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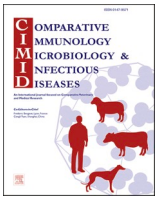

Disclosing coinfections: The interaction between *Toxoplasma gondii* and hemotropic agents in Colombian dogs and cats

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ABSTRACT

This study aimed to analyze the relationships between *Toxoplasma gondii* infection and hemotropic species (i.e., Rickettsiales, *Hepatozoon* spp., *Mycoplasma* spp., *Babesia* spp., *Bartonella* spp., and *Trypanosoma* spp.) via qPCR and to explore the associations between *T. gondii* monoinfection or coinfection with hemotropic species and the characteristics of dogs and cats in Antioquia (Colombia). A cross-sectional study was conducted with blood samples from dogs and cats positive for *T. gondii* by qPCR, with or without hemotropic coinfection. Hemogram results and demographic data were analyzed. Associations with monoinfection/coinfection were tested via Fisher's exact test or the χ^2 test ($p < 0.10$). Among the 590 animals (383 dogs, 207 cats), 262 (44.4 %) tested positive for *T. gondii* (dogs: 175/262, 66.8 %; cats: 87/262, 33.2 %). Among the dogs, 73.7 % had coinfections, including Rickettsiales (101/129), *Mycoplasma* spp. (55/129), *Hepatozoon* spp. (9/129), *Bartonella* spp. (7/129), and *Babesia* spp. (2/129); none tested positive for *Trypanosoma* spp. Coinfections were associated with breed, outdoor access, reticulocytes, lymphocytes, or reproductive status. In cats, 72.4 % had coinfections: *Bartonella* spp. (40/63), *Mycoplasma* spp. (23/63), Rickettsiales (11/63), and *Hepatozoon* spp. (3/63). All the cats tested negative for *Babesia* spp. and *Trypanosoma* spp. Coinfections were associated with leukocyte, neutrophil, lymphocyte, and protein alterations. *T. gondii* is prevalent in dogs and cats, with frequent coinfections. Environmental and biological factors influence these patterns, underscoring the importance of integrated diagnostics and surveillance. These findings suggest associations that warrant further investigation.

1. Introduction

Infectious diseases in canines and felines have attracted increasing interest in both veterinary medicine and human health, given the close relationship between humans and companion animals and the zoonotic potential of many of these agents [1]. These types of diseases can be caused by hemotropic agents, which are characterized by the development of one or several stages of their life cycle in blood cells [2]. Infections caused by these microorganisms commonly range from subclinical to severe illness in dogs and cats, and the severity of the disease depends on the specific pathogen involved and the host's conditions. Clinical manifestations can include anemia and/or thrombocytopenia, intermittent fever, depression, lethargy, anorexia, lymphadenomegaly, splenomegaly, and, in more severe cases, even death [3]. These infectious agents are also referred to as vector-borne pathogens since they are transmitted primarily to domestic animals through arthropods, including fleas, ticks, and flies, where they act as biological vectors [4], but the spread of these microorganisms can also occur through other transmission routes via infected blood (i.e., direct interactions between individuals or transfusions) [5]. In recent years, various prevalence studies performed in several countries have reported and confirmed the presence of these agents worldwide [6–9], with bacteria of the genus *Mycoplasma* being the most studied and reported pathogens, especially in felines [10], whereas *Ehrlichia* spp. constitute one of the genera among the rickettsial agents that are of greatest relevance and are most frequently detected in studies with canine populations [11].

Toxoplasmosis is considered a parasitic zoonosis; it is an infectious

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disease caused by *Toxoplasma gondii* (phylum Apicomplexa), an obligate intracellular protozoan that can infect nucleated and mitotically active cells of various mammals, with domestic cats and wild felines being its definitive hosts, essential for completing its life cycle where sexual reproduction of the microorganism takes place and the release of the infectious form of the agent into the environment occurs [2]. Mammals and some bird species, where asexual reproduction occurs, can act as intermediate hosts and can become infected by ingesting sporulated oocysts or meat contaminated with cysts as well as by vertical transmission (in humans) [12]. It is known that immunocompetent felines may remain asymptomatic, but in general terms, during the acute phase of infection, *T. gondii* causes digestive disorders, such as diarrhea or vomiting, fever, anorexia, or dyspnea, whereas in the chronic phase, it mainly causes neurological, respiratory and ocular disorders because bradyzoites, a slowly multiplying form, can be found in muscles or tissues [13–15]. Overall, *T. gondii* infection is associated with low morbidity and mortality rates in dogs and cats, but its clinical implications for primary care practices should be considered. It has been reported that clinical toxoplasmosis is more common in cats than in dogs, which more frequently suffer from neosporosis caused by *Neospora caninum*. In cats, susceptibility to clinical toxoplasmosis may be influenced by immunosuppression or a lack of prior immunity, particularly in animals that have not been vaccinated against core feline pathogens (such as herpesvirus, calicivirus, and panleukopenia virus), which can predispose them to opportunistic infections. Although no commercial vaccine against *N. caninum* is currently available for dogs, factors such as young age, immunosuppression, or underlying disease may increase their susceptibility to neosporosis [15]. Numerous studies have reported that the prevalence of the parasite in dogs and cats is between 6 % and 88 % worldwide [16]. Coinfections of *T. gondii* with different infectious agents, such as rickettsial bacteria, *Leptospira* spp., *Leishmania* spp., *N. caninum* and *Sarcocystis neurona,* have been reported [17–20]. In dogs, mixed infections with viruses such as canine distemper virus (CDV), canine adenovirus (CAdV) and canine parvovirus (CPV) are common, and in cats, coinfections with feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) are common [21,22]. These simultaneous infections by several microorganisms can mask the presence of some of the infectious agents causing important pathologies in the host; for this reason, a comprehensive diagnosis that allows investigation of the largest number of variables associated with the patient's status and clinical symptoms is essential.

Other hemotropic agents, such as *Mycoplasma* spp., *Bartonella* spp., *Hepatozoon* spp., *Babesia* spp., filariae, and some genera within the order Rickettsiales (*Anaplasma* spp., *Ehrlichia* spp., *Wolbachia* spp., and *Rickettsia* spp.), have been found in dogs and cats. These pathogenic microorganisms can be transmitted by vectors, mainly ticks and fleas, and as vectors can carry more than one infectious agent, there is a possibility of generating states of coinfection in animals [23].

The appearance of clinical signs in infected individuals with hemotropic agents usually precedes conditions that induce a state of immunosuppression in the animal, such as stress, concurrent and autoimmune diseases, and comorbidities [24]. The diagnosis of these pathogens can be made via indirect tests (ELISA, IFA) or direct tests, such as molecular assays and stool analysis. One of the main difficulties in diagnosis is that these kinds of diseases have very similar clinical signs and are often confused with other febrile pathologies in canines and felines; therefore, many cases remain undiagnosed [25]. Serological tests are widely used methods for detecting pathogens; however, as practical tools, they are unreliable both because of the cross-reactions that occur and because they cannot discriminate between a current infection and a previous exposure to the pathogen, as antibody titers tend to persist for several months or even years after treatment [26]. On the other hand, molecular techniques, such as PCR, are considered the most reliable diagnostic methods since they are highly sensitive tests for detecting blood-borne pathogens, allowing the identification of a wide range of microorganisms [27] and the detection of coinfections at different stages of the disease, an advantage for diagnosis because the transmission of these infectious agents is common in tropical and subtropical regions [28,29].

In Colombia, the frequency of hemotropic microorganisms in domestic animals is approximately 60 % in dogs and 70 % in cats. Within the group of canines positive for hemotropics, 43 %, 18 % and 1.3 % of those positive for one, two or three infectious agents, respectively, were infected. In the case of infected felines, a positive percentage of 56 % for one agent, 14 % for two agents and 2.3 % for three types of hemoparasites has been reported [30,31]. In canine patients, the main pathological alterations present in positive individuals with nonspecific clinical signs (fever, anorexia, myalgia, lethargy) are anemia and/or thrombocytopenia, presenting or not resulting in an inflammatory pattern in the leukogram. In a study carried out on felines, most individuals infected with some hemotropic agent presented blood counts without significant alterations, and only a small percentage presented leukocyte alterations and thrombocytopenia. Importantly, the absence of clinical signs in positive animals does not rule out the presence of infection with this type of pathogen, which may circulate subclinically [11,30,31]. In particular, with respect to *T. gondii* infection, there are reports of seroprevalence studies in the main cities of the country, where a parasite frequency in dogs and cats of 16.8 and 56.2 %, respectively, has been reported [32,33]. A literature review of *T. gondii* in Colombia revealed that, from published reports, 86 % are related to human infection, and the remaining 14 % are related to mammalian animals and other categories (one report associated with wildlife) [34].

Studies reported in the literature on toxoplasmosis in combination with other hemotropic agents are scarce. In a tropical country such as Colombia, where climatic conditions favor the presence and spread of infection by these pathogens [35], a timely diagnosis, together with the early detection of risk factors, is key to reduce the spread of these infectious agents. Further research on this type of zoonotic infection is needed to determine the prevalence and association of the presence of hemotropic microorganisms with clinical pathologies, which will help in the formulation of appropriate and effective treatments for patients in daily clinical practice, considering animal and human welfare by reducing the risk of infection to pet owners.

Therefore, this study has two objectives: first, to analyze the frequency and relationship between *T. gondii* infection and hemotropic species (i.e., Rickettsiales, *Hepatozoon* spp., *Mycoplasma* spp., *Babesia* spp., *Bartonella* spp., and *Trypanosoma* spp.), identified through molecular analysis (qPCR); second, to explore the associations between the outcomes of *T. gondii* monoinfection or coinfection with hemotropic species and specific animal-level characteristics and blood test results of dogs and cats domiciled in the metropolitan area of the Aburrá Valley and the municipality of Rionegro, Antioquia (Colombia), during 2023.

2. Materials and methods

2.1. Ethical considerations

No animals were manipulated during the study, as data from routine molecular diagnoses of infectious diseases in canine and feline samples sent to the TestMol© S.A.S laboratory were analyzed. The use of each patient's data for research was authorized through informed consent signed by their tutors as a routine process during veterinary consultations. Ethics committee approval for animal experimentation was not needed.

2.2. Study design and study population

A cross-sectional study was carried out, considering records of canines and felines that tested positive by real-time PCR for *T. gondii* from blood samples submitted by veterinary clinics and private veterinarians of the metropolitan area of the Aburrá Valley (consisting of 10 municipalities) and the municipality of Rionegro, Antioquia (Colombia), to TestMol© S.A.S., a molecular reference laboratory, from 2023 (January

1st to December 31st). All samples from owned pets were sent to the laboratory for molecular diagnosis to detect hemotropic agents; no stray, feral, or shelter animals were included. Each animal had undergone a prior clinical evaluation by the referring veterinarian according to its health status. On the basis of the obtained molecular results (profiles for hemotropics), in individuals with a positive result for *T. gondii*, individuals with coinfection by another species of hemotropic agent were evaluated. Blood samples were collected in EDTA tubes by the attending veterinarian.

2.3. Blood count and demographic data

Hemograms and demographic data were provided by the treating veterinarians with authorization from the owners of *T. gondii*-positive animals (both dogs and cats), with or without coinfection with hemotropic species. The referring veterinarians supplied the hemogram results from reference laboratories, where they had been processed according to established protocols.

Demographic variables such as species, breed, sex, and age group were collected from the form used to request sample shipping and processing at TestMol© S.A.S. Additional variables analyzed included the housing area, outdoor access, cohabitation with other animals, diet, water source, reproductive status, outcome on the basis of the diagnosis, and qPCR results. These data were obtained from medical records and phone interviews with pet tutors.

2.4. Genomic DNA isolation from blood samples

The DNA from bacteria and parasites was extracted from a 200 µL blood sample. Samples from 590 individuals were extracted via an automatic method with Kingfisher™ Duo open extraction equipment (Thermo Fisher Scientific Inc., MA, USA) and a MagMAX™ CORE M Express-96 nucleic acid purification kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the established conditions of the manufacturer for blood samples with frozen blood in EDTA (BD Vacutainer Systems, Preanalytical Solutions, Plymouth, UK) tubes. The final elution volume of the samples at the end of the extraction process was 100 µL. The DNA was stored at −80°C until processing. The purity and concentration of the DNA were analyzed with a Nanodrop spectrophotometer (Fisher Scientific, Madrid, Spain). Samples with a DNA concentration greater than 10 ng/µl and a purity ratio of 260/280 between 2.0 and 2.2 were considered suitable for processing via qPCR. The samples were re-extracted if they did not meet the criteria until they passed the required quality parameters.

2.5. qPCR method for identification of T. gondii and hemotropic agents

The DNA samples were processed with specific primers (Macrogen, Korea) targeting the 16S rRNA gene in bacteria (*Anaplasma* spp., *Ehrlichia* spp., *Wolbachia* spp., *Rickettsia* spp., *Mycoplasma* spp., Candidatus *Mycoplasma* spp., *Bartonella* spp.) and the 18S rRNA gene in parasites (*Babesia* spp., *Hepatozoon* spp., *Dirofilaria* spp., *Brugia* spp., *Acanthocheilonema* spp., *T. gondii*). For the amplification process, reactions of 19 µl were prepared containing 10 µl of SYBR Green real-time PCR MasterMix enzyme (2X) (Thermo Fisher Scientific Inc., MA, USA), 2 µl of each primer (100 nM) and 5 µl of DNA from each sample (20–25 ng/µL). The thermal profile was run with an initial denaturation of 3 min at 95°C, then 35 cycles of 30 s at 95°C, 1 min at 57°C, and 1 min at 72°C, followed by a final extension for 5 min at 72°C. The simple qPCRs for each infectious agent were performed in a Mic qPCR Cycler with 4 channels (BioMolecular Systems, Australia) according to the laboratory's protocols.

This study used both positive and negative controls to ensure the accuracy and validity of the results. The positive controls were provided by the TestMol© S.A.S laboratory for specialized molecular diagnosis and research, which consists of a qPCR mixture with the corresponding primers plus the plasmid for each evaluated infectious agent in the same volume as the sample DNA used in a reaction. As a negative control, DNase-RNase-free sterile water (Cat No. 129114; Qiagen, Germany) was used. For the internal controls used for DNA extraction and qPCR, specific primers for the cytochrome B gene in mammals were used [36].

The results were analyzed in terms of the reached threshold cycle (Ct) value reached and the dissociation curves (temperature range Tm °C established for each infectious agent). Samples with a Ct value < 35 were considered positive, and samples with values ≥ 35 were considered negative. The bacterial and parasitic loads in copies/µl were calculated on the basis of the laboratory's internal test validation process, where the theoretical copy number for each plasmid was established.

2.6. Data analysis

All the data generated during the study were entered into Excel spreadsheets (Microsoft Corp., Redmond, WA, USA) and then exported to Stata v.18 (StataCorp, 2023, College Station, TX, USA) for statistical analysis. Each demographic variable was categorized as follows: breed (purebred, mixed breed); sex (male, female); age group (puppies/kittens—<1 year, young adults—1–6 years, mature adults—7–10 years, seniors—>10 years); housing area (urban, rural, mixed); outdoor access (yes, no); cohabitation with other animals (yes, no); diet (commercial balanced feed, Biologically Appropriate Raw Food—B.A.R.F. [37], home-cooked feed, and mixtures); water source (potable, not potable, filtered, and mixtures); origin (breeder, adopted, rescued, offspring from household pets); reproductive status (spayed/neutered, intact); and outcome on the basis of the diagnosis (alive, deceased). To provide greater detail regarding the breeds of the animals in the study, the distribution was presented according to breed classification in dogs [38] and in cats [39]. Descriptive statistics were calculated for all the demographic variables of interest. Associations between the outcome of coinfection with *T. gondii* (presence or absence) and each of the variables of interest were analyzed via Fisher's exact test or the chi-square (χ^2) test, as appropriate. Fisher's exact test was applied when any expected cell count was less than 5, particularly in 2×2 tables, given its accuracy in small sample sizes. The chi-square test was used when all expected cell frequencies were 5 or greater, as it provides a valid approximation under these conditions and is computationally efficient for larger datasets. A significance level of $p < 0.10$ was chosen because of the exploratory nature of this study, which seeks to identify potential trends that may warrant further investigation. This more lenient threshold allows for the detection of patterns that could be overlooked under more conventional significance levels, providing a basis for future research.

3. Results

Among the 590 individuals (383 dogs and 207 cats), 262 (44.4 %) animals (dogs: 175/262, 66.8 %; cats: 87/262, 33.2 %) tested positive for *T. gondii* from 86 different sources (veterinary clinics and private veterinarians) in the 10 municipalities that make up the Aburrá Valley and the municipality of Rionegro, Antioquia (Colombia).

A total of 175 dogs were included in the study, representing 41 different breeds, which were categorized according to the Fédération Cynologique Internationale (FCI) classification system [38]. The most frequent group was Group 9 (companion dogs, e.g., Chihuahua, Shih Tzu) with 30 individuals, followed by Group 5 (Spitz and primitive types, e.g., Alaskan Malamute, Siberian Husky) with 24, Group 3 (terriers, e.g., Pitbull, Yorkshire Terrier, Bull Terrier) with 20, and Group 8 (retrievers, flushing dogs, and water dogs, e.g., Labrador Retriever, Golden Retriever, Cocker Spaniel) with 18. Group 1 (Sheepdogs and cattle dogs, e.g., Australian Shepherd, Border Collie, German Shepherd) included 13 dogs; Group 2 (Pinschers, Schnauzers, Molossians, and Swiss Mountain and Cattle Dogs, e.g., Bernese Mountain Dog, American Bully, Pinscher) included 11; and Group 4 (Dachshunds) included nine. The less frequent groups included Group 6 (Scent hounds and related

breeds, e.g., Rhodesian Ridgeback, Beagle) with five dogs, Group 7 (Pointing dogs, e.g., Brussels Griffon, Weimaraner) with two, and Group 10 (Sighthounds, e.g., Afghan Hound) with one individual. In addition, 40 dogs were classified as mixed breeds, and breed information was unavailable for two dogs.

The average age of dogs was 59.8 months (4.9 years), with a range of 2–228 months. Among the 175 *T. gondii*-positive dogs, 129 (73.7 %) were coinfected with at least one hemotropic species, whereas 46 (26.3 %) were infected with only *T. gondii*. Coinfections with *T. gondii* included Rickettsiales (101/129), *Mycoplasma* spp. (55/129), *Hepatozoon* spp. (9/129), *Bartonella* spp. (7/129), and *Babesia* spp. (2/129). All dogs tested negative for *Trypanosoma* spp.

Monoinfection with *T. gondii* as well as coinfection with Rickettsiales in dogs was associated with breed ($p = 0.040$ in both cases). Coinfection with *Hepatozoon* spp. was associated with outdoor access ($p = 0.007$), as were coinfection with blood reticulocytes ($p = 0.012$) and lymphocytes ($p = 0.072$). Coinfection with *Mycoplasma* spp. was associated with housing area ($p = 0.048$) and outdoor access ($p = 0.100$). Similarly, coinfection with *Babesia* spp. was associated with outdoor access ($p = 0.057$). Coinfection with *Bartonella* spp. was associated with breed ($p = 0.002$), reproductive status ($p = 0.030$), diagnosis outcome ($p = 0.024$), and all blood test results. No other significant associations were found for the variables explored in dogs. Table 1 presents a summary of the results, displaying only the significant findings for clarity. The complete table is available in Supplementary Material 1.

A total of four breed groups were represented among the cats included in the study, following the classification of the Fédération Internationale Féline (FIFe) [39]. The most frequent group was Group 1 (Exotic breeds, e.g., Persian, Ragdoll, Sacred Birman), with 10 individuals, followed by Group 4 (Abyssinian, Siamese, Sphynx, and similar breeds, e.g., Balinese, Cornish Rex, Oriental Shorthair), with six individuals, and Group 3 (Bengal, British Longhair, and similar breeds, e.g., Scottish Straight), with two individuals. Additionally, 69 cats were classified as mixed breeds.

The average age of the cats was 54.5 months (4.5 years), with a range from 3 to 166 months. Among the 87 *T. gondii*-positive cats, 63 (72.4 %) were coinfected with at least one hemotropic species of interest, whereas 24 (27.6 %) were infected with only *T. gondii*. Coinfections with *T. gondii* included *Bartonella* spp. (40/63), *Mycoplasma* spp. (23/63), Rickettsiales (11/63), and *Hepatozoon* spp. (3/63). All the cats tested negative for *Trypanosoma* spp. and *Babesia* spp. (Table 3).

Monoinfection with *T. gondii* in cats was associated with blood leukocytes, neutrophils, lymphocytes, proteins, and platelets ($p = 0.100$, 0.059, 0.085, 0.068, and 0.042, respectively), whereas coinfection with Rickettsiales was associated with diagnosis outcome and neutrophils ($p = 0.100$ in both cases). Coinfection with *Hepatozoon* spp. was associated with blood lymphocyte counts ($p = 0.085$). Coinfection with *Mycoplasma* spp. was associated with the housing area ($p = 0.045$). Coinfection with *Bartonella* spp. was associated with erythrocytes ($p = 0.015$), hematocrit ($p = 0.055$), lymphocytes ($p = 0.100$), proteins ($p = 0.028$), and blood platelets ($p = 0.035$). No other significant associations were found for the variables explored. Table 2 presents a summary of the results, displaying only the significant findings for clarity. The complete table is available in Supplementary Material 1.

To further characterize the infection dynamics observed in the studied population, we analyzed the frequency of coinfection combinations involving *T. gondii* and hemotropic agents in both dogs and cats. Table 3 summarizes the number of animals presenting with single or multiple hemotropic coinfections, helping to illustrate the diversity and overlap of hemotropic pathogens coexisting with *T. gondii*.

4. Discussion

T. gondii infection is generally associated with low morbidity and mortality rates in dogs. However, dogs often develop neurological signs similar to those observed with *Neospora caninum* infections [40]. Some cases of patients infected with *T. gondii* that generate clinical manifestations are triggered by immunosuppressive chemotherapy, as evidenced in dogs by disseminated toxoplasmosis with fatal complications, which presents after the administration of immunosuppressive combination therapy for the treatment of immune-mediated hemolytic anemia (reported case of a 16-month-old dog) [41]. On the other hand, coinfections in immunocompetent patients with *T. gondii* are usually associated with more severe clinical manifestations that worsen the patient's prognosis, probably due to the stress on the immune system that allows the simultaneous proliferation of the infectious agents involved [20,21]. Therefore, the evaluation of possible coinfections in individuals positive for *T. gondii* allows a more adequate and timely therapeutic approach to the patient. Nevertheless, the severity of the disease does not depend only on the patient's condition or on the virulence of the infectious agent, since, remembering the epidemiological triad, it is necessary to evaluate other variables related to the environment that may be involved in the development of the disease.

An association with breed was found in individuals who were infected with *T. gondii* and in individuals who presented coinfection by *T. gondii* with Rikettsial agents, and a greater frequency of this type of infection was observed in purebred dogs than in mixed breeds (Table 1). This observation may suggest that breed could be a contributing factor to the risk of *T. gondii* monoinfection and/or coinfection with other opportunistic pathogens. Although most published studies do not establish a direct causal relationship between breed and coinfection, several studies have reported breed-related patterns that may reflect underlying behavioral or environmental exposures. For example, coinfection with *T. gondii* and *E. canis* has been reported in specific breeds with neurological disorders [42], whereas other studies have reported correlations between breed and infections with *E. canis* and *Anaplasma* spp [43,44]. In a study conducted in Rio de Janeiro (Brazil), mixed breed dogs had a greater probability of being infected with *T. gondii* than did purebred dogs [45]. Conversely, in Egypt, anti-*T. gondii* antibodies are more frequently detected in purebred German Shepherds, particularly in males over 3 years of age [46]. These findings highlight the possible influence of breed-associated factors, such as housing, lifestyle, or outdoor access, on infection risk, which merits further investigation through well-controlled, breed-stratified studies.

We also found that coinfection with *Hepatozoon* spp. was associated with outdoor access (Table 1). This coinfection has been reported previously in only one dog with neurological signs that died [47]. Nevertheless, a variety of reports have demonstrated a significant association between the high presence of *T. gondii* antibodies in dogs that live outdoors [48,49]. On the other hand, staying longer at home is a protective factor against infection by *Hepatozoon* spp. in dogs [50], whereas being a hunting dog has been identified as a significant risk factor, likely due to increased exposure to ticks in outdoor environments [51]. The association between the presence of these pathogens and outdoor access is largely due to exposure to vectors and the consumption of food or water contaminated with oocysts or cysts of *T. gondii*. The most frequent hematological findings in hepatozoonosis-positive dogs are lymphopenia, anemia, thrombocytopenia, relative neutrophilia and neutrophilic leukocytosis [52,53], which is consistent with our findings.

In this study, we also found a statistically significant association of *T. gondii* and *Babesia* spp. coinfection in dogs with the variable or condition of "outdoor access" (Table 1). Although this type of coinfection has not been documented in canines in the literature to date, the coexistence of both parasites in ticks of the genus *Ixodes* spp. has been demonstrated [54].

The presence of *Bartonella* spp. has rarely been documented in dogs. However, a study in São Paulo, Brazil, detected antibodies against both *T. gondii* and other hemotropic pathogens, including *Bartonella* spp [55]. In the present study, we found that coinfection with *T. gondii* and *Bartonella* spp. in dogs appeared to be associated with breed (Table 1). A similar observation was noted by Henn et al. [56], who reported higher *Bartonella* seroprevalence in sporting and herding breeds; however, the

Table 1
Statistically significant animal-level characteristics and outcomes of dogs domiciled in the metropolitan area of the Aburrá Valley and the municipality of Rionegro, Antioquia (Colombia), during 2023 (n = 175).

					Hemotropic species coinfecting									
Variable	Categories n (%)	Coinfections with *Toxoplasma gondii*- n (%)*			Rickettsiales		*Hepatozoon* spp.		*Mycoplasma* spp.		*Babesia* spp.		*Bartonella* spp.	
		No	Yes	p value	Positive n (%)	p value	Positive n (%)	p value	Positive n (%)	p value	Positive n (%)	p value	Positive n (%)	p value
Dog features and pet ownership practices														
Breed														
Purebred	135 (77.1)	41 (30)	94 (7)	**0.040**	80 (85.1)	**0.004**	6 (6.4)	0.703	40 (42.6)	1.000	1 (1.1)	0.471	1 (1.1)	**0.002**
Mixed breed	40 (22.9)	5 (12.5)	35 (87.5)		21 (60)		3 (8.6)		15 (42.9)		1 (2.9)		6 (17.1)	
Housing area														
Urban	75 (42.9)	18 (24)	57 (76)	0.380	44 (77.2)	0.647	5 (8.8)	0.658	24 (42.1)	**0.048**	2 (3.5)	0.641	1 (1.8)	0.147
Rural	12 (6.8)	1 (8.3)	11 (91.7)		10 (90.9)		0 (0)		9 (81.8)		0 (0)		0 (0)	
Mixed (farms and/or pet care centers and urban)	13 (7.4)	4 (30.8)	9 (69.2)		6 (66.7)		1 (11.1)		3 (33.3)		0 (0)		0 (0)	
Not reported	75 (42.9)	23 (30.7)	52 (69.3)		41 (78.8)		3 (5.8)		19 (36.5)		0 (0)		6 (11.5)	
Outdoor access														
No	15 (8.6)	4 (26.7)	11 (73.3)	0.420	5 (45.4)	0.394	3 (27.3)	**0.007**	8 (72.7)	**0.100**	1 (9)	**0.057**	6 0 (0)	0.575
Yes	47 (26.8)	9 (19.2)	38 (80.8)		31 (81.6)		4 (10.5)		14 (36.8)		1 (2.6)		1 (2.6)	
Not reported	113 (64.6)	33 (29.2)	80 (70.8)		65 (81.2)		2 (2.5)		33 (41.2)		0 (0)		6 (7.5)	
Reproductive status														
Spayed/neutered	59 (33.7)	13 (22)	46 (78)	0.504	34 (73.9)	0.668	3 (6.5)	0.825	21 (45.6)	0.501	0 (0)	0.710	0 (0)	**0.030**
Intact	41 (23.4)	10 (24.4)	31 (75.6)		25 (80.6)		3 (9.7)		15 (48.4)		1 (3.2)		1 (3.2)	
Not reported	75 (42.9)	23 (30.7)	52 (69.3)		42 (80.8)		3 (5.8)		19 (36.5)		1 (1.9)		6 (30.8)	
Outcome based on the diagnosis														
Survived	86 (49.1)	19 (22.1)	67 (77.9)	0.384	49 (73.1)	0.211	6 (8.9)	0.701	33 (49.3)	0.264	1 (1.5)	1.000	1 (1.5)	**0.024**
Died	20 (11.4)	5 (25)	15 (75)		14 (93.3)		0 (0)		6 (40)		0 (0)		0 (0)	
Not reported	69 (39.5)	22 (31.9)	47 (68.1)		38 (80.8)		3 (6.4)		16 (34)		1 (2.1)		6 (12.8)	
Blood test results														
Erythrocytes														
Normal*	63 (36)	17 (27)	46 (73)	0.230	35 (76.1)	0.820	3 (6.5)	0.777	21 (26.1)	0.530	1 (2.2)	1.000	1 (2.2)	**0.019**
Lower	51 (29.2)	9 (17.6)	42 (82.4)		32 (76.2)		4 (9.5)		20 (47.6)		1 (2.4)		0 (0)	
Higher	2 (1.1)	1 (50)	1 (50)		1 (100)		0 (0)		0 (0)		0 (0)		0 (0)	
Not reported	59 (33.7)	19 (32.2)	40 (67.8)		33 (82.5)		2 (5)		14 (35)		0 (0)		6 (15)	
Hematocrit														
Normal*	60 (34.3)	16 (26.7)	44 (73.3)	0.419	35 (79.5)	0.585	3 (6.8)	0.835	20 (45.4)	0.675	1 (2.3)	1.000	1 (2.3)	**0.023**
Lower	49 (28)	9 (18.4)	40 (81.6)		30 (75)		4 (10)		19 (47.5)		1 (2.5)		0 (0)	
Higher	7 (4)	2 (28.6)	5 (71.4)		3 (60)		0 (0)		2 (40)		0 (0)		0 (0)	
Not reported	59 (33.7)	19 (32.2)	40 (67.8)		33 (82.5)		2 (5)		14 (35)		0 (0)		6 (15)	
Reticulocytes														
Normal*	46 (26.3)	8 (17.4)	38 (82.6)	0.231	27 (71)	0.519	5 (13.2)	**0.012**	19 (50)	0.569	0 (0)	0.351	1 (2.6)	**0.039**
Lower	58 (33.1)	15 (25.9)	43 (74.1)		36 (83.7)		0 (0)		18 (41.9)		0 (0)		0 (0)	
Higher	4 (2.3)	2 (50)	2 (50)		2 (100)		1 (50)		0 (0)		0 (0)		0 (0)	
Not reported	67 (38.3)	21 (31.3)	46 (68.7)		36 (78.3)		3 (6.5)		18 (39.1)		2 (4.3)		6 (13)	
Leukocytes														
Normal*	60 (34.3)	17 (28.3)	43 (71.7)	0.351	34 (79.1)	0.216	1 (2.3)	0.137	22 (56.9)	0.367	2 (51.2)	0.409	1 (2.3)	**0.021**
Lower	10 (5.7)	2 (20)	8 (80)		8 (100)		0 (0)		2 (25)		0 (0)		0 (0)	

(*continued on next page*)

Table 1 (*continued*)

		Hemotropic species coinfecting											
Higher	46 (26.3)	8 (17.4)	38 (82.6)		26 (68.4)		6 (15.8)		17 (44.7)		0 (0)		0 (0)	
Not reported	59 (33.7)	19 (32.2)	40 (67.8)		33 (82.5)		2 (5)		14 (35)		0 (0)		6 (15)	
Neutrophils														
Normal*	60 (34.3)	16 (26.7)	44 (73.3)	0.395	35 (79.5)	0.581	2 (4.5)	0.569	22 (50)	0.280	1 (2.3)	1.000	1 (2.3)	**0.019**
Lower	3 (1.7)	0 (0)	3 (100)		3 (100)		0 (0)		0 (0)		0 (0)		0 (0)	
Higher	53 (30.3)	11 (20.8)	42 (79.2)		30 (71.4)		5 (11.9)		19 (45.2)		1 (2.4)		0 (0)	
Not reported	59 (33.7)	19 (32.2)	40 (67.8)		33 (82.5)		2 (5)		14 (35)		0 (0)		6 (15)	
Lymphocytes														
Normal*	101 (57.7)	23 (22.8)	78 (77.2)	0.559	61 (78.2)	0.361	5 (6.4)	**0.072**	37 (47.4)	0.543	2 (2.6)	0.622	1 (1.3)	**0.027**
Lower	9 (5.2)	2 (22.2)	7 (77.8)		5 (71.4)		0 (0)		2 (28.6)		0 (0)		0 (0)	
Higher	6 (3.4)	2 (33.3)	4 (66.7)		2 (50)		2 (50)		2 (50)		0 (0)		0 (0)	
Not reported	59 (33.7)	19 (32.2)	40 (67.8)		33 (82.5)		2 (5)		14 (35)		0 (0)		6 (15)	
Proteins														
Normal*	68 (38.9)	18 (26.5)	50 (73.5)	0.465	42 (84)	0.189	2 (4)	0.280	19 (38)	0.117	1 (2)	0.758	0 (0)	**0.014**
Lower	7 (4)	1 (14.3)	6 (85.7)		4 (66.7)		1 (16.7)		2 (33.3)		0 (0)		0 (0)	
Higher	41 (23.4)	8 (19.5)	33 (80.5)		22 (66.7)		4 (12.1)		20 (60.6)		1 (3)		1 (3)	
Not reported	59 (33.7)	19 (32.2)	40 (67.8)		33 (82.5)		2 (5)		14 (35)		0 (0)		6 (15)	
Platelets														
Normal*	89 (50.9)	20 (22.5)	69 (77.5)	0.477	52 (75.4)	0.812	6 (8.7)	0.902	30 (43.5)	0.279	2 (2.9)	0.655	0 (0)	**0.005**
Lower	25 (14.3)	7 (28)	18 (72)		14 (77.8)		1 (5.6)		9 (50)		0 (0)		1 (5.6)	
Higher	2 (1.1)	0 (0)	2 (100)		2 (100)		0 (0)		2 (100)		0 (0)		0 (0)	
Not reported	59 (33.7)	19 (32.2)	40 (67.8)		33 (82.5)		2 (5)		14 (35)		0 (0)		6 (15)	

* Within the reference ranges [39].

authors did not establish breed as a definitive risk factor, and this association should be interpreted cautiously. Additionally, we observed an association between coinfection and outdoor access (Table 1), which is consistent with previous findings [57], where it has been reported that *Bartonella* spp. seropositive dogs are more likely to live in rural environments. With respect to the hematological alterations associated with coinfection of *T. gondii* and *Bartonella* spp., previous studies have shown that the main clinical changes in dogs with bartonellosis include anemia —sometimes immune-mediated anemia, along with leukocytosis, neutrophilia, eosinophilia, and thrombocytopenia. These alterations are likely driven primarily by *Bartonella* spp. and may be exacerbated by coinfection with *T. gondii* in dogs [58,59].

T. gondii infection is generally associated with low morbidity and mortality rates in cats. Toxoplasmosis is usually more common in cats than in dogs, and in felines, clinical signs may emerge under immunosuppression, as evidenced by reactivation of latent *T. gondii* infection secondary to cyclosporine-induced immunosuppression [60].

The associations between monoinfection with *T. gondii* and alterations in blood leukocytes, neutrophils, lymphocytes, proteins, and platelets (Table 2) align with previous studies that highlighted significant immunological and hematological changes linked to *T. gondii* infection in cats. For example, a decrease in neutrophil phagocytic activity, particularly in stray cats, alongside variations in T lymphocyte subpopulations, with seropositive domestic cats showing greater numbers of T-suppressors, whereas stray cats with a larger T-helper population have been previously reported [61]. Furthermore, a previous study reported that cats with elevated IgM titers presented increased packed cell volume, red blood cell counts, and monocyte counts. These findings underscore the correlation between *T. gondii* infection and blood cell profiles, suggesting its diagnostic value, especially in chronic toxoplasmosis. While these findings highlight the impact of *T. gondii* on blood cells, it is essential to consider that not all infected cats exhibit clinical symptoms, suggesting a complex interplay between the parasite and host immune responses [62].

An association was also observed between coinfection with *T. gondii* or Rickettsiales and neutrophil alterations and clinical outcomes in the cat population studied (Table 2). This finding is consistent with existing research, which suggests that *T. gondii* influences neutrophil behavior, including the release of extracellular traps (NETs) and changes in reactive oxygen species (ROS) production [63,64]. The presence of Rickettsiales coinfection may amplify host immune responses, with neutrophils already mobilized against *T. gondii* potentially exhibiting functional alterations that could influence the severity of clinical manifestations. Additionally, studies have shown that stray cats seropositive for *T. gondii* display reduced neutrophil phagocytic activity compared with domestic cats, highlighting the role of environmental factors in modulating immune responses during coinfection [61].

Coinfection with *T. gondii* and *Hepatozoon* spp. can significantly alter the immune response of lymphocytes in feline blood, as found herein, resulting in complex interactions that impact disease outcomes. This immune response is characterized by changes in cytokine profiles and lymphocyte activity, which may lead to increased mortality and altered immune dynamics. *T. gondii* triggers a strong Th1 immune response, whereas *Hepatozoon* spp. may elicit a different response, creating a dysregulated immune environment [62]. The presence of *T. gondii* can cause dysfunction in memory CD8 T cells, diminishing their

Table 2
Statistically significant animal-level characteristics and outcomes of cats domiciled in the metropolitan area of the Aburrá Valley and the municipality of Rionegro, Antioquia (Colombia), during 2023 (n = 87).

					Hemotropic species coinfecting							
Variable	Categories n (%)	Coinfections with *Toxoplasma gondii*-n (%)*			Rickettsiales		*Hepatozoon* spp.		*Mycoplasma* spp.		*Bartonella* spp.	
		No	Yes	p value	Positive n (%)	p value	Positive n (%)	p value	Positive n (%)	p value	Positive n (%)	p value
Cat features and pet ownership practices												
Housing area												
Urban	35 (40.2)	8 (22.9)	27 (77.1)	0.7465	3 (11.1)	0.278	1 (3.7)	1.000	8 (29.6)	**0.045**	19 (70.3)	0.511
Rural	4 (4.6)	1 (25)	3 (75)		1 (33.3)		0 (0)		3 (100)		2 (66.7)	
Mixed (farms and/or petcare centers and urban)	1 (1.2)	0 (0)	1 (100)		0 (0)		0 (0)		1 (33.3)		0 (0)	
Not reported	47 (54)	15 (31.9)	32 (68.1)		7 (21.9)		2 (6.2)		11 (34.4)		19 (59.4)	
Outcome based on the diagnosis												
Survived	38 (43.7)	7 (18.4)	31 (81.6)	0.3651	3 (9.7)	**0.100**	1 (3.2)	0.649	11 (35.5)	1.000	21 (67.7)	0.441
Died	3 (3.5)	1 (33.4)	2 (66.6)		1 (50)		0 (0)		1 (50)		2 (100)	
Not reported	46 (52.8)	16 (34.8)	30 (65.2)		7 (23.3)		2 (6.7)		11 (36.7)		17 (56.7)	
Blood test results												
Erythrocytes												
Normal*	25 (28.7)	4 (16)	21 (84)	0.115	3 (14.3)	0.164	0 (0)	0.257	6 (28.6)	0.707	17 (80.9)	**0.015**
Lower	14 (16.1)	2 (14.3)	12 (85.7)		0 (0)		0 (0)		4 (33.3)		10 (83.3)	
Higher	8 (9.2)	2 (25)	6 (75)		1 (16.7)		1 (16.7)		3 (50)		2 (33.3)	
Not reported	40 (46)	16 (40)	24 (60)		7 (29.2)		2 (8.3)		10 (41.7)		11 (45.8)	
Hematocrit												
Normal*	30 (34.5)	5 (16.7)	25 (83.3)	0.125	3 (12)	0.139	1 (4)	0.819	8 (32)	0.799	18 (72)	**0.055**
Lower	12 (13.8)	2 (16.7)	10 (83.3)		0 (0)		0 (0)		3 (30)		9 (90)	
Higher	5 (5.8)	1 (20)	4 (80)		1 (25)		0 (0)		2 (50)		2 (50)	
Not reported	40 (46)	16 (40)	24 (60)		7 (29.2)		2 (8.3)		10 (41.7)		11 (45.8)	
Leukocytes												
Normal*	32 (36.8)	5 (15.6)	27 (84.4)	**0.100**	3 (11.1)	0.277	1 (3.7)	0.788	8 (29.6)	0.699	19 (70.3)	0.156
Lower	7 (8)	1 (14.3)	6 (85.7)		1 (16.7)		0 (0)		2 (33.3)		5 (83.3)	
Higher	8 (9.2)	2 (25)	6 (75)		0 (0)		0 (0)		3 (50)		5 (83.3)	
Not reported	40 (46)	16 (40)	24 (60)		7 (29.2)		2 (8.3)		10 (41.7)		11 (45.8)	
Neutrophils												
Normal*	33 (38)	6 (18.2)	27 (81.8)	**0.059**	2 (7.4)	**0.100**	0 (0)	0.218	9 (33.3)	0.422	19 (70.4)	0.144
Lower	7 (8)	0 (0)	7 (100)		2 (28.6)		1 (14.3)		1 (14.3)		6 (85.7)	
Higher	7 (8)	2 (28.6)	5 (71.4)		0 (0)		0 (0)		3 (60)		4 (80)	
Not reported	40 (46)	16 (40)	24 (60)		7 (29.2)		2 (8.3)		10 (41.7)		11 (45.8)	
Lymphocytes												
Normal*	35 (40.2)	7 (20)	28 (80)	**0.085**	3 (10.7)	0.271	0 (0)	**0.100**	11 (39.3)	0.651	21 (75)	**0.100**
Lower	7 (8)	1 (14.3)	6 (85.7)		1 (16.7)		0 (0)		1 (16.7)		5 (83.3)	
Higher	5 (5.8)	0 (0)	5 (100)		0 (0)		1 (20)		1 (20)		3 (60)	
Not reported	40 (46)	16 (40)	24 (60)		7 (29.2)		2 (8.3)		10 (41.7)		11 (45.8)	
Proteins												
Normal*	32 (36.8)	7 (21.9)	25 (78.1)	**0.068**	2 (8)	0.234	1 (4)	0.819	8 (32)	0.935	21 (84)	**0.028**
Lower	6 (6.9)	0 (0)	6 (100)		1 (16.7)		0 (0)		2 (33.3)		3 (33.3)	
Higher	9 (10.3)	1 (11.1)	8 (88.9)		1 (12.5)		0 (0)		3 (37.5)		5 (62.5)	
Not reported	40 (46)	16 (40)	24 (60)		7 (29.2)		2 (8.3)		10 (41.7)		11 (45.8)	
Platelets												
Normal*	40 (46)	6 (15)	34 (85)	**0.042**	4 (11.8)	0.178	1 (2.9)	0.661	13 (38.2)	0.266	24 (70.6)	**0.035**
Lower	7 (8)	2 (28.6)	5 (71.4)		0 (0)		0 (0)		0 (0)		5 (100)	
Higher	0 (0)	0 (0)	0 (0)		0 (0)		0 (0)		0 (0)		0 (0)	
Not reported	40 (46)	16 (40)	24 (60)		7 (29.2)		2 (8.3)		10 (41.7)		11 (45.8)	

The numbers in bold refer to statistical significance ($p < 0.100$).
* Within the reference ranges [39].

Table 3
Distribution of coinfection combinations with hemotropic agents among *Toxoplasma gondii*-positive dogs and cats domiciled in the metropolitan area of the Aburrá Valley and the municipality of Rionegro, Antioquia (Colombia), during 2023 (n = 262).

Coinfection combination	Number of dogs	Number of cats
Toxoplasma gondii only	46	24
Bartonella spp.	2	29
Hepatozoon spp.	3	2
Hepatozoon spp. + *Bartonella* spp.	2	0
Hepatozoon spp. + *Mycoplasma* spp.	3	0
Mycoplasma spp.	15	14
Mycoplasma spp. + *Babesia* spp.	1	0
Mycoplasma spp. + *Bartonella* spp.	2	7
Rickettsiales	65	5
Rickettsiales + *Bartonella* spp.	1	3
Rickettsiales + *Hepatozoon* spp.	1	1
Rickettsiales + *Mycoplasma* spp.	33	1
Rickettsiales + *Mycoplasma* spp. + *Babesia* spp.	1	0
Rickettsiales + *Mycoplasma* spp. + *Bartonella* spp.	0	1
Total	175	87

effectiveness against both parasites [63]. Additionally, dendritic cells infected with *T. gondii* exhibit altered migration and adhesion properties, potentially hindering their ability to present antigens effectively [64]. Conversely, while coinfection can lead to immune suppression, some studies suggest that the immune system may adapt to manage both infections. For example, in models of coinfection with *T. gondii* and helminths, compensatory immune mechanisms have been observed that help balance inflammatory and regulatory responses, allowing partial control of both pathogens [65].

Since specific data on *T. gondii* and *Mycoplasma* spp. coinfection in cats are limited, further research is needed to explore their dynamics in populations living in rural or urban settings. The association between the coinfection of *T. gondii* and *Mycoplasma* spp. in cats, particularly concerning their housing conditions, reveals significant insights from recent studies but is related to care status (owned animals vs shelter animals). Research indicates that stray cats exhibit higher seroprevalence rates of *T. gondii* than owned cats do [61].

Another significant finding of our study was the associations between coinfection with *T. gondii* and *Bartonella* spp. in cats and hematocrit, proteins, lymphocytes, platelets and erythrocytes (Table 2). This coinfection is known to lead to anemia and other hematological disorders, with the interplay between these pathogens potentially exacerbating clinical symptoms, particularly in immuno-compromised or stressed cats. *Bartonella* spp. infections are commonly linked to chronic anemia and hematological abnormalities, such as monocytosis and inflammatory leukograms, in both animals and humans [66,67]. The presence of *Bartonella* spp. in cats is well documented, especially in regions where vector-borne diseases (VBDs) are prevalent [68]. While there is evidence that coinfection can lead to anemia and other disorders, more research is needed to fully understand the mechanisms and interactions between *T. gondii* and *Bartonella* spp. in feline health.

We address the main findings derived from the analysis of the relationship between *T. gondii* infection and the presence of hemotropic species (including Rickettsiales, *Hepatozoon* spp., *Mycoplasma* spp., *Babesia* spp., *Bartonella* spp., and *Trypanosoma* spp.) through molecular analysis (qPCR). Additionally, we aimed to explore the associations between the outcomes of *T. gondii* monoinfection or coinfection with hemotropic species and various animal-level characteristics of dogs and cats. Nevertheless, given the exploratory nature of the study, the associations reported herein should be interpreted with caution and confirmed in future research with larger, more controlled samples.

Although the qPCR panel included primers for *Dirofilaria* spp., *Brugia* spp., and *Acanthocheilonema* spp., these agents were not the focus of the present study and were included primarily for differential diagnostic purposes in the routine workflow of the diagnostic laboratory. This predefinition was based on known blood tropism, relevance to small animal clinical practice in Colombia, and the availability of validated qPCR curves for these pathogens in the internal protocol. In addition, in endemic areas, it is plausible that filarial nematodes and *Leishmania* spp. may coexist with other hemotropic pathogens, including *T. gondii*, given overlapping vector ecologies and host exposures. However, none of the animals included in this study tested positive for these agents, which precludes further epidemiological interpretation. Although not the focus of this study, the exclusion of these pathogens represents a limitation, particularly in the context of regions where polyparasitism may be common. Future research incorporating broader diagnostic targets may help clarify the potential interactions between *T. gondii* and these additional vector-borne pathogens.

Hemotropic agents —mostly zoonotic agents, are transmitted primarily by arthropod vectors such as ticks, mosquitoes, and fleas, which are major contributors to VBDs relevant to both veterinary medicine and public health [69]. These vectors are particularly prevalent in tropical and subtropical regions, with Colombia ranking among the top 10 countries at risk in Latin America and the Caribbean [70]. In Colombia, ticks and fleas are the most frequently reported vectors in companion animals, [71,72] and are associated with the transmission of multiple hemotropic pathogens [73,74]. Our findings highlight the importance of monitoring these vectors and their associated pathogens, especially in regions with high vector abundance. Although few studies in Colombia have examined environmental and sociocultural transmission factors, risk variables such as outdoor access, untreated water, bushmeat consumption, and human–animal proximity remain relevant [75]. *T. gondii* transmission may occur horizontally (via the ingestion of oocysts or tissue cysts) [12,76], or vertically (via tachyzoites during gestation or lactation) [77]. While no specific data exist on *T. gondii* oocyst contamination in Colombian soils, the high seroprevalence in cats suggests a significant environmental presence, as infected felids shed millions of viable oocysts during a brief excretion period [16,75]. Outdoor defecation and predation behavior in cats perpetuate this cycle [78], and oocysts remain viable after passing through the digestive tract of dogs, with *T. gondii* DNA detected in canine feces [32]. These complex dynamics reinforce the importance of evaluating all potential transmission pathways when interpreting infection patterns in companion animals and assessing public health risks.

The coinfection of *T. gondii* with hemotropic pathogens in dogs and cats presents significant gaps in knowledge, particularly regarding awareness among pet owners and the veterinary community. This lack of information can hinder effective management and prevention strategies. Research indicates that many pet owners lack essential knowledge about zoonotic diseases, including *T. gondii*. In Bangladesh, for example, one study reported that only 21.25 % of pet owners had a good understanding of these diseases [79], whereas another reported that 77.8 % of cat owners had poor knowledge of toxoplasmosis and its transmission routes, potentially increasing the risk of coinfection with hemotropic pathogens [80].

In terms of pathogen detection, a study conducted in Saudi Arabia identified multiple vector-borne pathogens in dogs and cats, noting coinfections in dogs but not in cats. This discrepancy suggests a potential area for further investigation regarding *T. gondii* coinfection [68]. In addition, the pathogenicity of hemotropic *Mycoplasma* species varies, complicating the understanding of their interactions with *T. gondii* in affected animals [8].

One limitation of this study is the lack of species-level identification within the Rickettsiales group. While the qPCR assay employed was designed to broadly detect members of this order (e.g., *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp.), it did not allow for differentiation among genera or species. This constraint limits the interpretability of the data, particularly in terms of pathogen-specific clinical relevance and zoonotic potential. Future studies should consider incorporating species-

specific primers or follow-up sequencing approaches to improve taxonomic resolution and diagnostic precision, enabling a more targeted understanding of the role these pathogens play in coinfection dynamics and their implications for both veterinary and public health.

Importantly, the proportion of *T. gondii*-positive animals was not stratified by species (i.e., proportion among all tested dogs or cats), as this study was not designed to estimate species-specific prevalence. The sample was drawn from submissions to a diagnostic laboratory without proportional representation or randomization by species. As such, reporting within-species proportions might lead to misinterpretation as prevalence estimates. Instead, the focus was placed on exploring coinfection patterns and associations in a mixed population of companion animals, without inferring differences in infection frequency between species.

Additionally, while reporting confidence intervals (CIs) for all descriptive proportions could offer insights into estimate precision, we chose not to include them in the current version owing to the exploratory scope of the study and the complexity of the resulting tables. Given the extensive stratification by species, pathogen, infection status, and host variables, adding CIs would have considerably reduced table readability without providing significant additional value for the descriptive objectives of the study. These findings are not intended for population-level inference and should be interpreted accordingly.

Despite our findings, comprehensive studies exploring the interplay between *T. gondii* and hemotropic pathogens are still lacking, necessitating further research to address these knowledge gaps. Furthermore, diagnosing these infections remains challenging because of the nonspecific nature of clinical signs and hematological changes [81]. In this sense, effective prevention and control of these zoonotic parasites require a multidisciplinary "One Health" approach, given its multifaceted presentation.

5. Conclusion

These preliminary findings highlight potential patterns of coinfection, which require further validation through longitudinal or hypothesis-driven studies. This study demonstrated significant associations between *T. gondii* infection and coinfection with hemotropic agents in dogs and cats in Colombia, identifying individual and environmental predisposing factors such as outdoor access and breed. Coinfection with agents such as Rickettsiales, *Hepatozoon* spp., *Bartonella* spp., and *Babesia* spp. is associated with hematological alterations and complex immune responses that impact clinical outcomes. These findings indicate that vector exposure and environmental factors can play critical roles in the transmission of these pathogens, with important public health implications due to the zoonotic potential of these agents. In addition, this study highlights the need for further research to explore coinfection dynamics and pathogenic mechanisms, as well as educational interventions to raise awareness among pet owners and veterinary professionals. Given the often-nonspecific clinical signs, a multidisciplinary "One Health" approach is essential for the effective control of these zoonotic infections and the prevention of public health risks.

CRediT authorship contribution statement

N.M. Correa-Valencia: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **I.L. Jaramillo-Delgado:** Writing – review & editing, Validation, Supervision, Methodology. **L.M. Rendón-Ramos:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **C. Ríos-Úsuga:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used OpenAI ChatGPT (October 2023 version, https://chat.openai.com/) to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the content of the published article.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cimid.2025.102385.

Data availability

The test conditions of the molecular processes are part of the intellectual property of the company TestMol© S.A.S. However, if there is a need for additional data or details, such information can be obtained through a request process that maintains complete confidentiality. Other data will be made available on request.

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