How to Use PCR and Serology in Vector Borne Disease Testing: Which Tests and When?

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Vector borne disease is an important cause of morbidity and mortality in dogs. Clinical findings caused by infection include those commonly associated with idiopathic immune mediated disease. Interestingly, like vector borne disease, the incidence of idiopathic immune mediated disease has been shown to be seasonal in some studies. It has been hypothesized that many patients with immune mediated disease suffer from undiagnosed underlying infection with vector borne and other infectious agents. Inappropriately declaring that vector borne disease has been ruled out in a patient with immune mediated disease because of a "negative tick panel" may significantly impact morbidity and mortality.

"Tick panels" and "vector borne disease panels" offered by diagnostic laboratories or in house test kits give clinicians the ability to test peripheral blood for multiple agents using serology, polymerase chain reaction (PCR) or a combination of the two. Choosing a panel requires the clinician to consider which organisms to test for, and which methodology (PCR or serology) to employ. Knowing the epidemiology and clinical findings that are most commonly associated with each organism help determine which agents should be included in a diagnostic panel. The sensitivity of serology as compared to PCR varies with characteristics of the host, the assays, and pathophysiologic characteristics of the organism. Therefore, using either PCR or serology exclusively may overlook the presence of infection. Knowing when clinical signs occur, when and if organisms circulate in peripheral blood, and whether they circulate in high or low number helps determine whether serology and/or PCR, or acute and convalescent serologic testing are most appropriate for an individual patient.

Which organisms should be included in a panel?

Knowing epidemiology and common clinical findings helps determine which organisms should be included in a panel for a particular ill patient. This consideration may be more important for serologic testing, as most serologic panels are not as comprehensive as widely available PCR panels. It is important to be sure that agents that are commonly associated with the clinical findings for a particular patient are included in diagnostic testing. For example, in a dog with IMHA, a negative result for a "tick panel" that tests for antibodies to Borrielia burgdorferi, Rickettsia rickettsii and Ehrlichia canis would not effectively rule out vector borne disease as a cause for IMHA, as E. canis is the only one of these three organisms that has been associated with IMHA in dogs. Babesia, Anaplasma, Bartonella and haemotropic Mycoplasma species would also be important to include in diagnostic testing for a dog with IMHA. Furthermore, serologic crossreactivity among different species within a genus does not always occur. It is important to be sure that all the species of a genus that are likely in a particular clinical scenario are included in a serologic panel. For example, a Staffordshire terrier with hemolytic anemia due to infection with B. gibsoni may test negative for antibodies to B. canis. Knowing that B. gibsoni is a likely cause of hemolytic anemia in this breed, helps guide the clinician to use a panel that includes this agent. Consideration of geographic locale is also important. For example, it would be theoretically more important for a clinician practicing in Wisconsin to include testing for A. phagocytophilum for a thrombocytopenic dog with no travel history than a clinician practicing in South Dakota. Summaries of common clinical signs and geographic distributions of infectious agents are available. Comprehensive testing can also be beneficial, because the geographic distribution of vector borne agents is expanding, as are the clinical descriptions of diseases associated with these agents. Restricting diagnostic testing to a few organisms may overlook infection in some patients.

Which methodology should I use, PCR or serology?

Most serologic tests for vector borne disease agents indirectly document infection by detecting antibodies. Whole organisms or select antigens are used as targets for antibodies in the assays. An important limitation of serology is that for most assays, a positive result for a single sample indicates exposure but not necessarily active infection for some agents. This makes it difficult to determine if clinical findings can be attributed to infection, particularly in endemic areas. Despite this limitation, serology has been the mainstay of diagnostic testing for vector borne disease for decades due to technical difficulties associated with isolating and culturing these organisms from patient samples. PCR detects DNA of organisms themselves without the need for culture. Recently, advances in molecular biology have made PCR panels easy to perform and widely available. PCR makes it possible to directly test for the presence of multiple organisms in peripheral blood and other samples with high sensitivity and specificity, assuming appropriate laboratory controls are in place. Despite these advances in molecular biology, serology still has an important role in the diagnosis of vector borne disease. Consideration of the pathophysiology of the organism and the strengths and limitations of the assays in the context of the clinical presentation can help the clinician determine which methodology is most appropriate and whether combination testing is indicated. Specifically, considering whether the organism is likely to be circulating in blood at the time of presentation, and the

significance of a positive or negative test result can help determine whether PCR or serology should be used or if additional testing is warranted.

Is the organism likely to be present in the blood sample at the time of presentation?

In order for PCR to be positive, the organism has to be present in the sample. Many current PCR assays are very sensitive and can reliably detect the presence of only one organism in a sample. Therefore, if organisms are circulating in blood in high numbers at the time they cause clinical signs, it is very likely that a PCR test will detect the organism. However, clinicians should keep in mind that the aliquot of blood used to extract DNA for PCR is small (eg. 200uL) and the volume of extracted DNA used in the assay is even smaller (eg. 5uL). Therefore the organism's DNA might not be present in a test sample even if the organism is present in peripheral blood in high numbers, and the PCR assay is exquisitely sensitive. For example, dogs infected A. phagocytophilum exhibit clinical signs acutely after infection. Anaplasma phagocytophilum infected granulocytes circulate in peripheral blood and can be present in relatively high numbers during this acute phase. Therefore PCR testing of peripheral blood is sensitive. However, if the organism does not happen to be present in a particular aliquot used for the assay, or the patient has received medication such as doxycycline that decreases circulating copy number, PCR can be "falsely" negative. Additional PCR testing (if doxycycline has not been given) of the acute sample or acute and convalescent serologic testing can confirm active infection if the initial PCR is negative.

Documenting seroconversion is particularly useful for diagnosing acute infection with agents that circulate in peripheral blood in low copy number. In this scenario, PCR is inherently insensitive due to the pathophysiology of the organism. For example, R. rickettsii causes acute illness in dogs, and these endotheliotropic organisms circulate in low copy number in peripheral blood. Therefore, PCR is not as sensitive as is demonstrating a four-fold change in titer.

Serologic testing can also help diagnose infection with agents that cause chronic infection. Interestingly, clinical signs of Lyme borreliosis do not occur in experimentally infected dogs until 2-6 months after tick exposure. Organisms do not circulate in peripheral blood in high numbers. Consequently, serologic testing rather than PCR is routinely used to document exposure to B. burgdorferi using peripheral blood samples. Ehrlichia canis can cause clinical signs during acute or chronic infection in dogs. This organism may circulate in blood in low copy number and/or intermittently during chronic infection. Similarly, Bartonella species also may circulate only intermittently or in low copy number during chronic infection. Serology can indicate exposure if PCR is negative and thereby facilitate diagnosis. Alternatively, to document infection with Bartonella, sterilely collected samples of whole blood can be grown in enrichment media prior to PCR to increase sensitivity. In summary, for organisms that circulate in low copy number or intermittently at the time clinical signs occur, serology may facilitate diagnosis.

What is the significance of a positive result in my patient?

Assuming appropriate laboratory controls are in place, a positive PCR result indicates active infection or the presence of circulating dead organisms. Response to therapy will help determine if presenting clinical signs can be attributed to infection or if coinfection or other underlying disease should be considered. PCR assays can be positive at the level of genus or species, depending on the specific DNA sequence that the primers target. If sequencing is not performed to verify the infecting species, it is possible, albeit unlikely, that a species is actually different from the reported species. This may have implications with regard to treatment and prognosis, among other factors. Considering whether or not infection with the specific agent makes sense given the signalment, clinical signs and geographic locale is helpful. If the results don't make sense for a patient, consulting the laboratory and further testing, including DNA sequencing can be considered.

When considering the significance of a positive titer on a single sample it is again useful to consider the pathophysiology of the organism in the context of the individual patient because a positive serologic test may indicate exposure and not necessarily active infection. For example, R. rickettsii and A. phagocytophilum primarily cause acute rather than chronic disease in dogs. However, previous infection may result in long lived antibody titers. If serologic testing is positive for these agents and clinical signs have been present for many months, previous exposure, but not active infection is likely. In this case co-infection with other vector borne agents, the presence of cross reacting antibodies, or the presence of other underlying disease should be considered. Additional testing of banked or newly obtained samples using PCR and/or serology may be useful in such cases.

Serologic crossreactivity among species within a genus and between genera also affects the interpretation of a positive serologic test. As is the case with PCR, considering whether or not infection with the specific agent makes sense given the signalment, clinical findings and geographic locale is helpful. For example, there is extensive serologic crossreactivity among spotted fever group (SFG) Rickettsia. In general the species of infecting Rickettsia is been presumed based on geographic locale, so if a dog from the South Eastern US seroconverts to R. rickettsii, Rocky Mountain spotted fever (RMSF) becomes the presumptive diagnosis. However, other species of SFG Rickettsia that infect people are present in the US and it is also likely that these organisms infect dogs and induce disease. Furthermore, exposure to nonpathogenic SFG Rickettsia, some of which are common endosymbionts in ticks, may be a common cause of positive titers, particularly low and persistent titers, to R. rickettsii in dogs. Serologic cross reactivity with SFG Rickettsia also appears to occur in dogs infected with Bartonella henselae. Therefore a single positive titer to R. rickettsii may not

represent infection with that organism. PCR testing on banked acute EDTA samples can facilitate species verification and acute PCR and convalescent serology can help confirm or rule out the presence of active infection in seropositive patients.

When interpreting results of positive serologic assays in individual patients, clinicians should also keep in mind that infection with novel species and expanding geographic distribution of known species occurs, as this phenomenon is responsible for many outbreaks of emerging infectious disease. For example, the geographic distribution of RMSF in the United States has increased beyond the distribution of its primary tick vectors, Dermacentor variabilis and D. andersonii. Rhipicephalus sanguineous, a ubiquitously distributed tick, recently caused an outbreak of RMSF with associated mortality in a non-endemic area of Arizona. Retrospectively, it was shown that infection existed in the dog population before the fatal outbreak in people. This illustrates how a positive titer in a nonendemic region does not necessarily represent a false positive result and may represent disease emergence.

What is the significance of a negative result in my patient?

The pathophysiology of an infecting agent can also affect the overall sensitivity of PCR testing. As alluded to previously, PCR may have a high sensitivity in that it can reliably detect one copy of target DNA in a sample (absolute sensitivity), but a low sensitivity with regard to determining whether an organism is infecting a patient (clinical sensitivity) if organisms normally circulate only transiently during a particular phase of infection or in low numbers in peripheral blood. Thus even the most sensitive PCR may not indicate that an animal is infected with a particular agent, and the presence of infection cannot be definitively ruled out based on a negative result. Therefore, repeating PCR or performing serologic testing is necessary to rule out infection in some cases. PCR may also be negative because the infecting species is not targeted by primers. Primers that target genus rather than species facilitate testing for a broad range of species in an individual patient.

Serologic testing on a single sample may also be negative in the face of infection if the organism causes clinical signs in the acute phase of disease, prior to the time when antibodies are formed. For example, serology might not indicate exposure in dogs acutely infected with agents like R. rickettsii, E. canis, Babesia species and A. phagocytophilum, because clinical signs can sometimes manifest prior to seroconversion. Convalescent testing documenting seroconversion or PCR on acute samples may confirm active infection in these acutely infected patients.

Surprisingly, and unfortunately, serologic testing can also be negative during chronic infection. For some organisms, such as Babesia and Bartonella, antibodies are not detectable in some chronically infected patients even after seroconversion should have already occurred. PCR is necessary to document infection in these cases.

Sensitivity can also differ among different types of serologic assays testing for the same organism. For example, some dogs actively infected with E. canis test seropositive using IFA and seronegative using ELISA. This may be related to differences in the nature of antigens used in the respective assays.

Finally, the presence of some organisms cannot be tested for using serology because they can't be grown in culture, they have never been isolated, or a commercial assay is not available. Examples would include haemotropic Mycoplasmas and Babesia conradae. PCR or microscopic examination of blood smears is needed to document infection with these agents.

The importance of banking samples.

In order to enable appropriate testing using PCR and serology, both serum and EDTA samples can be obtained at initial presentation. Based on the presenting scenario, a decision can be made to determine if testing using serology or PCR or both is most appropriate initially. If initial PCR or serologic testing is negative, and both testing methodologies were not employed, banked samples can be accessed to test using alternative methodology. Convalescent serologic testing can be considered in order to confirm active infection, particularly when there is an acute history of illness. Repeating PCR on initial or additional samples may also be advantageous in some cases.

Summary

Consideration of the epidemiology, associated clinical findings, and pathophysiology of vector borne disease agents is helpful in determining which organisms to include in testing and which methodologies should be employed for a given presenting scenario. Combination testing using PCR and/or acute and convalescent serologic testing may be necessary to accurately diagnose infection with vector borne agents in some patients and should be considered. Most importantly, clinicians should remember that a "negative panel" using either serology or PCR, does not necessarily rule out the presence of underlying vector borne disease in a patient with clinical signs of immune mediated disease.

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