PARASITOLOGY

Immune-Complex Dissociation for Heartworm Diagnosis

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Heartworm disease in dogs and cats is caused by infection with *Dirofilaria immitis*. The mosquito vector, most commonly belonging to the genus *Aedes, Culex,* or *Anopheles,* acquires *D immitis* microfilariae during a blood meal from an infected dog. After at least 10 days, depending on climatic conditions such as temperature and humidity, the microfilariae have molted into third-stage larvae (L3) and migrated to the mosquito's proboscis to be deposited next time the mosquito feeds.[1,2] Within a couple of days of entering a host, the L3 molt into fourth-stage larvae (L4) and migrate through the subcutis and muscles to the thorax.

In dogs, the L4 undergo a final molt into sexually immature worms (formerly known as fifth-stage larvae) 50 to 70 days after infection.[1] The immature worms are carried by the venous system to the pulmonary arteries to finish the maturation process. Six months after infection is typically when worms have matured to the point of reliable antigen detectability, and if enough mature worms of both sexes are present, microfilaria begin becoming detectable 180 to 210 days after infection.[1] In dogs, the heartworm life cycle takes 7 to 9 months.[1]

In cats, most worms at the L4 stage are eliminated by an immune response; the effects of this response can result in the development of respiratory signs known as heartworm-associated respiratory disease (HARD).[3] Any surviving worms molt into sexually immature adults 4 to 6 months after infection.[3] Microfilaremia is seldom reported and transient if present.[3]

Abstract

The gold standard for diagnosis of heartworm (*Dirofilaria immitis*) infection is the detection of circulating antigen. In most cases, antigen is reliably detectable with routine testing; however, when antigen is bound by circulating antibodies, a false-negative result can be obtained. Immune-complex dissociation (ICD) techniques are used to unbind antigen-antibody complexes, thus allowing detection of the antigen. Heat or acid treatment can be used for ICD. This article reviews the benefits of, and indications for, the use of ICD techniques related to heartworm testing.

Take-Home Points

- For dogs, routine antigen testing is considered the gold standard for diagnosis of heartworm infection and should be conducted in tandem with a microfilariae test on an annual basis. For cats, antigen testing is often used in combination with other diagnostics, particularly when heartworm disease is suspected.
- Circulating antibodies can form immune complexes with heartworm antigen, thereby reducing the level of antigen detectable by routine testing and potentially yielding a false-negative result.
- Certain techniques can improve antigen detection by dissociating antibody-antigen complexes using heat or acid.
- Using heat to perform immune-complex dissociation (ICD) has been shown to increase the detection of heartworm antigen in canine and feline serum and plasma samples.
- ICD testing should be considered for a dog with a suspected false-negative initial antigen test result and any of the following criteria: present microfilariae, clinical signs or abnormalities consistent with heartworm infection, discordant antigen test results, or high risk for infection (lifestyle or location).
- ICD testing should also be considered when performing antigen testing in a cat with suspected heartworm infection.

ROUTINE ANTIGEN DETECTION IN DOGS

Antigen-capturing enzyme-linked immunosorbent assays (ELISAs) have been considered the gold standard for heartworm diagnosis since 1985.[4] The antigens detected are derived from the heartworm reproductive tract, predominantly of the adult female worms, and are present in host blood, plasma, and serum.[5-7] Standard antigen-capturing ELISAs used without heat can detect antigens as early as 5 months and consistently by 7 months after infection and with a parasite burden as low as 1 or 2 female worms.[1,8-10]

Antigen testing is highly specific and confirms heartworm infection; however, this diagnostic method is fallible. False-negative results can be obtained when circulating levels of antigen are below detectable levels due to low parasite burden, infection with only male worms, early infection with L3 or L4, or sequestration through immune complexing with host antibodies.[1,5,6] ELISAs must be performed under the strict guidance of the manufacturer's instructions for optimal results; if the test's result is ambiguous or inconsistent with the clinical history, the test should be repeated (**FIGURE 1**).[2]

Although antigen testing is the most sensitive and specific method for heartworm diagnosis, it is recommended to be performed along with other diagnostic tests, including, at minimum, microfilariae testing; cardiac ultrasonography, radiography, blood analysis, or urinalysis may also be necessary.[2] In light of the issues associated with antigen testing, it is important to report negative ELISA results as "no antigen detected (NAD)," because it is not possible to know whether a dog is truly heartworm negative or experiencing a previously outlined scenario.

ROUTINE ANTIGEN DETECTION IN CATS

Antigen detection is still considered the gold standard for heartworm diagnosis in cats because it confirms infection with adult worms.[1-3,12-14] However, it has several of the same limitations in cats as described in dogs, including antigen sequestration by antibodies.[15,16] Furthermore, an NAD result is expected for cats experiencing disease related to HARD and the death of larval worms before worm maturation (low to no antigen yet present).[1-3,12-14] An NAD result does not rule out *D immitis* infection in cats, and ancillary testing should be performed as described for dogs, especially when heartworm infection or heartworm disease is suspected.[1-3,12-14] Antibody testing (available from reference laboratories) may prove useful in ruling heartworm infection in or out for feline patients.[1,3,17]

IMMUNE-COMPLEX DISSOCIATION TECHNIQUES

The use of immune-complex dissociation (ICD) techniques prior to antigen testing for *D immitis* has been described since 1985.[4] ICD techniques improve antigen detection by dissociating antibody-antigen complexes in the sample using heat or acid treatment before the antigen test is performed.[5] Until 1995, manufacturer instructions for several antigen tests

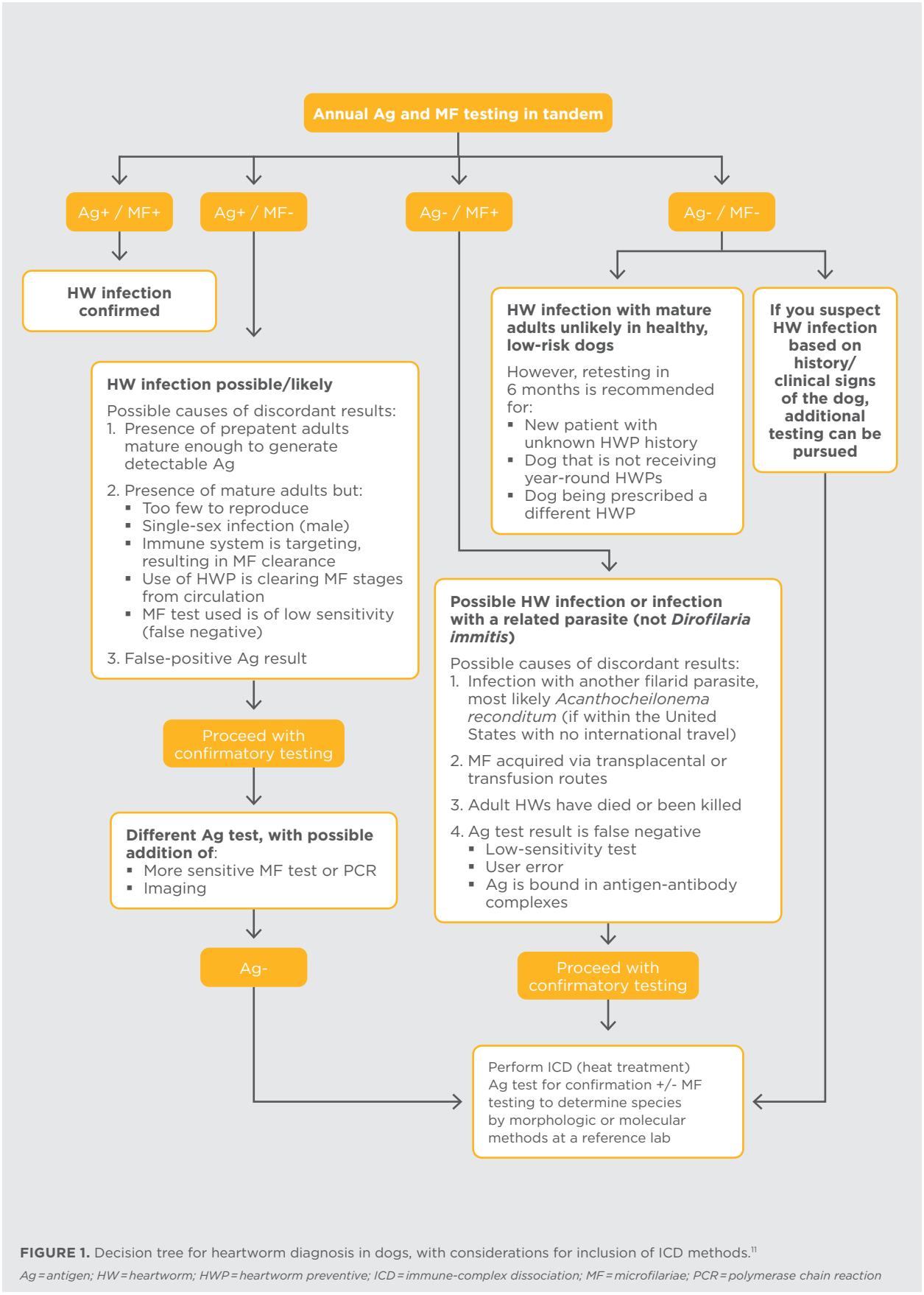

FIGURE 1. Decision tree for heartworm diagnosis in dogs, with considerations for inclusion of ICD methods.[11]

Ag = antigen; HW = heartworm; HWP = heartworm preventive; ICD = immune-complex dissociation; MF = microfilariae; PCR = polymerase chain reaction

included the use of chemical reagents or heat to disrupt these complexes[4]; for example, the original PetChek heartworm test (IDEXX, idexx.com) included an ICD method using an enzymatic technique with pepsin as a reagent.[5]

Heat Treatment

Temperatures above 65 °C (149 °F) promote antibody-antigen dissociation, thereby increasing the amount of detectable antigen. The proposed mechanism behind this dissociation is that the antibodies are denatured at these temperatures, while the heartworm antigen is heat stable.[18-22] In the past decade, heating 0.4 to 1.5 mL of serum or plasma to 103 °C to 104 °C (217 °F to 219 °F) for 10 minutes prior to antigen testing has improved heartworm detection in canine and feline serum and plasma samples.[4,15,16,18-20,23-30]

In 1 study, heating samples prior to antigen testing decreased the mean time to detection of *D immitis* in experimentally infected dogs to 98 days (range, 126.9 to 131.5 days depending on assay) compared with 133 days (range, 162.6 to 162.8 days depending on assay) when heat treatment was not performed.[9] In another study, heat treatment of canine samples led to antigen being consistently detected by 120 days after infection; unfortunately, earlier timepoints were unavailable for testing.[29]

Heat treatment benefits in increasing antigen detection have consistently been documented not only in experimental studies but also in regional and nationwide surveys using samples from shelter and pet dogs and cats.[20,31] In a recent large-scale nationwide survey, the antigen prevalence in serum samples from pet dogs increased from 3.8% to 7.3% when heat treatment was used.[23] Unfortunately, concurrent microfilariae testing was not possible with that study; furthermore, the authors reported that 0.5% of the samples that initially tested positive converted to NAD status after heat treatment,[23] indicating that a small percentage of the positive results were potentially false. Another recent study demonstrated that dogs infected with only male worms tested positive using antigen testing with heat treatment.[18] Heating 300 to 400 µL of serum or plasma to 104 °C (219 °F) for 10 minutes, followed by centrifugation for 5 minutes, increased antigen detection in feline samples from shelter and free-roaming cats from the South Central and Southeast regions of the United States and in 6 experimentally infected cats.[15,16]

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Heat treatment of samples before an antigen test does not incite a false-positive result due to cross-reactions with other parasites such as *Acanthocheilonema reconditum*; *Ancylostoma caninum*; *Ancylostoma braziliense*; *Trichuris vulpis*; *Toxocara canis*; *Dipylidium caninum*; *Spirometra mansonoides*; *Macracanthorhynchus ingens*; and *Cystoisospora*, *Giardia*, and *Sarcocystis* species.[4] However, false-positive results of antigen testing are possible, even with heat treatment, in dogs naturally infected with parasites that are rare in the United States (e.g., *Dirofilaria repens*, *Angiostrongylus vasorum*, *Acanthocheilonema dracunculoides*, *Spirocerca lupi*).[32-34]

Inclusion of heat-treatment ICD is considered beneficial in dogs with an initially negative antigen test that meet the following criteria: present microfilariae, clinical signs or abnormalities consistent with heartworm infection, discordant antigen test results, or high risk based on lifestyle and/or location.[25]

Acid Treatment

Acid treatment has been explored as an alternative ICD technique that could be better suited to in-clinic use because the required volume of sample is smaller and equipment needs are fewer.[28] Specifically, mixing trichloroacetic acid with canine plasma resulted in nearly as many conversions from NAD to positive status as heat treatment in 1 study.[28] While acid treatment has shown promising results, a recently published quantitative analysis comparing different ICD methods using heat, acid, or both concluded that heat treatment outperformed other ICD methods in consistently increasing antigen detection.[26]

The use of acid-treatment ICD has been explored in healthy pet cats in a large-scale nationwide survey; however, the results before and after ICD treatment were reported to be inconsistent.[17] Further exploration of acid-based ICD using well-characterized positive feline samples is needed to test the utility of acid treatment for diagnosis of heartworm infection in cats.

SUMMARY

The currently recommended annual heartworm screening protocol in dogs includes routine antigen testing performed in tandem with microfilariae testing. Heat-treatment ICD should be considered for dogs with an initially negative antigen test that meet the following criteria: present microfilariae, clinical signs or abnormalities consistent with heartworm infection, discordant antigen test results, or high risk based on lifestyle and/or location. For cats, heat-based ICD testing may be beneficial and should be considered for any cat suspected of having heartworm infection or disease. TVP

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Dr. Starkey recently rejoined the Oklahoma State University (OSU) College of Veterinary Medicine as an associate professor after several years at Auburn University. In 2007, she earned her B.S. degree in animal science from the University of Arkansas. She completed both her DVM and PhD degrees in veterinary biomedical sciences at OSU. She is a diplomate of the American College of Veterinary Microbiology with a subspecialty in parasitology, completing her residency training through the National Center for Veterinary Parasitology at OSU in 2015. She is involved in various research projects involving vectors, vector-borne pathogens, and diagnostic parasitology. She also teaches parasitology to veterinary students and has received 2 teaching awards, most recently the Zoetis Distinguished Teacher Award. She currently serves as a codirector of the National Center for Veterinary Parasitology and a board member of the American Heartworm Society.