

Novel Diagnostics for Exotic Mammals

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Abstract: Specialized testing for exotic mammals is available but often unknown to most practitioners. It is important to understand the principle behind the test and the limits of the technique, as well as the validation process, in order to best use them in clinical cases. These concepts will be reviewed in conjunction with a presentation and discussion of testing available for rabbits, ferrets, and rodents.

Introduction

Traditional testing methods and new and improved assays have greatly revolutionized veterinary diagnostics over the past 20 years. ELISA-based immunoassays have become commonplace not only at the laboratory levels but at the point of care for many companion animal species. PCR tests for the detection of RNA and DNA have been developed for many viruses and bacteria and are available at a growing number of laboratories. Test options for diseases of exotic animals are available, but their wide use is still retarded by the lack of information in regards to clinical applicability. The goal of this summary is to provide a general review the current diagnostic tests and how they may be used in the exotic veterinary practice.

Diagnostic Tools

Laboratory infectious disease diagnostics rely upon the following principles: detection by culture, detection of seroconversion (antibodies), detection of antigen (by serology), and detection of DNA/RNA (by PCR).^{1,2} Culture continues to be limited by issues related to the infectious agents themselves. Bacterial culture has not greatly changed over the past 10 years with the exception of the addition of molecular technology. Growth from specialized cultures (ie, for *Mycobacterium* species) can now be screened using PCR to improve both sensitivity and specificity. Adaptations to virus isolation can similarly be made.

There have been considerable improvements in immunoassays. Examples of serological assays include ELISA, virus neutralization, complement fixation, indirect immunofluorescence, and gel immunodiffusion. The use of recombinant antigens (vs crude antigen preparations) has aided in the development of more sensitive and specific assays. Western blot testing, often regarded as to technically difficult for use as a routine test is now more readily available as a primary test option or for confirmatory testing. In specific regard to exotic diagnostics, the “older” techniques of agglutination, immunodiffusion, and neutralization continue to provide an important base for testing as they do not require species specific reagents that are often needed for ELISA testing. However, with added pressure to produce good exotic tests, more monoclonal antibodies will likely become available to improve the existing serodiagnostic test options.

Decision Analysis and Test Validation

Decision analysis is a process for analyzed test choices by the use of a flowchart or decision tree.³ This perhaps is a fancy way of going through a diagnostic rule-out list, but it forces an acknowledgement of an action to be taken for any positive or negative result. That is, by formulating such an analysis for a clinical case, it questions the value of each diagnostic test in making a treatment plan or final diagnosis.

Sensitivity and specificity are very relative terms. They are extremely useful for comparing different tests for the same agent but do not have direct use in clinical practice. They are often based on ideal cases—sometimes with experimental infection—with animals at a particular timepoint along infection or disease. They do not address a large uncertainty about diagnostic parameters such as the following.⁴

1. Test properties and performance. There is no or very little standardization in veterinary diagnostics. Techniques, cut-off values (for positive vs. negative results), and controls will all vary between laboratories.
2. Clinical context. In some cases, the likelihood of receiving a positive result is greater in a clinically ill patient. However, complicating issues maybe present that may affect results, including other concurrent diseases, previous treatments, and poor immune status.
3. Prevalence of the disease. Antibody-based testing in an area or population with higher prevalence will be complicated in terms of determining possible infection vs exposure or post treatment re-exposure.
4. Multiple testing. In general, paired or serial testing is considered to be helpful but in particular disease processes, increases in titers are not always seen during infection.

Assay validation is complicated by similar criteria whereby validation cannot be limited to a few reference samples and ideal cases.⁵ Validation is truly an experimental process involving the optimization of a technique to detect an analyte with accuracy and precision. Reference samples can then be employed to determine an initial sensitivity and specificity. Importantly, this must be extended by what is referred to as conditional, incremental, and continuous processes. As a group, these analyses refer to changes relative to assay that should improve the ability to interpret the test results. For instance, validity may increase with use of additional confirmed reference samples over time, sensitivity and specificity for a test may be variable dependent on the phase of infection, and the assay must be continuously monitored through statistical verification. Test validation and design improvements do not end with the publication or presentation of a new test. It continues through a repetitive process to assess test performance for each population of animals to which it is applied.

Your Role in Diagnostic Testing

It is important to recognize that analytical errors can occur while the sample is still at the clinic or in transit to the lab. Thus, in addition to choosing a lab that you feel is competent, you must minimize any sample issues that could result in pre-analytical errors. A fundamental part of this process is having the most current lab information to ensure that the proper sample is collected—not only proper sample volume but proper sample type (ie, correct anticoagulant, swab type). This continues with storing and shipping the sample by the preferred method. Ideally, a standard operating procedure should be available and periodically reviewed to have the most accurate information. Keep a list of contact names, numbers, and websites to update this information and expedite answering of questions on sample collection and test interpretation.

Diagnostic for Exotic Mammals

Excellent general reference materials on this subject are readily available including the *VCNA Exotic Animal Practice publication*, *Exotic DVM*, the *Journal of Exotic Pet Medicine*, and others.⁶⁻⁷ The current review will state available testing options and issues relevant to the particular disease agent. Table 1 presents a non-comprehensive list of laboratories which offer specialized testing that would be of use to the exotic veterinary practitioner.

1. *Fungal diseases*. Serologic options remain limited for the diagnosis of various fungal diseases.⁸ Agglutination-based assays for antibody can be applied to any species as they depend only on a final read out of precipitation of antibody-antigen complexes. Similarly, antigen-based assays for *Cryptococcus* and *Aspergillus* species can also be employed. However, specific application of these tests to many exotic animals has not been described in the literature.
2. *Cryptosporidium* and *Giardia* species. Traditional light microscopy has been supplanted by test options including ELISA, immunofluorescence, and PCR. *Giardia* ELISA and PCR testing has been reported to have similar sensitivity and specificity in humans, although PCR was able to detect positive samples earlier in infection.⁹ ELISA antigens are believed to be conserved among *Giardia* species. *Cryptosporidium* ELISA has been applied with success in animal samples with a good sensitivity for potentially zoonotic *C parvum* isolates but less sensitivity for non-zoonotic species.¹⁰ To this reviewer's knowledge, there have been no publications regarding the application of PCR to routine diagnostic animal samples for these parasites, although test services are available.
3. *Pasteurella multocida*. Two test options are available and should be considered to be used in conjunction. An ELISA using purified specific antigen has been described in the literature and found to give highly reproducible data and significant titer differences between negative and positive experimentally infected animals.¹¹ Significant antibody differences were also described in naturally infected animals.¹² PCR has been applied in human medicine but this reviewer could not find any description in veterinary medicine, although PCR has been described as a monitoring tool in *P pneumotropica* in lab animals. The value of the test is likely high as the reliability of nasal cultures is not great. The extra sensitivity and specificity of the molecular technique should aid in detection.
4. *Tyzzer's disease/Clostridium piliforme*. Serological testing for this agent in rabbits (and rodent species) is available through most rodent clinical pathology labs. PCR testing has been described in the literature using feces of rodents.¹³ PCR services are available commercially (through lab animal labs) but this reviewer could find no comprehensive description of the application to rabbits.
5. *Rodent serology and PCR testing*. A wide host of test services are available for all species of rodents due to their need in lab animal medicine. All the diagnostic centers listed in Table 1 are receptive to samples from private practitioners. Serological assays are IgG based so care must be taken in test interpretation in acutely ill animals. PCR tests have been well documented in lab animals and if samples are prepared and shipping properly, results will be reliable.
6. *Rabbit rotavirus*. ELISA techniques are described in the literature but could not be found for use in veterinary diagnostics by this reviewer. Notably, in experimental infection, rabbits were found to strongly seroconvert and remain positive through 2 years postinoculation.¹⁴ PCR analysis was recently described in lab rabbits and demonstrated that a human PCR kit provided ample cross reaction.¹⁵ This test is available for fecal samples at Michigan State University (MSU).

7. *Rabbit hemorrhagic disease virus*. Antigen detection tests are the primary screening test for this virus and involves the use of liver or spleen homogenates in an ELISA technique.¹⁶ More recently, a more sensitive PCR test has been described.¹⁷ This test is available for postmortem use at MSU.
8. *Encephalitozoon cuniculi*. Diagnosis of *E cuniculi* is troublesome. Seropositive animals can be found in the absence of clinical signs. Most serostudies that have been published are from non-U.S. locations which does not aid in understanding the prevalence of this agent in routine exotic practice. In the United Kingdom, 23% of asymptomatic rabbits were found to be seropositive vs. 69% of symptomatic animals. Titers were found to be detectable 3–4 weeks after exposure.¹⁸ In an experimental model of infection, antibody was not found to correlate with disease.¹⁹ Thus IgG seropositive status may reflect chronic infection, clearance but seroconversion, incidental exposure, or possible cross reaction. There are 2 prospective studies that may have some worth in furthering ECUN diagnostics. First, IgM titers were found in experimentally infected animals from day 17 through 38.²⁰ Additionally, ECUN spores can be recovered in the urine of some infected animals. One study found spores present between week 4 and 12 post exposure.²¹ Relative insensitive techniques have been used thus far but PCR should prove to enhance both sensitivity and specificity. Rodent laboratories offer PCR testing, but practitioners should note that no controlled studies have been conducted using IgM titers or PCR in rabbits with natural infection.
9. *Ferret epizootic catarrhal enteritis (ECE)*. Detection of this agent is the key to diagnosis. Molecular techniques using tissues, saliva, and feces have been published.²² MSU offers PCR testing on feces.
10. *Ferret influenza*. Ferrets can be naturally infected by (human) influenza A and B. No specific or ferret-validated tests for the virus have been described, but the virus is known to induce seroconversion in experimental models of disease. PCR testing is available at most human laboratories and, if accessible, could be considered for use in ferret cases.
11. *Ferret (canine) distemper virus*. Standards known to canine clinical pathology can be applied to ferret infection. Seroconversion can be monitored by serum virus neutralization assays with the use of paired samples. In experimental infection, blood was found to carry viral antigen from day 2 to 6 postinfection and conjunctival scrapings were found to be positive after day 9.²³ Direct fluorescent testing of these samples as well as the use of PCR would be of diagnostic value. PCR has been described in the literature where over 50% of clinically ill dogs and one ferret were found positive.²⁴
12. *Helicobacter mustelae*. A limited study comparing immunohistochemistry and PCR was performed and showed a good sensitivity of the molecular technique with the final recommendation of submitting swabs from oral mucosa, stomach, and rectum whenever possible.²⁵ Antibody ELISA techniques have also been described but are not commercially available.²⁶ Seroconversion was found to increase with age but the titer was found to decrease with a positive response to treatment. Practitioners should note that other *Helicobacter* species are prevalent in rodents and PCR testing at most rodent labs is readily available.
13. *Aleutian disease virus*. Three types of tests have been presented for ADV testing: serum protein electrophoresis, serology, and PCR. Protein electrophoresis allows for the quantitation of specific acute phase protein fractions and immunoglobulins (gamma fraction). There are several literature citations including Porter and colleagues who reported that naturally infected ferrets have a significantly higher gamma globulin levels (0.49g/dl vs 1.06g/dl).²⁷ In experimentally infected animals, increases were found by day 63 postinfection and were present throughout the termination of the experiment at day 182.²⁷ Palley reported more marked increases (up to 54% of the total protein) in 2 naturally infected ferrets.²⁸ As protein electrophoresis is more commonplace in diagnostic labs, the practitioner should take care to submit non-hemolyzed, non-lipemic samples to a lab that has in-house established reference ranges.

The application of serological testing for antibody was first documented for application to infection in mink species. Counter immunoelectrophoresis (CEP or CIEP) involves the use of an agarose gel medium and an electric current. Antibody-antigen complexes are formed when the sample has ADV antibody and read out as a precipitin. Serum samples are not diluted for analysis and there is no quantitation of titer normally given in this test. The antigen used in this test is a tissue culture preparation of whole virus lysate representing a spectrum of ADV antigens. Two ELISA tests have been produced using recombinant antigen. In general, the use of specific recombinant antigens in ELISA tests greatly eliminates any potential false cross reaction that may be present in tissue culture prepared antigen. However, as the normal antibody response is polyclonal during infection, the use of specific antigen(s) may not fully gauge the humoral immune response and result in lower overall sensitivity. Avecon uses combination of capsid (VP) and non structural (NS) recombinant antigens (B. Stephon, personal communication, April 2006). UGA-IDL uses a capsid ELISA (K. Pennick, personal communication, April 2006). Notably, the application of the tests to ferrets in controlled studies (including the CEP) have not been described in the literature. Also, although anti-ferret IgM and IgG are commercially available, the test services do not report specific titers. Quantitation of such may not be clinically helpful (B. Stephon, personal communication, April 2006).

There has been very little information published regarding ADV serology in ferrets and little more in mink. What has been documented is as follows and does support perhaps questioning titers for NS vs VP antigens in clinical cases:

- a. CEP, immunofluorescence, and complement fixation have been found to be reliable and specific in detecting mink antibody to ADV.²⁹
- b. By Western blot analysis in minks, when the hypergammaglobulinemia was exhibited, 10/12 animals reacted preferentially with VP antigens and 2/12 reacted preferentially to NS antigen.³⁰ Seroconversion by indirect immunofluorescence and increases in gamma globulins were present by day 30 postinfection.
- c. In mink species, vaccination with VP proteins enhanced the disease whereas the use of NS antigens in the vaccines promoted partial protection.³¹ Hypothetically, if the same is true in ferrets, the titers to nonstructural antigens during infection may shed some light on the severity of disease.
- d. Porter et al reported that 28/71 ferrets with ADV showed equal titers to VP and NS antigens, whereas 42/71 reacted preferentially with NS antigens and 1/71 reacted only with VP antigens.³² Furthermore, low antibody titer animals were found to be more reactive to NS antigens versus high titer animals which were more reactive to VP antigens.

The application of PCR testing has been proposed in the literature. Jackson et al found that after initial infection of mink species, ADV DNA was initially found high levels in the blood but decreased when the animals seroconverted.³³ The amount of DNA was also found to correlate with the severity of the lesions. The PCR technique was applied to formalin-fixed tissues by Une in a fatal case of ADV in a ferret.³⁴ More recently, Pennick reported the presence of ADV DNA in serum, urine, feces, and blood of an antibody-positive ferret over a 1.5-year period.³⁵ Necropsy tissues were later analyzed by in situ hybridization and found positive for ADV. Thus, PCR will likely be a viable technique to complement antibody testing. More detailed reports of natural and experimental infection of ferrets should be completed to understand the time course of positive results for both assays.

Table 1. List of laboratories with exotic mammal test services.

Laboratory	Test Services
Avecon www.avecon.com (610) 837-8400	ELISA for ADV
Avian Biotech www.avianbiotech.com (800) 514-9672	PCR for <i>Mycobacterium</i> , <i>Candida</i> , <i>Cryptosporidium</i> , <i>Giardia</i> , <i>Salmonella</i>
Bioreliance www.bioreliance.com (800) 553-5372	Rodent and rabbit serology, rodent PCR
Blue Cross Animal Hospital (208) 678-5553 Contact: Dr. Blau	CEP for ADV
Charles River Labs www.criver.com (877) CRIVER1	Serology and PCR for rodents
Cornell University http://www.diaglab.vet.cornell.edu/ (607) 253-3900	Serum neutralization and direct fluorescence for CDV, <i>Giardia</i> and <i>Cryptosporidium</i> antigen ELISA, fungal serology
Infectious Disease Lab-University of Georgia (706) 542-8092	PCR for <i>Salmonella</i> , <i>Pasteurella</i> (also serology), Anticipates ADV ELISA and PCR for summer 06
MSU www.animalhealth.msu.edu/ (517) 353-1683 Contact: Dr. Maes (maes@dcpah.msu.edu)	PCR for CDV, ferret enteric coronavirus, ferret rotavirus A and C, rabbit rotavirus, rabbit hemorrhagic disease virus, Aleutian disease virus
RADIL-University of Missouri http://www.radil.missouri.edu/ (800) 669-0825	Serology and PCR testing for rodents and serology for rodents and rabbits
RAL, Inc. www.vetdna.com (972) 960-2221	PCR for <i>Helicobacter</i> , <i>Cryptosporidium</i> , <i>Giardia</i> , <i>Salmonella</i> , interests in receiving samples for ADV and ECUN
Taconic Anmed www.taconic.com (301) 762-0366	Serology for rodents and rabbits, PCR for rodents and also <i>Helicobacter</i>
University of Miami – Comparative Pathology http://pathology.med.miami.edu/cpl/ (800) 596-7390	Serology for rodents, interested in receiving samples for rabbit ECUN testing, <i>Giardia</i> and <i>Cryptosporidium</i> antigen ELISA
Veterinary Molecular Diagnostics www.vmdlabs.com (513) 576-1808	PCR for <i>Helicobacter</i> , <i>Cryptosporidium</i> , interest in receiving samples for <i>Salmonella</i> , <i>Giardia</i> , <i>Mycobacterium</i> , and ADV
Zoologix www.zoologix.com (818) 717-8880	Extensive menu of avian, primate, wildlife and rodent PCR tests

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