A MANUAL OF METHODS FOR LABORATORY DIAGNOSIS OF AVIAN CHLAMYDIOSIS

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PREFACE

Avian species as pets have become increasingly popular over the past several years. Every indication is that this trend will continue. The value of such birds and the emotional attachment of owners have resulted in pet birds becoming an important part of the practice of many veterinarians.

Chlamydiosis is an increasing problem for the bird owner, the veterinarian and the public health officials. The need for a confirmed diagnosis has placed increased pressure on the practitioner and on diagnostic laboratories to provide this service. Many laboratories do not provide diagnostic aid for chlamydiosis in birds because of the danger of human infection, lack of facilities and lack of interested or trained personnel.

It is the purpose of this publication to 1) acquaint the veterinary practitioner with the services and limitations of laboratory assistance, 2) explain some of the problems of interperting laboratory results, 3) provide a guide to labcratories not now conducting examinations for chlamydiosis and 4) foster more uniform methods and interpretation of results amoung laboratories doing examinations for chlamydiosis. A further purpose is to begin to establish standard laboratory procedures in the likelihood that chlamydiosis will become a part of a control program under the National Cage and Aviary Bird Improvement Program.

There is no intent to make this publication a complete coverage of chlamydiosis in birds, therefore, topics such as clinical hematology, treatment and public health procedures are

not included. The reader is referred to other publications for a more comprehension coverage of chlamydiosis.

INTRODUCTION

Chlamydiosis (psittacosis - ornithosis) is a frequent infection in several species of birds. The disease in birds ranges from subclinical to fatal infections. There is variation in the disease among species of birds and also varying pathogenicity among different isolates of the causative agent.

Chlamydiosis has been recognized in psittacine and non-psittacine birds for many years. According to some investigators the incidence in cage and aviary birds and in man from contact with these birds has been on the increase over the past several years (McDonald & Boyer, Grimes et al., and Panigrahy), Much of this increase has been in the large psittacine birds and may be related to the requirement that all imported birds be quarantined. Suffice it to say that chlamydiosis is an economic problem in cage and aviary birds and that there is a significant public health risk, because the infection is readily transmitted from birds to man.

Chlamydiosis first became a problem in turkeys in early 1950 and was a major problem between 1951 and 1956. The disease was transmitted from turkeys to workers in processing plants where infected flocks were processed.

It is not within the scope of this publication to review the history of chlamydiosis. For more information the reader is referred to numerous review articles and text books.

It is important to realize that chlamydiosis is a major disease problem for the cage and aviary bird industry, the pet store, the pet bird owner, the veterinarian, public health

agencies and the public. Programs and acceptable procedures for certifying that flocks as well as individual birds are free of chlamydiosis must be developed. The increase in pet birds in facilities for elderly citizens is of major concern because such populations are especially susceptible to severe chlamydial infections.

ETIOLOGY

The etiologic agent of chlamydiosis is Chlamydia psittaci. Chlamydia are non-motile, spheroid, bacterial organisms that are obligate intracellular parasites. They have a cell wall and a metabolism that is similar to bacteria but have a more complex replication cycle. The infectious particle is an elementary body. Within the host cell it enlarges to a reticulated spheroid, known as an initial body. The initial body divides by binary fission creating clusters of daughter cells which reduce in size and become the elementary bodies, thus completing the life cycle. Chlamydia psittaci can be cultivated only in susceptible animals, avian embryos or cell cultures.

Chlamydia are susceptible to chemicals and are readily inactivated by quanternary ammonia compounds and lipid solvents. In tissue homogenates the organisms are inactivated by 56 C for 5 minutes, 37 C in 48 hours, but at 4 C the organisms will live for up to 50 days (Page). C. psittaci in yolk sac membranes or cell suspensions can be preserved by freezing at -20 C or below.

Different isolates of C. psittaci vary greatly in pathogenicity; however, antigenic differences among avian isolates are only minor, thus for practial purposes all avian isolates are considered to be of a single antigenic type.

All avian strains can be propagated in chicken embryos and apparently in a number of types of cell lines of murine, avian or human origin. There is some evidence that cell cultures will not detect small numbers as well as avian embryo. Laboratory animals usually used include guinea pigs and mice. C. psittaci is

susceptible to some antibiotics such as tetracycline, chloramphenicol, enythromycin and penicillin. Certain antibiotics have no effect on chlamydia, thus, can be useful in inhibiting other bacteria or fungi during primary isolation.

SAMPLE COLLECTION

Serology

Whole clotted blood may be submitted to the laboratory in a single tube. Do not submitt the sample in a capillary tube because it is difficult to harvest the serum from a capillary tube. If submitting whole clotted blood send at least 0.5 ml. preferably serum should be separated from the blood clot prior to submission to the laboratory. If serum is submitted provide the laboratory with at least 0.2 ml of nonhemolized serum. Plasma may be submitted instead of serum. More blood or serum should be provided if the size of the bird permits collecting a larger sample.

Isolation

A. Live bird:

1. In the live bird collect a cloacal swab using a steril cotton swab or a calcium alginate swab depending on the size of the bird. Swab the mucosa of the cloaca and collect as much fecal material as will adhere to the swab. Swirl the swab in 2 ml of a transport broth containing appropriate antibiotics (appendix). Discard the swab, tightly stopper the tube, label and send by the fastest method to the laboratory. If the sample can not be transported immediately it should be frozen. If there is to be any delay during transportation, pack with dry ice or one of the refreezable type refrigerant.

2. Freshly passed feces may be collected and placed in an appropriate sterile tube, whirl pack bag, or in a tube of the transport medium. The transport medium will be specified or

furnished by the laboratory doing the examination. A satisfactory medium is brain-heart infusion broth with antibiotics (appendix). The tube containing any sample must be tightly stoppered and parafilmed or taped to prevent leakage and transported in styrofoam shipping cartons.

B. Dead birds:

Most laboratories would prefer to recieve the unopened carcass of a freshly dead bird. The owner should be discouraged from making any effort to open the bird. If the veterinarian wishes to perform a necorpsy, he should aseptically collect tissues for submission to the laboratory. The carcass or samples should be appropriately packaged and transported to the laboratory under refrigeration but not forzen.

Necropsy Procedures:

A. Safety: When a necropsy is performed on a bird suspected of having chlamydiosis, safety precautions should be taken. At a minimum:

1. Wear protective clothing and gloves, and work under a hood if possible or wear a surgical mask.

2. Wet or dip the carcass in a solution of disinfectant.

3. Provide safe, covered disposal trays or other procedures for disposing of contaminated instruments or other materials.

B. Necropsy procedure:

1. Observe for signs such as diarrhea and soiling of the feathers around the vent, emaciation and nasal or ocular

discharges.

2. Open the bird carefully using usual necropsy techniques. Observe for gross lesions such as airsacculitis, pericarditis, splenomegaly, hepatitis or peritonitis.

3. Using clean glass slides make impression smears of a) air sacs b) pericardium c) the uncut surface of the spleen and d) liver. Air dry the slides and lightly fix with heat or with methanol for 5 minutes. Save for staining to look for chlamydia.

4. For C. psittaci isolation attempts, collect tissues as outlined below and place a sample of approximately 1 gm from each organ in a grinder. The two main systems used for isolation are the chicken embryo and cell cultures; the mouse is used less frequently. The tissues to be collected will differ to some extent according to the system to be used. In a diagnostic laboratory differential diagnosis is also important and additional samples may be collected for that purpose. Collect and process samples as follows for isolation attempts in:

A. Cell cultures - collect air sacs, pericardium, the apex of t heart and spleen. Liver is avoided because of possible toxicity for cells in cultures. If the examination is to be restricted to chlamydia, pool the tissues, mince and grind using a mortar and pestle. Suspend tissues in Hanks, MEM or some suitable broth medium to make a 20% suspension weight/volume.

B. Avian embryos - to isolate chlamydia as well as other pathogens such as paramyxo, influenza or herpes viruses, the following pools may be prepared.

1. Pooled spleen and lung

2. Pooled liver and pericardium

3. Pooled cloacal and tracheal swabs

A 20 to 30% wt/vol tissue suspension is prepared and used undiluted for inoculation of embryos.

5. Histopathology - collect appropriate pieces of varius organs and fix in 10% formalin.

6. Complete the examination and dispose of carcass by incineration and make certain that the working area and all materials are safely decontaminated.

LABORATORY PROCEDURES AND INTERPRETATION

Serology

Serologic procedures may aid in the diagnosis of chlamydiosis but there are many limitations. The most widely used test is the complement fixation (CF) test. It has been reported that the sera from some avian species do not fix guinea pig complement. Modifications of the CF test such as the indirect CF test were developed to overcome this problem. It is now apparent however that the direct CF test does work in certain avian species if appropriate antigens are used. Although it was reported that turkey sera would not fix complement, the direct CF test works well when using antigen prepared by the method of Grimes. This same antigen has been found to work satisfactorily for testing psittacine birds. For some as yet unexplained reason, the direct CF does not appear to work well in some small

psittacines such as parakeets and possibly young African greys. To restate, the preparation of the antigen for the direct CF test in birds is critical.

Serology is best used as a diagnostic procedure in a flock or population of birds. Serologic titers in individual birds may be a guide to diagnosis. If the titer is "high" over 1:32 in most laboratories then it probably indicates a current infection. Low titers or even negative titers may not be that meaningful in the individual bird.

The antigen produced by Grimes is from cell cultures and has a more universal application for the direct CF test than antigens previously available. (See appendix - for preparation of antigen and detailed procedures for conducting the test). This or some other similar antigen must be made available so all laboratories doing serology can use the direct CF test and avoid having to become involved with the more complex indirect CF test. Other tests such as ELISA may replace the CF test, but must await more research before being recommended for testing birds.

ISOLATION AND IDENTIFICATION

The three host systems that are most often used for isolating C. psittaci are mice, chicken embryos and cell cultures and mice. A combination of chicken embryo - cell culture is sometimes used to an advantage. The avian embryo is considered more sensitive in detecting small numbers of organisms and is also useful in detecting viruses that may be essential to a differential diagnosis. The cell culture system allows a more

rapid result with a short "turn around" on samples.

Accepted procedures now being used by some laboratories doing examinations for C. psittaci are given as minimum standards.

Samples: The samples or tissue suspensions as outlined under "Sample Collection" are centrifuged for 20 minutes at 2000 RPM (approximately 800 g) in a safety centerfuge carrier under a hood if possible. The supernate is collected for inoculation and the pellet is discarded.

Chicken Embryos:

1. Use mycoplasma free eggs (specific pathogen free eggs are recommended) and incubate fo 6 to 8 days.

2. Inoculate each of 5, 6 to 8 day old embryos with 0.2 to 0.3 ml by the yolk sac route.

3. Incubate inoculated embryos at 37 C and candle daily.

4. If embryos die on the 2nd or 3rd day post inoculation harvest amnionic - allantoic fluid (AAF) as in a standard Newcastle disease virus isolaton procedure.

5. Harvest the yolk sac from embryos that die on the 4th through 9th day. If no embryo mortality has occured by the 9th day post inoculation, harvest the yolk sac from the live embryos.

6. Make impressions from the yolk sacs after as much yolk as possible has been removed. Stain the impressions by the Giemsa and Gimenez methods (see appendix for staining procedure) and examine microscopically for the typical organisms.

6.A. As an alternate procedure:

a. Freeze yolk sacs at -20 C until hard (about 2 hrs) thaw

and grind in Tenbroeck homogenizer or other suitable homegonizer. Add sufficient broth with antibiotics to make a 10% suspension, centrifuge as previously described and inoculate cell cultures (procedure as outlined under "Cell culture").

b. After 48 to 72 hrs of incubation stain by Gimenez or procedure prepare and examine with fluorescent antibody procedures. (see "Cell Culture" for procedure).

Cell culture:

1. Prepare monolayers of mouse fibroblast cells, clone L-929 or McCoy cells that are free of mycoplasma or other suitable cell lines on coverslips in wells in multiwell plates or one gram shell vials.

2. Pretreat each cell monolayer on coverslips with diethlaminoethyldextran using 20 g per ml.

3. Inoculate four wells or vials with the suspensions outlined under "sample collection" diluted 1:25. Other wells or vials should be inoculated wth a reference culture of C. psittaci to serve as a positive controls.

4. Centrifuge at 800 x g for 1 hour.

5. Incubate stationary for one hour at 35 to 37 C.

6. Remove excess inoculum and replace with fresh cell culture medium. Incubate at 35 to 37 C.

7. After 48 hours incubation remove 2 coverslips and stain one by Gimenez and one by Giemsa method.

Examine with a light microscope. Use the high dry objective to locate a field that has numerous stained cells. Use oil emersion to examine for chlamydia which will be located

intracellularly. With the Gimenez stain the elementary bodies will stain red or reddish-purple. The intermediate and larger forms will stain blue or bluish-green. With the Giemsa stain the elementary bodies will stain reddish-purple while the other forms will stain bluish.

If the 48 hour cultures are negative, examine the other two coverslips after 72 hrs post inoculation.

7.A. The fluorescent antibody procedure may be used instead of the Gimenez staining procedre. Anti-chlamydial conjugate is used for direct examination of coverslips after 48 to 72 hours of incubation.

The procedure is:

a. Incubate cultures at 37 C for 48 to 72 hours.

b. Aspirate medium and rinse cultures with 0.01 M phosphate buffered saline (PBS), pH 7.2.

c. Add 1.0 ml of acetone and hold at room temperature for 10 minutes.

d. Remove acetone and add 0.2 ml of anti-chlamydial conjugate. Incubate at 37 C for 30 minutes.

e. Remove conjugate, rinse with PBS, and then rinse with 0.005% Evans blue stain.

f. Remove coverslip and mount cell side down with 1 to 2 drops of 50% glycerol in PBS. Remove excess fluid from slide.

g. Examine by fluorescent microscopy. The Chlamydia will be observed as bright green inclusions in the cytoplasm of the cells.

Mouse inoculation:

Using undiluted samples as outlined under "sample collection" for avian embryos or cell culure:

1. Inoculate weanling or young adult mice intraperitoneally using 0.2 ml/mouse.

2. Make impressions of peritoneal fluid (also spleen if desired) from mice that became sick (ruffled with pasty eyes) or die and stain by the Gimenez or Giemsa method for microscopic examination.

3. If mice fail to become sick or die in 7 to 10 days, passage spleen suspensions to other mice, chicken embryos or cell cultures.

APPENDIX

To include detail procedures such as:

Dubeceos phosphate buffered saline wth diethyaminoethyl (DEAE) - Dextran.

Gimenez stain and procedure

Giemsa stain and procedure

Media for cell culture and procedure for preparing monolayers on coverslips.

Procedure for CF test FA conjugate and procedures

Source for supplies

Antigen preparation for CF test

Antibiotics to use and concentration - instructions for preparation

Transport media

Others

List of Laboratories