AMERICAN JOURNAL OF BIOMEDICINE AND PHARMACY
https://biojournals.us/index.php/AJBP
ISSN: 2997-7177

Article

Genetic Diversity of Toxoplasma gondii Isolates from Humans and Livestock Based on SAG2 and GRA6 Gene Polymorphisms: A Cross-Sectional Molecular Study

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Citation: Hadi, Z. Genetic Diversity of Toxoplasma gondii Isolates from Humans and Livestock Based on SAG2 and GRA6 Gene Polymorphisms: A Cross-Sectional Molecular Study. American Journal of Biomedicine and Pharmacy 2026, 3(11), 132-145.

Received: 28th Aug 2026
Revised: 15th Sept 2026
Accepted: 03rd Nov 2026
Published: 24th Nov 2026

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Abstract: Toxoplasma gondii is an obligate intracellular parasite infecting humans and animals worldwide. SAG2 and GRA6 are important molecular markers for identifying its genetic lineages. However, molecular data from Al-Shatra district, Thi-Qar Governorate, Iraq, remain limited.Objective: To determine the SAG2/GRA6 genotype distribution of T. gondii in human and livestock isolates and investigate its association with clinical presentation in human cases. This cross-sectional study included 96 suspected human toxoplasmosis cases and 84 livestock animals from Al-Shatra district, Iraq, between March and September 2026. Human blood, amniotic fluid, cerebrospinal fluid, and placental tissue, as well as livestock tissue specimens, were screened using PCR. Positive samples were genotyped using nested PCR-RFLP targeting SAG2 and GRA6, with sequencing of ambiguous results. T. gondii DNA was detected in 72/96 (75.0%) human samples and 51/84 (60.7%) livestock samples. Successful genotyping was achieved in 64 human and 44 livestock isolates. Type II predominated in humans (53.1%), followed by type III (21.9%), mixed/atypical (20.3%), and type I (4.7%). In livestock, type III was most frequent (43.2%), followed by type II (36.4%) and mixed/atypical genotypes (20.5%). Genotype distribution did not differ significantly between hosts (P = 0.057). SAG2 and GRA6 showed 79.6% concordance. Mixed/atypical genotypes were more frequent in severe human cases (30.0%) than in mild or congenital cases (11.8%), although the association was not statistically significant (P = 0.118). Types II and III were the predominant genotypes in human and livestock isolates. The findings provide valuable regional molecular data and highlight the need for further studies investigating genotype diversity and clinical outcomes.

Keywords: Toxoplasma Gondii, Genetic Diversity, SAG2, GRA6, PCR-RFLP

Introduction

Among zoonotic infections, toxoplasmosis—caused by the protozoan Toxoplasma gondii—ranks as one of the most widely distributed worldwide. Serology indicates that close to one-third of people globally have encountered the parasite at some point [1], though this figure conceals wide variation: fewer than 10% in parts of northern Europe and East Asia are seropositive, against more than 60% in some populations of Latin America and sub-Saharan Africa [2]. Most infections in people with

intact immunity pass without symptoms or resolve on their own, but the parasite can still do serious harm—to a fetus infected in utero, to the eye, or to an immunocompromised host, in whom dormant tissue cysts may reactivate and trigger fatal encephalitis. Livestock are affected differently: in sheep and goats especially, T. gondii drives substantial reproductive loss through abortion and neonatal death [3], and the tissue cysts persisting in the meat of infected animals form one of the principal routes by which humans become infected [4].

A flexible transmission biology largely explains this parasite's ecological success. Only felids act as definitive hosts, releasing oocysts that resist degradation in the environment, while essentially any warm-blooded animal can serve as an intermediate host carrying bradyzoite-filled tissue cysts. Ingestion of either oocysts or tissue cysts can establish infection in an intermediate host, and the parasite also crosses the placenta to infect offspring directly. What sets T. gondii apart from most parasites is its ability to pass carnivorously from one intermediate host to another, bypassing the sexual cycle altogether—a route that has been put forward as the mechanism behind the clonal expansion seen in a limited set of genotypes [5].

Analyses of restriction fragment length polymorphisms and isoenzymes in the early years of this field pointed to a simple picture: isolates from Europe and North America fell almost entirely into one of three clonal lineages, types I, II and III [6]. Virulence in laboratory mice sharply distinguishes these lineages—type I kills at even the lowest inocula, whereas types II and III are of milder or intermediate virulence and tend instead to settle into chronic infection [7]. That three-lineage picture turned out to be an artefact of where sampling had taken place rather than a true account of the species' diversity: once wildlife and domestic animals in South America, Africa and Asia were examined, a far richer population structure emerged [8], [9]. Genome-scale studies now distinguish at least fifteen haplogroups falling into six major clades, alongside a great many recombinant genotypes that circulate only locally [9], [10].

Knowing which genotypes circulate among people and among the animals that feed them therefore carries direct public-health weight. Genotype affects how readily the parasite spreads within a host, how much immunopathology it provokes, and how efficiently it is transmitted by the oral route, and particular genotypes recur in reports of severe congenital, ocular and disseminated disease [11]-[13]. Establishing whether human and livestock genotypes coincide also offers indirect evidence about transmission pathways—evidence that control programmes depend on.

Genotyping large numbers of isolates and clinical specimens in practice calls for markers that amplify reliably by PCR even from material with a low parasite burden, that are cheap to analyse, and that are polymorphic enough to tell lineages apart. SAG2, the gene for a major tachyzoite surface antigen, was among the first such markers and has underpinned PCR-RFLP genotyping since the late 1990s [14]. GRA6, encoding a polymorphic dense-granule antigen, joined it more recently as a complementary marker, valued both for its lineage-specific polymorphisms and for its direct functional bearing on host–parasite interaction [15], [16]. A substantial number of studies now type both loci together, which yields a dataset reasonably comparable across different regions and host species. Human and livestock genotype data from Iraq and the wider Middle East, however, remain sparse [17], [18], despite the high reported seroprevalence in some local populations and the centrality of sheep, goats and cattle to the region's rural economy.

This study was carried out to help close that gap. Three objectives were set: first, to determine the SAG2 and GRA6 genotype distribution of T. gondii isolates recovered from human patients and from livestock (sheep/goat and cattle) at Al-Shatra district/Thi-Qar Governorate,Iraq; second, to examine the degree of concordance between the two loci and the overlap in dominant genotypes between the human and livestock isolates; and third, to test whether mixed or atypical genotypes were associated with more severe clinical presentation among the human cases.

Background: Biology and Population Structure of Toxoplasma gondii

Life cycle and modes of transmission

Sexual reproduction takes place nowhere but the intestinal epithelium of felids, where it culminates in oocysts shed in an unsporulated state; these mature in the environment over days to

weeks and then stay infective for months or even years in damp soil and water [19]. Once an intermediate host ingests either sporozoites or bradyzoites, the parasite invades the gut epithelium and switches into the rapidly dividing tachyzoite form, which spreads through the body. As the host's immune system presses back, tachyzoites slow their division and convert into bradyzoites walled inside tissue cysts, favouring the brain, the retina, and skeletal and cardiac muscle. Such cysts endure for the host's lifetime and become the source of infection whenever the meat is eaten raw or undercooked.

Three routes dominate human infection: eating undercooked meat harbouring tissue cysts, ingesting food, water or soil contaminated by oocysts, and transplacental spread following a mother's primary infection during pregnancy [20]. How much each route contributes differs from one population to another, and this is partly mirrored in genotype distribution, since strains are not equal in their capacity to generate oocysts, to form lasting cysts in a given host species, or to cause disease once inside an intermediate host.

Clonal lineages and the global population structure

Before the early 2000s, T. gondii was viewed as a strikingly clonal species, its population structure accounted for almost entirely by three lineages across Europe and North America [6]. These lineages are believed to trace back to a single genetic cross between two ancestral strains, followed by rapid clonal expansion—a history that explains why sequence divergence between them is so low [11], [22]. Experimental and genomic evidence has since shown that sexual recombination, although rare in the temperate zones where early sampling concentrated, happens routinely in tropical ecosystems, where a greater variety of wild felids and prey species supplies more opportunity for it [5], [8].

Since then, larger-scale multilocus genotyping has delineated a worldwide population structure composed of a small number of main clades. North America contributes, beyond the familiar types II and III, a separate clade heavily represented in wildlife and designated haplogroup 12 [23]. South America hosts a wide array of recombinant and divergent strains circulating among both domestic and wild animals, strains linked to comparatively severe disease in people [24]. Africa carries both lineages found elsewhere and haplogroups that appear to be locally distinct [25], whereas China is dominated by what is essentially a single genotype—often called Chinese 1—recovered across a wide range of host species [9]. Population-genomic analysis has shown that these mosaic genomes result from local admixture among amplified and diversified secreted pathogenesis determinants [26], which explains why a small set of loci—among them those coding for rhoptry and dense granule proteins—display exceptional polymorphism even though genome-wide divergence between the archetypal lineages is otherwise low.

One practical upshot follows directly: a marker system built around the archetypal lineages will perform best precisely where those lineages predominate, and will grow steadily less reliable as recombination becomes more common in a region. Keeping this caveat in mind is essential whenever two-locus genotyping data are interpreted, including those generated in the present study.

Genotype and phenotype

The parasite's polymorphic effector proteins, which it secretes into host cells via its rhoptries and dense granules, are the primary driver of the virulence phenotype, which has been extensively investigated in laboratory mice. Differences in acute mouse virulence trace largely to allelic combinations at the polymorphic kinases ROP18 and ROP5 [27]- [29], while the dense-granule effector GRA15 shapes host NF-kB signalling in a way that depends on the infecting strain [30]. Mouse virulence, however, predicts human disease only imperfectly: type II, essentially avirulent in mice, is nonetheless responsible for most congenital toxoplasmosis in Europe, while atypical genotypes are behind much of the severe ocular and disseminated disease seen in parts of South America [12], [13]. Host genetics, the size of the parasite burden, the stage of gestation at which infection occurs, and immune status all clearly modify how any single genotype plays out clinically — a relationship this study also examines directly.

Materials and Methods

Study design, setting and period

This was a cross-sectional molecular epidemiological study of human and livestock T. gondii isolates, conducted at Al-Shatra district/Thi-Qar Governorate,Iraq between March and September 2026 and written informed consent was obtained from all human participants or their guardians prior to sample collection.

Study population and sample collection

Human participants comprised patients with a clinical suspicion of toxoplasmosis (congenital infection, ocular disease, or disease in an immunocompromised host) presenting consecutively during the study period. Blood, amniotic fluid, cerebrospinal fluid or placental tissue was collected according to the clinical indication from Al-Shatra hospital. Livestock specimens were collected from sheep/goat and cattle carcasses or tissue samples at Veterinary Hospital in Thi-Qar, comprising brain, heart, diaphragm, skeletal muscle, or aborted foetal tissue and placenta, as available. Both human and animal specimens were selected because direct tissue typing, rather than typing of isolates recovered by mouse bioassay or cell culture, avoids the selection bias toward virulent strains inherent to isolation-based approaches, at the cost of requiring a parasite burden high enough to amplify at single-copy loci [31].

Rationale for SAG2 and GRA6 as genotyping targets

SAG2 encodes a GPI-anchored surface antigen related to the SAG1 family, thought to contribute to tachyzoite attachment and immune modulation; its bidirectional polymorphism pattern (changes at the 5′ end distinguishing type II from types I/III, and changes at the 3′ end distinguishing type I from types II/III) allows each archetypal lineage to be read from the combined 5′/3′ profile, while discordant combinations flag a recombinant or unusual strain [14]. An additional polymorphic stretch, alternative SAG2 (alt. SAG2), was included to improve discrimination of non-archetypal genotypes [32]. GRA6 encodes a roughly 32-kDa dense-granule protein whose lineage-distinguishing amino-acid variants arise from single-nucleotide polymorphisms and small indels across its coding sequence [15]; because it lies elsewhere in the genome and appears to be subject to immune-mediated selection rather than the comparatively neutral selection acting on SAG2, agreement between the two loci increases confidence in a lineage call, while disagreement flags a possible recombinant or mixed infection. Table 1 sets out the allelic profiles used to assign lineage, and Table 2 compares the two markers' technical and biological features.

Table 1. Expected allelic profiles of the three archetypal T. gondii lineages at the SAG2 and GRA6 loci, used for lineage assignment in this study, with typical virulence and distribution reported in the literature

Lineage	SAG2 5′ end	SAG2 3′ end	GRA6	Virulence in mice	Typical distribution
Type I	I/III allele	I allele	Type I allele	High (lethal even at very low inocula)	Found in clinical cases worldwide; disproportionately represented among severe disease in some regions
Type II	II allele	II/III allele	Type II allele	Intermediate to low	Predominant among human and animal isolates in Europe and North America
Type III	I/III allele	II/III allele	Type III allele	Low	Widespread in animals globally; common in livestock

Lineage	SAG2 5′ end	SAG2 3′ end	GRA6	Virulence in mice	Typical distribution
Mixed / atypical	Discordant combination or novel allele	Discordant combination or novel allele	Any, often discordant with SAG2	Variable	Most frequent in South America, parts of Africa and Asia

Table 2. Comparison of the technical and biological characteristics of SAG2 and GRA6 as genotyping markers.

Feature	SAG2	GRA6
Gene product	Surface antigen (GPI-anchored), SAG1-related family	Secreted dense granule protein (approx. 32 kDa)
Pattern of polymorphism	Split between the 5′ and 3′ ends; alt. SAG2 adds extra resolution	SNPs and small indels distributed across the coding sequence
Lineage resolution	Resolves types I, II and III once both ends are analysed	Resolves types I, II and III; divergent natural variants identified by sequencing
Typical method	Nested PCR followed by RFLP; sequencing for confirmation	PCR followed by RFLP or direct sequencing
Biological relevance	Attachment and immune modulation	Immunodominant antigen; strain-specific host signalling (NFAT4)
Main limitation	Cannot resolve most atypical genotypes on its own	Allelic combinations alone do not identify recombinant haplotypes

DNA extraction, nested PCR-RFLP and sequencing

Genomic DNA was extracted from all specimens using a commercial kit, with an extraction blank included in every batch. A validated real-time PCR assay was used first to screen all specimens for T. gondii DNA; PCR-positive specimens were then genotyped by nested PCR-RFLP at SAG2 (5′ end, 3′ end and alt. SAG2) and at GRA6, following the primer sets and amplification conditions described by [14], [32]. Amplicons were digested with locus-specific restriction enzymes (SAG2 with NciI and HpaII; GRA6 with MseI) [32], and digestion products were resolved on agarose or polyacrylamide gel alongside reference strains of types I, II and III run in every batch, with independent reading by two observers. Amplicons that produced a discordant, ambiguous, or non-archetypal RFLP profile were sequenced bidirectionally by the Sanger method and compared against curated reference alleles; all novel sequences were submitted to a public sequence database. An isolate was classified as concordant/archetypal when both SAG2 and GRA6 indicated the same lineage, and as mixed/atypical when the two loci disagreed or when either locus carried an allele outside the three archetypal profiles (Table 1).

Quality control

Table 3 summarises the quality-control measures applied at each step of sample handling and genotyping.

Table 3. Key steps and quality-control measures in SAG2/GRA6 PCR-RFLP genotyping.

Step	Description	Quality-control measure
1. Sample I selection	solates, human clinical D samples or livestock tissue; record host, tissue and geographical origin	ocument sample metadata; confirm T. gondii positivity by a validated PCR before typing
2. DNA C extraction	ommercial kit or I phenol-chloroform method	nclude extraction blank; assess DNA yield and purity
3. Nested PCR E	xternal and internal primer S pairs for SAG2 (5′ and 3′ ar ends), alt. SAG2 and GRA6	eparate pre- and post-PCR eas; positive and negative controls in every run

Step	Description	Quality-control measure
4. Restriction L digestion	ocus-specific enzymes R according to the published protocol	eference strains of types I, II and III in each run; verify complete digestion
5. A Electrophoresis	garose or polyacrylamide C gel with molecular size marker	ompare banding patterns with reference lanes; independent reading by two observers
6. Sequencing S	anger sequencing of B selected or discordant amplicons	idirectional reads; submit sequences to a public database
7. A Interpretation	ssign lineage by concordant R alleles; classify discordant profiles as mixed or atypical	e-test discordant results; consider mixed infection before declaring recombinants

Statistical analysis

Categorical variables are reported as counts and percentages. The distribution of SAG2/GRA6 lineage categories (type I, type II, type III, mixed/atypical) between human and livestock isolates was compared using the chi-square test. The association between mixed/atypical genotype and severe clinical presentation (ocular or disseminated disease versus congenital or mild disease) among human isolates was assessed using Fisher's exact test, with the odds ratio and its 95% confidence interval calculated from the 2×2 table. A two-sided P value below 0.05 was considered statistically significant. Analyses were performed in software SPSS version 26.

Results

Sample flow and genotyping yield

Of 96 human patients enrolled, T. gondii DNA was detected in 72 (75.0%), and both loci were successfully typed in 64 of these (88.9% of PCR-positive samples). Of 84 livestock specimens (40 sheep/goat, 24 cattle), 51 were PCR-positive (60.7%), with the highest positivity in sheep/goat and the lowest in cattle (9/24, 37.5%); 44 of the 51 PCR-positive livestock specimens (86.3%) were successfully typed at both loci (Table 4). Overall, 108 of 123 PCR-positive specimens (87.8%) yielded a complete SAG2/GRA6 profile; the remainder amplified at only one locus and were excluded from the lineage-assignment analysis.

Table 4. Sample flow from enrolment to successful genotyping.

Group	Enrolled, n	PCR-positive, n (%)	Successfully genotyped (both loci), n (%)
Human	96	72 (75.0%)	64 (88.9%)
Livestock – sheep/goat	40	28 (70.0%)	25 (89.3%)
Livestock – cattle	24	9 (37.5%)	7 (77.8%)
Livestock – all	84	51 (60.7%)	44 (86.3%)
Total	180	123 (68.3%)	108 (87.8%)

Lineage distribution in human and livestock isolates

Among the 64 fully typed human isolates, type II was the most frequent genotype (34, 53.1%), followed by type III (14, 21.9%), mixed/atypical profiles (13, 20.3%) and type I (3, 4.7%). Among the 44 fully typed livestock isolates, type III predominated (19, 43.2%), followed by type II (16, 36.4%) and mixed/atypical profiles (9, 20.5%); no type I isolate was identified in any livestock species. Mixed/atypical profiles were proportionally most frequent in cattle (3/7, 42.9%) and least frequent in sheep/goat (4/25, 16.0%), although the cattle subgroup was small (Table 5). The overall lineage distribution did not differ significantly between human and livestock isolates ($\chi^2 = 7.52$, df = 3, P =

0.057), though the human group showed a numerically higher share of type II and the livestock group a higher share of type III.

Table 5. SAG2/GRA6 lineage distribution among human and livestock isolates.

Host group	n typed	Type I, n (%)	Type II, n (%)	Type III, n (%)	Mixed/atypical, n (%)
Human	64	3 (4.7%)	34 (53.1%)	14 (21.9%)	13 (20.3%)
Livestock – sheep/goat	25	0 (0.0%)	12 (48.0%)	9 (36.0%)	4 (16.0%)
Livestock – cattle	7	0 (0.0%)	1 (14.3%)	3 (42.9%)	3 (42.9%)
Livestock – all	44	0 (0.0%)	16 (36.4%)	19 (43.2%)	9 (20.5%)
Total	108	3 (2.8%)	50 (46.3%)	33 (30.6%)	22 (20.4%)

$\chi^2 = 7.52$, df = 3, P = 0.057 (human vs. livestock – all, 4-category comparison).

SAG2–GRA6 concordance

The two loci agreed on an archetypal lineage call in 86 of the 108 fully typed isolates (79.6%); the remaining 22 (20.4%) were classified as mixed/atypical because of discordance between SAG2 and GRA6 or because one locus carried a non-archetypal allele. Concordance was similar in human (51/64, 79.7%) and livestock isolates (35/44, 79.5%).

Genotype and clinical presentation among human cases

Among the 64 human isolates, clinical presentation was congenital infection in 22, ocular disease in 18, disseminated disease or immunocompromise in 12, and mild or other presentation in 12. Mixed/atypical genotypes were more frequent among isolates from patients with a severe presentation (ocular or disseminated disease combined, 9/30, 30.0%) than among those with congenital infection or a mild presentation (4/34, 11.8%) (Table 6). This association did not reach statistical significance (Fisher's exact P = 0.118; odds ratio 3.21, 95% CI 0.87–11.83).

Table 6. Mixed/atypical genotype by clinical presentation among human isolates ().

Clinical presentation	n	Mixed/atypical genotype, n (%)
Congenital infection	22	2 (9.1%)
Ocular disease	18	6 (33.3%)
Disseminated disease / immunocompromise	12	3 (25.0%)
Mild / other presentation	12	2 (16.7%)
Severe (ocular + disseminated) subtotal	30	9 (30.0%)
Non-severe (congenital + mild) subtotal	34	4 (11.8%)

Fisher's exact test, severe vs. non-severe: OR = 3.21, 95% CI 0.87–11.83, P = 0.118.

Human–livestock genotype overlap

The two most frequent genotypes overall — type II and type III — were shared between human and livestock isolates, consistent with an ecological overlap between the two host groups at Al-Shatra district/Thi-Qar Governorate,Iraq. This pattern was not statistically distinguishable from chance at the sample sizes achieved here and two-locus concordance of this kind does not by itself establish a direct transmission link between the two groups.

Discussion

Principal findings in the context of the published literature

Considerable variation in design runs through the studies available for comparison. Most genotype either isolates grown by mouse bioassay or cell culture, or DNA pulled directly from clinical and animal tissue, and the majority rely on nested PCR-RFLP at SAG2 (its 5′ and 3′ ends, with or without alt. SAG2) and GRA6, with a smaller subset confirming or extending these profiles through sequencing [14], [32]. Sample sizes are all over the map, many reports cover only a few isolates, and the share of positive samples that ended up fully typed often goes unstated. In contrast to animal data, which mostly draws from aborted foetuses, abattoir tissue, and retail meat, human data in the literature are primarily based on congenital, ocular, and immunocompromised patients — the same clinical categories sampled in the present study.

The great majority of human infections in Europe, including congenital toxoplasmosis and disorders in immunocompromised persons, are consistently caused by type II strains [12], [14]. Based on an analysis of French congenital infection isolates, the majority were type II, with just a handful of out-of-the-ordinary genotypes; these isolates frequently exhibited more severe symptoms [12]. Clinical samples from North America do contain type III and recombinant strains, but type II is still the dominant trend [23], [33]. There is a complete change in tone in South American narratives, where atypical or recombinant genotypes are common vectors for human infections in Brazil and neighbouring countries, associated with an elevated incidence of severe ocular disease and disseminated infection even in individuals with normally functioning immune systems [13], [21], [24]. Iranian and other Middle Eastern studies have shown human samples with a mixture of type II, type III, and mixed or atypical profiles, though typically in small samples with uneven geographic coverage [17], [18]. The predominance of type II among the human isolates in the present study (53.1%, Table 5), alongside a sizeable minority of mixed/atypical profiles (20.3%), sits between the strongly type II-dominated pattern reported in Europe and the more diverse pattern reported in South America, and is broadly in line with the mixed picture previously reported for the wider Middle East.

Livestock genotyping studies show a broadly similar range: in Europe and North America, sheep most often carry type II, with type III to a lesser degree [17], [34], whereas sheep and goats in South America and reportedly in parts of the Middle East and North Africa carry a higher share of type III and mixed/atypical profiles [17], [18]. Genotyping of cattle is comparatively rare in the literature, since cattle are generally resistant to clinical toxoplasmosis and seldom contain live tissue cysts [34]; the small cattle subgroup in the present study (n = 7) likewise yielded the highest proportional share of mixed/atypical profiles among the three livestock species (Table 5), though this estimate should be treated cautiously given the small denominator.

Table 7 summarises the dominant genotypes reported by region in previously published human and livestock studies, for comparison with the findings of the present study.

Table 7. Dominant T. gondii genotypes reported in human and livestock isolates by region in previously published studies (literature synthesis, for comparison with the present study's findings in Table 5).

Region	Human isolates	Livestock isolates	Remarks
Europe	Predominantly type II, including congenital and immunocompromised cases; atypical genotypes in a few severe cases	Predominantly type II in sheep and cattle; type III less frequent; a small share mixed or atypical	High concordance between host groups
North America	Predominant type II in clinical samples; type III and recombinants also detected	Types II and III in sheep and cattels; a minority atypical	A further wildlife-associated clade lies outside the archetypal sets

Region	Human isolates	Livestock isolates	Remarks
South America	Atypical and recombinant genotypes frequent; severe ocular and disseminated disease reported	Recombinant genotypes common in sheep and goats; atypical genotypes in cattles	Archetype-based panels likely to misassign many isolates
Africa	Types II and III alongside distinct local haplogroups	Types II and III with mixed profiles in small ruminants (North Africa)	Data limited and geographically uneven
Asia	A single dominant genotype reported in China across hosts; heterogeneity elsewhere	Dominant genotype shared with humans in China; atypical genotypes in extensively reared cattle	Strong national predominance of one lineage in China
Middle East	Mix of type II, type III and mixed or atypical profiles reported	Types II and III with mixed profiles; type III often more frequent than in human cases	Small samples, few loci, uneven geographical coverage in the existing literature

The gradient summarised in Table 7, running from clonal, type II-dominated populations in Europe and North America to highly diverse, recombinant populations in South America and parts of Africa and Asia, finds a ready explanation in the transmission ecology of T. gondii: infrequent recombination paired with carnivorous transmission between intermediate hosts is thought to have favoured clonal expansion of a few genotypes in temperate zones, whereas tropical ecosystems, with their richer array of wild felids and prey species, offer far more scope for sexual recombination [5], [8]. Sampling history also plays a part in producing this gradient, since early surveys concentrated on Europe and North America [9]. The present study's mixed type II/type III/atypical pattern is consistent with a setting that does not fall cleanly into either extreme of that gradient.

Livestock as a reservoir and the interpretation of overlap

It has been suggested that consuming meat from infected animals is a significant way for humans to acquire the disease, given the genetic overlap often reported between human and livestock isolates from the same area [20], [35]. The overlap observed here between the dominant human and livestock genotypes (Human–livestock genotype overlap) is consistent with that possibility, but such overlap is exactly what would be expected whenever only a handful of genotypes circulate widely in a region, and it says nothing about which particular animals or food items caused which human cases. Several explanations could also account for the slightly higher frequency of type III observed in livestock than in human isolates in this study, a pattern also reported in some Middle Eastern and North African surveys [17], [18]: humans tend to be genotyped only once symptomatic or identified as part of a risk group, so genotypes that cause overt disease may be over-represented in the human data; detection methods also differ, with bioassay favouring virulent strains while direct PCR simply recovers whichever genotype is abundant enough to detect [31]; and exposure routes themselves may differ between the two hosts. None of these explanations rules out the others, and the present cross-sectional data cannot tease them apart. Independent epidemiological evidence, such as a shared food supply, or very high-resolution typing showing that isolates from linked animals and humans match across several additional loci, would be required to establish a true livestock-to-human transmission relationship; two-locus concordance alone cannot substantiate such a claim, since several unrelated isolates may share an identical profile at just two loci.

Genotype, SAG2/GRA6 and clinical outcome

The non-significant association observed here between mixed/atypical genotype and severe clinical presentation (Genotype and clinical presentation among human cases; OR = 3.21, 95% CI 0.87–11.83, P = 0.118) should be interpreted with the same caution the literature recommends for this kind of

comparison. Alleles at non-linked loci are nonetheless passed down as a block in clonal archetypal lineages, so that SAG2 and GRA6 stand in for the whole lineage, including linked virulence determinants such as ROP18 and ROP5 [27], [28]. That block of linkage breaks down in recombinant isolates, where a two-locus profile loses much of its power to predict virulence or clinical outcome. GRA6 is something of an exception, since its own allelic variants can contribute directly to phenotypic differences through their effects on host signalling and T-cell responses [36], [37]. The direction of the association found here — more mixed/atypical profiles among ocular and disseminated cases than among congenital or mild cases — mirrors the pattern previously reported from South America, where atypical genotypes are disproportionately represented among severe ocular and disseminated disease [12], [13], [21], even though it did not reach significance here, most likely reflecting the limited sample size in the severe-disease subgroups (Table 6). Larger, prospective studies pairing typing with clinical and immunological data, and where feasible with additional genetic loci, would be needed to confirm this trend.

Relevance for [study area] and the wider Middle East

The mixed type II/type III/mixed-atypical pattern found here in both human and livestock isolates is broadly consistent with the limited existing reports from the Middle East [17], [18], and extends that literature with region-specific, paired human-and-livestock data responding directly to the data gap previously identified for Iraq and neighbouring countries. Given how central sheep, goats and cattle are to the local rural economy and how high reported seroprevalence can be in some populations, these findings point to the value of larger, systematically sampled studies pairing human and livestock isolates from the same area and period, applying the tiered genotyping approach described in A tiered genotyping framework.2: SAG2 and GRA6 first as a screening tool, followed by extended multilocus or sequence-based typing of any isolate that proves discordant, atypical, or clinically significant.

Strengths and limitations

The principal strength of this study is that it pairs human and livestock sampling from the same setting and period using an identical genotyping protocol, which allows the kind of direct host-group comparison that most of the published literature, built from heterogeneous individual studies, cannot support (Principal findings in the context of the published literature). Its main limitations mirror those of the wider field: the sample, especially the cattle subgroup (n = 7) and the severe-disease human subgroups (Table 6), is too small to detect modest effect sizes with confidence, as reflected in the wide confidence interval around the genotype–clinical-presentation association Genotype and clinical presentation among human cases); genotyping success depended on parasite burden, which may have skewed the typed subset toward higher-burden infections [31]; and, as with any two-locus panel, SAG2/GRA6 typing cannot fully resolve recombinant haplotypes or distinguish a true recombinant from a mixed infection (Limitations). These limitations argue for the larger, multi-site studies recommended in (Research Gaps and Future Directions).

Limitations of Two-Locus Typing and a Proposed Tiered Framework

Limitations

It is important to recognise the limitations of SAG2 and GRA6, even though they remain informative and cost-effective. A recombinant strain may be mistakenly classified as archetypal if it shares the same SAG2 and GRA6 alleles with an archetypal lineage but differs everywhere else in its genome; conversely, a discordant two-locus profile cannot on its own differentiate between a true recombinant and a mixed infection involving two distinct strains, because very little genealogical information is carried by just two loci. Another constraint is that neither locus traces the whole genome's evolutionary history, likely because of immune-mediated selection acting at GRA6 and because SAG2 belongs to a surface-antigen gene family. Thirdly, the diversity among atypical genotypes cannot be captured by marker sets built around the archetypal lineages; this can only be resolved with additional loci or sequencing [32], [38]. Because of these caveats, the lineage calls reported in (Results) should be read as a provisional, first-pass classification rather than a definitive genomic

characterisation, and the proportion of isolates that could not be fully typed (Table 4) is reported explicitly rather than folded silently into a single summary figure.

A tiered genotyping framework

Building on these limitations, a multi-level strategy is recommended for future research on Toxoplasma gondii in both people and livestock. At the first level, isolates are provisionally sorted into archetypal, mixed, or atypical categories using nested PCR-RFLP performed alongside reference-strain controls, with SAG2 (both its 5′ and 3′ ends, plus alt. SAG2) and GRA6 serving as the inexpensive screen, as applied in the present study. Isolates considered to be of epidemiological or clinical significance — such as those associated with congenital defects, eye disease, or outbreaks, or, as in this study, with the more severe clinical presentations examined in (Genotype and clinical presentation among human cases) — are then subjected to a second layer of testing comprising extended multilocus PCR-RFLP or multilocus sequence typing across additional loci [32]. A third level, reserved for isolates of particular representative or epidemiological interest, would involve whole-genome sequencing placed in the context of the global population framework [10], [26]. Structured this way, the approach balances cost against discriminatory power and allows comparisons across studies that, at present, the use of mismatched marker panels makes difficult.

Research Gaps and Future Directions

A handful of priorities stand out from this study and the wider literature it draws on. One is the need for larger, systematically sampled studies that genotype human and livestock isolates from the same geographic area and time period using identical methods, extending the paired design used here beyond its present sample size, so that valid, adequately powered comparisons become possible — much current data instead come from convenience samples of different tissue types analysed by different methods, which prevents any meaningful comparison across host species. Another is the continuing under-representation of the Middle East and surrounding regions in the published literature; the present findings are a step toward addressing that gap but would need replication, ideally at multiple sites across Iraq, to be generalisable. A third priority favours direct genotyping of clinical and animal samples over bioassay-based isolation wherever feasible, so as to minimise selection bias, drawing on sensitivity-enhancing methods such as nested PCR and, increasingly, targeted amplicon sequencing.

The functional consequences of GRA6 and SAG2 polymorphism also merit closer attention than they have so far received. Evidence that GRA6 variants activate host signalling differently and shape T-cell responses in distinct ways [36], [37] hints that some of these marker polymorphisms carry real phenotypic weight, and argues for genotype-phenotype studies in humans and livestock — building on the exploratory association reported in (Genotype and clinical presentation among human cases) — to incorporate immune response and clinical outcome data alongside genotype itself, in larger samples powered to detect the effect size suggested here. Standardising nomenclature and data reporting — depositing sequences, agreeing on common reference alleles — would go a long way toward making studies more comparable. Integrating environmental and food-chain sampling with genotyping, detecting oocysts in soil and water in addition to tissue cysts in retail meat, would shed light on the relative importance of various transmission channels in this setting [19].

Conclusion

In this cross-sectional molecular study, type II and type III were the dominant SAG2/GRA6 genotypes among both human and livestock T. gondii isolates at [setting], with mixed/atypical profiles present in roughly one in five fully typed isolates and no significant difference in lineage distribution between the two host groups (χ^2 = 7.52, df = 3, P = 0.057). SAG2 and GRA6 were concordant in approximately four out of five isolates. Mixed/atypical genotypes were numerically, though not statistically significantly, more common among human cases with severe clinical presentation than among those with congenital infection or mild disease (OR = 3.21, 95% CI 0.87–11.83, P = 0.118), a pattern consistent with previous reports from regions where atypical genotypes predominate. These findings extend the sparse existing molecular data from Iraq and the wider Middle East and support the use of

SAG2 and GRA6 as a practical, low-cost first-line screen within the tiered genotyping framework proposed in (A tiered genotyping framework), with larger, multi-site, prospective studies needed to confirm the genotype–clinical-outcome association observed here and to clarify the extent of true transmission overlap between human and livestock populations.

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