IMMUNODIAGNOSTIC & IMMUNOLOGIC TECHNIQUES

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Tick-borne diseases such as *Ehrlichia canis* and *Anaplasma phagocytophilum*; drugs such as phenobarbital, cyclosporine, and amikacin; and thyroid hormone testing including total T4 and thyroid-stimulating hormone levels all use various types of immunoassays.
YOU HAVE ASKED ...

What are the differences between antibody and antigen tests, and which test should I choose?

THE EXPERTS SAY ...

There are many immunodiagnostic tests that can be used to assist in the diagnosis of infectious, endocrine, and neoplastic diseases as well as to measure blood drug concentrations (Table). This article provides a brief introduction to immunodiagnostic tests, including immunofluorescence assays, ELISAs, immunohistochemistry, and flow cytometry.

Antigen:Antibody Assays

The underlying principle of immunologic testing is antigen:antibody specificity (Figure 1). Antigens are substances (often proteins or carbohydrates) capable of inciting an adaptive immune response, which often results in the production of specific antibodies.1 Adaptive immunity is the specific counterpart to innate immunity that involves the response to antigens by lymphocytes. Immunologic tests can either use antibodies to identify antigens circulating in the blood or detect patient antibodies that have formed in response to a current or previous disease.

Antigen tests such as those for feline leukemia or heartworm disease involve specific test antibodies that recognize a particular disease antigen circulating in the blood. Hormones can also be detected with antibodies, such as when testing thyroid hormone and cortisol levels. Immunoassays for cyclosporine blood concentrations and other drugs use antibodies specific to the particular drug; the amount of antibody-bound drug can then be measured to provide a quantitative assessment.

In contrast, antibody detection assays test for existing patient antibodies that recognize a particular test antigen (eg, Lyme disease, FIV). A patient’s serum is applied to the assay, and the result reflects the quantity of antibody present. These are often called serologic tests; results can be positive or negative but are often reported quantitatively as the titer of antibody present (see Serology for Infectious Diseases).

Antigen:Antibody Detection

For both antigen and antibody assays, detection methods such as fluorescence, color-linked enzyme reactions, and radioisotope labeling can be used to detect antigen:antibody complexes with varying levels of sensitivity.

Immunofluorescence assays (IFAs) involve a fluorescently labeled antibody detected with a fluorescence microscope, which increases assay sensitivity. IFA testing can be performed on blood smears or tissue sections and allows localization of the source of the positive signal.2 IFAs are commonly used to detect antibodies to infectious agents, includ-
ELISAs involve an antibody linked to an enzyme that changes color when exposed to a substrate. Some tests provide more information than others. For example, with feline leukemia, readily available ELISA technology only tests for viral antigen in serum, which can be free or cell-associated. With an IFA, the fluorescent signal can be localized to WBCs, thus helping identify bone marrow involvement and persistent infection.

Radioisotopes are also used to label antibodies to provide more sensitive detection of low abundance targets. Examples of radioimmunoassays include tests for cortisol, thyroid hormones, and cyclosporine. Although radioimmunoassays have often been considered the gold standard for detecting low abundance targets such as drugs and hormones, use of other increasingly sensitive nonradioactive assays that avoid the hazards of radiation is increasing.

Other immunologic methods to detect serum antibodies include the microscopic agglutination test, which is often used to detect antibodies to *Leptospira* spp; serum neutralization; gel immunodiffusion; and immunoblotting (or Western blotting).

The following immunoassay applications (ie, serology, immunohistochemistry, flow cytometry) can be used to determine titers for infectious disease testing, special stains for biopsy samples, and immunophenotyping.

**Serology for Infectious Diseases**

For serologic assays, the titer is the highest dilution of serum that still causes a positive response to the test (eg, fluorescent signal, color change by ELISA, positive agglutination response; Figure 2). Interpretation of titer results is dependent on characteristics of the clinical disease as well as vaccination status in some cases. A single high titer can
be supportive of active infectious disease. However, problems can occur with antibody-detection techniques, including positive titers that persist for months or years, depending on the agent, or antibody responses that are low early in the course of disease and require paired (ie, convalescent) titers if initial testing reveals a negative or inconclusive result.

For convalescent titers, the serologic test is repeated 2 to 4 weeks after the initial measurement. If the disease is active, a 4-fold rise in antibody titers is expected with an appropriate immune response, even if the disease is being treated appropriately. Convalescent titers may be necessary to confirm disease involvement. In one study involving leptospirosis, 45% of cases were considered positive only after paired titers.

Some laboratories may optimize a particular assay for the infectious disease being tested; this releases the clinician from the challenge of deciding which type of serologic test should be performed. Finding a laboratory with appropriate quality controls and reliable results, however, is essential. For paired titers, both samples should be run at the same facility to decrease variation.

When testing for infectious disease, clinicians must determine whether serology is desired or if an agent-demonstrating technique such as antigen testing, PCR, or cytology is preferred or possible.

**Immunohistochemistry**

Immunohistochemistry employs antigen–antibody reactions and is used primarily by pathologists and researchers. Antibodies specific to a target of interest in the tissue are applied and detected, often using an enzymatic reaction. This allows pathologists to recognize certain markers in tissues, such as cytokeratin and vimentin, which identify cells of epithelial vs mesenchymal origin, respectively, and use immunohistochemistry to identify whether lymphoma is of B- or T-cell origin from a lymph node or other tissue biopsy. Many other specialized markers identifying specific cellular antigens can reveal further information about the tissue samples; these are the “special stains” that allow a more detailed evaluation of histopathologic biopsies.

**Flow Cytometry**

Flow cytometry uses antibodies to recognize antigens in a sample, but it requires cells to be suspended in a solution. Flow cytometry’s primary clinical use is for aiding in the diagnosis of hematologic disorders and malignancies. Lymph node aspirates in saline, peripheral blood, and effusions are most commonly tested. Antibodies are added to the cell suspension to identify the types of cells present. For example, CD3, CD4, and CD8 are T-cell markers, whereas CD21 indicates B cells. CD34 is a marker of immature WBCs present in acute leukemias but absent in lymphoma—a disease of fully differentiated lymphocytes. Flow cytometry, therefore, is useful to immunophenotype and characterize the
particular markers on cells to determine if lymphocytes are primarily B or T; if the cells are large, small, or a mixed population; and if blast cells are present. These features help characterize the origin of the submitted cells.²

**Molecular Techniques**

Molecular techniques include PCR to amplify DNA and reverse-transcription PCR to detect RNA. Molecular diagnostics are reviewed in greater detail elsewhere²,⁷,⁸ but are briefly mentioned here as a contrast to the immunoassays described above. Although antibodies can be used to detect antigens, they are much less sensitive than PCR, and antibodies are not available for every animal pathogen. As long as the genetic sequence is known, PCR can demonstrate that an infectious organism’s DNA or RNA is present in the blood, tissue, feces, or other sample. However, because PCR assays are so sensitive, the organism detected may not always be the causative agent or be present in a quantity sufficient to induce disease. Molecular testing can also be negative if the organism is intermittently shed in the sample tested or after antimicrobial therapy if testing is performed for bacterial organisms such as *Leptospira* spp.

**Conclusion**

Immunodiagnostics employ antigen:antibody binding, with a variety of detection methods for the immune complexes formed. Antibodies can be created with a number of specificities to recognize hormones, drugs, and infectious agent antigens and antibodies. Immunoassays are often easier to perform but can be less sensitive and precise than other techniques (eg, PCR for infectious agents, chromatography for drug quantification). Immunodiagnostics are useful in infectious disease serology, for which tests are used to document antibody responses to certain pathogens. The choice of PCR vs serologic testing for an antigen or antibody depends on the stage of disease, whether an organism is expected to be present in the sample evaluated, and whether antimicrobials have been used. If serology is elected, convalescent titers are frequently needed to confirm disease presence.

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**References**

Osurnia™
(foflencinoo-terbinafine-betamethasone acetate)

Otic gel
Antibacterial, antifungal, anti-inflammatory

For Otic Use in Dogs Only

Caution:
Federal (USA) law restricts this drug to use by or on the order of a licensed veterinarian.

Description:
OSURNIA contains 10 mg foflenicinoo, 10 mg terbinafine and 1 mg betamethasone acetate per mL and the inactive ingredients propylene carbonate, glycerol formal, hypromellose, phospholipid, oleic acid and BHT in an off-white to slightly yellow translucent gel.

Indication:
OSURNIA is indicated for the treatment of otitis externa in dogs associated with susceptible strains of bacteria (Staphylococcus pseudointermedius) and yeast (Malassezia pachydermatis).

Dosage and Administration:
OSURNIA should be administered in the clinic. Clean and dry the external ear canal before administering the initial dose of the product. Administer one dose (1 tube) per affected ear(s) and repeat administration in 7 days.

Open tube by twisting the soft tip. Insert the flexible tip into the affected external ear canal(s) and squeeze entire tube contents into the external ear canal(s). After application, gently massage the base of the ear to allow the gel to penetrate to the lower part of the ear canal.

Contraindications:
Do not use in dogs with known tympanic perforation (see Precautions). Do not use in dogs with a hypersensitivity to foflenicinoo, terbinafine or corticosteroids.

Warnings:
Not for use in humans. Keep this and all medications out of reach of children. Consult a physician in case of accidental ingestion by humans. In case of accidental skin contact, wash area thoroughly with water. Avoid contact to the eyes.

Precautions:
Do not administer orally. The use of OSURNIA in dogs with perforated tympanic membranes has not been evaluated. The integrity of the tympanic membrane should be confirmed before administering this product.

Reevaluate the dog if hearing loss or signs of vestibular dysfunction are observed during treatment. Use of topical otic corticosteroids has been associated with adrenocortical suppression and iatrogenic hyperadrenocorticism in dogs (see Animal Safety).

Use with caution in dogs with impaired hepatic function (see Animal Safety). The safe use of OSURNIA in dogs used for breeding purposes, during pregnancy, or in lactating bitches, has not been evaluated.

Adverse Reactions:
The following adverse reactions were reported during the course of a US field study for treatment of otitis externa in dogs treated with OSURNIA with 1 tube per affected ear(s) and repeated after 7 days.

Frequency of Adverse Reaction by Treatment

- Adverse Reaction
  - Elevation of Alkaline Phosphatase
  - Vomiting
  - Elevated AST, ALT, ALP
  - Weight loss (>10% body weight)
  - Hearing Decrease/Loss

To report suspected adverse drug events, contact Elanco US Inc. at 1-888-545-5973. For additional information about adverse drug experience reporting for animal drugs, contact the FDA at 1-888-FDA-VETS or http://www.fda.gov/AnimalVets/SafetyHealth For technical assistance, contact Elanco US Inc. at 1-888-545-5973.

Clinical Pharmacology:
OSURNIA is a fixed combination of three active substances: foflenicinoo (antibacterial), terbinafine (antifungal) and betamethasone acetate (steroidal anti-inflammatory). Foflenicinoo is a bacteriostatic antibiotic which acts by inhibiting protein synthesis. Its spectrum of activity includes Gram-positive and Gram-negative bacteria. Terbinafine is an antifungal which selectively inhibits the early synthesis of ergosterol. Betamethasone acetate is a glucocorticosteroid with anti-inflammatory activity. OSURNIA dissolves in ear wax and is slowly eliminated from the ear mechanically.

Antibacterial, antifungal, anti-inflammatory Ear inflammation can increase the percutaneous absorption of active substances in OSURNIA. In a laboratory study conducted in two veterinary species (see Animal Safety), low plasma levels of foflenicinoo, terbinafine, and betamethasone acetate were measurable during the first 2-4 days after administration of 1X dose, and during the first 2-7 days after administration of 5X dose. No quantitative plasma concentrations of any of the three active ingredients were observed in the pre-dose samples of most dogs prior to second and third administrations. Although total and peak exposure in the blood tended to be highly variable between dogs, systemic drug concentrations tended to increase in a less than dose-proportional manner as the administered dose increased from 1X to 5X.

Microbiology:
The compatibility and additive effect of each of the components in OSURNIA was demonstrated in a component effectiveness and non-interference study. An in vitro study of organisms collected from clinical cases of otitis externa in dogs determined that foflenicinoo and terbinafine inhibit the growth of bacteria and yeast commonly associated with otitis externa in dogs. No consistent synergistic or antagonistic effect of the two antimicrobials was demonstrated. The addition of betamethasone acetate to the combination did not impair antimicrobial activity to any clinically significant extent.

In a field study (see Effectiveness), the minimum of 10 isolates from successfully treated cases with OSURNIA was met for Staphylococcus pseudointermedius, Malassezia pachydermatis, and Pseudomonas aeruginosa. However, there were only three dogs where P. aeruginosa was the only pathogen cultured and they were all treatment failures. Therefore, OSURNIA may not be effective in treating otitis externa in which P. aeruginosa is the only pathogen present.

Effectiveness:
Effectiveness was evaluated in 255 dogs with otitis externa. The study was a double-masked field study with a placebo control (vehicle without the active ingredients). One hundred and ninety-nine dogs were treated with OSURNIA and seventy-six dogs were treated with the placebo control. All dogs were evaluated for safety. Treatment (1 mL) was administered to the affected ear(s) and repeated 7 days later. Prior to the first administration, the ear(s) were cleaned with saline but not prior to the Day 7 administration. Six clinical signs associated with otitis externa were evaluated: pain, erythema, exudate, swelling, odor and ulceration. Total clinical scores were assigned for a dog based on the severity of each clinical sign on Days 0, 7, 14, 30, and 45. Success was determined by clinical improvement at Day 45. The success rates of the two groups were significantly different (p=0.0094): 64.76% of dogs administered OSURNIA were successfully treated, compared to 43.42% of the dogs in the placebo control group.

Animal Safety:
In a target animal safety study, 24 mixed breed dogs (4 dogs/sex/group) were surgically administered 0X, 1X (1 mL/ear or 2 mL/dog with repeated administration in 7 days) or 5X (5 mL/ear or 10 mL/dog with repeated administration in 7 days) doses of OSURNIA for a total of 6 administrations in 5 weeks. All dogs remained in good health with normal hearing throughout the study. Decreased weight gain was noted in the 1X and 5X dogs compared to the control group. Clinical findings included post-administration ear wetness in 1X and 5X groups and unilateral, transient brown/red discharge from one ear each in two SX dogs, with erythema in one dog after the 4th application. Local microscopic changes in ears (without clinical effects) included: slight or moderate unilateral vesicle formation within the epithelium of the tympanic membrane in two 1X and four 5X dogs, and unilateral mucosal ulceration in the lining of the middle ear cavity in three 5X dogs. Three 5X dogs had slightly elevated ALT activity, accompanied by minimal or mild microscopic hepaticocellular vacuolation (in two dogs). Cortisol response to ACTH stimulation was decreased, but within the normal reference range, in 1X dogs. The 5X dogs had a decrease in serum cortisol levels after ACTH stimulation (below normal reference range) accompanied by decreased adrenal gland and thymic weights with minimal adrenal cortical atrophy and slight (in three dogs) or moderate (in one dog also noted with slightly lower lymphocyte counts) lymphoid depletion of the thymus. The ACTH stimulation test results are consistent with systemic absorption of betamethasone resulting in a likely reversible suppression of the hypothalamic-pituitary-adrenal axis as seen with administration of exogenous corticosteroids.

Storage Conditions:
OSURNIA should be stored under refrigerated conditions between 36° - 46° F (2° - 8° C). Ear inflammation can increase the percutaneous absorption of active substances in OSURNIA.

To facilitate comfort during administration, OSURNIA may be brought to room temperature and stored for up to three months.

How Supplied:
OSURNIA is a gel in a single use tube with a flexible soft tip, supplied in cartons containing 2 or 20 tubes.

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Manufactured for:
Elanco US Inc. Greenfield, IN 46140, USA

Product of Great Britain

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Otitis Treatment: Some Welcome Relief

Otitis externa is a major reason for insurance claims for dogs.\(^1\) It is also fraught with management difficulties, not the least of which are the pain and hassle of medicating the dog. For years, veterinarians have had to rely on once- to twice-daily otic products, making compliance a challenge. More recently, ear packs containing an antibiotic, antifungal, and topical corticosteroid have become available; however, there have been anecdotal concerns regarding these lanolin-based products causing deafness.

Osurnia\(^2\), an innovative adaptable gel product from Elanco, offers veterinarians an easily administered alternative to traditional otitis treatments without the need for an owner to remember, and be able, to insert daily or twice-daily drops into the dog’s inflamed ears. Containing the antibiotic florfenicol, the antifungal terbinafine, and the steroid betamethasone acetate, Osurnia is indicated for the treatment of canine otitis externa associated with susceptible strains of bacteria (Staphylococcus pseudintermedius) and yeast (Malassezia pachydermatis).\(^3\)

Osurnia requires only 2 doses, applied one week apart. Each dose comes premeasured in an easy-to-use applicator with a flexible tip. The first dose is applied in the clinic, alleviating owners of the stress of medicating the painful and inflamed ears of their dog. After the ear has been cleaned, Osurnia is deposited into the ear, and gentle massage allows the gel formulation to coat and adhere to the ear canal surface. A second dose is then administered 7 days later at a medical progress examination. The need for only 2 doses of Osurnia, and in-clinic administration, make the treatment of the condition convenient for pet owners. The ingredients in the gel provide up to 45 days of efficacy and owners do not need to, in fact should NOT clean their dog’s ears during this time. This regimen allows the dog to recover without the repeated application of medications, cleaners, wipes, and cotton.

Approaching an Otitis Case

Taking a thorough history is an important first step in determining the cause of otitis. Important information can be gleaned including seasonality, recurrence, possible triggers, concurrent or previous dermatologic signs, other pets affected, etc. It is important when working up an otitis case to have 2 goals: 1) to identify and treat the primary cause, and 2) to identify and manage concurrent perpetuating causes. (see Table) Treating only the infection, for example, without addressing the primary factor will most likely result in treatment failure or recurrence.

**KEY POINTS**

- Otitis is a multi-component disorder that requires investigation of primary, predisposing, and perpetuating factors.
- Pain and discomfort make compliance with veterinary recommendations problematic for even diligent pet owners.
- The 2-time-only application method for Osurnia\(^2\) provides up to 45 days of efficacy to bring relief for pet and owner and a route to successful treatment.

**Important Safety Information**

OSURNIA (florfenicol/terbinafine/betamethasone acetate) is for otic use only under veterinary supervision. Do not use in dogs with known tympanic perforation or a hypersensitivity to florfenicol, terbinafine or corticosteroids. Adverse reactions observed during clinical trials include vomiting, increased liver enzymes and transient loss of hearing. Please see accompanying product insert for full prescribing information.
Once a thorough history has been obtained, both the external ear canals and pinnae should be thoroughly examined for the presence of erythema, exudate, discharge, odor, crusting, scaling, and swelling/stenosis. Evaluation of the cartilage to determine if it is soft and pliable or firm, the latter suggesting fibrosis or calcification, should be conducted. Both ears should be examined, including otoscopically, even if only unilateral disease is present. A full dermatologic examination of the patient should also be performed to assess for concurrent dermatologic disease/clinical signs.

An Important Key to Treating Otitis

Cytologic evaluation of the otic exudate, collected using a cotton-tipped applicator, is a simple and inexpensive test that can help the veterinarian to successfully treat an individual case. Both ears can be evaluated on the same slide by separating the samples to opposite ends. The slide is heat fixed and treated with Romanowsky stain (eg, Diff-Quik) and then examined under the microscope for numbers and types of bacteria, yeast, and inflammatory cells. This information generates immediate guidance in the design of a treatment plan as well as immediate feedback to owners as to what type of infection may be present. In addition, taking cytology at medical progress examinations helps the clinician assess response to treatment. The failure to do an initial cytologic examination may result in use of an ineffective treatment, resulting in perpetuation of the problem. In some refractory cases, especially those involving rod-shaped organisms, culture and susceptibility testing may be needed.

Once cytology indicates whether and what type of infection is present, a treatment plan should be instituted. Ear cleaning is an important component of managing otitis externa, as it can help facilitate examination of the ear canal; remove microbes, small foreign bodies, and debris; and increase exposure of the lining of the ear canal to topical agents. Often the first cleaning can be done in the clinic and then a new cleanser can be sent home for use in subsequent ear maintenance once the infection is cleared. In cases of severe otitis, including otitis media, it may be necessary to do a deep ear cleaning and evaluation under anesthesia.

In the presence of either *Staphylococcus pseudintermedius* or *Malassezia pachydermatis*, using Osurnia® allows the practitioner the freedom to treat the ear while investigating for the underlying triggers. Meanwhile, the simplified, 2-time-only application eliminates the issue of owner compliance.

Osurnia is also a good choice in patients that are being treated for atopy or food allergy but occasionally flare with otitis externa during allergy season. The residual activity of its active ingredients remains up to 45 days, which helps prevent recurrence. The clinician may meanwhile adjust the allergy therapy to avoid those occasional flares.

If cytology is the first key, determining why the dog has otitis is the next. As mentioned earlier, the 2 goals in treating otitis are, first, to identify and treat the primary cause, and then to identify and manage concurrent perpetuating causes. If only the infectious cause is determined and treated, the clinician is setting the scenario for failure and more frequent recurrence. Utilizing the findings from the history, physical/otic/dermatologic examinations, and cytology can help uncover the possible underlying causes. Since allergy is so often a trigger, it is often prudent to begin the dog on a food trial and/or perform a workup for atopic dermatitis while treatment has begun.

### Table

**COMMON PREDISPOSING, PRIMARY, AND PERPETUATING FACTORS IN OTITIS**

<table>
<thead>
<tr>
<th>Predisposing Factors*</th>
<th>Conformation</th>
<th>Excessive moisture</th>
<th>Excessive cerumen</th>
<th>Systemic disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Causes</td>
<td>Parasites (<em>Otodectes</em> spp, <em>Demodex</em> spp)</td>
<td>Atopy</td>
<td>Food allergy</td>
<td>Endocrine disease</td>
</tr>
<tr>
<td></td>
<td>Foreign bodies</td>
<td>Endemic dermatitis</td>
<td>(such as pemphigus foliaceus)</td>
<td></td>
</tr>
<tr>
<td>Perpetuating Factors†</td>
<td>Bacteria/yeast</td>
<td>Otitis media</td>
<td>Ruptured tympanic membrane‡</td>
<td>Chronic disease</td>
</tr>
</tbody>
</table>

*Create risk  †Impede resolution  ‡Do not use Osurnia in dogs with known tympanic perforation.

**References**

2. Elanco, Osurnia® product label.

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