trūRapid™ FOUR Test Product Bulletin

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Key Points

- Canine heartworm disease caused by the parasite *Dirofilaria immitis*, is a life-threatening disease.
- The prevalence of heartworm disease is increasing in North America and Europe.
- Lyme disease caused by the spirochete *Borrelia burgdorferi* (Bb) and related *Borrelia* strains results in lameness, fever, lethargy, anorexia and renal disease in dogs.
- The prevalence and geographic range of Lyme disease is increasing in North America, the United Kingdom (UK), and continental Europe.
- U.S. Dogs living in the South and in the Atlantic Coast region are at high risk for ehrlichiosis.
- Granulocytic anaplasmosis is an emerging disease of dogs, cats, horses, and humans.
- The Companion Animal Parasite Council (CAPC) recommends screening dogs for exposure to the agents of ehrlichiosis and anaplasmosis to identify dogs at risk of disease.
- The trūRapid FOUR test detects:
  - *Dirofilaria immitis* (heartworm) antigen
  - Canine antibodies to *Borrelia burgdorferi* (Lyme) antibody
  - Canine antibodies to *Anaplasma* spp.
  - Canine antibodies to *Ehrlichia* spp.
  - The trūRapid FOUR tests have excellent sensitivity and specificity relative to reference laboratory testing and current in-market rapid tests for heartworm, Lyme, *Anaplasma*, and *Ehrlichia*.

Introduction

Dirofilaria immitis, or heartworm, is a filarial parasite with an indirect life cycle, spreading from host to host through the bites of mosquitoes.[1] The parasite commonly resides in the pulmonary arterial system as well as in the heart. A major effect on the health of infected animals is a manifestation of damage to the lung vessels and tissues.[2] Diagnosis of *Dirofilaria immitis* infection in dogs is based on many factors, including the detection of an adult female worm antigen in serum, plasma, or anticoagulated whole blood.[3]

Lyme disease is a bacterial infection caused by *Borrelia burgdorferi* (Bb) sensu lato complex, which are bacterial spirochetes transmitted through the bite of an *Ixodes* sp. tick. In North America, *B. burgdorferi* is the main cause of Lyme disease; but in Europe, *B.* afzelii and *B.* garinii are the primary etiological agents.[4,5] Clinical signs of Lyme disease are most commonly reported in humans and in dogs. Asymptomatic infection is common in many domestic species.[6–8] Wildlife, especially deer and mice, are reservoir hosts for Bb.

Anaplasma spp. and *Ehrlichia* spp. are Gram-negative, obligate intracellular, tick-borne rickettsial bacterial pathogens infecting dogs and other hosts.[9] Multiple species of *Ehrlichia* and *Anaplasma* are reported to infect dogs in North America and Europe. In the USA, *E. canis*, and *E. ewingii* are the primary agents responsible for causing canine ehrlichiosis.[10] *A. phagocytophilum* and *A. platys* cause anaplasmosis in the canine population. *A. phagocytophilum*, the most common etiological agent of the disease, is transmitted by *Ixodes scapularis* (deer tick or Eastern black-legged tick) bites and infects white blood cells. *A. platys*, transmitted by *Rhipicephalus sanguineus* (brown dog tick), infects platelets.[11]

Antech™ trūRapid FOUR is a combination test that detects anine antibodies to heartworm antigen, Lyme C6 antibody, *Ehrlichia*, and *Anaplasma* antibodies on one device via lateral flow immunoassay technology.

Clinical Significance

Heartworm

Heartworm disease, caused by adult stages of *Dirofilaria immitis*, is a globally dispersed and clinically significant cardiopulmonary disease seen in canids and felids, as well as other genera (e.g., Mustelidae, Otariidae). Endemic areas in the United States have shown a prevalence up to 45 percent, with tropical climates conferring the chance of even higher infection rates.[1,12] Over the last ten years, heartworm has spread from Southern Europe into eastern and northeastern countries.[13] Furthermore, isolates resistant to macrocyclic lactones, the drug class to which all heartworm preventives belong,[14] have been reported in the United States[15] and Southern Europe.[16]

Clinical signs of *D. immitis* infection in dogs include respiratory distress, epistaxis, hemoptysis, ascites, exercise intolerance, and anorexia. But during the early stages of infection and disease, dogs typically present asymptomatically. Disease progression tends to lead to worsening clinical signs and prognosis.[3]

Medical and surgical management of canine heartworm disease can be difficult for a myriad of reasons, including the life cycle of *D. immitis* (Table A), side effects and efficacy of medical therapies, and clinical presentations in practice. Furthermore, even with complete resolution of the infection, some animals experience long-term effects from the damage inflicted by the infection. Prevention and monitoring remain key aspects of eradication programs, regardless of levels of endemicity.[3]

D. IMMITIS LIFE STAGE	MICROFILARIA, L1, L2, L3	L3	L4	DEVELOPING ADULT	ADULT
Stage Complete	10–14 days	3–4 days	45–65 days	4–5 months	6–7 months (post-infection)
Location	Mosquito	Host tissue	Host tissue	Host bloodstream	Host bloodstream

Table A: Life Cycle of *D. immitis*

Point-of-care heartworm antigen tests can detect *D. immitis* in whole blood, serum, or plasma and provide veterinary professionals with vital tools in diagnosing and monitoring canine heartworm infection and disease. Antigen tests are developed to recognize adult female heartworm antigen, which can complicate diagnosis in the presence of low worm burdens, prepatent infections with only larvae or immature stages of the worms, or all-male infections. Sensitivity and specificity remain high for antigen tests broadly, with as much as 99% sensitivity and 100% specificity, depending on the test type and worm burden.[3]

Borrelia burgdorferi and Canine Lyme Disease

The prevalence and geographical range of Lyme disease is increasing, although precise estimates are difficult due to variation in case definition and surveillance methods.[5,8,17–28] The Mid-Atlantic, Northeast, and Upper Midwest regions of the United States have the greatest prevalence of Lyme disease in the United States.[25] In Canada, most cases are reported in Ontario, Quebec, and Nova Scotia.[28] In the UK, the majority of cases are reported from Scotland and from South and Southwest England.[22,23] In continental Europe, the prevalence of Lyme disease is greatest in Scandinavia, the Baltic States, Austria, the Czech Republic, Germany, and Slovenia.[5] Expansion of the range of the *Ixodes* sp. tick vectors associated with climate change, encroachment of suburban into rural areas, and increased human activities in tick environments are important factors in the increased number of *B. burgdorferi* (Bb) infections.[7]

Diagnosis of Lyme disease depends on detection of Bb antibodies in the blood of dogs with characteristic clinical signs. The clinical signs and syndromes most associated with Lyme disease are lameness, fever, lethargy, anorexia, lymph node enlargement, and glomerulonephritis.[6,7] However, mostly Bb infections in animals are asymptomatic which presents challenges in confirming a diagnosis. Serology for the Bb C6 antibody is the recommended assay for supporting a diagnosis of Lyme disease and eliminates interference resulting from vaccine associated antibodies.[7] The C6 peptide is highly specific and antigenically conserved among various species of *B. burgdorferi* sensu lato complex. The C6 peptide derived from *B. burgdorferi* detects the infection regardless of infecting strains.[29–32] Levels of C6 antibody decrease in infected animals during treatment. The trūRapid FOUR test detects the C6 antibody.

Routine serologic screening of dogs is recommended in areas endemic for Lyme disease, followed by screening seropositive dogs for proteinuria.[7] The presence of Bb antibody indicates tick exposure and a risk for not only Lyme disease but also for a variety of other tick-borne diseases.[18,20,24,26] Confirming a diagnosis of Lyme disease in a symptomatic dog requires the detection of C6 antibody and the ruling out of other tick-borne diseases or other potential causes of the presenting clinical signs. Initiation of measures to limit tick exposure and enhance tick control is warranted in any dog seropositive for Bb.

Anaplasma spp. and *Ehrlichia* spp.

In the United States, canine granulocytic anaplasmosis is an emerging tick-borne disease that affects cats, dogs, horses, and humans. *Anaplasma phagocytophilum* is transmitted by *Ixodes* spp. predominantly found in the Upper Midwest, New England, parts of the Mid-Atlantic States, and Northern California. The 2024 forecast for ehrlichiosis in dogs in the United States suggests that there will continue to be a high risk of the disease in specific regions including the Southeast, Southwest, South Central, and Atlantic Coast. CAPC says it's important to note that the prevalence of ehrlichiosis can vary within a 200-mile range, emphasizing the significance of local awareness and understanding of the disease.[33]

The prevalence of *A. phagocytophilum* in Europe is estimated to be 19.91 percent. However, the prevalence varies by region, ranging from 0 to 61 percent.[34] While ehrlichiosis is not endemic in the UK, it's been reported in dogs that have traveled abroad.[35] One study found that 25 out of 76 dogs with tick-borne diseases were diagnosed with ehrlichiosis, and three of those dogs hadn't traveled outside the UK.[36] However, in other European countries, the test positivity rate for *Ehrlichia* decreased from 4.3 percent in 2016 to 3.4 percent in 2020.[24]

Dogs infected with *A. phagocytophilum* may have no signs of illness or only minor symptoms but often manifest lameness, joint pain, fever, lethargy, and anorexia. Clinical disease occurs in the acute phase of infection when morulae are more likely to be found in neutrophils in peripheral blood smears. *Anaplasma platys* infects canine platelets and is the causative agent of infectious canine cyclic thrombocytopenia (ICCT).[37,38] *Rhipicephalus sanguineus* has been identified as the vector for transmission of this organism. Although the infection is often mild or asymptomatic, it can potentially become fatal due to severe thrombocytopenia and subsequent hemorrhaging.[39]

Multiple species of *Ehrlichia* can infect dogs in North America. The clinical signs associated with *Ehrlichia* infections are often nonspecific and may include fever, lethargy, poor appetite, lymph node enlargement, and, in severe cases, abnormal bruising, bleeding, acute renal injury, acute respiratory distress syndrome, and neurological symptoms. *Ehrlichia ewingii* is the most common species infecting dogs in the United States. Clinical signs in dogs infected with *E. ewingii* can be similar to those of *E. canis*, including fever, lethargy, anorexia, lameness (polyarthritis), and in some cases, severe thrombocytopenia.[40]

Symptoms of ehrlichiosis in dogs include fever, lethargy, rapid wasting, nosebleeds, skin hemorrhages, breathing difficulties, abnormal lung sounds, swollen lymph nodes, and an enlarged spleen. Early treatment during the acute phase leads to favorable outcomes. However, if left untreated, dogs can progress to the subclinical phase, where they remain infected but asymptomatic. During this phase, the bacteria can persist in the spleen for months or even years. Anaplasmosis can also cause serious health complications for dogs if left untreated. Symptoms include fever, chills, muscle aches, nausea, vomiting, diarrhea, loss of appetite, and more.

Ehrlichia canis has been cultured in canine macrophage cell lines and tick cell lines, but this method isn't typically used for routine agent detection.[41] *Anaplasma platys* hasn't been cultured in vitro to date. Both pathogens are obligate intracellular bacteria, and the gold standard for confirming bacterial infections — routine bacterial culture — isn't a practical option for diagnosing these pathogens.

Anaplasma platys and *E. canis* invade canine platelets and monocytes, respectively, and replicate in the cytoplasm inside a parasitophorous vacuole forming a structure called the "morula." In *A. platys* infections, morulae within the platelet cytoplasm may be visible in stained blood films, but observing *E. canis* morulae in monocytes is rare.[42] *Ehrlichia ewingii* morulae can be observed in granulocytes (also true for *A. phagocytophilum* morulae), and *E. chaffeensis* can be seen in monocytes in Giemsa-stained blood smears, particularly during the acute infection phase.[43] Dogs infected with *A. platys* are often found in areas where *E. canis* infection is prevalent, and co-infection with both organisms has been documented in dogs.

Diagnosing ehrlichiosis and anaplasmosis involves assessing a combination of clinical signs, as well as positive serology, PCR testing, and hematology with a platelet count. Indirect fluorescent antibody (IFA) assays are used to determine IgM and IgG titers for *E. canis*, *E. chaffeensis*, and *A. phagocytophilum*. After the treatment and resolution of clinical signs, antibody titers may persist for months to years, indicating either a persistent infection or reinfection, particularly in *E. canis* infections. Therefore, the presence of antibodies alone doesn't indicate a need for treatment in the absence of clinical disease evidence.[44]

Technology

The Antech trūRapid FOUR in-house test strips are sandwich immunoassays intended for the visual, qualitative detection of heartworm (*D. immitis*) antigen and canine *B. burgdorferi*, *Ehrlichia* spp., and *Anaplasma* spp. antibodies in canine serum, plasma, or anticoagulated whole blood. Each trūRapid FOUR test is comprised of one cassette with three nitrocellulose membrane-based test strips.

The heartworm assays are set up as follows: two antibodies are used in the test strip for the detection of the *D. immitis* antigen from the female reproductive tract. One antibody is coupled with gold nanoparticles, and a second, high-specificity antibody is immobilized in the heartworm test line area (H).

The Lyme C6, *Ehrlichia*, and *Anaplasma* antibody assay methodologies are similar. A combination of a protein and highly specific Lyme C6, *Ehrlichia* spp., and *Anaplasma* spp. peptides are used in the test strip for antibody detection. The specific peptides are coupled with gold nanoparticles and the protein is immobilized in the Lyme (L), *Ehrlichia* (E), and *Anaplasma* (A) test line areas. The control gold nanoparticles bind to the antigen, a protein immobilized in the control line area, forming a control line (Figures 1 and 2).

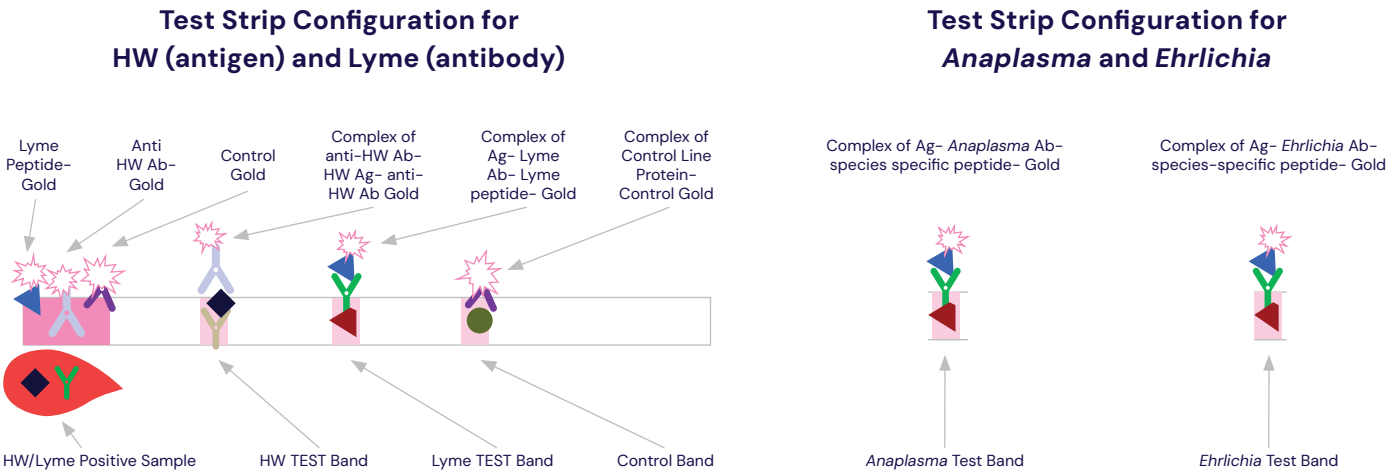

Figure 1: The technology behind trūRapid FOUR test strips

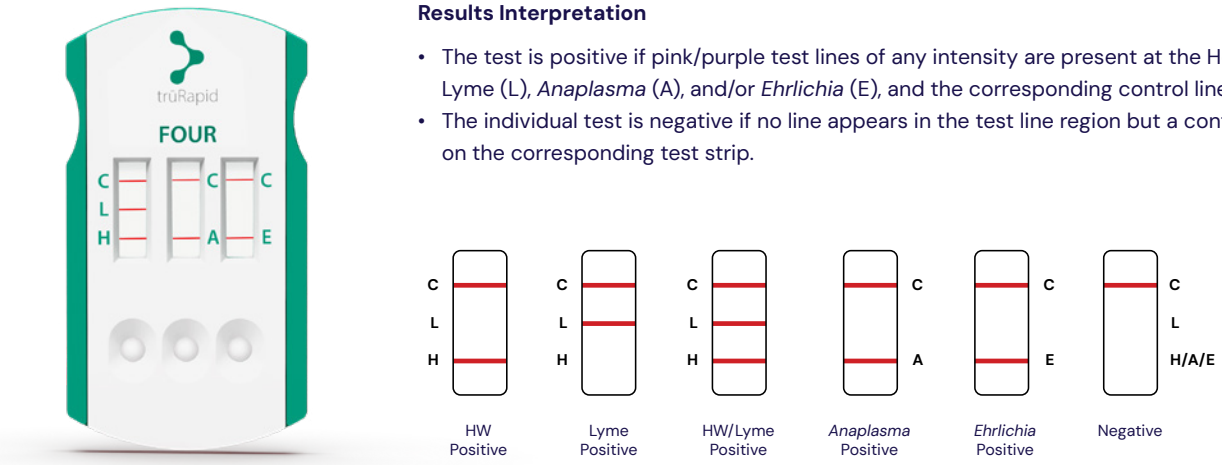

Figure 2: Results interpretation for trūRapid FOUR. Refer to the Instructions for Use for further details.

When the sample, followed by the sample buffer, is put into the sample wells, this will be absorbed into the pad of the test strip. The fluid mixes with the gold-labeled antibodies and peptides in the conjugate pad. Due to the capillary action of the test strip, the fluid will run up over the test strip, crossing the test line regions — (H) and (L) on the heartworm and Lyme strip, (A) on the *Anaplasma* strip, and (E) on the *Ehrlichia* strip — and then, subsequently, the control line regions. The control line should always appear to signify that each test is functioning correctly.

If the patient sample contains the antigen of even a single adult female heartworm, a line will appear in the heartworm test line region (H). This happens by building a sandwich between the gold-labeled antibodies from the conjugate pad, the antigen from the sample, and the immobilized antibody of the heartworm test line region. Even a faint line is considered positive for heartworm infection. If no antigen is in the sample, the gold-labeled antibodies cannot connect to the immobilized antibodies in the heartworm test line region, so no heartworm test line appears.

If the patient sample contains the *B. burgdorferi* C6, *Ehrlichia*, or *Anaplasma* antibodies, lines will appear in the respective areas of the indicated test strips (L, E, A). This happens by building a sandwich between the gold-labeled peptide from the conjugate pad, the Lyme, *Ehrlichia*, or *Anaplasma* antibody from the sample, and the immobilized protein of the Lyme, *Ehrlichia*, and *Anaplasma* test line regions. If the test is positive for *B. burgdorferi* C6, *Ehrlichia*, or *Anaplasma* antibodies, it means the animal has antibodies from natural exposure to these infectious agents.

If no *B. burgdorferi* C6, *Ehrlichia*, or *Anaplasma* antibody is in the sample, the gold-labeled peptide cannot connect to the immobilized protein, so no lines appear in the respective Lyme, *Ehrlichia*, or *Anaplasma* sections.

Material and Methods

Antech's trūRapid FOUR test strips were tested in this performance evaluation. All serum samples were collected from naturally infected dogs.

A total of 270 samples were tested in two comparison studies comparing the trūRapid heartworm and Lyme results to other methods. The heartworm performance data was generated by testing samples using a commercial enzyme-linked immunosorbent assay (ELISA) and a rapid antigen test. The comparisons for Lyme were generated by testing against an indirect fluorescent antibody assay (IFA).

A total of 200 samples were tested in comparison with *E. canis* IFA test (gold standard reference for both *E. canis* and *E. chaffeensis*) and in-house *E. canis, E. chaffeensis* and *E. ewingii* species-specific ELISAs. The 100 *Ehrlichia* spp. positive samples were obtained from various sources, including North Carolina State University (N=15); veterinary animal clinics in North Carolina (N=48); veterinary animal clinics in Michigan (N=13); Antech Diagnostics laboratory in Brownsburg, Indiana (N=9); University of Texas Medical Research (UTMB) (N=9); and the Animal Clinic of Bay Ridge, New York (N=6). The 100 *Ehrlichia* IFA-negative serum samples were sourced from various veterinary animal clinics in North Carolina.

The serum sample panel of 220 samples, which were characterized using the Anaplasma IFA assay was used. The *Anaplasma* spp. seropositive samples are obtained from various consisted sources, including veterinary animal clinics in North Carolina (N=36); North Carolina State University (N=6); veterinary animal clinics in Michigan (N=11); the Animal Clinic of Bay Ridge, New York (N=6); Antech Diagnostics laboratory in Brownsburg, Indiana (N=30); Colorado State University (N=10); and the University of Texas Medical Branch (UTMB), Galveston, Texas (N=15). The 106 negative serum samples were sourced from various veterinary animal clinics in North Carolina.

An analysis of Anaplasmosis prevalence in dogs in the United States in 2024 conducted by the Companion Animal Parasite Council (CAPC) showed that as of November 27, 2024, the overall canine Anaplasmosis national prevalence is 6.05 percent.[45] The positive prevalence rate in the study sample set largely exceeds the national incidence of Anaplasmosis in the US; however, it is appropriate for this study to ensure the diagnostic sensitivity of the kit is adequate to perform in the field as needed.

Results

Sensitivity and Specificity

The sensitivity and specificity of the trūRapid heartworm and Lyme tests were evaluated by dog serum samples and compared to the DiroCHEK® HW Antigen test and the Lyme IFA test results, as shown with a 2x2 analysis (Tables 1 and 2). All data is from naturally infected animals.

TRŪRAPID HEARTWORM TEST	DIROCHEK® HEARTWORM ANTIGEN TEST		TOTAL
	DETECTED	UNDETECTED	
Detected	150	2	**152**
Undetected	0	118	**118**
Total	150	120	**270**
Sensitivity: 100% (95% CI: 97.6 – 100%)			
Specificity: 98.3% (95% CI: 94.1 – 99.8%)			

Table 1: Comparison of heartworm test results: trūRapid versus DiroCHEK®.

TRŪRAPID LYME TEST	LYME IFA TEST*		TOTAL
	DETECTED	UNDETECTED	
Detected	103	3	**106**
Undetected	1	163	**164**
Total	104	166	**270**
Sensitivity: 99.0% (95% CI: 94.8 – 100%)			
Specificity: 98.2% (95% CI: 94.8 – 99.6%)			

Table 2: Comparison of Lyme test results: trūRapid versus IFA.

*VMRD - SLD-IFA-LD test

The sensitivity and specificity of the trūRapid Ehrlichia and Anaplasma tests were evaluated using dog serum samples and compared to the respective immunofluorescence assay (IFA) test results, and in-house, species specific ELISA for Ehrlichia test, as shown with 2x2 analysis Tables 3 and 4. All data is from naturally infected animals.

TRŪRAPID *EHRLICHIA* TEST	*EHRLICHIA* IFA* AND SPECIES-SPECIFIC ELISA** TEST		TOTAL
	DETECTED	UNDETECTED	
Detected	100	1	**101**
Undetected	7	92	**99**
Total	107	93	**200**
Sensitivity: 93.5% (95% CI: 87.0 – 97.3%)			
Specificity: 98.9% (95% CI: 94.2 – 100.0%)			

Table 3: Comparison of *Ehrlichia* test results: trūRapid versus IFA and ELISA.

*VMRD SLD-IFA-EC test
**Species-specific ELISA test performed for *E. canis, E. chaffeensis,* and *E ewingii*

TRŪRAPID *ANAPLASMA* TEST	*ANAPLASMA* IFA* TEST		TOTAL
	DETECTED	UNDETECTED	
Detected	110	2	**112**
Undetected	4	104	**108**
Total	114	106	**220**
Sensitivity: 96.5% (95% CI: 91.3 – 99.0%)			
Specificity: 98.1% (95% CI: 93.4 – 99.8%)			

Table 4: Comparison of *Anaplasma* test results: trūRapid versus IFA.

*VMRD SLD-IFA-AP test

Correlation studies were carried out to document the agreement between the trūRapid FOUR tests and the IDEXX® SNAP® 4DX® tests, as shown in Table 5. All data is from naturally infected animals.

PARAMETER	NUMBER OF SAMPLES TESTED (A)	TOTAL NUMBER OF SAMPLES DETECTED BY BOTH TESTS (B)	TOTAL NUMBER OF SAMPLES UNDETECTED BY BOTH TESTS (C)	TOTAL NUMBER OF SAMPLES WITH EXACT CORRELATION BETWEEN BOTH METHODS (D) = (B+C)	AGREEMENT OF TRŪRAPID FOUR VERSUS SNAP 4DX TEST (D) ÷ (A)
Heartworm	278	149	127	276	99.3%
Lyme	128	49	76	125	97.7%
Ehrlichia	228	93	125	218	95.6%
Anaplasma	138	99	38	137	99.3%

Table 5. Overall correlation summary when comparing trūRapid FOUR to SNAP 4Dx test results.

Conclusion

The study demonstrates that the Antech trūRapid FOUR test delivers excellent sensitivity and specificity for heartworm antigen, Lyme C6 antibody: *Ehrlichia* spp. canine antibodies, and *Anaplasma* spp. canine antibodies when compared to reference laboratory and in-house, rapid test methods. It's a reliable test that provides rapid results for the screening and detection of these vector-borne disease pathogens.

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