Revista Brasileira de Parasitologia Veterinária
Brazilian Journal of Veterinary Parasitology
ISSN 1984-2961 (Electronic)
www.cbpv.org.br/rbpv

Original Article

Evaluation of quantitative polymerase chain reaction for the detection of *Toxoplasma gondii* oocysts shed by cats

Avaliação da reação em cadeia da polimerase quantitativa para a detecção de oocistos de *Toxoplasma gondii* eliminados por gatos

Ana Carolina Miura[1]; Luiz Daniel de Barros[1]*; Ana Flávia Minutti[1]; Thais Agostinho Martins[1]; João Pedro Sasse[1]; Beatriz de Souza Lima Nino[1]; João Luis Garcia[1]

[1]Departamento de Medicina Veterinária Preventiva, Laboratório de Protozoologia Animal, Universidade Estadual de Londrina – UEL, Londrina, PR, Brasil

How to cite: Miura AC, Barros LD, Minutti AF, Martins TA, Sasse JP, Nino BSL, et al. Evaluation of quantitative polymerase chain reaction for the detection of *Toxoplasma gondii* oocysts shed by cats. *Braz J Parasitol Vet* 2021; 30(4): e016621. https://doi.org/10.1590/S1984-29612021091

Abstract

Felines are definitive hosts of *Toxoplasma gondii* and can shed oocysts in their feces, contaminating the environment. Sporulated oocysts are highly resistant to the environment and have higher infectivity, which are attributed to many toxoplasmosis outbreaks. The aim of the present study was to evaluate a quantitative polymerase chain reaction (qPCR) technique for the detection of *T. gondii* oocysts shed by cats. Twelve cats from a previous vaccine experiment were challenged orally with 600 cysts of the TgDoveBr8 strain on day 72. Fecal samples were collected daily using the centrifugal flotation technique, with microscopic examination (Sheather technique) and qPCR for 20 days after the challenge. Cats from all groups shed oocysts in their feces. Five negative cats in the Sheather were positive according to qPCR on the 3rd day post-inoculation (dpi). Oocysts were detected on the 4th dpi using the Sheather; however, there was no statistical difference between the two methods (p=0.1116). In addition, there was no statistically significant difference in oocyst shedding between the groups according to the Sheather technique (p=0.6534) and qPCR (p=0.9670). In conclusion, these results demonstrate that qPCR can be used as an alternative to the Sheather to detect and quantify *T. gondii* oocysts.

Keywords: Toxoplasmosis, real-time PCR, Sheater, cats.

Resumo

Felinos são hospedeiros definitivos do *Toxoplasma gondii* e podem eliminar oocistos nas fezes, contaminando o meio ambiente. Oocistos esporulados são altamente resistentes ao meio ambiente com elevada infectividade, sendo atribuído a muitos surtos de toxoplasmose. O objetivo do estudo foi avaliar a reação em cadeia da polimerase quantitativa (qPCR) para a detecção de oocistos de *T. gondii* eliminados por gatos. Doze gatos de um experimento prévio de vacina foram desafiados por via oral com 600 cistos da cepa TgDoveBr8 no dia 72. Amostras fecais foram coletadas diariamente pela técnica de centrifugo-flutuação seguida de exame microscópico (técnica de Sheather) e qPCR por 20 dias após desafio. Gatos de todos os grupos eliminam oocistos nas fezes. Cinco gatos negativos na técnica Sheather foram positivos de acordo com a qPCR no 3º dia pós-inoculação (dpi). Oocistos foram detectados no 4º dpi no Sheather; entretanto, não houve diferença estatística entre os dois métodos (p=0,1116). Não houve diferença estatisticamente significativa na eliminação de oocistos entre os grupos de acordo com a técnica de Sheather (p = 0,6534) e qPCR (p = 0,9670). Em conclusão, esses resultados demonstram que qPCR pode ser usada como uma alternativa ao Sheather para detectar e quantificar oocistos de *T. gondii*.

Palavras-chave: Toxoplasmose, PCR em tempo real, Sheather, gatos.

Received September 23, 2021. Accepted November 10, 2021.
*Corresponding author: Luiz Daniel de Barros. E-mail: daniel_vetuel@hotmail.com

Introduction

Felines, including domestic cats, are definitive hosts of *Toxoplasma gondii* and can shed oocysts in their feces, thus contaminating the environment (Frenkel et al., 1970). A cat can shed millions of oocysts by ingesting a single bradyzoite, which, in ideal environmental conditions, become infectious within one to five days during the sporulation process, although this event is not synchronized, as some oocysts can sporulate early while others can take more time (Dubey, 2001). Sporulated oocysts are more resistant than those that are not, and have a double wall, making them as strong as ordinary plastics, resistant to low and high temperatures, or treatments, such as chlorination, ozonation, ultraviolet radiation, and freezing, and to disinfectants (Jones & Dubey, 2010).

Studies investigating *T. gondii* oocysts have demonstrated their viability in soil for up to 18 months (Frenkel et al., 1975), under refrigeration at 4ºC for up to 4.5 years, in seawater for 2 years, and frozen at -10ºC for 106 days (Dubey, 1998; Lindsay & Dubey, 2009). Worms, flies, and cockroaches can also spread oocysts in the environment, directly contaminating food (Chinchilla et al., 1994). In addition, oocysts possess a high infective capacity for intermediate hosts, in which a single oocyst can cause infection in pigs (Dubey et al., 1996).

The epidemiology of human toxoplasmosis outbreaks has changed over the past few decades and, currently, oocyst ingestion is the primary source of infection in outbreaks of human toxoplasmosis (Pinto-Ferreira et al., 2019). Livestock animals may also become infected by oocysts, and many studies have reported the presence of cats as a risk factor for *T. gondii* infection; domesticated animals are more exposed to the parasite than farms without cats (García-Bocanegra et al., 2010; Vieira et al., 2018).

Centrifugal flotation followed by optical microscope examination (Sheather technique) is the most common method used to detect *T. gondii* oocysts in feline feces. However, limiting factors, such as similar morphology to other coccidian oocysts, lead to false-positive results, and samples with few oocysts can lead to false-negative results because the detection threshold is between 250 and 1000 oocysts/g of feces (Jones & Dubey, 2010). A modified Kato–Katz technique with Kinyoum's staining demonstrated sensitivity for a more extended period than centrifugal flotation. It can generate a permanent record of oocysts (stained slides); however, it is a semi-quantitative technique, much like centrifugal-flotation (Meireles et al., 2008). The mouse bioassay is the only technique capable of detecting viable and infectious oocysts; however, it is expensive and requires resources, time, and infrastructure (Salant et al., 2007).

Molecular approaches that detect DNA are highly specific and widely used in scientific research (Dabritz et al., 2007). An experimental study reported a detection sensitivity of 1–2 oocysts in 200 µL of feces by amplifying a repetitive 529 base pair (bp) DNA fragment (Salant et al., 2007). However, detection in fecal samples from naturally infected animals and environmental samples is not very sensitive due to the difficulty of breaking the oocyst wall and the presence of polymerase chain reaction (PCR) inhibitors (Dabritz et al., 2007).

Most cats have free access to the street, even those that are domiciled, and the number of wandering animals is high, while many others are abandoned. These factors perpetuate the *T. gondii* life cycle because these animals do not have a proper place to defecate and bury their feces in the environment. The seroprevalence of *T. gondii* is higher in older animals (Schares et al., 2008); however, this is not a protective factor because a previous study demonstrated that seropositive adult cats can re-shed oocysts in large numbers, especially when re-infected with a different strain, which may be relevant in sites with high clonal diversity and genetic recombination (Zulpo et al., 2018). Although oocysts shed by cats are epidemiologically important in the spread of *T. gondii*, few studies have investigated methods to control oocyst shedding. Moreover, high-sensitivity and high-specificity techniques are required to detect and quantify oocysts shed by cats to estimate environmental contamination. Therefore, the aim of this study was to evaluate a molecular approach for the detection of *T. gondii* oocysts shed by cats.

Material and Methods

Animals

Twelve short-haired domestic cats (*Felis catus*; 8 males, 4 females; 6 to 9 months of age) from a previous vaccine study (unpublished data) were used in the experiment. The animals were examined, vaccinated against feline viral diseases (rhinotracheitis, calici, and panleukopenia viruses; Feligen, Virbac, Carros, France), and dewormed (Vetmax plus, Vetnil, Louveira, Brazil). None of the cats included in the study exhibited detectable anti-*T. gondii* antibodies according to indirect fluorescent antibody test or *T. gondii* oocysts shedding before the study.

The cats were randomly allocated to individual cages and fed a commercial dry food diet with water *ad libitum* access to water. Monitoring was performed for two months, which consisted of daily physical examination, cleaning, feeding, and welfare checks.

All procedures involving the animals were performed according to current Brazilian regulations and approved by the Ethics Committee on Animal Use at the State University of Londrina, Paraná, Brazil (No. 102/12).

Challenge

The cats were divided into three groups, each comprising four animals: G1 (25 µg of rROP2 plus 25 µg of rHSP70 and 20 µg of Quil-A); G2 (25 µg of *Escherichia coli* and 20 µg of Quil-A); and G3 (control, only isotonic sodium chloride solution). All cats were challenged on day 72 with 600 cysts of the TgDoveBr8 strain (ToxoDB#1), isolated from a dove (Barros et al., 2014). Inoculation was performed via stomach tube, after which the cats were injected with 5 mL of isotonic sodium chloride solution to avoid residuals. The animals were anesthetized with tiletamine plus zolazepam (3.15 mg/kg [intramuscular], Zoletil, Virbac) to perform the challenge.

Fecal examination

Fecal samples from each cat were examined daily for 20 days using the centrifugal flotation technique to detect *T. gondii* oocysts, as previously described (Sheather, 1923). Each day, the total volume of feces from each cat was pooled, homogenized, and weighed. Two grams of this homogenate was mixed with 10 mL of sucrose solution (density 1.18 g/cm^3), filtered using gauze, and centrifuged (1,200 × g for 10 min). One drop of the solution was removed from the meniscus and examined under a microscope. Then, the supernatant was collected, mixed with 40 mL of water in a 50 mL tube, and centrifuged (1,200 × *g* for 10 min). The supernatant was discarded, the pellet was resuspended in water (1 mL). This yield solution was used for oocyst quantification and stored in labeled microtubes at -20ºC until molecular analysis. Oocyst quantification was performed using a four WBC chambers (i.e., Neubauer) hemocytometer.

DNA extraction

For DNA extraction, 100 µL of the yield solution from centrifugal-flotation in the sucrose solution was homogenized by vortexing with 900 µL TE buffer (100 mM Tris HCl; 10 mM EDTA, pH 8.0) followed by centrifugation at 10,000 × *g* for 5 min at room temperature. The supernatant was discarded, and the pellet was resuspended by vigorous vortexing in 300 mL lysis buffer (T1 buffer, Macherey-Nagel GmbH, Duren, Germany). Five freeze-thaw cycles (-80ºC for 10 min and 65ºC for 5 min) were performed, and lysis was achieved using overnight incubation with proteinase K (2 mg/mL) at 65ºC.

Total DNA was extracted by adding ultrapure buffered saturated phenol (v/v), homogenized, and centrifuged (10,000 × *g*) for 5 min at room temperature. The supernatant was transferred to a new clean tube with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The samples were mixed and centrifuged again, as described above. The translucent aqueous phase (250 µL) was transferred to a clean tube, and DNA was precipitated using cold 100% ethanol (3:1) and ammonium acetate (10 M). The mixture was homogenized and stored at -20°C for 1 h. The sample was then centrifuged (10,000 × *g*) for 15 min at 4°C, the supernatant was discarded, and DNA purification was performed by washing with 1 mL of 70% ethanol and centrifuging for 15 min at 10,000 × *g*. After drying the pellet at 37ºC, DNA was eluted using ultrapure water (25 µL) and stored at -20ºC until PCR.

Quantitative PCR (qPCR)

The qPCR reaction included 5 µL of SYBR Select Master Mix (Thermo-Fisher Scientific, Waltham, MA, USA), 0.2 µL (10 µM) each of forward and reverse primer, 0.4 µL of bovine serum albumin (BSA), 3.2 µL ultrapure water, and 1 µL of DNA template, resulting in a total volume of 10 µL. BSA (10 µg/µL) was added to the reaction mix to mitigate the effects of PCR inhibitors without affecting the PCR reaction. The forward (Tox-9) and reverse (Tox-11) primer set targeted a repetitive 529 bp DNA fragment, as described previously (Reischl et al., 2003).

Cycling conditions (ABI 7500 StepOnePlus™ Real-Time PCR system, Thermo-Fisher Scientific) were as follows: 95°C for 10 min; 40 cycles at 94°C for 15 ; 59°C for 30 s; and 72°C for 30 s. Fluorescence was measured at the end of each cycle. An additional step, 95°C for 15 s and 60°C to 95°C for 1 min (0.3°C/s), was added to obtain the melting

curve. Data were analyzed using StepOne™ version 2.2.2 (Thermo-Fisher Scientific). Each sample was tested in duplicate, and each qPCR plate contained a negative control.

The standard curve was constructed using genomic DNA isolated from 1.4×10^6 *T. gondii* oocysts (five points at 1:10 dilution) and included on each plate. The slope of the standard curve was used to calculate amplification efficiencies using the formula E=10(−1/s) −1, in which E represents the amplification efficiency and s the slope.

Statistical analysis

The Kruskal–Wallis test was used to compare oocyst shedding among the groups, while the *t*-test was used to evaluate differences between the techniques (i.e., Sheather versus qPCR). All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Inc, San Diego, CA, USA); differences with $p<0.05$ were considered to be statistically significant.

Results

All the cats shed *T. gondii* oocysts in feces; however, none of the animals exhibited showed significant clinical signs. Post-amplification melting curve analysis revealed one peak (Figure 1). According to qPCR, it was possible to quantify oocysts in all samples analyzed based on a standard curve (Figure 2). Five animals that were negative

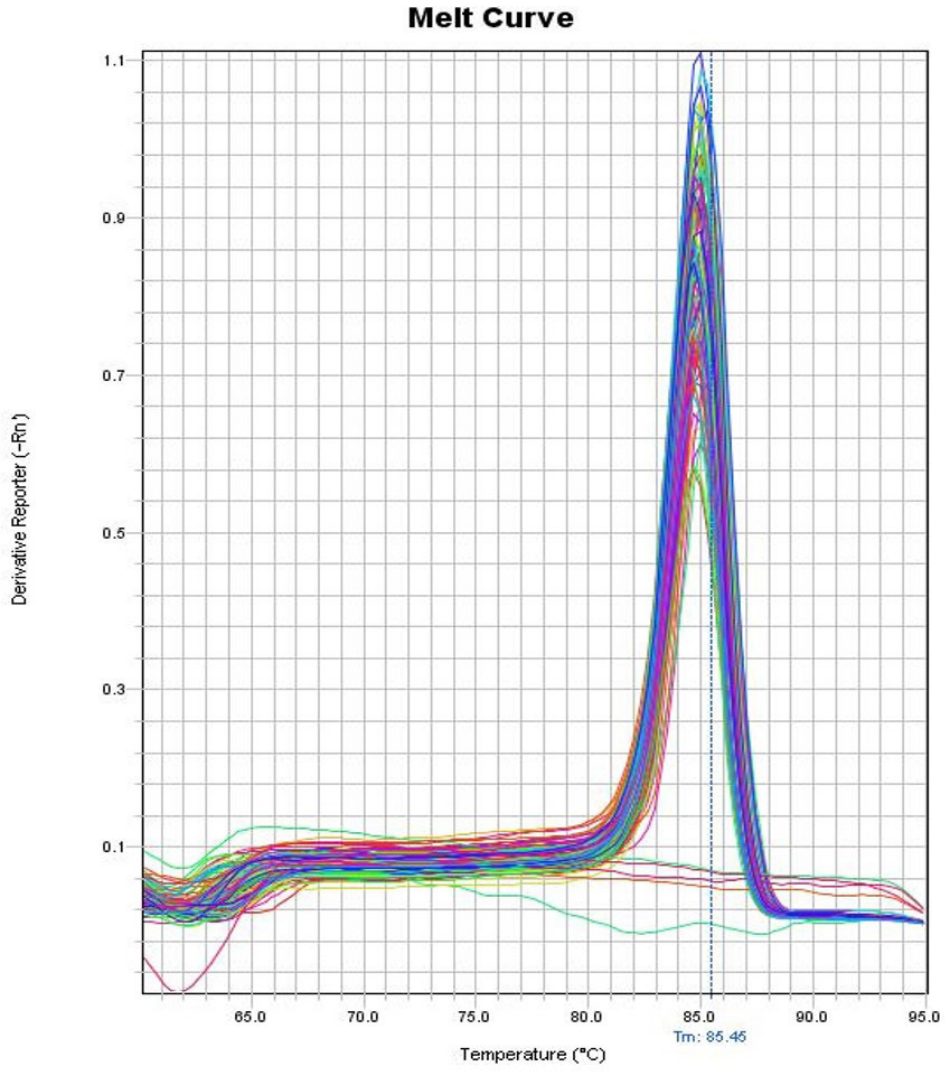

Figure 1. Post-amplification melting curve using SYBR Green dye (Thermo-Fisher Scientific, Waltham, MA, USA) *Toxoplasma gondii*-positive samples yielded one peak.

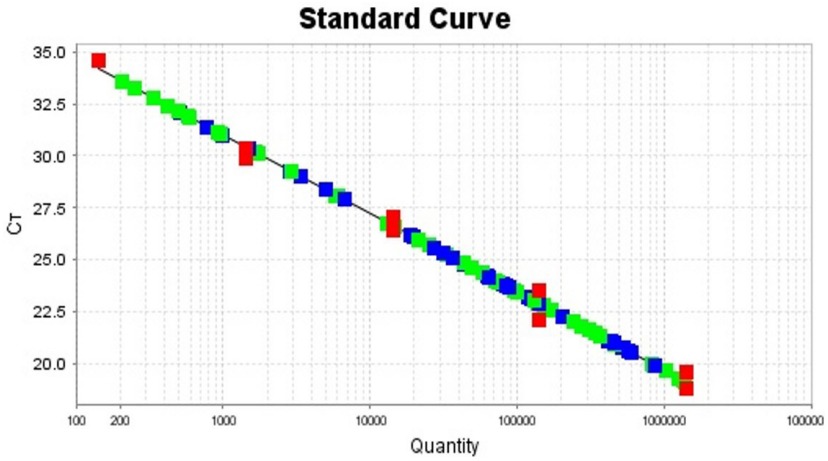

Figure 2. Standard curve for a serial 10-fold dilutions of *Toxoplasma gondii* oocysts from cat feces. Slope, -3.772; Y-intercept, -42.3089; correlation (i.e., r^2), 0.991.

according to the centrifugal flotation technique on the 3rd dpi were positive according to qPCR, with 328, 450, 470, 509, and 1828 oocysts shed per gram of feces (OOPG). Using the Sheather technique, oocyst detection and quantification were only possible on the 4th dpi. Although it was possible to detect and quantify oocysts using qPCR earlier than the Sheather technique, there was no statistical difference between the two techniques (p=0.1116).

Oocyst shedding per group according to each of the techniques is reported in Figure 3. G2 animals shed fewer OOPG compared to the other groups. The average number of OOPG according to the Sheather technique was

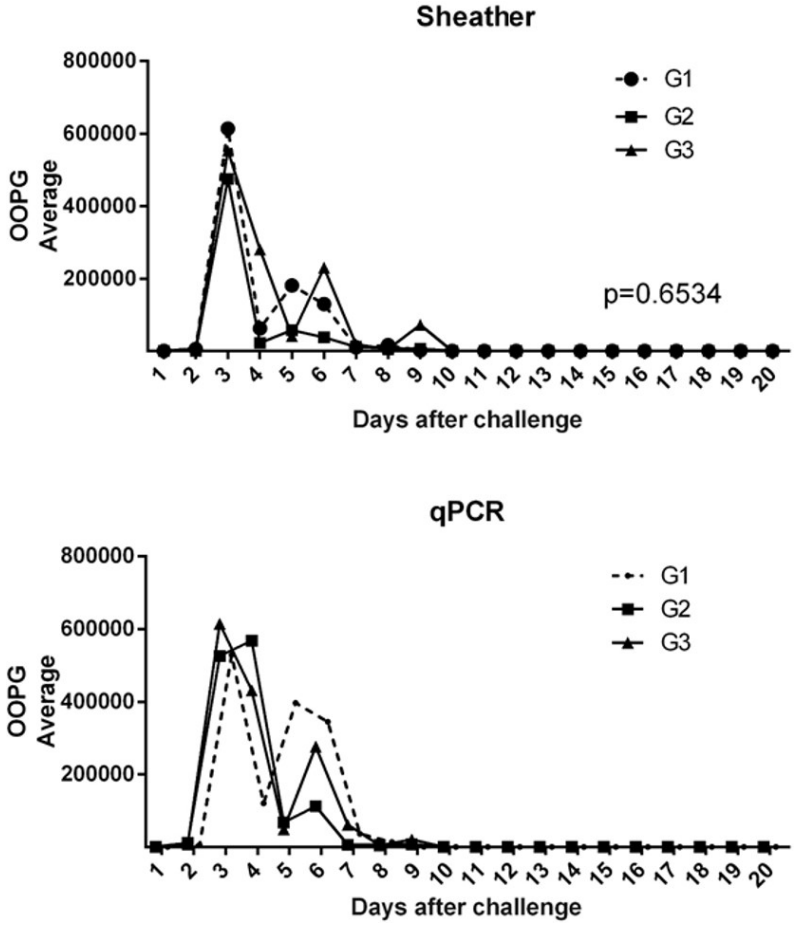

Figure 3. Evaluation of quantitative polymerase chain reaction and the Sheather technique for detection of *Toxoplasma gondii* oocysts shed by cats immunized with rROP2 and HSP70 (G1), adjuvant (G2), and control (G3).

90,888 and 144,976 for qPCR. Cats in G1 shed an average OOPG of 114,120 and 160,760 according to the Sheather technique and qPCR, respectively. Cats in G3 had an average OOPG of 134,263 according to the Sheather technique and 162,907 according to qPCR. However, there was no statistically significant difference between the groups according to Sheather technique and qPCR results (p=0.6534 and p=0.9670, respectively).

Discussion

We detected *T. gondii* DNA detection using the centrifugal flotation technique, while no DNA was detected using total feces. This may be due to fewer impurities and a higher concentration of oocysts, thus facilitating DNA extraction and mitigating the negative impact of PCR inhibitors usually found in total feces. The oocyst wall consists of a proteinaceous structure with a lipid coating, providing high environmental resistance, and the freeze-thaw cycling has been reported as a necessary step to yield a greater DNA concentration (Cornelissen et al., 2014; Freppel et al., 2018).

In this study, cats that were positive according to qPCR were negative according to the centrifugal flotation technique. This technique is widely used and considered to be inexpensive because specialized equipment is not required. However, it requires training and experience for oocyst identification and quantification, and has a high detection threshold. We found that samples that were negative according to the Sheather technique yielded 1828 OOPG according to qPCR, indicating that Sheather detection threshold is likely ≥ 1828 OOPG; however, previous studies have indicated that coproparasitological examination requires ≥ 1000 oocysts to be positive (Jones & Dubey, 2010). Moreover, it requires training and experience for oocyst identification and quantification (Lalonde & Gajadhar, 2011). Furthermore, we observed that centrifugal flotation is necessary for qPCR detection, corroborating a previous study that demonstrated the utility of flotation with saturated $NaNO_3$ before microscopic and molecular detection of *T. gondii* oocysts (Sroka et al., 2018).

The qPCR was able to detect oocysts in the feces earlier than coproparasitological methods. This molecular technique has advantages over conventional PCR because it yields better sensitivity and specificity, and primarily quantifies parasitic DNA (Hunt, 2011). However, caution should be exercised because this technique may detect *T. gondii* DNA from infected prey instead of oocysts (Poulle et al., 2016). Cornelissen et al. (2014) also suggested that qPCR can detect DNA fragments in feces without oocysts and should be carefully analyzed, especially in studies evaluating vaccine efficacy and varying challenge doses.

According to both techniques used in the present study, oocyst shedding was detected on the 3rd dpi to the 11th dpi. Previous studies have also reported oocyst shedding at a similar interval (Zulpo et al., 2012, 2017; Cornelissen et al., 2014); however, characteristics related to the cats' immunity, genotype, and infectious dosage can influence this interval (Garcia et al., 2007; Zulpo et al., 2018).

Cats in G3 had an OOPG average higher than those in the other groups; however, the difference was not statistically significant. In contrast, cats in G2 had the lower OOPG average, according to both techniques. These differences were not statistically significant and are merely casual. A previous study also demonstrated the immunomodulatory effect of Quil-A, indicating that it is a useful adjuvant for vaccines (Zulpo et al., 2012).

Oocysts quantification is commonly performed in experimental studies; however, few investigations have quantified the shedding of oocysts in the feces of naturally infected cats (Dubey et al., 2020). In addition, the used molecular methods, such as PCR-RFLP, would contribute to the similar parasites in felines stools (Da Silva et al., 2009). Thus, molecular and coproparasitological techniques, such as qPCR and the Sheather technique, can be used concomitantly to estimate potential environmental contamination by *T. gondii* oocysts in feline stool samples.

Conclusions

qPCR method was comparable to centrifugal flotation for detection of oocysts in experimentally infected cats; thus, it may be used as an alternative tool for the detection and quantification of *T. gondii* oocysts in stool samples from infected cats.

References

Barros LD, Taroda A, Zulpo DL, Cunha IA, Sammi AS, Cardim ST, et al. Genetic characterization of *Toxoplasma gondii* isolates from eared doves (*Zenaida auriculata*) in Brazil. *Rev Bras Parasitol Vet* 2014; 23(4): 443-448. http://dx.doi.org/10.1590/s1984-29612014073. PMid:25517521.

Chinchilla M, Guerrero OM, Castro A, Sabah J. Cockroaches as transport hosts of the protozoan *Toxoplasma gondii. Rev Biol Trop* 1994; 42(1-2): 329-331. PMid:7480943.

Cornelissen JBWJ, van der Giessen JWB, Takumi K, Teunis PFM, Wisselink HJ. An experimental *Toxoplasma gondii* dose response challenge model to study therapeutic or vaccine efficacy in cats. *PLoS One* 2014; 9(9): e104740. http://dx.doi.org/10.1371/journal.pone.0104740. PMid:25184619.

da Silva RC, Su C, Langoni H. First identification of *Sarcocystis tenella* (Railliet, 1886) Moulé, 1886 (Protozoa: Apicomplexa) by PCR in naturally infected sheep from Brazil. *Vet Parasitol* 2009; 165(3-4): 332-336. http://dx.doi.org/10.1016/j.vetpar.2009.07.016. PMid:19647370.

Dabritz HA, Miller MA, Atwill ER, Gardner IA, Leutenegger CM, Melli AC, et al. Detection of *Toxoplasma gondii*-like oocysts in cat feces and estimates of the environmental oocyst burden. *J Am Vet Med Assoc* 2007; 231(11): 1676-1684. http://dx.doi.org/10.2460/javma.231.11.1676. PMid:18052801.

Dubey JP, Cerqueira-Cézar CK, Murata FHA, Kwok OCH, Hill D, Yang YR, et al. All about *Toxoplasma gondii* infections in pigs: 2009-2020. *Vet Parasitol* 2020; 288: 109185. http://dx.doi.org/10.1016/j.vetpar.2020.109185. PMid:33271424.

Dubey JP, Lunney JK, Shen SK, Kwok OCH, Ashford DA, Thulliez P. Infectivity of low numbers of *Toxoplasma gondii* oocysts to pigs. *J Parasitol* 1996; 82(3): 438-443. http://dx.doi.org/10.2307/3284082. PMid:8636849.

Dubey JP. Oocyst shedding by cats fed isolated bradyzoites and comparison of infectivity of bradyzoites of the VEG strain *Toxoplasma gondii* to cats and mice. *J Parasitol* 2001; 87(1): 215-219. http://dx.doi.org/10.1645/0022-3395(2001)087[0215:OSBCFI]2.0.CO;2. PMid:11227895.

Dubey JP. *Toxoplasma gondii* oocyst survival under defined temperatures. *J Parasitol* 1998; 84(4): 862-865. http://dx.doi.org/10.2307/3284606. PMid:9714227.

Frenkel JK, Dubey JP, Miller NL. *Toxoplasma gondii* in cats: fecal stages identified as Coccidian Oocysts. *Science* 1970; 167(3919): 893. http://dx.doi.org/10.1126/science.168.3929.353.b. PMid:4903651.

Frenkel JK, Ruiz A, Chinchilla M. Soil survival of *Toxoplasma* oocysts in Kansas and Costa Rica. *Am J Trop Med Hyg* 1975; 24(3): 439-443. http://dx.doi.org/10.4269/ajtmh.1975.24.439. PMid:1098494.

Freppel W, Ferguson DJP, Shapiro K, Dubey JP, Puech P-H, Dumètre A. Structure, composition, and roles of the *Toxoplasma gondii* oocyst and sporocyst walls. *Cell Surf* 2018; 5: 100016. http://dx.doi.org/10.1016/j.tcsw.2018.100016. PMid:32743133.

Garcia JL, Navarro IT, Biazzono L, Freire RL, Guimarães JS Jr, Cryssafidis AL, et al. Protective activity against oocyst shedding in cats vaccinated with crude rhoptry proteins of the *Toxoplasma gondii* by the intranasal route. *Vet Parasitol* 2007; 145(3-4): 197-206. http://dx.doi.org/10.1016/j.vetpar.2007.01.007. PMid:17296268.

García-Bocanegra I, Simon-Grifé M, Dubey JP, Casal J, Martín GE, Cabezón O, et al. Seroprevalence and risk factors associated with *Toxoplasma gondii* in domestic pigs from Spain. *Parasitol Int* 2010; 59(3): 421-426. http://dx.doi.org/10.1016/j.parint.2010.06.001. PMid:20601110.

Hunt PW. Molecular diagnosis of infections and resistance in veterinary and human parasites. *Vet Parasitol* 2011; 180(1–2): 12-46. http://dx.doi.org/10.1016/j.vetpar.2011.05.027. PMid:21700392.

Jones JL, Dubey JP. Waterborne toxoplasmosis – recent developments. *Exp Parasitol* 2010; 124(1): 10-25. http://dx.doi.org/10.1016/j.exppara.2009.03.013. PMid:19324041.

Lalonde LF, Gajadhar AA. Detection and differentiation of coccidian oocysts by real-time PCR and melting curve analysis. *J Parasitol* 2011; 97(4): 725-730. http://dx.doi.org/10.1645/GE-2706.1. PMid:21506835.

Lindsay DS, Dubey JP. Long-Term Survival of *Toxoplasma gondii* Sporulated Oocysts in Seawater. *J Parasitol* 2009; 95(4): 1019-1020. http://dx.doi.org/10.1645/GE-1919.1. PMid:20050010.

Meireles LR, Tsutsui VS, Carmo CV, Galisteo AJ Jr, Hiramoto RM, Terentowicz HC, et al. Quantitative *Toxoplasma gondii* oocyst detection by a modified Kato Katz test using Kinyoun staining (KKK) in ME49 strain experimentally infected cats. *Rev Inst Med Trop São Paulo* 2008; 50(3): 187-190. http://dx.doi.org/10.1590/S0036-46652008000300010. PMid:18604418.

Pinto-Ferreira F, Caldart ET, Pasquali AKS, Mitsuka-Breganó R, Freire RL, Navarro IT. Patterns of transmission and sources of infection in outbreaks of human Toxoplasmosis. *Emerg Infect Dis* 2019; 25(12): 2177-2182. http://dx.doi.org/10.3201/eid2512.181565. PMid:31742524.

Poulle M-L, Forin-Wiart M-A, Josse-Dupuis É, Villena I, Aubert D. Detection of *Toxoplasma gondii* DNA by qPCR in the feces of a cat that recently ingested infected prey does not necessarily imply oocyst shedding. *Parasite* 2016; 23: 29. http://dx.doi.org/10.1051/parasite/2016029. PMid:27449050.

Reischl U, Bretagne S, Krüger D, Ernault P, Costa J-M. Comparison of two DNA targets for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. *BMC Infect Dis* 2003; 3(1): 7. http://dx.doi.org/10.1186/1471-2334-3-7. PMid:12729464.

Salant H, Markovics A, Spira DT, Hamburger J. The development of a molecular approach for coprodiagnosis of *Toxoplasma gondii. Vet Parasitol* 2007; 146(3-4): 214-220. http://dx.doi.org/10.1016/j.vetpar.2007.02.022. PMid:17395378.

Schares G, Vrhovec MG, Pantchev N, Herrmann DC, Conraths FJ. Occurrence of *Toxoplasma gondii* and *Hammondia hammondi* oocysts in the faeces of cats from Germany and other European countries. *Vet Parasitol* 2008; 152(1-2): 34-45. http://dx.doi.org/10.1016/j.vetpar.2007.12.004. PMid:18226453.

Sheather AL. The detection of intestinal protozoa and mange parasites by a floatation technique. *J Comp Pathol Ther* 1923; 36: 266-275. http://dx.doi.org/10.1016/S0368-1742(23)80052-2.

Sroka J, Karamon J, Dutkiewicz J, Wójcik-Fatla A, Cencek T. Optimization of flotation, DNA extraction and PCR methods for detection of *Toxoplasma gondii* oocysts in cat faeces. *Ann Agric Environ Med* 2018; 25(4): 680-685. http://dx.doi.org/10.26444/aaem/97402. PMid:30586968.

Vieira FEG, Sasse JP, Minutti AF, Miura AC, De Barros LD, Cardim ST, et al. *Toxoplasma gondii*: prevalence and characterization of new genotypes in free-range chickens from south Brazil. *Parasitol Res* 2018; 117(3): 681-688. http://dx.doi.org/10.1007/s00436-017-5730-5. PMid:29344803.

Zulpo DL, Headley SA, Biazzono L, Da Cunha IAL, Igarashi M, De Barros LD, et al. Oocyst shedding in cats vaccinated by the nasal and rectal routes with crude rhoptry proteins of *Toxoplasma gondii. Exp Parasitol* 2012; 131(2): 223-230. http://dx.doi.org/10.1016/j.exppara.2012.04.006. PMid:22542988.

Zulpo DL, Igarashi M, Sammi AS, Santos JR, Sasse JP, Cunha IA, et al. rROP2 from *Toxoplasma gondii* as a potential vaccine against oocyst shedding in domestic cats. *Rev Bras Parasitol Vet* 2017; 26(1): 67-73. http://dx.doi.org/10.1590/s1984-29612017007. PMid:28403374.

Zulpo DL, Sammi AS, dos Santos JR, Sasse JP, Martins TA, Minutti AF, et al. *Toxoplasma gondii*: a study of oocyst re-shedding in domestic cats. *Vet Parasitol* 2018; 249: 17-20. http://dx.doi.org/10.1016/j.vetpar.2017.10.021. PMid:29279081.