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BACTERIOLOGICAL EXAMINATION OF PET BIRDS' FECES FOR MYCOBACTERIOSIS

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Summary. The article reports findings from a bacteriological study on 232 fecal samples from 29 different companion bird species, searching for mycobacteriosis. The results of the study revealed the detection of atypical mycobacteria in 161 samples, namely *M. scrofulaceum* (n = 3), *M. avium* (n = 4), *M. genavense* (n = 154), which amounted to 1.3, 1.7, and 66.4% of the samples examined, respectively. Co-infections with other pathogens were detected in 62% of the examined fecal samples, independent of a mycobacterial agent's presence. Among these co-infections, *Cryptosporidium* was detected in 34.0% of cases, non-acid-resistant bacteria in 32.6%, and molds and yeast-like fungi in 48.4%

Keywords: *M. avium*, *M. scrofulaceum*, *M. genavense*, *Cryptosporidium*

Introduction. Avian tuberculosis is a very serious disease affecting domestic, exotic and agricultural birds, with pet birds being at increased risk of contracting the disease. There are currently over 130 types of mycobacteria acknowledged, of which ten have been verified as bird pathogens. These mycobacterial species comprise *M. avium* subsp. *avium*, *M. genavense*, *M. tuberculosis*, *M. bovis*, *M. gordonae*, *M. nonchromogenicum*, *M. fortuitum* subsp. *fortuitum*, *M. avium* subsp. *hominissuis*, *M. intracellulare*, *M. scrofulaceum*, and *M. kansasii* (Schrenzel, 2012; Shivaprasad and Palmieri, 2012; Buur and Saggese, 2012).

In a study conducted in Switzerland, *M. genavense* was detected in 71% of cases while *M. avium* complex was only detected in 17%. Other isolates included *M. fortuitum* (4%), *M. tuberculosis* (4%), *M. gordonae* (2%), and *M. nonchromogenicum* (2%) (Hoop, Böttger and Pfyffer, 1996).

Molecular-genetic methods were employed to determine that *M. genavense* was responsible for the majority of mycobacterial infections (up to 80%) in companion birds, particularly within Passeriformes and Psittaciformes species, while MAC was identified in just 5–10% of cases (Tell, Woods and Cromie, 2001; Hoop, 1997; Manarolla et al., 2009).

M. genavense has been found in Coraciiformes, Piciformes, Columbiformes, Ciconiiformes, and Galliformes in previous studies (Tell, Woods and Cromie, 2001; Schmitz et al., 2018a, 2018b), but there have been no reported instances of *M. genavense* detection in large commercial poultry farms.

In birds, *M. genavense* causes a disseminated disease with clinical and histopathological signs that do not differ from the infection caused by *M. avium* (Antinoff et al., 1996; Van Der Heyden, 1997). Nevertheless, a study conducted from 2017 to 2019 observed that mycobacteriosis caused by *M. avium* subsp. *avium* resulted in more common pathological changes, particularly the formation of granulomas. On the other hand, the absence of these signs is more frequently associated with cases caused by *M. genavense* (Schmidt et al., 2022).

In addition to the above two primary avian tuberculosis pathogens, exotic captive birds have been reported with rare infections that result in distinctive pathological organ lesions. These infections are caused by *M. avium* subsp. *hominissuis*, *M. fortuitum*, *M. intracellulare*, *M. scrofulaceum*, *M. gordonae*, *M. celatum*, *M. nonchromogenicum*, and *M. intermedium* (Schmidt et al., 2022; Bertelsen, Grøndahl and Giese, 2006; Kik, Houwers and Dinkla, 2010; Pfeiffer et al., 2017). Furthermore, Psittacine parrots are the sole avian species in which cases of tuberculosis caused by *M. tuberculosis* and *M. bovis* are frequently reported (Schmidt et al., 2008; Washko et al., 1998; Fulton and Sanchez, 2013). Generally, these infections are traced back to human patients with tuberculosis.

The high prevalence of mycobacterial infections among parrots, especially those caused by *M. genavense*, may be due to genetic factors, specific susceptibility of species, as well as exogenous causes (housing conditions, crowding, stress, etc.). In addition, concomitant infections (*Macrorhabdus ornithogaster*, circovirus, polyomavirus, avian bornavirus, adenovirus, *Mycoplasma* species, *Salmonella* species, *Escherichia coli*, *Aspergillus* species and various parasites), by disrupting the immune status, contribute to the activation of *M. genavense* in the host (Manarolla et al., 2009; Schmitz et al., 2018a, 2018b; Manarolla et al., 2007). The danger of mycobacterial infections is that many infected birds often do not show clinical signs of the disease, as mycobacterioses develop slowly and cause a chronic course of the disease, and in most cases in older birds. Due to the high prevalence of mycobacteriosis among domestic parrots and their high susceptibility to mycobacterial infections, purchasing a parrot from an unknown breeder carries a significant risk of infection. The disease is most likely to occur in densely populated areas with poor sanitation and hygiene. Based on the fact that the above mycobacteria are of great

importance in human pathology, domestic parrots can represent a potentially dangerous source of mycobacterial infection for owners, especially for immune compromised individuals, children and the elderly (Realini et al., 1999).

The aim of the study is to determine the species composition of mycobacteria that cause mycobacteriosis in companion birds.

Materials and methods. The research was conducted in 2019–2022. The bacteriological (cultural, bacterioscopic) method was used to analyze 232 fecal samples from companion birds encompassing 29 distinct species of Psittaciformes, Strigiformes, Corvidae, Columbiformes, and Passeriformes. The analyzed samples included 50 from Jaco, 7 — from Cockatoos, 1 — from Suriname parrot, 6 — from Senegalese parrots, 17 — from Amazon (Venezuelan, Cuban, and White-fronted), 3 — from Pyrhurra, 11 — from Ara, 50 — from Corella, and 22 — from Budgerigar. 26 — from Quaker monk, 1 — from Eclectus, 7 — from Alexandrian parrot, 8 — from Cyanoramphus, 4 — from Aratinga, 2 — from rosella, 5 — from lovebird, 10 — from Necklace Parrot, 1 — from Mountain Patagonian, 1 — from Black-capped lory, 1 — from Lord Derby's Parakeet, 1 — from Rüppel's Parrot, 1 — from Amadina, 1 — from Watersluger Canary, 1 — from Eurasian Hobby, 1 — from Eurasian Scops Owl, 1 — from Owl, 1 — from Eagle Owl, 4 — from Crow (gray and black), 1 — from Starling, 4 — from Pigeon. Fecal samples were collected in sterile plastic containers by bird owners. Samples were stored in a refrigerator at the temperature of 4 °C for no more than one day, after that pre-inoculation treatment was performed.

Pre-inoculation treatment of excrement. 1.0 g of excrement was added to a plastic container with 20.0–25.0 cm^3 of sterile distilled water. The mixture was shaken to a homogeneous suspension and settled for 30 min. To a sterile centrifuge tube with 0.08 g of crystalline N-cetylpyridinium chloride, with a sterile pipette 10.0 cm^3 of the supernatant layer of the excrement suspension was added. The tube was hermetically sealed with a rubber stopper, shaken thoroughly until the preparation was dissolved, and left at 18–25 °C for 20 hours. The next day, the tube with the sample was centrifuged for 15 min at 3,000 rpm, the supernatant was removed, and the precipitate was washed once with sterile distilled water by centrifugation (15 min, 3,000 rpm). The supernatant was poured off, and the precipitate was resuspended in 2.0–2.5 cm^3 of 0.85% sodium chloride solution. The suspension was inoculated 0.5 cm^3 into two tubes with nutrient medium for cultivation of mycobacteria and two tubes with nutrient medium with growth factor (mycobactin). The tubes with the cultures were placed in a thermostat at 37–38 °C for 3 days in a horizontal position with not hermetically sealed stoppers, and then paraffinized. Incubation was carried out at a temperature of 37.5–38.5 °C. The presence of colony growth was recorded once a week for 5 months.

The genus identification of *Mycobacterium* and *Cryptosporidium*, tinctorial and morphological characteristics of the detected microorganisms were determined in smears stained by the Ziehl-Nielsen method.

The species affiliation of the isolated mycobacterial culture was determined in biochemical (hydrolysis reaction of Tween-80, amidase and catalase activity, reduction of tellurite) and cultural (growth rate, growth ability at 22 and 45 °C, tolerance to 5% sodium chloride in the medium) tests. To do this, a culture suspension at a concentration of 1.0 mg of bacterial mass in 1.0 cm^3 of 0.85% sodium chloride solution was inoculated onto different media for each test.

Results and discussion. According to the anamnesis, all the birds exhibited diverse health issues including lung and skin lesions, feather loss, joint inflammation, gastrointestinal problems, lethargy, and depression. Furthermore, approximately 50% of the examined birds had positive PCR results for mycobacteria or lung darkening observed on radiographs. Certain birds had bacterial infections as well.

According to study results, 71 (30.6%) fecal samples contained no mycobacteria, while 161 samples contained *Mycobacterium* spp. The number of samples positive for mycobacteria was 69.4%, of which 4.3% (7 cultures) were slow-growing cultures of atypical mycobacteria, namely: 4 non-chromogenic cultures from 2 Jacos, 1 crow, 1 Corella and 3 scotochromogenic cultures from 2 Jacos and 1 Quaker monk. The remaining 95.7% of isolates (n = 154) were identified as *M. genavense*. According to the results of the cultural study, the primary growth of colonies was detected after 25–30 days on both media in the form of round, smooth and shiny white (n = 4) or bright orange (n = 3)colonies. These colonies subsequently merged to form a continuous growth over the entire surface of the medium, as shown in Figs 1, 2.

Microscopy of isolated cultures of microorganisms showed straight or slightly curved, with grains at the poles, acid-resistant rods, which were located individually or in clusters (Figs 3, 4).

According to the Runyon classification, 4 nonchromogenic cultures of atypical mycobacteria were assigned to group III, and 3 scotochromogenic cultures were assigned to group II.

When determining the species affiliation of isolated mycobacterial cultures, it was found that the optimal temperature for colony growth was 37–38 °C, at 22 °C the growth of colonies was slower and less intense, at 45 °C no growth of orange cultures was observed, and nonchromogenic cultures grew, but less intensively.

Figure 1. Growth of nonchromogenic (Corella, Jaco) and scotochromogenic cultures (Jaco 1, Quaker).

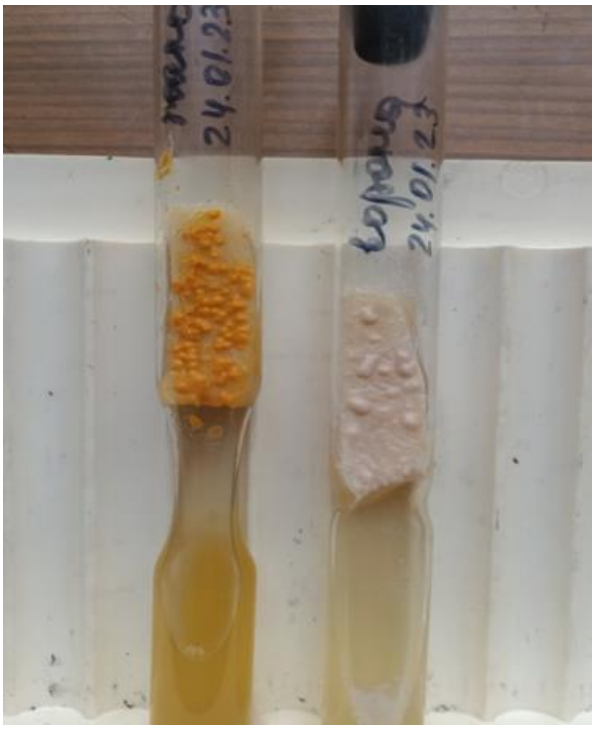

Figure 2. Growth of scotochromogenic culture (Jaco 2) and a nonchromogenic culture (crow) on Pavlovsky's medium.

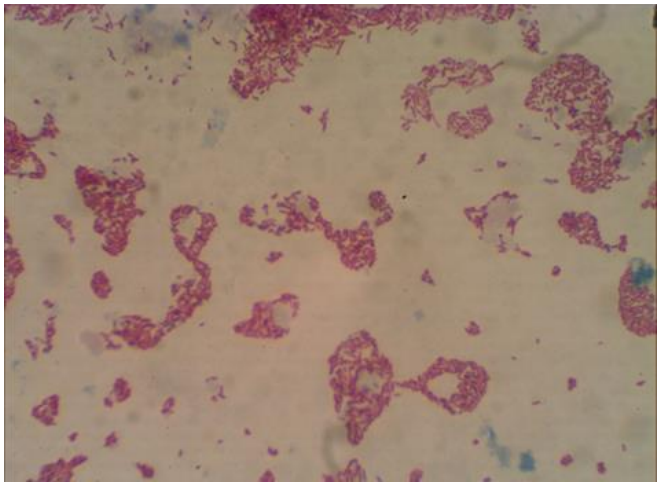

Figure 3. Microscopy of a nonchromogenic mycobacterial culture.

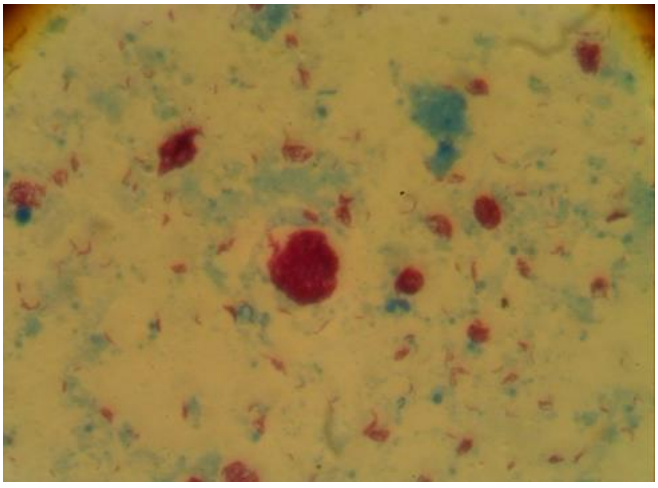

Figure 4. Microscopy of a scotochromogenic culture of mycobacteria.

None of the cultures grew on medium containing 5% NaCl. When determining enzymatic activity in scotochromogenic mycobacterial cultures, positive reactions to nicotinamidase, pyrisinamidase, carbamidase, and catalase were found. The cultures did not reduce tellurite from potassium tellurite and did not hydrolyze Tween-80.

Based on cultural, morphological and biochemical characteristics, 3 scotochromogenic cultures were identified as *M. scrofulaceum*.

It should be noted that mycobacteriosis caused by *M. scrofulaceum* caused death in 2 Jaco parrots. This mycobacterial species is an environmental organism and is frequently present in water and soil, as well as in potable water. The data on the potential of *M. scrofulaceum* to cause infection in birds are debatable. Bertelsen et al. (2006), Hoop et al. (1996), Kik et al. (2010), Pfeiffer et al. (2017) and Abd El-Ghany (2022) have reported that *M. scrofulaceum*, among other non-tuberculous mycobacteria, can cause avian tuberculosis-like pathological lesions in poultry. Marco et al. (2000) found *M. scrofulaceum* in an aviary housing exotic birds. However, their data indicates that *M. scrofulaceum* is not a recognized source of mycobacterial infection (Marco,

Domingo and Lavin, 2000). However, the death of two out of the three birds, namely the Jaco parrots, from which *M. scrofulaceum* was isolated, contradicts this finding. Further data is required to confirm the pathogenicity of this species in parrots.

Regarding nonchromogenic mycobacterial cultures (n = 4), the results of culture, morphological and biochemical tests showed that they had positive reactions to nicotinamidase and pyrisinamidase, restored tellurite on the 9th day, had negative reactions to catalase, carbamidase and hydrolysis of Tween-80. These findings led to the identification of these cultures as *M. avium*. Once diagnosed, the birds were euthanized by their owners. Unfortunately, no necropsies were conducted, resulting in a lack of pathological lesion data.

In the subsequent cultural examination of the remaining samples after 5 months of cultivation in growth factor medium (pH = 6), 154 (66.4%) samples showed very small, transparent, nonphotochromogenic disgonal colonies measuring less than 0.5 mm in size, which did not increase in size regardless of the cultivation period. Microscopy of the scrapings from the surface of this medium or swabs revealed a large number of agglomerates of small short acid-resistant rods (ARR) and cocci (ARC) (Fig. 5), in some cases ARR were found in the cytoplasm of macrophages (Fig. 6).

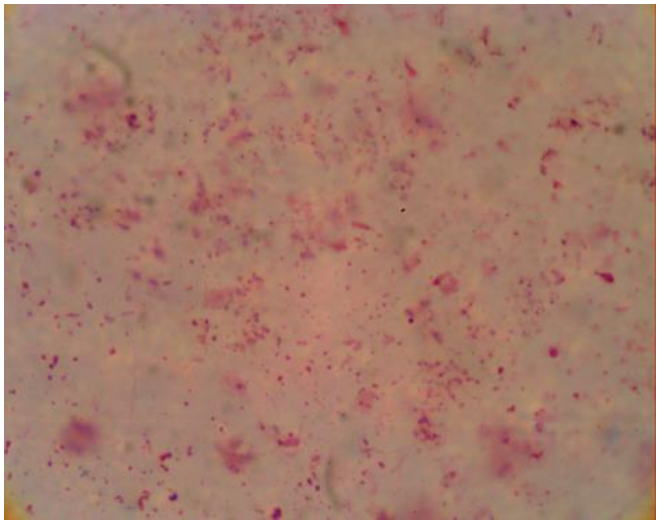

Figure 5. Swabbing off the surface of the medium (Jaco).

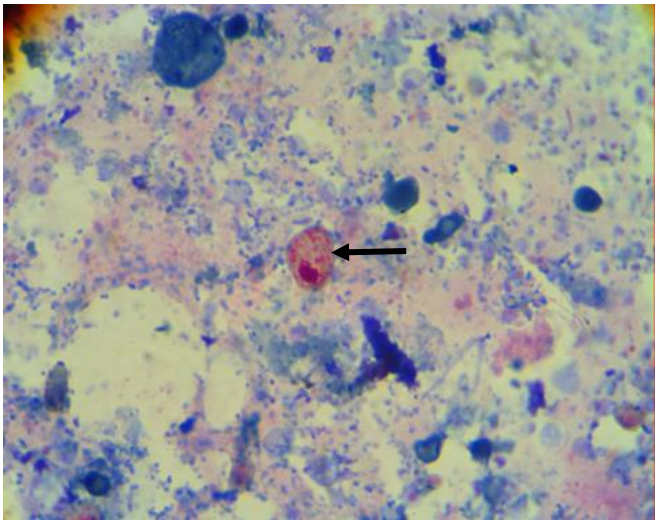

Figure 6. Flushing from the surface of the medium (Cockatoo). Macrophage with ARR and ARC.

The presence of infected macrophages in the feces indicated gastrointestinal inflammation. Under standard cultivation conditions, i.e., on conventional mycobacterial culture medium without growth factor and neutral pH, no colony growth was observed, these microorganisms remained 'unculturable', but microscopy revealed single small clusters of ARR and ARC. In addition, these mycobacteria did not grow on medium with 5% NaCl and at a temperature of 22 °C. Due to 'unculturability' or very long-term growth, it was not possible to obtain a sufficient amount of bacterial mass to study biochemical properties. Based on the results obtained, namely: dependence on growth factor, very slow cell replication and, accordingly, colony growth, cell morphology (small coccoidal forms), tendency to acidic environment (pH = 6.0), and high specific sensitivity of parrots of the genus Psittacinae to this species of mycobacteria, it is reasonable to believe that the detected microorganisms belong to the species *M. genavense.*

It should be noted that a total of 62% of the fecal samples examined revealed concomitant infections caused by other pathogens, regardless of the presence of a mycobacterial agent. Thus, microscopy of mycobacterium-positive smears revealed cryptosporidium co-infection in 15.7% of cases, and cryptosporidium was detected in 18.3% of mycobacterium-negative samples (Fig. 7). Moreover, *Cryptosporidium* was detected at different stages of development in the form of pink spherical oocysts or merozoites. Non-acid-resistant bacteria were detected in 32.6% of the samples, and molds and yeast-like fungi in 48.4% (Figs 7, 8).

Thus, a high prevalence of co-infections indicates a compromised immune status among the studied birds.

During the study, some age dependence was found in the infection of birds with *M. genavense*. The age of the studied birds ranged from 1.3 months to 42 years. But, among the infected birds, the number of birds under the age of 2 years was only 22.1%. The fact that mycobacteriosis in young birds is observed much less frequently was noted by other authors (Tell, Woods and Cromie, 2001; Manarolla et al., 2009; Shivaprasad and Palmieri, 2012).

In addition, among the infected birds, there was a slight correlation between age and the number of bacteria observed during microscopy.

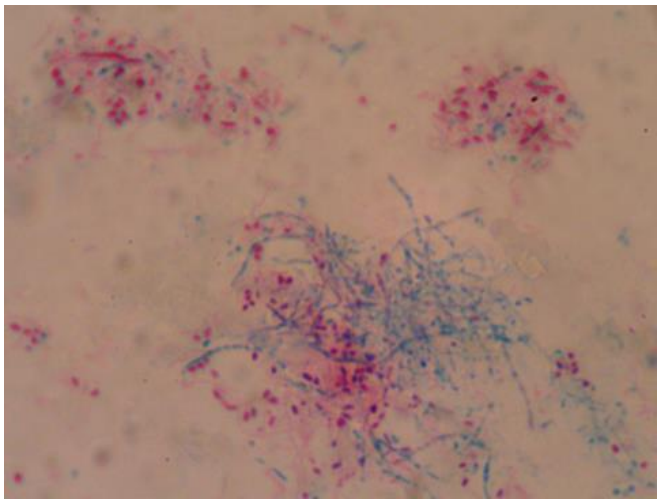

Figure 7. *Cryptosporidium* (pink) and non-acid-resistant bacteria (blue).

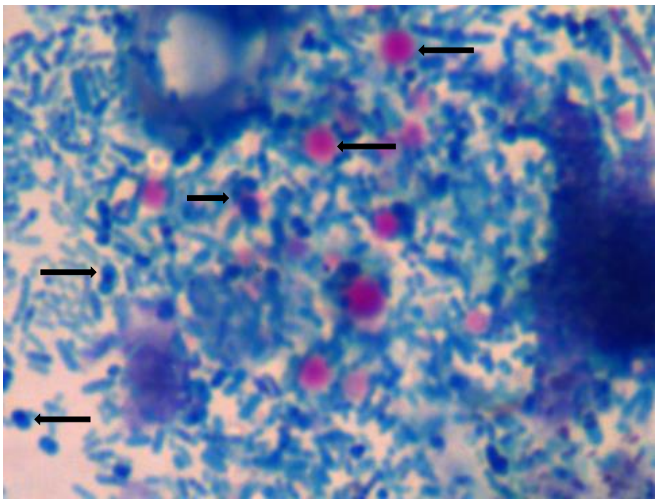

Figure 8. *Cryptosporidium* (oocysts) (pink) and yeast-like fungi (dark blue).

Thus, a higher number of mycobacteria was detected in older birds. It can be assumed that a high coefficient of microscopic detection of mycobacteria indicates the activation of the pathological process. Thus, mycobacteriosis caused by *M. genavense* causes a chronic, latent disease, which is more pronounced in adult birds.

The isolation of *M. avium*, *M. genavense*, and *M. scrofulaceum* suggests that these mycobacterial species circulate and persist among companion birds in Ukraine. Fecal excretion of nontuberculous mycobacteria is a significant mode of transmission for other birds, animals, or humans, directly or indirectly through environmental, feed, or water contamination. Isolated species of mycobacteria, such as *M. scrofulaceum*, are known to cause lymphadenitis, while *M. avium* and *M. genavense* can lead to disseminated infection, particularly in immunocompromised individuals, children, and the elderly.

Close contact with infected birds, whether kept as pets or in zoological gardens, also poses a potential risk of human infection.

Conclusions. The study of feces from companion birds revealed that mycobacteriosis caused by *M. scrofulaceum* was 1.3%, *M. avium* — 1.7%, and *M. genavense* — 66.4%. It is imperative to examine poultry for these mycobacterial species to identify and type the pathogen, considering their potential danger to bird's owners.

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