Detection of *Chlamydia psittaci* Infection in Pet Birds Using a Molecular Based Diagnostic Assay

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Summary: *Chlamydia psittaci* infection is well established in our pet bird population. Infections range from a clinically inapparent state, with intermittent shedding of the organism, to overt clinical disease and mortality. Establishing a confirmatory diagnosis of infection in the live bird is difficult as no single test can accurately diagnose the disease in all species or at all times. Conventional diagnostic methods have inherent problems in detection (sensitivity & specificity) and interpretation of results which are often equivocal. A molecular based DNA assay was developed to test clinical samples for the presence of the chlamydial organism. Test performance was evaluated both *in vitro* and *in vivo* via a controlled infectivity study. The molecular based assay was extremely sensitive and specific in detecting *Chlamydia psittaci* in infected birds.

Introduction

Avian chlamydiosis is an important disease that has historically affected aviculture for decades. Disease impact ranges directly from overt clinical disease and mortality to the often non-diagnosed effects on growth, health, and reproduction. The *Chlamydia psittaci* organism exhibits a pronounced variability in host susceptibility, pathogenicity, course of disease, and diagnostic parameters. While numerous diagnostic methods have become available over the years, test results are often equivocal, making a confirmed diagnosis elusive.

Chlamydial Infection

The elementary body is the infective form of the Chlamydial organism which reproduces by infecting a host cell. Generally this occurs at the initial site of infection. The columnar epithelium of the mucous membranes (and macrophages) lining the respiratory or digestive route is generally the first area involved. During this localized phase, the chlamydial organism enters the host cell, undergoes transition to a large reticulate body (inclusion) and through growth and division, produces between 100-500 chlamydial bodies per cell. Approximately 48 hours post infection, the cell may rupture, releasing these infective organisms. Sometimes however, cells do not lyse, causing them to remain chronically infected for long periods of time.

The organism has been isolated from over 120 species of birds with infection in parakeets, lovebirds, cockatiels, amazon parrots, and macaws being the most prevalent. In general, young birds are more susceptible to chlamydial infection than adults. New World species, especially amazon parrots and macaws appear to be most susceptible while cockatoos and African gray parrots are fairly resistant

Chlamydia psittaci infections can persist in a clinically inapparent state with intermittent shedding of the organism over long periods of time. Many birds, especially parakeets, lovebirds and cockatiels, remain asymptomatically infected. These "carrier" birds appear normal but can shed large numbers of organisms during periods of stress. Shedding occurs in the droppings, nasal and ocular discharges, and oVMD and pharyngeal secretions. Feather dust, droppings, and secretions are frequent sources of transmission as they dry and become dispersed through the air. Other birds become infected by either inhaling or ingesting these sources of the organism. Vertical transmission through the egg has also been shown to occur.

Incubation Period
The time between exposure and clinical disease is highly variable. The diverse response to infection among the wide range of avian species and variable strain virulence account for differences in the incubation period. Estimates of this period in caged birds vary from days to weeks and longer. Most commonly, this period is at least 3 to 10 days in length. Latent infections are common and active disease may occur years after exposure. Estimates have ranged from 42 days up to several years. Incubation period is difficult to assess due to these chronically infected birds that develop persistent, asymptomatic infections.

Clinical Disease
The course of Chlamydial infection in pet birds is extremely variable. The pathogenicity of the organism and relative susceptibility of the host species are major factors in determining the outcome of infection. A considerable diversity exists in the pathogenicity and virulence of different strains of the avian Chlamydial organism. Some may produce only sub-clinical infections while others almost always produce severe, fatal disease. Infections in pet birds may range from showing no clinical signs to subacute, acute, or chronic disease. The species of bird involved also affects the outcome of infection. One particular strain may cause severe disease in one species while causing mild or no clinical signs in another. Route of exposure and stress on the individual are other determining factors. The virulence of a particular strain of *Chlamydia* and the disease that it produces is also dependent upon certain hepatic and nephrotoxins. The high number of birds with significant chlamydial antibody titers suggests that most infections occur without the development of obvious clinical disease.

Chlamydia Diagnosis
Chlamydial infections can generally be adequately treated provided they are accurately diagnosed. Accurate diagnosis however can represent a considerable challenge. Establishing a confirmatory diagnosis of infection in the live bird is difficult as no single test can accurately diagnose the disease in all species or at all times. In one study, several commonly used diagnostic tests each run on 246 individual birds, gave total positive test results ranging from 2.5% to 58.5%.[1] Alternately, no single test can guarantee that a bird is free from the infection.

Traditionally, diagnostic tests for avian chlamydiosis have relied on the direct detection of the organism in clinical samples or indirect detection by measuring a host response to the organism. Antigen and organism tests detect physical characteristics of the organism being present in various clinical samples or the organism itself (cell culture, enzyme linked immunosorbent assay, & immunoflourescent stains). Host response tests often measure some type of serologic response of the bird to infection (latex agglutination, blocking enzyme linked immunosorbent assay, elementary body agglutination).

Conventional methods have inherent problems in detection (sensitivity & specificity) and interpretation of results. No one particular method is "ideal." While most available tests can support a tentative diagnosis of chlamydiosis, results are often equivocal and open to interpretation. As in most diseases, test results must be interpreted in light of a thorough medical evaluation involving a complete history, physical exam, routine diagnostic tests, and response to therapy to arrive at a correct diagnosis. Current commonly used methods of Chlamydia diagnosis are as follows:

Culture
Chlamydial culture, where the organism is grown in mice, chick embryos, or tissue culture, directly demonstrates the presence of the chlamydial organism in a clinical sample.[2] It historically has been the most specific diagnostic procedure and a considered "gold standard" for chlamydial diagnosis. The method allows for the detection of a small number of organisms making it a sensitive diagnostic tool. Tissue samples (liver, spleen, kidney) and droppings can be tested in this manner. A positive result reliably predicts a chlamydia-infected bird. However, a negative result is not as reliable in determining that a bird is not infected. Disadvantages include false-negative results, which can be due to intermittent shedding and/or loss of the organism's viability. Specialized handling of the clinical sample is imperative to insure a reliable test. An additional disadvantage of cell culture is that it can take up to 2 weeks to determine a result and is often expensive.

When screening live birds for *C. psittaci*, the microorganism may not be shed daily. Serial specimens should be collected over a 3 to 5 consecutive day period. Specimens should be refrigerated and transported with wet ice. Diagnosis by isolation of *C. psittaci* in cell culture remains difficult and requires sufficient numbers of viable, infectious organisms to confirm infection.[2]

ELISA

Enzyme linked immunosorbent assay methods (ELISA) for the detection of *C. psittaci* relies on a monoclonal antibody binding to a specific antigen of the chlamydial organism.
Two of the ELISA tests currently being used to detect C. psittaci were developed for human Chlamydia detection (IDEIA, Johnson & Johnson Surecell). While their sensitivity and specificity for detecting *C. psittaci* in birds is unknown, they have had some benefit as an in-clinic screening procedure.[3] These results must be evaluated in light of other clinical findings. If a bird is ELISA positive but appears clinically normal, other tests should be run to verify the infection. Conversely, chlamydiosis cannot be ruled out in a clinically ill bird with a negative ELISA result.

Antibodies generally react weakly with the chlamydial elementary body because the antigen is often not readily accessible for interaction. False negative results will occur when insufficient numbers of organisms exist in the clinical sample. Alternately, high levels of certain contaminating bacteria (*Staphylococcus* sp.) can cause false positive results. ELISA methods, in general, are less sensitive than cell culture isolation.

Immunofluorescent Stains

Antibodies produced against Chlamydial antigens can be combined with fluorescein stains and used to identify elementary bodies in a test sample. Choanal, cloacal or fecal smears from live birds or impression smears from necropsy tissues can be tested. This method is most useful if large amounts of antigen are present in the sample. Non-specific fluorescence in cloacal or fecal smears reduces the specificity of this test. The test does have the advantage that it can aid in a rapid diagnosis of infection and does not require the organism to be in a viable state.

Serologic Tests

Serology tests detect antibody produced by the bird in response to chlamydial infection. Many methods are available including direct complement fixation (DCF), indirect complement fixation, latex agglutination (LA), blocking enzyme linked immunosorbent assay (BELISA) and elementary body agglutination (EBA). The major problem with serologic testing is the interpretation of results. A positive serologic test result is evidence that the bird probably was exposed to *C. psittaci* in the past, but does not prove the bird is currently infected. Conversely, a negative serologic test result is not proof that the bird is free of infection. [4]

Direct Complement Fixation

The DCF test has been the most commonly used method in the past. The test is sensitive in detecting antibody activity but it only detects one type of antibody, IgG. IgG titers persists for long periods of time and do not usually change dramatically.[5] They indicate a possible past exposure to chlamydia but are not very useful in differentiating an active, current infection. DCF is diagnostic if a clinically ill bird is initially DCF negative, then shows a significant increase in IgG levels 5-7 days later. The test is therefor useful, only if paired serum samples are tested.

Another disadvantage is that some birds such as parakeets, lovebirds, and African Grey parrots produce antibody very irregularly. They may not develop sufficient antibody levels that can be readily detected. This will result in a false-negative test result. The indirect or modified complement fixation test is more sensitive than the DCF however it has the same disadvantages.

Latex agglutination

The LA test detects mainly serum IgM antibody.[6] It is not as sensitive in detecting IgG as DCF. IgM usually is present during the acute and active stage of chlamydial infection and does not persist for a long time like the DCF antibody. The LA test can be used to determine treatment efficacy because IgM levels generally drop after the infection has been eliminated. False-negative results are possible on specimens from birds with acute stage infections when antibody activity is not yet detectable and for certain species

that may not produce detectable antibody (lovebirds, parakeets, cockatiels). While positive test results are usually accurate, the LA has a low test senstivity.

BELISA

A chlamydia blocking antibody test has been developed in Europe. The BELISA test identifies serum antibodies in the blood of the bird. It is extremely sensitive, but test specificity has been questioned [(1)]. BELISA is not currently available or approved in the U.S.

EBA

Like the latex agglutination test the elementary body agglutination test determines active infection by detecting IgM antibody. It is however much more sensitive. It is an effective test to screen for *Chlamydia*-infected birds especially in birds with low grade infections.[4] Although the specificity of EBA is lower than desired, it is probably the most useful current serology test now available.

Molecular Based Diagnostics

Over the last 15 years, advances in the field of molecular biology have allowed for the development of extremely sensitive and specific nucleic acid (DNA, RNA) detection methods. The first applied use of viVMD specific DNA technology in avian disease diagnosis was marked by the development of tests for the Psittacine Beak and Feather Disease (PBFD) and avian polyoma (APV) viruses (Psittacine Research Group, University of Geaorgia). Research Associates Laboratory (VMD) has commercially offered these tests since 1992.

These DNA based diagnostics use specific nucleic acid probes, to identify distinct nucleic acid sequences, unique to the desired microorganisms' genome. The sequences are detected in DNA extracts from submitted blood and tissue swab samples. DNA amplification techniques coupled with internal sequence probe detection allows for diagnostic tests of <u>extreme specificity</u> and <u>sensitivity6</u>.[7]

The efficiency of molecular diagnostic techniques generally exceeds that of other methodologies. With the goal of improved diagnosis of avian Chlamydial infections and detection of non-clinical, "carrier" birds, VMD has developed an applied molecular based Chlamydia assay.

Materials and Methods

Molecular Based Assay

The VMD *Chlamydia psittaci* Assay was developed to quantify the amplification product derived from a conserved major outer membrane protein (MOMP) gene segment of the avian strains of *Chlamydia psittaci.*[8] DNA was extracted from blood, choanal swab, and cloacal swab samples utilizing a modification of standard methods.[2] Specific DNA primer sequences were determined from analysis of a conserved MOMP gene segment of the *Chlamydia psittaci* genome.[8,10,11] The MOMP gene sequence was selected as the target for PCR-based detection and differentiation of *C. psittaci* as it comprises the major exposed structural protein of the infectious elementary body.[8,12,13] A semi-nested polymerase chain reaction (PCR*) was developed and used to asymmetrically amplify a 199 base pair sequence of the MOMP gene fragment of *Chlamydia psittaci*. An internal 58 bp fragment was further amplified and detected from within the asymmetric amplification products. Based upon the limiting assay on serially diluted positive samples, the assay sensitivity was optimized for the detection of 10 copies of target DNA. The assay was further validated by comparison of results with known positive test samples. Assay specificity was assessed by testing negative control samples containing genomic DNA of human origin and that of major species of psittacine pet birds

Controlled Infectivity Study

A controlled infectivity, pilot study was conducted at a university laboratory, to evaluate the performance of the VMD chlamydia assay. Six cockatiels (*Nymphicus hollandicus*) were selected for study based on normal findings on physical examination, choanal and cloacal cultures, complete blood counts, and direct

*PCR is a patented process, owned and licensed under agreement with Roche Molecular Systems.

and flotation fecal examination. Choanal and cloacal swabs, and whole, uncoagulated blood were tested for *Chlamydia psittaci* by the VMD assay.

All six birds were inoculated with a pathogenic strain of *Chlamydia psittaci* by an intra-conjunctival route. Whole unclotted blood, choanal swabs, and cloacal swabs were collected on days 5, 10, and 15 post-inoculation. All clinical samples were tested for Chalamydia psittaci using the VMD molecular based assay. On day 15, the birds were euthanitized and tissues collected for histopathological exam. Pooled organ samples the birds were cultured for the presence of *Chlamydia psittaci*.

Results

PCR assay

Within 43 cycles of each assay, nonspecific amplification was insignificant for negative control samples containing genomic DNA of human origin or major species of psittacine pet birds. The assay also consistently detected Chlamydia psittaci nucleic acid in positive laboratory control samples. The observed target sensitivity of the VMD assay, based upon the limiting assay on serially diluted positive samples, was consistently < 10 copies of Chlamydia psittaci gene sequence with detection limits down to four copies. An independent, molecular biology laboratory validated this assay sensitivity.

The molecular based assay results on clinical samples taken during the controlled infectivity study are shown in Table 1. On day 5 post-infection, all the test birds showed positive choanal swabs whereas all the cloacal swabs and blood samples tested negative. On day 10 testing, two birds showed positive cloacal swab results. All remained positive on choanal swabs and negative on blood sample testing. By day 15 post-infection, all birds showed positive choanal, cloacal, and blood test results.

TABLE 1. Molecular based assay test results in study birds

	DAY 5			DAY 10			DAY 15		
ID	Choanal	Cloacal	Blood	Choanal	Cloacal	Blood	Choanal	Cloacal	Blood
A	+	-	-	+	-	-	+	+	+
B	+	-	-	+	+	-	+	+	+
C	+	-	-	+	-	-	+	+	+
D	+	-	-	+	-	-	+	+	+
E	+	-	-	+	+	-	+	+	+
F	+	-	-	+	-	-	+	+	+

Days listed are days post infection; (-) = negative molecular based assay test result; (+) = positive test result

The six test birds remained clinically normal throughout the 15-day study period. Histopathology revealed splenomegaly resulting from lymphoid hyperplasia, fibrinoid necrosis of the spleen, and a mild necrotizing hepatitis. Several birds showed a few aggregates of leukocytes comprised of heterophils, lymphocytes, and mononuclear cells. No pulmonary lesions were identified. The abnormal lesions were consistent with a diagnosis of chlamydiosis. No significant bacteria were isolated on pooled tissue culture however the pooled sample was culture positive for *Chlamydia psittaci*.

Discussion

PCR amplification of chlamydial DNA using primers specific for conserved regions of the MOMP gene has enabled the detection of fewer than 10 elementary bodies in clinical samples.[8]

The VMD molecular assay compares favorably with the results from other researchers as it consistently detected <10 copies of target DNA sequence in positive control samples. Test specificity was also very good, as the test did not show non-specific amplification of control DNA or negative clinical samples.

The first post-infection clinical sampling taken on day 5 showed positive choanal swab test results on all the study birds. This represents a logical progression of the infection as the conjunctival fluids are naturally drained through to the oral cavity. The fact that all the birds showed negative blood test results on day 5 & 10 most likely indicates that the infection was still in the "localized phase" and had not yet become systemic. By day 10, two birds showed positive cloacal swab results while the infection continued in the oral cavity (positive choanal swab test) on all birds. Although all the study birds remained clinically normal, they all showed evidence of systemic infection by positive blood and swab test results on day 15. The molecular assay was consistent in identifying sub-clinical chlamydial infection in these normal appearing birds. Pathology examination revealed mild changes in some tissues that were compatible with chlamydial infection. Pooled tissue samples supported systemic infection as they cultured positive for *Chlamydia psittaci* at the end of the study period (Day 15).

The high degree of sensitivity and specificity of this test is consistent with the other avian molecular based diagnostic assays. False positive test results are extremely unlikely. False negative test results may occur but appear to be minimal.

Obtaining a confirmed diagnosis of chlamydial infection in pet birds has often been difficult for the avian practitioner. While other current diagnostic methods can identify chlamydia-infected birds, the molecular biology approach is a potentially superior technique. It appears ideally suited to detecting states where infectivity is low or where a rapid assay is desired. It can provide a <u>confirmed</u> diagnosis of Chlamydial infection in the clinically inapparent and/or persistent infected state. It also has the advantage of providing a sensitive method for chlamydia detection, which is not dependent upon a host immune response.

The pilot study demonstrates the potentiality of molecular techniques in the diagnosis and differentiation of *Chlamydia psittaci* infection. Due to the initial localized phase of chlamydia infections, a combined choanal/cloacal swab may be the best sample to submit in early exposures. The blood test appears to reliably detect the non-clinical, infected individual within a short time (15 days) following infection. Further correlation of test results with other diagnostic methods and observed clinical cases in the field are needed to further validate this test

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