Diagnosis, Treatment, and Prevention of Megabacteriosis in the Budgerigar (*Melopsittacus undulatus*)

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Abstract: "Megabacteriosis" is a condition affecting many psittacine and non-psittacine birds for which a consistently effective 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 has also been detected in clinically normal, thriving birds. We have demonstrated that removing eggs from the nest 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 was also 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. In a related drug trial, lufenuron was shown to have no beneficial effect against megabacteriosis in the budgerigar. Infection was established in the domestic chicken (*Gallus gallus*) by oral inoculation of organisms derived from infected budgerigars. The domestic chicken was shown to be a useful model for performing infectivity studies and antimicrobial drug trials against this organism.

Key words: megabacteria, proventriculus, ventriculus, isthmus, Calcofluor, amphotericin B, lufenuron, budgerigar, *Melopsittacus undulata,* domestic chicken, *Gallus gallus*

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 (PAS)-positive microbes that have been found at the isthmus (the junction of the proventriculus and ventriculus) in many psittacine and non-psittacine birds. Although originally thought by most investigators to be a bacterium, recent studies suggest that this organism is actually a yeast.[1] Megabacteria have been shown to have a eukaryotic nucleus,[1,2] stain distinctly with Calcofluor White-M2R (a stain for chitin and cellulose), and have a ribosomal RNA genetic sequence consistent with other yeasts.[1,2]

Megabacteriosis is reported to be widespread in budgerigars (*Melopsittacus undulatus*),[3,4,5] where it has been attributed to causing a chronic, progressive wasting condition termed "going light." Commonly reported signs of this disease include chronic weight loss, dysphagia, vomiting/regurgitation, diarrhea, and death.[4,5,6,7] Megabacteria-associated disease has also been

described in lovebirds (*Agapornis* species),[3] parrotlets (*Forpus* species), canaries (*Serinus* species),[8] various species of finch,[3,9] ostrich (*Struthio camelus*),[10] and domestic chickens (*Gallus gallus*).[11,12] However, megabacteria have been detected in birds, particularly budgerigars, that fail to demonstrate any clinical signs of disease.[13] Conclusions of the pathogenicity of this organism have in many cases been based upon anecdotal information. Additional study is needed in this area using purified megabacteria in specific-pathogen-free (SPF) birds. However, experimental infection has been hampered by the lack of a repeatable method of culturing this organism in vitro. Perhaps the recent determination that this organism is a yeast will allow a method of laboratory culture to be developed.

Megabacteriosis is most commonly diagnosed antemortem by demonstration of the organism in feces, either by gram stain of fecal smears, or wet-mount analysis of fresh droppings. In ongoing studies performed at Texas A&M University, megabacteria are consistently demonstrable on fecal wet-mount preparations, and viewed at 100X and 400X. For contrast enhancement, Calcofluor is used, either on fecal preparations or tissue histopathology slides. Postmortem diagnosis is made by microscopic examination of isthmus mucosal scraping or histologic examination of the proventricular-ventricular junction. When viewed in situ, in most instances organisms are numerous and are stacked side to side, analogous to "logs floating down a river" or having a "haystack" appearance. Megabacteria are most commonly confined to the koilin layer, rarely extending deeper than the mucosa. However, an interesting observation is that megabacteria organisms frequently are detected between the "columns" of koilin being produced by the underlying epithelium. As koilin is being constantly produced in the budgerigar, the finding of megabacteria in this location implies that this organism is able to withstand the gradient caused by the growing koilin.

Megabacteria have also reportedly been detected in the liver of a severely infected bird, and may also be detected in the mucosal glands of the distal proventriculus.[17] In fecal analyses or in scrapings of the koilin layer of the ventriculus, megabacteria are most commonly adhered to fragments of koilin, gastrointestinal contents, or other debris. It is less common to observe megabacteria organisms freely floating without aggressive agitation of the sample, or inclusion of some detergent in the solution. No significant correlation has been noted in studies thus far between the number of organisms detected in fecal preparation and the degree of colonization of the gastrointestinal tract.

In studies at Texas A&M University, the youngest budgerigar that has been diagnosed with megabacteriosis was 10 days old. The vast majority of birds in the collection at Texas A&M University that are diagnosed with megabacteriosis fail to display any clinical signs of ill health, nor any gross lesions on necropsy. Occasionally a lymphocytic or lymphoplasmacytic infiltrate in the distal proventriculus is noted on histopathologic analysis; however, this is not limited to megabacteria-positive budgerigars. The cause of this observation is unknown, and further analysis of this is underway.

Attempts at treating birds infected with megabacteria have met with limited success. Filippich found amphotericin B, a polyene macrolide antifungal drug, to be effective at ameliorating fecal shedding of megabacteria organisms in infected budgerigars.[14] However, in one trial, several treated birds were observed to return to fecal shedding of megabacteria, suggesting either that reinfection occurred, or that therapy was incomplete. Treatment with amphotericin B is also complicated by the need for long-term administration, considerable cost in treating large flocks of birds, and can be difficult to obtain in oral formulation. Presently, amphotericin B administered orally at 100 mg/kg twice daily is the antimicrobial drug most commonly used in treatment of this

condition. Other treatments that are reported to have shown some degree of success include oral nystatin and oral lactobacillus.[11]

The purpose of the study summarized herein 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 for use in aviculture.

Experiment

Megabacteria-positive adult budgerigars were used in this study to produce offspring that were managed in one of two ways. Eggs were either hatched and raised by the parents (n=6), or eggs were removed from the nest box 1 to 2 days prior to expected hatch, and were hand-raised under isolation conditions (n=18). Microscopic examination of individual unstained wet-mount fecal specimens had previously demonstrated that all adult budgerigars were actively shedding megabacteria. Hatchlings were moved to sterile microisolator cages with autoclaved recycled paper bedding. Hatchlings were housed under strict isolation conditions, with all handling performed aseptically.

Chicks were initially fed a psittacine hand-feeding formula (Exact™ Hand-Feeding Formula, Kaytee Products, Inc., Chilton, WI). However, due to poor growth of SPF chicks in early trials, the hand-feeding formula was supplemented with approximately 25% peanut butter (v/v) to increase the caloric content of the diet. Chicks were fed using sterile syringes 6 times daily, approximately 10-15% of their weight at each feeding. At approximately 4 to 5 weeks of age, the budgerigars were weaned onto a seed diet identical to that fed to the adult budgerigars.

At 8 weeks of age the birds were humanely euthanized. The isthmus was resected, formalin-fixed, and embedded in paraffin. Five-micron-thick sections were deparaffinized and stained using Calcofluor White-M2R (Calcofluor) fluorescent stain (Product No. F-3397, Sigma Chemical Corp., St. Louis, MO).[15,16] Calcofluor was prepared as a 0.5% solution in phosphate-buffered saline (pH 7.2) and stored in the dark at room temperature. Slides were examined with a Nikon Labphot-2 microscope with UV capability and excitation barrier filters (380 to 420 nanometers) using 100X and 400X magnifications. Results of ultraviolet light microscopic analysis were recorded as either positive or negative for the presence of megabacteria without quantitation. Statistical analysis of the results of this study was performed using the Chi-square test of independence. Results were considered statistically significant at $P<0.01$.

Results

In early trials, the SPF birds that were fed solely the Exact™ hand-feeding formula failed to thrive, and several died. When the hand-feeding formula was supplemented with 25% (v/v) peanut butter, the SPF chicks grew at a rate comparable to that of the parent-raised chicks, gaining approximately 1 gram in body weight per day. All birds appeared healthy at the time of euthanasia, and weights and physical body conditions were determined to be essentially identical between the two groups.

Fluorescent microscopic examination of Calcofluor-stained tissues revealed that 100 percent (6/6) of the parent-raised budgerigar chicks were positive for megabacteria. Megabacteria were found in the lumen and in 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. Using the Chi-square test of independence, the prevalence of infection was found to be significantly less ($P<0.01$) in the SPF birds.

Megabacteria were not shed consistently in the feces of birds that were subsequently found to be megabacteria-positive. In weekly examination of fecal smears from megabacteria-positive budgerigars, there was no consistent finding of megabacteria in the droppings, nor was there any correlation to the degree of colonization of the proventricular-ventricular junction as seen on necropsy.

Discussion

Megabacteriosis continues to be an elusive condition in many respects. The taxonomic identity of this organism, its role as a disease agent, methods of culturing the organism in the laboratory, and a treatment regimen that is completely effective and reliable remain undetermined. Because of the work that has recently been performed on determination of the genetic sequence of this organism, a taxonomic identity may soon be proposed. However, infectivity studies using SPF birds constitute one necessary direction of investigation, while additional drug trials using novel chemotherapeutic agents represent another focus of study. If future study proves that megabacteria are pathogenic, elimination of this organism by interrupting the transmission, as opposed to widespread use of antimicrobial drugs, may be necessary. In this study, we have taken the first step in producing megabacteria-free birds. By removing eggs from the nest immediately prior to hatch, disinfecting them, and raising the young in isolation, we were able to prevent infection. These data demonstrate that vertical (in ovo) transmission of megabacteria does not occur in the budgerigar. Furthermore, data suggest 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. However, 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 is not yet known what potential environmental reservoirs, if any, exist for megabacteria. Furthermore, if future investigation demonstrates that 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 there is no consensus 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. There is a report of a megabacteria-positive budgerigar being housed with an uninfected budgerigar, with no apparent transmission of the condition.[14]

Megabacteria appear to be shed only intermittently in the feces. It is not yet known which factors may contribute to shedding of this organism. Therefore, the predictive value of using the number of organisms on fecal smears or fecal wet-mount preparations to assess the degree of "infection" would appear to be low in the budgerigar. Similarly, concluding that a bird is cleared of infection likely requires serial fecal collection, since even in megabacteria-positive birds (from necropsy findings) negative fecal examinations may be obtained.

The Calcofluor staining method was found to rapidly and inexpensively facilitate the visualization of megabacteria in fecal smears and histologic tissue specimens. Though megabacteria are

demonstrable with other staining methods, Calcofluor was shown to be highly specific for this organism, thereby minimizing confusion with other microorganisms. A drawback of this method, however, was the requirement of a microscope with ultraviolet fluorescent illumination and barrier filter capabilities.

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

Drug Trial in the Budgerigar

In ongoing drug trials at Texas A&M University, novel antimicrobial agents are being evaluated for their efficacy against megabacteria in the budgerigar. One of these drugs, lufenuron, was recently studied. Lufenuron was included in this study due, in part, to its reported efficacy in treating dermatophytoses and dermatomycoses in the dog and the cat.[18] Lufenuron's mechanism of action that makes it effective against both the flea and cutaneous fungi is that it inhibits a pathway necessary for chitin synthesis. Given that megabacteria appear to be a type of fungus, it was hypothesized that lufenuron may be effective in the treatment of megabacteriosis as well. The dosage used in the budgerigar was derived by metabolic scaling from the feline dosage used for the treatment of fleas. Budgerigars were assembled into treatment groups, with one group serving as a control (n=15), one group administered lufenuron orally once daily at 100 mg/kg (n=20), and one group administered the identical dosage of lufenuron once weekly (n=10). Fecal shedding of megabacteria was monitored once weekly for each group. At the end of the four-week course of therapy, birds were humanely euthanized and tissues were collected for histopathologic analysis. Examination of the proventricular-ventricular junction indicated that 8/15 (53%) of the control birds were positive for megabacteria, 15/20 (75%) of the daily-treated birds were positive, and 7/10 (70%) of the weekly-treated birds were positive. No significant gross abnormalities were noted in any of the birds on necropsy. However, in many of the birds, a lymphocytic to lymphoplasmacytic infiltrate was noted in the mucosal layer of the ventriculus. The cause of this is presently unknown, as this inflammation was not confined to megabacteria-positive budgerigars. Further analysis of these findings is presently underway.

The results of fecal monitoring in the budgerigars demonstrated no consistency between the shedding of megabacteria organisms, and the ultimate findings on necropsy. Birds that demonstrated a heavy burden of megabacteria on histopathologic analysis typically shed no more organisms in their droppings than those with a lesser burden. In addition, a small number of budgerigars occasionally had negative fecal examinations, but were still found to harbor organisms in the proventricular-ventricular junction on histopathologic examination.

The Domestic Chicken (*Gallus gallus*) as a Study Model

Recently the chicken was examined as a potential species in which to conduct additional investigations of megabacteriosis. If successful, the chicken model would be advantageous over the budgerigar or other species in that chicks can be readily and inexpensively obtained from a local poultry producer and are comparatively easy to maintain and medicate.

In the initial experiment, three budgerigars previously proven to be infected with megabacteria were humanely euthanized, and the mucosal surface of the isthmus and proventriculus of each bird

was scraped and placed in phosphate-buffered saline. The mucosal scrapings of the three birds were composited and quantitated to be at a concentration of approximately $4x10^6$ megabacteria organisms per milliliter. Six one-day-old *Gallus gallus* chicks were orally inoculated with a 0.2 ml dosage ($8x10^5$ organisms) of this suspension. The six chicks were housed communally in a wire-bottomed cage and fed a standard chicken starter and water ad lib. After three weeks, the chicks were humanely euthanized and proventricular and ventricular tissues were examined identically to those of the budgerigars. All six of the chickens demonstrated active infection with megabacteria in a pattern that was very similar to that seen in the budgerigars. Based upon these findings, interspecies postmortem passage of megabacteria was determined to be possible and constitutes a feasible method of maintaining an actively infected population of birds for continued study of this organism. The proventricular and isthmus mucosal scrapings from these birds were subsequently used to inoculate an additional set of *Gallus gallus* chicks for antimicrobial drug trials.

Ongoing inoculation and infectivity trials at Texas A&M University are aimed at determining the lowest infective dosage of megabacteria organisms in the chicken, and also whether naïve birds housed communally with inoculated birds become infected with megabacteria. The results of this investigation are expected to be available at the time of the conference.

Preliminary Drug Trials in the Domestic Chicken

Additional antimicrobial drug trials are underway using the chicken as a study model. These drug trials are focused on the use of novel antifungal agents such as amphotericin B, flucytosine, fluconazole, thiabendazole, silver sulfadiazine, and sodium iodide. Most of these drugs are being administered orally by gavage. Other drugs are also proposed for future study. Drugs that appear to exhibit efficacy in the chicken will subsequently be studied in the budgerigar. The results of these investigations are expected to be available at the time of the conference.

At this time, the significant unknowns regarding megabacteriosis are whether or not this organism is a primary pathogen, opportunistic invader, or nonpathogenic organism, and whether there is species variation in the degree of pathogenicity. Studies focusing on answering these questions are complicated by the fact that this organism has not been successfully cultured in the laboratory. Once a method of either culturing megabacteria in vitro has been developed, or of purifying the organism adequately and in sufficient quantity to support infection trials, these studies can be undertaken and shed more light on this interesting organism.

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