Detection of *Toxoplasma gondii* in Cattle using Molecular Markers

Irsa Saleem[1,*], Irsa Shahab[1], Sijia Lu[2], Musfira Jabeen[1] and Saneela Kauser[1]

[1]University of Agriculture, Faisalabad
[2]College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, China
*Corresponding author: irsasaleemiqbal@gmail.com

Abstract

The obligate parasite *Toxoplasma gondii* is a zoonotic organism that can be spread from animals to other living organisms. Animals consume raw food from various sources, which can trigger the transmission of zoonotic diseases. Due to its extremely high prevalence and long-term chronicity of contamination, *T. gondii* is a well-known parasite that exploits any compromise in the host's immune status. Restriction fragment length polymorphism (RFLP) markers help to make important limitations on rudimentary genetic linkage maps. They are useful for identification and high-intention studies using experimental samples. Moreover, various methods are used, like microsatellites, multi-locus sequence typing (MLST), and nested PCR (n-PCR). Nested PCR is the most suitable technique to detect *T. gondii* DNA by pointing the B1 gene, which is genotyped by the molecular markers. According to various detection techniques and livestock sources, different locations and nations may have a varied prevalence of *T. gondii* infection in cattle. Only one (Tg16) out of nine positive samples could be genotyped with complete data for every locus. Except for the GRA6 locus, which belonged to type III, all of Tg16's loci were of type I. All of the loci in the current investigation were categorized into clonal type I based on the available data, except for type I.

Keywords: Molecular markers, Cattle, *T. gondii*, Sero-prevalence, Nested PCR, Restriction fragment length polymorphism (RFLP)

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Introduction

Toxoplasma gondii is an apicomplexan and intracellular parasite that contaminates all warm-blooded vertebrates, including mammals and birds. It is the only known species in the genus Toxoplasma and is considered to be one of the most successful eukaryotic pathogens in terms of the number of host species and percentage of animals infected worldwide. Up to one-third of the human population in the world is frequently infested (Shwab et al., 2018). Due to its tremendously high occurrence and long-term chronicity of infection, *T gondi* is well suited to take advantage of any cooperation in the host immune status. Newly attained infections are an important cause of spontaneous miscarriage in domestic animals and of inborn disease in humans (Moncada & Montoya, 2012). Human infections are primarily caused by ingesting undercooked meat containing feasible tissue cysts or by ingesting food or water contaminated with oocysts shed in the feces of infected cats (Zhou et al., 2011).

T. gondii has subpopulation groups in many geographic locations. Their population arrangement in Europe and North America appears to be colonial, with three lineages predominating (types I, II, and III). These populations were identified through microsatellite analysis, polymerase chain reaction-restriction fragment length polymorphism (PCRRFLP), and Multi-locus Enzyme Electrophoresis (MLEE) (Wendte et al., 2011). According to recent research, the same clonal organization exists. These colonial structures are varied when they are isolated from animal and human bodies in South America and especially dissimilar from those which are isolated in North America and Europe. These different parasites which are not types I, II, or III have historically been referred to as "atypical" or "exotic" isolates. These isolates have low linkage disequilibrium across genomic loci implies that the parasites have frequently experienced sexual recombination (Dardé et al., 2020).

Molecular Genetics

Most of the life *T. gondii* multiplies mitotically and arise to be haploid cells because they are obligate intracellular parasites. Epithelial cells are the site where coupling occurs which leads to the beginning of oocysts. After that oocyst is covered with waste material and transferred to the production of a haploid individual while the result shows meiosis of a single round. When the new investigation of cross established in cats, the result shows coupling type is not encoded in *T. gondii* (Blader et al., 2015). Respectively states that recovery is possible if cat mixing unlike parental clones leads to both parental genotype and recombinant after self-fertilization and coupling. Restriction fragment length polymorphism (RFLP) markers help to make important limitations on rudimentary genetic linkage maps. The strategy of making a map by the available phenotype of an exact chromosome using linkage is restricted by the labor and time-intensive nature of RFLP mapping (Shwab et al., 2014).

Methods of Detection and Identification for Molecular Marker

Molecular methods are used to study the DNA of *T. gondii* and identification of exact findings. Mainly there are two sets of molecular

methods. The first set is for studying *T. gondii* DNA in organic trials. This group consists of conventional PCR (c-PCR), nested PCR (n-PCR) and quantitative real-time PCR (qPCR). The other set depends on the high intention of the study of separated *T. gondii*. This group is associated with microsatellites, multi-locus sequence typing (MLST) and multi-locus PCR-RFLP (Hanafiah et al., 2016).

Molecular Detection by Conventional, Nested, and Quantitative PCR

Due to the better effectiveness of amplifying a tiny DNA fragment compared to a larger one. Small DNA sequences is recommended to obtain high sensitivity in PCR. Additionally, repetitive sequences of every organism have more template copies. The one hundred and ten copies of internal transcribed spacer (ITS-1), thirsty five copies of the B1 gene and the three hundred-copies, 529 base pair of repeat elements are three repetitive DNA sequences that are frequently utilized to identify *T. gondii* in the organism blood sample. Using cPCR as a molecular detection approach for *T. gondii* and focusing on the B1 gene (Slana et al., 2021).

A repetitive sequence, 529 bp repeat element was recognized to be 10-100 times more responsive than the B1 gene. A few researchers have employed that 110-copy ITS-1 or 18S rDNA responded to B1 gene as a target and in certain investigations, n-PCR of the ITS-1 and B1 gene has been used to increase sensitivity. Both these markers demonstrated remarkable sensitivity in identifying just one parasite (Su et al., 2010).

To study a specific repetitive sequence, n-PCR is considered more sensitive than a regular PCR. For example, the n-PCR of B1 gene can detect *T. gondii* with more sensitivity than the traditional PCR in the samples of amniotic fluid of patients with congenital human toxoplasmosis. Recently 18S rDNA was used in n-PCR which appeared to be even more sensitive and exclusive than B1 gene. It is of special relevance as a marker because it can discriminate closely related parasitic protozoans with *T. gondii* (Dubey, 2010).

A rather better technique than n-PCR in terms of sensitivity was discovered recently to be qPCR to target repetitive DNA sequences (Ivović et al., 2012). It became popular because along with the identification of *T. gondii*, this technique also quantifies it from the biological samples. It means it not only detects *T. gondii* within a 6-7 order of magnitude and detects parasites in animal tissue, blood or amniotic fluids. but also estimates the intensity and progression of infection of *T. gondii* in patients (Bajnok, 2017). The qPCR of 529 bp region is most sensitive for the detection of *T. gondii* as it provides 1/50 genome equivalent or 6 repeat equivalents of sensitivity (Su et al., 2010).

The above-mentioned techniques are useful in the detection of *T. gondii* infection but do not provide further information except detection. To treat clinical toxoplasmosis effectively, it should be accurately and rapidly being detected and to understand parasite epidemiology, there is an increased interest in its genetic classification. The following methods are used to study genetic classification and identification of *T. gondii*.

Molecular Characterization by Microsatellite, Multi-locus PCR-RFLP or Multi-locus Sequence Typing (MLST)

Epidemiological studies require the identification of isolates and tracking of contamination sources of *T. gondii*. Microsatellite, multi-locus PCR-RFLP or MLST typing are frequently used techniques for these purposes. The PCR-RFLP works on the principle of restriction endonucleases' capacity to identify single nucleotide polymorphisms followed by displaying DNA banding on agarose gels by the process of electrophoresis (Shwab et al., 2014). The microsatellite analysis is based on the DNA sequence length polymorphisms of the short-nucleotide tandem repeats. *T. gondii* tandem repeats work as dinucleotide repeats that frequently occur 2-20 times (Majumdar, 2010). The MLST method is used for the insertions, deletions and SNPs in DNA sequence length polymorphisms. Microsatellites and SNPs are expected to evolve at various rates of mutation. SNP mutation rates for eukaryotes as a whole are expected to range from 10^{-9} to 10^{-10} per nucleotide location for each replication, while mutation rates for microsatellites are between 10^{-2} and 10^{-5} per replication per locus (Joeres, 2024).

Microsatellites help differentiate *T. gondii* isolates that are genetically similar. In comparison to MLST typing, microsatellite, and multi-locus PCR-RFLP typing are easier to use and less expensive, making them the favored methods for characterizing *T. gondii* in epidemiological research. A landmark investigation of the epidemiological studies and population dynamics of *T. gondii* was carried out on 106 isolates of animals and humans in North America and Europe more than ten years ago. In this work, 3 dominant lineages (types I, II, and III) were found using 6 PCR-RFLP markers. It was determined that *T. gondii* population was clonal (Shwab et al., 2014). Three prominent lineages were identified using six PCR-RFLP markers in this study. More microsatellites and multi-locus PCR-RFLP markers were used to conduct epidemiological studies which revealed the concluding findings that *T. gondii* which was isolated from South America were markedly varied from those of Europe and North America. *T. gondii* in South America was determined to have an epidemic pattern of population(Wendte et al., 2011). But these investigations do not allow the comparison of genotypes because different markers are used in other laboratories.

The sensitivity of a single-copy gene and genomic sequence is less than a highly repetitive sequence. Multi-locus typing is sometimes a technical challenge because samples are usually in less volume and less DNA amount. We created the multiplex multi-locus nested PCR-RFLP (Mn-PCR-RFLP) technique to solve this issue. It uses 10 genetic markers having BTUB, SAG1, SAG2, SAG3, GRA6, L358, c22-8, c29-2, Apico and PK1. Based on recent research using 4 of these 10 markers (SAG2, SAG3, GRA6 and BTUB), the sensitivity of this approach is predicted to be at least 10 *T. gondii* genome equivalents (Dubey, 2010). Comparatively, a conventional PCR-RFLP provides ≥100 genome equivalents of sensitivity. Mn-PCR-RFLP typing allows all markers to be pre-amplified in a single reaction by using multiple PCR adding external primers. These pre-amplified products serve as templates by n-PCR to amplify individual markers. This technique requires very small amounts of so-called 'precious samples.

For the unlimited number of isolates of DNA, MLST typing is considered the best method because of its highest resolution. Recently, studies have shown unique alleles of the parasite in Brazil (Joeres, 2024), depicting the importance of this method for genetic and molecular phylogeny studies of *T. gondii*.

Risk Factors and Economic Impact of Farm Animals-Prevalence of Infection in different animals

Sheep and Goats

In small ruminants, reproductive losses are due by protozoan pets and also the origin of infection of *T. gondii* through sheep and goats all over world. The transfer of infection in humans is due to zoonosis and small ruminants by toxoplasmosis (Belluco et al., 2016; Opsteegh et al.,

2016). Goats and sheep can produce antibodies against *T. gondii*. These demonstrate infections of *T. gondii* are relatively widespread in smaller ruminants, despite variances in research design, study goal, serological technique, and cut-off points utilized making it tough to compare results (Dubey, 2010). From 1969 to 2016 data is summarized as meta-analysis which shows the total occurrence of goat is 22.9% (12.3-36.0%) and sheep is 26.1 (17.0-37.0%) record from Africa (Tonouhewa et al., 2017). Sheep contains about 41. and 26% and goats contain 62% antibodies according to Egypt (Al-Kappany et al., 2018).

Sheep and lamb contain a 22.0% level of pathogens of *T. gondii* which is higher than goats, which is 30.7% (Guo et al., 2016). The estimation of sheep that contain seroprevalence is about 11.8% (2305/19,565) according to Asia. In China, the estimation of goats is about 17.6% (3260/ 18,556) (Dong et al., 2018). If we look at an area of Pakistan goats (42.8%) have higher seroprevalence in comparison sheep (26.2%) (Ahmed et al., 2016). Small ruminants are the main important host for parasites for *T. gondii* when it is isolated from tissues of small ruminants. Some other study shows the presence of *T. gondii* in the heart, muscles, diaphragm, and brain in sheep (Dubey, 2010; Opsteegh et al., 2016). The brain and heart are among the goats' preferred organs, while the muscular tissues scored highly in the research.

Table 1: Comparison of different methods of detection and characterization of *T. gondii*

Method	Sensitivity (Parasite Count per PCR Reaction)	Advantages	Limitations	Appropriate Uses	References
qPCR of repetitive sequences	0.02 to 1 genome	Highly sensitive	Not suitable for typing.	Diagnosis	(Khanaliha et al., 2021)
n-PCR of repetitive sequences	1 to 5 genomes	Sensitive	It cannot be used for typing.	Diagnostic applications	(Su et al., 2010)
Mn-PCR-RFLP	10 genomes	High-resolution	Less sensitive than qPCR or n-PCR.	Epidemiology, population genetics	(Liu et al., 2015)
Mn-PCR-based sequencing	10 genomes	Isolation not needed	Less sensitive compared to other	Epidemiology, population genetics	(Su et al., 2010)
Conventional PCR-based sequencing	100 genomes	High resolution	Low sensitivity	Epidemiology, population	(Su et al., 2010)
Multiplex PCR of microsatellite markers	not specified.	Allows for high-resolution	Insensitive	Epidemiology, population genetics	(Ajzenberg et al., 2010)

The most vital route for the infection transmission is through oral uptake of environment-friendly oocysts via polluted hay and water. In goats, rans-placental endogenous transfer of the parasite to their offspring and the recurrence of chronic infection were both documented (Osman, 2024). *T gondi* DNA found in the semen of male goats and rams in various examinations also from cases of infection that occurred naturally (Bezerra et al., 2014) or from animals who were vaccinated in research (Santana et al., 2010; Lopes et al., 2013). In the case of milk, also has a risk for transmission of infection to the goat babies and limbs from infected parents.

Diseases

10 to 23% of ovine abortions are caused by toxoplasmosis according to the estimation in USA and Europe. the major signs of the horizontal transmission shown after the experiment importantly as lack of appetite and the occurrence of higher temperatures. In divergence, inherited spreading has severe significance for the fetus. The gestational stage at which the illness manifests itself partially determines the trans-placental transmission results in fetus death. Gestational age is revealed after the result of infection examination while pathogenesis causes a major effect on the fetal immune system during the maturation stage(Castaño et al., 2016). In small ruminants early abortion occur after infection if the toxoplasmosis occurs these all are studied when sheep and goats are injected in the experiment. Hormonal disturbance or high fever is assumed to be the major cause of early abortion. (Dubey, 2010). Some expect like leukomalacia in the fetal brain and placenta-carrying vascular lesions are described in recent studies (Castaño et al., 2014).

Chickens and Poultry

T. gondii oocysts present in the environment are indicated by gondii infection in free-ranging fowl (Dubey, 2010). The kind of husbandry appears to be crucial. *T. gondii* incidence is typically greater in chickens raised outdoors or on tiny home farms than in poultry d indoors (N. Yang et al., 2012; Guo et al., 2015; Schares et al., 2017;). Because farms that are sampled may differ in size and farm type feed supply, the presence or absence of cats, rodent or bird management, and water quality, prevalence estimates are frequently not comparable (Dubey, 2010; Bangoura et al., 2011; Schares et al., 2017). Generally, chickens had *T. gondii* seroprevalence between 0 to 100% (Dubey, 2010; Bangoura et al., 2011; Ayinmode & Olaosebikan, 2014; Matsuo et al., 2014; Deng et al., 2018). There hasn't been much published research on *T. gondii* infection prevalence in the turkeys. Between 1.7 and 21% and 5.9 and 43%, respectively, of ducks and geese had *T. gondii* sero-prevalence (Bangoura et al., 2011).

The primary method of infection is probably oral consumption of materials or through water contaminated with oocysts of *T. gondii* s since chickens eat on the ground (Dubey, 2010). Turkeys, chickens and ducks are examples of poultry that are omnivorous, which means they may also consume other insects like cockroaches and earthworms that may contain or be infected with oocysts(Geuthner et al., 2019). In the past, vertical *T. gondii* transmission in poultry has been explored, however comprehensive chicken tests have shown that this route of infection may be ignored(Malkwitz, 2019).

Diseases

In general, clinical indications of *T. gondii* are uncommon in chickens, turkeys, ducks and geese (Dubey, 2010). However, some of the clinical cases attributed to *T. gondii* may have been brought on by additional infections (such as viral ones) or worsened by additional illnesses

(Dubey, 2010). There is no evidence of genotype-dependent virulence of *T. gondii* in adult chickens, and American strains that are extremely virulent to mice appear to be a virulent in chickens (Vaudaux et al., 2010; Hamilton et al., 2017). Young hens contaminated with (*T. gondii* Type I (GT1 strain) oocysts showed clinical indications of toxoplasmosis, whereas those contaminated with Type II (ME49) oocysts showed no such symptoms(Geuthner et al., 2019).

Cattle

There is no obvious evidence of *T. gondii* in cattle due to huge diversity of sero-positive. Only a few cases are reported as positive *T. gondii* infection in naturally visible cattle (Maia et al., 2012). The presence of it is difficult to find in small ruminants. The geographical region of cattle can alter the ratio of *T. gondii* in them, it was revealed after great analysis. *T. gondii* genotype is different in a different region of the world, which indicates the prevalence of T. gondii in cattle (Castaño et al., 2014).

T. gondii oocytes are transmitted orally into the cattle by digestion of polluted hay and water. Cattle infected through the digestion of tissue cysts because they are herbivores. For example, when cattle consume freshly infected organisms during feeding the result will show abortion of bovine fetuses after detecting genome fragments of *T. gondii* (Castaño et al., 2014).

Diseases

There are very few clinical reports of presence of toxoplasmosis in spontaneously infected cattle. The experiment expresses that cattle are restricted to toxoplasmosis and infection in experimental form. But it is not confirmed that toxoplasmosis can lead to abortion. *T. gondii* can cause parasitemia in cattle. Later on, it can lead to hyperemia, respiratory distress, and nasal discharge in recent studies. The rate of mortality is uncommon in vaccine animals. It happens when calves are injected with oocysts and tachyzoites but after some time infection will be disclosed (Dubey et al., 2020).

Potential Risk Factor

The most common cause of *T. gondii* infections in humans are eating undercooked or raw meat. The data from the literature is simply classified on specific risk variables as “statistically significant” or “not statistically significant”. The most critical channels by which an illness might spread among animals must be understood to pinpoint particular risk factors for infection (Khan et al., 2023). These include consuming tissues from rodents or other intermediate hosts that have been infected, as well as oocyst contamination of the environment, food, or water. The risk of infection includes elements linked to various livestock group-raising practices. There are no overarching guidelines for defining or rating such risk factors.

General Factors

- Age
- Gender
- Regional and geographical characteristics
- Farm management, which includes
  - Production system
  - Specific farming conditions
  - Herd and flock size
  - Contact of cattle with other species
  - Animal restocking, biosecurity, staff hygiene and farm buildings
  - Cleaning and disinfection measures
  - Maintaining herd health by treatment of diseases and other veterinary care

Factors affecting life cycle of *T. gondii*

- Final host
- Intermediate hosts on form like rodents
- Feed related parameters
- Water related parameters
- Soil contact, pasturing and outside access

Effect of Toxoplasmosis on Economy of Livestock

The possibility of infection due to *T. gondii* in food producing animals affecting human health and incurring expenditures must be emphasized. The most common mode of transmission of *T. gondii* infection from parasite to human is small ruminants. It is recognized that the major cause of reproductive losses in small ruminants is *T. gondii* infection (Belluco et al., 2016; Opsteegh et al., 2016). There is just one instance of serious clinical symptoms at a Chinese farm that is rearing pigs (Li et al., 2010). Demonstrating economic significance of toxoplasmosis on these farms. Small ruminants may become infected with *T. gondii*, which can result in financial losses for farms.

Generally, the illness has great influence on economy that must be interpreted such as cost of disease(C), costs for disease prevention (P), treatment cost of animal (T) and losses (L):

- Wastage of resources due to disease (L) and expected loss value.
- The cost of treatment of infected animals (T)
- The cost of disease prevention (P)(Rashid et al., 2019)

Molecular Markers Associated with Toxoplasmosis in Cattle

Toxoplasmosis is a serious parasitic zoonosis that is spread by contaminated food and is brought on by the obligatory intercellular protozoan *T. gondii*. Numerous nations have documented *T. gondii* infections in cattle, which can result in miscarriage, significant financial loss and the possibility of transfer to humans and other animal (Shapiro et al., 2019).

The frequency of infection in cattle that are killed for human consumption is poorly understood. Therefore, the goal of the current investigation was the identification of sero-prevalence and genotype of *T. gondii* infection in cattle using enzyme linked immunosorbent assay (ELISA) and PCR restriction fragment length polymorphisms (RFLP).

Conclusion

The key aspect of this study is that the molecular marker is effective for the detection of disease. According to this research, the basic molecular markers best for detecting this disease are a group of 11 molecules, GRA6 being the most effective one. Others include SAG (1,2,3), BTUB, Apico,s etc. Observations suggested that intermediate hosts for *T. gondii* are cats, which can transfer the pathogen to cattle and others. However, it is still unknown how cattle influence the epidemiology of actual *T. gondii* in both humans and animals. The samples used in this research were the clonal type of *T. gondii*. Sero-prevalence of this disease depends on environmental conditions.

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