Article
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DIAGNOSTIC METHODS USED TO DETECT *TOXOPLASMA GONDII* INFESTATION IN CATS - CASE REPORT

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Abstract

The results regarding the prevalence of toxoplasmosis in humans in the city of Iasi during one year, show a rate of 6,6% of cases detected with positive IgM, and 32.7% of cases detected with positive IgG, compared to the prevalence of toxoplasmosis in cats which shows a rate of 0.7% of positive cases detected with positive IgM; which denotes that toxoplasmosis is underdiagnosed in veterinary medicine. A very important role is played by the diagnostic method used. The article deals with a case study, a 1,8-year-old cat with cerebellar ataxia, dysmetria and hypermetria, with moderate opacification of the entire corneal surface, panuveitis, chorioretinitis and corneal edema. Following the paraclinical investigations, the diagnosis of toxoplasmosis was made, using the Welltest *Toxoplasma* IgG/IgM immunochromatographic test, confirming the acute phase of the disease with positive IgM and negative IgG. Using the molecular detection techniques through qRT PCR, the result was negative, emphasizing the fact that the protozoan *Toxoplasma gondii* uses the blood as a way of spreading in the body, the relatively short phase that can induce a negative result, despite the presence of severe symptoms. The conclusions emphasize the importance of using a correct diagnostic method, molecular techniques, despite their high sensitivity, are not always recommended. In toxoplasmosis, the recommended diagnostic method is the serological one to detect IgG/IgM antibodies.

Key words: *Toxoplasma gondii*, qRT PCR, IgG/IgM antibodies

Introduction. Toxoplasmosis is a very important zoonosis caused by the intracellular protozoan *Toxoplasma gondii*, having as its intermediate host almost all warm-blooded animals, including humans, in which transplacental transmission can be life-threatening to the fetus causing death or severe neurological damage, inflammation and retinochoroiditis (Molaei S. *et al*., 2022; Torrey, E.F.; Yolken, R.H. 2013). Immunocompromised patients are associated with severe central nervous system damage, lethal encephalitis and myocarditis (Galvan-Ramirez M., 2013).

The reproductive sexual cycle of *Toxoplasma gondii* occurs only in definitive hosts, represented by domestic and wild felids, as they may also be intermediate hosts. First, tachyzoites develop an active multiplication in tissues, associated with a rapid invasion that produces harmful effects. They have a tropism especially for the central nervous system and striated muscles, where they remain dormant as bradyzoites, leading to a long-term chronic infection until a definitive host ingests the tissue. After 16-21 days of infection, cats excrete oocysts in feces, contaminating soil and water (Calero-Bernal, R. & Gennari, S. 2019; Dubey, J. 2004; Dubey, J. & Jones, J. 2008; Weiss, L. M. & Dubey, J. P. 2009; Silva, J. C. R. *et al*., 2001; Dubey, J. *et al*., 2020).

Toxoplasma infection in cats is in most cases asymptomatic, complicating the diagnosis of the disease, increasing the risk of infection in humans and animals.

This parasitosis has repercussions on public health, producing a wide range of symptoms in humans, as well as repeated abortions, significant economic losses in animals (Hatam-Nahavandi K. *et al.*, 2021; Dubey J.P. *et al*., 2020). Clinical signs are not conclusive to distinguish toxoplasmosis from other infections (Molaei S. *et al.*, 2022). Serological methods are the most commonly used in the diagnosis of toxoplasmosis (Shieh M. *et al*., 2017; Dard C. *et al.*, 2016). New techniques with higher selectivity and accuracy are needed for direct determination of biomarkers for *Toxoplasma gondii*.

Toxoplasma gondii infection has an acute phase of manifestation, which in immunocompetent patients is often asymptomatic,

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and a chronic phase (Pittman K.J. *et al*., 2014). In recent years biosensors have been defined as ideal for the diagnosis of toxoplasmosis due to their sensitivity and selectivity compared to formal procedures (Anik Ü. *et al*., 2017).

In terms of the clinical picture, there is a variety of symptoms, correlating with the different categories of toxoplasmosis, including the acquired form in immunocompetent patients, during pregnancy and congenitally, a reactive form in immunocompromised patients, as well as ocular infections. But these symptoms are not specific and vary from asymptomatic forms in immunocompetent patients to ocular forms, congenital neurotoxoplasmosis being often fatal. Thus, the use of several different diagnostic methods is necessary for a diagnosis of certainty (Ybanez R.H.D. *et al*., 2020; Strharsky J. *et al*., 2009).

Diagnosis is crucial for surveillance, prevention and control of toxoplasmosis. Laboratory diagnostic approaches include immunological, molecular and immunohistochemical methods. Thus, diagnosis is divided into indirect methods by immunological tests and direct methods of parasite detection by microscopy and molecular methods (Müller de Barros R.A. *et al*., 2022). Thus, we have the Sabin-Feldman dye test (DT) enzyme-linked immunosorbent assay (ELISA), immunosorbent agglutination assay (ISAGA), indirect hemagglutination test (IHA), indirect fluorescence antibody test (IFAT), modified agglutination test (MAT), latex agglutination test (LAT) and Western blot (WB) (Liu *et al.*, 2015; Sun *et al.*, 2015; *Dard et al*., 2016).

In human medicine, the detection of specific anti-toxoplasma antibodies (IgM, IgE, IgG and IgG progress) in serum samples of affected patients is a priority (Montoya J.G. *et al.*, 2002; Rostami A. *et al*., 2018; Wassef R. *et al*., 2019). Direct molecular methods are commonly used for the diagnosis of toxoplasmosis, especially in immunocompromised patients with serum antibody deficiency or prenatal and congenital transmission. Thus, various molecular methods, including conventional PCR, RAPD-PCR, RT-PCR, high-resolution melting and microsatellite analysis, are used to improve diagnostic methods (Teixeira L.E. *et al.*, 2013; G. Saadatnia *et al*., 2012; K. Khanaliha *et al*., 2021; Witter R. *et al.*, 2020).

The sensitivity and specificity of PCR techniques depend on a number of factors such as the gradients of the amplification reaction, the primers used and the method of DNA extraction from biological samples such as whole, pleural and peritoneal blood (Montoya J.G. *et al.*, 2002; Molaei S. *et al*., 2022). Sensitivity and specificity between PCR techniques, were between 70-95% and 85-100%, respectively, in several studies performed on different samples (Khanaliha K. *et al*., 2021; Ferreira Ade M. *et al.*, 2004; Soltani Tehrani B. *et al*., 2020). In humans the PCR technique, allowed the detection of *Toxoplasma gondi* DNA in clinical samples such as amniotic fluid, aqueous humor, cerebrospinal fluid, bone marrow and blood (Edvinsson B. *et al*., 2008; Mattos C.C. *et al*., 2011; Camilo L.M. *et al*., 2017), the technique being recommended especially in AIDS patients, who have a poor immunological status.

MATERIAL AND METHOD

The study aimed to establish a diagnostic protocol for toxoplasmosis in veterinary medicine, given that this disease is under-diagnosed in animals as compared to the high number of positive cases in humans. Thus, we consulted the data on toxoplasmosis cases diagnosed in Iasi County in humans, in the Praxis laboratory, in order to demonstrate that the incidence of this disease in cats in Iasi County is not real and the need for caution in diagnosis is required.

Case description

A case was presented in the Faculty of Veterinary Medicine Iasi: cat, common breed, 1 year and 8 months with apathy, inappetence, cerebellar ataxia, dysmetria and hypermetria.

Ocular ultrasound showed a reduction in the size of the eyeball, of the anterior and posterior chamber; areas of increased hyperechogenicity in the uvea, and of the projection area of the optic nerve papilla with an increase in size of these structures. In both eyes there is thickening of the posterior structures with increased echogenicity (choroid and retina). The diagnosis of panuveitis, chorioretinitis and corneal oedema was established. Nervous signs and ocular damage led to the suspicion of toxoplasmosis. The acute stage fades in a few days to months, leading to the latent stage. Latent infection is normally asymptomatic; however, in immunocompromised patients (such as those infected with HIV or transplant recipients on immunosuppressive therapy), toxoplasmosis may develop. The most notable manifestation of toxoplasmosis in immunocompromised patients is toxoplasmic encephalitis, which can be fatal. If infection with *T. gondii* first occurs during pregnancy, the parasite can cross the placenta, possibly leading to hydrocephalus, intracranial calcification and chorioretinitis, with the possibility of miscarriage or intrauterine death. It has also been tested for FIV and FELV, with negative results.

The *Toxoplasma* IgG/IgM Antibody (TOXO Ab) rapid test, using the double layer sandwich lateral flow immunochromatographic method, was used for the diagnosis. The test aims at the qualitative detection of *Toxoplasma* IgG and IgM antibodies in animal blood samples, serum or plasma.

The rapid test for veterinary use - Well Test Toxoplasma gondii Ag- a rapid test using the double-layer, sandwich, lateral flow immunochromatographic method was also used for the qualitative detection of *Toxoplasma gondii* antigens in faecal, serum or plasma samples.

Microscopic examination of blood smears and diagnosis by RT-PCR were used as direct methods. Real-time PCR is the fastest and most reliable method to achieve accurate detection of *T. gondii.* The DNA extraction, was made using BioMagPure 12 Plus (Biosan, Latvia). Concisely, genomic DNA was extracted from 200 µl whole blood using Blood DNA Extraction Kit 200, according to the manufacturer's protocol.

For RT-PCR diagnosis, Nzytech's *Toxoplasma gondii* quantitative qPCR kit was used, which is a highly specific product designed for real-time PCR (qPCR) applications. The qPCR method serves as the gold standard in molecular diagnostics due to its exceptional accuracy, specificity and sensitivity. A *T. gondii* specific primer and probe mix is provided and can be detected through the FAM channel in a Real-time PCR experiment.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the T. gondii DNA. A fluorogenic probe, which consists of a DNA sequence labelled with a 5`-dye and a 3`-quencher, is included in the same reaction mixture to hybridize specifically in the DNA target region between the two primers. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The kit includes a positive control template that allows controlling the PCR set-up and is also useful for copy number determination. This can be used to generate a standard curve of T. gondii copy number / quantitation Cycle (Cq) value.

BioRad's CFX96 equipment was used, using the thermal cycling conditions recommended by the kit (*table 1*).

Tabel 1

RT-PCR program used

Cycles	Temperature	Time	Notes
1	95°C	2min	Polymerase activation
40	95°C	5 s	Denaturation
	60°C	30 s	Annealing/Extension

RESULTS AND DISCUSSIONS

Prevalence of toxoplasmosis in Iasi County

Diagnosis in the Praxis laboratory is performed on request, using immunochromatographic tests for qualitative determination of IgG and IgM antibodies. But the diagnosis of toxoplasmosis is much more complex and must combine both direct and indirect methods for a certainty result.

The results of epidemiological investigations carried out at the Praxis laboratory, Iasi County in the period July 2022-July 2023 showed 226 requests for anti-*Toxoplasma gondii* IgM antibody screening (ELISA), of which 208 in women and 18 in men, with 15 positive cases in women and no positive cases in men (*figure 1*).

Figure1 **Distribution of toxoplasmosis by sex in humans**

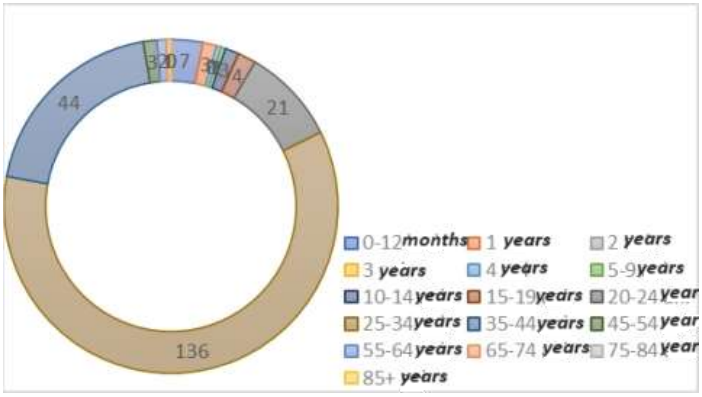

Figure 2 **Distribution of toxoplasmosis by age groups for the presence of IgM**

Out of a total of 226 tests performed for women and men, 60.17% (136) were performed in the age category 25-34 years, 19.46% (44) were performed in the age category 35-44 years, 9.29% (21) of the tests were performed in the age category 20-24 years, 3.09% (7) of the tests were performed in the age category 0-12 months, and the rest of the tests were performed in the other age categories (*figure 2*). The high proportion of testing in the 25-34 age group is strictly related to the number of pregnant women in this age group.

Out of a total of 220 positive tests, 134 (60.90%) were performed in the age category 25-34 years, 37 (16.81%) were performed in the age category 35-44 years, 20 (9.09%) in the age category 20-24 years, and the remaining 29 (13.18%) in other age categories (*figure 3*). The presence of the majority of IgG-positive cases in the age category 25-34 years is also directly proportional to the high number of cases tested in this category, closely related to the testing during pregnancy.

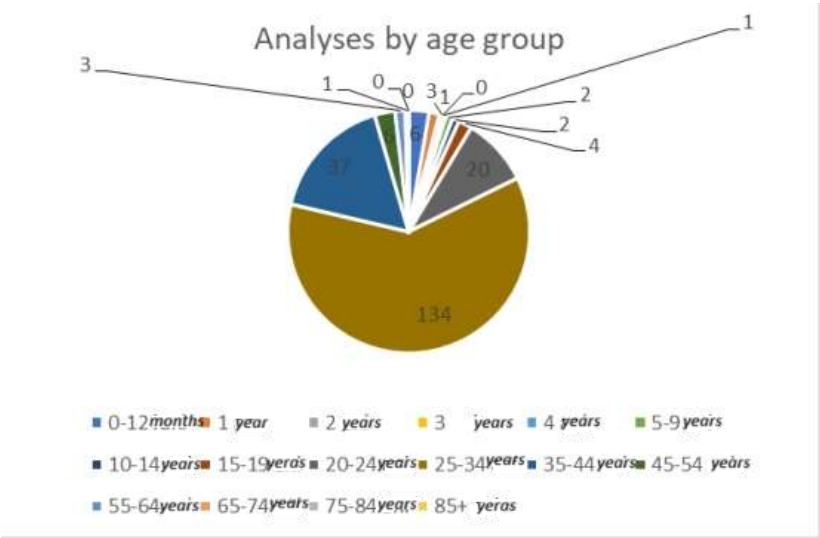

Figure 3 **Distribution of toxoplasmosis by age groups for the presence of IgG**

Studies show that IgM antibodies peak at 2 months post-infection, making the sensitivity and specificity of serological tests dependent on the timing of post-infection testing.

The case presented at the Faculty of Veterinary Medicine underlines the need to combine diagnostic methods in toxoplasmosis.

The *Toxoplasma* IgG/IgM Antibody (TOXO Ab) rapid test was weakly positive for IgM and negative for IgG. The WELLTEST *Toxoplasma gondii* Antigen test was negative (*figure 4*).

Cytological examination - Smear exam (MGG/DQ stain):

A. Schizocytes, erythrocyte agglutination, anisocytosis (reduced degree);
B. Nf reactive, Nf total 88.6% (35.90x 10^3/µl), Nf young (1-2 lobes) 62.4%, Nf (3 lobes) 29.4%, Nf (4-5 lobes) 8.2, %, Eo 0% (x10^3/µl), Mo 4.1% (1.66x 10^3/µl), Lf 7.3% (2.96x10^3/µl).
C. Giant platelets.

Cytological and haematological diagnosis (reference values The Merck Veterinary Manual,

ed 8): anaemia, leukocytosis, neutrophils, eosinopenia, lymphopenia.

Figure 4 **Results of the WELLTEST *Toxoplasma gondii* Antigen *and Toxoplasma* IgG/IgM Antibody (TOXO Ab) rapid test respectively**

Diagnosis by RT-PCR was negative, which underlines the importance for diagnosis of using an appropriate biological sample and of collecting it at the right time in the course of the disease. Although RT-PCR is considered the gold standard in diagnosis, in toxoplasmosis it is only useful in the dissemination phase in the body, when blood is used as a biological sample.

The detection of oocysts in the faeces is not a reliable method of diagnosis because they look similar to those of some other parasites. Additionally, cats can also shed oocysts for only a short period of time and often are not shedding oocysts when they are showing signs of disease. Clinically manifested toxoplasmosis occurs during dissemination and intracellular replication of tachyzoites. It usually occurs as a reactivation of a latent infection, and more rarely after a newly acquired infection. If a carrier cat is immunosuppressed, bradyzoites in tissue cysts rapidly replicate and disseminate again as tachyzoites.

CONCLUSIONS

Following a 2020 study by Mahbobeh Montazeri et al. on the basis of official reports it is estimated that more than one billion people would be infected with *T. gondii*, mainly through consumption of food: water, vegetables and fruit contaminated with sporulated oocysts shed by cats and through consumption of raw or undercooked contaminated meat. CDC (Center for Disease Control and Prevention) reports toxoplasmosis as the second most common cause of death due to food-borne diseases - approximately 327 deaths and the fourth leading cause of hospitalizations attributed to food-borne diseases (approximately 4428 hospitalizations) in the US in the mid to late 2000s.

Regarding the definitive host, a global survey conducted between 1967 and 2017 estimated the seroprevalence of *T. gondii* at 35% in domestic cats and 59% in feral cats. It is most widespread in Australia and Africa, where the seroprevalence of *Toxoplasma gondii* in domestic cats reaches 52% and 51% respectively. Asia ranks last with a seropositivity of 27% in domestic cats. As for *Toxoplasma gondii* seroprevalence values in feral cats, it is estimated at 74% in Africa, 67% in Asia, 67% in Europe and 66% in South America.

The article points out that this disease is under-diagnosed in both human and veterinary medicine. Although the number of cases is much higher in human medicine, it is found that the requests are correlated with the period of pregnancy, when this toxoplasmosis screening test is mandatory, and is not suspected or monitored in the human population.

Also, toxoplasmosis in cats is often asymptomatic, with few requests for diagnosis, except in cases of severe symptoms.

The importance of using the correct diagnostic method in toxoplasmosis according to the stage of the disease has been highlighted in this case study. Thus, molecular methods using blood as a biological sample are not recommended, as they may be false negatives.

The protozoan is rarely found in blood, and occasionally in CSF, fine needle aspirates of organs (e.g., lymph nodes) and transtracheal or bronchoalveolar lavages and are common in peritoneal fluids of animals developing ascites, but collection of these biological samples is invasive and poses risks to the patient. Detection of tachyzoites confirms the diagnosis.

We conclude a need for the use of combined methods in the diagnosis of toxoplasmosis, recommending the use of serological methods, but still considering the development of IgM antibodies, differently depending on the immune status of the host, considering that it is necessary to use both direct and indirect methods.

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