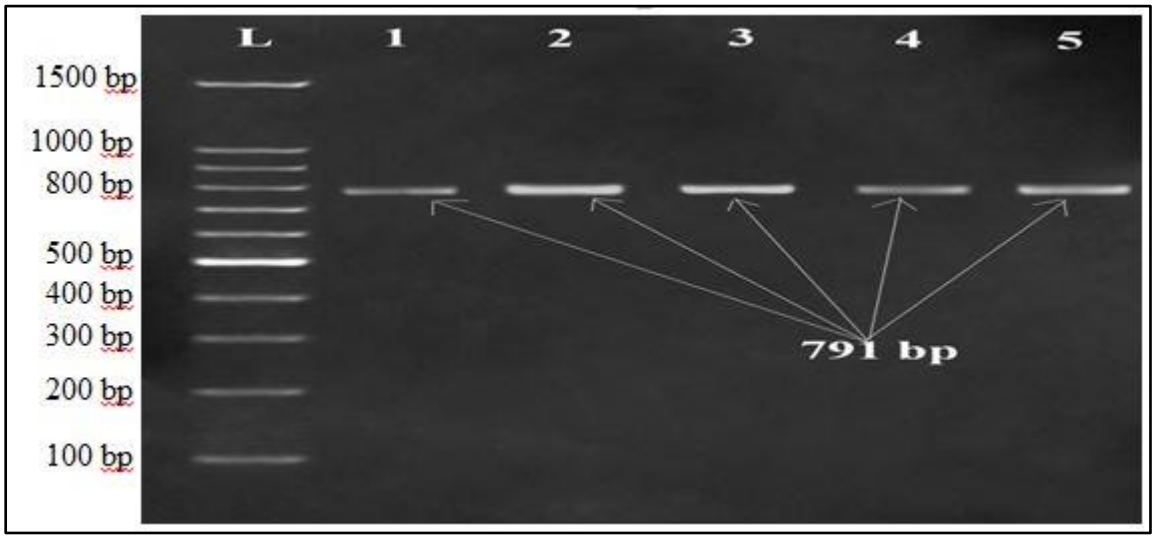

Figure 1.Nested-PCR detection of GRA6 gene in HBV-infected patients. Gel electrophoresis showing GRA6 gene amplification in five randomly selected samples from patients with hepatitis B virus infection. All samples (lanes 1-5) yielded positive amplicons at the expected size of 791 bp, confirming the presence of T. gondii DNA. M: 100 bp molecular weight marker.

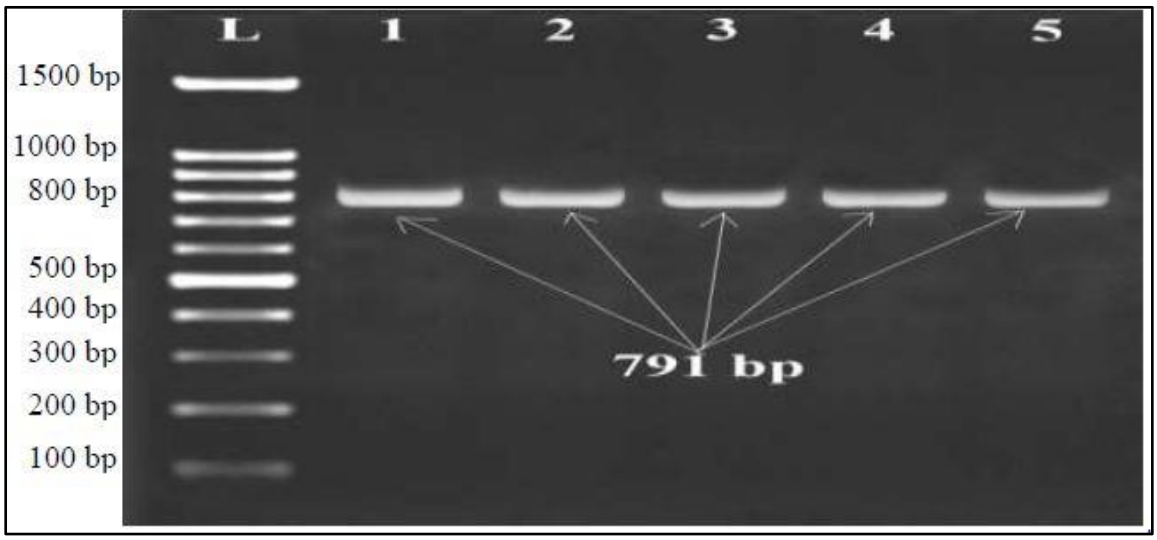

Figure 2.Amplification of GRA6 gene of T. gondii in patients with Hepatitis C Virus (HCV) Infection. Nested-PCR analysis targeting the GRA6 gene in blood samples from HCV-positive individuals. All five tested samples (lanes 1-5) show clear and specific bands at 791 bp, indicating consistent detection of T. gondii DNA. M: DNA ladder (100 bp).

The genotype of Toxoplasma samples was determined by utilizing RFLP-PCR to assess Toxoplasma gondii genotypes after gene-specific amplification of the GRA6 product was digested by restriction enzymes. Following PCR-RFLP, all GRA6 positive samples were treated with MseI endonuclease, and the type of samples was identified based on patterns of digestion. Under UV light, bands were found on all positive DNA fragments that had two cut sites for the MseI restriction enzyme. Following enzymatic digestion, the endonuclease generated distinct DNA fragment patterns corresponding to the different Toxoplasma gondii genotypes: 168 bp and 544 bp fragments indicated type I, 75 bp and 623 bp fragments

were characteristic of type II, while type III was identified by the presence of 97 bp and 544 bp fragments

Table 1. Nested-PCR Results and RFLP Patterns for GRA6 Gene

Sample No.	Disease Type	Nested-PCR Result	Genotype (MseI digestion)	Fragment Sizes (bp)
1	HBV	Positive	Type I	168 bp, 544 bp
2	HBV	Positive	Type I	168 bp, 544 bp
3	HBV	Positive	Type II	75 bp, 623 bp
4	HBV	Positive	Type II	75 bp, 623 bp
5	HBV	Positive	Type III	97 bp, 544 bp
6	HCV	Positive	Type II	75 bp, 623 bp
7	HCV	Positive	Type II	75 bp, 623 bp
8	HCV	Positive	Type II	75 bp, 623 bp
9	HCV	Positive	Type II	75 bp, 623 bp
10	HCV	Positive	Type I	168 bp, 544 bp

The genotyping results based on restriction enzyme digestion of the nested-PCR amplicons for the GRA6 gene revealed that, among HBV-infected patients, Toxoplasma gondii genotypes I and II were each identified in two isolates, while genotype III was detected in a single isolate.

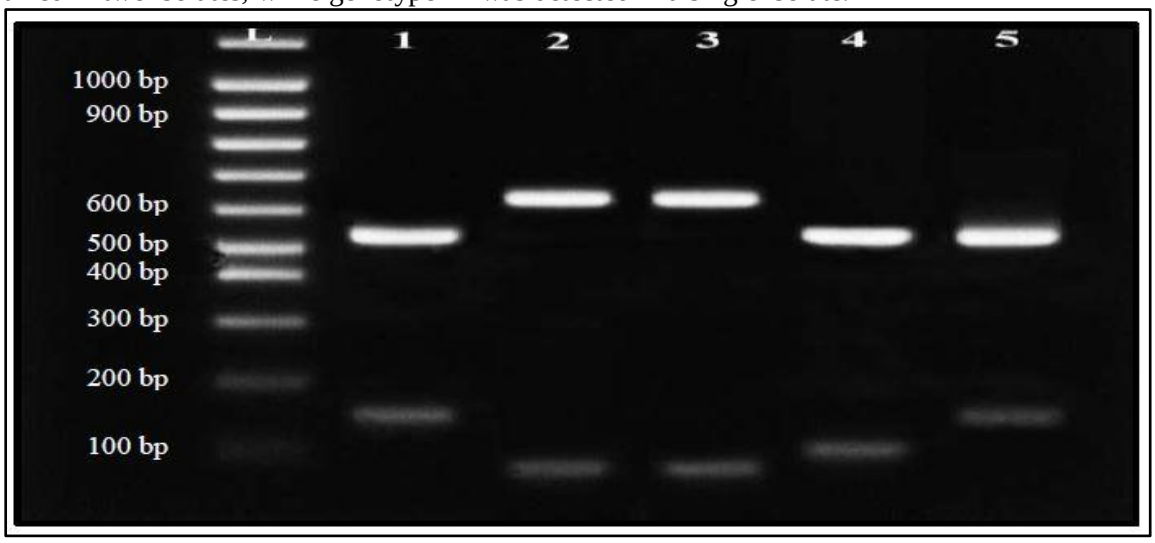

Figure 3. Genotypic differentiation of Toxoplasma gondii isolates from Hepatitis B patients based on GRA6 gene digestion with MseI. Agarose gel electrophoresis of GRA6 gene PCR products digested with MseI from T. gondii DNA. Lanes (1 and 5) Type I; Lanes (2 and 3) Type II and Lane (4) Type III; L; Ladder (100 bp).

In HCV-infected patients, genotype II was the most frequently detected, appearing in four isolates. Genotype I was identified in only one isolate, whereas genotype III was not detected in any of the examined samples (Figure 4).

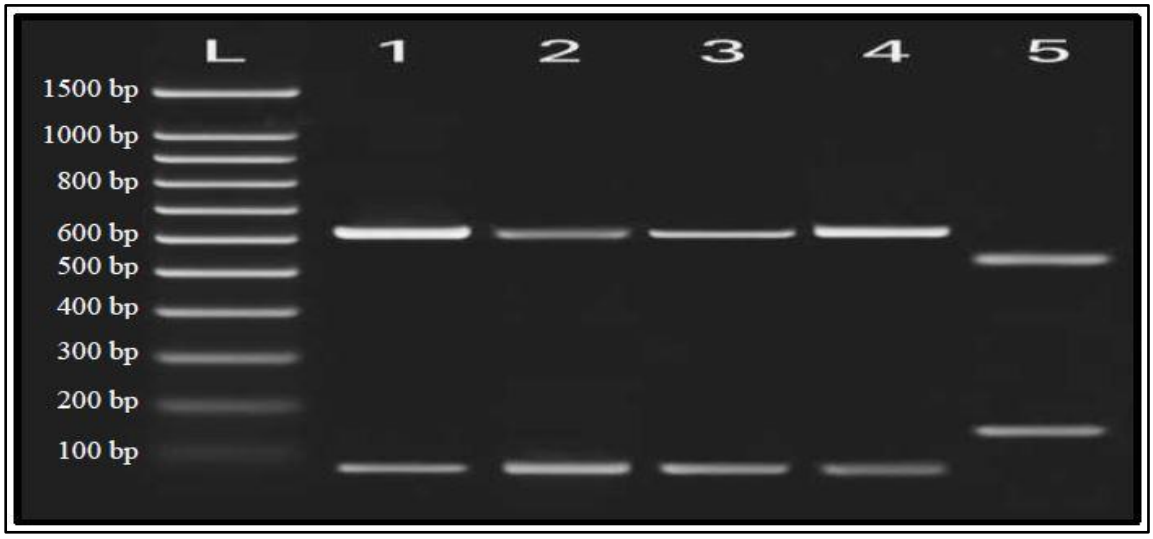

Figure 4.Molecular genotypic of Toxoplasma gondii in Hepatitis C via GRA6 gene PCR-RFLP using MseI enzyme. Electrophoresis profile of GRA6 gene PCR products digested with MseI from T. gondii DNA. Lane (5) Type I; Lanes (1, 2, 3 and 4) Type II; No Type III was detected; L; Ladder (100 bp).

DISCUSSION

Several genetic markers have been utilized for T. gondii strain typing, among these markers, GRA6 gene is generally used for genetic characterization and typing of T. gondii isolated from humans, animals, and meat products (12–14). To the best of our knowledge, there is no study on genotyping of T. gondii isolates from hepatitis patients using PCR-RFLP methods, in Iraq; therefore, this study was designed for the characterization and analysis of the genetic variation of T. gondii. GRA6 was used to genotype the parasite in this study because its coding region is highly polymorphic, and even when compared to other T. coding genes that were examined, such as SAG1, SAG2, and GRA4, it is more variable. The rate at which amino acids change from non-synonymous to synonymous is high, indicating that variation in GRA6 genes amongst T. gondii isolates may affect the parasite's survival, especially in the parasitophorous vacuole [8,10]. Similarly, a recent investigation emphasized the relevance of the GRA6 gene as a reliable genetic marker for strain differentiation, particularly in clinical samples [15]. Due to its high polymorphism, the GRA6 gene is frequently utilized as a marker for strain distinction, particularly in conjunction with RFLP analysis [16]. Potential variations in genotype prevalence between individuals infected with HBV and HCV may be indicated by the distribution of Toxoplasma gondii genotypes seen in the current investigation. Following digestion with the MseI enzyme and nested PCR targeting the GRA6 gene, Type II was the most common genotype found in both groups, with Type I and Type III following in decreasing order of frequency. These results provide important new information about the prevalence of T. gondii in the study region's healthy controls and patients with hepatic diseases. Our most recent study's greatest frequency of type II is consistent with several recent studies from the Middle East and other regions that have demonstrated that Toxoplasma gondii genotype II is the most common strain among patients with liver illness in the Middle East. Genotype II was found to be prevalent in this community in Saudi Arabia, accounting for about 80.6% of positive cases [17].

Similarly, a study of pregnant women in northwest Iran found that 80.6% of infected people possessed Genotype II, suggesting that this group is widely afflicted [18]. The preponderance of Genotype II is further supported by other molecular characterizations of T. gondii from household cats and their owners in Tehran, Iran. This genotype was the most prevalent in both humans and cats, according to the study [19]. Our study's Type II genotype prevalence is in line with the global epidemiological landscape. Toxoplasma gondii genotype II is the most common genotype infecting people, according to numerous studies from different continents. Several recent investigations from different geographic locations have consistently shown that Toxoplasma gondii genotype II is more common in human infections. According to a study, Genotype II was found in 29% of isolates from congenital toxoplasmosis patients in Brazil, indicating the genotype's high frequency in the afflicted community [20]. Likewise, 78.57% of human Toxoplasma gondii isolates in China were of Genotype II, highlighting its regional dominance [21]. Numerous genotyping investigations conducted in Europe have further supported this pattern, showing that the prevalence of Genotype II in clinical human samples exceeds 85% [22]. This genotype's broad distribution has been ascribed to its intermediate virulence, which permits the parasite to survive inside

the host without producing severe or lethal acute infections, thereby promoting prolonged cycles of transmission. Type II strains establish a balance that favors persistence and transmission, in contrast to the extremely virulent Type I strains, which frequently cause acute, occasionally fatal infections and may be underrepresented in chronic illness cohorts. Type III strains, while less virulent, are less commonly isolated in human infections globally and appear less adapted to long-term persistence in humans [23]. This balance of virulence is particularly relevant in liver diseases, where immune dysregulation and tissue damage may predispose patients to opportunistic infections or allow latent parasites to reactivate. The chronic presence of T. gondii Type II in hepatic tissues might exacerbate liver inflammation or fibrosis via parasite-induced immune responses or direct cytopathic effects [24]. While the present study and numerous others from the Middle East and Europe have consistently reported Type II as the dominant Toxoplasma gondii genotype in human infections particularly in chronic cases and liver-related conditions, some studies conducted in other geographic regions have reported contrasting patterns, with a predominance of Type I, Type III, or atypical genotypes. For example, through a multilocus PCR-RFLP genotyping approach, documented the circulation of a diverse set of Type III strains across Central and South America [25]. Their findings highlight that the global T. gondii population structure is not uniform and that regional ecological and host factors may drive the emergence of distinct genetic lineages. In another study identified that Type III as the most frequent genotype among human toxoplasmosis cases in France, contrasting with the dominance of Type II reported [26]. This variation was suggested to result from different exposure sources and environmental reservoirs. Moreover, a study analyzed congenital toxoplasmosis in Egypt and revealed a predominance of Type I. The authors speculated that Type I strains may have greater neurotropism and are more likely to cause reactivation in immunocompromised hosts [27]. These discrepancies can be attributed to several factors. Firstly, the geographic location plays a pivotal role in the distribution of T. gondii genotypes. In Latin America, especially Brazil and Mexico, the presence of wild feline and avian reservoirs supports the circulation of genetically diverse and virulent strains, unlike the more clonal populations observed in Europe and the Middle East [28]. Secondly, host immunity and clinical manifestation influence strain detection; studies focusing on acute or neuroinvasive toxoplasmosis are more likely to isolate highly virulent strains such as Type I. Thirdly, the diagnostic technique matters: while this study used GRA6-based nested PCR and MseI digestion for genotyping, others employed multilocus or whole-genome sequencing, which enables more precise identification of hybrid and atypical lineages [29]. The limited presence of type I and III may indicate relatively uniform exposure to various strains or comparable host susceptibility also may reflect a regional or host-specific restriction in strain distribution or geographical strain distribution or selective immune pressures [30]. Our use of the GRA6 gene and MseI digestion for genotyping provides robust discrimination among the clonal lineages. The polymorphic nature of GRA6 and the specificity of MseI restriction sites allow precise genotype differentiation, lending credibility to the reported genotype distribution. These results demonstrate the value of molecular genotyping for studying strain-specific pathogenicity and how it interacts with host immunological state, in addition to epidemiological surveillance.

In summary, Toxoplasma gondii's genetic variety and its possible influence on the course of the disease and the effectiveness of treatment, especially in immunocompromised persons, are highlighted by the prevalence of Genotype II among hepatitis patients. The geographical genetic distribution of T. gondii is better understood thanks to these findings, which also highlight the significance of continued molecular surveillance and additional study to clarify the connection between parasite genotypes and chronic liver disorders.

CONCLUSIONS

It is advised that future research use more genetic markers and larger sample sizes to better understand the distribution of Toxoplasma gondii genotypes and their clinical consequences in liver disease patients. Such studies may aid in the creation of more potent therapeutic and preventative plans for this susceptible patient group.

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AUTHOR CONTRIBUTIONS

All authors had equal roles in design, work and manuscript writing.

ETHICAL APPROVAL

All procedures were in accordance with the ethical standards of the research committee at Al-Qadisiyah University with certificate NO. 57436.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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