Acta Parasitologica
https://doi.org/10.1007/s11686-024-00905-z

ORIGINAL PAPER

First Report on the Molecular Detection and Genetic Characterization of *Toxoplasma gondii* From Donkeys in Kenya

Fredrick O. Obonyo[1,2] · Ndichu Maingi[1] · Samuel M. Githigia[1] · Kevin O. Ochwedo[3] · Anne A. Owiti[4] · Evans N. Nyaboga[4]

Received: 23 June 2024 / Accepted: 5 August 2024

Abstract

Purpose The present study was conducted to determine the presence of *Toxoplasma gondii* in donkeys by molecular tests and genetic diversity analysis of the obtained DNA samples from central Kenya.

Method A total of 363 blood samples were collected from donkeys in Meru and Kirinyaga Counties, and 96 samples that were previously seropositive for *T. gondii* using indirect ELISA were subjected to nested PCR based on the amplification of the internal transcribed spacer 1 (ITS-1) gene followed by DNA sequencing and phylogenetic analysis. Genotyping was performed on 15 selected positive samples using multilocus nested polymerase chain reaction restriction fragment length polymorphism (Mn-PCR-RFLP) with eight genetic markers (’SAG 2, 5’SAG 2, Alt. SAG 2, SAG 3, GRA 6, C29-2, BTUB and L358).

Results *Toxoplasma gondii* DNA was detected in 36.5% (35/96) of the blood samples. The sequences obtained exhibited 98.2–99.5% homology with those deposited in GenBank. Phylogenetic analysis demonstrated that the obtained sequences are conserved and clustered with those of infecting animals from other regions of the world. Eighteen distinct *T. gondii* haplotypes were identified to be circulating in donkeys from central Kenya. The *T. gondii* DNA samples exhibited high haplotype diversity (Hd: 0.915) and limited genetic diversity ($\pi = 0.01027$). PCR-RFLP of *T. gondii* DNA-positive samples revealed three different genetic combinations that consisted of alleles I, II and III, indicating the dissemination of atypical genotypes.

Conclusion This study demonstrated that *T. gondii* is widespread in donkeys from Kenya and could be a possible source of infection in humans. These findings are important for designing control strategies for this parasite to improve the livestock sector, which is one of the main sources of livelihood for farmers in Kenya.

Keywords Donkeys · Genotypes · Haplotypes · ITS-1 gene · Kenya · *Toxoplasma gondii*

Introduction

Toxoplasma gondii (*T. gondii*) is an intracellular protozoan parasite that infects a wide range of hosts, including humans, livestock and wild animals [1]. The parasite has a complex life cycle that involves sexual and asexual phases in domestic and wild hosts found in different ecosystems. The sexual phase occurs in the intestines of domestic and wild felids, which are the definitive hosts and result in the excretion of non sporulated oocysts in feces into the environment. Part of the asexual phase takes place in the intestines of definitive hosts, resulting in the formation of schizonts with merozoites, while the other part takes place in tissues of intermediate hosts, which include a wide range of warm blooded animals [2] in which tachyzoites undergo

✉ Fredrick O. Obonyo
fobonyo@must.ac.ke

1 Department of Veterinary Pathology, Microbiology and Parasitology, University of Nairobi, P.O. Box 29053, Kangemi 00625, Kenya

2 Department of Animal Science, Meru University of Science and Technology, P.O. Box 972, Meru 60200, Kenya

3 Department of Entomology, Plant Pathology and Nematology, University of Idaho, 492 W Taylor Ave, Apt 6, Moscow, Russian Federation

4 Department of Biochemistry, University of Nairobi, P.O. Box 30197, Nairobi 00100, Kenya

Published online: 19 August 2024

development and result in the formation of intracellular cysts in various organs [3].

Humans can be infected by the consumption of food and water contaminated with sporulated oocysts, ingestion of infective cysts in raw or undercooked meat containing bradyzoites from infected intermediate hosts and congenital transmission from pregnant mothers to the unborn fetus [4]. Additionally, humans may be infected via contact with cat litter, blood transfusions and organ transplantation from infected individuals [5] and less commonly ingestion of non pasteurized goat milk or milk products [6, 7]. Approximately 2 billion people are estimated to be infected with *T. gondii* worldwide [8]. Although *T. gondii* infection in healthy individuals is asymptomatic, infection in pregnant women, fetuses, neonates and immunocompromised patients can have serious consequences [9]. The main sources of *T. gondii* infection for domestic animals, including donkeys, are water or feed contaminated with oocysts sporulated after previous elimination from cat feces and transplacental transmission [4]. Most domestic animals are asymptomatic, but in sheep and goats, *T. gondii* infection is associated with reproductive failure, resulting in great economic losses [7]. In equines, *T. gondii* infection is asymptomatic [4], but patients may exhibit unusual clinical signs such as fever, incordination, degerenation of the retina and inflammatory changes in the central nervous system [10].

In Kenya, donkey is ranked as one of the most important livestock species compared to cattle, sheep, goats, chickens and ducks, with more than 7 million people benefiting directly from working donkeys. This is due to its ability to enhance transport and accessibility to hard-reach areas and provide livelihoods to communities through income generation. The legalization of donkey meat by the Government of Kenya has made the country one of the largest exporters of donkey meat to Asia [11]. Donkey meat and milk are locally consumed by some communities in Kenya [12]. Since *T. gondii* is nonhost specific, intermediate hosts such as infected donkeys may be a source of infection for humans and other animals. Humans may be infected by the consumption of milk and undercooked meat from donkeys, creating a possible source of acquired human toxoplasmosis [13]. Additionally, toxoplasmosis is an occupational disease for slaughterhouse workers and butchers who may be infected during the evisceration process [14]. This makes it necessary to monitor the distribution of *T. gondii* genotypes circulating in donkeys in Kenya.

The clonal genetic structure of *T. gondii* comprises three worldwide distributed clonal lineages, namely, types I, II and III [15]. Recent molecular studies have reported greater genetic diversity in *T. gondii* after the discovery of atypical genotypes that cannot be classified within the three main lineages [16]. These *T. gondii* lineages differ in their phenotypes, which are associated with virulence, persistence and ability to migrate and induce an immune response to infection [17]. Knowledge of the genetic structure of *T. gondii* is important for evaluating the effects of genotypes on the clinical manifestations of *T. gondii* infection, understanding the risk of spread of the parasite in the food chain and predicting the possibility of the parasite being able to infect humans [18].

In Kenya, studies have been conducted on *T. gondii* infection in pigs, humans and chickens using different detection methods, and the prevalence of *T. gondii* has been reported to reach 34.53%, 84.0% and 79% in pigs, humans and chickens, respectively [19–21]. However, only one study has been performed on the genetic diversity of *T. gondii* in chickens, in which the type II clonal lineage was identified [22]. Data on the genetic diversity and *T. gondii* genotypes circulating in donkeys are not available. The scarcity of this information prevents the identification of infection sources and compromises the development of effective measures to control *T. gondii* infections. This study was therefore conducted to determine the genetic diversity and identify the genotypes of *T. gondii* strains circulating in donkeys from Kirinyaga and Meru Counties in central Kenya.

Materials and Methods

Study Area

The study was conducted in Kirinyaga and Meru counties in central Kenya (Fig. 1). These two Counties were selected for the study because they have a high population of donkeys [11]. Kirinyaga County at latitudes 001′ and 00 40′S and longitudes 37° and 38° E is located on the southern slopes of Mount Kenya and the Aberdare Ranges, respectively, with an altitude ranging from 1158 to 5380 m. The county has a tropical climate and an equatorial rainfall pattern with two rainy seasons, long rains, which average 2146 mm, and short rains, which average 1212 mm per year. The temperature ranges from a mean of 8.1 °C in the upper zones to 30.3 °C in the lower zones during the cold and hot seasons, respectively [23]. Meru County lies at longitudes of 0° 6′ N and 0° 1′S and between latitudes 37°W and 38°E. The county covers the northern to eastern slopes of Mount Kenya and has a warm and temperate climate. Rainfall ranges from 300 to 2500 mm per year, while the average temperature in these counties ranges from 8 to 32 °C during the cold and hot seasons, respectively [24].

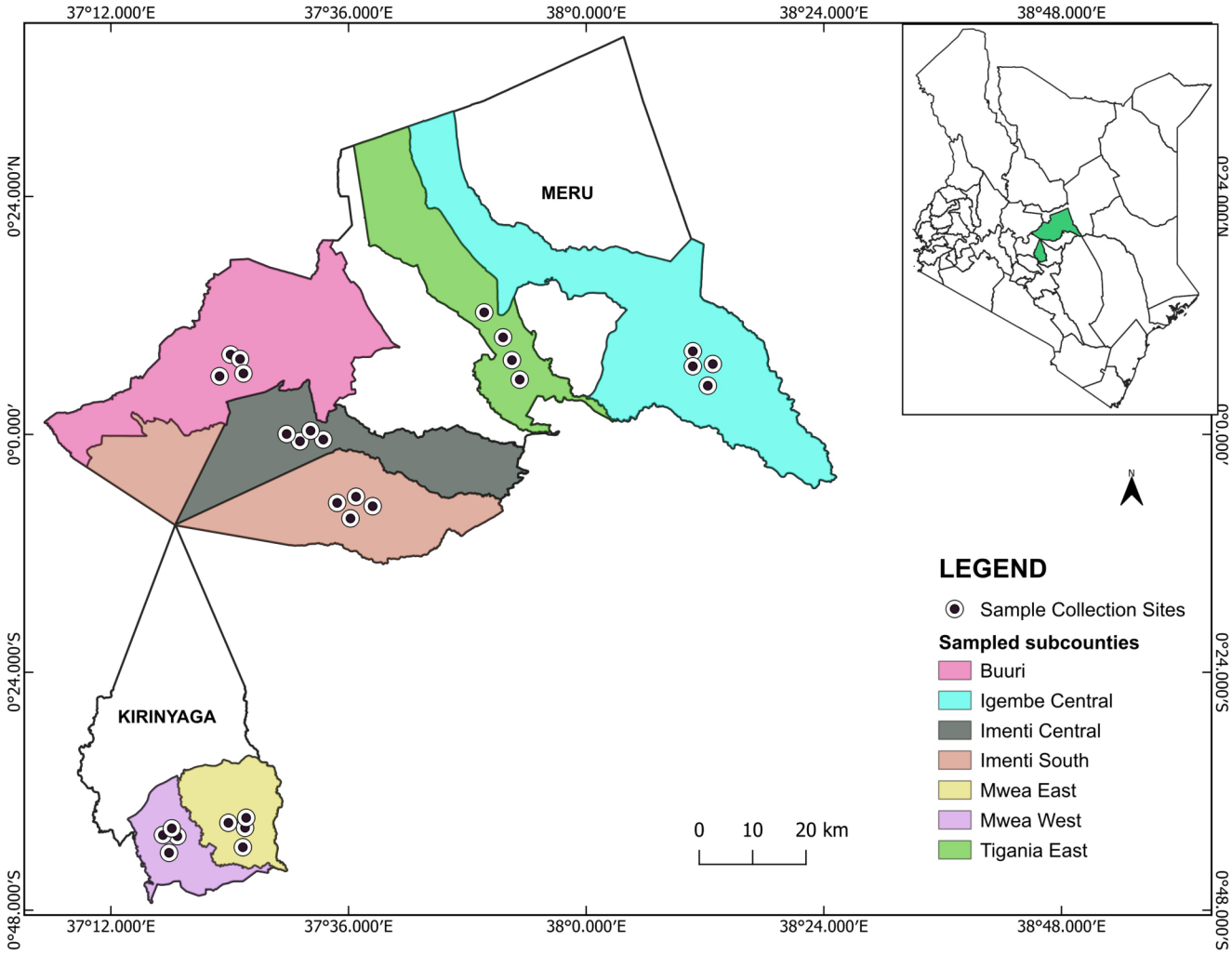

Fig. 1 Map of Kirinyaga and Meru Counties showing sample collection sites in different sub-Counties

Study Design and Blood Sampling

This study was part of a larger project in which the occurrence and risk factors for *T. gondii* infection in donkeys in Kenya were investigated and reported by [25]. This cross-sectional study was conducted on samples ($n = 363$) obtained from the blood of donkeys between September 2019 and December 2020. Approximately 5 ml of blood was collected aseptically from each animal by venipuncture through the jugular vein in sterile 10 ml tubes (Vacutainer® Beckton-Dickinson, USA) containing EDTA (anticoagulant). Blood samples were labeled and stored in an ice box during the day and transported to the laboratory at Meru University of Science and Technology, where vacutainer tubes were removed from the ice box and stored at -20 °C until use.

Genomic DNA Extraction and Nested Polymerase Chain Reaction (PCR) Targeting the *Toxoplasma gondii* ITS-1 Gene

Genomic DNA was extracted from the 96 seropositive blood samples using a Wizard® Genomic DNA Kit (Promega, Madison Wisconsin, USA) following the manufacturer's instructions and stored at -20 °C until use for PCR amplification. Nested PCR targeting 227 base pairs (bp) of the internal transcribed spacer 1 (ITS-1) gene was performed using external and internal primers in two subsequent amplifications as previously described by [26]. PCR amplification was performed in a PTC-100 PELTIER-MJ research thermocycler (Bio-Rad, Hercules, CA, USA). The first round of amplification was carried out using forward external primer NN1 (5'-CCT TTG AAT CCC AAG CAA AAC ATG AG-3') and reverse external primer NN2 (5'-GCG AGC CAA GAC ATC CAT TGC TGA-3') in a total of 20 µl of PCR mixture. The reaction mixture contained

2.0 µl of DNA template (50 ng/µl), 0.3 µl of each forward and reverse primer, 0.3 µl of My Taq polymerase (5 U/µl; Bioline, UK), 4 µl of 5× PCR buffer (Bioline, UK) and 13.1 µl of double-distilled nuclease-free water. The amplification procedure was as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C for 30 s and extension at 72 °C for 30 s; and a final extension at 72 °C for 3 min.

The second round of amplification was carried out using forward internal primers Tg-NP1 (5'- GTGATAGTATCGA AAGGTAT-3') and reverse internal primer Tg-NP2 (5'-A CTCTCTCTCAAATGTTCCT-3'). The PCR products from the first round of amplification were diluted 1:10 using double-distilled nuclease-free water and used as a template for nested PCR with internal primers. The protocol for nested PCR was as follows: initial denaturation at 94 °C for 5 min, followed by 39 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 1 min and extension at 72 °C for 30 s. The final extension was performed at 72 °C for 3 min. In all the experiments, negative controls (2 µl of nuclease-free water and a DNA sample of blood from a healthy donkey) and a positive control (DNA from *Toxoplasma gondii*) were included. The nested PCR products were electrophoresed on a 1.5% agarose gel and visualized with a UV gel documentation system (Bio-Rad Gel Doc XR imaging system, California, USA).

Sequencing, Sequence Alignment and Phylogenetic Analysis

Following nested PCR, the amplicons were purified and subjected to Sanger sequencing (Macrogen Europe, The Netherlands). The sequences from forward and reverse primer sequencing were assembled, manually edited, trimmed and aligned to obtain a consensus sequence. The resulting ITS-1 consensus sequences were subjected to a Basic Local Alignment Search Tool (BLAST) search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the nucleotide database in the National Center for Biotechnology Information (NCBI) GenBank nucleotide database to determine nucleotide similarity. A Basic Local Alignment Search Tool (BLAST) search was used to determine the percentage identity of the generated sequences against previously published sequences. Multiple sequence alignment was carried out using the BioEdit (v7.3.1.0) sequence alignment editor 7.3.1.0. Phylogenetic analysis of the ITS-1 consensus sequence was performed using MEGA 6 software. The maximum likelihood method using the Tamura–Nei model was used to infer evolutionary history. The maximum composite likelihood was used to estimate the pairwise distance of a matrix of the phylogenetic tree that was constructed using the neighbor joining method. *Hammondia hammondi* (accession no. AH008381) was used as an outgroup species for the ITS-1 gene. Natural selection, genetic diversity between sequences and neutrality tests were conducted to assess the genetic distances using DnaSP (v5.10.01 software). The number of haplotypes and polymorphic sites and the ratio of nonsynonymous substitution (dN) to synonymous substitution (dS) were evaluated via DnaSP (v5.10.01) software. The genetic relationships of the aligned ITS-1 gene sequences were determined using TSC (v1.21). All the sequences obtained in this study were submitted to the National Center for Biotechnology Information under accession numbers PP531375-PP531404.

Genotyping Using Multilocus Nested PCR-RFLP

Samples that were positive for the *T. gondii* ITS-1 gene according to nested PCR were selected for further genetic characterization. Genotyping was performed using eight genetic markers (3'SAG 2, 5'SAG 2, Alt. SAG 2, SAG 3, GRA 6, C29-2, BTUB and L358) as described previously by [27]. The standard protocol for multilocus nested PCR previously described by [27] was modified to include individual nested PCR in single reactions by the use of the same primers for individual *T. gondii* genetic markers. Briefly, for each genetic marker, nested PCR was carried out with a set of external primers for each of the eight markers in a PTC 100 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). The first round of PCR was carried out in a final volume of 20 µl containing 0.3 µl of My Taq polymerase (5 U/µl; Bioline, UK), 4 µl of 5× PCR buffer (Bioline, UK), 13.1 µl of molecular water, 0.3 µl of each external forward and reverse primer and 2.0 µl of target DNA. The PCR conditions used for each marker were the same except for the annealing temperature, which was different (Table 1). The thermal cycling conditions were 95 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s and annealing at 72 °C for 2 min; and a final extension at 72 °C for 10 min. The PCR products from the first round of PCR were diluted 1:1 with nuclease-free water and used as templates for the second round of PCR. Briefly, 12.5 µl of My Taq PCR Master Mix (Bioline, UK), 0.3 µl of each internal primer and 3 µl of template (first-round PCR product diluted with nuclease-free water at a ratio of 1:1) were used. The thermal cycling conditions were 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s and annealing at 72 °C for 1 min; and a final extension at 72 °C for 10 min for all the genetic markers. In each reaction, 2 µl of eight positive controls, GT1, PTG, CTG, TgCgCal, MAS, TgCtBr5, TgCtBr64, and TgRsCr1, with *T. gondii* DNA (kindly provided by Prof. Chunlei Su, University of Tennessee, Knoxville, USA), and negative controls (double-distilled nuclease-free water and blood samples from a healthy donkey) were included. The PCR products from the second step were visualized after 1.5% agarose

Table 1 Genetic markers, primers and restriction enzymes for PCR-RFLP used for genotyping of *T. gondii* in donkeys from Kirinyaga and Meru counties

Genetic marker	Primers	Annealing temperature (°C)	Restriction enzymes and digestion conditions	Expected amplicon size
3' SAG 2	External (5'-3)		HhaI, 37°C 3 hours 2.5% gel	222
	F: TCTGTTCTCCGAAGTGACTCC			
	R: TCAAAGCGTGCATTATCGC			
	Internal (5'-3)	57.2		
	F: ATTCTCATGCCTCCGCTTC			
	R: AACGTTTCACGAAGGCACAC			
5'SAG 2	External (5'-3')		MboI, 37°C 3 hours 2.5% gel	242
	F: GGAACGCGAACAATGAGTTT	57.2		
	R: GCACTGTTGTCCAGGGTTTT			
	Internal (5'-3')			
	F: GAAATGTTTCAGGTTGCTGC			
	R: GCAAGAGCGAACTTGAACAC	51.9		
Alt. SAG 2	External		HinfI + TaqI, 37°C, 30 minutes, 65°C 30 minutes 2.5% gel.	546
	F: GGAACGCGAACAATGAGTTT	57.2		
	R: GCACTGTTGTCCAGGGTTTT			
	Internal			
	F: ACCCATCTGCGAAGAAAACG			
	R: ATTTCGACCAGCGGGAGCAC	51.9		
SAG3	External		NciI, 37°C 3 hours, 2.5% gel.	225
	F: CAACTCTCACCATTCCACCC	50.0		
	R: GCGCGTTGTTAGACAAGACA			
	Internal			
	F: TCTTGTCGGGTGTTCACTCA	54.0		
	R: CACAAGGAGACCGAGAAGGA			
GRA 6	External		MseI, 37°C 3 hours, 2.5% gel.	344
	F: ATTTGTGTTTCCGAGCAGGT	57.0		
	R: GCACCTTCGCTTGTGGTT			
	Internal			
	F: TTTCCGAGCAGGTGACCT	51.9		
	R: TCGCCGAAGAGTTGACATAG			
C29-2	External		HpyCH4IV + RsaI, 37°C 3 hours, 2.5% gel.	446
	F: ACCCACTGAGCGAAAAGAAA	51.9		
	R: AGGGTCTCTTGCGCATACAT			
	Internal	51.9		
	F: AGTTCTGCAGAGTGTCGC			
	R:TGTCTAGGAAAGAGGCGC			
BTUB	External		BsiEI + TaqI, 37°C 3 hours, 2.5% gel.	411
	F: TCCAAAATGAGAGAAATCGT	51.0		
	R: AAATTGAAATGACGGAAGAA			
	Internal			
	F: GAGGTCATCTCGGACGAACA	60.0		
	R: TTGTAGGAACACCCGGACGC			
L358	External		HaeIII + NlaIII, 37°C 3 hours, 2.5% gel.	418
	F: TCTCTCGACTTCGCCTCTTC	60.0		
	R: GCAATTTCCTCGAAGACAGG			
	Internal			
	F: AGGAGGCGTAGCGCAAGT	60.0		
	R: CCCTCTGGCTGCAGTGCT			

gel electrophoresis using 0.05% ethidium bromide-stained agarose gel in 1× Tris-acetic-EDTA (TAE) buffer (HiMedia, India).

The nested PCR products for the eight successfully amplified markers were digested using restriction endonucleases (New England Biolabs, USA) specific for each genetic marker according to the manufacturer's guidelines (Table 1). The restriction enzyme digestion mixture for six markers (3'SAG 2, 5'SAG 2, Alt. SAG 2, SAG3, GRA 6, and C29-2) were made up of 10 µl of nested PCR product, 9 µl of 10× Cut smart buffer and 1 µl of restriction enzymes, except for BTUB and L358 markers, which required double digestion, and whose mixture was made up of 10 µl of nested PCR product, 8 µl of 10× Cut smart buffer and 1 µl of each restriction enzyme and whose mixture was incubated at 37 °C for 3 h. Twenty microliters of the digested products were electrophoresed on 2.5% agarose gels and visualized via a UV gel documentation system (Bio-Rad, Hercules, CA, USA). The banding patterns of the digested products from each sample were compared with those of the genotypes deposited in ToxoDB (http://toxodb.org.toxo/).

Results

Molecular Detection of the ITS-1 Gene of *T. gondii*

The 96 *T. gondii* seropositive blood samples were subjected to nested PCR amplification targeting the ITS-1 gene. Successful amplification of the 277 bp product of the ITS-1 gene was observed in 35 (36.5%) out of the 96 DNA samples (Supplementary Table S1). A representative agarose gel image of the PCR amplicons is presented in Supplementary Fig. S1.

Sequence, BLAST and Multiple Sequence Alignment Analysis

Sanger sequencing of the 35 PCR amplicons was performed and confirmed the presence of *T. gondii* DNA in 85.71% (30/35) of the blood samples from the donkeys used in the present study. A BLAST analysis of the partial sequences of the ITS-1 gene revealed 99.5% nucleotide homology. BLAST analysis of the 30 nucleotide sequences obtained in this study indicated 98.2–99.5% sequence homology with reported *T. gondii* sequences from goats (OL461229) in Pakistan, European bison (KX459518) in Poland, cats (KP895872) in Thailand, Eurassian otter (KM657806) in Norway, Sparrows (GQ160468) in Brazil, cats (EU25025) in Germany and sea otters (KX999999) in California, USA (Supplementary Table S2). Multiple sequence alignment of 43 nucleotide substitutions in *T. gondii* isolates from the present study. The ITS region (219 sites/positions) of the 30 sequences had a conservation index of 92.7%. Eighteen mutations were distributed within 16 polymorphic sites. Of the 16 polymorphic sites, 11 were singletons (only one sequence out of 30), while 5 were parsimony informative (more than one sequence). The singleton sites were at nucleotide positions 25, 60, 86, 102, 133, 134, 159, 172, 194, 198, and 210. The parsimony informative sites were at nucleotides 16, 96, 112, and 191 (Supplementary Fig. S2). On the basis of multiple sequence alignments of the ITS-1 gene amino acid sequences, 54 nonsynonymous amino acid substitutions of *T. gondii* codon positions 11 [tryptophan (W) and arginine (R) instead of glycine (G)], 29 [arginine (R) instead of histiidine (H)], 38 [leucine (L) instead of isoleucine (I)], 45 [proline (P) instead of leucine (L)], 59 [serine (S) instead of histidine (H)], 64 [arginine (R) instead of glutamine (Q)] and 70 [leucine (L) instead of phenylalanine (F)] (Supplementary Fig. S2) were observed in the sequences obtained in this study.

Analysis of partial ITS-1 gene sequences of the 30 Kenyan *T. gondii* isolates and other *T. gondii* sequences retrieved from the GenBank revealed 11 genetic variants. These genetic variants were selected on the basis of their differences from each other by the presence of at least one mutation in their nucleotide sequence that occurred as a substitution (Supplementary Fig. S4).

Phylogenetic Analysis of the ITS-1 Gene Sequence of *T. gondii*

Phylogenetic analysis revealed that the 30 sequences obtained in this study formed one cluster with other *T. gondii* strains retrieved from GenBank, revealing no discrimination of the different genotypes. A phylogenetic tree constructed using partial ITS-1 gene sequences of *T. gondii* in this study and sequences from GenBank (Fig. 2) confirmed that six sequences (T47 ITS, T61 ITS, T64 ITS, T65 ITS, T71 ITS, and T12 ITS) were closely related to *T. gondii* sequences reported in Norway, China, Brazil, Japan, the USA, Pakistan, Germany, Poland, Iraq and Tunisia. Seven sequences, namely, the T54 ITS, T59 ITS, T62 ITS, T89 ITS, T92 ITS, T46 ITS, and T94 ITS, were also closely related to the sequences of isolates reported in Thailand, Brazil and the USA. Four *T. gondii* isolates, namely, the T55 ITS, T58 ITS, T85 ITS and T93 ITS, were homologous to sequences of *T. gondii* isolates from Malaysia, Brazil and Iraq.

Genetic Diversity Analysis

Genetic diversity indices and neutrality test analyses indicated that the *T. gondii* population in the present study exhibited low genetic diversity ($\pi = 0.01027$). The analysis

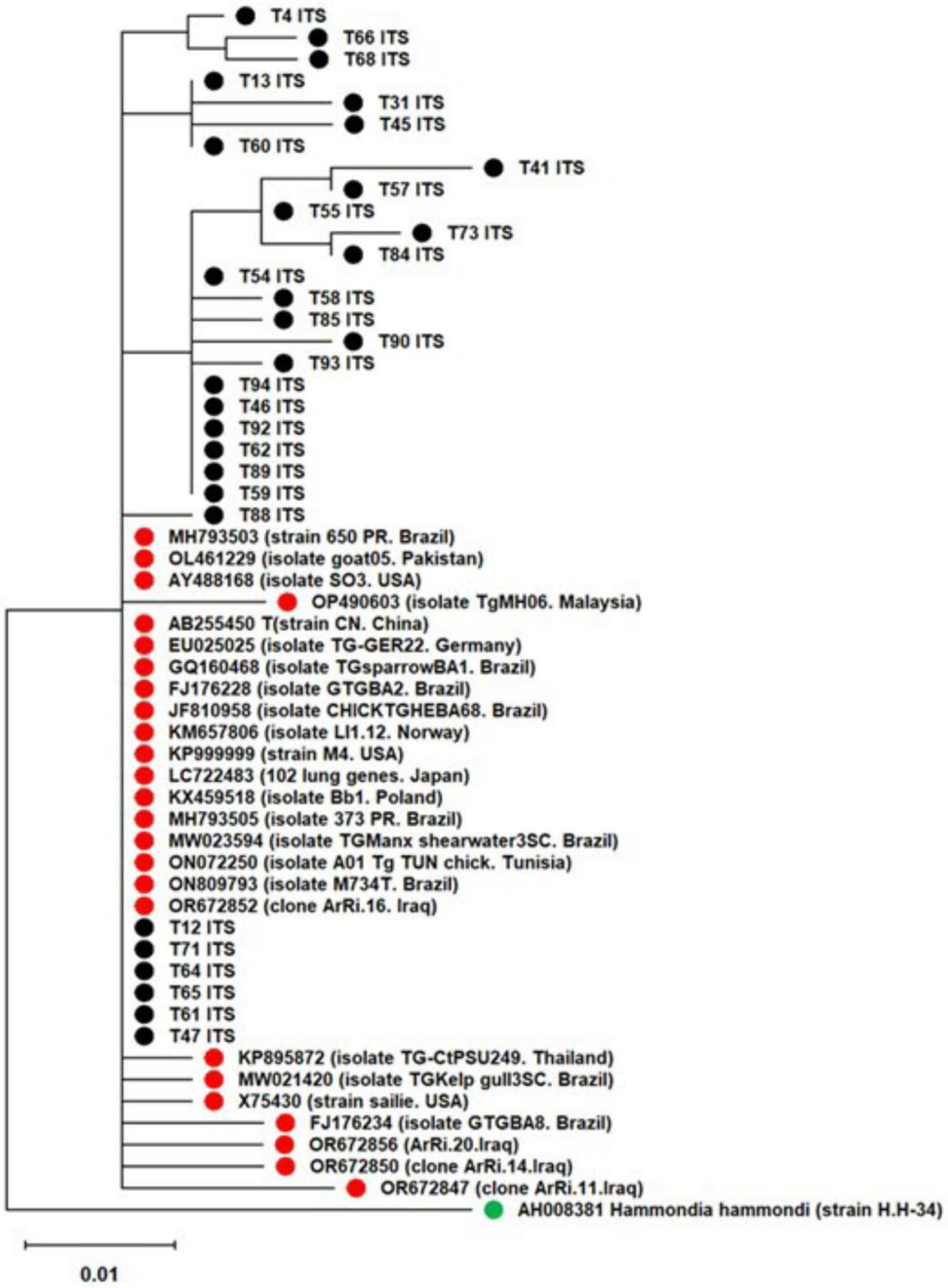

Fig. 2 Phylogenetic tree inferred using the sequenced ITS-1 region of *T. gondii* and sequences from the GenBank database. The tree was constructed using the maximum likelihood method and the Tamura–INei model. The numbers above the branches are bootstrap values as a percentage of 1000 replicates supporting each evolutionary branch. Because none of the bootstrap values exceeded 70%, they are not shown. The sequences marked with black circles are local isolates, while the sequences marked in red are accessions from the GenBank database. *H. hammondi accessions AH008381* served as an outgroup

of polymorphic sites confirmed the presence of 18 distinct haplotypes circulating in donkeys in Kenya, with a Hd of 0.915. The other genetic diversity (segregating sites, nucleotide diversity and average number of pairwise nucleotide differences) and neutrality test (Tajima's D, Fu, Li's Fs, Fu's Fs, and Ramos-Onsins and Rozas$_{R2}$) results are presented in Supplementary Table S3.

Haplotype Network Analysis

In total, 18 different haplotypes were recorded across the sampling sites in Kirinyaga and Meru Counties. Fifteen haplotypes (Hap_1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 18) and 5 haplotypes (Hap_2, 7,15, 16 and 17) were confirmed to be circulating with *T. gondii* strains in Kirinyaga and Meru Counties, respectively (Fig. 3). According to the TSC network, five haplotypes, namely, Hap_1, 2, 6, 7 and 16, were shared across the populations but were

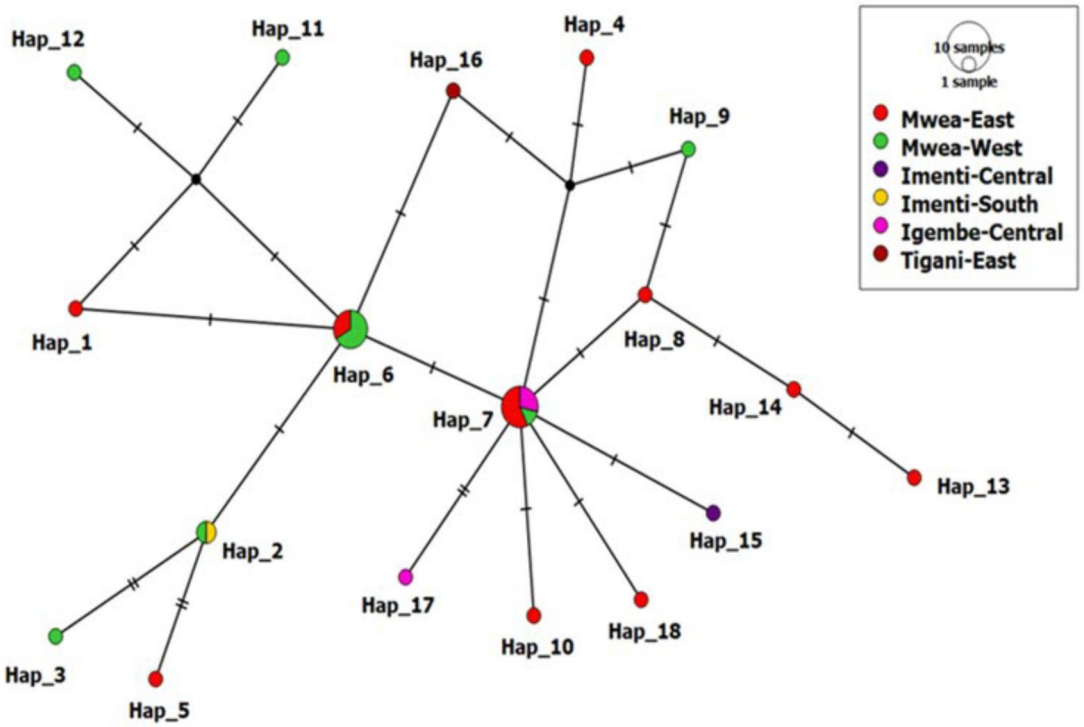

Fig. 3 HaplotypeTCS network analysis of the local relationships of *T. gondii* isolates from different subcounties in Kenya based on the sequenced ITS-1 region. The hatch marks are the number of polymorphisms resulting in a specific haplotype, while the size of each circle corresponds to the frequency of sequences under a specific haplotype

frequent in the Mwea East and Mwea West subcounties. Hap_1, 4, 5, 8, 10, 13 and 18 were exclusively recorded in Mwea West, while Hap_3, 9, 11 and 12 were exclusively recorded in Mwea East in Kirinyaga County. Hap_7 was recorded in Igembe Central, while Hap_15 and 16 were recorded in Imenti Central and Tigania East, respectively. Two distinct haplotypes (Hap_6 and 7) were widely spread among the populations, and these two *T. gondii* haplotypes had the highest number of isolates with the corresponding sequences. Analysis of the 18 haplotypes via the TSC network revealed that Hap_3, 5 and 7 were connected to two mutation steps, while the majority of them were connected to one mutation step.

Genotyping of *T. gondii* in Donkeys Using Multilocus Nested PCR-RFLP (Mn-PCR-RFLP)

In the present study, complete genotyping results were obtained for only five *T. gondii* DNA samples (54, 59, 60, 89 and 94) using all eight genetic markers (Table 2 and Supplementary Fig. S5), while partial genotyping was obtained for 10 of the remaining samples. When the results of this study were compared to those in ToxoDB, the five DNA samples that were successfully amplified were mixed (atypical) types, and they exhibited combinations of alleles I, II and III. It was not possible to determine the genotypes of the ten isolates because the targeted sequences were not amplified for any of the markers.

Discussion

Toxoplasma gondii infection in donkeys was confirmed by ITS-1 amplicon sequencing. BLAST analysis of the partial sequences obtained from our PCR-positive samples confirmed *T. gondii* infection. These results showed that the amplified sequences were conserved and closely related to other identified *T. gondii* sequences that were obtained from goats in Pakistan [28], European bison in Poland [29], cats in Germany and Thailand [30, 31] Eurassian otters in Norway [32], sparrows in Brazil [33] and sea otters in California, USA [34]. Phylogenetic analysis revealed one variant that clustered together with those from Asia, Africa, Europe, South America and North America.

The haplotype diversity of the *T. gondii* sequences in the present study was high, suggesting that there was a selective mutation where some haplotypes were likely subjected to selective pressure within a small size of the expanding population. The nucleotide diversity was low, suggesting the presence of limited genetic diversity of *T. gondii* DNA samples infecting donkeys in central Kenya. Limited genetic diversity of *T. gondii* strains has also been reported

Table 2 A genotyping summary of 8 genetic markers (3' SAG 2, 5' SAG 2, (3'+5') SAG 2, Alt. SAG 2, SAG 3, GRA6, C-29-2, BTUB, L358) on 15 blood samples from donkeys

Donkey ID	Sub-County (Location)	3' SAG 2	5' SAG 2	(3'+5') SAG 2	Alt. SAG 2	SAG 3	GRA6	C-29-2	BTUB	L358
46	Mwea West	II	II	II	II	II	nd	II/III	III	nd
54	*Mwea East*	*II*	*I*	*III*	*II**	*II*	*III**	*II/III**	*III**	*I**
59	*Mwea East*	*II*	*II*	*II**	*II**	*II*	*III**	*II/III*	*III**	*I**
60	*Imenti South*	*II*	*II*	*II**	*II**	*II**	*II*/III*	*II*/III*	*III*	*I**
62	Igembe Central	II	II	II	nd	II	II/III	II/III	III	I
65	Mwea East	II	II	II	II	II	II/III	II/III	III	nd
66	Mwea West	nd	II	II	II	nd	II	II/III	III	nd
84	Mwea East	II	II	II	II	II	II	nd	III	nd
85	Imenti Central	II	III	III	nd	II	II/III	III	III	nd
88	Tigania East	II	II	II	II	nd	II	III	III	I
89	*Mwea East*	*II*	*II*	*II**	*II**	*II*	*II/III**	*III*	*III**	*I**
90	Igembe Central	II	II	II	II	nd	II	nd	III	nd
92	Mwea East	II	III	II	II	II	II/III	nd	II/III	nd
93	Mwea East	II	III	II	II	II	II/III	nd	III	nd
94	*Igembe Central*	*II*	*III*	*II*	*II**	*II*	*II/III**	*III**	*III**	*I**

*= alleles that match the genotype in ToxoDb; nd = not determined

in chickens and pigs from Malaysia based on the ITS-1 gene sequence [35]. In the present study, low genetic diversity, as opposed to high haplotype diversity, could indicate the rapid expansion of *T. gondii* strains from a small population, which may lead to selective sweeping by a highly successful lineage of *T. gondii* mutants, favoring the accumulation of genetic diversity [36].

Genetic studies involving *T. gondii* haplotypes in donkeys using ITS-1 are rare, making it difficult to compare studies using haplotype diversity in donkeys. Despite the scarcity of information on *T. gondii* haplotypes in donkeys, the number of haplotypes identified in this study is greater than that reported in other studies, such as that of [35], who recorded six haplotypes in tissue samples from village chickens and pigs using the ITS-1 gene of *T. gondii* in Malaysia. Using DNA analysis of the B1 gene, [37] recorded 13 distinct *T. gondii* haplotypes in naturally infected sheep in Mexico. In Nigeria, using the PCR-RFLP method with 5 markers (5 + 3 sag 2, sag 3, btub and gra 6 and Apico), [38] recorded nine distinct haplotypes in humans, free-range chickens and pigs. These findings indicate that the *T. gondii* strain circulating in the current study regions is genetically unstable. However, the use of only ITS-1 gene may not provide detailed understanding of the genetic diversity of *T. gondii* infections in donkeys in Kenya [39].

Molecular analysis of the sequences obtained in the present study suggested limited genetic diversity, which resulted in the identification of eighteen distinct haplotypes circulating in donkeys in Kenya. The limited genetic diversity could be because the samples used in this study were obtained from closely located sampling sites. Previous studies in Malaysia using the ITS-1 gene [35] and in Nigeria using PCR-RFLP markers [38] have documented the limited genetic diversity of *T. gondii*. The findings of this study suggest that rare sexual recombination occurred in the definitive hosts (cats in this case) in the study area and donkeys from central Kenya may have acquired the infection via the ingestion of contaminated feed and water [14], which might have resulted in the limited genetic diversity of the *T. gondii* DNA samples obtained in this study. However, a limitation for this study is that only ITS-1 gene was used which is not sufficient to determine the genetic diversity and therefore there is need to use multiple target genes for a detailed understanding of the genetic diversity of *T. gondii* infections in donkeys in Kenya.

Network analysis suggested that the *T. gondii* strains circulating in Meru County originated from Kirinyaga County, suggesting that there is a gene flow between the Kirinyaga and Meru DNA samples, which could explain the limited genetic diversity of the DNA samples observed in this study. This could presumably be due to donkey trade patterns and the movement of donkeys between Meru and Kirinyaga through the livestock chain linking the two Counties. In this context, an infected donkey from Kirinyaga County could be slaughtered or die in Meru County. The carcass and offal of this donkey could be consumed by local cats leading to the introduction of new *T. gondii* strains. This highlights the need to educate donkey owners on the importance of donkeys in the transmission of the parasite through consumption of donkey products such as meat. The ITS-1 mutant strains of *T. gondii* from Kenya, particularly Haplotype 6, appear to share lineages with strains from Norway, China, Brazil, Japan, the USA, Pakistan, Germany, Poland, Iraq and Tunisia, which were retrieved from the GenBank as suggested by phylogenetic analysis. The fact that haplotype

6 was also detected in these countries indicates that *T. gondii* is not specific to certain geographical areas or hosts.

Studies on the genotypic characterization of *T. gondii* in donkeys are limited, and they include those of [40], who recorded ToxoDB #1 and ToxoDb #9 in Shadong, Eastern China; [41], who recorded ToxoDB #9 and ToxoDB# 10 in Jilin and Liaoning, China; and [42], who recorded ToxoDB #163 in Brazil. Other studies on the genetic characterization of *T. gondii* in donkeys include those of [13], who identified type II and III genotypes in the blood and milk of donkeys in Tuscany, Italy. The *T. gondii* isolates recorded in this study are genetically different from the isolates recorded in donkeys worldwide. The differences in the genotypes recorded may be attributed to variations in the population structure of *T. gondii* in different localities [43]. Regardless of the genotype recorded, all the studies indicate that donkeys can harbor *T. gondii* and be a source of infection in humans.

Previous genotyping studies using nested PCR-RFLP in Kenya with one genetic marker (at the SAG 2 locus) identified the allele II genotype as the most predominant genotype in chickens from Kisumu [44]. The results of genotypic characterization of this study indicate that atypical genotypes circulate in food animals such as donkeys in central Kenya. The findings of atypical genotypes in this study are consistent with the results of a previous study by [45] who demonstrated atypical genotypes in the brain tissues of food animals in Ghana. A limitation of this study is that accurate genotypes of *T. gondii* could not be revealed because genotype analysis was not performed with all 10 genetic markers. The genotyping of *T. gondii* with all the 10 markers allows the identification of the three genotypes including any new *T. gondii* genotype [46]. However, successful genotyping with many different RFLP markers can only be possible after bioassays in mice to increase the concentration of *T. gondii* DNA before genotyping with PCR-RFLP technique [47]. In this study, we did not have animal bioassay facilities and therefore negative amplification products could be observed when genotyping directly from DNA samples. Further studies are therefore required to determine the specific genotypes of *T. gondii* circulating in Kenya and the association between pathogenicity and genotype.

Conclusion

This is the first report on the presence of *T. gondii* DNA and distinct genotypes in donkeys located in central Kenya. The detection of *T. gondii* in donkey blood suggested that the meat and milk of these animals are potential sources of *T. gondii* infection transmission to humans. The study has also demonstrated limited genetic diversity of *T. gondii* haplotypes circulating in donkeys. The five DNA samples that were successfully genotyped from Meru and Kirinyaga were identified as atypical genotypes. However, further studies are needed to determine the association between pathogenicity and genotype. These new findings can be used as a basis for future studies on the genetic diversity and molecular epidemiology of *Toxoplasma gondii*. Further studies using different molecular markers and larger sample sizes should be conducted in different geographical areas to determine the distribution patterns of *T. gondii* haplotypes and genotypes in Kenya. Since *T. gondii* is a zoonotic parasite and since donkey meat has been legalized in Kenya, these results emphasize the need for donkey owners and donkey meat handlers in the value chain to be educated about the role played by donkeys in the transmission of *T. gondii* to humans. Emphasis should be given to the importance of controlling this parasite in donkey meat to protect consumers. We recommend a nationwide *T. gondii* detection program to prevent its spread to other domestic animals and humans in Kenya.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11686-024-00905-z.

Acknowledgements The authors would like to thank the donkey owners from the study areas for their participation and cooperation and the Department of Biochemistry, University of Nairobi, for providing the necessary facilities to carry out this research. We wish to acknowledge Dr. Chunlei Su (Department of Microbiology, University of Tennessee, Knoxville, USA) for kindly providing the reference strains.

Author Contributions F.O.O. designed the study, conducted fieldwork, laboratory work, statistical analyses, wrote and edited the original manuscript. N.M. conceived the study, reviewed and edited the manuscript, and provided supervision. S.M.G. conceived the study, reviewed and edited the manuscript, and provided supervision. A.A.O. conducted laboratory work. K.O.O. interpreted the data. E.N.N. conceived the study, reviewed and edited the manuscript, and provided supervision. All authors reviewed the manuscript.

Funding This study was partly funded by a grant from Meru University of Science and Technology (MUST), 6th call for individual research proposal.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethical Statement This study was approved by the Biosafety, Animal Use and Ethics Committee, Faculty of Veterinary Medicine, University of Nairobi, Kenya, reference number FVM BAUEC/2019/240, and informed consent was obtained before sampling at the household level from the donkey owners.

Competing Interests The authors declare no competing interests.

References

1. Dubey JP (2009) History of the discovery of the life cycle of Toxoplasma gondii. Int J Parasitol 39:877–882. https://doi.org/10.1016/j.ijpara.2009.01.005
2. Tenter AM (2009) Toxoplasma gondii in animals used for human consumption. Mem Inst Oswaldo Cruz 104:364–369. https://doi.org/10.1016/j.fawpar.2019.e00037
3. Dubey JP, Lindsay DS, Speer CA (1998) Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clin Microbiol Rev 11:267–299. https://doi.org/10.1128/CMR
4. Dubey JP (2010) Toxoplasmosis of animals and humans. Boca Raton, Florida
5. Koutsoumanis K, Allende A, Alvarez-Ord´o˜nez A, Bolton D, Bover-Cid S, Chemaly M, Davies R, de Cesare A, Herman L, Hilbert F, Lindqvist R, Nauta M, Peixe L, Ru G, Simmons M, Skandamis P, Suffredini E, Cacci`o S, Chalmers R, Deplazes P, Devleesschauwer B, Innes E, Romig T, van der Giessen J, Hempen M, van der Stede Y, Robertson L (2018) Public health risks associated with food-borne parasites. EFSA J 16. https://doi.org/10.2903/j.efsa.2018.5495
6. Chiari CA, Neves DP (1984) Human toxoplasmosis acquired by ingestion of goat's milk. Mem Inst Oswaldo Cruz 79:337–40. https://doi.org/10.1590/s0074-02761984000300007
7. Stelzer S, Basso W, Benavides Silván J, Ortega-Mora LM, Maksimov P, Gethmann J, Conraths FJ, Schares G (2019) *Toxoplasma gondii* infection and toxoplasmosis in farm animals: risk factors and economic impact. Food Waterborne Parasitol 15:e00037. https://doi.org/10.1016/j.fawpar.2019.e00037
8. Marquis ND, Bishop TJ, Record NR, Countway PD, Fernandez Robledo JA (2019) Molecular epizootiology of *Toxoplasma gondii* and *Cryptosporidium parvum* in the Eastern Oyster (Crassostrea virginica) from Maine (USA). Pathogens 8:125. https://doi.org/10.3390/pathogens8030125
9. Weiss LM, Dubey JP (2009) Toxoplasmosis: a history of clinical observations. Int J Parasitol 39:895–901. https://doi.org/10.1016/j.ijpara.2009.02.004
10. Miao Q, Wang X, She LN, Fan YT, Yuan FZ, Yang JF, Zhu XQ, Zou C (2013) Seroprevalence of *Toxoplasma gondii* in horses and donkeys in Yunnan Province, Southwestern China. Parasit Vectors 6:168. https://doi.org/10.1186/1756-3305-6-168
11. Maichomo MW, Karanja Lumumba T, Olum MO, Magero J, Oketch T, Nyoike SN (2019) The status of donkey slaughter and its implication on community livelihoods. KALRO report submitted to the Brook East Africa
12. Gichure M, Onono J, Wahome R, Gathura P (2020) Analysis of the benefits of production challenges of working donkeys in small holder farming systems in Kenya. Vet World 13(11):2356–2352. https://doi.org/10.14202/vetworld.2020.2346-2352
13. Mancianti F, Nardoni S, Papini R, Mugnaini L, Martini M, Altomonte I, Salari F, D'Ascenzi C, Dubey JP (2014) Detection and genotyping of Toxoplasma gondii DNA in the blood and milk of naturally infected donkeys (Equus asinus). Parasit Vectors 7:165. https://doi.org/10.1186/1756-3305-7-165
14. Symeonidou I, Sioutas G, Lazou T, Gelasakis AI, Papadopoulos E (2023) A review of *Toxoplasma gondii* in animals in Greece: a FoodBorne. Pathogen Public Health Importance Anim 13:2530. https://doi.org/10.3390/ani13152530
15. Sharif M, Amouei A, Sarvi S, Mizani A, Aarabi M, Hosseini SA, Daryani A (2017) Genetic diversity of *Toxoplasma gondii* isolates from ruminants: a systematic review. Int J Food Microbiol 258:38–49. https://doi.org/10.1016/j.ijfoodmicro.2017.07.007
16. Costa ME, Oliveira CB, Andrade JM, Medeiros TA, Neto VF, Lanza DC (2016) An alternative nested-PCR assay for the detection of toxoplasma gondii strains based on GRA7 gene sequences. Acta Trop 159:120–124. https://doi.org/10.1016/j.actatropica.2016.03.035
17. Ning HR, Huang SY, Wang JL, Xu QM, Zhu XQ (2015) Genetic diversity of *Toxoplasma gondii* strains from different hosts and geographical regions by sequence analysis of GRA20 gene. Korean J Parasitol 53(3):345–348. https://doi.org/10.3347/kjp.2015.53.3.345
18. Sibley LD, Khan A, Ajioka JW, Rosenthal BM (2009) Genetic diversity of *Toxoplasma gondii* in animals and humans. Philos Trans R Soc Lond B Biol Sci 364(1530):2749–2761. https://doi.org/10.1098/rstb.2009.0087
19. Mose JM, Kagira JM, Kamau DN, Maina NW, Ngotho M, Karanja SM (2020) A review on the present advances on studies of toxoplasmosis in Eastern Africa. Biomed Res Int 2020:1–12. https://doi.org/10.1155/2020/7135268
20. Cook EAJ, Gitahi N, de Glanville WA, Thomas LF, Kariuki S, Kang'ethe E, Fèvre EM (2021) Prevalence and risk factors for exposure to *Toxoplasma gondii* in slaughterhouse workers in western Kenya. BMC Infect Dis 21(1):944. https://doi.org/10.1186/s12879-021-06658-8
21. Chepyatich D, Sentamu DN, Bor N, Onono J, Gathura PB, Akoko JM, Thomas LF (2023) Seroprevalence of *Toxoplasma gondii* in slaughtered pigs in Kiambu, Kenya. Zoonotic Dis 3(4):301–306. https://doi.org/10.3390/zoonoticdis3040024
22. Velmurugan GV, Dubey JP, Su C (2008) Genotyping studies of *Toxoplasma gondii* isolates from Africa revealed that the archetypal clonal lineages predominate as in North America and Europe. Vet Parastiol 155:314–318. https://doi.org/10.1016/j.vetpar.2008.04.021
23. Government of Kenya (GoK) (2018a) Kirinyaga County Government. County integrated development plan. 2018–2022
24. Government of Kenya (GoK) (2018b) County Government of Meru. County integrated development plan, 2018–2022
25. Obonyo FO, Maingi N, Githigia SM, Kimeli P, Nyaboga EN (2022) Occurrence of serum antibodies to *Toxoplasma gondii* and associated risk factors in donkeys from central Kenya. Trop Anim Health Prod 54(1):77. https://doi.org/10.1007/s11250-022-03079-w
26. Hurtado A, Aduriz G, Moreno B, Barandika J, Garcia-Perez AL (2001) Single tube nested PCR for the detection of *Toxoplasma gondii* in fetal tissues from naturally aborted ewes. Vet Parasitol 102:17–27. https://doi.org/10.1016/s0304-4017(01)00526-x
27. Su C, Shwab EK, Zhou P, Zhu XQ, Dubey JP (2010) Moving toward an integrated approach to molecular detection and identification of *Toxoplasma gondii*. Parasitology 137(1):1–11. https://doi.org/10.1017/S0031182009991065
28. Aziz MN, Iqbal RK, Irfan M, Parveen A, Asif M, Ozubek S, Aktas M, Said MB, Iqbal F (2022) First report on molecular epidemiology, seasonality and phylogeny of *Toxoplasma gondii* infecting goats from Khanewal district in Punjab, Pakistan. Acta Trop 228:106304
29. Moskwa B, Bień J, Kornacka A, Cybulska A, Goździk K, Krzysiak MK, Reiterova K, Cabaj W (2017) First *Toxoplasma gondii* isolate from an aborted fetus of European bison (*Bison bonasus bonasus L*). Parasitol Res 116(9):2457–2461. https://doi.org/10.1007/s00436-017-5549-0
30. Schares G, Vrhovec MG, Pantchev N, Herrmann DC, Conraths FJ (2008) Occurrence of *Toxoplasma gondii* and *Hammondia Hammondi* oocysts in the feces of cats from Germany and other European countries. Vet Parasitol 152(1–2):34–45. https://doi.org/10.1016/j.vetpar.2007.12.004
31. Chemoh W, Sawangjaroen N, Nissapatorn V, Sermwittayawong N (2016) Molecular investigation on the occurrence of *Toxoplasma gondii* oocysts in cat feces using TOX-element and ITS-1

region targets.Vet J. 215:118–122. https://doi.org/10.1016/j.tvjl.2016.05.018. https://doi
32. Gjerde B, Josefsen TD (2015) Molecular characterization of Sarcocystis lutrae n. sp. and *Toxoplasma gondii* from the musculature of two eurasian otters (Lutra lutra) in Norway. Parasitol Res 114(3):873–886. https://doi.org/10.1007/s00436-014-4251-8
33. Gondim LS, Abe-Sandes K, Uzêda RS, Silva MS, Santos SL, Mota RA, Vilela SM, Gondim LF (2010) *Toxoplasma gondii* and *Neospora Caninum* in sparrows (*Passer domesticus*) in the Northeast of Brazil. Vet Parasitol 168(1–2):121–124. https://doi.org/10.1016/j.vetpar.2009.09.055
34. Miller MA, Grigg ME, Kreuder C, James ER, Melli AC, Crosbie PR, Jessup DA, Boothroyd JC, Brownstein D, Conrad PA (2004) An unusual genotype of *Toxoplasma gondii* is common in California sea otters (*Enhydra lutris nereis*) and is a cause of mortality. Int J Parasitol 34(3):275–84. https:https://doi.org/10.1016/j.ijpara.2003.12.008
35. Leong SD, Hassan L, Sharma RSK, Toung OP, Musa HI (2023) Prevalence and haplotypes of *Toxoplasma gondii* in native village chickens and pigs in Peninsular Malaysia. Vet Sci 10:334. https://doi.org/10.3390/vetsci10050334
36. Bertranpetit E, Jombart T, Paradis E, Pena H, Dubey J, Su C, Mercier A, Devillard S, Ajzenberg D (2017) Phylogeography of *Toxoplasma gondii* points to a south American origin. Infect Genet Evol 48:150–155. https://doi.org/10.1016/j.meegid.2016.12.020
37. Martínez-Flores WA, Palma-García JM, Caballero-Ortega H, Del Viento-Camacho A, López-Escamilla E, Martínez-Hernández F, Vinuesa P, Correa D, Maravilla P (2017) Genotyping *Toxoplasma gondii* with the B1 gene in naturally, infected Sheep from an endemic region in the Pacific Coast of Mexico. Vector Borne Zoonotic Dis 17(7):495–502. https://doi.org/10.1089/vbz.2016.2085
38. Nzelu IN, Kwaga JKP, Kabir J, Lawal IA, Beazley C, Evans L, Blake DP (2021) Detection and genetic characterization of *Toxoplasma gondii* circulating in free-range chickens, pigs and seropositive pregnant women in Benue state, Nigeria. PLoS Negl Trop Dis 15(6):e0009458. https://doi.org/10.1371/journal.pntd.0009458
39. Sibley LD, Khan A, Ajioka JW, Rosenthal BM (2009) Genetic diversity of Toxoplasma gondii in animals and humans. Philos Trans R Soc Lond B Biol Sci 364(1530):2749–2761. https://doi.org/10.1098/rstb.2009.0087
40. Cong W, Chen L, Shan XF, Qian AD, Meng QF (2018) First genetic characterization of Toxoplasma gondii infection in donkey meat slaughtered for human consumption in Shandong province, eastern China. Infect Genet Evol 61:1–3. https://doi.org/10.1016/j.meegid.2018.03.008
41. Zhang XX, Shi W, Zhang N-Z, Shi K, Li JM, Xu P, Zhao Q, Du R (2017) Prevalence and genetic characterization of Toxoplasma gondii in donkeys in northeastern China. Infect Genet Evol 54:455–457. https://doi.org/10.1016/j.meegid.2017.08.008
42. Gennari SM, Esmerini P, de O, Lopes MG, Soares HS, Vitaliano SN, Cabral AD, Villalobos E, M C (2015) Occurrence of antibodies against *Toxoplasma gondii* and its isolation and genotyping in donkeys, mules, and horses in Brazil. Vet Parasitol 209(1–2):129–132. https://doi.org/10.1016/j.vetpar.2015.01.023
43. Dubey JP, Velmurugan GV, Rajendran C, Yabsley MJ, Thomas NJ, Beckmen KB, Sinnett D, Ruid D, Hart J, Fair PA, McFee E, Shearn-Bochsler V, Kwok OCH, Ferreira LR, Choudhary S, Faria EB, Zhou H, Felix TA, Su C (2011a) Genetic characterization of Toxoplasma gondii in wildlife from North America revealed widespread and high prevalence of the fourth clonal type. Int J Parasitol 41:1139–1147. https://doi.org/10.1016/j.ijpara.2011.06.005
44. Dubey JP, Karhemere S, Dahl E, Sreekumar C, Diabaté A, Dabiré KR, Vianna MC, Kwok OC, Lehmann T (2005) First biologic and genetic characterization of *Toxoplasma gondii* isolates from chickens from Africa (Democratic Republic of Congo, Mali, Burkina Faso, and Kenya). J Parasitol 91:69–72. https://doi.org/10.1645/GE-410R
45. Pappoe F, Cheng W, Wang L, Li Y, Obiri-Yeboah D, Nuvor SV, Ambachew H, Hu X, Luo Q, Chu D, Xu Y, Shen J (2017) Prevalence of *Toxoplasma gondii* infection in HIV-infected patients and food animals and direct genotyping of *T. gondii* isolates, Southern Ghana. Parasitol Res 116(6):1675–1685. https://doi.org/10.1007/s00436-017-5442-x
46. Su C, Zhang X, Dubey JP (2006) Genotyping of *Toxoplasma gondii* by Multilocus PCRRFLP markers: a high resolution and simple method for identification of parasites. Int J Parasitol 36:841–848
47. Bezerra RA, Carvalho FS, Guimarães LA, Rocha DS, Silva FL, Wenceslau AA, Albuquerque GR (2012) Comparison of methods for detection of *Toxoplasma gondii* in tissues of naturally exposed pigs. Parasitol Res 110:509–514