# PCR Diagnostics: The New Millennia Kendal Harr, DVM, DACVP URIKA, LLC Mukilteo, WA

Within the past 20 years, there has been a dramatic increase in the numbers of tests available and the ability to make diagno sis in veterinary medicine using polymerase chain reaction (PCR). Applications of this analytic approach are seen in several areas, including cancer testing, inherited diseases, and infectious disease including viruses, bacteria, and drug resistance. There is variation in results between laboratories that perform the same assays (e.g. PCR testing for morbillivirus or Chlamydophila in birds) as they frequently use different primers and assay conditions. Detail on sensitivity and specificity of the tests are required for appropriate interpretation as the likelihood of false positives and negatives must be considered prior to final diagnosis. Many of the tests are advertised as being "the most sensitive analysis available". But what does the word sensitive really mean? There are actually two different scientific meanings for this word in laboratory medicine that must be clearly differentiated in order to understand the assay. These are not emotional words but only one is specifically analytical.

#### Analytical sensitivity

Is synonymous with the lower limit of detection (LLOD). This is required as part of assay validation and all manufacturers should be able to tell you the lowest concentration, number of strands, etc. that the assay can measure. PCR is exquisitely analytically sensitive and can frequently detect even just a couple of organisms. Therefore, many of these advertizements are true. But does this always help you diagnostically?? The answer is frequently no, it does not. Diagnostic sensitivity is different that analytical sensitivity. This is what is taught to you in veterinary school and should not be confused with the language used in diagnostic advertising. Diagnostic sensitivity is the likelihood that healthy animals in a population are diagnosed as healthy.

### True negatives/true negatives + false negatives

So this reflects the number of false negatives in a population. Yes, diagnostic sensitivity may also be low but this encompasses many more issues such as sampling and transport as well as appropriate selection of primers for disease state.

For example, PCR for feline corona virus that may cause feline infectious peritonitis is available. This may be useful as we all know that FIP can be difficult to diagnose definitively even when strongly suspected. It is frequently necessary to use all the diagnostics in the armamentarium to be sure that the diagnosis is correct prior to the frequently inevitable euthanasia. Howe ver, as with the other diagnostics used with FIP, detailed knowledge of the assay is important for correct interpretation. Corona virus is an RNA virus which means that it is far more likely to degrade over time if not frozen at -80 which rarely happens in diagnostic laboratories. Therefore, it is quite possible that a sample that is positive when initially extracted from an animal will be negative when analyzed due to nucleic acid degradation. This results in a relatively high false negative rate. Additionally, the primers that are correctly identifying feline coronavirus cannot differentiate between corona virus that causes mild diarrhea and coronavirus the causes FIP. On top of that, the primer will identify all remnant nucleic acid regardless of whether the nucleic acid is contained in a live virus that can actually cause disease or not. Within this assay, there is a great deal of "real-life" issues which may cause false positives and false negatives.

This may lead to further misunderstanding due to the use of similar words. Lets define specific. In laboratory medicine. Analytical Specificity is defined as the assays ability to identify only the target nuclei acid in the face of interferents and other cross reactive nucleic acid (e.g. as one changes species and therefore genomes or potentially when there is insect contamination, etc.). Dia gnostic Specificity has a very different meaning: the likelihood that an animal with disease is correctly diagnosed with disease.

#### True positives/true negatives + false negatives

So this reflects the number of false positives in a population.

#### Neoplasia

There are several mutations which have been explored in veterinary medicine which include the p53 mutation in sarcomas and other neoplasias, the Philadelphia translocation in myelogenous leukemia, as well as others. This talk will focus on PCR used in lymphoma as this is offered by most veterinary oncologists at this time. PARR or polymerase chain reaction for antigen receptor rearrangement is now commonly used for the diagnosis and staging of lymphoma. While this is standard practice in human medicine, this has still to be diagnostically validated in dogs and cats and is poorly understood in horses. PARR amplifies the highly variable T or B cell antigen receptor gene in the different species. As these regions are unlikely to be exactly the same, clonal arrangement of the T or B cell antigen receptor gene is abnormal and indicates probably neoplasia. Different primers are used for different species in different laboratories, so as usual there is devil in the detail.

PARR is useful when there are low numbers of lymphocytes such as staging hematogenous spread of a lymphoma and in the diagnosis of gastrointestinal lymphoma. The diagnostic sensitivity and specificity of this assay have been assessed though this should be considered further from a study design and "real-life" perspective. It is important to note that when diagnostic specificity and

sensitivity are calculated, the gold standard used is histopathology of gastrointestinal tissue. As many veterinarians who have worked with animals over the course of a GI lymphoma may tell you, histopathology is likely not the final answer when it comes to this diagnosis. Diagnosis of gastrointestinal lymphoma in human medicine includes histopathology with immunohistochemistry including CD20, cytokeratin, CD3 and additional CD markers are considered baseline and standard for diagnosis. Additional diagnostics typically include ultrasound for masses, bone marrow assessment, PET scan, and other diagnostic imaging. PCR is also frequently performed as an ancillary diagnostic to categorize and further assess lymphoma, though this is becoming a more standardized diagnostic.

As in humans, infectious diseases such as Ehrlichia and purely immune mediated hypersensitivities may cause false positives due to clonal proliferation.

There are several laboratories investigating detection of stage V lymphoma as a means of noninvasive diagnosis. Neoplastic lymphocytes defined by clonal proliferation may be detected in the peripheral blood of dogs with lymphoma using PCR, in numbers below the threshold of microscopic detection. One study reports approximately 2.5 times the numbers of animals (89% versus 32% the number of animals) were positive for hematogenous neoplastic cells in animals with previously diagnosed lymphoma when using PARR in comparison to microscopy. The sensitivity of PARR for detecting lymphoma cells in peripheral blood may provide a noninvasive method for detecting lymphoma in its early stages, determining phenotype, monitoring therapy, screening breeds at risk for lymphoma, and possibly for assessing prognosis. Studies examining these uses of PARR are ongoing. (Keller, 2004)

In one recent study, PARR had a 100% diagnostic sensitivity (likely due to low n) and approximately 95% diagnostic specificity for diagnosis of alimentary lymphoma with division of the complicating diseases such as enteritis (IBD) and carcinoma based on histopathology. Diagnostic sensitivity of PARR in diagnosis of alimentary lymphoma ranges down to 80% when intestinal biopsies are used. Diagnostic specificity may be a bit lower. Again many studies compare to histopathology samples as the gold standard which may not be appropriate. Importantly in this study, time to survival/euthanasia was also assessed which was highly statistically significant. "All samples were categorized into three groups [lymphoma (n=4), adenocarcinoma (n=5) and enteritis groups (n=69)] based on the histopathological diagnosis. In the lymphoma group, one case was IgH major-positive, and three cases were TCRypositive, representing clonal expansion of B- and T-cells, respectively. PARR produced negative results for all cases in the adenocarcinoma group. In the enteritis group, six cases were TCRy-positive. Two of the six TCRy-positive enteritis cases were cytologically diagnosed as lymphoma by fine needle aspiration during a laparotomy. In the enteritis group, the survival times were compared between the TCRy-positive and TCRy-negative cases. The overall survival time of the TCRy-positive enteritis cases was significantly shorter than that of the TCR $\gamma$ -negative enteritis cases according to a log-rank test (p<0.0001). With regard to other factors, such as age, clinical signs and the serum albumin concentration, there were no significant differences between the T CRypositive and TCRy-negative enteritis cases. In conclusion, PARR is capable of detecting alimentary lymphoma and latent alimentary lymphoma, which cannot be histopathologically diagnosed using endoscopic biopsy specimens. Furthermore, aT CRy-positive result in PARR may imply a poor prognosis."(Kaneko et al., 2009)

### Infectious disease

There are numerous tests for the various infectious diseases. It should be noted that in veterinary medicine, unlike human medicine, the diagnostic laboratories are not required to publish diagnostic specificity and sensitivity of these analyses. This leaves the veterinary practitioner to interpret tests which must have error rates, potentially with verbal discussion with a specialist alone.

## Common pathogens tested for in the gastrointestinal tract include

- Campylobacter coli
- Campylobacter jejuni
- C. difficile toxins A/B
- C. perfringens enterotoxin
- Cryptosporidium spp.
- Feline parvovirus
- Giar dia spp.
- Salmonella spp.
- Tritrichomonas foetus

- 1. What is the difference between standard PCR and real time PCR or TAQ and why does it matter?
- 2. How much does real time PCR help us with gastrointestinal panels?

# Do you really care about 2 Giardia or Tritrichomonas organisms in an animals GI tract?

- 1 Polymerase chain reaction (PCR) is a method that allows exponential amplification of short DNA sequences (usually 100 to 600 bases) within a longer double stranded DNA molecule. PCR entails the use of a pair of primers, each about 20 nucleotides in length, that are complementary to a defined sequence on each of the two strands of the DNA. These primers are extended by a DNA polymerase so that a copy is made of the designated sequence. After making this copy, the same primers can be used again, not only to make another copy of the input DNA strand but also of the short copy made in the first round of synthesis. This leads to exponential amplification. Since it is necessary to raise the temperature to separate the two strands of the double strand DNA in each round of the amplification process, a major step forward was the discovery of a thermo-stable DNA polymerase (Taq polymerase) that was isolated from Thermus aquaticus, a bacterium that grows in hot pools; as a result it is not necessary to add new polymerase in every round of amplification. After several (often about 40) rounds of amplification, the PCR product is analyzed on an agarose gel and is abundant enough to be detected with an ethidium bromide stain. For reasons that will be outlined below, this method of analysis is at best semi-quantitative and, in many cases, the amount of product is not related to the amount of input DNA making this type of PCR a qualitative tool for detecting the presence or absence of a particular DNA. In order to measure messenger RNA (mRNA), the method was extended using reverse transcriptase to convert mRNA into complementary DNA (cDNA) which was then amplified by PCR and, again analyzed by agarose gel electrophoresis. In many cases this method has been used to measure the levels of a particular mRNA under different conditions but the method is actually even less quantitative than PCR of DNA because of the extra reverse transcriptase step. Reverse transcriptase-PCR analysis of mRNA is often referred to as "RT-PCR" which is unfortunate as it can be confused with "real time-PCR".
  - Real-Time PCR is similar to a simple PCR except that the progress of the reaction is monitored by a camera or detector in "real-time". Additionally, a secondary probe must also bind with a flurescent marker which improves precision and minimizes the need for post PCR sequencing. There are a number of techniques that are used to allow the progress of a PCR to be monitored. Each technique uses some kind of fluorescent marker which binds to the DNA. Hence, as the number of gene copies increases during the reaction so the fluorescence increases. This is advantageous because the efficiency and rate of the reaction can be seen. There is also no need to run the PCR product out on a gel after the reaction. The main advantage of TaqMan® based chemistry is that a fluorescent signal is generated only when there is specific hybridization of the probe to the target sequence and this is quantified as the reaction is running. No signal is generated from any non-specific amplification products that may be formed during the reaction. Therefore, you can tell if there is a single organism, 100s, or 1000s of organisms in the sample if well-validated. Another advantage is that probes can be labeled with different, spectrally distinct reporter dyes, which allows for the amplification of multiple target sequences within a single tube (multiplex real-time PCR). The main disadvantage of TaqMan® based chemistry is that design and synthesis of different dual-labeled probes is required for each target sequence, which increases assay setup and cost.
- 2. If real-time results were reported quantitatively, this would enable the practitioner to better decide what is diagnostically relevant. Unfortunately, this is often not done. Questions such as do I care about one or two Giardia organisms can then be asked by the practitioner and answered by experts. In my mind, typically the answer is no, but lets discuss this further during our lecture.

### References

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