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Detection of toxoplasmosis by serology and nested-PCR in kidney transplant recipients and patients on hemodialysis from Sri Lanka

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Abstract

Toxoplasmosis, the parasitic disease caused by *Toxoplasma gondii (T.gondii*) affects approximately one-third of the global population. In immunocompromised patients who had been previously infected with *T.gondii,* the parasite can reactivate to cause infection. This study aimed to determine the seroprevalence of *T.gondii* among kidney transplant recipients (KTR) and hemodialysis patients (HD) by enzyme-linked immunosorbent assay (ELISA) and to detect *T.gondii* Deoxyribonucleic Acid (DNA) by nested-Polymerase Chain Reaction (nPCR). Of the 342 patients (114 KTR and 228 HD), 64 (18.7%) and 123 (36%) showed evidence of acute and past infection, respectively, by ELISA, while two (0.6%) had indeterminate results and 153 (44.7%) were negative. In nPCR, there were 28 (8.2%) positives, of which 6.1% only IgG positives, 1.2% only IgM positives, 0.6% both IgM and IgG positives, and 0.3% were indeterminates. The importance of using a combination of serology and molecular methods to determine toxoplasmosis status before commencing treatment in patients awaiting KTR and undergoing HD is indicated by these results. This is the first study that determined toxoplasmosis seroprevalence and targeted the B1 gene using nPCR method to detect toxoplasmosis among KTR and HD patients in Sri Lanka.

Keywords Toxoplasmosis, Renal dialysis, Kidney transplant, PCR, Immunocompromised, Seroprevalence

Introduction

Toxoplasmosis is an infectious disease caused by the parasite *Toxoplasma gondii (T.gondii)* [5]. Definitive hosts of *T.gondii* are felids including cats while warm- blooded animals including humans serve as intermediate hosts [26]. The disease is transmitted from animals to humans, from mother to fetus, and rarely through blood transfusion and organ transplantation [7]. Laboratory handling of infected materials is another way of disease transmission [35].

The global prevalence of toxoplasmosis is 25%–30% in humans and varies in different geographical settings [6]. The immunocompetent individuals are mostly asymptomatic, while a minority of about 10% show mild flu-like

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symptoms and lymphadenopathy [14, 24]. In immunocompromised patients, new infections or reactivation of previously infected parasites can lead to severe, life-threatening conditions [9, 10]. The majority (80%) of kidney transplant recipients (KTR) show fever with other clinical presentations such as pneumonia and generalized neurological signs such as headaches, lethargy, and drowsiness [38]. The complications of toxoplasmosis increase when both the donor and the recipient are *T.gondii*-positive [29]. Transplant recipients show symptoms within the first three months of the transplant [18]. In both KTR and haemodialysis (HD) patients, the reactivation of the chronic toxoplasmosis can cause brain abscesses, pneumonia, fever of unknown origin, diffuse encephalitis, myocarditis, skin rashes, and hepatosplenomegaly [13, 17]. Despite the high risk and clinical significance, there is a notable lack of data and a gap in knowledge regarding the prevalence of toxoplasmosis among immunocompromised categories, including KTR and HD patients in Sri Lanka. No data is available on toxoplasmosis in any category in the recently published (2022–2023) annual health bulletin of Sri Lanka [39]. The reported prevalence of toxoplasmosis among pregnant women in Sri Lanka ranges from 12.3% to 29.9% [8, 15]. No other research data exists on other immunocompetent groups of populations from Sri Lanka. When considering the immunocompromised group of patients, a study done among 321 cancer patients from Sri Lanka, reported that 30 (9.3%) patients were nPCR positive, 31 (9.7%) were IgM positive, 153 (47.7%) were IgG positive and 18 (5.6%) were both IgM and IgG positive for *T.gondii* [36].

Antibody detection is the most common method used to diagnose toxoplasmosis, which may not be accurate in immunocompromised patients as they fail to produce high levels of antibodies [12, 25] Therefore, the use of molecular biological techniques is preferred in immunocompromised category of patients, with target genes of *T.gondii* such as dense granule protein 1 (GRA1), dense granule protein 6 (GRA6), Surface antigen 1 (SAG 1), Surface antigen 2 (SAG2), surface antigen 3 (SAG3) and B1 genes [3, 21]. In the current study, the B1 gene was amplified as it is the most conserved gene in *T. gondii* as it has been repeated 35 times in the genome compared to other genes [4]. Due to the repetitive sequences, it has a high specificity [20]. This study aimed to determine the seroprevalence of *T.gondii* among KTR and HD patients by enzyme-linked immunosorbent assay (ELISA) and to detect *T.gondii* Deoxyribonucleic Acid (DNA) by Polymerase Chain Reaction (PCR) and nested-PCR (nPCR). This study also aims to compare Enzyme-linked Immunosorbent assay (ELISA) test results with molecular biology test (nPCR) results. This is the first study that determined toxoplasmosis seroprevalence and targeted the B1 gene using nPCR method to detect toxoplasmosis among KTR and HD patients in Sri Lanka.

Method

This study was conducted among a group of 18 to 80-year-old (including both cut-off ages) adult female and male renal patients ($n=342$) that included 114 (33.3%) KTR and 228 (66.7%) HD patients. The study included only the transplant recipients on immunosuppressant drugs or steroids and end-stage renal patients on hemodialysis who were admitted to the medical wards at the National Institute of Nephrology and Dialysis and Transplantation, Maligawatte, Colombo, Sri Lanka, from 1st of November 2022 to 30th June 2023. Patients newly diagnosed with kidney diseases but not yet confirmed, patients admitted with urinary calculi, and those unable to communicate or understand were excluded from the study. First, informed written consent was taken from all patients. Then, an interviewer-administrated questionnaire in trilingual format (Sinhala, English, and Tamil) that was developed for this study and piloted before use was utilized to collect socio-demography and clinical manifestations of toxoplasmosis. Then five milliliters of blood were drawn from each patient, two milliliters for a plain tube and three milliliters for EDTA-containing tubes. Plain tubes with blood samples were centrifuged for 10 min at 3000 rpm, serum separated and stored at -20^{0}C. Anti *T.gondii* IgG and IgM antibodies were tested within two weeks of sample collection using ELISA kits (Calbiotech, El Cajon, CA, USA) following the manufacturer's instructions. To perform ELISA test positive, and negative controls and calibrators were added to ELISA plates along with 10 µl of patients' serum samples diluted in 200 µl of diluent. Patients were categorized into four groups according to Center for Disease Control and Prevention (CDC) guide, considering ELISA results. The categories were those with possible acute infection (IgM and IgG positive, IgM positive and IgG negative, IgM positive and IgG borderline, IgM borderline and IgG negative), possible past infection (IgM negative and IgG positive, IgM borderline and IgG positive), indeterminate (IgM negative and IgG borderline) and negative (both IgG and IgM negative) [7].

DNA was extracted from the three milliliters of blood in EDTA tubes of all 342 patient samples using a QIAamp® blood mini kit (Qiagen, Germany), and nPCR was performed on all. Initially, the first round of the nPCR was performed on all 342 extracted DNA samples to amplify a 200 bp region of the *T.gondii* B1 gene. We did not design but used the primers already published by previous researchers [4],outer primers OB1/F (5'-GGAACTGCATCCGTTCATGAG-3') and OB1/R (5'-TCTTTAAAGCGTTCGTGGTC-3') synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). Each 25

μL reaction contained 1 μL of each primer (10 μM), 12.5 μL GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA), 2 μL DNA template, and nuclease-free water to complete the volume. The thermocycling protocol included initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min.

Then the second round of the nPCR was performed on all 342 samples targeting a 100 bp region using already published [4] inner primers IB1/F (5'-TGCATAGGTT GCAGTCACTG-3') and IB1/R (5'-GGCGACCAATCT GCGAATACACC-3') (IDT). The reaction mixture and cycling conditions were the same as in the first round of the nPCR. The template for the nPCR was a 1:5 dilution of the primary PCR product.

Positive controls consisted of *T. gondii* RH strain DNA (ATCC Number: 50174D, ATCC, ATCC, Manassas, VA, USA), while negative controls contained distilled water. Amplicons were visualized by electrophoresis on 2% agarose gels prepared with 1 × TBE buffer and stained with ethidium bromide. A hundred-base pair ladder (DM001-R500, GeneDirex® INC, Taiwan) was used to identify the DNA bands. Electrophoresis was performed at 100 V for 20 min, and bands were visualized using a gel documentation system. Data were analyzed using the Python statistical programme (https://www.python.org).

All laboratory diagnostic tests and data analysis were conducted at the Department of Parasitology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Nugegoda, Sri Lanka. Research ethics approvals were obtained from the Ethics Review Committee, University of Sri Jayewardenepura (ref no: 13/21) and the Ministry of Health, Sri Lanka (ref no: ETR/AC/M3/38/2021) in accordance with the Declaration of Helsinki. Administration approvals were obtained from National Institute for Nephrology, Dialysis and Transplantation (NINDT) in Maligawatte, Sri Lanka. Confidentiality of data was maintained throughout, and primary data was accessible only to the members of the research group. Data were stored securely.

Table 1 Sociodemographic data of the KTR and HD patients

Variable	Category	Frequency (%) *N* = 342
Renal Patient Category	Kidney Transplant Recipients (KTR)	114 (33.3%)
	Hemodialysis (HD)	228 (66.7%)
Gender	Male	232 (67.8%)
	Female	110 (32.2%)
Province	Western	280 (81.8%)
	Southern	19 (5.6%)
	North Western	20 (5.8%)
	Sabaragamuwa	11 (3.2%)
	Central	6 (1.8%)
	Uva	4 (1.2%)
	Eastern	2 (0.6%)
Age Group (years)	18–40	64 (18.7%)
	41–60	193 (56.4%)
	61–80	86 (25.2%)
Education Level	Primary	8 (2.4%)
	Secondary	334 (97.6%)
Monthly Income (LKR)	< 20,000 or no income	126 (36.8%)
	≥ 20,000	216 (63.2%)
Cat Ownership	Yes	196 (57.3%)
	No	146 (42.7%)
Raw Meat Consumption	Yes	0 (0.0%)
	No	342 (100.0%)
Clinical Manifestations	Present	0 (0.0%)
	Absent	342 (100.0%)

Table 2 IgM and IgG results of the hemodialysis patients and renal transplant recipients

	Number of positive patients (%)	Number of negative patients (%)	Number of patients in the borderline (%)
ELISA- IgM	59 (17.3%)	275 (80.4%)	08 (2.3%)
ELISA- IgG	153 (44.7%)	187 (54.7%)	02 (0.6%)

Results

Socio-demography of the population

The Socio-demography data of the patients summarised in Table 1.

Toxoplasmosis seroprevalence by ELISA

IgM and IgG ELISA results of both KTR and HD patients summarised in Table 2.

Patient categorization considering the IgM and IgG ELISA results The acute infection category included 23 (6.7%) females and 41(12.0%) males, distributed among the age groups 18–40, 41–60, and 61–80 as 8 (2.3%), 39 (11.4%), and 17 (5.0%), respectively. Acute infection was found among 31 (9.1%) KTR and 33 (9.6%) HD patients.

nPCR results of HD and KTR patients Among all 342 patients, 28 (8.2%) were positive by the first and second rounds of the nPCR, while 314 (91.8%) were negative. Figure 1 shows an image of the first round of the nPCR, and Fig. 2 shows an image of the second round of the nPCR. Among these 28 PCR positives, there were 9 (32.1%) females and 19 (67.9%) males, 2 (7.1%), 21 (75%), and 5 (17.9%) in the 18–40, 41- 60, and 61–80 age groups, respectively. Among the nPCR positives, 6 (21.4%) were KTR patients and 22 (78.6%) were HD patients.

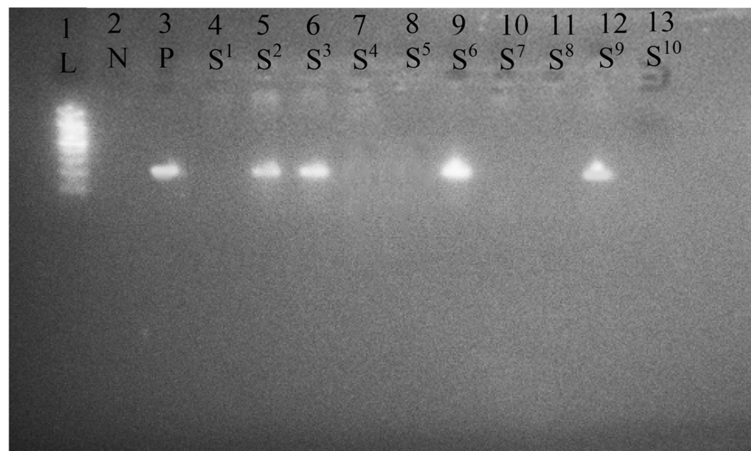

Fig. 1 A gel image of the first PCR results of B1 gene amplification. L: 100 bp ladder, N: negative control, P: positive control, S1: patient sample with negative results, S2 and S3: patient samples with *T.gondii* positive results, S4 and S5: patient samples with PCR negative results, S6: patients sample with PCR positive results, S7and S8: patients samples with PCR negative results, S9: patient sample with PCR positive results and S10: patient sample with *T.gondii* PCR negative results

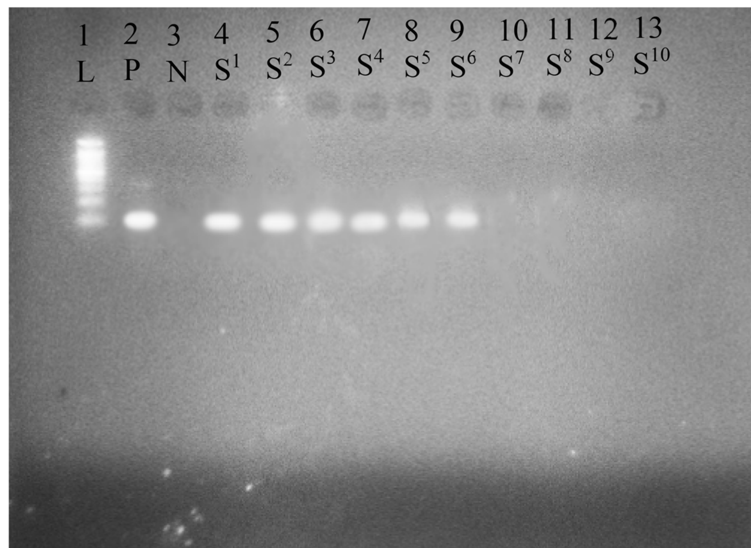

Fig. 2 Gel image of *T.gondii* second round nested PCR B1 gene amplification. L: 100 bp ladder, P: positive control, N:negative control. S1 to S6: patient samples with *T.gondii* second round nested PCR positive results and S7 to S10: patient samples with *T.gondii* second round nested PCR Negative results

ELISA and PCR results

Association of risk factors with ELISA and PCR results

The comparison of the ELISA and PCR results of the KTR and HD patients is summarized in Table 5.

The association of risk factors with the ELISA and PCR results of the patients is summarized in Table 3.

Discussion

Cellular immunity, which acts against intracellular parasites such as *T. gondii,* is impaired among immunocompromised patients [33]. The phagocytic activity and the number of natural killer cells are also very low in them [34]. As a result, *T. gondii* leads to severe clinical manifestations such as cerebral toxoplasmosis among immunocompromised patients. Furthermore, the immunosuppressive drugs received by transplant recipients, such as KTR patients to prevent graft rejection, make these patients more susceptible to reactivations of latent forms such as *T. gondii* bradyzoites [16]. Therefore, routine screening for toxoplasmosis of patients awaiting organ transplants is worthwhile irrespective of the presence or absence of clinical manifestations.

The present study aimed to determine the prevalence of toxoplasmosis among the immunocompromised patients, taking a group of KTR and HD as a sample population. There are no published studies on toxoplasmosis among KTR and HD patients in Sri Lanka.

Table 3 Risk association of risk factors with ELISA and PCR results

Risk factor	Laboratory test	Chi-square value	*P*-value
Age group	ELISA	4.536	0.604
	PCR	4.645	0.098
Sex	ELISA	1.52	0.678
	PCR	0	1.0
Renal condition (Dialysis vs RTR)	ELISA	9.481	0.024
	PCR	1.405	0.236
Residential district	ELISA	27.9	0.953
	PCR	19.84	0.135
Education level	ELISA	0.568	0.904
	PCR	0.0	1.0
Income level	ELISA	1.072	0.784
	PCR	0.111	0.739
Presence of cats	ELISA	3.508	0.32
	PCR	4.728	0.030
Raw meat consumption	ELISA	3.17	0.366
	PCR	0.172	0.679
Use of gloves when gardening	ELISA	9.32	0.156
	PCR	0.341	0.843
Washing fruits and vegetables	ELISA	7.675	0.263
	PCR	0.623	0.554
Raw meat handling	ELISA	0.932	0.818
	PCR	0.514	0.473
Swimming	ELISA	0.581	0.901
	PCR	0.008	0.930
Washing hands before eating	ELISA	7.285	0.295
	PCR	4.933	0.085
Washing hands after bathroom use	ELISA	4.112	0.662
	PCR	0.545	0.762

A *p*-value less than 0.05 is considered statistically significant

There are no existing data from Sri Lanka on the prevalence of toxoplasmosis among patients with kidney diseases. Thus, we had to rely on global literature, which reported 20 to 70 years [1] as the common age groups with toxoplasmosis among kidney transplant recipients and hemodialysis patients. Thus, we decided to focus our study on adults within this age group. In Sri Lanka, persons over the age of 65 years have increased from 4% in 1970 to 11% in 2019 [28] and chronic kidney disease is more prevalent in the 10 to 85-year age group [37]. Considering those factors, we decided to include patients up to the age of 80 years. However, the upper age limit was kept at 80 to minimize the influence of age-related physiological and immune system variations. The legal age for consent is 18 years in Sri Lanka, and that was taken as the lower limit of age cut-off for the study. Individuals under 18 were excluded due to the complexity and practical difficulty in obtaining consent from parents and kids (Table 1).

When considering the risk factors, in our study (Table 3), the majority (81.8%) of the patients were from the western province, possibly because the sample collection was done in a hospital located in the western province. A significant association was identified between the status (hemodialysis and renal transplant recipients) of the renal patient and the ELISA test results. It showed a significantly higher prevalence of toxoplasmosis among hemodialysis patients compared to renal transplant recipients ($P = 0.024$). The PCR results of renal patients showed a significant association with cat ownership ($P = 0.030$). No other significant association with PCR results was found. Thus, the use of a molecular biological test as a complementary test in addition to antibody detecting serology (such as ELISA) should be considered even in resource-limited settings to reduce over-treatment in the best interest of the patient.

In the current study, both the ELISA and PCR techniques were used to determine *T. gondii* infection among immunocompromised individuals. The ELISA test detects antibodies such as IgM and IgG produced by the host in response to infection, providing information on prior exposure or immune status. The molecular biology techniques, such as PCR, directly identify the genetic material of the parasite, providing evidence of active infection at the time of sample collection [19, 22]. These two tests complement each other in different stages of the infection, as serology or ELISA is more reliable in detecting chronic and latent infections, while PCR is more reliable for identifying current infection as it directly detects the parasite DNA. Similarly, PCR is suitable to diagnose early or reactivated infections in patients with compromised immunity, as they may not generate a strong antibody response [2].

The ELISA test is the common method of identifying *T. gondii*-specific antibodies [5]. This is available in both quantitative and qualitative but neither of them can accurately predict the timing of the infection. In the quantitative ELISA test, the four-fold rise of IgG antibody titer is assessed by taking a second sample [27].

In our study, patients in categories A and B of ELISA test results were cross-verified with the clinical symptoms, but none of them had symptoms.

The data given in Tables 2 and 4 are based on serological evidence only. Table 5 compares serological evidence with molecular biology tests. According to a study done in Turkey among 173 HD patients, 97 (56.1%) had IgG antibodies, and three (1.73%) were IgM positive. In a study done in central Iran among 90 HD patients, 54 (60%) and 3 (3.3%) were positive for IgG and IgM antibodies, respectively. Five (6%) patients were positive for the *T.gondii*-specific DNA amplification by PCR [30]. In another study from Iran conducted among both KTR and HD patients, IgG antibodies were found among (26/50) 52% KTR and (85/135) 63% HD patients. None of the KTR patients were IgM positive, but one of the HD patients had IgM antibodies. Altogether, nine (5%)

Table 4 Patient categorization considering the IgM and IgG ELISA results; based on Center of Disease Control (CDC) categories. For ease of reference, they were labeled as A to D

Categories	
Category A: Evidence of acute infection 64 (18.7%)	IgM-positive and IgG-positive 30 (8.7%)
	IgM positive and IgG negative 29 (8.5%)
	IgM positive and IgG borderline 0 (0%)
	IgM borderline and IgG negative 5 (1.5%)
Category B: Evidence of past infection 123 (36%)	IgM negative IgG positive 120 (35.1%)
	IgM borderline and IgG positive 03 (0.9%)
Category C: No evidence of toxoplasmosis 153 (44.7%)	IgM negative and IgG negative 153 (44.7%)
Category D: Indeterminate results 02 (0.6%)	IgM negative and IgG borderline 02 (0.6%)
	IgM and IgG borderline 0 (0)

Table 5 ELISA and PCR results

Patient category based on serology* and ELISA Results $n=342$ (%)	PCR results $n=342$ (%)	
	PCR Positive	PCR Negative
Category A: Evidence of acute infection- 64 (18.7%)	**6 (1.8%)**	**58 (17.0%)**
IgM positive and IgG positive- 30 (8.8%)	2 (0.6%)	28 (8.2%)
IgM positive and IgG negative- 29 (8.5%)	4 (1.2%)	25 (7.3%)
IgM positive and IgG borderline- 0	0	0
IgM borderline and IgG negative- 5 (1.5%)	0	5 (1.5%)
Category B: Evidence of past infection- 123 (36.0%)	**21 (6.2%)**	**102 (29.8%)**
IgM negative IgG positive- 120 (35.1%)	21 (6.2%)	99 (28.9%)
IgM borderline and IgG positive- 3 (0.9%)	0	3 (0.9%)
Category C: No evidence of toxoplasmosis- 153 (44.7%)	**0**	**153 (44.7%)**
IgM negative and IgG negative- 153 (44.7%)	0	153 (44.7%)
Category D: Indeterminate results- 2 (0.6%)	**1 (0.3%)**	**1 (0.3%)**
IgM negative and IgG borderline- 2 (0.6%)	1 (0.3%)	1 (0.3%)
IgM and IgG borderline- 0	0	

*Patient categories were from CDC (CDC—Toxoplasmosis, 2023)

patients were PCR positive, where six were KTR patients and three of them were HD patients [29].

In the current study, if only serology was used to decide on the treatment plan, 58 (17.0%) would have received treatment unnecessarily as having an acute infection, while only six (1.8%) were detected as positives by nPCR. There were 21(6.1%) patients who were nPCR positive but in the category of past infection by serology, and thus these patients would not have received treatment and suffered from complications if the nPCR had not been performed. The patient who was positive by nPCR but had indeterminate serology results would not have been treated either (Table 5). The discrepancy observed between ELISA and PCR results in the current study and the previous studies can be attributed to a combination of biological and technical limitations in both diagnostic methods. The sensitivity of the ELISA results can be lowered due to low antibody production in patients undergoing immunosuppressive therapies [2].

Screening tests are developed to identify potential diseases in asymptomatic individuals, with the primary objective of early detection to mitigate associated risks. The ideal screening test should exhibit high sensitivity and specificity, be cost-effective, simple to administer, safe, and cause minimal discomfort. However, in practice, these tests may yield false positives and false negatives, necessitating a thorough evaluation of their benefits and limitations [23].

The PCR is considered a direct method that detects parasitic DNA, while the ELISA detects antibodies indirectly [4, 11]. According to a review, PCR is reported as the most sensitive and specific method in diagnosing *T.gondi* [31].

Treatment and follow-up of patients were not part of the study. However, the copies of laboratory reports were handed over to the clinician treating the patients for future management. In immunocompromised patients, there is high parasitaemia as they fail to produce antibodies, and thus, antibody detecting tests are less dependable, while direct tests, including DNA detection techniques, can be considered more dependable to detect the acute phase of infection [2, 32]. However, the DNA-based test results can be affected by the DNA degradation risk depending on the time from collection to processing of the sample. Therefore, in the clinical setting, IgM-positive patients (even with PCR-negative results) may still warrant treatment based on the clinical manifestations and overall risk profile, including the possible complications, and the treating clinician should consider all these aspects when deciding treatment.

Quantitative or qualitative ELISA tests detecting serum antibodies are the main diagnostic test for the detection of toxoplasmosis in Sri Lanka. Similar to the existing literature, results of this study also indicate that the ELISA test is useful for identifying previous exposure rather than active infections, making it unsuitable as a standalone diagnostic tool. This sheds light on the importance of using molecular methods (such as PCR techniques) to detect parasitic DNA, which is a more reliable indication of current infection [21]. However, given the relatively high cost of molecular biology tests and the low budgetary allocation to the healthcare sector in low or middle income countries such as Sri Lanka (total health expenditure as a share of GDP is less than 4%) [41], the PCR techniques currently have only a complementary role

as a diagnostic tool in different phases of infection and other groups of patients such as immunocompromised category. Furthermore, local availability of DNA-based diagnostic tests is low, partly due to the cost. Therefore, the implementation of a cost-effective diagnostic strategy for toxoplasmosis in immunocompromised patients is important in resource-limited settings. For future research, we propose creating a diagnostic algorithm for real-world practice for immunocompromised categories, performing a cost–benefit analysis of different diagnostic tests currently in use, extending to other forms of toxoplasmosis, and developing in-house ELISA assays.

Despite the valuable findings are some limitations in this study that should be addressed in future studies. One of the major limitations of the current study was the use of a qualitative ELISA kit due to insufficient funding. Furthermore, there was an average of a two-week delay in processing the collected sample for PCR after the DNA extraction due to unavoidable logistical reasons, and this could have affected the results due to damage to the DNA during storage.

In conclusion, first, according to the data of the current study, the use of a molecular biological test is vital to detect DNA, considering the results of serology before the commencement of treatment to reduce over-treatment. Secondly, toxoplasmosis in this population is more than 8% by molecular biological tests, and that reminds clinicians to be vigilant about the infection among this immunocompromised group of KTR and HD patients, specially in the current context of rising incidence and mortality rates of renal patients in Sri Lanka [40].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12879-025-11353-z.

Supplementary Material 1

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Authors' contributions
Weerasooriya GPC (W.G.P.C.), Manamperi A (M.A.), Banneheke BMHA (B.B.M.H.A.). W.G.P.C. wrote the research proposal and grant application, collected the data, conducted the practical experiments, analysed the data, and prepared the original draft. M.A. contributed intellectually to prepare the research proposal and grant application, helped in planning the research methodology, guided laboratory procedures in molecular biology, participated in data representation and analysis of data, and revised the article. B.B.M.H.A. suggested the research concept and topic, contributed intellectually to prepare the research proposal and grant application, and edited them, planned the research methodology, guided laboratory procedures, provided clinical input, participated in data representation and analysis of data, and edited the article and revised final draft of the manuscript.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Research ethics approvals were obtained from the Ethics Review Committee, University of Sri Jayewardenepura (ref no: 13/21) and the Ministry of Health, Sri Lanka (ref no: ETR/AC/M3/38/2021) in accordance with the Declaration of Helsinki. Administration approvals were obtained from NINDT, Maligawatte, Colombo, Western Province, Sri Lanka. Data were accessible only to the members of the research group. Data were stored and maintained confidentially. Informed written consent was taken from all patients.

Consent for publication
When informed, written consent was obtained for sample collection. Consent was also taken for the dissemination of data by presenting at seminars, symposia, and workshops, and including in the postgraduate thesis and publishing journal articles.

Competing interests
The authors declare no competing interests.

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