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Original Study

Comparison of In-Clinic Diagnostic Testing Methods for *Macrorhabdus ornithogaster*

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Abstract: Macrorhabdus ornithogaster is an ascomycete yeast often found at the isthmus of the ventriculus and proventriculus of infected birds. Antemortem diagnosis has traditionally involved direct visualization of organisms on wet-mount or gram-stained fecal preparations, cloacal and crop swabs, or by both methods; however, different in-clinic diagnostic techniques have never been compared to establish an optimum test for the identification of *M ornithogaster* in an avian patient. We compared 5 microscopically evaluated diagnostic testing methods: fecal Gram's stain, direct fecal wet preparation, macro suspension technique, macro suspension with Gram's stain, and macro suspension stained with new methylene blue. Each technique was performed on 96 fecal samples collected during the treatment of *M ornithogaster*–infected budgerigars with water-soluble amphotericin B. The macro suspension technique produced statistically higher organism counts than the other 4 techniques and was always estimated to have the largest detection probability. We recommend that the macro suspension technique be implemented as the most efficacious diagnostic test for in-clinic assessment of avian patients possibly infected with *M ornithogaster*.

Key words: Macrorhabdus ornithogaster, diagnosis, cytology, feces, avian, budgerigars

INTRODUCTION

Macrorhabdus ornithogaster (MO) is a yeast found primarily in a narrow zone between the proventriculus and ventriculus (isthmus) of birds.[1] *M ornithogaster* causes significant morbidity and mortality in pet birds and birds in avicultural collections worldwide. The microorganism has been identified in more than 40 bird species, including Psittaciformes, Passeriformes, Galliformes, Struthioniformes, Anseriformes, Pelecaniformes, and Rheiformes.[1–4] *M ornithogaster* ranges in size from 20 to 80 μm long and 2 to 3 μm wide and are slender, straight rods with rounded ends when found in the feces.[5,6] When the organism is viewed directly through a microscope in a wet mount preparation, small oblong refractile nuclei are found at regular intervals within the organism.[5,6] *M ornithogaster* are readily identified in suspensions of scrapings of the isthmus obtained during postmortem examination of infected birds. Histologically, the organisms are palely eosinophilic and are present on the surface of the isthmus and between the glands of the proventriculus and isthmus, stacked in parallel. In severe cases, MO can grow deep between the glands of the proventricular isthmus and penetrate into the koilin lining of the ventriculus causing a lymphoplasmacytic inflammatory response.[5,7,8]

Macrorhabdus ornithogaster causes disease characterized by vomiting, dark tarry feces, chronic wasting, and, in severe cases, death.[2,3] Birds may also be infected with MO without exhibiting any clinical disease signs.[7,9] It is becoming increasingly evident that concurrent comorbidities influence the host's immune competence and gastrointestinal

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flora, resulting in the manifestation of clinical signs associated with MO infection.[7,8,10,11] When disease does occur, MO can cause significant morbidity and mortality in avicultural collections. Therefore, detecting infected birds early and accurately is essential, so one can isolate and treat affected birds to prevent transmission through the avicultural collection. Similarly, birds that are infected without showing signs of disease may act as a persistent source of environmental contamination or expose progeny, perpetuating MO in future generations.[12]

Antemortem, the most common diagnostic test used to obtain a diagnosis of MO, is microscopic examination of the bird's droppings. Traditionally, a direct wet mount slide is made with fresh feces mixed with 0.9% sodium chloride solution and scanned at ×40 magnification.[5] The Gram's and quick stains (eg, Diff-Quik, Astral Diagnostics, Freeway Park, NJ, USA) are often used to examine fixed fecal smears for the presence of MO, but poor stain uptake with variable staining characteristics have been reported.[10] When stained appropriately, the organisms are gram positive and stain dark blue with Diff-Quik stains.[10] A rapid method of concentrating MO and separating the organisms from other solid fecal matter is described by homogenizing the droppings with approximately 20 times their volume of physiologic saline in a small tube, after which the homogenized feces are rested for 10 seconds before placing a small drop of the suspension collected from the meniscus on a microscope slide to examine through a microscope. Because MO takes longer to settle than the particulate matter, the organism is reportedly easier to see by this concentration technique.[10]

Recently, other diagnostic modalities have been evaluated, including polymerase chain reaction (PCR), isolating and amplifying MO DNA with MO-specific primers in fecal or isthmus samples, and the Mini-FLOTAC device (FLOTAC Group, University of Naples, Napoli NA, Italy), which allows visualization of MO by direct microscopy.[10,13] Cloacal swabs were tested by PCR technology and compared with fecal Gram's stain in the diagnosis of MO in a flock of budgerigars (*Melopsittacus undulatus*). The PCR testing of the cloacal swabs was positive for MO in approximately 50% more birds than identified with the Gram's stain.[14] The Mini-FLOTAC device was also compared with fecal Gram's stain and found to be equally successful in the diagnosis of MO in a variety of species.[13] Postmortem examinations were not performed on the sampled birds in either study; therefore, the sensitivity and specificity of the PCR, Mini-FLOTAC, and fecal Gram's stain diagnostic tests to identify MO in birds remains unknown.

Despite the general availability of in-clinic MO diagnostic tests to the veterinary community, there is no substantial information regarding the validity of the tests in determining the sensitivity and specificity of each. This research investigation evaluated the efficacy of 5 commonly used in-clinic diagnostic tests for MO and provides recommendations for the most effective clinical diagnostic technique to identify infected avian patients.

MATERIALS AND METHODS

Source of samples

Fecal samples were collected from budgerigars maintained at an aviary in New South Wales, Australia, after reports of a mortality event attributed to MO. In the preceding 2 weeks, 14 birds had died. Three out of 14 dead birds were submitted for postmortem examination, with only 1 having tissues submitted for histopathology. The 2 birds that were only examined grossly had large numbers of MO identified on direct wet mount preparations of scrapings obtained from the proventricular isthmus. Histopathology of the remaining bird identified glandular dysplasia with mixed inflammatory cellular infiltration and large numbers of MO deep within the glands of the proventricular isthmus.

Before seeking veterinary assistance, the aviary owner had administered various treatments, including trimethoprim-sulfonamide administered in the drinking water for 5 days at an unknown dose (Sulfa AVS, Carlingford Veterinary Hospital, Sydney, New South Wales, Australia), a 5-in-1 in-water medication containing amprolium, ronidazole, tylosin, levamisole, and probiotics (13 g/1 L drinking water × 3 days; Vita King 5 in 1, Vita King Inc, Granger, IN, USA) and an iodophore-based germicide (30 mL/100 L drinking water × 7 days; Sani-Chick, Ruakura Pty Ltd, New South Wales, Australia). The aviary owner did not see any positive treatment effect from the medications administered to the budgerigars.

Ninety-six fecal samples were collected from 16 infected birds. One sample from each infected bird was analyzed, 6 times over 10 days, as part of the diagnostic investigation and treatment at the aviary. Treatment group birds were randomly selected in the order they were caught from both breeding cabinets and flight aviaries and isolated in a standard budgerigar exhibition cage at the breeder's facility until feces were passed onto a

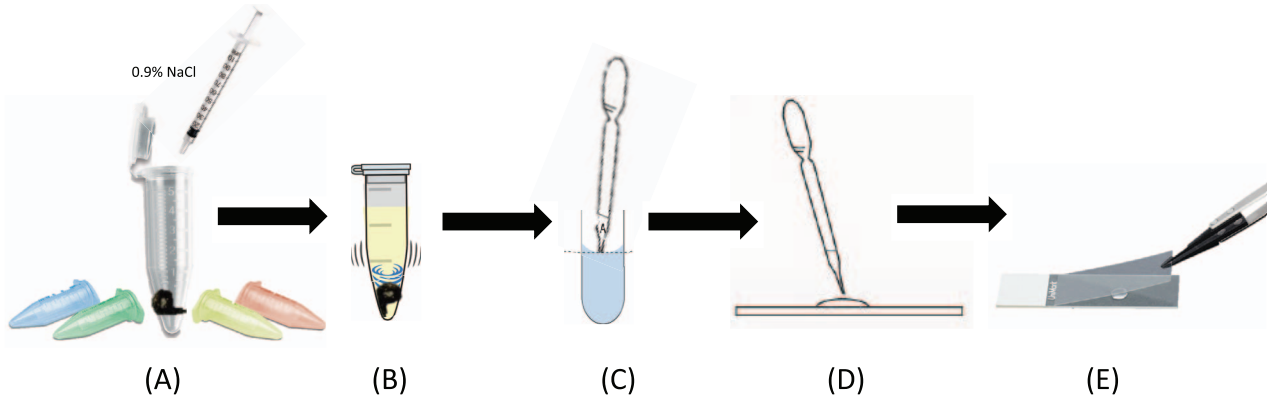

Figure 1. A pictorial representation of the macro fecal suspension technique: (A) 0.3 mL of 0.9% sodium chloride is placed in a microcentrifuge tube with 0.5 g of feces; care must be taken to avoid contaminating the suspension with the uric acid portion of the droppings. (B) The lid is closed and a slurry formed by shaking the microcentrifuge tube manually for 20 seconds. (C) The slurry is placed in an upright position for 3 seconds to allow particulate matter to settle before removing 0.06 mL from the meniscus with a 1-mL syringe. (D) A drop is placed on a microscope slide and (E) covered with a coverslip for analysis.

clean plastic liner within the cage. Positive birds were identified and grouped together in a 1.2 × 1.0 × 0.6-m flight cage to undergo treatment with amphotericin B 0.9 mg/mL water-soluble powder (Megabac-S, Vetafarm Pty Ltd, Wagga Wagga, New South Wales, Australia). Feces were collected from each bird every second day during treatment and then again, once, 3 weeks after the treatment with amphotericin B was discontinued. To collect samples, the birds were housed individually in an exhibition cage to ensure each sample collected was confirmed to belong to the individual bird being tested at that time. The first fecal pellet passed was collected with a cotton-tip swab (Swisspers, McPherson's Consumer Products, Kingsgrove, New South Wales, Australia).

Detection techniques

Five microscopically evaluated detection techniques for MO were compared in this research study. Direct fecal wet mount preparations were made with a 2 × 2-mm piece of fresh fecal material that was placed on a glass slide with 0.03 mL of 0.9% sodium chloride. A glass coverslip was placed over the feces in the 0.9% sodium chloride and used to agitate the sample gently to facilitate mixing until the feces were spread across the coverslip. Gram staining was performed on the fecal wet mount preparation samples by removing the coverslip and allowing the slides to air dry. The slides were heat-fixed and gram stained (Australian Biostain Pty Ltd, Victoria, Australia). For the macro suspension preparations, 0.3 mL of 0.9% sodium chloride was placed in a 1.5-mL microcentrifuge tube with 0.5 g of feces.[15] Care was taken to avoid the uric acid portion of the droppings when the feces were collected. The microcentrifuge tube lid was closed, and a slurry was formed by shaking the tube manually for 20 seconds. The slurry was placed in an upright position and 0.06 mL from the meniscus was immediately removed with a 1-mL syringe (Fig 1). One 0.03-mL aliquot was placed onto a glass slide and covered with a coverslip and microscopically examined without staining. After assessment of the unstained suspension sample, the coverslip was removed, and the sample was air-dried, heat-fixed, and gram stained. A second 0.03-mL aliquot from the fecal suspension slurry was placed onto a glass slide, and 0.01 mL of methylene blue was dispensed onto the sample before placement of a glass coverslip and microscopic evaluation. The wet mount samples (fecal wet preparation, macro suspension preparation, and macro suspension preparation with methylene blue) were microscopically examined immediately upon collection, and the gram-stained samples were reviewed at the conclusion of the sample collection by the same investigator, blinded to the results of the previous MO counts from that fecal sample.

Each slide was microscopically examined by the same technique, with the stage diaphragm reduced for unstained samples. Two passes of the ×40 microscope objective over the coverslip were made over the first and the last quarters of the coverslip. Each MO organism visualized was counted and recorded as a continuous numerical value.

Difficulties interpreting the slides were encountered for any of the diagnostic techniques evaluat-

ed when uric acid crystal contamination was present in the fecal sample. When contaminated, the uric acid crystals dominated the microscopic field, thus reducing the ability to evaluate the slides. Slides that contained uric acid crystals were discarded, and a second fecal pellet was immediately collected from the same bird, as previously described, to replace the nondiagnostic sample.

Data collection and statistical analysis

For each bird, counts of MO by the 5 diagnostic tests were taken on selected days. Let y_{ijk} be the number of MO observed for the ith bird on the jth day its samples were taken with the kth test. Standard regression models, such as generalized linear models, are not appropriate to analyze these data because measurements from the diagnostic tests conducted on the same bird at the same time were not independent, nor were measurements obtained from the same bird at different points in time. If a bird was shedding a large number of organisms, then all 5 tests were likely to detect many MO organisms; likewise, a bird shedding a large number of MO will continue shedding high numbers of the organism at the next testing time point. Consequently, sequential measurements on different days would be correlated, a common feature of time-series data like these.

A generalized linear state-space model was developed and fitted to account for both forms of correlation. The state process of this model comprises latent variables related to the unknown shedding rates of the birds, which we hereafter refer to as the shedding measure. A negative binomial distribution was fitted to each observed count, which is commonly used for count data with high variance, as in this study. In the model, the observed count y_{ijk} has the expectation $\lambda_{ijk} = \exp\{\beta_{0k} + s_{ij}\}$, where s_{ij} is the latent shedding measure of the ith bird on the jth day its samples were taken, and β_{0k} controls the relationship between this shedding measure and the expected number of organisms detected by the kth method. A diagnostic test with a large β_{0k} is capable of detecting many organisms and may therefore be more effective at diagnosing an MO infection.

The latent shedding measure of a bird may vary over time because of both treatment and random day-to-day fluctuations caused by other processes, such as bird health and immune response. The model allows the shedding measure to change over time differently during treatment and nontreatment periods (ie, to accommodate a decrease in shedding during treatment but a potential increase upon its cessation). The model uses $s_{ij} = \alpha_1 t_j + u_j$ for measurements taken during the treatment period, where t_j is the number of days since the beginning of treatment on the jth day the bird's samples were taken, and $s_{ij} = \alpha_1 t_e + \alpha_2(t_j - t_e) + u_j$ for measurements after the cessation of treatment, where t_e is the total number of treatment days. The latter includes a piecewise-linear relationship over time, where the shedding measure changes with one slope during treatment and another after treatment; the parameters α_1 and α_2 are slopes controlling the per-day change in the bird's shedding measure during treatment and after cessation of treatment, respectively. Finally, u_j accounts for changes in shedding from other factors (eg, bird health), which is modeled by a Gaussian process to account for correlation between 2 counts from samples from the same bird taken close together in time.

Comparing β_{0k} parameters between different diagnostic tests identifies, on average, which tests detect larger numbers of organisms than others. Moreover, the model allows calculation of the probability of successfully diagnosing a MO infection given a particular shedding measure and diagnostic test, achieved by calculating the probability of a nonzero count under the fitted negative binomial distribution. To further assess the diagnostic performance of the tests, the probability of a successful diagnosis from the different tests was calculated, on the basis of the estimated shedding measures at end-of-treatment and follow-up of the birds that were known to have remained infected throughout the experiment.

The model was fitted in R (R Foundation for Statistical Computing, Vienna, Austria) with the package Template Model Builder.[16] Code used to fit the model is available online (https://github.com/b-steve/macrorhabdus), where further technical details about the model can also be found.

RESULTS

Detection techniques

The macro suspension technique provided the clearest identification field for visualizing MO compared with all other techniques because the preparations contained little background debris. Indeed, this technique consistently visualized more MO organisms when compared with all other techniques (Fig 2). When the methylene blue stain was added, many of the MO organisms were found to be associated with small accumulations of debris, making direct visualization more difficult. When using the direct fecal wet mount and Gram's

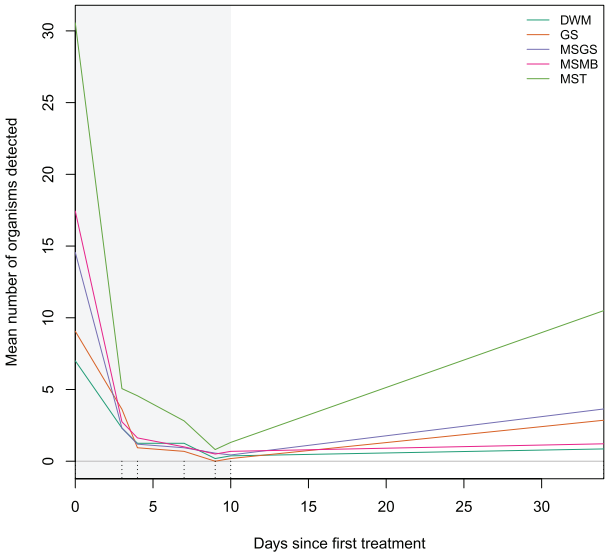

Figure 2. The average number of *Macrorhabdus ornithogaster* organisms detected across birds for each day samples were taken with each of the different diagnostic testing methods (direct fecal wet mount [DWM], fecal Gram's stain [GS], macro suspension Gram's stain [MSGS], macro suspension new methylene blue stain [MSMB], and macro suspension technique [MST]), demonstrating that MST, on average, identified more organisms than any of the other diagnostic techniques. The grey region represents the treatment period, and the vertical dotted lines show the days on which samples were taken between first treatment (day 0) and follow-up (day 34).

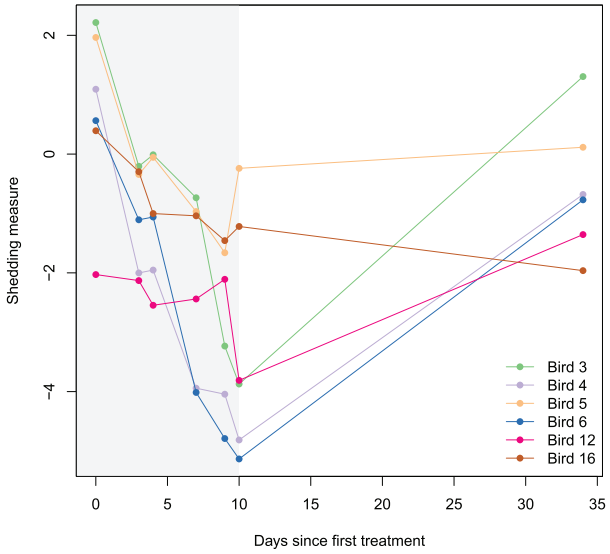

Figure 3. Shedding measures estimated by the model for the 6 birds that returned positive tests at the conclusion of the study on days samples were taken. A shedding measure of 0 corresponds to the average shedding rate across birds at first treatment. The shedding measure can be interpreted multiplicatively following exponentiation; for example, a shedding measure of –0.5 indicates that expected detection counts are exp(–0.5) = 0.61 times as high as that of the average bird at first treatment. Estimated shedding rates for all birds decreased during the treatment period, shown in grey, although birds 5 and 16 were still shedding at moderate rates at end-of-treatment. The remaining 4 birds were shedding at low rates at end-of-treatment but had returned to moderate levels by follow-up.

stain techniques, the majority of the MO organisms were visualized on the periphery of the smear where there was less fecal material.

The estimate of α_1 was negative and statistically significantly different from zero ($P < .001$), confirming that shedding decreased during the treatment period. The change in counts between the end-of-treatment and follow-up varied between birds: Some did not shed any further organisms, whereas others saw a substantial increase in shedding rates (eg, Fig 3).

The expected number of organisms detected between different test types was statistically significantly different ($P < .001$). In particular, evidence suggested that the expected number of organisms detected by the macro suspension technique was more than that of all other diagnostic tests evaluated: direct fecal wet mount ($P < .001$), macro suspension Gram's stain ($P < .001$), fecal suspension with methylene blue ($P < .001$), and fecal Gram's stain ($P < .001$). Evidence also suggested that the expected number of organisms detected by macro fecal suspension with methylene blue was more than that of direct fecal wet mount ($P < .001$) and Gram's stain ($P = .006$). All other pairwise comparisons between diagnostic testing methods were not statistically significant ($P > .05$).

Six birds returned positive tests at follow-up, with 4 estimated to have low shedding rates at the end of treatment, despite remaining infected (Fig 3). Consequently, the estimated probabilities of detecting MO organisms were low (<.5) at these shedding rates for all diagnostic tests (Fig 4), indicating that false negative diagnoses are likely for some infected birds because of low shedding rates immediately after treatment. All 4 birds with low shedding rates at end-of-treatment had substantially higher estimated shedding rates at the end of follow-up (Fig 3), with correspondingly higher probabilities of detecting MO organisms, close to 1 for the macro suspension technique (Fig 4), indicating that for some birds diagnosis is more sensitive at follow-up than at the end of treatment.

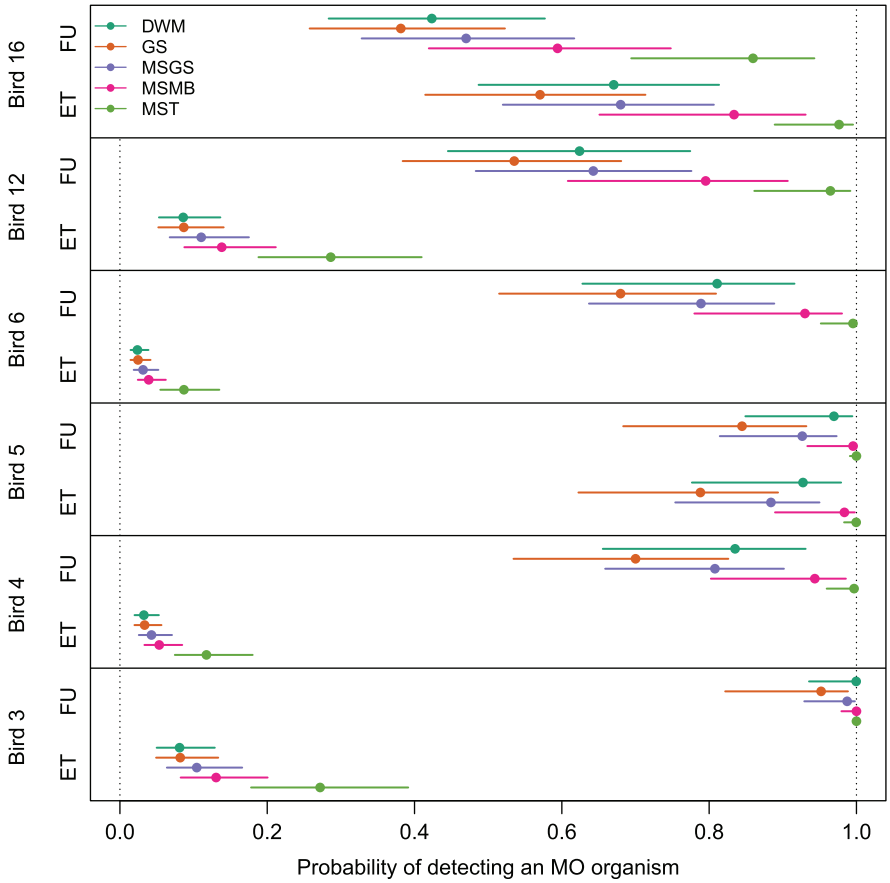

Figure 4. Point estimates and 95% confidence limits for the probabilities of detecting at least 1 organism, when shedding measures were set at values estimated by the model for the 6 birds that returned positive tests at the conclusion of the study. Birds' shedding measures for both end-of-treatment (ET) and follow-up (FU) were considered. Probabilities are shown separately for direct fecal wet mount (DWM), fecal Gram's stain (GS), macro suspension Gram's stain (MSGS), macro suspension new methylene blue stain (MSMB), and macro suspension technique (MST). Estimated detection probabilities were highest by MST. Birds 5 and 16 were shedding at high rates at end-of-treatment and thus had high detection probabilities at that stage, whereas the remaining 4 birds' shedding rates were low, making a successful diagnosis unlikely by any method. These 4 birds had higher detection probabilities at follow-up, after their shedding rates had increased.

The remaining 2 birds had relatively high estimated shedding measures at the end of treatment, but the shedding rate did not change substantially during the follow-up period (Fig 3). Consequently, estimated MO detection probabilities were similar at the 2 points in time (Fig 4).

The macro suspension technique method had estimated MO detection probabilities that were higher than the other diagnostic tests, and the 95% confidence intervals around the estimates indicate significant differences with fecal Gram's stain, macro suspension Gram's stain, and direct fecal wet mount methods for many of the scenarios (bird 3 at end-of treatment, birds 4 and 6 at follow-up, and birds 12 and 16 at both end-of-treatment and follow-up). However, evidence was not found to suggest a difference in detection probabilities between the macro suspension test and macro suspension stained with methylene blue or between any other pairwise comparisons.

In some cases the difference between the macro suspension technique and other methods had substantial practical significance: At shedding rates similar to bird 16 at follow-up, for example, the probability of detecting an MO organism was estimated (with 95% confidence limits) to be 0.86 (0.69, 0.94), whereas that for the fecal Gram's stain was 0.38 (0.25, 0.52). This result corresponds to the finding stated above, that the macro suspension technique detects significantly more MO organisms than the other testing methods evaluated.

DISCUSSION

Macrorhabdus ornithogaster has a worldwide distribution and is a significant pathogen of pet, aviculture, and wild birds. At present, no "best practice" is accepted for veterinary clinicians attempting to diagnose MO in avian patients. We have evaluated the most common in-clinic diagnostic tests to establish the most effective technique for identifying MO organisms in diseased avian patients. Of the 5 diagnostic tests evaluated in this study, the macro suspension technique was found to identify a greater number of MO organisms than the others and, with stained methylene blue fecal suspension, was the most likely to identify at least 1 organism in the feces. We therefore recommend using the macro suspension technique over fecal Gram's stain, macro suspension Gram's stain, direct fecal wet mount, and the macro suspension technique stained with methylene blue.

Not only did the macro suspension technique consistently identify the highest number of organisms, but they were easily visualized by this technique because of minimal background interference of digested plant material and other particulate matter. The same technique with methylene blue stain as a contrast agent resulted in, on average, fewer organisms being visualized. The methylene blue stained the background liquid, but also clearly highlighted the background of bacteria and digested plant material, complicating the diagnosis of MO.

The direct fecal wet mount preparation technique is the most commonly used in-clinic diagnostic modality recommended for the visualization of MO in feces[10] but proved less effective in identifying MO organisms. This method consistently recorded lower counts and, more importantly, often missed birds that were found to be positive by the macro suspension technique. Despite a very thin fecal smear preparation, the dense consolidation of fecal material and digested plant cell wall, feathers, and other ingesta prevented clear visualization of MO organisms and was likely the reason for fewer organisms being detected by the direct fecal wet mount preparation technique.

The fecal gram-stained samples consistently produced lower MO counts than the majority of the wet mount techniques, despite that fecal gram-stained slides were made from the same feces and preparations as the wet preparation techniques. The reason for this result is unclear, but it is postulated that removing the coverslip from the previously examined slide may have also removed some MO organisms held in suspension and decreased the number of MO on the stained slides. It is also possible that because the birds were undergoing treatment when samples were being collected, the MO organisms may have stained differently or not at all with Gram's stain. This idea is supported by the findings of Gestier,[17] who identified variable staining MO organisms during a treatment trial with amphotericin B, and Phalen,[10] who confirmed the difficulty in variable stain uptake, and proposed that the organism sticks poorly to glass slides, something that is partially resolved with heat fixing.

The statistical model was used to investigate the probability of each diagnostic test detecting at least 1 shed MO organism in birds that remained infected throughout the study, both after 10 days of treatment (the recommended treatment duration with water-soluble amphotericin B) and at follow-up. The better the diagnostic testing method, the higher the probability of detecting at least 1 MO organism, although detection probabilities for the same test vary depending on the number of organisms a bird is shedding. For example, any diagnostic test is likely to detect MO in birds shedding many organisms, but unlikely to detect MO in birds that are shedding few organisms. The differences between diagnostic testing methods are small for birds that are shedding few organisms (ie, birds 4 and 6 at end-of-treatment) or a large number of organisms (ie, birds 3 and 5 at follow-up). In these scenarios, all diagnostic testing methods are either very unlikely (for birds shedding low numbers of organisms) or very likely (for birds shedding high numbers of organisms) to detect an organism; however, the differences are more pronounced for birds between these 2 extremes (ie, birds 12 and 16 at follow-up). It is important to note that a larger than expected number of organisms detected from 1 sample does not imply that a diagnostic test is more likely to detect routinely at least 1 shed organism from an infected bird; the model fitted different mean-to-variance relationships across the different tests, and it is possible for a diagnostic test with a larger expectation to nevertheless have a lower probability of detecting at least 1 organism if its variance is also larger.

Previous studies evaluating fecal Gram's stain and its efficacy compared with a diagnostic test using PCR technology and the Mini-FLOTAC method found that the PCR test identified significantly more positive results, whereas the Mini-FLOTAC method only had a slight increase in the likelihood of positive birds being identi-

fied.[13,14] Cloacal swabs tested by PCR returned 57% positive results for MO birds in a mixed budgerigar aviary, whereas the same birds evaluated by fecal Gram's stain were found to be 24% positive for the presence of MO, a difference of 33%.[14] In comparison, our study found that 27 of 81 (33.3%) of the samples tested, which were positive by the macro suspension technique, were negative by fecal Gram's stain, a figure that is in agreement with the difference between fecal samples tested for MO using PCR technology and Gram's stain. Unfortunately no birds from either of these 2 studies were available for postmortem examination, and without sensitivity and specificity data for PCR, Mini-FLOTAC, or Gram's stain MO diagnostic tests, it is unclear whether all of the birds that returned PCR-positive results, were indeed infected.[14]

In conclusion, this study evaluated 5 different in-clinic diagnostic testing methods to determine which is the most effective in making a definitive diagnosis of MO in an avian species. We established that the macro suspension technique is superior in identifying the greatest number of organisms, as well as the most effective at isolating organisms if they are present. The results of this study should aid veterinarians in their effort to detect and diagnose MO in avian patients. Work is ongoing to assess the sensitivity and specificity of assays using feces for the detection of MO in birds known to be infected, and further research is required to compare the success of the macro suspension technique with diagnostic tests that use PCR technology.

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