Care of Spiny-tailed Lizards

Application of Lab Animal Immunoassays
Application of Laboratory Animal Immunoassays to Exotic Pet Practice*

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Laboratory animal veterinarians frequently use immunoassays (serology) to diagnose infection in colony rabbits and rodents. Immunoassays are valuable when an infectious agent is difficult to culture (e.g., viruses or fastidious bacteria, such as mycoplasma), difficult to isolate (e.g., microsporidia), or dangerous to handle. Three infectious agents seen commonly in exotic pet practice that are ideal for serologic testing are *Mycoplasma pulmonis*, *Pasteurella multocida* and *Encephalitozoon cuniculi*.

**Mycoplasma Infection in Rats**

*Mycoplasma pulmonis*, in combination with other respiratory pathogens, causes chronic respiratory disease (CRD) in rats (Fig 1). Severity of CRD in rats varies greatly according to the mycoplasma strain and environmental and host factors. Concurrent infection with Sendai virus, sialodacryoadenitis virus, pneumonia virus of mice, rat respiratory virus and cilia-associated respiratory bacillus exacerbates CRD. Variability in the genetic susceptibility of rats and in the virulence of different mycoplasma strains affect the host-pathogen relationship. Vitamin A or E deficiency and ammonia levels within the cage also contribute to expression of CRD.

**Diagnosis of Mycoplasma Infection**

*Mycoplasma* is difficult to grow in a laboratory, requiring special media such as SP-4 formula or A7 agar (Fig 2). For *M. pulmonis* culture, viable organisms are collected in mycoplasma broth from affected sites, such as the upper and lower respiratory tract via nasopharyngeal washes. However, cultures fail to detect the organism in 25-30% of infected animals. Serology is the preferred choice for diagnostic testing, especially in older animals. Younger rats naturally exposed to *M. pulmonis* may be seronegative for up to 4 months post-exposure.

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Pasteurella Infection in Rabbits

In rabbits, most cases of rhinitis or “snuffles” arise from multibacterial infections. *Pasteurella multocida* is involved in 55% of cases of upper respiratory tract infection with nasal discharge and sneezing in pet rabbits. *Bordetella bronchiseptica* is present in 52% of cases, *Pseudomonas* spp. in 28% and *Staphylococcus* spp. in 17% of cases. The most frequently found combination (in 29% of rabbits) is *P. multocida* with *B. bronchiseptica.* Multibacterial and viral infections are also associated with pneumonia. Rabbits dying from pneumonia often have *P. multocida* isolated, although *B. bronchiseptica* and *P. aeruginosa* are isolated from lungs as well. In Europe, myxomatous myxoma virus strains have also been isolated from rabbits dying of acute hemorrhagic pneumonitis.

*Pasteurella* infection can be related to poor husbandry, overcrowding and nutritional deficiencies, although these problems are more common in colonies of rabbits raised for meat than in pet rabbits. Housing, particularly air quality, is important; chemical injury to the respiratory mucosa through exposure to ammonia increases susceptibility of rabbits to *P. multocida* infection.

Genetic susceptibility to pasteurellosis varies among rabbit strains. For example, chinchilla rabbits are more susceptible than blue beveran rabbits, and pneumonia and pleuritis in experimental pasteurellosis are more severe in Flemish giant rabbits than in New Zealand white rabbits.

Some strains of *P. multocida* are more pathogenic than others, and certain serogroups are associated with specific types of disease, due to virulence factors. Type A strains are more adhesive to respiratory mucosa than are type D strains, and this adhesion may explain the association of Type A strains with pneumonia. Other virulence factors include phagocyte resistance and endotoxin production.

**Diagnosis of Pasteurella Infection**

Isolation and identification of *P. multocida* by bacteriology (Fig 3) is difficult and can result in both false-negative and false-positive results. A deep nasal swab is required for culture, but it can be difficult to obtain satisfactory swabs. In addition, infection can be absent from nasal passages but present in paranasal sinuses or middle ears. *P. multocida* does not survive well in transport media and survives for less than 24 hours at room temperature. Although Cary-Blair medium is recommended for the transport of *P. multocida* in rabbit nasal specimens, few laboratories advise...
Identification of *Pasteurella* (*P. multocida* and other species) can differ significantly among laboratories, depending on the isolation and identification methods used. Some strains of *P. multocida* grow best at 35°C, which is lower than most routine cultures, and some strains require 5% CO₂ for growth. In one study, using the API 20 NE (BioMérieux) kit (Fig 4) in 4 laboratory animal diagnostic laboratories, there was only 22.5% agreement in speciation of *Pasteurella* strains. In fact, the API 20 NE kit misclassified 42% of *P. multocida* strains, and 38% of profiles obtained with *Pasteurella* strains not in the test kit database were misidentified as *P. multocida*.

In contrast, serology testing reliably diagnoses *Pasteurella* infection in young rabbits. Ideally, rabbits should be tested when they are 5 months or older, as the incidence of infection does not increase significantly until that age. Predisposing factors for infection in young rabbits include the presence of maternal antibodies, maternal vaginal infection, the age of weaning and the prevalence of infection within a colony. Serology is of limited use in older rabbits because they develop antibodies to gram-negative core antigens shared by *P. multocida* and Enterobacteriaceae, and false-positive results occur. Serology in older rabbits is thus useful only to rule out absence of infection, not to identify infected rabbits.

The best method for diagnosis of *P. multocida* is a combination of polymerase chain reaction (PCR) on nasal swabs and serology. Serology testing for antibodies to *P. multocida* extracellular sialidase detects both immunoglobulin M (IgM) and IgG. The presence of IgG antibodies identifies carriers, while clinically ill rabbits have more IgM antibodies.

Encephalitozoon Infection in Rabbits

*Encephalitozoon cuniculi* is a microsporidium that infects rabbits (Fig 5). Surveys of encephalitozoonosis in pet rabbits in Europe have shown high rates of infection in healthy animals (7-42%) and in those with neurologic signs (40-85%). No large-scale study of encephalitozoonosis in pet rabbits in the US has been conducted.

Three forms of encephalitozoonosis may occur individually or combined. The renal form (Figs 5, 6) is associated with chronic progressive renal disease; the ocular form is associated with cataracts, extensive lens damage and uveitis (Fig 7); and the neurologic form varies from mild change in behavior to severe vestibular disease.

Many rabbits infected with *E. cuniculi* remain healthy or show only minor clinical signs, such as slower reactions to stimuli. In more serious cases, clinical signs are often neurologic, with some rabbits developing ocular lesions and cataracts. Lethargy and head tilt are usually the first signs, and ataxia, paresis and hind limb paralysis follow. This presentation can...
be confused with vestibular disease caused by bacterial infection (usually *P. multocida*). Full paralysis and death may occur in severe cases. Chronic renal infection leads to a characteristic pitted appearance of the kidneys (Fig 8). Although gross lesions of the brain are not usually visible, meningencephalitis can be observed upon histologic examination (Fig 9).

**Diagnosis of Encephalitozoon Infection**

A definitive diagnosis of encephalitozoonosis is difficult. While rabbits with encephalitozoonosis are seropositive, many seropositive rabbits are apparently healthy and do not show any signs of disease. Immunoassays are not specific, and results must be considered in combination with other diagnostic findings. However, the absence of antibody (seronegativity) indicates that other differential diagnoses must be considered in sick rabbits.

Experimental subcutaneous immunization of rabbits with inactive *Encephalitozoon* spores generates
a high-titer antibody response, with the humoral immune response predicted to last 7 years. Although antibody titers decline over time, this means a rabbit infected with *E. cuniculi* 2 or 3 years previously but not showing clinical signs can still have a high antibody titer. In other infectious diseases, high antibody titers often indicate active or recent infection, but they are meaningless for *E. cuniculi* infection.

Several methods for detecting antibodies against *E. cuniculi* include indirect immunofluorescence (IIF), enzyme-linked immunoabsorbence assay (ELISA) and carbon immunoassay (CIA or India-ink assay). Any of these assays is suitable for routine health monitoring of rabbits. While there is no difference in the ability of each assay to detect antibodies in serum, a comparison of the techniques showed that IIF (rather than ELISA) is the best for quantitative measurement of antibodies. PCR detection of *E. cuniculi* DNA in cerebrospinal fluid from seropositive rabbits with clinical signs is of no help in the diagnosis of encephalitozoonosis.

### Laboratory Test Operating Characteristics

In order to determine whether a laboratory test will be clinically useful, several parameters must be assessed. Individual diagnostic tests must be reliable and accurate, while sensitivity and specificity are important for determining the relevance of the test result to the diagnosis.

Test reliability (or precision) measures the reproducibility obtained by running the test many times on the same specimen. An unreliable test produces results that vary considerably due to technical error or chance. Test accuracy measures the reproducibility of the test result compared with the true or known value. Test reliability and accuracy can vary independently of each other; that is, a test may be inaccurate but reliable (Fig 10).

Sensitivity and specificity allow evaluation of how test results affect diagnostic probabilities (Table 1). The sensitivity of a test is the proportion of diseased animals in a screened population that is identified as being diseased by the test. That is, sensitivity is a measure of the probability of correctly diagnosing a condition. The specificity of a test is the proportion of non-diseased animals identified by the screening test. That is, specificity is a measure of the probability of correctly identifying a healthy animal.

Immunooassays have been developed primarily for either screening a population to identify infected individuals or diagnosing correctly that an animal presenting with clinical signs of a particular infection is really infected.

Screening tests are designed to detect all infected animals, and as such, must possess exquisite sensitivity. Diagnostic tests, in contrast, must be positive in a large number of animals with the infection (high sensitivity) and negative in a large number of animals without the infection (high specificity). The ideal diagnostic test would have 100% sensitivity and specificity. Every animal with the specific infection would have a positive test (no false negatives). Similarly, positive tests would occur only in animals with the specific infection (no false positives).
Unfortunately, tests with such high sensitivity, specificity and predictive value do not exist. Rather, tests with high sensitivity tend to have low specificity and tests with high specificity have low sensitivity.

For any given test, the ranges of results for normal and abnormal individuals will overlap (Fig 11a). As such, the sensitivity and specificity of many tests cannot be maximized simultaneously. If the “cutoff point” is the value below which the test result is abnormal, then a percentage of healthy individuals will be found infected (false positives), and a percentage of infected individuals will be found healthy (false negatives).

If an attempt is made to improve the test sensitivity by changing the cutoff point (moving it to the right), the numbers of false negatives are decreased and sensitivity is increased. However, in doing so, the number of false positives increase, thereby decreasing specificity (Fig 11b).

Specificity can be increased by moving the cutoff point to the left, decreasing false positives. However, this decreases sensitivity by simultaneously increasing the number of false negatives (Fig 11c).

As long as normal and abnormal distributions overlap, any attempt to improve sensitivity or specificity by changing the cutoff point will result in a lowering of the other characteristic. This relationship can be depicted by the receiver operating characteristic (ROC) curve. The ROC curve is a graphic representation of the trade-off between false negative and false positive rates for every possible cut off point, showing the trade-offs between sensitivity and specificity (Fig 12).

It is important to have a specific purpose in mind when choosing a test, because very few tests have the operating characteristics required to satisfy every purpose. In general, to rule out a particular diagnosis, choose a test with high sensitivity; to confirm a diagnosis, choose a test with high specificity (Table 2). Because sensitivity and specificity tend to be inversely related, a test chosen for ruling out disease will not be particularly valuable for confirmation if it is unexpectedly positive. Similarly, a negative test, if chosen to confirm the disease, is less helpful for ruling the disease out. For monitoring an animal over time, reliability is the most important characteristic so that any change in status will be detected.


**Fig 11. a)** The ranges of normal and abnormal individuals overlap in any given test. The “cutoff point” is the value below which the test result is abnormal. Results from individuals that lie in the shaded areas will be false positives (FP) or false negatives (FN).

**b)** Sensitivity (numbers of false negatives) is improved by moving the cut-off point to the right, but this increases false positives (lowering specificity).

**c)** Specificity is increased by moving the cutoff point to the left, decreasing false positives, but this also increases false negatives (lowering sensitivity).
## Selected Laboratory Animal Diagnostic Laboratories

| USA | Comparative Pathology Laboratory  
University of Miami  
1600 NW 10th Avenue  
RMSB 7101A  
Miami, FL 33136  
Tel: 800-596-7390 or 305-243-6700  
Fax: 305-243-5662  
E-mail: compathlab@med.miami.edu  
Web: pathology.med.miami.edu/cpl |
| --- | --- |
| IDEXX | sends rabbit *Pasteurella* samples to:  
Infectious Diseases Laboratory  
Department of Medical Microbiology  
College of Veterinary Medicine  
University of Georgia  
Athens, GA 30602-7386  
Tel: 706-542-5812  
Fax: 706-542-5233  
* Pasteurella Test Codes  
#940 (Serum ELISA)  
#99991 (DNA nasal swab)  
#99992 (DNA nasal swab + serum ELISA)  
| --- | --- |
| IDEXX | sends rabbit *Encephalitozoon* samples to:  
College of Veterinary Medicine  
Texas A&M University  
College Station, Texas 77843  
* Encephalitozoon Test Code  
#941 (Serum IFA)  
| --- | --- |
| Australia | Cerberus Sciences  
39 Winwood Street  
Thebarton, SA 5031 Australia  
Tel: +61 (08) 8234-8780  
Fax: +61 (08) 8234-8712  
Email: cereberus@cereberus.net.au  
www.cereberus.net.au/cerberus.htm |
| CZECH REPUBLIC | AnLab, Ltd.  
Videnska 1083  
Prague 4, 142 20 Czech Republic  
Tel: +42 0261 711667  
Fax: +42 0261 711719  
Email: info@anlab.cz  
www.anlab.cz |
| Denmark | Taconic Europe  
PO Box 1079  
Ry, 8680 Denmark  
Tel: +45 70 23 04 05  
Fax: +45 86 84 16 99  
Email: TaconicEurope@taconic.com  
www.m-b.dk |
| Germany | BioDoc  
Fedder-Lynen-Strasse 23  
Hannover, 30625 Germany  
Tel: +49 511 548884  
Fax: +49 511 548886  
Email: biodoc_machler@web.de  
www.biodoc-online.de |
| The Microbiology Laboratories (Deutschland)  
Postfach 100  
Gross Trebbow, 19069 Germany  
Tel: +49 3867 530056  
Fax: +49 3867 530057  
Email: microlabs.deutschland@t-online.de |
| Italy | Charles River Laboratories Italia S.r.l.  
Via Indipendenza 11  
Calco (Lecco), 23885 Italy  
Tel: +39 039 509915  
Fax: +39 039 508696  
www.criver.com |
| NETHERLANDS | Harlan Netherlands  
Kreuzelweg 53, PO Box 6174  
Horst, 5960 AD Netherlands  
Tel: +31 478 578 300  
Fax: +31 478 571 117  
Email: hnlcsd@harlan.nl  
www.harlaneurope.com/index2.html  
| Swiss Diagnostics  
Central Animal Laboratory  
Geert Grooteplein 29  
Nijmegen, 6525 EZ Netherlands  
Tel: +31 (0)24 361 54 33  
Fax: +31 (0)24 361 63 75  
Email: qmdiagnostics@mmb.umcn.nl  
www.qmdiagnostics.org/index.php |
| Switzerland | MicroBioS GmbH  
Dammstrasse 36  
Muuenchenstein, 4142 Switzerland  
Tel: +41 61 4169610  
Fax: +41 61 4169619  
Email: wilcke@microbios.ch |
| The Microbiology Laboratories (Scandinavia)  
PO Box 8146 Dep, Oslo 0033 Norway  
Tel: +47 22 96 47 75  
Fax: +47 22 96 45 35  
Email: needham@microlabs.demon.co.uk |
| Norway | The Microbiology Laboratories  
PO Box 156  
PO Box 156 Northumberland Rd.  
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Tel: +44 2088 668050  
Fax: +44 2088 666100  
Email: needham@microlabs.demon.co.uk |

**T.M. Donnelly, DVM**
Fig 12. Receiver operating characteristic (ROC) curve, depicting the sensitivity (y axis) and specificity (x axis) of an ELISA immunoassay for antibodies to human herpesvirus 8. Sensitivity was determined for 135 true-positive individuals and specificity for 178 individuals in the true-negative group. Sensitivity and specificity were calculated at each optical density (OD) value observed. Numeric OD values are given alongside the points. (Reproduced with permission from Reference 8.)

Table 2. Guidelines for Laboratory Test Selection

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Essential Operating Trait</th>
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<tr>
<td>Screening</td>
<td>Sensitivity and specificity</td>
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<td>Diagnostic:</td>
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<td>Confirmation</td>
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<td>Ruling out</td>
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<td>Monitoring</td>
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Table 3. Laboratory Animal Immunoassays for Exotic Pet Mammals

<table>
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<tr>
<th>Agent</th>
<th>Serology</th>
<th>PCR</th>
<th>Recommended Assay</th>
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<tr>
<td>Mycoplasma pulmonis</td>
<td>ELISA, IFA</td>
<td>Tracheal and/or nasal swab</td>
<td>Serology or PCR</td>
</tr>
<tr>
<td>Pasteurella multocida</td>
<td>ELISA</td>
<td>Tracheal and/or nasal swab</td>
<td>Combined serology and PCR</td>
</tr>
<tr>
<td>Encephalitozoon cuniculi</td>
<td>ELISA, IFA</td>
<td></td>
<td>Serology</td>
</tr>
</tbody>
</table>

Conclusion

Commercial ELISA kit manufacturers usually set a low cutoff point (low optical density) in their tests (e.g., OD = 0.06, Fig 12), thereby ensuring high test sensitivity. This means that the test will identify all infected animals but also identify some uninfected animals as positive. Manufacturers do this to avoid litigation for failure to correctly identify an infected animal. Animals that test positive should be verified by a second test (in case it is a false positive).

In contrast, laboratory animal diagnostic laboratories usually set a high cut-off point (high optical density) in their tests (e.g., OD = 0.49, Fig 12) to ensure high specificity. Although some animals with infection are missed, false positive animals will not be reported. The consequences of a positive immunoassay to a transgenic mouse colony or laboratory animal breeder could mean destruction of the entire mouse colony or loss of business. Hence, a second immunoassay (usually immunofluorescence) is typically run on samples that fall between the high cutoff point and a slightly lower cutoff point to confirm or refute the results. This way, initial ELISA results can be reported in a short turnover time without risk of declaring a colony or breeder falsely positive, and confirmatory tests can be reported later.

For the exotic pet veterinarian, a list of recommended tests for the diagnosis of *M. pulmonis*, *P. multocida* and *E. cuniculi* is presented in Table 3. A list of recommended diagnostic laboratories that will perform these tests is given on page 25.

References and Further Reading

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**Clinical Results Naturally**

**Uses for HEALx from the Exotic DVM Forum**

**HEALx for itchy ferrets**

“In some ferrets with itchy skin we have been using HEALx Sunshine Factor and in some cases Booster HEALx if there is any infectious suspicion. I suppose their content of Omega 3 and 6 together with vitamin A and E help with any problem affecting skin. Apart from that, they get great fur and accept it amazingly well. We use a daily dose of 0.3-0.5 ml/kg.”

Sergio Sarmiento Valiente, DVM
Exoticos Vet Clinic, Spain

**HEALx a possible help with kidney disorder therapy**

“We see the same results here with improvement in pruritus and coat quality—and many of our ferrets have been on it for over a year on a daily basis—most of them accept it readily out of a syringe, bowl, or off a finger.”

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