

A Method of Preventing Transmission of So-called “Megabacteria” in Budgerigars (*Melopsittacus undulatus*)

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Abstract: “Megabacteriosis” is a condition affecting many psittacine and nonpsittacine birds for which an effective, reliable therapy and means of prevention have not been developed. Megabacteriosis has been associated with a chronic wasting condition in the budgerigar (*Melopsittacus undulatus*) termed “going light,” but the organism also has been detected in clinically healthy, thriving birds. In this study, removing eggs from the nests of megabacteria-positive adult budgerigars and hand-raising hatchlings under isolation conditions prevented transmission of megabacteria in all offspring. Staining fecal smears and histologic tissues with Calcofluor White-M2R also was shown to be a reliable means of demonstrating megabacteria. Hand-raising budgerigar hatchlings, and those of other avian species in which megabacteriosis is considered to be of concern, is a potentially valuable method of producing offspring that are free of this organism.

Key words: megabacteria, proventriculus, ventriculus, avian, budgerigar, *Melopsittacus undulatus*

Introduction

“Megabacteria” are organisms or a group of organisms whose classification has historically been unclear. They are rod-shaped to filamentous, gram-positive, and periodic acid-Schiff-positive microbes and have been found in the ventriculus at its junction with the proventriculus in many psittacine and nonpsittacine birds. Although originally thought to be a bacterium, recent studies suggest that this organism is actually a yeast (B. Tomaszewski, K. F. Snowden, D. N. Phalen, unpublished data, August 2000). Megabacteria have been shown to have a eukaryotic nucleus (B. Tomaszewski, K. F. Snowden, D. N. Phalen, unpublished data, August 2000),¹ stain distinctly with Calcofluor White-M2R (a stain for chitin and cellulose, substances present in fungi), and have the ribosomal RNA gene sequence of a yeast (B. Tomaszewski, K. F. Snowden, D. N. Phalen, unpublished data, August 2000).¹

Megabacteriosis is widespread in budgerigars (*Melopsittacus undulatus*),²⁻⁴ where it has been associated with a chronic, progressive wasting condition termed “going light.” Commonly reported

signs of disease include chronic weight loss, dysphagia, vomiting or regurgitation, diarrhea, and death.³⁻⁶ Megabacteria-associated disease has also been described in lovebirds (*Agapornis* species),² parrotlets (*Forpus* species), canaries (*Serinus* species),⁷ various species of finch,^{2,8} ostrich (*Struthio camelus*),⁹ and domestic chickens.^{10,11} However, megabacteria have been detected in birds, including those budgerigars used in this study, that fail to demonstrate any clinical signs of disease (B. Tomaszewski, K. F. Snowden, D. N. Phalen, unpublished data, August 2000).¹² Therefore, conclusions of the actual pathogenicity of this organism require experimental infection studies with purified megabacteria in specific-pathogen-free (SPF) birds.

Megabacteriosis is typically diagnosed before death by demonstration of the organism in feces, and after death by microscopic examination of ventricular scrapings or histologic examination of the proventricular-ventricular junction. Attempts at treating flocks of birds infected with megabacteria with both antibiotics and antifungals largely have been unsuccessful. Amphotericin B, a polyene macrolide antifungal drug, has been reported to be effective.¹³ However, treated birds have been observed to return to fecal shedding of megabacteria, suggesting either that reinfection occurred, or that therapy was incomplete. Treatment with amphotericin B also is complicated by the need for long-term

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administration and considerable cost in treating large flocks of birds.

The purpose of this study was to determine whether incubator-brooding and hand-rearing of hatchlings from megabacteria-positive adult budgerigars would prevent transmission of megabacteria to offspring. If successful, this technique may be used to produce SPF budgerigars for subsequent infection trials as well as provide a management technique in aviculture.

Materials and Methods

Budgerigars used in this study were selected from a conventional breeding colony maintained for academic research purposes at Texas A&M University. All animal care practices and experimental procedures were performed in accordance with University Laboratory Animal Care Committee, Animal Use Protocol 8-337.

Fifteen adult breeding pairs of budgerigars were housed communally in conventional wire-mesh flight cages with wooden nest boxes. All breeding pairs were similarly aged, originally obtained from a single source, and maintained identically before and throughout this study. Five breeding pairs were housed in each of 3 cages. Fecal samples were collected monthly by placing clean papers beneath each flight cage and collecting the first 20 fecal samples that were produced. Direct smears of these 60 samples were made on glass slides, air-dried and saved for staining with Calcofluor White-M2R. The remaining feces were examined individually for the presence of megabacteria by microscopic examination of unstained wet-mounts. Adult birds were fed a commercial seed diet consisting of millet, flax, and hulled oats supplemented with a pelleted budgerigar diet (Kaytee Products, Inc, Chilton, WI, USA). Food and fresh water were available free-choice and were replaced daily.

Nest boxes were monitored once daily for nesting activity and egg production. Eggs from productive nest boxes were arbitrarily designated as 1 of 2 categories: those whose chicks would be parent-raised ($n = 6$), and those whose chicks would be raised under isolation conditions without parent contact ($n = 18$). Eggs and chicks were collected as necessary over an approximate 6-week period. Eggs for isolation were removed from the nest box 1–2 days before expected hatch. The exterior of each egg was cleansed with a warm (35.0°C) 5% povidone iodine solution (Betadine Solution, Purdue Frederick Co, Norwalk, CT, USA) prepared with sterile water. Eggs were subsequently placed into an incubator (Turn-X Model TX7 Automatic Incubator, Lyon

Electric Company, Inc, Chula Vista, CA, USA). The incubator was set at 37.2°C and 88% relative humidity. Hatchlings were moved to sterile microisolation cages with autoclaved recycled paper bedding. Hatchlings were housed under strict isolation conditions, with all handling performed under aseptic conditions.

Chicks were initially fed a psittacine hand-feeding formula (Exact Hand-Feeding Formula, Kaytee Products, Inc). However, because of poor growth of SPF chicks in early trials, the hand-feeding formula was supplemented with approximately 25% (vol/vol) peanut butter to increase the caloric content of the diet. Chicks were fed with sterile syringes 6 times daily. Chicks were fed 10–15% of their weight at each feeding. At approximately 4–5 weeks of age, the budgerigars were weaned onto a seed diet identical to that fed to the adult budgerigars. Fresh food and water were offered free-choice daily in sterile containers. Daily weights were recorded for all chicks in this study.

At 8 weeks of age, the birds were humanely euthanized by intramuscular injection of an anesthetic dosage of ketamine (50 mg/kg; Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, USA), followed by exsanguination via venipuncture of the right jugular vein. The proventricular–ventricular junction was formalin-fixed and embedded in paraffin. Five-micron-thick sections were deparaffinized and stained with Calcofluor White-M2R (Calcofluor) fluorescent stain (product F-3397, Sigma Chemical Corp, St Louis, MO, USA).^{14,15} Calcofluor was prepared as a 0.5% solution in phosphate-buffered saline (pH 7.2) and stored in the dark at room temperature. Immediately before use, the Calcofluor solution was centrifuged at 500g for 5 minutes to remove precipitates. Slides were fixed in methanol for 5 minutes and air-dried. Approximately 3 drops of Calcofluor solution were added to each slide. After 3 minutes, the slides were rinsed with distilled water and then counterstained with 0.1% Evans Blue in phosphate-buffered saline for 1 minute. Slides were again rinsed with distilled water and allowed to air-dry. A slide containing spores of *Encephalitozoon hellem* from tissue culture was included as a positive control. Slides were examined with a Nikon Labphot-2 microscope (Nikon Instrument Group, Inc., Melville, NY, USA) with ultraviolet capability and excitation barrier filters (380–420 nm) at $\times 100$ and $\times 400$ magnifications. Results of ultraviolet light microscopic analysis were recorded as either positive or negative for the presence of megabacteria without quantitation. Air-dried fecal smears were similarly fixed with methanol and stained with Calcofluor.



Figure 1. Photomicrograph of the junction of the proventriculus and ventriculus from a budgerigar infected with megabacteria. Note the typical "haystack" appearance of megabacteria organisms in situ (hematoxylin and eosin, original magnification $\times 400$).

Statistical analysis of the results of this study was performed by the chi-square test of independence. Results were considered statistically significant at $P < .01$.

Results

In early trials, the SPF birds that were fed solely the Exact Hand-Feeding Formula failed to thrive, and several died. When the Exact Hand-Feeding Formula was supplemented with 25% (vol/vol) peanut butter, the SPF chicks grew at a rate comparable to that of the parent-raised chicks, gaining approximately 1 g in body weight per day (data not shown). All birds appeared healthy at the time of euthanasia, and weights and physical body conditions were determined to be essentially identical between the 2 groups.

Individual examination of wet-mount fecal specimens collected from parent budgerigars routinely revealed that all parent birds were actively infected with and shedding megabacteria organisms in their droppings. Review of egg-laying and hatching records determined that the control group of parent-raised chicks ($n = 6$) was produced from no fewer than 3 different pairs of megabacteria-positive adult budgerigars, whereas the chicks raised in isolation were produced from a minimum of 7 different megabacteria-positive adult pairs.

Fluorescent microscopic examination of Calcofluor-stained proventricular tissues revealed that

100% (6/6) of the parent-raised budgerigar chicks were positive for megabacteria (Figs 1 and 2). Megabacteria were found in the lumen and within the koilin at the junction of the proventriculus and the ventriculus. In contrast, megabacteria were not found in any (18/18) of the SPF budgerigar chicks. All positive-control slides demonstrated adequate fluorescence. By using the chi-square test of independence, the prevalence of infection was found to be significantly less in the SPF birds.

Discussion

Megabacteriosis continues to be an elusive condition. Although study is ongoing, the taxonomic identity of this organism, its role as a disease agent, and consistently effective treatment regimens remain unknown. Attempts at laboratory culture of the organism also have largely been unrewarding,¹⁶ hampering study of this organism and the associated condition. Infectivity studies with SPF birds constitute 1 direction of investigation, whereas additional drug trials with novel chemotherapeutic agents represent another focus of study. If future studies prove that megabacteria are pathogenic, elimination of this organism by interrupting transmission, as opposed to widespread use of antimicrobial drugs, may be necessary.

In this study, we produced megabacteria-free birds. By removing eggs from the nest before hatch, disinfecting them, and raising the young in isola-



Figure 2. Photomicrograph of the junction of the proventriculus and ventriculus from a budgerigar infected with megabacteria. Calcofluor-White M2R stain with fluorescence demonstrates the bacteria (original magnification $\times 320$).

tion, we were able to prevent infection. These data demonstrate that vertical (in ovo) transmission of megabacteria does not occur in the budgerigar. Furthermore, analysis of data suggests that regurgitant feeding from parent to offspring or fecal contamination in the bird's environment constitute the most likely routes of transmission of megabacteria.

Hand-raising chicks is labor-intensive, but is commonly performed by aviculturists, and therefore may prove to be an acceptable means of establishing megabacteria-free colonies of birds. Several factors need to be investigated before this approach can be advocated for the budgerigar and other bird species. It is not known how long this organism can persist in the environment, if a contaminated environment can ever be fully disinfected, or if birds become infected by environmental exposure to the organism. It also is not known what potential environmental reservoirs, if any, exist for megabacteria. If infection can occur in SPF birds that are housed in clean environments and fed standard, untreated diets, then neither management nor treatment efforts will ever be successful at keeping a flock free of megabacteriosis. At present, no consensus exists on the method of transmission of this organism. Specific studies with SPF birds housed in close proximity or in direct contact with infected birds will help assess the degree of lateral transmission between birds or infection from the environment.

The Calcofluor staining method was found to rap-

idly and inexpensively facilitate the visualization of megabacteria in fecal smears and histologic tissue specimens. Although megabacteria are demonstrable with other staining methods, Calcofluor was shown to be highly specific for this organism, thereby minimizing confusion with other microorganisms. However, a drawback of this method was the requirement of a microscope with ultraviolet fluorescent and barrier filter capabilities.

Although not a primary focus of this study, we determined that supplementation of the commercial hand-feeding formula with peanut butter at 25% (vol/vol) was necessary to maintain growth rates in hand-raised budgerigar chicks that were consistent with those of their parent-raised counterparts. We postulate that the fat in the peanut butter provided the additional caloric density necessary for the growth of these nestlings.

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