CHAPTER 4

Immunodiagnostic and Molecular Diagnostic Tests in Veterinary Parasitology

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IMMUNODIAGNOSTIC METHODS IN PARASITOLOGY

Immunodiagnostic methods for a range of parasitologic infections are selectively available at fee-for-service diagnostic laboratories and as point-of-care tests that can be conducted in a clinical setting.

There are two basic approaches in designing an immunologic test. **Antigen detection** tests identify specific parasite-associated compounds in blood, serum or fecal suspensions that indicate the presence of the organism in the host. Alternatively, **antibody detection** tests show the host immune response to a parasite through the production of specific antibodies. In order to have a positive test result, it is assumed that the host animal is immunologically competent to react to the pathogen and that a sufficient time of exposure has occurred for the animal to produce detectable antibodies.

There are a variety of test formats for immunodiagnostic tests. The enzyme-linked immunosorbent assay (ELISA) is designed with a series of wells in a plate or tray with an end result indicated colorimetrically (Fig. 4.1). The lateral flow immunochromatographic assay uses similar principles and reagents in a cassette format, and works by capillary action with a series of reagents moving along a membrane with the end result indicated as a colored dot or line on the membrane (Fig. 4.2). Both of these test formats can be designed as antigen or as antibody detection assays, and both test formats have been developed for use with blood, serum, or feces. Most ELISA tests are designed for processing sample batches in a diagnostic lab setting. One benefit of this test format is that the intensity of color generated in the reaction is measurable using a spectrophotometer and is generally proportional to the antigen/antibody that is being detected. Therefore the ELISA may be used as a quantitative measure in a carefully calibrated test. The immunochromatographic tests are designed to give

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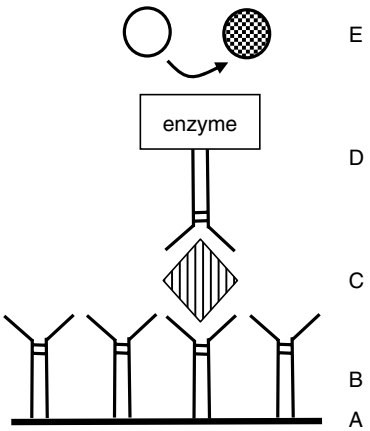

Fig. 4.1 Schematic ELISA antigen detection procedures: (A) test surface: polystyrene well; (B) parasite-specific capture antibody (may be monoclonal or polyclonal); (C) parasite antigen in serum of the animal patient; (D) detecting reagent parasite-specific antibody labeled with an enzyme; (E) visualizing step: if enzyme is present, it acts on soluble substrate to produce color, which can be evaluated visually or measured spectrophotometrically.

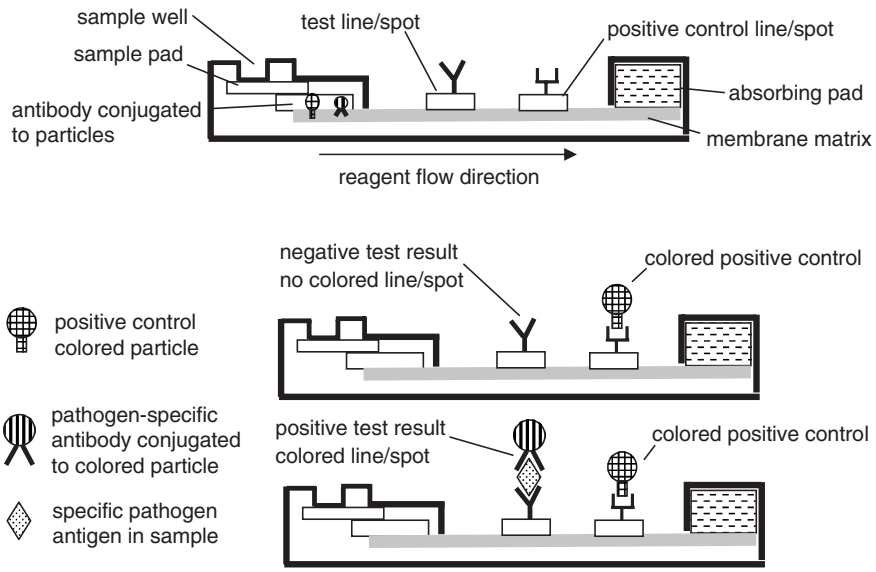

Fig. 4.2 Schematic of antigen detection using an immunochromatographic lateral flow test.

positive/negative results and are not generally designed to be quantitative; therefore the intensity of the colored dot/line is not necessarily proportional to the amount of antigen/antibody detected.

Immunochromatographic tests and selected ELISA assays are available as point-of-care tests that can be performed in a clinical setting on one or several samples in a relatively rapid time frame. Two of the most widely used parasitologic immunochromatographic or ELISA tests in companion animal medicine are the heartworm antigen test, which detects antigens primarily produced by adult female *Dirofilaria immitis* and

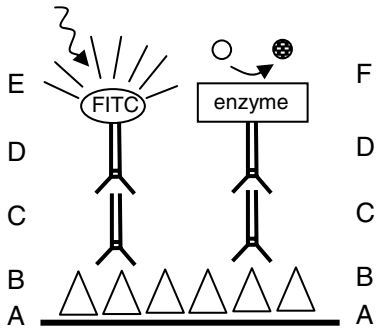

Fig. 4.3 Comparison of common antibody detection procedures: indirect fluorescent antibody (IFA, *left*) test and enzyme-linked immunosorbent assay (ELISA, *right*). A, test surface: glass slide (IFA) or polystyrene well (ELISA); B, parasite antigen: whole parasite, such as cultured tachyzoites and promastigores (IFA), or soluble parasite antigen, which can be a crude homogenate, a purified protein, or a recombinant protein (ELISA); C, serum of the animal patient, which may contain parasite-specific antibodies; D, detecting reagent for host-specific antibody: host-specific antibody labeled with a fluorochrome such as fluorescein (IFA) or host-specific antibody labeled with an enzyme (ELISA); E, visualizing step: specific UV wavelight from microscope causes fluorescein (FITC) to emit yellow-green fluorescence (IFA); F, visualizing step: if enzyme is present, it acts on soluble substrate to produce color, which can be evaluated visually or measured spectrophotometrically.

detected in blood, serum, or plasma, and the fecal antigen test for *Giardia duodenalis*. Since these types of assays are convenient, generally inexpensive, and relatively easy to perform in a clinical setting, it is likely that more point-of-care tests for additional parasites may become commercially available in the future.

Another commonly used format is the indirect immunofluorescent assay (IFA), which is an **antibody detection** test designed for use with serum or plasma (Fig. 4.3). These tests are routinely performed in the diagnostic lab setting because a compound microscope equipped with appropriate barrier filters and a UV light source is needed to conduct the test. The test result is typically expressed as a "titer" and the IgG antibody isotype is usually the immunoglobulin that is detected in the test. The titer value is the reciprocal of the highest dilution of serum/plasma where the test remains positive. Different test formats (ELISA vs. IFA most commonly) have different thresholds of antibody detection. Therefore, the positive/negative cutoff value for each standardized assay should be provided by the laboratory performing the test in order to adequately interpret the meaning of the antibody titer as positive/negative or high/low. For example, the cutoff value for an IFA test might be a 1 : 10 dilution, while a similar test in an ELISA format might have a 1 : 100 dilution cutoff value as positive.

Other less common immunodiagnostic test formats include direct or indirect hemagglutination (HA or IHA), complement-fixation tests (CF) and western blot tests for antibodies that react to selected parasites/pathogens. These tests are conducted at fee-for-service labs since reagents and equipment to conduct these assays are not routinely available in a clinical setting.

It is important to have an understanding of the life cycle and pathogenicity of a specific parasite in order to interpret antibody titers in a specific test. A helpful memory tool is the acronym, "PIE,", which represents Protected, Infected, or Exposed. For some pathogens (usually viruses, but parasites in a few cases), having a high antibody titer is considered protective. For instance, if a cat has a significant antibody titer against

Toxoplasma gondii, that animal is very unlikely to shed oocysts, and it is a low-risk animal as a pet for an immunocompromised or pregnant owner. However, having antibodies against a parasite is not protective in most cases. With most parasites, having a positive titer may indicate current infection or previous exposure without current active infection. Having a measurable antibody titer can be of significant diagnostic value in confirming a parasite infection as the cause of clinical disease. For example, if a dog has chronic dilated cardiac disease with a titer to *Trypanosoma cruzi*, a probable diagnosis is Chagas disease. Alternatively, having an antibody titer may indicate exposure, but not necessarily an active parasite infection. An example of this situation is a domestic cat with an antibody titer to *Dirofilaria immitis*. The cat can produce detectable antibodies to the parasite after exposure to larvae from an infected mosquito bite, but those parasites do not necessarily develop successfully to adult worms causing a patent infection. In summary, interpretation of serologic results is diagnostically helpful in the context of understanding the role that antibodies play in the host–pathogen relationship for each parasite.

In a clinical setting, the veterinarian must interpret the diagnostic test results in the context of the clinical data available for an individual patient or on a group basis for a herd/flock. The diagnostic accuracy of immunodiagnostic tests has traditionally been framed in the context of **sensitivity** and **specificity**). Sensitivity is defined as the probability that an animal that has the infection/disease will test positive, and a sensitive test indicates few false negative results in an infected population (Table 4.1). Specificity is defined as the probability that an animal that does not have the disease will have a negative test result, and a specific test yields few false positive test results in an infection-free population. Typically, commercial point-of-care tests will make sensitivity and specificity data available for each type of test/kit, which provides overall information on the accuracy of the test. However, the use of these terms may have limited applications in a clinical setting when dealing with a problematic or confusing test result for an individual patient.

Other terms that may aid in test result interpretation are **positive and negative predictive values**. These values correspond to the probability that an individual truly is or is not infected, given a positive or negative test result (Table 4.1). These values differ from sensitivity and specificity since predictive values are dependent on the prevalence of infection/disease in the population of interest. The positive predictive values increase as the prevalence of infection increases in the tested population. Conversely, negative predictive values increase as the prevalence of infection decreases in the tested population.

Table 4.1. Calculation of the diagnostic accuracy of a test (sensitivity, specificity, predictive values)

	True infection status		
Test result	Infection present	Infection absent	Total
positive	a	b	a + b
negative	c	d	c + d
total	a + c	b + d	a + b + c + d

sensitivity = a / (a + c)
specificity = d / (b + d)
positive predictive value = a / (a + b)
negative predictive value = d / (c + d)
Source: Modified from table in Timsit et al., 2018.

MOLECULAR DIAGNOSTIC METHODS IN PARASITOLOGY

The advent of molecular diagnostic methods has resulted in an expanding selection of sensitive and specific tests for a range of infectious organisms and genetic diseases. Experimental molecular diagnostic tests for parasitic infections are frequently reported in the scientific literature, and an increasing number of specific tests are commercially available to detect parasitic pathogens. Generally, molecular diagnostic tests are able to detect pathogen DNA at very low concentrations when compared with typical antigen detection immunodiagnostic tests. The high sensitivity of these molecular tests makes them especially attractive in cases of low parasite burden.

Molecular diagnostic assays detect DNA (or sometimes RNA) from a specific parasite/pathogen generally indicating active infection. Assays can be performed with many types of samples including blood, urine, tissues, feces, and other body fluids as well as environmental samples. Regardless of the sample matrix, the first step in a protocol is to isolate the nucleic acid for testing. The polymerase chain reaction (PCR) technique is a widely used molecular technique that amplifies and detects a specific short piece of target nucleic acid (such as from a parasite) (Fig. 4.4). In a well-validated assay, the test may be considered positive if the appropriately sized DNA amplicon is visualized by gel electrophoresis. In other cases, in order to confirm the identity of the parasite/pathogen, the amplified DNA fragment must be further analyzed by nucleic acid sequencing for comparison with previously determined target gene sequences from a known pathogen.

Currently, the most popular version for molecular diagnostic tests is the quantitative PCR (qPCR, sometimes called "real-time" or rtPCR), which is a rapid test that is sensitive and reliable for the detection of molecular targets. The technique is quantitative

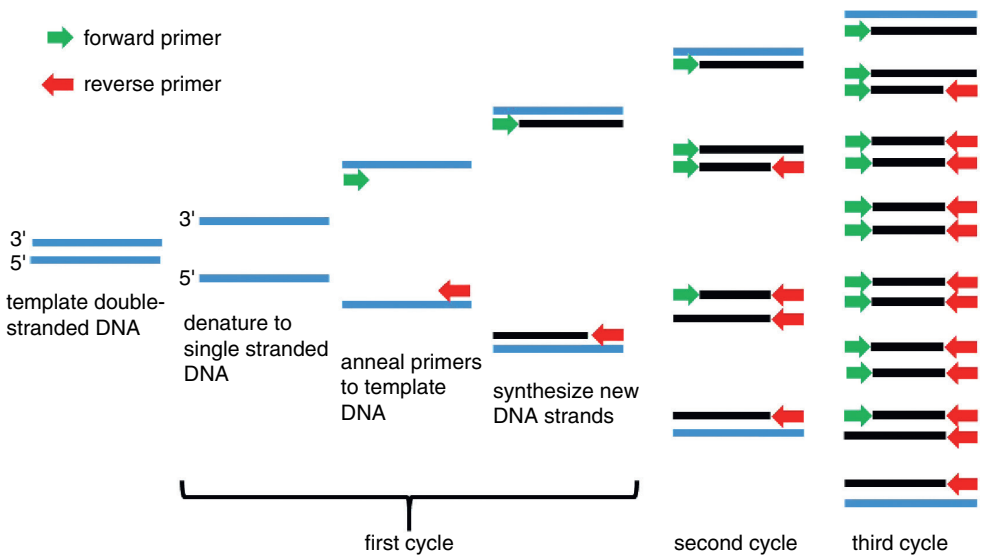

Fig. 4.4 Schematic of a conventional PCR assay. PCR is a process used to selectively amplify a targeted section of double-stranded DNA in a sequence of repeated steps. Double-stranded template DNA is first heated to denature to single-stranded DNA, and then cooled so that pathogen-specific short DNA primers can anneal to their complementary targets on the template DNA. By adding dNTP nucleotides and a DNA polymerase enzyme, new strands of DNA are synthesized. By repeating the cycles of heating and cooling the target DNA segment is amplified in an exponential manner.

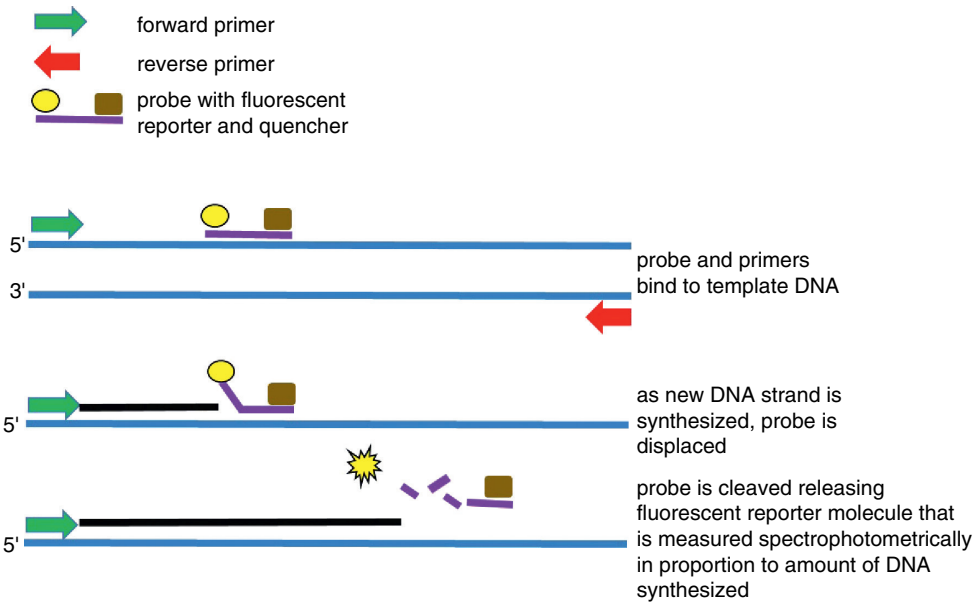

Fig. 4.5 Simplified illustration of how a qPCR assay differs from a conventional PCR assay. The qPCR uses primers to amplify a targeted section of template DNA similar to conventional PCR (see Fig. 4.4), but it also adds a sequence-specific DNA probe with a fluorescent reporter molecule and a quencher molecule that bind to the single-stranded DNA template. As the new strand of DNA is synthesized, the probe is cleaved releasing the fluorescent reporter molecule. The fluorescence intensity is measured spectrophotometrically at each cycle indicating the amount of DNA synthesized. Since a larger amount of template DNA in the starting sample will result in greater fluorescence in fewer cycles, the method is called "real-time" PCR or "quantitative" PCR.

and real-time because the DNA amplification product is measured repeatedly after each cycle of DNA amplification through the detection and quantification of a fluorescent reporter molecule (Fig. 4.5). An advantage of the qPCR assay is that it can be performed in a shorter time period than a conventional PCR. Also, the technique can be designed to simultaneously detect several pathogens by using a mixture of pathogen-specific primers along with sequence-specific probes that are labeled with fluorochromes of various detection wavelengths (Fig. 4.6). The accuracy of the test depends on the quality and quantity of DNA or RNA extracted as well as the presence/absence of inhibitors that can negatively impact the assay. A detractor to PCR methodology is that very small samples of a few microliters of fluid or a few milligrams of tissue or feces are processed for this assay, so it is possible that parasite DNA is not included in the tested sample. Therefore, a positive PCR test result indicates the presence of parasite DNA, while a negative test does not rule out possible infection with the target pathogen.

A parasitologic molecular diagnostic test widely used at this time detects the venereal protozoan of cattle, *Tritrichomonas foetus*, in preputial swabs or specialized short-term *in vitro* cultures. Since this parasite has become a regulatory issue in many states, the validation and comparison of molecular and *in vitro* culture methods has become a topic of interest to many diagnostic laboratories, and to veterinary practitioners and their clients as well. New test offerings, particularly in companion animal medicine, are also becoming available from an expanding number of commercial labs. Illustrating the potential for multi-pathogen molecular tests, canine vector-borne disease agent panels

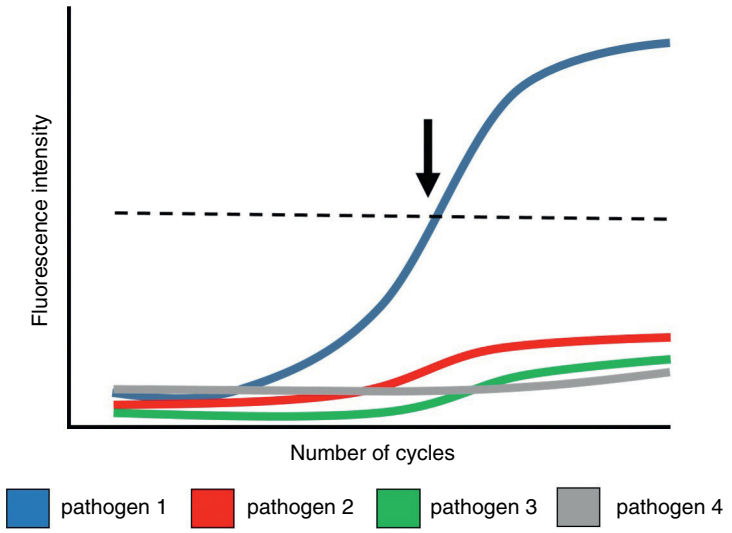

Fig. 4.6 Simple schematic of fluorescence plot of results from a multiplex qPCR assay that potentially detects four pathogens in a single test. For each pathogen, specific primers and probes labeled with fluorescent reporter molecules that emit light at different wavelengths are included in a single assay (see Fig. 4.5). If pathogen template DNA is present in the test sample, then an increasing amount of fluorescence at a specific wavelength is detected as the number of heating/cooling cycles increases. Based on previous standardization of the test with known pathogen DNA, a sample (blue pathogen 1) is considered positive if the fluorescence intensity reaches a minimum threshold at the completion of a pre-determined number of cycles (arrow). If no pathogen template DNA is included in the test sample, then no new DNA is synthesized and no fluorescence is produced for those pathogens, and the test is considered negative (pathogens 2, 3, and 4).

are offered by a number of commercial and university-associated diagnostic laboratories on a fee-for-service basis. These panels may include *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., *Trypanosoma cruzi*, *Leishmania* spp., and sometimes hemoprotozoan parasites such as *Babesia canis*, *B. gibsoni*, *Cytauxzoon felis*, or *Hepatozoon* spp. Similarly, diarrheal disease molecular panels for a variety of animal hosts that are readily available commercially sometimes include *Giardia duodenalis* or *Cryptosporidium* spp. In the future, it is likely that molecular tests for additional parasites will be available as individual assays or as part of clinically relevant panel screens from commercial laboratories based on clinical demands and the need to develop better diagnostic techniques for problematic parasitic pathogens. At this time, molecular diagnostic tests are conducted in fee-for-service labs or research labs due to the expense of the necessary equipment and the technical expertise used in conducting the tests. However, it is likely that in the foreseeable future, simplified benchtop PCR equipment and pathogen-specific reagent kits will be available for diagnostic use in a clinical setting.

As with any diagnostic testing, false positive or false negative results may occur for any individual immunodiagnostic or molecular diagnostic test. To minimize the likelihood of errors and to ensure accurate results it is important that all involved personnel are well trained and are monitored for consistent performance in following procedures and/or performing assays. When sending out samples for testing remember to: (1) collect samples in appropriate, accurately labeled containers, (2) store/ship/submit samples at the correct temperature using packaging containment appropriate for biological samples, (3) include appropriate submission forms with adequate clinical

history and patient information, (4) when test results are received, promptly enter information into the appropriate medical record. Similarly, best practices for point-of-care tests should include: (1) carefully collect, label, handle, and store samples correctly, (2) only use nonexpired, properly stored and handled reagents and kit/assay contents, (3) follow a standard written protocol for collecting samples and performing each assay according to the manufacturer's instructions, (4) promptly and accurately record test results in appropriate medical records.