SPLITTING STRANDS
Polymerase chain reaction testing is mainly used as a confirmatory test for detecting patient parasitic infection through DNA derived from parasite stages.

PARASITOLOGY

Molecular Testing for Parasite Detection and Disease Diagnosis

***Manigandan Lejeune**, BVSc&AH, MVSc, PhD, DACVM (Parasitology)*
Cornell University College of Veterinary Medicine

Diagnostic parasitology is an important discipline in the field of animal disease diagnosis and an integral component of animal health screening. This discipline thrived in the 20th century with the development of classic parasitology techniques that allowed simple antemortem detection of parasites, such as the Cornell-Wisconsin centrifugal flotation technique. Variants and modifications of these time-tested techniques remain the mainstay of many diagnostic parasitology labs.

While morphological evaluation of parasite life stages (e.g., eggs, cysts, oocysts) in fecal samples can often aid identification to a taxonomic level (e.g., species, genus, family) relevant to make informed clinical treatment and control decisions, classic parasitology tools have limitations for definite identification of some species.

Molecular techniques may provide the much-needed diagnostic resolution critical for effective parasitic disease management. Many serologic and molecular tests, such as polymerase chain reaction (PCR), are currently being used for specific parasite detection. These tests are more common in research/academic settings but are also offered through commercial diagnostic laboratories.

BASIC PCR APPROACHES FOR PARASITE DETECTION

PCR is increasingly used to confirm parasite species when morphologic detection is a challenge (**BOX 1**). There are 2 basic approaches: species-specific assays and universal assays.

Species-Specific Assays

Species-specific assays allow exclusive detection of parasite species from among even close relatives. For these tests, prior research has identified a DNA region that is unique to the parasite species of interest (say, species X). Specific primers are then designed to precisely amplify this region. With this approach, PCR amplification alone confirms parasite identification as species X, and no further sequencing of PCR products is required. This, in turn, improves the turnaround time for these assays (usually the same day).

Universal Assays

Universal assays typically amplify a "variable region" of DNA (unique to each parasite) that is flanked by "conserved regions" (identical across all related species). Universal primers based on the conserved DNA regions allow amplification of the variable region from all

related species. Amplified PCR products are further sequenced and searched against publicly available databases to determine parasite identity. This approach enables identification of either the parasite of interest or other closely related species in a single PCR run.

The sequencing requirement for this approach increases the turnaround time for universal assays (2 to 5 days) compared with species-specific testing. The best-known DNA regions (markers) used in universal assays are the internal transcribed spacers (ITS), cytochrome c oxidase (CO), and 18S small-subunit rRNA gene (18S).

WHEN TO USE PCR

In diagnostic parasitology laboratories, PCR is strategically used to complement traditional techniques, mainly to address the diagnostic limitations of the latter. Appropriate use of molecular techniques for definitive parasite detection depends on the need to distinguish between morphologically similar species.

For example, 9–13 × 7–9 µm cysts (**FIGURE 1**) observed on a zinc sulfate fecal flotation test of a cat fecal sample can be morphologically identified as belonging to the genus *Giardia*. Further identification to species or assemblages A through G is not possible, as *Giardia* cysts are indistinguishable. Cats can be infected with *Giardia duodenalis* assemblage F and/or assemblage A, and the latter is of zoonotic concern.[1] PCR testing based on primers designed for the 2 conserved regions flanking the variable region of β-giardin locus (i.e., a universal assay) is generally performed to differentiate *Giardia* assemblages.[2]

BOX 1 Overview of Polymerase Chain Reaction

What is it?
Polymerase chain reaction (PCR) is a process used to selectively amplify (reproduce) a specific section of double-stranded DNA for which the flanking sequence is known.

How does it work?
Basic PCR consists of 3 steps:
1. Denaturation of the double strands of template DNA
2. Annealing of primers to each strand of template DNA
3. Extension of the primer to synthesize a new DNA strand

In the ***denaturing*** step, the template DNA is heated to 94°C to separate the complementary strands. Next, the temperature is lowered (usually to 50°C to 65°C) to allow the primers to recombine with, or ***anneal*** to, their complementary targets on the template DNA. The annealing temperature is determined by the melting temperature (T_m) of the 2 primers. The last step is ***extension,*** during which the DNA polymerase produces a complementary copy of the template DNA strand—starting from where the PCR primer is attached—using the deoxynucleotide triphosphates (dNTPs) in the reaction mixture (PCR buffer, $MgCl_2$). The temperature for extension is determined by the specific thermostable polymerases and is commonly around 72°C. The reaction mixture is heated to specific temperatures for set times and a set number of cycles (35 to 45 cycles) using a thermocycler. The parent DNA template is amplified in an exponential manner such that a single template DNA can be amplified 35 billion times by the end of 35 cycles of PCR.

PCR COMPONENT	PURPOSE
DNA template	Contains the region of DNA that is to be amplified
Forward primer	Synthetically designed small sequence of DNA (20–25 base pairs) that complements the beginning sequence of the region of DNA that is to be amplified
Reverse primer	Synthetically designed small sequence of DNA (20–25 base pairs) that complements the end sequence of the region of DNA that is to be amplified
Taq DNA polymerase	A temperature-stable enzyme that creates a complementary strand of DNA
dNTPs	The nucleotides (dGTP, dCTP, dATP, dTTP) with which the polymerase can synthesize a new strand of DNA
PCR buffer	Provides an ideal chemical environment that promotes the stability and fidelity of the Taq DNA polymerase
$MgCl_2$	Mg^{2+} forms soluble complexes with dNTPs to produce the actual substrate that the Taq polymerase recognizes. Required for Taq to function.

In contrast, the egg packets of *Dipylidium caninum* (flea tapeworm; **FIGURE 2**) are morphologically distinct, and only one *Dipylidium* species infects dogs.[3] Therefore, identifying this egg packet on microscopic examination of a dog fecal sample should be confirmatory for *D caninum* infection, and further molecular analysis/confirmation is not warranted.

Species-Specific Assays

Species-specific PCR is generally employed to rule a target parasite species in or out, especially when a quicker turnaround is desired. For example, species-specific PCR would be indicated for a dog with a taeniid tapeworm infection to determine whether *Echinococcus multilocularis* (a zoonotic species) is present. The eggs of *E multilocularis* (**FIGURE 3**) are morphologically indistinguishable from those of other members of the family Taeniidae; therefore, morphologic diagnosis of these eggs allows parasite identification as Taeniidae but not genus or species. However, the concerned owner would expect the diagnostic laboratory to expeditiously identify the species. The species-specific PCR assay based on the unique nucleotide region in the NADH (nicotinamide adenine dinucleotide hydrogen) dehydrogenase subunit-1 gene (*ND1*) of *E multilocularis* has proven to have 100% detection specificity.[4]

Other examples in which specific PCR would be used include identifying *Dirofilaria immitis* (canine heartworm) in a dog blood sample and *Toxoplasma gondii* in cat feces. A species-specific PCR assay targeting a region in the *CO1* gene of *D immitis* overcomes the need for expertise to visually differentiate heartworm microfilariae from other parasites.[5] Similarly, the oocysts of *T gondii* (a zoonotic protozoan) shed in cat feces (**FIGURE 4**) resemble those of *Hammondia hammondi*, a lesser-known protozoan parasite of cats. A specific PCR assay based on the unique 529–base pair repeat element is used for definite detection of *T gondii*.[6]

The drawback of species-specific PCR is that it does not detect infection with related parasites. Despite this limitation, species-specific PCR is preferred by diagnostic laboratories to address specific requests.

Universal Assays

If more comprehensive parasite detection is the goal rather than rapid turnaround, universal assays can come in handy. For the scenario mentioned above for *E multilocularis*, if owners can be convinced of the

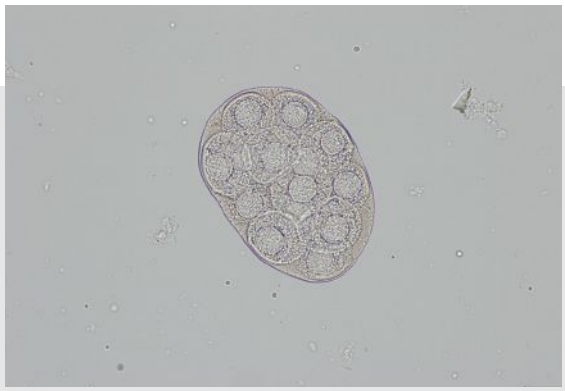

FIGURE 2. Egg packet retrieved from a segment of *Dipylidium caninum* voided in dog feces.

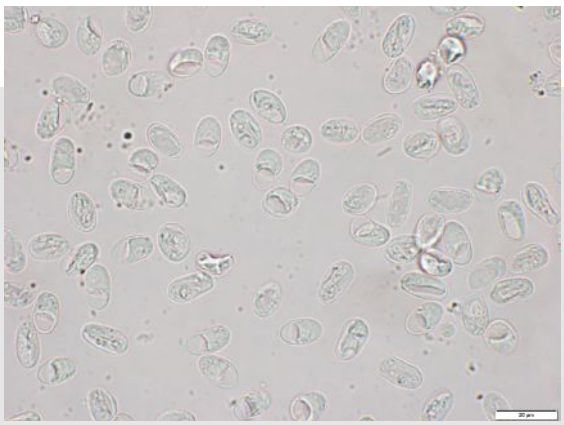

FIGURE 1. Cysts of *Giardia* observed in cat feces processed by zinc sulfate flotation. They cannot be differentiated morphologically to species or assemblages.

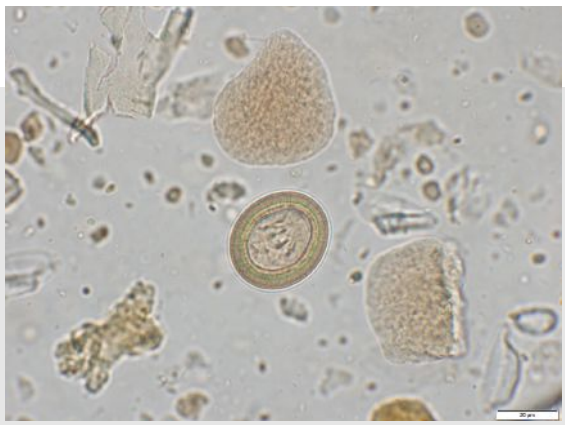

FIGURE 3. Egg from the family Taeniidae, which includes *Echinococcus* and *Taenia* genera. The eggs of these species are morphologically indistinguishable.

diagnostic advantage of detecting Taeniidae species other than *E multilocularis*, a universal assay for taeniids based on the *CO1* gene exists.[7] This assay was designed from the evolutionarily conserved regions of *CO1*, which flank a variable region that provides the required resolution to differentiate all members of the genera *Echinococcus* (including *E granulosus* and *E canadensis*) and *Taenia*. Similarly, universal PCR (based on the *18S* gene) is used to identify *Babesia canis vogeli* and *Babesia canis canis* in dog blood.[8] Blood smear examination cannot differentiate these 2 large *Babesia* species, and a universal PCR assay is more decisive.

Investigative Parasitology

Despite the lack of complete DNA sequence information for many parasites of veterinary importance, the universal PCR approach has generated much-needed DNA marker information for common parasites that is available through publicly accessible sequence databases. This, in turn, has facilitated the use of universal PCR as a predominant tool for investigative diagnostic parasitology.

In general, parasitologists employ classic skills to narrow down parasite identification to the lowest taxonomic level possible and, if needed, resort to universal PCR for specific species identification. For example, 5- to 6-µm oocysts (**FIGURE 5**) belong to the genus *Cryptosporidium*. However, there are more than 26 valid species in this genus and the oocysts of most are morphologically indistinguishable. Neither a flotation test nor the available *Cryptosporidium* enzyme-linked immunosorbent assay (ELISA) helps with species identification. Dogs can be infected with *C canis* and/or *C parvum,* both of which have zoonotic potential.[9] At times, oocysts of other *Cryptosporidium* species may pass through the dog's gut as spurious parasites. The genuine concern of *Cryptosporidium* transmission to dog owners/handlers cannot be addressed if the species is not identified. In this situation, the universal PCR assay based on the *18S* gene locus can provide the necessary resolution to differentiate each species of *Cryptosporidium*.[9]

Spurious Parasitism

PCR has been effectively used to address parasite diagnostic challenges pertaining to companion animals, such as identifying spurious parasitism in dogs; that is, finding parasites in dog feces for which dogs are not the proper host. Dogs are notorious for consuming feces of other animals.[10]

Failure to identify spurious parasitism may end up in unnecessary treatment. For example, observing ~60 × 40 µm strongyle-type eggs (**FIGURE 6**) in dog feces is generally interpreted as infection with *Ancylostoma caninum* (dog hookworm). If, in fact, the dog has consumed cat feces and is passing *Ancylostoma tubaeforme* as a spurious parasite, microscopic differentiation of species is not possible. Extracting DNA from these eggs and subjecting it to universal PCR to amplify and sequence the ITS-1 marker will establish parasite identity.[11] Similarly, employing ITS-2 PCR will identify whether the spurious strongyle-type eggs are of ruminant or equine origin.[12]

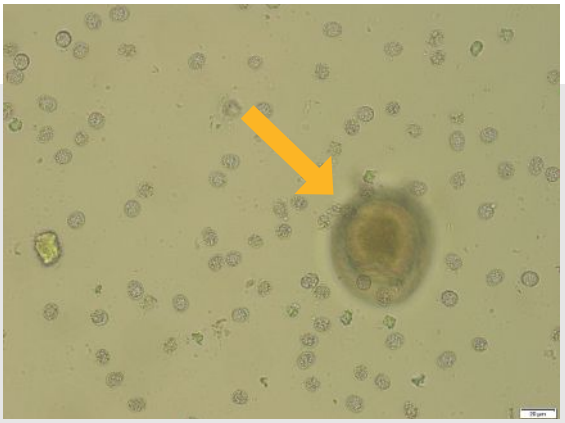

FIGURE 4. *Toxoplasma gondii*–like oocysts retrieved from cat feces that resemble oocysts of *Hammondia hammondi*. An egg of *Toxocara cati* **(yellow arrow)** is in the background away from the plane of focus.

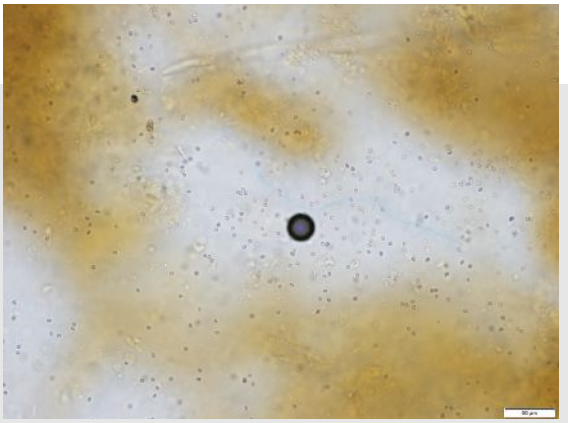

FIGURE 5. Oocysts of *Cryptosporidium* with the characteristic pink hue on a sugar flotation test. Oocysts of most valid *Cryptosporidium* species cannot be differentiated morphologically.

Left: Courtesy Jaime Hazard, AHDC, Cornell University

Other Applications

PCR can also be used to identify/confirm gross parasite specimens that are voided in the feces or retrieved from the host. For example, a worm removed from the anterior chamber of a dog's eye is usually suspected to be *Thelazia*, *Dirofilaria*, or *Onchocerca*. If the specimen is not suitable for morphology, using a universal PCR assay based on the NADH dehydrogenase subunit-5 gene (*ND5*) may help with identifying any of these species in a single PCR run.[13-15]

Specimens collected at necropsy can also be tested using PCR. In these cases, pathologists frequently seek molecular assistance to identify parasites in histologic sections, typically to confirm species of protozoans in genera such as *Sarcocystis*, *Toxoplasma*, *Neospora*, and *Hammondia*, the tissue stages of which are a challenge to differentiate. It is possible to identify many common parasites (including helminths and arthropods) using PCR, provided clues regarding broader taxonomy of the parasite are obtained by histology.

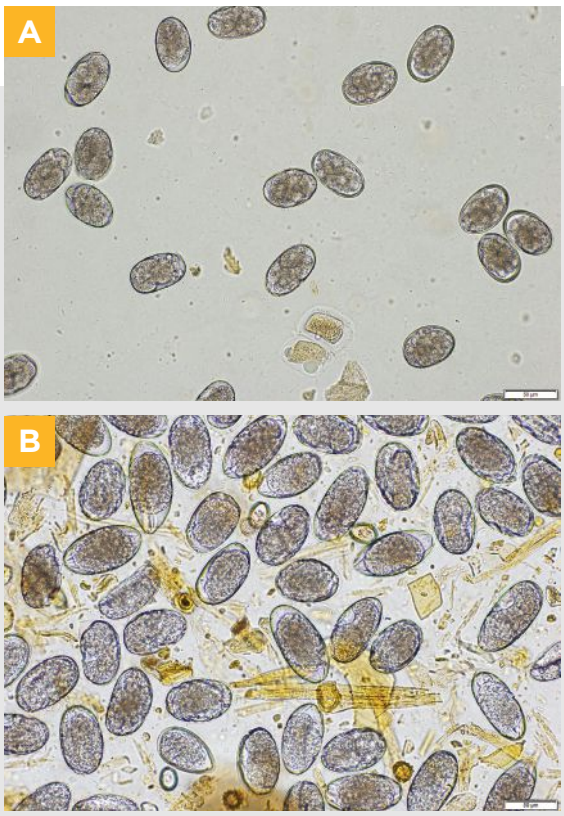

FIGURE 6. (A) Strongyle-type eggs in dog feces presumed to be *Ancylostoma caninum*. **(B)** Strongyle eggs from a domestic ruminant. The eggs of *A caninum* are difficult to differentiate from *Ancylostoma tubaeforme* of cats and other strongyle-type eggs of ruminants and horses, which are common spurious parasites in dogs.

SPECIMEN PREPARATION FOR PCR TESTING

The success of PCR for parasite detection depends on various factors, the foremost of which is the sample used for initial DNA extraction. Using fixatives such as formalin impairs DNA strand dissociation, which is a necessary step for PCR amplification. Therefore, for PCR testing, it is critical to submit fecal samples as fresh/refrigerated and gross parasites in 70% ethanol preservative. Formalin-fixed parasite specimens, including paraffin-embedded tissue scrolls, require a special DNA extraction protocol that may limit the quality of DNA required for successful PCR.

LIMITATIONS OF PCR

At present, molecular parasite detection is not considered an alternative to classic testing. Although used in a few instances to diagnose prepatent infections in dogs, it is mainly used as a confirmatory test for detecting patent parasitic infection through DNA derived from parasite stages. The obstacles to adopting molecular parasitology diagnostics are many. Developing molecular assays as a sole diagnostic tool for parasite detection would require tedious validation processes that are beyond the realm and reach of many diagnostic parasitology labs. More importantly, the risk of overdiagnosis looms. For example, a study found PCR evidence of *T gondii* DNA in the feces of a cat devoid of active intestinal infection; prey infected with a "-zoite" stage of the parasite was present, serving as the source of the DNA.[16] Such results must be interpreted with caution.

CONCLUSION

Unlike employing advanced diagnostics for detecting bacterial or viral etiologies, many clinicians see molecular testing for parasites as ancillary and unnecessarily expensive. Any new, advanced parasitology tests, even those meant for rapid diagnosis, require cost justification and clinical relevance. Therefore, it is prudent to use molecular advancements to augment classic identification methods for efficiently addressing the clinician's diagnostic needs. TVP

References

1. Ballweber LR, Xiao L, Bowman DD, et al. Giardiasis in dogs and cats: update on epidemiology and public health significance. *Trends Parasitol* 2010;26(4):180-189.
2. Lalle M, Pozio E, Capelli G, et al. Genetic heterogeneity at the b-giardin locus among human and animal isolates of *Giardia duodenalis* and

identification of potentially zoonotic subgenotypes. *Int J Parasitol* 2005;35(2):207-213.

3. Zajac AM, Conboy GA. Fecal examination for the diagnosis of parasitism. In: *Veterinary Clinical Parasitology.* 8th ed. Chichester, West Sussex: Wiley Blackwell; 2012:72-73.
4. Trachsel D, Deplazes P, Mathis A. Identification of taeniid eggs in the faeces from carnivores based on multiplex PCR using targets in mitochondrial DNA. *Parasitology* 2007;134(Pt 6):911-920.
5. Oh IY, Kim KT, Sung HJ. Molecular detection of *Dirofilaria immitis* specific gene from infected dog blood sample using polymerase chain reaction. *Iran J Parasitol* 2017;12(3):433-440.
6. Reischl U, Bretagne S, Kruger D, et al. 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;3:7.
7. Bowles J, Blair D, McManus DP. Molecular genetic characterization of the cervid strain ("northern form") of *Echinococcus granulosus*. *Parasitology* 1994;109(Pt 2):215-221.
8. Rucksaken R, Maneeruttanarungroj C, Maswanna T, et al. Comparison of conventional polymerase chain reaction and routine blood smear for the detection of *Babesia canis*, *Hepatozoon canis*, *Ehrlichia canis*, and *Anaplasma platys* in Buriram Province, Thailand. *Vet World* 2019;12(5):700-705.
9. Xiao L, Escalante L, Yang C, et al. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. *Appl Environ Microbiol* 1999;65(4):1578-1583.
10. Sloss MW. Spurious parasites in a dog. *Iowa State University Veterinarian* 1939;1(2): Article 8.
11. Liu Y, Zheng G, Alsarakibi M, et al. Molecular identification of *Ancylostoma caninum* isolated from cats in Southern China based on complete ITS sequence. *BioMed Res Int* 2013;2013:868050.
12. Gasser RB, Chilton NB, Hoste H, Beveridge I. Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. *Nucleic Acids Res* 1993;21(10):2525-2526.
13. Verocai GG, Hassan HK, Lakwo T, et al. Molecular identification of *Onchocerca* spp. larvae in *Simulium damnosum* sensu lato collected in Northern Uganda. *Am J Trop Med Hyg* 2017;97(6):1843-1845.
14. Liu GH, Gasser RB, Otranto D, et al. Mitochondrial genome of the eyeworm, *Thelazia callipaeda* (Nematoda: Spirurida), as the first representative from the family Thelaziidae. *PLoS Negl Trop Dis* 2013;7(1):e2029.
15. Hu M, Gasser RB, Abs El-Osta YG, Chilton NB. Structure and organization of the mitochondrial genome of the canine heartworm, *Dirofilaria immitis*. *Parasitology* 2003;127(Pt 1):37-51.
16. Poulle ML, Forin-Wiart MA, Josse-Dupuis E, et al. 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.

Manigandan Lejeune

Dr. Lejeune holds a bachelor's degree in Veterinary Science & Animal Husbandry and master's degree in Veterinary Parasitology. He completed his PhD at the University of Calgary in 2010. Dr. Lejeune worked as a wildlife parasitologist at the Alberta node of the Canadian Wildlife Health Cooperative before moving to Cornell University in 2016, where he oversees the parasitology section at the Cornell Animal Health Diagnostic Center (AHDC). Dr. Lejeune is a diplomate of the American College of Veterinary Microbiologists with a certification in veterinary parasitology.