NEW METHODS FOR DIAGNOSIS OF *MYCOBACTERIUM AVIUM* INFECTION IN BIRDS

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Introduction

Avian tuberculosis (ATB) is a chronic disseminated granulomatous disease caused by the organism *Mycobacterium avium*. Once a scourge of the poultry industry, ATB is now less prevalent there due to changes in husbandry practices. However, it continues to cause mortalities in exotic birds kept as companion animals, captive wild birds in zoological collections, and free-ranging wild birds. ATB is reported to be more prevalent in certain avian species, for instance, wild populations of the endangered whooping crane.7 Certain species are thought to be more susceptible to infection with *M. avium*, for example the Micronesian kingfisher.5

Epidemiologic evidence, including the isolation of *M. avium* from avian fecal samples, suggests ATB is transmitted by ingestion of food and/or water contaminated with the feces of infected birds. *M. avium*-infected birds, including those shedding the pathogen in their feces, usually do not show any clinical signs until late in the disease course and none of these signs is pathognomonic for ATB.

Diagnosis of ATB is most often made postmortem and based on observation of yellowish-white nodules 1 mm to several cm in diameter in multiple organs, and acid-fast organisms in tissue smears and histologic sections. Many other methods are employed in an effort to achieve an antemortem diagnosis however. Hematologic findings in ATB vary from normal to marked leukocytosis with or without a left shift, monocytosis and/or reactive lymphocytosis, polychromasia, and decreased hematocrit. Serum chemistry changes reported in severe disseminated disease include increased liver enzymes and bile acids, and hyperproteinemia with hypoalbuminemia and hyperglobulinemia.10 Radiography may reveal hepatomegaly, splenomegaly, pulmonary nodules, or bone involvement in ATB. Enlarged or granulomatous organs may be observed by endoscopy of the body cavity. Difficulty in finding a suitable site for skin testing in birds without wattles, the need to handle each bird being skin tested twice, and the poor agreement between tuberculin reactions and necropsy findings in avian species other than chickens all limit the usefulness of skin testing as a means of diagnosis of ATB.6 A whole-blood agglutination test for ATB reportedly has poor sensitivity in avian species other than domestic fowl.8 Enzyme-linked immunosorbent assays (ELISAs) have been described for detecting anti-mycobacterial antibodies in both experimentally infected chickens9 and naturally infected feral Barnacle geese.2,3 A lymphocyte transformation test has been investigated as a potential diagnostic tool for mycobacterial infections in birds, however there reportedly is poor correlation between the lymphocyte transformation response and necropsy findings.4 *M. avium* has been isolated from avian fecal and tissue samples using conventional culturing techniques,6 and there is a single
report of isolation of *M. avium* from blood from a known *M avium*-infected bird using a radiometric culturing method established for human samples.\textsuperscript{10}

The goals of our research have been to develop radiometric culture for isolation of *M avium* from avian fecal and tissue biopsy samples, and to develop an ELISA for detection of serum antibody against *M. avium* as tools for antemortem diagnosis of *M. avium* infection in birds.

**Radiometric Culture of *Mycobacterium avium* from Fecal and Tissue Biopsy Samples**

Radiometric culture offers several advantages over conventional culture methods for the isolation of mycobacteria, including its speed, sensitivity, and suitability for quantitative studies. A method for culturing *M paratuberculosis* from animal fecal and tissue samples using a modified BACTEC system (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD) has been described.\textsuperscript{1} Using this method as a starting point, we optimized radiometric culture for isolation of *M avium* from avian fecal and tissue samples. Radiometric culture involves the following steps: (1) sample decontamination to prevent the overgrowth of normal microbial flora or other contaminating, nonacid-fast organisms; (2) filter concentration of mycobacteria to increase the culture sensitivity; (3) inoculation and incubation of the growth medium; and (4) mycobacterial growth detection and species identification. The growth medium used in our lab for the culture of *M paratuberculosis* is Middlebrook 7H12 broth (BACTEC 12B) enriched with egg yolk and mycobactin-J, and supplemented with an antibiotic cocktail to control contamination. Pilot studies showed *M. avium* to be sensitive to a component of the cocktail used to isolate *M. paratuberculosis* and so alternatives were investigated. Most effective control of contaminants, with minimal inhibition of *M avium* growth, was achieved using an antibiotic cocktail composed of bacitracin (40 \( \mu \)g/ml), amphotericin B (20 \( \mu \)g/ml), and nalidixic acid (30 \( \mu \)g/ml). Due to differences between *M avium* and *M. paratuberculosis* in their tendency to aggregate, filter concentration of samples was not found to increase the sensitivity of the radiometric culture method for *M. avium*.

The protocol for radiometric culture of *M. avium* from avian fecal samples is presently as follows. Fecal samples are first decontaminated in a 1% hexadecylpyridinium chloride solution (HPC) (Sigma Chemical Co., St. Louis, MO) for 15 min or less, then filtered through two layers of gauze to remove large particulate material, and then inoculated directly into the enriched and antibiotic-supplemented growth medium. Inoculated growth vials are incubated at 37\(^\circ\)C under 5% CO\(_2\) without shaking for three weeks.

Growth is measured daily by a gas ionization detector instrument called the BACTEC 460 and reported as raw growth index (GI) value. When the cumulative GI exceeds 100, a few drops of the culture broth are plated on a blood agar plate (BAP) and observed for growth for 48 hrs. In addition, a drop of culture is placed on a slide and acid-fast stained. If no growth is detected on the BAP and acid-fast organisms are seen on the slide, then a *Mycobacterium* sp. is assumed to have been isolated, and identification is attempted using a DNA probe that hybridizes with *M. avium* 16S rRNA (Accuprobe, Gen-Probe, San Diego, CA). The protocol is the same for tissue biopsy samples, with the exception of the first step in which tissues are homogenized in saline before exposure to HPC.

This protocol has been used successfully to isolate *M. avium* from the feces of experimentally infected chickens and quail, starting as early as eight weeks after either a single intravenous or oral challenge with \(10^6\) colony forming units of a field isolate of *M. avium*, and also from tissue samples collected postmortem. We are using this radiometric culture protocol to isolate *M. avium* from clinical samples from avian and some non-avian animal species, however, further refinements are necessary before it can be used extensively as a diagnostic tool in a clinical setting. The sensitivity of different strains of *M. avium* to the decontaminant HPC and the use of alternative decontaminants are current areas of investigation to optimize the radiometric culture technique.
ELISA for Detection of Serum Antibody Against *Mycobacterium Avium*

An ELISA for detection of serum antibody against *M. avium* in chickens challenged intramuscularly with *M. avium*, reported in 1978, and another for detection of antibody in naturally infected feral Barnacle geese, reported in 1993, served as the starting point for our ELISA work. We developed a solid-phase, antibody-capture ELISA for detection of antibody against *M. avium* in serum collected weekly from chickens and quail challenged either orally or intravenously with *M. avium*.

Assays are performed in polystyrene 96-well microtiter plates coated with a whole cell homogenate of a field isolate of *M. avium*. Diluted serum samples are added to the antigen-coated wells and antigen-bound antibody is detected using a commercially available goat anti-chicken IgG (H+L) horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), in the case of chicken serum, and a goat anti-turkey IgG (H+L) horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), in the case of quail serum. The substrate for the enzyme conjugates is TMB (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Using this system, we were able to detect a specific antibody response to experimental challenge with *M. avium* in both chickens and quail. The extent of cross reactivity among IgGs of diverse avian species will determine the usefulness of ELISAs for diagnosis of *M. avium* infection in birds. As diagnostic methods, radiometric culture of avian fecal and tissue biopsy samples for *M. avium* and an ELISA for detection of serum antibody against the pathogen are still primarily research tools. However, with further refinement, both have potential clinical applications as means of identifying birds infected with *M. avium* and excreting the pathogen in their feces. Early diagnosis of ATB is critical for control of this disease in captive avian populations.

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LITERATURE CITED

5. Junge, R. E. (Personal communication).