HUMORAL RESPONSE TO *Mycobacterium avium* subsp. *avium* IN NATURALLY INFECTED RING-NECK DOVES (*Streptopelia risoria*)

A Thesis

by

PATRICIA LARA-LYNN GRAY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2007

Major Subject: Veterinary Microbiology

HUMORAL RESPONSE TO *Mycobacterium avium* subsp. *avium* IN NATURALLY INFECTED RING-NECK DOVES (*Streptopelia risoria*)

A Thesis

by

PATRICIA LARA-LYNN GRAY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Chair of Committee,	Ian Tizard
Committee Members,	David Phalen
	David McMurray
Head of Department,	Gerald Bratton

December 2007

Major Subject: Veterinary Microbiology

ABSTRACT

Humoral Response to *Mycobacterium avium* subsp. *avium* in Naturally Infected Ring-Neck Doves (*Streptopelia risoria*). (December 2007)

Patricia Lara-Lynn Gray, D.V.M., Aristotle University of Thessaloniki

Chair of Advisory Committee: Dr. Ian Tizard

Creation of a reliable and easy to use serologic test would greatly improve ante mortem diagnosis of *Mycobacterium avium* subsp. *avium* and aid in the control of avian mycobacteriosis, particularly in captive birds. In order to determine whether serodiagnostics could be of value in testing ring-neck doves (*Streptopelia risoria*) for *M. a. avium* infection, Western blot analysis was used to assess the humoral response of ring-neck doves exposed to *M. a. avium*, and to evaluate whether an association could be made between humoral response and necropsy findings, histopathology, culture, and PCR testing. Western blot results were examined for reactivity patterns associating the humoral response with infection status, severity and type of lesions (diffuse vs multifocal granulomatous inflammation) and phenotype (white vs non-white). A sensitivity of 88.24% and a specificity of 100% were achieved utilizing Western blot analysis to detect *M. a. avium* infection in ring-neck doves, offering a negative predictive value of 93% and a positive predictive value of 100%. While Western blot analysis results did not reflect lesion severity, lesion type did partially correspond with the humoral response. The findings of the present study indicate that serologic testing

can be used as a valuable ante mortem screening tool for identifying ring-neck doves infected with *M. a. avium*.

DEDICATION

This work is dedicated to Panos, whose unwavering support, encouragement and understanding make all things possible.

ACKNOWLEDGEMENTS

I would like to thank, first and foremost, my committee chair and mentor Dr. Ian Tizard. Not only did Dr. Tizard offer me the opportunity to pursue a Master of Science as well as his support throughout, he also made every effort to ensure that during the process I was able to take advantage of every opportunity available in order to grow as a professional. I am grateful as well to my committee members Dr. David Phalen and Dr. David McMurray for their valuable time, suggestions and encouragement. Deserving of many thanks are Dr. Miguel Saggese and Debra Turner for their continuous support and guidance in the lab, and more importantly, for their highly valued friendship. Finally, I would like to thank my family, both in Canada and in Greece, for always believing in me.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Avian mycobacteriosis is an insidious disease of wild and captive birds all over the world. Although losses due to mycobacteriosis appear to be sporadic in most wild bird populations, mycobacterial infections can cause significant morbidity and mortality in zoo and wildlife collections (Cromie et al., 1993; Tell et al., 2003b; Converse, 2007, pp. 289-302). While many species of mycobacteria can cause disease in birds, *Mycobacterium avium* subsp. *avium* is the most commonly isolated species in the majority of affected populations, with the exception of some captive populations of *Passeriformes* and *Psittaciformes* for which *Mycobacterium genavense* appears to be the predominant mycobacterial pathogen (Hoop et al., 1996; Portaels et al., 1996; Converse, 2007, pp. 289-302). *M. avium* is a ubiquitous environmental saprophyte and is the most widely distributed of the mycobacteria, and as such it is commonly encountered by birds (Cromie et al., 2000; Falkinham, 2004, pp. 27-33). Avian mycobacteriosis is a chronic disease causing granulomatous inflammation most often found in the liver, spleen, gastrointestinal tract, lung and/or air sacs. Infection is usually acquired following exposure via the oral route and less commonly via inhalation (Thoen, 1997, pp. 167-178; Cromie et al., 2000; Tell et al., 2001; Schmidt et al., 2003, pp. 76-77, 138-139; Converse, 2007, pp. 289-302). Birds infected with *M. a. avium* may appear normal until the disease is advanced, or only show non-specific signs of a wasting disease from months to days before death (Tell et al., 2001 Pollock, 2006, pp. 681-688).

This thesis follows the style of Veterinary Immunology and Immunopathology.

Rapid identification of infected birds is crucial for the effective control of mycobacteriosis (Tell et al, 2003a; Zsivanovits et al., 2004). Due to the lack of practical, sensitive and specific diagnostic tools available, ante-mortem diagnosis of avian mycobacteriosis is challenging and in some instances not possible. Clinical presentation is highly variable, and although hematologic findings, clinical chemistries and radiographic findings can be useful, they are often non-specific (Tell et al., 2003a; Pollock, 2006, pp. 681-688). Isolation via culturing and PCR testing remain the most definitive means of diagnosing mycobacterial infection in birds (Tell et al., 2003a; Converse, 2007, pp. 289-302). Both methods have considerable limitations, particularly when fecal samples - the only samples that can be obtained non-invasively - are used, and procuring appropriate tissues for testing can be difficult or impractical (Tell et al., 2001; Tell et al., 2003a; Saggese, 2007). Although it is unlikely that antibody-based tests can replace identification of the pathogen responsible for disease using culture methods or PCR, management and control of avian mycobacteriosis would greatly benefit from early, rapid, and reliable serodiagnosis (Aranaz et al., 1997; Phalen, 2001; Zsivanovits et al., 2004).

The use of serology in the diagnosis and evaluation of disease status in humans infected with *M. tuberculosis* has been a major research focus for the past several years, as has the use of serology in diagnosing human infection with the *M. avium* complex (Laal et al., 1997; Kitada et al., 2002; Beck et al., 2005b; Semret et al., 2006). Early studies based on crude antigen preparations showed that healthy humans possess antibodies that

cross react with many *M. tuberculosis* antigens (Kolk et al., 1989; Beck et al., 2005b). In an effort to develop sensitive and specific serodiagnostic assays, as well as to better understand the human humoral response to mycobacterial infection, many *M. tuberculosis* and *M. avium* complex antigens have been isolated and characterized (Young et al., 1992). Western blotting is now being used to evaluate antigen recognition patterns in individuals infected with or exposed to *M. tuberculosis*, and serologic tests are being created for diagnosing *M. tuberculosis* infection and determining disease status in patients (Laal, et al., 1997; Lysachenco et al., 2000; Olsen et al., 2000; Franco et al., 2001; Beck et al., 2005a). These techniques have also been applied to investigating the humoral response to *M. bovis* in badgers, cattle, white-tailed deer, and reindeer, as well as for the diagnosis and monitoring of *M. tuberculosis* infection status in elephants (Greenwald et al., 2003; Lysachenko et al., 2004; Waters et al., 2004; Waters et al., 2005; Lysachenko et al., 2006).

The avian immune response to mycobacterial infection is poorly understood, and this lack of knowledge has hindered the use of diagnostic serology. Antibody production over the course of mycobacterial infection has yet to be characterized. Though there have been indications that some species of bird may cease antibody production during the later stages of disease (Phalen et al., 1995; Cromie et al., 2000; Zsivanovits et al., 2004), the immune response likely differs among avian species (Tell et al., 2001).

The earliest ante mortem method used to diagnose mycobacteriosis in birds was the intradermal tuberculin test (ITT), whereby avian tuberculin is injected intradermally and the site is examined for soft swelling after 48 hours (Feldman, 1938, pp. 444-469). The test is convenient for use in chickens as it can be administered on the wattles, but it requires handling of the birds a second time in order to obtain the result. Despite reports of sensitivity as low as 50%, this method has been widely employed in the poultry industry, and it is in part due to the ITT that avian mycobacteriosis is no longer a concern in most commercial poultry flocks (Pavlas et al., 1993; Tell et al., 2001). The ITT is not convenient for use in most other bird species because of their lack of easily accessible featherless areas, and it has been shown to be ineffective in detecting *M. avium* infection when tested in turkeys, pigeons, quail, geese and ducks (Hinshaw et al., 1932; Svrcek et al., 1966; Karlson et al., 1970; Pavlas et al., 1983; Thoen, 1997, pp. 167-178). Ensly et al. (1975) reported using the ITT to test a number of birds in a zoo collection (Charadriiformes, Galliformes, Passeriformes, Gruiformes and Anseriformes) known to be infected with *M. avium*. All birds tested negative by this method, suggesting extreme insensitivity.

Serology has been examined as a diagnostic method in several bird species with varying levels of success, false positives being attributed to cross reactivity due to exposure to environmental mycobacteria and other prokaryotes, and false negatives being attributed to cessation of antibody production late in the disease process (Pavlas et al., 1993; Phalen et al., 1995; Cromie et al., 2000). Moses et al. (1943) created a rapid

agglutination test for avian mycobacteriosis, employing a concentrated suspension of avian tubercle bacilli to be tested against a serum sample and examined for agglutination. Karlson et al. (1950) attempted to improve this rapid agglutination test by using a drop of whole blood added to a drop of antigen on a white tile, and later Rozanska (1965) created a staining technique allowing better visualization of fine agglutination. Rapid agglutination has been used with varying levels of success in poultry, geese, ducks, pheasants, as well as raptors and cranes (Moses et al., 1943; Karlson et al., 1950; Hiller et al., 1967; Rozanska, 1967; Kwatra 1972; Hawkey et al., 1990; Cromie et al., 1993). It was later demonstrated by Pavlas et al. (1993) that, while fast and convenient, rapid agglutination tests are not always highly sensitive and often give false positive results, and that greater sensitivity and specificity could be achieved using the ITT together with rapid agglutination to confirm *M. avium* infection. Rapid agglutination has also been examined in exotic zoo collections, where the test was found to be unreliable for use in some species (Vizy et al., 1964; Montali et al., 1976).

Complement fixation, a test that detects complement fixing antibodies in serum, has also been used to detect *M. avium* infection in birds. Preliminary data on a complement fixation test used to screen over eighty samples from birds suspected of being infected with *M. avium* indicated that a more refined version of the test may prove valuable for detecting *M. avium* infections (Phalen et al., 1995). The same test was later used to evaluate the serologic response of doves naturally infected with *M. a. avium* (Saggese & Phalen, 2005).

Enzyme-linked immunosorbent assays offer high throughput serologic testing using a small quantity of serum. They are easy to standardize and they are relatively fast and simple to use. Cromie et al. (1993) investigated an ELISA and found it useful for screening some species of wildfowl for mycobacterial infection. In a later study the same group (Cromie et al., 2000) reported using an ELISA to evaluate humoral immune responsiveness after vaccination with mycobacterial antigens in several species of ducks, and reported that antibody responses were useful for evaluating exposure. Similar studies have been carried out using chickens (*Gallus domesticus*) (Thoen et al., 1977) and quail (*Coturnix* species) (Clark et al., 1995). Cray et al. (2001) reported testing several hundred samples from a variety of bird species for avian mycobacteriosis using an ELISA, with mixed results. One of the disadvantages to using ELISAs is the need for purified reagents in order to develop a sensitive and specific test. Many of the problems in the aforementioned studies associated with sensitivity and specificity using ELISAs were possibly due to the use of crude antigen preparations or inadequately specific secondary antibodies.

Western blot is a serologic test that can be used to examine the reactivity of individual antigens within a mixture when tested against serum containing antibodies. This technique enables the visualization of the antibody response, making it possible to determine which antigens are suitable for use in diagnosing infection. Certain pigeon and dove species (Order Columbiformes) have been found to be susceptible to mycobacterial

infection (Heijlicek & Treml, 1993; Friend, 2001, pp. 93-98), and differences in antibody production have been noted among doves naturally infected with *M. a. avium* when evaluated by complement fixation (Saggese & Phalen, 2005). The purpose of this study was to determine whether a serologic technique, Western blotting, can be used to accurately diagnose *M. a. avium* infection in ring-neck doves (*Streptopelia risoria*). Western blot analysis employing *M. a. avium* crude cell wall, cell membrane, cytosol and culture filtrate antigen preparations was used to assess the responses of ring-neck doves exposed to *M. a. avium*, and to evaluate whether an association could be made between the antibody recognition of specific *M. a. avium* antigens and post mortem diagnostic findings in infected individuals. Also, the usefulness of Western blot analysis employing purified *M. tuberculosis* culture filtrate native protein antigens as a tool for detecting ring-neck doves infected with *M. a. avium* was evaluated.

MATERIALS AND METHODS

Mycobacteria

The *Mycobacterium avium* subsp. *avium* strain employed was isolated from a granulomatous lesion in the spleen of a naturally infected dove used in this study. A sample taken from the granulomatous lesion was inoculated into 5ml of Middlebrook 7H9 liquid medium with 10% (v/v) oleic acid-albumin-dextrose-catalase (OADC) (Becton Dickinson, Sparks, MD, USA) and was incubated for 14 days at 37ºC without shaking. The resulting bacterial culture was monitored for growth characteristics of *M. a. avium* and examined microscopically using Ziehl-Neelsen staining in order to establish the presence of acid-fast bacteria and the absence of visible contaminants. Culturing on blood agar was performed in order to rule out the presence of fast growing contaminants. The cultures were centrifuged at 3,000 x *g* for 20 min and the bacteria were inoculated into one liter flasks containing 500ml modified Sauton medium without Tween 80. The cultures were incubated for 15 days at 40ºC without shaking. PCR was used to confirm the identity of the organisms isolated from each of the infected doves as part of a separate project (Saggese, 2007).

Bacterial antigen preparations

Approximately 1g of wet bacteria was harvested by centrifugation at 4,000 x *g* for 20 min, washed and resuspended in PBS, pH 7.4. In order to retrieve the culture filtrate proteins, the culture supernatant was sterile filtered using a 0.22 micron Easy Flow^{TM} filter (Becton Dickinson Labware, Lincoln Park, NJ, USA) and concentrated approximately ten times using a 5,000 molecular weight cut-off (MWCO) Amicon Ultra 15 filter (Millipore, Carrigtwohill, Co.Cork, Ireland). The concentrate was precipitated with 80% ammonium sulfate, and the precipitate was dissolved in PBS, pH 7.4 and dialyzed three times in this buffer. For preparation of the sub-cellular fractions the bacteria were heat inactivated at 67 ºC for 90 min, washed and resuspended in PBS, pH 7.4 containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The killed bacteria were ruptured by being passed three times through a French pressure cell at 17,000 lb/in^2, the lysate kept on ice throughout. Intact bacteria were removed after centrifugation at 4,000 x *g* for 20 min. The lysate was centrifuged at 27,000 x *g* for 20 min and the pellet (representing the crude cell wall) was suspended in PBS, pH 7.4. The supernatant was subjected to ultracentrifugation at 100,000 x *g* for 2 hours and the pellet (representing the cell membrane component) was suspended in PBS, pH 7.4. The supernatant containing the soluble cytosol was concentrated approximately ten times using a 5,000 MWCO Amicon Ultra 15 filter (Millipore, Carrigtwohill, Co.Cork, Ireland). All manipulations were performed at 4ºC. Protein content was measured using the Bradford dye-binding assay (Bio-Rad, Hercules, CA, USA). Proteins were visualized using SDS-

PAGE with a 6.5 to 200 kDa broad range marker (Bio-Rad, Hercules, CA, USA) and staining with Coumassie Brilliant Blue R-250 Staining Solution (Bio-Rad, Hercules, CA, USA). Culture filtrate *M. tuberculosis* antigens GroES, 45kDa and Ag85 Complex (Mycobacteria Research Laboratories, Colorado State University, Ft. Collins, CO, USA) were also employed.

SDS-PAGE and Western blot analysis

Protein electrophoresis was carried out as described by Laemmli (1970) using 15% polyacrylamide gels and a Mini-Protean®II gel electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). The antigen preparations were diluted in sample loading buffer containing β-mercaptoethanol at a ratio of 2:1 and heated to 95ºC for 3 minutes before being loaded onto the gel (7µg/slot). A prestained SDS-PAGE standard covering the 6.5 to 200 kDa range was used for molecular weight estimation (Bio-Rad, Hercules, CA, USA). The SDS-PAGE fractionated antigen preparations were transferred to Immobilon polyvinylidene difluoride (PVDF) transfer membranes (Millipore, Bedford, MA, USA) as described by Towbin et al., (1979). Transfer efficiency was monitored by the presence of prestained bands on the membranes. After transfer, the membranes were incubated for 2 hours in PBST blocking buffer (PBS, 0.05% Tween-20, 3% skimmed milk), for 2 hours with dove serum diluted 1:500 in PBST blocking buffer, and for 1 hour with HRP-conjugated goat anti-dove IgG (Bethyl Inc., Montgomery, TX, USA) diluted 1:10000 in PBST blocking buffer. The goat anti-dove antibodies employed were affinity purified

using serum collected from ring-neck doves used as negative controls in this study. Membranes were washed with PBST after each step, and all steps were performed at room temperature under constant shaking. Finally, the membranes were incubated for 30 min in Sigma-Fast^{TM} 3,3'-diaminobenzidine developing substrate (Sigma-Aldrich Inc., St. Louis, MO, USA) and then rinsed in distilled water. Western blot analysis results were initially determined prior to examination of post mortem diagnostic results on the birds. Taking into account the high probability of encountering cross-reactivity with the crude mycobacterial antigen preparations used, during initial evaluation of Western blot results, those indicating reactivity with solitary bands were considered non-specific and therefore negative (Bermudez et al., 1989; Laal et al., 1997; Cromie et al., 2000). Western blot results indicating reactivity with clearly visible grouped bands were considered positive. These criteria were re-evaluated when the results of the post mortem diagnostics were known. Using the results of necropsy findings, histopathology, culture, and PCR testing, Western blot results were re-examined for reactivity patterns correlating humoral response with health and infection status, severity and type of lesions (diffuse vs multifocal granulomatous inflammation) and phenotype (white vs non-white doves). Effect of phenotype on humoral response in diseased doves (both infected and uninfected) was examined using Fisher's exact test, and the odds ratio with 95% confidence intervals (95% CI) for mounting a humoral response was calculated. All statistical analyses were performed using a statistical software package (Prism5, GraphPad, San Diego, CA), and a p-value of <0.05 was considered significant. True positive Western blot results were evaluated to determine which antigen preparation

provided the most consistent positive result with the least cross reactivity. Test specificity and sensitivity, as well as positive and negative predictive values, were also calculated.

Ring-neck dove sera

Serum samples were obtained from 28 ring-neck doves (*Streptopelia risoria*) exposed to high levels of *M. a. avium*. These birds were members of a flock of 70 adult ring-neck doves from an aviary in Texas where more than 60 doves had died during the previous months. Mycobacterial infection had been confirmed at the Texas Veterinary Medical Diagnostic Laboratory, and the condition of some of the surviving birds was consistent with a chronic infectious disease such as mycobacteriosis. The birds were housed in a small, confined aviary with access to an outdoor flight, and there was excessive fecal contamination of both feed and water. The surviving birds were donated and transferred to an isolation building at the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University, TX. Serum was also obtained from 6 healthy ring-neck doves housed for 4 years in an aviary with no history of avian mycobacteriosis. These 6 doves were used as negative controls. Post mortem diagnostics were performed as part of a separate project (Saggese, 2007). All doves were euthanized and necropsies were performed. Birds were examined for gross lesions and representative samples from liver, spleen, lung, trachea, gonads, thyroid, adrenal gland, bone marrow, brain and intestines were investigated by histopathology and Ziehl-Neelsen staining. Samples from liver,

spleen and bone marrow were cultured, and samples from liver, spleen, bone marrow and duodenal aspirates were additionally investigated by PCR testing. Doves were considered "diseased" if histopathologic lesions consistent with mycobacterial infection were identified, and "healthy" if no histopathologic lesions were identified. Doves were considered "infected" if at least one of the above tests (Ziehl-Neelsen staining, culturing, and/or PCR) detected the presence of mycobacteria, and "uninfected" if no mycobacteria were detected. Following these criteria each dove was placed into one of 4 categories (Saggese, 2007):

Diseased/Infected – lesions present, mycobacteria detected

Diseased/Uninfected – lesions present, no mycobacteria detected

Healthy/Infected – no lesions present, mycobacteria detected

Healthy/Uninfected – no lesions present, no mycobacteria detected

RESULTS

Combined data on results of post mortem diagnostics and Western blot analysis in individual doves can be found in Table 4 (Appendix).

Based on the results of post mortem diagnostics the 34 ring-neck doves used in this study fell into 4 categories. Of the 28 exposed ring-neck doves, 17 were shown to be infected with *M. a. avium*. In the remaining 11 doves, no mycobacteria were detected in any of the tissues evaluated. All 6 control doves were also negative for mycobacterial infection using the above diagnostic methods. The results of Western blot analysis in relation to health/infection status can be found in Table 1.

Table 1. Results of Western blot analysis in relation to health/infection status in 28 ring-neck doves exposed to *M. a. avium* and 6 ring-neck doves used as negative controls.

Status (all doves, n=34)	Positive on Western blot	Negative on Western blot	Total
Diseased/Infected	15	1	16
Diseased/Uninfected	0	4	4
Healthy/Infected	0	1	1
Healthy/Uninfected	0	13	13

Diseased/Infected=lesions observed, mycobacteria detected; Diseased/Uninfected=lesions observed, no mycobacteria detected; Healthy/Infected=no lesions observed, mycobacteria detected; Healthy/Uninfected=no lesions observed, no mycobacteria detected

Of the 17 infected doves, 15 (88.24%) had antibodies that reacted with multiple *M. a. avium* antigens, and were considered positive for *M. a. avium* infection based on Western blot analysis. The remaining 2 (11.76%) infected doves had few or no

circulating antibodies recognizing *M. a. avium* antigens and were considered negative based on Western blot analysis. The sensitivity of Western blot analysis to detect infection with *M. a. avium* was 88.24%.

Of the 11 exposed doves that were considered uninfected, none were positive for circulating antibodies showing reactivity with multiple *M. a. avium* antigens based on Western blot analysis. The sera of all 6 negative control doves also tested negative for circulating antibodies to *M. a. avium* antigens. Based on these results, the specificity of Western blot analysis in detecting *M. a. avium* infection was 100%.

Based on the 62% prevalence of *M. a. avium* infection in the ring-neck doves used for this study, the positive predictive value using Western blot analysis to detect *M. a. avium* infection was 100%, and the negative predictive value was 93%. The prevalence of mycobacteriosis in captive bird collections is usually not higher than 15% (VanDerHeyden, 1997), in which case the positive predictive value of the test evaluated here would remain 100%, and the negative predictive value would be approximately 71%.

Serum from 20 of the exposed doves and all 6 negative control doves was tested against the *M. tuberculosis* culture filtrate GroES, Ag85 Complex and 45kDa antigens. No reactivity was seen with the GroES and Ag85 Complex antigens. Of 12 confirmed infected doves tested, 9 tested positive on Western blot employing the 45kDa antigen,

while the remaining 3 confirmed infected doves as well as the 6 negative control doves tested negative. The sensitivity and specificity of the Western blot using the 45kDa antigen to diagnose *M. a. avium* infection were 75% and 100%, respectively.

Depending on the intensity of the pattern seen, the Western blot analysis results were divided into weak positive (Fig. 1, Appendix) and strong positive (Fig. 2, Appendix). No association was found between the severity of lesions and the pattern seen on Western blot analysis. Results of Western blot analysis in relation to lesion type can be found in Table 2.

Table 2. Results of Western blot (Wb) analysis in relation to lesion type in 20 diseased ring-neck doves exposed to *M. a. avium*.

Lesion type (n=20)	**Diffuse**	**Multifocal**	**Both**
True negative on Wb	0	4	0
False negative on Wb	0	1	0
Weak positive on Wb	4	2	1
Strong positive on Wb	8	0	0
Total	12/20	7/20	1/20

The influence of color morph (white or non-white) on humoral response in diseased/infected and diseased/uninfected doves was examined using Fisher's exact test (Table 3). The results showed a significant difference in the number of diseased doves that developed a positive antibody response depending on phenotype (p=0.031). In addition, there was a significant odds ratio (OR = 16; 95% CI 1.32201; p=0.031) for the non-white doves to develop a positive response when compared to the white doves.

Table 3. Results of Western blot analysis in relation to color morph in 20 diseased/infected and diseased/uninfected ring-neck doves exposed to *M. a. avium.*

Color Morph and Status (n=20)	Negative on Western blot	Positive on Western blot
White Diseased/Infected	1	3
White Diseased/Uninfected	3	0
Non-white Diseased/Infected	0	12
Non-white Diseased/Uninfected	1	0

Faint cross-reactivity with a 40 kDa cell wall antigen, a 39 kDa cytoplasm antigen and a 42 kDa cytoplasm antigen was seen when testing the sera of 4 healthy/uninfected doves. All positive Western blot results indicated reactivity with antigens in the cell wall, cell membrane and cytoplasm antigen preparations. All Western blot positive sera reacted with cell wall antigens in the 34-35 kDa range as well as the 39-41 kDa range, cell membrane antigens in the 34-36 kDa range as well as the 39-43 kDa range, and cytoplasm antigens in the 39-45 kDa range. Few or no antibodies were present for culture filtrate antigen fractions, and there was no reactivity seen with the same antigen in multiple serum samples.

DISCUSSION AND CONCLUSIONS

Discussion

The present study was the first in which Western blot analysis was utilized to examine the humoral response in birds with avian mycobacteriosis. It was also the first study to evaluate the usefulness of serologic diagnostics in avian mycobacteriosis which was coupled with such a thorough post mortem diagnostic investigation of the birds tested. As such, it has helped to improve our understanding of the immune response of doves to mycobacterial infection. Although there have been indications that some species of birds may cease antibody production during the later stages of disease in which case antibody screening would be of little diagnostic value (Phalen et al., 1995; Cromie et al., 2000; Zsivanovits et al., 2004), this does not appear to be the case in the doves with mycobacteriosis. Many of these doves were in a very advanced state of disease. Surprisingly, despite the inevitable exposure of the doves used in this study to environmental mycobacteria and the reports of problems with false positive results due to cross reactivity in previous studies, very little cross reactivity was seen with *M. a. avium* antigens when testing negative doves.

Antibodies cross reacting with individual antigens in uninfected individuals were rarely detected and no cross reactivity was detected with the antigens found in the cell membrane fraction. This coupled with the consistent reactivity seen with multiple

antigens in the cell membrane preparation make it the most interesting preparation for potential use in creating a more practical serologic assay (i.e. ELISA). While it was possible to see differing intensities in reactivity using Western blot analysis, densitometry could likely have been used to determine more accurately just how intense the different bands really were, at least at a semi-quantitative level. A far more accurate measurement of intensity can be achieved using an ELISA, allowing for a quantitative assessment of antibody production, as well as for an accurate evaluation of antibody titers in serial dilutions. As the cell membrane fraction of *M. a. avium* appears to be the least cross reactive of the antigen preparations examined, a sensitive and specific ELISA can now be created using the cell membrane fraction. This would allow for high throughput testing of samples and serial dilutions, something that would require prohibitively large quantities of serum to test using Western blot analysis.

The inconsistent and infrequent reactivity seen with the *M. a. avium* culture filtrate antigens was unexpected in light of the fact that many of the most promising antigens being studied for use in screening for *M. tuberculosis* in humans are culture filtrate proteins (Samanich et al., 1998; Rosenkrands et al., 2000; Samanich et al., 2000). This may be due to the absence of true tubercle formation as seen in mammals infected with *M. tuberculosis,* within which high numbers of mycobacteria replicate extracellularly and the expression of certain antigens is likely enhanced (Samanich et al., 2000). It should be noted that not only can protein expression by mycobacteria differ depending on the medium used, but also that the *in vivo* and *in vitro* protein secretion likely differ

as well, and that these factors offer a possible explanation for the low level of reactivity seen with culture filtrate antigens (Pais et al., 2000). No reactivity was seen with *M. tuberculosis* culture filtrate antigens GroES and Ag85 Complex, and inconsistent reactivity was noted using the 45kDa antigen, making these antigens unsuitable for use in the diagnosis of *M. a. avium* infection in ring-neck doves.

The affinity purified goat anti-dove IgG antibodies used detected predominantly IgY antibodies reacting with mycobacterial antigens, but may also have detected IgM antibodies. The detection of both IgY and IgM dove antibodies may be advantageous as it makes it possible to gauge antibody production levels even when only one antibody type is being produced.

Two forms of granulomatous inflammatory lesions are typically seen in avian tissues affected by *M. a. avium*, the focal form in which macrophages, multinucleated giant cells, histiocytes and plasma cells surround an often necrotic center, and the diffuse form in which there is extensive infiltration of the affected tissue with macrophages, heterophils and sheets of histiocytes (Schmidt et al., 2003, pp. 76-77, 138-139). Comparison of our Western blot results with the form of granulomatous inflammation observed suggested an association between lesion type and the immune response. It is possible that this may reflect Th1 or Th2 reactivity. All 4 of the doves in the diseased/uninfected category tested negative on Western blot analysis, and only multifocal inflammatory lesions were seen, suggesting that the doves were at one point

infected with *M. a. avium*, but were able to mount an effective cell-mediated response and had successfully cleared the infection. It is also possible that there may have been antibody production as the infection was being cleared but that the antibodies were no longer detectable at the time of testing. The single dove from the diseased/infected category which tested negative on Western blot also exhibited only multifocal lesions, the lack of antibody production possibly indicating that the immune system was still in the process of attempting to clear infection by a Th1 response. The remaining 3 doves with multifocal lesions all showed a weak positive result on Western blotting and were diseased/infected, which suggests that these doves may have been tested at a point where there was recently a switch from a Th1 to a Th2 response, and antibodies had begun to appear. The antibodies produced offer no protection from invading mycobacteria and may even be detrimental to the host (Boom, 2004, p. 104). While it is difficult to say whether the diseased/infected doves with diffuse lesions had at any point mounted a predominantly cell-mediated response, all of the doves showing strong positive and the majority of the doves showing weak positive results on Western blot analysis had diffuse lesions, suggesting a strong association between diffuse lesion type and high levels of antibody production, or a Th2 response. The use of an ELISA to examine antibody titers over the course of disease would provide valuable information on this matter. The only infected dove with no discernable lesions was negative on Western blot analysis, possibly due to very recent or subclinical infection.

In a previous study white ring-neck doves were found to be less susceptible to *M. a. avium* infection than were other color morphs (Saggese, 2007). Fisher's exact test was employed to examine the Western blot results from both infected and uninfected diseased doves in order to determine whether the white doves' resistance to infection was reflected in their humoral response. Based on the results of the Fisher's exact test, diseased non-white doves were 16 times more likely to develop a humoral response compared to the diseased white doves ($p=0.031$). Given that antibody production is probably not protective, this finding is in agreement with previous findings suggesting that white doves are more resistant to *M. a. avium* infection than non-white doves (Saggese, 2007).

Controlled studies using Western blot analysis to investigate the immune response to *M. a. avium* infection in susceptible bird species would be of value, as would studies examining the avian immune response at different time points over the course of *M. a. avium* infection. Very little cross reactivity was seen when evaluating ring-neck dove serum using Western blot analysis. Using the methods described here, bird species already examined using other serologic methods can be tested in order to discern whether the causes of cross reactivity can be identified. The antigen preparations used in this study were created from an *M. a. avium* serotype isolated from the infected doves. Future studies examining the use of different *M. a. avium* isolates to test ring-neck doves are necessary, as are studies examining the use of the serotype employed in this study to

test other bird species. These studies will provide the information needed to discern how versatile future serologic assays will be for use in testing birds of different species.

Conclusions

Taking into account the high level of specificity and sensitivity (100% and 88.24%, respectively), as well as the high positive and negative predictive value (100% and 93%, respectively) of Western blotting when used to detect *M. a. avium* infection, we conclude that this method can be employed as a reliable ante mortem screening tool for identifying infected ring-neck doves. Because of the importance of advancing serodiagnostics for use in numerous avian species, further controlled studies investigating the avian immune response to mycobacterial infection are warranted, as are studies using Western blot analysis to evaluate and improve serologic testing for *M. a. avium* infection in other susceptible avian species.

REFERENCES

Aranaz, A.E., Liebana, A.M., Mateos, A., Domínguez, L., 1997. Laboratory diagnosis of avian mycobacteriosis. Semin. Avian Exot. Pet Med. 6, 9-17.

Beck, S.T., Leite, O.M., Arruda, R.S., Ferreira, A.W., 2005a. Combined use of Western blot/ELISA to improve the serological diagnosis of human tuberculosis. Braz. J. Inf. Dis. 9(1), 35-43.

Beck, S.T., Leite, O.M., Arruda, R.S., Ferreira, A.W., 2005b. Humoral response to low molecular weight antigens of *Mycobacterium tuberculosis* by tuberculosis patients and contacts. Braz. J. Med. Biol. Res. 38, 587-596.

Bermudez, L.E.M., Wu, M., Enkel, H., Young, L.S., 1989. Naturally occurring antibodies against *Mycobacterium avium* complex. Ann. Clin. Lab. Sci. 19(6), 435-443.

Boom, H.W., 2004. The acquired immune response to *M. tuberculosis*. In: Schlesinger, L.S., DesJardin, L.E. (Eds.), Tuberculosis: The Microbe Host Interface, Horizon Bioscience, Norfolk, UK, p. 104.

Clark, S.L., Collins, M.T., Price, J.I., 1995. New methods for diagnosis of *Mycobacterium avium* infection in birds. Proceedings, American Association of Zoo

Veterinarians, Wildlife Disease Association, American Association of Wildlife Veterinarians Joint Conference, East Lansing, Michigan, pp. 151-154.

Converse, K.A., 2007. Avian Tuberculosis. In: Thomas N. J., Hunter D. B., Atkinson C. T. (Eds.), Infectious Diseases of Wild Birds, Blackwell Publishing, Ames, Iowa, pp. 289-302.

Cray, C., Crim, A., Clubb, S., 2001. Serological and molecular diagnosis of mycobacteriosis. Proceedings, Association of Avian Veterinarians Conference, Orlando, Florida, pp. 83-85.

Cromie, R.L., Brown, M.J., Forbes, N.A., Morgan, J., Stanford, J.L., 1993. A comparison and evaluation of techniques for diagnosis of avian tuberculosis in wildfowl. Avian Pathol. 22, 617-630.

Cromie, R.L., Ash, N.J., Brown, M.J., Stanford, J.L., 2000. Avian immune responses to *Mycobacterium avium*: The wildfowl example. Dev. Comp. Immunol. 24, 169-185.

Ensly, P.K., Montali, R.J., Smith, E., Bush, M., 1975. Avian tuberculosis at the National Zoological Park. Proceedings, American Association of Zoo Veterinarians, San Diego, California, pp. 203-206.

Falkinham, J.O., 2004. Environmental sources of *Mycobacterium avium* linked to routes of exposure. In: Pedley, S., Bartram, J., Rees, G., Dufour, A., Cotruvo, J. (Eds.), Pathogenic Mycobacteria in Water: A Guide to Public Health Consequences, Monitoring and Management, IWA Publishing, London, UK, pp. 27-33.

Feldman, W.H., 1938. Dissemination and control of avian tuberculosis. In: Avian Tuberculosis Infections, Williams & Wilkins Co., Baltimore, Maryland, pp. 444-469.

Franco, J., Camarena, J.J., Nogueira, J.M., Blanquer, R., Ruiz, M.J., Marin, J., 2001. Serological response (Western blot) to fractions of *Mycobacterium tuberculosis* sonicate antigen in tuberculosis patients and contacts. Int. J. Tuberc. Lung Dis. 5(10), 958-962.

Friend, M., 2001. Tuberculosis. In: Friend, M., Franson, J.C. (Eds.), Field Manual of Wildlife Disease: General Field Procedures and Diseases of Birds, United States Geological Service, Washington D.C., pp. 93-98.

Greenwald, R., Esfandiari, J., Lesellier, S., Houghton, R., Pollock, J., Aagaard, C., Andersen, P., Hewinson, R.G., Chambers, M., Lysachenko, K., 2003. Improved serodetection of *Mycobacterium bovis* infection in badgers (*Meles meles*) using multiantigen test formats. Diagn. Microbiol. Infect. Dis. 46, 197-203.

Hawkey, C., Kock, R.A., Henderson, G.M., Cindery, R.N., 1990. Haematological changes in domestic fowl (*Gallus gallus*) and cranes (Gruiformes) with *Mycobacterium avium* infection. Avian Pathol. 19, 223-234.

Heijlicek, K., Treml, F., 1993. Epizootology and pathogenesis of avian mycobacteriosis in the doves (*Streptopelia sp*.). Vet Med (Praha) (in Czech). 38, 619-628.

Hiller, K., Schliesser, T., Fink, G., Dorn, P., 1967. Zur serologischen Diagnose der Huhnertuberkulose. Berl Munch Tierarztl Wochenschr (in German). 80, 212-216.

Hinshaw, W.R., Niemann, K.W., Busic, W.H., 1932. Studies of tuberculosis in turkeys. J. Am. Vet. Med. Assoc. 80, 765-777.

Hoop, R.K., Bottger, E.C., Pfyffer, G.E., 1996. Etiological agents of mycobacteriosis in pet birds between 1986 and 1995. J. Clin. Microbiol. 34, 991-992.

Karlson, A.G., Zinober, M.R., Feldman, W.H., 1950. A whole blood, rapid agglutination test for avian tuberculosis – a preliminary report. Am. J. Vet. Res. 11, 137-141.

Karlson, A.G., Thoen, C.O., Harrington, R., 1970. Japanese quail: Susceptibility to avian tuberculosis. Avian Dis. 14, 39-44.

Kitada, S., Maekura, R., Toyoshima, N., Fujiwara, N., Yano, I., Ogura, T., Ito, M., Kobayashi, K., 2002. Serodiagnosis of pulmonary disease due to *Mycobacterium avium* complex with an enzyme immunoassay that uses a mixture of glycopeptidolipid antigens. Clin. Inf. Dis. 35, 1328-1335.

Kolk, A.H., Evers, R., Groothus, D.G., Gilis, H., Kuijper, S., 1989. Production and characterization of monoclonal antibodies against specific serotypes of *Mycobacterium avium* and the *Mycobacterium avium – Mycobacterium intracellulare – Mycobacterium scrofulaceum* complex. Inf. Immun. 57(8), 2514-2521.

Kwatra, M.S., Sharma, G.L., Singh, G., 1972. Passive agglutination test in diagnosis of experimental tuberculosis in ducks, compared with other fowl. Avian Dis. 16(5), 1035-1041.

Laal, S., Samanich, K.M., Sonnenberg, M.G., Zolla-Pazner, S., Phadtare, J.M., Belisle, J.T., 1997. Human humoral responses to antigens of *Mycobacterium tuberculosis*: Immunodominance of high-molecular-mass antigens. Clin. Diagn. Lab. Immunol. 4(1), 49-56.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 226, 680-685.

Lysachenko, K.P., Singh, M., Colangeli, R., Gennaro, M.L., 2000. A multi-antigen print immunoassay for the serological diagnosis of infectious diseases. J. Immunol. Meth. 242, 91-100.

Lysachenko, K.P., Whelan, A.P., Greenwald, R., Pollock, J.M., Andersen, P., Hewinson, R.G., Vordermeier, H.M., 2004. Association of tuberculin-boosted antibody responses with pathology and cell-mediated immunity in cattle vaccinated with *Mycobacterium bovis* BCG and infected with *M. bovis*. Infect. Immun. 72, 2462-2467.

Lysachenko, K.P., Greenwald, R., Esfandiari, J., Olsen, J.H., Ball, R., Dumonceaux, G., Dunder, F., Buckley, C., Richard, M., Murray, S., Payeur, J.B., Andersen, P., Pollock, J.M., Mikota, S., Miller, M., Sofranko, D., Waters, W.R., 2006. Tuberculosis in elephants: Antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. Clin. Vaccine Immunol. 13(7), 722-732.

Montali, R.J., Bush, M., Thoen, C.O., Smith, E., 1976. Tuberculosis in captive exotic birds. J. Am. Vet. Med. Assoc. 169, 920-927.

Moses, H.E., Feldman, H.W., Mann, F.C., 1943. Mycobacterial rapid agglutination antigens and their diagnostic value in tuberculous fowl. Am. J. Vet. Res. 4, 390-394.

Olsen, I., Reitan, L.J., Wiker, H.G., 2000. Distinct differences in repertoires of low-molecular-mass secreted antigens of *Mycobacterium avium* complex and *Mycobacterium tuberculosis*. J. Clin. Microbiol. 38(12), 4453-4458.

Pais, T.F., Feijo Cunha, J., Appelberg, R., 2000. Antigen specificity of T-cell response to *Mycobacterium avium* infection in mice. Inf. Immun. 68(8), 4805-4810.

Pavlas, M., Patlokova, V., Zajicek J., 1983. Morphological changes in geese after experimental and natural infection with Mycobacterium avium serotype 2. Acta vet. (Brno). 52, 163-167.

Pavlas, M., Michalska, A., Hunady, M., 1993. Diagnosis of avian tuberculosis-mycobacteriosis by rapid agglutination. Acta vet. (Brno). 62, 63-69.

Phalen, D.N., Grimes, J.E., Phalen, S.W., McMurray, D.N., Frahm, M., 1995. Serologic diagnosis of mycobacterial infections in birds (a preliminary report). Proceedings, Association of Avian Veterinarians Conference, Philadelphia, Pennsylvania, pp. 67-73.

Phalen, D.N., 2001. The use of serologic assays in avian medicine. Sem. Avian Exot. Pet Med. 10(2), 77-89.

Pollock, C.G., 2006. Implications of mycobacteria in clinical disorders. In: Harrison G.J., Lightfoot, T.L. (Eds.), Clinical Avian Medicine, Spix Publishing, Palm Beach, Florida, pp. 681-688.

Portaels, F., Realini, L., Bauwens, L,. Hirschel, B., Meyers W.M., DeMeurichy, W., 1996. Mycobacteriosis caused by *Mycobacterium genavense* in birds kept in a zoo: 11-year survey. J. Clin. Microbiol. 34(2), 319-323.

Rosenkrands, I., Weldingh, K., Jacobsen, S., Veggerby Hansen, C., Florio, W., Gianetri, I., Andersen, P., 2000. Mapping and identification of *Mycobacterium tuberculosis* proteins by two-dimensional gel electrophoresis, microsequencing and immunodetection. Electrophoresis. 21, 935-948.

Rozanska, M., 1965. Preparation of antigen for whole blood rapid agglutination test and its specificity for diagnosis of avian tuberculosis. Bull. Vet. Inst. Pulway. 9(1), 20-25.

Rozanska, M., 1967. Serological diagnosis of tuberculosis in ducks and geese. Medycyna Wet. (in French). 23, 584-587.

Saggese, M.D., Phalen, D.N., 2005. Serological and histological findings in doves with mycobacteriosis. Proceedings, Association of Avian Veterinarians Conference, Monterey Bay, California, pp. 71-73.

Saggese, M.D., 2007. Studies on the molecular epidemiology, pathogenesis, diagnosis and treatment of avian mycobacteriosis. PhD dissertation, Department of Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station.

Samanich, K.M., Belisle, J.T., Sonnenberg, M.G., Keen, M.A., Zolla-Pazner, S., Laal, S., 1998. Delineation of human antibody responses to culture filtrate antigens of *Mycobacterium tuberculosis*. J. Inf. Dis. 178, 1534-1538.

Samanich, K.M., Keen, M.A., Vissa, V.D., Harder, J.D., Spencer, J.S., Belisle, J.T., Zolla-Pazner, S., 2000. Serodiagnostic potential of culture filtrate antigens of *Mycobacterium tuberculosis*. Clin. Diagn. Lab. Immunol. 7(4), 662-668.

Schmidt, R.E., Reavill, D.R., Phalen, D.N., 2003. Pathology of pet and aviary birds, Iowa State Press, Ames, Iowa, pp. 76-77, 138-139.

Semret, M., Bakker, D., Smart, N., Olsen, I., Haslov, K., Behr, M., 2006. Genetic analysis of *Mycobacterium avium* complex strains used for producing purified protein derivatives. Clin. Vaccine Immunol. 13(9), 991-996.

Svrcek, S., Vritiak, O.J., Kapitancek, B., Pauer, T., Koppel, Z., 1966. Wild birds and domestic pigeons as sources of avian tuberculosis. Rozhl. Tuberk. 26, 659-667.

Tell, L.A., Woods, L., Cromie, R.L., 2001. Mycobacteriosis in birds. Rev. Sci. Tech. Off. Int. Epiz. 20, 180-203.

Tell, L.A., Foley, J., Needham, M.L., Walker, R.L., 2003a. Diagnosis of avian mycobacteriosis: Comparison of culture, acid-fast stains, and polymerase chain reaction for the identification of *Mycobacterium avium* in experimentally inoculated Japanese Quail (*Coturnix coturnix japonica*). Avian Dis. 47, 444-452.

Tell, L.A., Woods, J.F., Needham, M.L., Walker, R.L., 2003b. A model of avian mycobacteriosis: Clinical and histopathologic findings in Japanese quail (*Coturnix coturnix japonica*) intravenously inoculated with *Mycobacterium avium*. Avian Dis. 47, 433-443.

Thoen, C.O., Eacret, W.G., Himes, E.M., 1977. An enzyme-labeled antibody test for detecting antibodies in chickens infected with *Mycobacterium avium* serotype 2. Avian Dis. 22(1), 162-166.

Thoen, C.O., 1997. Tuberculosis. In: Calnek, W.B. (Ed), Diseases of Poultry, Iowa State University Press, Ames, Iowa, pp. 167-178.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings, National Academy of Sciences, USA, 76, pp. 4350-4354.

VanDerHeyden, N., 1997. Clinical manifestations of mycobacteriosis in pet birds. Semin. Avian Exot. Pet. Med. 6(1), 18-24.

Vizy, L., Dozsa, I., Pasztor, L., 1964. Serological slide agglutination tests using an antigen prepared from decapsulated tuberculosis bacteria on zoo birds. Proceedings, The Vth International Symposium on Diseases in Zoo Animals, Amsterdam, pp. 214-219.

Waters, W.R., Palmer, M.V., Bannantine, J.P., Whipple, D.L., Greenwald, R., Esfandiari, J., Andersen, P., McNair, J., Pollock, J.M., Lysachenko, K.P., 2004. Antigen recognition by serum antibodies in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis*. Clin. Diagn. Lab. Immunol. 11, 849-855.

Waters, W.R., Palmer, M.V., Bannantine, J.P., Greenwald, R., Esfandiari, J., Andersen, P., McNair, J., Pollock, J.M., Lysachenko, K.P., 2005. Antibody responses in reindeer (*Rangifer tarandus*) infected with *Mycobacterium bovis*. Clin. Diagn. Lab. Immunol. 12, 727-735.

Young, D.B., Kaufmann, S.H.E., Hermans, P.W.M., Thole, J.E.R., 1992. Mycobacterial protein antigens: A compilation. Molec. Microbiol. 6(2), 133-145.

Zsivanovits, H.P., Neumann, U., Brown, M.J., Cromie, R.L., 2004. Use of an enzyme-linked immunosorbent assay to diagnose avian tuberculosis in a captive collection of wildfowl. Avian Pathol. 33(6), 571-575.

APPENDIX

Table 4. Data on 28 ring-neck doves exposed to *M. a. avium* and 6 ring-neck doves used as negative controls.

Dove	Color Morph	Infected	Diseased	Lesions	Wb Result
A	white	+	+	M	WP
B	non-white	+	+	D	WP
C	non-white	+	+	D	SP
D	non-white	+	+	D	SP
K	non-white	+	+	D	SP
L	non-white	+	+	D	WP
M	non-white	+	+	D	SP
N	non-white	-	-	-	-
Ñ	non-white	+	+	M	WP
O	white	+	+	B	WP
P	white	-	-	-	-
Q	non-white	+	+	D	SP
R	white	-	-	-	-
S	white	+	+	D	SP
T	white	-	+	M	-
U	non-white	+	+	D	SP
W	non-white	+	+	D	WP
100	non-white	+	-	-	-
103	white	-	+	M	-
106	non-white	-	-	-	-
112	non-white	-	-	-	-
113	white	-	-	-	-
120	non-white	+	+	D	SP
123	white	-	+	M	-
136	non-white	+	+	D	WP
139	white	+	+	M	-
141	non-white	-	+	M	-
144	white	-	-	-	-
C1	non-white	-	-	-	-
C2	non-white	-	-	-	-
C3	non-white	-	-	-	-
C4	non-white	-	-	-	-
C5	non-white	-	-	-	-
C6	non-white	-	-	-	-

+=positive; -=negative; M=multifocal; D=diffuse; B=both; Wb=Western blot; WP=weak positive; SP=strong positive

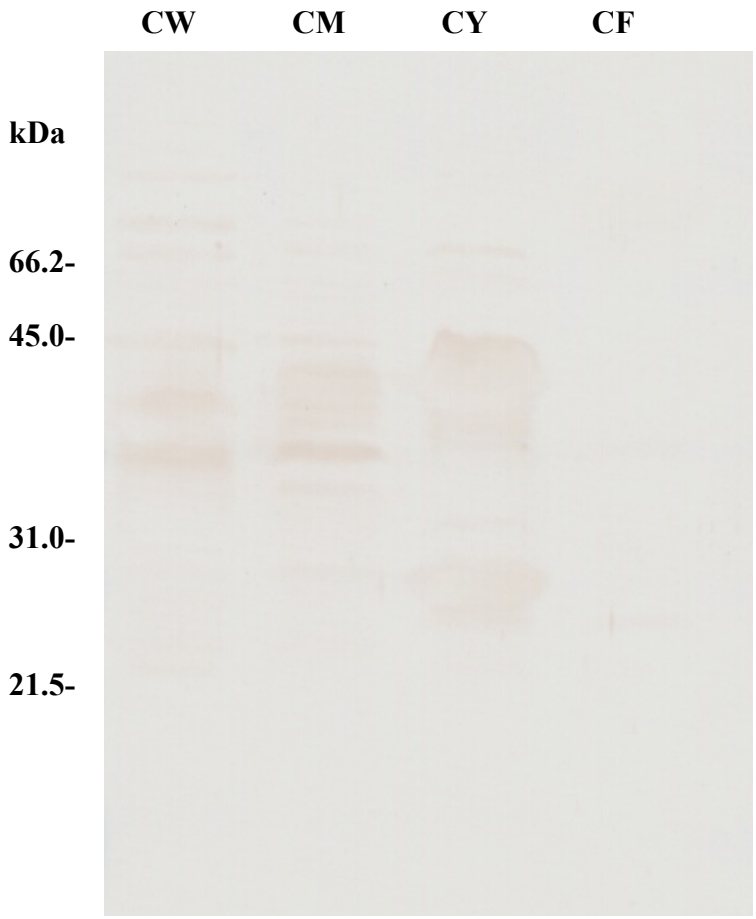

Fig. 1. Weak positive Western blot result.

CW=cell wall; CM=cell membrane; CY=cytoplasm; CF=culture filtrate

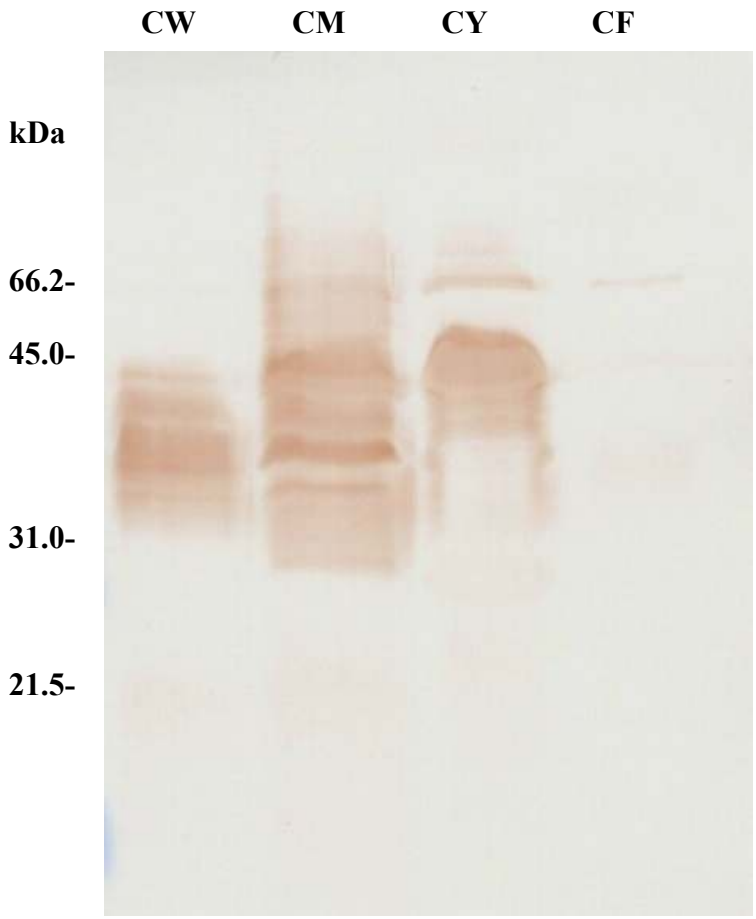

Fig. 2. Strong positive Western blot result.

CW=cell wall; CM=cell membrane; CY=cytoplasm; CF=culture filtrate

VITA

Name:	Patricia Lara-Lynn Gray
Address:	The Schubot Exotic Bird Health Center, Department of Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, MS 4467 TAMU
Email Address:	pgray@cvm.tamu.edu
Education:	D.V.M., Aristotle University of Thessaloniki, 2003 M.S., Texas A&M University, 2007