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Molecular prevalence and genetic diversity of *Toxoplasma gondii* in free-range chicken in Northeastern Libya

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Abstract

Background: *Toxoplasma gondii* is one of the zoonotic protozoa parasites. It can prevalently infect humans and warm-blooded animals, causing human health problems and substantial economic losses to the livestock industry worldwide. Chicken is one of the potential sources of toxoplasmosis, but there is no report of the prevalence of toxoplasmosis and their genotypes in free-range chickens in Libya.

Aim: This study aims to conduct a survey of molecular prevalence and identify the *T. gondii* genotype in free-range chickens and its association with the risk factors of age, gender, and region in Northeastern Libya.

Methods: This study was conducted by examining a total of 315 free-range chicken organs (brain and heart) derived from three administrative districts in Northeastern Libya. The molecular prevalence was determined by PCR technique using B1 gene amplification. and the *T. gondii* genotype was determined by nested PCR-RFLP of GRA6 gene amplicon with restriction enzymes (*Mse*I).

Results: The overall molecular prevalence of *T. gondii* in free-range chicken in all three districts was 9.5% (30/315), and the highest (15.4%) was in the Al-Marj district ($p = 0.01$; $x^2 = 9.238$). The highest prevalence of *T. gondii* by age was in chickens aged more than 2 years ($p = 0.001$; $x^2 = 15.530$). The difference in *T. gondii* prevalence in male and female chickens was not significant ($p = 0.372$; $x^2 = 0.798$). The predominant genotype I (93.3%) had identified at position 544 and 194 bp at the GRA6 marker, and only two positives were from genotype II (6.7%) at 700 and 100 bp fragments.

Conclusion: The molecular prevalence of toxoplasmosis in free-range chicken in three districts in Northeastern Libya was 9.5%, and the highest rate was shown in the Al Marj district. Chicken by age more than 2 years had more risk to transmit toxoplasmosis in human. There was no different infection risk by consuming male or female free-range chicken. It is the first report to determine the predominant genotype, which was genotype I.

Keywords: B1 gene, GRA6 gene, Genotyping, Free-range chicken, *Toxoplasma gondii.*

Introduction

Toxoplasma gondii is a zoonotic parasite ranked among the most important foodborne pathogens worldwide (Fabian *et al*., 2020). Food animals are reservoirs of *T. gondii* and one of the sources of parasite transmission to humans giving *T. gondii* its medical and veterinary importance (Tonouhewa *et al.*, 2017). Humans get infected by consuming raw or uncooked meat containing tissue cysts, contaminated food or water with oocysts; or, from mother to fetus through vertical transmission in a primary infection (Hamilton *et al.*, 2019; Salinas *et al.*, 2021). More than a third of the world's population has been infected with *T. gondii* which can have serious consequences for immunodeficient or immunocompromised people (Mao *et al.*, 2021).

First, genotyping studies have suggested the existence of *T. gondii* clonal populations with three main genotype lineages types I, II, and III, and rare recombinant strains (Howe and Sibley, 1995). Following that, the three genotypic variations of *T. gondii* were found to exist in Western Europe and America which differ in phenotype, including pathogenicity (Liu *et al.*, 2015). Recently, more significant genetic variability has been shown using multilocus markers. The worldwide

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distribution of *T. gondii* genotypes is well-known in Western Europe and America, while there is only a little information on it in Asia and Africa. In Africa, lineages type II and type III, African lineages named Africa 1,2,3, and recently Africa 4 have been identified using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or microsatellite (MS) markers (Lachkhem *et al*., 2021; Galal *et al*., 2022).

Chicken is one of the most consumed meats in Libya, and free-range chicken meat is frequently consumed, as it is supposed to be healthier than that of caged chickens. However, consuming raw or undercooked chicken meat may expose an individual to human toxoplasmosis (Vieira *et al.*, 2018). As per their feeding habit, free-range chickens have been used widely in ascertaining the environmental contamination with *T. gondii* oocyst; and identifying the genetic variation of *T. gondii* worldwide (Hamilton *et al.*, 2019), but there is no information of the prevalence of *T. gondii* infection and their genotypes in free-range chickens in Libya. This research aimed to assess the molecular prevalence and identify the *T. gondii* genotype in free-range chicken and its association with the risk factors of age, gender, and region in Northeastern Libya.

Materials and Methods

Study period and location

This cross-sectional study was undertaken in the Al Jabal Al Akhdar region of Northeast Libya at different farms in three districts. It lasted from March 2021 to February 2022.

Sample collsection

A total of 315 samples were taken and grouped according to the origin of the materials. Those were three administrative districts in the Al Jabal Al Akhdar region (Al Marj, Al Bayda, and Derna districts). All purchased free-range chicken were kept by farmer. Based on information from veterinarian, chickens were grouped by gender into males and females and were categorized into four groups by age of chicken (Table 1). The chickens were slaughtered under sterile conditions, and tissue samples (heart and brain) were obtained. Samples were stored at −20°C before being transported in cold boxes to the laboratory of Animal Health Research in the Ministry of Agriculture in Egypt.

Sample preparation and DNA extraction

About one gram of each chicken tissue (heart and brain) was homogenized individually by tissue lyser (Qiagen, Hilden, Germany), in sterile Phosphate Buffered Saline (PBS) with two glass beads (5 mm). About 180 µl of ATL buffer and 20 µl of proteinase K were added to 200 µl homogenate into a 1.5 ml microcentrifuge tube, then incubated at 56°C till tissue lysis. DNA was extracted using a commercial kit (QIAamp DNA Mini kit, Animal Tissue protocol, catalog number 51304) following the manufacturer's instructions. The extracts were stored at −20°C for further use.

Molecular detection of B1 gene by PCR

Emerald Amp GT PCR Master Mix (Takara, code number. RR310A) kit to amplify the *T. gondii* B1 gene, was performed using the specific primers described by Lin *et al.* (2000). Briefly, the final volume was 25 µl of PCR reaction as follows: 5 µl DNA template, 12.5 µl of Emerald Amp GT PCR, 5.5 µl of PCR grade water (dH_2O), 1 µl of each primer (20 pmol). The cycling conditions were standardized as primary denaturation of strands at 95°C for 5 minutes, followed by 35 cycles of amplification (at 94°C for 30 seconds, at 60°C for 30 seconds, at 72°C for 30 seconds), and the final extension at 72°C for 7 minutes.

Genotyping by nested PCR

The nested PCR assay was performed to amplify the GRA6 gene. Briefly, the specific primers pair were described in the previous study (Armand *et al.*, 2017). The first round of a 25 µl PCR reaction includes (20 pmol) forward primer (1 µl), (20 pmol) reverse primer (1 µl), (5 µl) of extracted DNA, Emerald Amp GTPCR master mix (12.5 µl), PCR grade water (5.5 µl). The cycling conditions were set up for 95°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 40 seconds, and 72°C at 50 seconds, with a final extension at 72°C for 10 minutes. In the second round performed, the first amplified GRA6 product was cycled 35 times by the primary denaturation of strands at 95°C for 5 minutes, denaturation at 94°C for 30 seconds, followed by annealing at 58°C for 40 seconds, extension at 72°C for 45 seconds), and the final extension step at 72°C for 10 minutes. The success of amplicons was electrophoresed on 1.5% agarose gel and visualized under UV.

Nested PCR-RFLP

A nested PCR-RFLP assay was performed to digest the 750 bp nested PCR amplified GRA6 product using 1.5 U of *Mse*I enzyme for genotyping. To prepare the RFLP reactions of 7 µl of PCR product was mixed with 1 µl of restriction enzyme and 1 µl of enzyme buffer, and the total volume was adjusted to 15 µl by adding distilled water. The preparation was then incubated at 37°C for 20 minutes. The digested products were separated by electrophoresis on 1.5% agarose gel at 60 minutes in ethidium bromide-stained and visualized on a trans-illuminator under UV light.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows, version 28.0. (IBM Corp., Armonk, NY). A chi-square test was used to determine whether there were significant differences between disease occurrence and independence. Variable (area, sex, and chicken age), statistical significance was defined as a p-value ≤0.05.

Table 1. Risk Factor and its Association with the Molecular Prevalence of *T. gondii* in Positive Chickens.

Risk Factors	Positive Samples	Percentage (%)	*p* value and X^2
Al Jabal Al Akhdar			
Al Marj district	19/123	15.4	
Al Bayda district	6/104	5.8	($p = 0.01$; $X^2 = 9.238$)
Derna district	5 /88	5.7	
Chicken age			
4–6 months	5/77	6.5	
≥6 months–1 year	3/65	4.6	($p = 0.001$; $X^2 = 15.530$)
≥ 1 year– 2 years	2/64	3.1	
≥ 2 years	20/109	18.3	
Chicken sex			
Male	7/96	7.3	($p = 0.372$; $X^2 = 0.798$)
Female	23/219	10.5	

Ethical approval

The free-range chicken sampling started after obtaining approval from the Libyan National Committee for Biosafety and Bioethics (approval number: SH/3/2021).

Results

Molecular prevalence of T. gondii and the associated risk factors

The overall molecular prevalence of *T. gondii* in the 315 free-range chicken brain and heart tissues was 9.5% (30/315). The PCR amplification products of the B1 gene were the predicted amplicon size of 196 bp as in (Fig. 1).

The highest prevalence was revealed of *T. gondii* in the chicken tissue obtained from the Al Marj district 15.4% (19/123), followed by Al Bayda district at 5.8% (6/104) and then Derna district at 5.7% (5/88), the prevalence rate among districts was a significantly different ($p = 0.01$; $x^2 = 9.238$). The prevalence rate among age groups was significantly different ($p = 0.001$; $x^2 = 15.530$), The highest prevalence rate was 18.3% (20/109) for the chickens aged (≥2 years;) followed by those in the age group 4–6 months at 6.5% (5/77). Chickens aged ≥6 months to 1 year and ≥1 year to 2 years, had prevalence rates of 4.6% (3/65), and 3.1% (2/64), respectively. As per the sex, the prevalence was higher in the female group at 10.5% (23/219), compared to the male group at 7.3% (7/96). However, it was not significant ($p = 0.372$; $x^2 = 0.798$) (Table 1, Fig. 2).

Genetic characterization

Nested-PCR result

To determine the genetic characterization of isolates, all positive B1 gene PCR products samples (30) were subjected to GRA6 nested-PCR amplification. As a result, all samples were detected positive by visualizing the 750 bp fragment on the agarose gel (Fig. 3).

Nested PCR-RFLP results

All 30 positive samples of the second nested-PCR GRA6 were genotyped (100%) using the RFLP method and restriction enzymes (*Mse*I). According to the PCR-RFLP, there are two genotypes among all positive samples; 28 samples were from genotype I (93.3%), which showed 544–bp and 194–bp fragments and only two samples were positive from genotype II (6.7%). This sample was detected at 700-bp and 100-bp fragments (Fig. 4).

Discussion

Ascertaining the scope of genetic diversity of *T. gondii* in Libya is important in assessing its potential impact on public health. However, there was no information about the genotypes of *T. gondii* in domestic animals, including chickens, in Libya. Therefore, this is the first study to use PCR assay and PCR-RFLP to diagnose and identify *T. gondii* strains, which report their molecular prevalence in chickens in Libya.

Most of the studies used serological methods to investigate the high prevalence of this parasite in different domestic hosts around Libya. However, these methods have low sensitivity because of low antibody levels (Mose *et al.*, 2016). Although some previous reports that used different serological methods for diagnosis, displayed a high prevalence of *T. gondii* antibodies (Al-mabruk *et al*., 2013; Fadiel *et al*., 2021). The difference in the prevalence as per the area of origin of the chicken may be related to the number of chickens examined, and the contaminated environment. The higher prevalence of chicken-positive toxoplasmosis in the Al Marj district than in the Al Bayda and Derna districts could be due to the differences in agricultural activity, and livestock keeping, which is more common in the Al Marj district. As it is known, cats play a key role in spreading *T. gondii* oocysts in the environment,

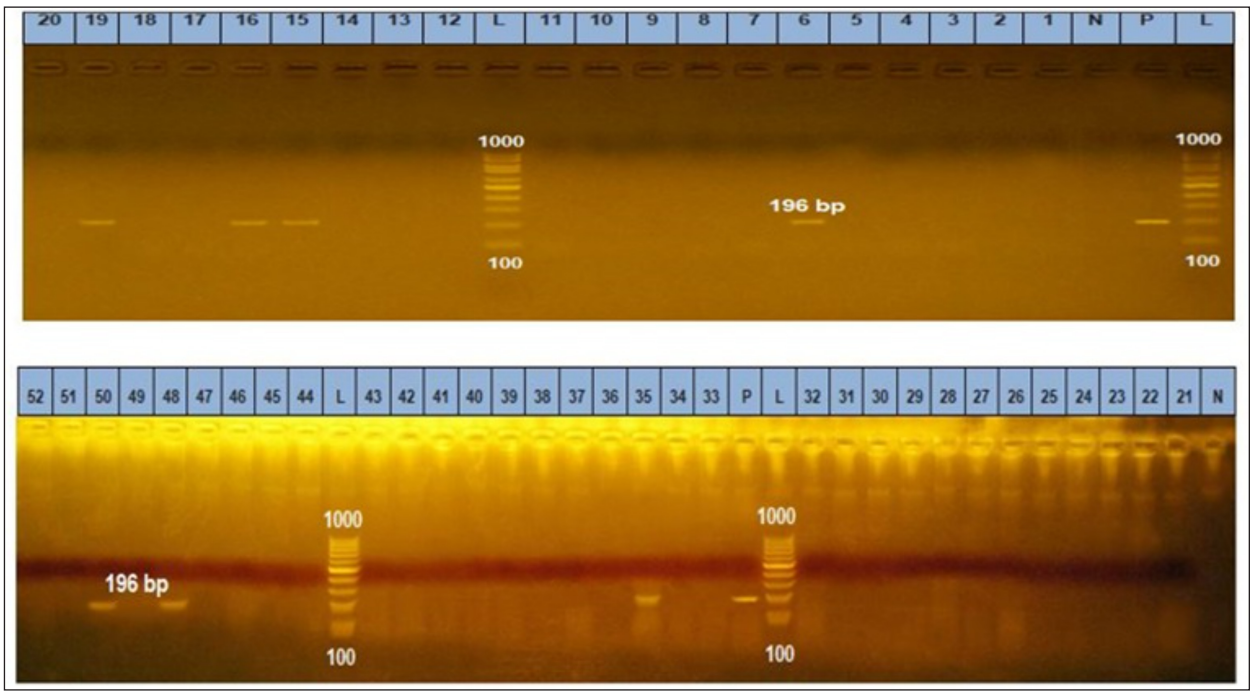

Fig. 1. Gel electrophoresis of PCR amplification products of B1 in the brain and heart (L): DNA size marker 100–1,000 bp., N: Negative control; P: Positive control. Lanes 6, 15, 16, 19, 35, 48, 50 are positive samples (196 bp).

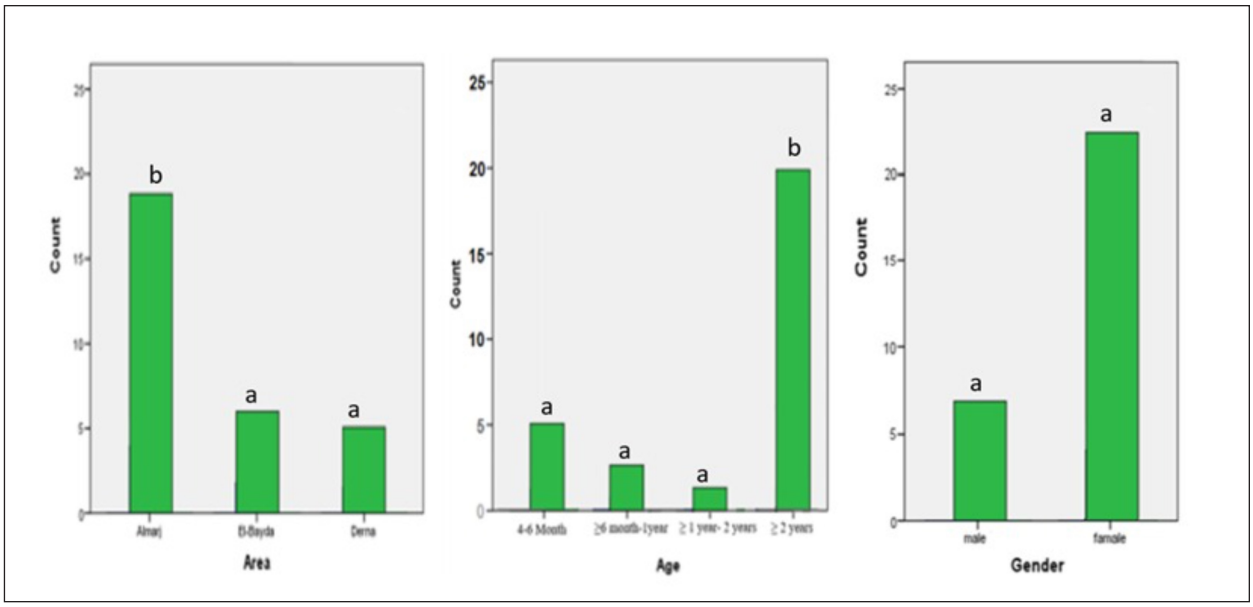

Fig. 2. Percentage of positive samples based on the area, chicken ages, and sex of chicken. Different notation means significant different (p-value ≤ 0.05, chi-square test).

so we assume that the number of cats in the Al Marj district may be more than in other areas. Thus, this could explain the high prevalence of infection in free-range chicken in that region compared to other regions. Al Marj district's surrounding environment is a rural area, as in the case of Brazil, where a high prevalence between 46.0% and 50% was free-range chickens in rural areas, indicating widespread contamination of the rural environment of that country with *T. gondii* oocysts (Sá *et al*., 2017; Tonouhewa *et al*., 2017).

There is a significant association between the prevalence of *T. gondii* in different chickens ages. A significantly higher prevalence was observed in older chickens (more than 2 years) than in younger chickens. A high prevalence of toxoplasmosis in older chickens (≥2 years) was also reported in Kenya (Mose *et al*.,

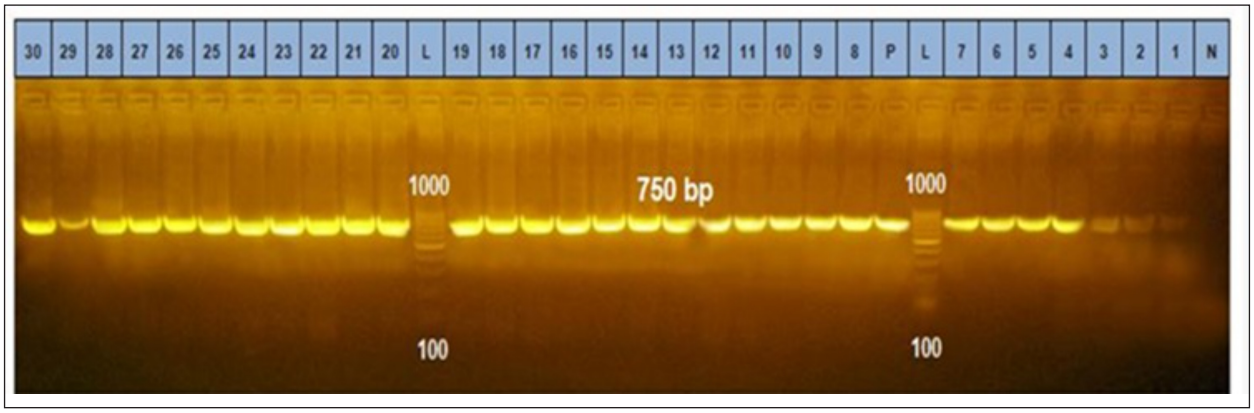

Fig. 3. Gel electrophoresis after secondary PCR amplification product with GRA6 primers. N: Negative control; *p*: Positive control. The positive band is at 750 bp. Ladder weight: DNA size marker (L):100–1,000 bp.

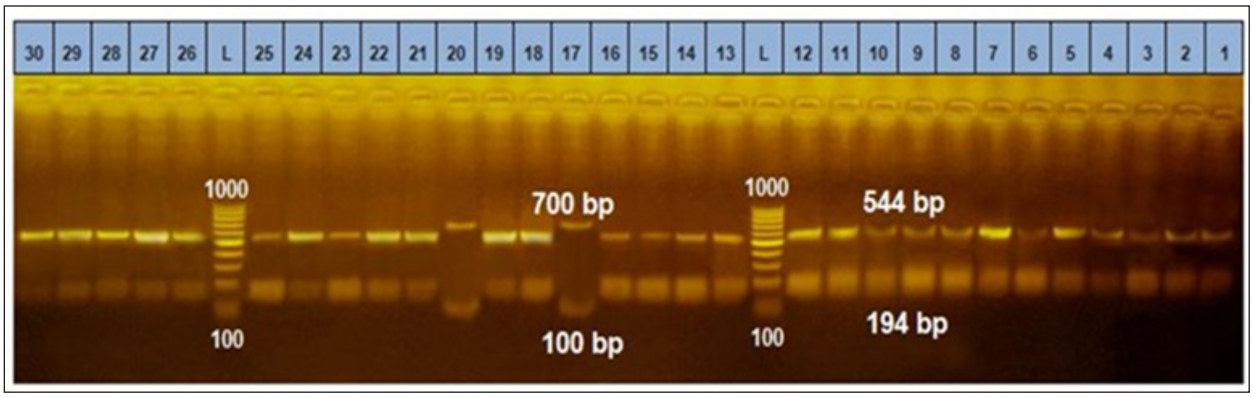

Fig. 4. PCR-RFLP analysis of GRA6 gene coding region with restriction enzymes (MseI). Lanes: L DNA size markers. (Between 1,000bp and 100 bp), Lanes: 17, and 20 digested PCR products of GRA6 for Type II & lanes: 1–16, 18, 19 and lanes: 21–30 digested PCR products of GRA6 gene for Type I.

2016), and Pakistan (Khan *et al.*, 2020). This could be attributed to older chickens having more outdoor access, considered a risk factor, and therefore being more predisposed to *T. gondii* infection. Being outdoors will expose them to soil contaminated with *T. gondii* oocysts.

Although the sex risk factor in our study was not significant, the prevalence of female infection compared to males was high, which resembles the results of a previous study done in China on ducks (Lv *et al.*, 2021); these results also match what was mentioned by Mose *et al*. (2016).

The brain and heart tissues were used to detect DNA because both organs are included in the most common predilection sites for *T. gondii* (Lüder and Rahman, 2017). Unfortunately, in this study, a low prevalence of 9.5% was indicated in chicken brains and hearts, which may explain the false-negative results due to the small amount of chicken tissue taken for DNA extraction, whereas, only one gram of host tissue was used.

A study conducted in Pakistan, using chicken heart targeting the B1 *T. gondii* gene, gave the same prevalence of 9.5% (Khan *et al*., 2020). However, our finding expressed a higher prevalence compared with the previous study in China that reported, the prevalence of *T. gondii* in chicken was only 8.17% (Zou *et al*., 2017). In contrast, previous study using PCR to detect the *T. gondii* B1 gene on three Caribbean islands showed a higher prevalence (24.4%, 17.1%, and 17.1%) in the free-range chicken (Hamilton *et al.*, 2019). Another previous study in northern Iran reported a high prevalence of the B1 gene of *T. gondii* that reached 40% in free-range chicken heart samples (Abbaszadeh *et al*., 2022). Our result also expressed a lower prevalence compared to the Tunisian study performed by PCR target B1 which showed a much higher prevalence of 43.3% (Zrelli, 2022). According to our search in the literature, the highest prevalence of *T. gondii* in livestock and poultry animals was found in Asia in 2014 with 89.8% (Hajimohammadi *et al*., 2022).

Molecular diagnostics of toxoplasmosis is generally based on the detection of a specific DNA sequence, using various assays and protocols, mostly from highly conserved regions such as the B1 gene, internal

transcribed spacer–1 and 18S rDNA gene sequences (Ivovic *et al*., 2012).

It is important to note, however, that molecular diagnostics, as a constantly evolving modern methodology, is not standardized even among the world's leading laboratories. The differences are significant and numerous, and they span all aspects of the methodology, including target genes for parasite detection and markers for genotyping, equipment manufacturers, and protocols (Ivovic *et al*., 2012).

In our study, the most common genotype using PCR-RFLP of GRA6 gene is the *T. gondii* type I. This study is consistent with the previous study in Iran, which genotype type I was reported as the most prevalent genotype based on the SAG2 gene using RFLP method (Mahami-Oskouei *et al*., 2017). Similar study showed *T. gondii* type I was predominant in Iran based on the GRA6 and SAG2 markers (Armand *et al*., 2017). A study in China revealed a high prevalence of *T. gondii* in chicken hearts from farmers' markets, On the 77 positive samples, nested PCR-RFLP genotyping was performed using the *T. gondii* SAG3 and GRA6 gene loci. SAG3 genotyping revealed a mixed infection rate of 89.6% for type I and type I/II strains, while GRA6 genotyping revealed a type I strain with infection rate of 98.7% (Wang *et al*., 2020).

In Libya, Several studies carried out on humans have shown a high prevalence of human toxoplasmosis (Gamal, 2015; Gashout *et al*., 2016). One study was undertaken in the area close to our study area in the economic capital city in Northeastern Libya (Benghazi). This particular study determined the genotype for ocular toxoplasmosis which showed (25%) of type I based on a single locus PCR-RFLP analysis based upon the SAG2 gene (Ali *et al.*, 2018). The above study's findings appear to be consistent with ours in this study, the predominance of the clonal type I lineage of *T. gondii*. Therefore, our findings may spur exploring the risk of human infection with *T. gondii* through consuming undercooked infected chicken meat.

In contrast to our findings, the first report on the chicken population of India indicated *T. gondii* lineage as type III based on PCR-RFLP of the GRA6 gene (Biradar *et al*., 2014). A recent survey by Gorgani-Firouzjaee *et al*. (2022) using PCR-RFLP of SAG2 and GRA6 genes demonstrated the alleles of clonal type III in all isolates. These different results are likely because of various genotypes of *T. gondii* in different geographic regions. This may explain the high diversity of *T. gondii* lineages in these regions.

The GRA6 gene is widely used as an appropriate marker for *T. gondii*. It can identify and distinguish its three and some atypical genotypes with a single PCR reaction, followed by an endonuclease (*Mse*I) digestion (Danehchin *et al.*, 2016). In this study, the PCR-RFLP assay on the products of the second steps of nested-PCR of GRA6 genes had been used to identify the *T. gondii* genotypes (Gorgani-Firouzjaee *et al.*, 2022). The use of single molecular markers may represent a problem due to a large extent diversity may be lost or genotypically different parasites may not be distinguished efficiently (Fernández-Escobar *et al*., 2022). Therefore, this is the main limitation of this research for reliable genotype classification. To date, there is no commonly used set of markers to genotype *T. gondii* strains (Su *et al*., 2006). However, only loci GRA6 and SAG3 had enough high-quality sequences to perform a robust phylogenetic analysis (Fernández-Escobar *et al*., 2022).

Most *T. gondii* isolates from human and animal sources in Northern America and Europe were grouped into one of three clonal lineages by multilocus enzymes electrophoresis, PCR-RFLP and MS typing. The rapid development of multilocus-sequencing methods as well as the description of a diverse panel of new PCR-RFLP and MS markers resulted in firm observations on the predominance of three clonal/archetypal types or lineages (Su *et al*., 2006). Unfortunately, traditional *T. gondii* typing methodologies have significant limitations because only very specific and restricted sites within a large *T. gondii* genome are assessed. Whole-genome sequencing data analysis has emerged as the most suitable approach for a thorough analysis of the genetic diversity in *T. gondii* (Fernández-Escobar *et al*., 2022).

Conclusion

This study determined the *T. gondii* DNA for the first time and genotyped the *T. gondii* strain in free-range chickens in Libya, with type I as the most predominant genotype. Because of its potential to impact public health, the relatively high prevalence of infected chickens especially in Al Marj District, and those of chickens more than 2 years old, further studies need to be done. Studies on other domestic animals are also important to determine their genetic diversity to gain more knowledge of its control and prevention.

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Authors' contributions

All authors contributed to making the completion of this manuscript possible. Conceptualization: H.A.A., T.W.S., L.E.F., M.A.S., and A.A. Collecting samples and conducting the research trials: H.A.A., data curation: H.A.A., T.W.S., L.E.F., and M.A.S.; writing original draft preparation: H.A.A, supervised the research; T.W.S., L.E.F., M.A.S., and A.A., supervised the field research: M.A.S. Finishing the manuscript: H.A.A., T.W.S., L.E.F., M.A.S., A.A.

Conflict of interest

The authors declare that there is no conflict of interest.

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