Veterinary Parasitology: Regional Studies and Reports 18 (2019) 100345

Contents lists available at ScienceDirect

Veterinary Parasitology: Regional Studies and Reports

journal homepage: www.elsevier.com/locate/vprsr

Original Article

Development of a real-time PCR assay for detection of *Cryptosporidium canis* in dog fecal samples

Camila Guariz Homem, Isabela Garcia do Nascimento, Bruna Nicoleti Santana, Marcelo Vasconcelos Meireles*

Universidade Estadual Paulista (UNESP), Faculdade de Medicina Veterinária, Rua Clóvis Pestana, n° 793, CEP 16050-680, Bairro Dona Amélia, Araçatuba, SP, Brazil

ARTICLE INFO

Keywords:
Cryptosporidium canis
Dogs
Diagnosis
Real-time PCR
HSP70 gene

ABSTRACT

Cryptosporidiosis is an emerging zoonotic disease caused by the worldwide distributed parasitic protozoa *Cryptosporidium* spp. The host-adapted species *Cryptosporidium canis* is most frequently found in dogs, although human infections with this species have been described. This study aimed to develop a real-time PCR targeting the *HSP*70 protein gene for *C. canis* DNA detection in dog fecal samples collected from two municipalities in the state of São Paulo, Brazil. Furthermore, the occurrence of *Cryptosporidium* spp. and. *C. canis* was also determined by nested PCR. Fecal samples from 367 dogs (21 puppies and 346 adults) were purified by water-ether sedimentation. A real-time PCR protocol targeting the HSP70 gene for the species-specific detection of *C. canis* was developed and compared with nested PCR results. Real-time PCR identified *C. canis* in 15.3% (58/367) samples. Nested PCR revealed that 10.4% (38/367) of samples were positive for *Cryptosporidium* spp. All sequenced 18S rRNA amplicons were *C. canis*. There was a higher prevalence of *Cryptosporidium* spp. and *C. canis* in puppies compared to adult dogs. No non-specific amplification was observed in *C. canis* specific real-time PCR assay.

1. Introduction

Cryptosporidiosis is an emerging zoonotic disease caused by the worldwide distributed parasitic protozoa *Cryptosporidium* spp. *Cryptosporidium canis* is the most frequent species found in dogs (Uehlinger et al., 2013; Itoh et al., 2014; Li et al., 2015; Osman et al., 2015; Xu et al., 2016).

Infection with *C. canis* is usually asymptomatic, with shedding of few oocysts per gram of feces. In contrast, immunosuppressed dog may present long patent period and clinical signs such as diarrhea, lethargy, and physical deterioration (Santín, 2013; Cui et al., 2018).

Dogs have close contact with humans and can also act as reservoirs for zoonotic *Cryptosporidium* species (Bowman and Lucio-Forster, 2010). The infection in humans by *C. canis* has been described in several countries, including, Brazil (Lucca et al., 2009), Kenya (Gatei et al., 2006), Peru (Xiao et al., 2007), Jamaica (Gatei et al., 2008), Jordan (Hijjawi et al., 2010), England, Wales (Elwin et al., 2012), Dominican Republic (Lalonde et al., 2013), Ethiopia (Adamu et al., 2014), Mexico (González-Díaz et al., 2016), Nigeria (Molloy et al., 2010), Cambodia (Moore et al., 2016), and Angola (Dacal et al., 2018).

Several diagnostic techniques are used to detect *Cryptosporidium* spp. in fecal samples, as conventional microscopy and immunological assays. However, such methods do not allow species-specific diagnosis (Jex et al., 2008). Although it is time-consuming and expensive, nested PCR followed by genetic sequencing has been extensively used for *Cryptosporidium* spp. detection and species characterization (Adeyemo et al., 2018).

Real-time PCR assays are valuable tools for epidemiological studies of cryptosporidiosis in humans and animals and species-specific detection (Burnet et al., 2012; Mary et al., 2013; Bouzid et al., 2016).

The detection of *C. canis* in fecal samples of dogs has been performed using nested PCR followed by amplicon sequencing. This study aimed to develop a real-time PCR targeting the HSP70 gene for the specific detection of *C. canis* DNA in dog fecal samples. Furthermore, we aimed to determine the occurrence of *Cryptosporidium* spp. and *C. canis* in dog fecal samples from two municipalities from the state of São Paulo, Brazil.

2. Material and methods

2.1. Fecal samples and DNA extraction

A total of 367 fecal samples of dogs were collected from residences, pet stores, non-governmental organizations, and animal control centers

* Corresponding author.
E-mail address: m.meireles@unesp.br (M.V. Meireles).

https://doi.org/10.1016/j.vprsr.2019.100345
Received 20 December 2018; Received in revised form 16 October 2019; Accepted 23 October 2019
Available online 01 November 2019

in the municipalities of Araçatuba and Jaboticabal, in the state of São Paulo, Brazil, regardless of breed or gender. The collected samples were divided in two groups: puppies (< 12 month-old) and adults (> 12 month-old). Twenty-one and 346 samples were collected from puppies and adult dogs, respectively.

Samples were harvested immediately after natural defecation, stored at 4 °C in 50 ml plastic containers in a potassium dichromate solution (at a final concentration of 2.5%), strained with disposable sieves and subjected to water–ether sedimentation technique (Meloni and Thompson, 1996).

An aliquot of 200 mg of sediment from the purification process was washed with deionized water/Tween 20 (0.1%) and stored at −20° C in a 2 ml Eppendorf tube for the subsequent extraction of genomic DNA (Silva et al., 2010).

2.2. Real-time PCR

Nucleotide sequences of the HSP70 gene of *C. canis* were aligned with sequences belonging to all *Cryptosporidium* species and genotypes available in GenBank database. This information was used to determine a sequence specific to *C. canis*, which was employed to design *C. canis* specific semi-nested PCR and real-time PCR primers using the Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) tool (Table 1).

The reaction was standardized with a standard regression curve using a 303 bp DNA fragment corresponding to a partial HSP70 gene sequence from *C. canis* (GenBank accession number EU754842), which was amplified by semi-nested PCR using primers specific for *C. canis* and genomic *C. canis* DNA previously isolated from a dog. Hemi-nested amplicons were cloned using the TransformAid® Bacterial Transformation Kit (Thermo Fisher Scientific) and CloneJET PCR Cloning Kit (Thermo Fisher Scientific). Plasmid DNA was purified with GenElute® HP Five-Minute Plasmid Miniprep Kit (Sigma-Aldrich). Semi-nested plasmid dilutions were made ranging from 10^7 to 10^3 DNA molecules (Applied Biosystems, 2003) to obtain five points in the regression curve. Each dilution was tested in triplicate.

Real-time PCR consisted of 1 × SsoFast™ EvaGreen® Supermix (Bio-Rad), 250 nM of each primer and 2.0 µL of sample DNA, in a final volume of 20 µL. Amplification conditions were: initial denaturation for 2 min at 98 °C, followed by 50 denaturation cycles at 98 °C for 5 s, annealing at 60 °C for 5 s and extension at 72 °C for 25 s, followed by dissociation curve analysis, with temperature ranging from 65 °C to 95 °C and increase of 0.5 °C every 5 s. Reactions were performed using the CFX96® real-time PCR system (Bio-Rad).

Analytical specificity of real-time PCR assay was verified using genomic DNA from *C. andersoni*, *Cryptosporidium* avian genotype II, *C. bovis*, *C. felis*, *C. galli*, *C. parvum*, *C. ryanae*, *C. serpentis*, and *Cryptosporidium suis*-like genotype. Each sample was tested in duplicate. Electrophoresis using a 3% agarose gel was performed to check the sizes of the amplified fragments.

Analytical sensitivity of real-time PCR was determined using serial plasmid dilutions containing 10^7 to 10^0 copies of *C. canis* DNA. Each sample was tested in triplicate.

2.3. Nested PCR and sequencing

Nested PCR targeting the 18S rRNA gene was used to detect *Cryptosporidium* spp. DNA in fecal samples (Xiao et al., 2000). The amplified DNA was imaged via electrophoresis on 1.5% agarose gels stained with GelRed (Biotium).

Nested PCR amplicons were purified using the QIAquick Gel Extraction kit (Qiagen) and sequenced using ABI Prism DyeTerminator 3.1 on an ABI 3730XL automatic sequencer (Applied Biosystems). Sequencing reactions were performed in both directions, with oligonucleotide primers of nested PCR.

2.4. Statistical analysis

Differences in proportions of positive samples between puppies and adult dogs were assessed using Fisher's exact probability test, with values of $p < .05$ considered significant. Prevalence rates with 95% confidence intervals were calculated using Wilson (score) intervals (Sergeant, 2018).

2.5. Nucleotide sequence accession number

Nucleotide sequence generated in this study was submitted to GenBank database under the accession number MK240325.

2.6. Ethical approval

Ethical approval for this study was granted by the Committee for Ethical Use of Animals (CEUA) of São Paulo State University (UNESP), School of Veterinary Medicine, Araçatuba (approval number 00126-2013).

3. Results and discussion

The gene coding for the HSP70 protein (Sulaiman et al., 2000) was chosen because of its high polymorphism between the different species of *Cryptosporidium*, which allowed the design of primers specific for *C. canis*. The efficiency of real-time PCR was 111.8% (Fig. 1). The dissociation curve analysis identified the mean dissociation temperature of 84.75 °C (± 0.75 °C) for *C. canis* real-time PCR amplicons (Fig. 2).

Overall positive rate for *C. canis* obtained by real-time PCR was 15.3% (56/367; CI: 11.9–19.3). *C. canis* positive samples were 7/21 (33.3%; CI: 17.2–54.6) and 49/346 (14.2%; CI: 10.9–18.2%) for puppies and adult dogs, respectively ($p = 0.027$) (Table 2).

Cryptosporidium spp. were detected in 38/367 (10.4%; CI: 7.6–13.9) samples. The frequency of *Cryptosporidium* spp. in puppies and dogs was 6/21 (28.6%; 95% CI: 13.8–50)

and 32/346 (9.2%; CI: 6.6–12.2), respectively ($p = 0.014$) (Table 2). All sequenced 18S rRNA gene amplicons (6 samples) had 100% genetic similarity with *C. canis* sequences from GenBank (GU365876, MG516774).

The significant higher prevalence of *Cryptosporidium* spp. and *C. canis* in puppies compared to adult dogs was in accordance with other reports (Rimhanen-Finne et al., 2007; Uehlinger et al., 2013; Jian et al.,

Table 1
Primers used for semi-nested PCR and real-time PCR targeting the HSP70 gene in *Cryptosporidium canis*.

Primers		Position[a]	Sequence 5′- 3′	Amplified product (bp)
Semi-nested PCR	Can-F	1244–1263	TCTACGAGGGTGAGAGAGCC	496
	Can-R	1720–1739	ATGAGTGGGTTCATGTGGGC	
	SemCan-F	1437–1456	CAAGGGCAGACTCTCCAAGG	303
	Can-R	1720–1739	ATGAGTGGGTTCATGTGGGC	
Real-time PCR	CanisHSP70-F	1567–1586	AGAAACACCATCCAGGAGCC	150
	CanisHSP70-R	1697–1716	AACCTCCTTTTGCTGGTGCT	

[a] Annealing position in the HSP70 gene of *C. canis* (GenBank EU754842).

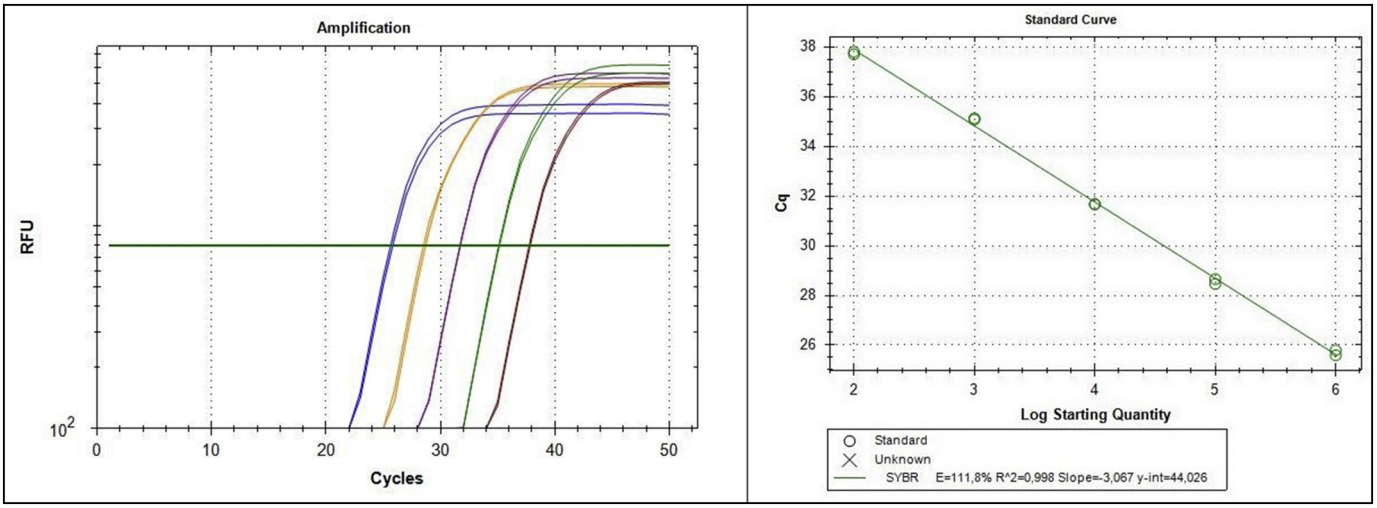

Fig. 1. Standard regression curve. Standardization of the real-time polymerase chain reaction with real-time amplification of dilutions containing 10^7 to 10^3 copies of *C. canis* DNA.

2014). In contrast, other surveys did not find statistical significance among prevalence rates between puppies and adult dogs (Itoh et al., 2014; Osman et al., 2015).

In several countries, the prevalence of cryptosporidiosis in dogs ranges from 0.0% to 44.8% (Bowman and Lucio-Forster, 2010; Yoshiuchi et al., 2010; Bajer et al., 2011; Itoh et al., 2014; Jian et al., 2014; Xu et al., 2016; Rosanowski et al., 2018). In Brazil, the prevalence of *C. canis* in dogs ranges from 1.4% to 26.2% (Lallo and Bondan, 2006; Mundim et al., 2007; Katagiri and Oliveira-Sequeira, 2008; Balassiano et al., 2009; Sevá et al., 2010; Grecca et al., 2013; Gizzi et al., 2014). The only study performed in the Araçatuba municipality identified *Cryptosporidium* spp. in 2.4 and 1% of dogs' fecal samples using ELISA and Kinyoun acid fast staining, respectively (Bresciani et al., 2008).

The average cycle threshold (Ct) observed in the real-time PCR assay was 37.5 and 39.4 for puppies and adult samples, respectively; the lowest and the highest Ct were 29.5 and 44.8, respectively. Samples that were negative by nested PCR and positive by real-time PCR showed higher Ct values when compared to samples positive by both assays. Fecal samples that were negative by nested PCR probably had few oocysts, as observed by Yang et al. (2009), and were diagnosed only by real-time PCR at high Ct values. Several studies have also reported a greater sensitivity of real-time PCR when compared to nested PCR (Sunnotel et al., 2006; De Waele et al., 2011).

Almost all samples (85%; 6/7) of puppies positive for *C. canis* by real-time PCR were also positive by nested PCR. In contrast, only 65% (32/49) of samples of adult dogs positive by real-time PCR were also positive by nested PCR. Oocysts of *Cryptosporidium* in stools are more commonly detected in puppies than in older dogs (Santín, 2013), which explains the finding of higher positivity rates in adult dogs using a technique with higher sensitivity, as real-time PCR.

Nested PCR assay targets the 18S rRNA gene of *Cryptosporidium* spp. and real-time PCR targeting the HSP70 gene is specific for *C. canis* diagnosis. Since dogs were asymptomatic, a low number of fecal oocysts, a small amount of DNA amplified by PCR, and failure to identify *Cryptosporidium* species by sequencing reaction were expected in

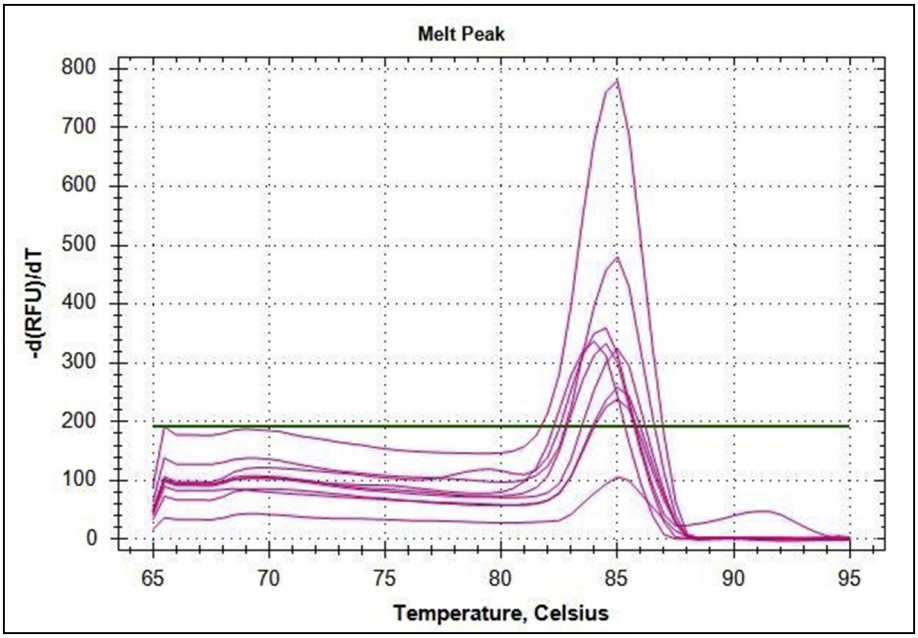

Fig. 2. Dissociation curve obtained from DNA samples positive for *C. canis* from real-time PCR assay targeting the HSP70 gene.

Table 2
The frequency distribution of *Cryptosporidium* spp. and *C. canis* in dogs.

Fecal sample	N° sampled	Real-time PCR			Nested PCR		
		N° positive	% positive (95% confidence interval)	*P*-value	N° positive	% positive (95% confidence interval)	P-value
Puppies	21	7	33.3 (17.2–54.6)	0.027	6	28.6 (13.8–50)	0.014
Adult dogs	346	49	14.2 (10.9–18.2)		32	9.2 (6.6–12.2)	
Total	367	56	15.3 (11.9–19.3)		38	10.4 (7.6–13.9)	

several PCR positive samples. This fact hampered the determination of epidemiological sensitivity and epidemiological specificity of real-time PCR using nested PCR/sequencing as a gold standard test. However, the sequencing of nested PCR amplicons from six samples, which were also positive by real-time PCR, allowed the identification of *C. canis*.

Two samples positive by nested PCR were negative by real-time PCR. Since nested PCR amplifies fragments of *Cryptosporidium* spp. 18S rRNA gene, real-time PCR negative results for *C. canis* are possibly related to the infection with other *Cryptosporidium* species previously detected in dog fecal samples such as *C. andersoni* (Rosanowski et al., 2018), *C. muris* (Lupo et al., 2008; Ellis et al., 2010), *C. meleagridis* (Hajdusek et al., 2004), *C. parvum* (Rosanowski et al., 2018), and *C. ubiquitum* (Li et al., 2015).

The analytical sensitivity test showed that up to one DNA copy of *C. canis* was detected per reaction. Electrophoresis of the fragments amplified by real-time PCR demonstrated the amplification of a 150 bp amplicon. Analysis of the analytical specificity showed no unspecific amplification for tested *Cryptosporidium* species or genotypes DNA. Although there was no availability of DNA from all species of *Cryptosporidium* for analysis of analytical specificity of real-time PCR, the determination of in silico specificity demonstrated that the oligonucleotide primers are specific for *C. canis*.

In conclusion, real-time PCR assay developed in this study is rapid, sensitive and specific and may serve as an alternative method to nested PCR/sequencing as a gold standard test for the diagnosis of infection by the *Cryptosporidium* species that most commonly infects dogs (*C. canis*). However, if the aim is to detect other species of *Cryptosporidium* that infect dogs, PCR followed by sequencing is the most recommended method.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Acknowledgments

We would like to thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for the following grants: financial support (2011/08814-1) and a Doctor of Science scholarship to C.G. Homem (2011/09614-6).

References

Adamu, H., Petros, B., Zhang, G., Kassa, H., Amer, S., Ye, J., Feng, Y., Xiao, L., 2014. Distribution and clinical manifestations of *Cryptosporidium* species and subtypes in HIV/AIDS patients in Ethiopia. PLoS Negl. Trop. Dis. 8, e2831.

Adeyemo, F.E., Singh, G., Reddy, P., Stenström, T.A., 2018. Methods for the detection of *Cryptosporidium* and *Giardia*: from microscopy to nucleic acid based tools in clinical and environmental regimes. Acta Trop. 184, 15–28.

Applied Biosystems, S.D., 2003. Creating Standard Curves With Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR. Available in. https://assets.thermofisher.com/TFS-Assets/LSG/Application-Notes/cms_042486.pdf>.

Bajer, A., Bednarksa, M., Rodo, A., 2011. Risk factors and control of intestinal parasite infections in sled dogs in Poland. Vet. Parasitol. 175, 343–350.

Balassiano, B.C.C., Campos, M.R., Menezes, R.C.A.A., Pereira, M.J.S., 2009. Factors associated with gastrointestinal parasite infection in dogs in Rio de Janeiro, Brazil. Prev. Vet. Med. 91, 234–240.

Bouzid, M., Elwin, K., Nader, J.L., Chalmers, R.M., Hunter, P.R., Tyler, K.M., 2016. Novel real-time PCR assays for the specific detection of human infective *Cryptosporidium* species. Virulence 7, 395–399.

Bowman, D.D., Lucio-Forster, A., 2010. Cryptosporidiosis and giardiasis in dogs and cats: veterinary and public health importance. Exp. Parasitol. 124, 121–127.

Bresciani, K.D.S., Amarante, A.F.T., Lima, V.M.F., Marcondes, M., Feitosa, F.L.F., Táparo, C.V., Serrano, A.C.M., Ishizaki, M.N., Tome, R.O., Perri, S.H.V., Meireles, M.V., 2008. Infecções por *Cryptosporidium* spp. em cães de Araçatuba, SP, Brasil. Vet. Zootec. 15, 466–468.

Burnet, J.B., Ogorzaly, L., Tissier, A., Penny, A., Cauchie, H.M., 2012. Novel quantitative TaqMan real-time PCR assays for detection of *Cryptosporidium* at the genus level and genotyping of major human and cattle-infecting species. J. Appl. Microbiol. 114, 1211–1222.

Cui, Z., Dong, H., Wang, R., Jian, F., Zhang, S., Ning, C., Zhang, L., 2018. A canine model of experimental infection with *Cryptosporidium canis*. Exp. Parasitol. 195, 19–23.

Dacal, E., Saugar, J.M., De Lucio, A., Hernández-De-Mingo, M., Robinson, E., Köster, P.C., Aznar-Ruiz-De-Alegría, M.L., Espasa, M., Ninda, A., Gandasegui, J., Sulleiro, E., Moreno, M., Salvador, F., Molina, I., Rodríguez, E., Carmena, D., 2018. Prevalence and Molecular Characterization of *Strongyloides Stercoralis*, *Giardia Duodenalis*, *Cryptosporidium* spp., and *Blastocystis* spp. vol. 11. Isolates in School Children in Cubal, Western Angola. Parasit. Vectors, pp. 67.

De Waele, V., Berzano, M., Berkvens, D., Speybroeck, N., Lowery, C., Mulcahy, G.M., Murphy, T.M., 2011. Age-stratified bayesian analysis to estimate sensitivity and specificity of four diagnostic tests for detection of *Cryptosporidium* oocysts in neonatal calves. J. Clin. Microbiol. 49, 76–84.

Ellis, A.E., Brown, C.A., Miller, D.L., 2010. Diagnostic exercise: chronic vomiting in a dog. Vet. Pathol. 47, 991–993.

Elwin, K., Hadfield, S.J., Robinson, G., Chalmers, R.M., 2012. The epidemiology of sporadic human infections with unusual cryptosporidia detected during routine typing in England and Wales, 2000-2008. Epidemiol. Infect. 140, 673–683.

Rimhanen-Finne, R., Enemark, H.L., Kolehmainen, J., Toropainen, P., Hänninen, M.L., 2007. Evaluation of immunofluorescence microscopy and enzyme-linked immunosorbent assay in detection of *Cryptosporidium* and *Giardia* infections in asymptomatic dogs. Vet. Parasitol. 145, 345–348.

Gatei, W., Wamae, C., Mbae, C., Waruru, A., Mulinge, E., Waithera, T., Gatika, S.M., Kamwati, S.K., Revathi, G., Hart, C.A., 2006. Cryptosporidiosis: prevalence, genotype analysis, and symptoms associated with infections in children in Kenya. Am. J. Trop. Med. Hyg. 75, 78–82.

Gatei, W., Barrett, D., Lindo, J.F., Eldemire-Shearer, D., Cama, V., Xiao, L., 2008. Unique *Cryptosporidium* population in HIV-infected persons, Jamaica. Emerg. Infect. Dis. 14, 841–843.

Gizzi, A.B.R., Oliveira, S.T., Leutenegger, C.M., Estrada, M., Kozemjakin, D.A., Stedile, R., Marcondes, M., Biondo, A.W., 2014. Presence of infectious agents and co-infections in diarrheic dogs determined with a real-time polymerase chain reaction-based panel. BMC Vet. Res. 10, 23–30.

González-Díaz, M., Urrea-Quezada, A., Villegas-Gómez, I., Durazo, M., Garibay-Escoba, R.A., Hernández, J., Xiao, L., Valenzuela, O., 2016. *Cryptosporidium canis* in two mexican toddlers. Pediatr. Infect. Dis. J. 35, 1265–1266.

Grecca, M., Thomaz-Soccol, V., Ribeiro, M.C.C., Truppel, J.H., Pereira, J.T., Osaki, S.C., 2013. Occurrence of *Cryptosporidium* sp. in dogs and cats from Curitiba and its metropolitan area. Arch. Vet. Sci. 18, 1–6.

Hajdusek, O., Ditrich, O., Slapeta, J., 2004. Molecular identification of *Cryptosporidium* spp. in animal and human hosts from the Czech Republic. Vet. Parasitol. 122, 183–192.

Hijjawi, N., Ng, J., Yang, R., Atoum, M.F.M., Ryan, U., 2010. Identification of rare and novel *Cryptosporidium* GP60 subtypes in human isolates from Jordan. Exp. Parasitol. 125, 161–164.

Itoh, N., Oohashi, Y., Ichikawa-Seki, M., Itagaki, T., Ito, Y., Saeki, H., Kanai, K., Chikazawa, S., Hori, Y., Hoshi, F., Higuchi, S., 2014. Molecular detection and characterization of *Cryptosporidium* species in household dogs, pet shop puppies, and dogs kept in a school of veterinary nursing in Japan. Vet. Parasitol. 200, 284–288.

Jex, A.R., Smith, H.V., Monis, P.T., Campbell, B.E., Gasser, R.B., 2008. *Cryptosporidium* – biotechnological advances in the detection, diagnosis and analysis of genetic variation. Biotechnol. Adv. 26, 304–317.

Jian, F., Qi, M., He, X., Wang, R., Zhang, S., Dong, H., Zhang, L., 2014. Occurrence and molecular characterization of *Cryptosporidium* in dogs in Henan Province, China. BMC Vet. Res. 10, 26–29.

Katagiri, S., Oliveira-Sequeira, T.C.G., 2008. Prevalence of dog intestinal parasites and risk perception of zoonotic infection by dog owners in São Paulo state, Brazil. Zoonoses Public Health 55, 406–413.

Lallo, M.A., Bondan, E.F., 2006. Prevalence of *Cryptosporidium* sp. in institutionalized dogs in the city of São Paulo, Brazil. Rev. Saúde Pública. 40, 120–125.

Lalonde, L.F., Reyes, J., Gajadhar, A.A., 2013. Application of a qPCR assay with melting curve analysis for detection and differentiation of protozoan oocysts in human fecal samples from Dominican Republic. Am. J. Tropic. Med. Hyg. 89, 892–898.

Li, W., Song, M., Lu, Y., Yang, J., Tao, W., Jiang, Y., Wan, Q., Zhang, S., Xiao, L., 2015. Prevalence and genetic characteristics of *Cryptosporidium, Enterocytozoon bieneusi* and *Giardia duodenalis* in cats and dogs in Heilongjiang province, China. Vet. Parasitol. 208, 125–134.

Lucca, P., Gaspari, E.M., Bozzo, L.M., Funada, M.R., Silva, S.O.S., Luliano, W., Soares, R.M., 2009. Molecular characterization of *Cryptosporidium* spp. from HIV infected patients from an urban area of Brazil. Rev. Inst. Med. Trop. São Paulo. 51, 341–343.

Lupo, P.J., Langer-Curry, R.C., Robinson, M., Okhuysen, P.C., Chappell, C.L., 2008. *Cryptosporidium muris* in a Texas canine population. Am. J. Trop. Med. Hyg. 78, 917–921.

Mary, C., Chapey, E., Dutoit, E., Guyot, K., Hasseine, L., Jeddi, F., Menotti, J., Paraud, C., Pomares, C., Rabodonirina, M., Rieux, A., Derouin, F., 2013. Multicentric evaluation of a new real-time PCR assay for quantification of *Cryptosporidium* spp. and identification of *Cryptosporidium parvum* and *Cryptosporidium hominis*. J. Clin. Microbiol. 51, 2556–2563.

Meloni, B.P., Thompson, R.C.A., 1996. Simplified methods for obtaining purified oocysts from mice and for growing *Cryptosporidium parvum* in vitro. J. Parasitol. 82, 757–762.

Molloy, S.F., Smith, H.V., Kirwan, P., Nichols, R.A.B., Asaolu, S.O., Connelly, L., Holland, C.V., 2010. Identification of a high diversity of *Cryptosporidium* species genotypes and subtypes in a pediatric population in Nigeria. Am. J. Trop. Med. Hyg. 82, 608–613.

Moore, C.E., Elwin, K., Phot, N., Seng, C., Mao, S., Suy, K., Kumar, V., Nader, J., Bousfield, R., Perera, S., Bailey, J.W., Beeching, N.J., Day, N.P., Parry, C.M., Chalmers, R.M., 2016. Molecular characterization of *Cryptosporidium* species and *Giardia duodenalis* from symptomatic cambodian children. PLoS Negl. Trop. Dis. 10, e0004822.

Mundim, M.J.S., Rosa, L.A.G., Hortêncio, S.M., Faria, E.S.M., Rodrigues, R.M., Cury, M.C., 2007. Prevalence of *Giardia duodenalis* and *Cryptosporidium* spp. in dogs from different living conditions in Uberlândia, Brazil. Vet. Parasitol. 144, 356–359.

Osman, M., Bories, J., El Safadi, D., Poirel, M.T., Gantois, N., Benamrouz-Vanneste, S., Delhaes, L., Hugonnard, M., Certada, G., Zenner, L., Viscogliosi, E., 2015. Prevalence and genetic diversity of the intestinal parasites *Blastocystis* sp. and *Cryptosporidium* spp. in household dogs in France and evaluation of zoonotic transmission risk. Vet. Parasitol. 214, 167–170.

Rosanowski, S.M., Banica, M., Ellis, E., Farrow, E., Harwood, C., Jordan, B., James, C., Mckenna, D., Fox, M., Blake, D.P., 2018. The molecular characterisation of *Cryptosporidium* species in relinquished dogs in Great Britain: a novel zoonotic risk? Parasitol. Res. 117, 1663–1667.

Santín, M., 2013. Clinical and subclinical infections with *Cryptosporidium* in animals. N. Z. Vet. J 61, 1–10.

Sergeant, E.S.G., 2018. Epitools Epidemiological Calculators. Ausvet Pty Ltd. Available at: http://epitools.ausvet.com.au.

Sevá, A.P., Funada, M.R., Souza, S.O., Nava, A., Richtzenhain, L.J., Soares, R.M., 2010. Occurrence and molecular characterization of *Cryptosporidium* spp. isolated from domestic animals in a rural area surrounding Atlantic dry forest fragments in Teodoro Sampaio municipality, state of São Paulo, Brazil. Braz. J. Vet. Parasitol. 19, 249–253.

Silva, D.C., Homem, C.G., Nakamura, A.A., Teixeira, W.F.P., Perri, S.H.V., Meireles, M.V., 2010. Physical, epidemiological, and molecular evaluation of infection by *Cryptosporidium galli* in Passeriformes. Parasitol. Res. 107, 271–277.

Sulaiman, I.M., Morgan, U.M., Thompson, R.C.A., Lal, A.A., Xiao, L., 2000. Phylogenetic relationships of *Cryptosporidium* parasites based on the 70-kilodalton heat shock protein (HSP70) gene. Appl. Environ. Microbiol. 66, 2385–2391.

Sunnotel, O., Snelling, W.J., Xiao, L., Moule, K., Moore, J.E., Millar, C., Dooley, J.S.G., Lowery, C.J., 2006. Rapid and sensitive detection of single *Cryptosporidium* oocysts from archived glass slides. J. Clin. Microbiol. 44, 3285–3291.

Uehlinger, F.D., Greenwood, S.J., Mcclure, J.T., Conboy, G., O'Handley, R., Barkema, H.W., 2013. Zoonotic potential of *Giardia duodenalis* and *Cryptosporidium* spp. and prevalence of intestinal parasites in young dogs from different populations on Prince Edward Island, Canada. Vet. Parasitol. 196, 509–514.

Xiao, L., Alderisio, K., Limor, J., Royer, M., Lal, A.A., 2000. Identification of species and sources of *Cryptosporidium* oocysts in storm waters with a small subunit rRNA-based diagnostic and genotyping tool. Appl. Environ. Microbiol. 66, 5492–5498.

Xiao, L., Cama, V.A., Cabrera, L., Ortega, Y., Pearson, J., Gilman, R.H., 2007. Possible transmission of *Cryptosporidium canis* among children and a dog in a household. J. Clin. Microbiol. 45, 2014–2016.

Xu, H., Jin, H., Wu, W., Li, P., Wang, L., Li, N., Feng, Y., Xiao, L., 2016. Genotypes of *Cryptosporidium* spp., *Enterocytozoon bieneusi* and *Giardia duodenalis* in dogs and cats in Shanghai, China. Parasit. Vectors 9, 121–129 (2106).

Yang, R., Jacobson, C., Gordon, C., Ryan, U., 2009. Prevalence and molecular characterization of *Cryptosporidium* and *Giardia* species in pre-weaned sheep in Australia. Vet. Parasitol. 161, 19–24.

Yoshiuchi, R., Matsubayashi, M., Kimata, I., Furuya, M., Tani, T., Sasai, K., 2010. Survey and molecular characterization of *Cryptosporidium* and *Giardia* spp. in owned companion animal, dogs and cats, in Japan. Vet. Parasitol. 174, 313–316.