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Short Title: Mycobacterium infection of budgerigars

Figure 2 to be in colour in printed version (free of charge)

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Abstract

The aim of the present study was to determine the susceptibility of budgerigars (*Melopsittacus undulatus*) to infections with different *Mycobacterium* species. For inoculations the following *Mycobacterium* species were used: *M. avium* subsp. *avium*, *M. bovis* subsp. *bovis*, *M. tuberculosis* subsp. *tuberculosis*, *M. intracellulare* and *M. fortuitum* subsp. *fortuitum*. The bacterial suspension was administrated intramuscularly and all the birds were monitored for 70 days starting from the day of inoculation. During the experiment clinical examination, x-rays, plate agglutination tests, tuberculin tests, feces smear preparations and culture of mycobacteria were performed. The study showed, that *M. bovis* subsp. *bovis* was the most pathogenic *Mycobacterium* species for budgerigars. After inoculation the bacilli induced tuberculosis-typical, clinical signs and necropsy findings. In two out of six birds infected with *M. bovis* subsp. *bovis* radiological changes were also visible. Birds inoculated with other *Mycobacterium* species did not show any typical symptoms of infection and only the results of histopathological and bacteriological examinations indicated the presence of infection.

Introduction

Infections with *Mycobacterium* spp. in birds are still a problem, particularly in ornamental fowl (Motali *et al.,* 1976; Tell *et al*., 2001) and pet birds (Keymer *et al*., 1982; Hoop *et al*., 1996). The *M. avium-intracellulare* complex and *M. genavense* are the species most often reported as pathogenic for birds (Hoop *et al*., 1996; Tell *et al*., 2003a; Tell *et al*., 2003b).

Until 2002, when it was described in canary (Hoop*,* 2002), *M. tuberculosis* subsp. *tuberculosis* infections had been recognized only in *Psittaciformes* birds (Washko *et al.,* 1998; Motali *et al.,* 2001). Several cases of *M. bovis* infections in parrots have also been presented (Altman *et al.,* 1997).

In studies on *Mycobacterium* prevalence in feces of parrots from Polish zoological gardens the most frequently isolated species was *M. fortuitum* subsp. *fortuitum* (Ledwon, 2005). Earlier studies had revealed this bacterium as a frequent etiological factor of mycobacterioses in exotic pet birds (Hoop *et al.,* 1996).

The diagnosis of mycobacterioses or tuberculosis in *Psittaciformes* is based mostly on the results of the necropsy and histopathological examinations (Dolfin *et al.,* 1979; Snyder, 1979; Britt *et al.,* 1980; Panigrahy *et al.,* 1983; Clippinger *et al*., 1998). Antemortem diagnoses are rare and in majority of cases are the result of biopsy of the organs with lesions, direct Ziehl-Nielsen stained smears and culture (Kiehn *et al.,* 1996; Washko *et al.,* 1998; Clippinger *at al.,* 1998; Foldenauer *et al*., 2007). Despite the lack of calcification of tubercles in birds, radiography is also recognized as a useful diagnostic tool (Rosskopf *et al.,* 1981; Clippinger *et al*, 1998; Tell *et. al.*, 2001). In poultry some *in vivo* tests, such as the tuberculin and plate agglutination tests, are also used (Różańska *et al*, 1967).

Earlier studies indicated the low practical value of the tuberculin tests in domestic birds (Montali *et al.,* 1976). The plate agglutination test has not been used until now in *Psittaciformes* The studies performed aimed primarily at assessing the pathogenicity of selected *Mycobacterium* species for budgerigars. Additional studies were performed in order to check their potential usefulness for the diagnosis of mycobacterioses and tuberculosis in *Psittaciformes*. Selection of *Mycobacterium* species for our experiment was based on the documented cases of mycobacterioses and tuberculosis in parrots.

Materials and Methods

A total of 48 three-to six-month-old budgerigars were used. Birds were randomly assigned to experimental groups, each consisting of 6 individuals and inoculated with the following *Mycobacterium* species: *M. avium* subsp. *avium* strain 35716, *M. avium* subsp. *avium* strain 23W, *M. intracellulare* strain 13950, *M. fortuitum* subsp. *fortuitum* strain 6841, *M. tuberculosis* subsp. *tuberculosis* strain 27294 (H37Rv) and *M. bovis* subsp. *bovis* strain 19210. After inoculation birds had been monitored for 70 days and subsequently euthanized. All *Mycobacterium* strains (except for 23W) originated from the collection of the National Tuberculosis and Lung Diseases Research Institute, Warsaw. Strain 23W was isolated from feces of clinically healthy parrots.

The inoculum was administrated into the pectoral muscles (i. m.) at a dose of 5×10^5 CFU kg^{-1} body weight. The decision on intramascular route of the mycobacteria administration was made based on the positive results of earlier studies, indicating shorter time of lesion development after i.m. administration (Heilicek & Treml 1993, 1994; Heilicek *et al.* 1994). This route of inoculation reduced the risk of environment contamination as compared to the natural route of infection (oral, spray).

During the experimental period the monitoring of activity and appetite was performed and feces consistency was evaluated. Feces consistency of budgerigars was measured on an integer scale of 1 to 5. The body weight and the nutritional status were also determined. A week before and eight weeks after the inoculation, a tuberculin test was carried out with avian PPD Tuberculin (Avituberculin® 1012991, BIOWET Puławy, Puławy, Poland) and human PPD Tuberculin (RT 23 SSI 1445F, Statens Serum Institute, Copenhagen, Denmark). In the experiment involving inoculation with *M. bovis* subsp. *bovis*, *M. intracellulare* and *M. fortuitum* subsp. *fortuitum*, instead of human PPD tuberculin the bovine PPD tuberculin (Bovituberculin® 1042000, BIOWET Puławy, Puławy, Poland) was used. The tuberculin tests were performed on propatagium. Consecutive measurements were performed twice, after 48 and 72 hours. Radiological examinations were performed one day before the inoculation and on the 70th day of the experiment. Each time all the birds were first subjected to general anesthesia with ketamine and xylasine. Right and left lateral and ventrodorsal whole-body radiographs were obtained. The plate agglutination test (Różańska, 1967) was performed using reagent prepared in our lab based on the protocol of Tuberculognost (BIOWET Puławy, Puławy, Poland). Each reagent contained a strain of homologous to the strain used for

inoculation. A drop of fresh blood and a drop of diagnostic reagent were put on a plate and the drops were mixed together with a glass rod and the result was read after 2 minutes. The test was performed a day before the inoculation of budgerigars and on the last day of the experiment. Necropsy was performed on both birds that died and the birds subjected to euthanasia by i.v. injection of concentrated barbiturane solution.

During necropsy, samples of the liver, spleen, lungs, kidneys, intestines, tibiotarsal, ulnar and radial bones as well as pectoral muscles were collected for bacteriology and histopathological examinations. Tissue samples for histopathological examinations were fixed in 10% buffered formalin. The fixed tissue samples were stained with hematoxylin and eosin (H&E) stain or according to Ziehl- Neelsen (Z-N) method.

The aim of the bacteriological cultures was to determine the presence of acid-fast bacilli in feces taken once a day for the first seven days and then once a week. Cultures were also performed on samples of all internal organs evaluated during necropsies. The samples were decontaminated using oxalic acid (Yajko *et al*., 1993). Microscopic slides were prepared according to Z-N method (Tb-color, Merck, Darmstadt, Germany). All samples were also cultured on Löwenstein-Jensen medium (Löwenstein, 1931; Jensen, 1932). Additionally, Stonebrink medium was used for culturing feces and organs samples of the budgerigars infected by *M. bovis* subsp. *bovis* (Stonebrink, 1951). Media were incubated in 37ºC for 10 weeks.

To assess whether the budgerigars infected with different *Mycobacterium* species varied in body weight and nourishing condition, an assumption was made that environmental conditions had been equal for all the birds. The multivariate analysis of variance (MANOVA) was applied under completely randomized design. After applying the Box-Cox transformation (Quinn & Keough, 2002), MANOVA was applied for the transformed variables to study whether *Mycobacterium* species influenced feces consistency of budgerigars. When a hypothesis from MANOVA was rejected, discriminant analysis (Quinn & Keough, 2002) was applied to determine differences among the groups as well as find which variables caused these differences. Statistical analysis was carried out with the use of the program SPSS 12.0 (SPSS Inc., 2003).

Results

During first two weeks of the monitoring period the birds did not exhibit any behavioral changes and showed good appetite and vitality. Starting from the third week after inoculation in the group of budgerigars infected with *M. bovis* subsp. *bovis* a decrease of appetite was observed. On the sixteenth day of the experiment one bird from the group infected with *M. bovis* subsp. *bovis* became visibly dejected with feathers ruffled. A scant amount of discharge from nostrils and extravasation under corneous layer on the right side of the beak were also observed. Two days later the budgerigar was not able to sit on a perch and stayed on the cage floor. On the following day the bird died.

The multivariate analysis of variance revealed no significant differences in average body weight and feces consistency of budgerigars infected with different *Mycobacterium* species. The discriminant analysis showed that the nutritional status of birds depended on the *Mycobacterium* species used for inoculation. The differences in nutritional status were especially evident in the ninth week of the study and to a lesser degree it was also observed in the third, fourth and tenth week of the study. Generally, the influence of the *Mycobacterium* species challenge was most evident during last week of the study. The discriminant analysis revealed that similar nutritional status was observed in birds challenged in following groups: (i) *M. avium* subsp. *avium* and *M. bovis* subsp. *bovis*; (ii) Control, *M. avium* subsp. *avium*, *M. intracellulare*, and *M. tuberculosis* subsp. *tuberculosis*; and (iii) *M. fortiutum* subsp. *fortuitum*.

Results of tuberculin and plate agglutination tests were negative in every budgerigar examined.

Radiological lesions typical for mycobacteriosis or tuberculosis were evident in the group of budgerigars infected with *M. bovis* subsp. *bovis* only (Fig. 1). In several birds, however, pathological obesity was observed and two budgerigars infected with *M. bovis* subsp. *bovis* showed a severe emaciation. In one of them lesions suggesting ongoing inflammatory process in bones were identified; in the other a considerable unilateral enlargement of the pectoral muscle was observed. Moreover, in both birds the cloudiness of air sacks was observed (Fig 1).

Necropsy of budgerigars infected with *M. avium* subsp. *avium* or *M. tuberculosis* subsp. *tuberculosis* did not reveal any lesions typical for mycobacteriosis or tuberculosis. A minor tubercles were identified only in pectoral muscles (where inoculum were administered) of three birds. In budgerigars infected with *M. fortuitum* subsp. *fortuitum* and *M. intracellulare* no post mortem mycobacteriosis-induced lesions were identified. Necropsy

examination of budgerigars infected with *M. bovis* subsp. *bovis* showed the presence of tuberculosis-induced lesions of different intensity.

Table 1 shows the results of histopathology examinations. Acid fast bacilli (AFB) were visible only in few organs of *M. bovis*-infected budgerigars. Lesions typical for mycobacteriosis were observed only in ovary, bones (Fig. 2) and heart muscle of budgerigars infected with *M. bovis*.

Table 2 shows the results of bacteriological examination of feces sampled during the experiment and internal organs sampled during necropsy. Positive results of feces bacteriological and microscopical examinations were observed mainly in budgerigars infected with *M. avium* subsp. *avium.* Positive results of culture and presence of AFB bacilli in necropsies organs were observed mainly in *M. bovis* subsp. *bovis* infected budgerigars.

Discussion

Although some studies describing experimental inoculations of birds with mycobacteria have already been published, none of them concerned budgerigars. Hejlicek *et al*. (1993, 1994) intramuscularly inoculated sparrows and rooks with *M. avium*. The authors observed typical tuberculosis-induced lesions in organs in 21 and 12-35 days after the inoculation of sparrows and rooks, respectively. In the present study we used the same inoculation mode and the same *Mycobacterium* species for inoculation of budgerigars, but we were not able to observe any macroscopic lesions even 70 days after *Mycobacterium avium* inoculation. Unlike in other reports we also did not observe any typical infection-induced changes such as weight loss or considerable pectoral muscles atrophy (Montali *et al.,* 1976). Our results indicate a low susceptibility of budgerigars to inoculations with the *M. avium* subsp. *avium* strain used.

Fitzgerald *et al*. (2003) inoculated pigeons with *M. bovis* by intratracheal administration while Butler *et al*. (2001) inoculated crows and starlings to the crop or intraperitoneally. Results of these studies showed high resistance of the examined bird species to *M. bovis*. On the contrary, budgerigars inoculated intramuscularly with *M. bovis* in the present investigation demonstrated typical tuberculosis-induced signs and radiography revealed the presence of lesions in bones in one third of the birds examined. However, the direct comparison of our results with results of other authors is difficult because of different inoculation modes applied.

Although *M. tuberculosis* has not been used for experimentally inoculation of birds so far, the susceptibility of *Psittaciformes* to this bacillus is well known (Washko *et al.,* 1998; Montali *et al.,* 2001). Till the isolation of *M. tuberculosis* in 2002 from a canary (Hoop, 2002), *Psittaciformes* had been the only group of birds reported to suffer from tuberculosis caused by this agent.

The results of the present study performed on budgerigars are rather unexpected. In fact we were not able to identify any tuberculosis-induced clinical signs or necropsy findings. Histopathology of internal organs showed only the presence of some minor inflammatory infiltrations and lesions within the liver and lungs of some individuals. Similarly, *M. intracellulare* and *M. fortuitum* subsp. *fortuitum* with known pathogenicity to birds (Hoop *et al*., 1996; Tell *et al*., 2001) did not cause any lesions in budgerigars inoculated, except for few inflammatory infiltrations observed at histopathology.

The present study confirmed that antemortem diagnosis of mycobacteriosis and tuberculosis in birds is not easy. Radiological changes were found only in two out of six birds with active tuberculosis.

Positive results of bacteriology were very uncommon and mostly involved the budgerigars infected with *M. avium* subsp. *avium*. Tuberculin test and plate agglutination test, despite their usefulness in diagnosing mycobacteriosis in poultry, turned out to be useless in diagnosing of the same disease in budgerigars. Histopathology and bacteriology appeared to be useful tools for both antemortem and post mortem diagnosis of mycobacteria in budgerigars.

In conclusion *M. bovis* subsp. *bovis* to be the most pathogenic to budgerigars and the only one that caused clinical signs and necropsy findings typical for tuberculosis out of the *Mycobacterium* species tested in the present study.

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Figure Legends

Fig. 1. *Ventrodorsal radiograph of a budgerigar infected with M. bovis. The radiograph indicates radial (R) and tibiotarsal (T) left bones lesions and marked atrophy of pectoral muscles (P) as well as opacity of thoracic air sacs (A).*

Fig. 2. *Radial bone tuberculosis due to M. bovis infection showing non-encapsulated granulomatous inflammation after H&E staining. The bar is 400 µm. The insert shows multinucleated giant cells after H&E staining. The bar is 100 µm.*

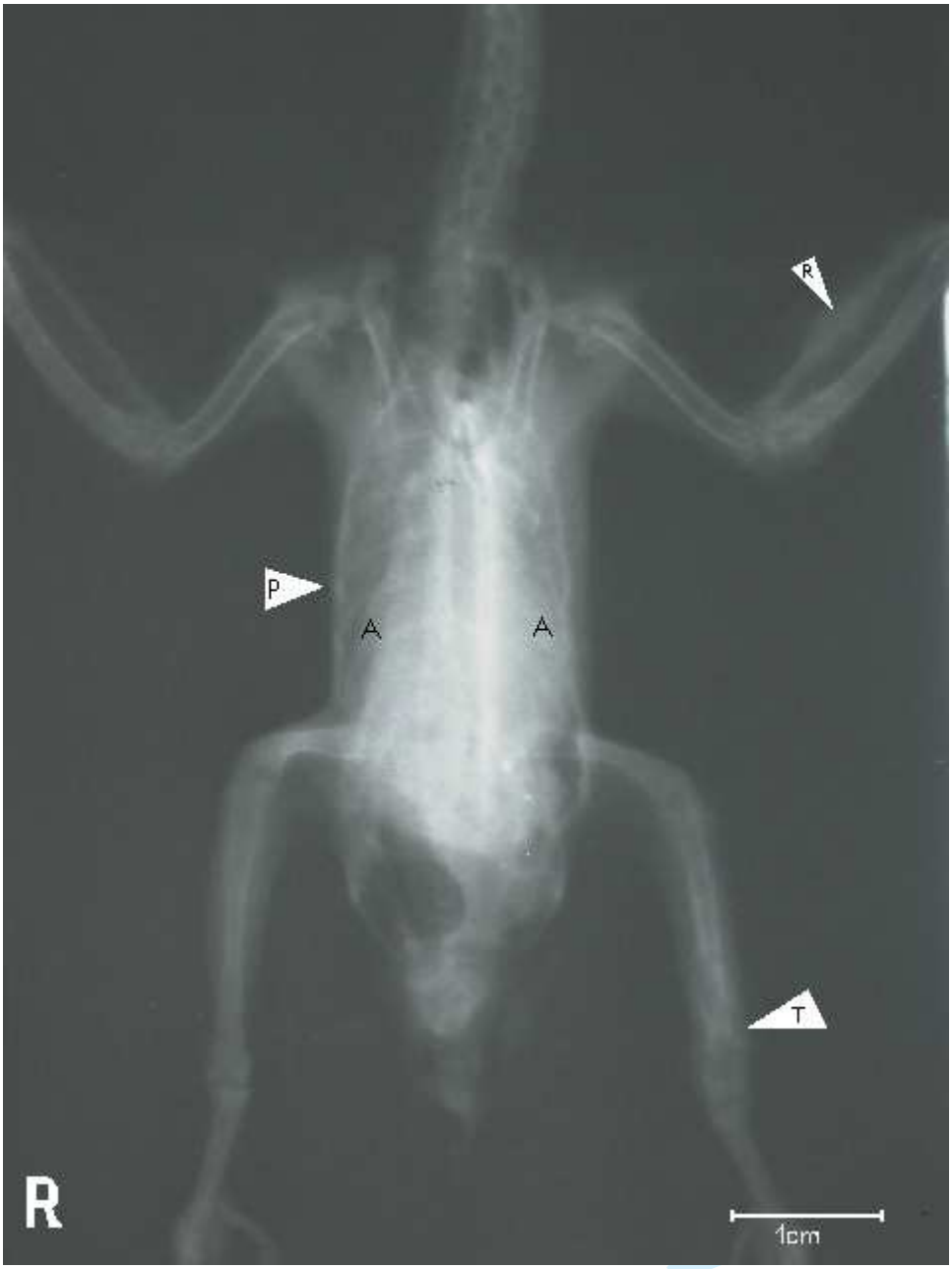

Fig 1. Ventrodorsal radiograph of budgerigar infected with *M. bovis*. Radiograph indicate radial (R) and tibiotarsal (T) left bones' lesions and marked atrophy of pectoral muscles (P) as well opacity of thoracic air sacs (A).

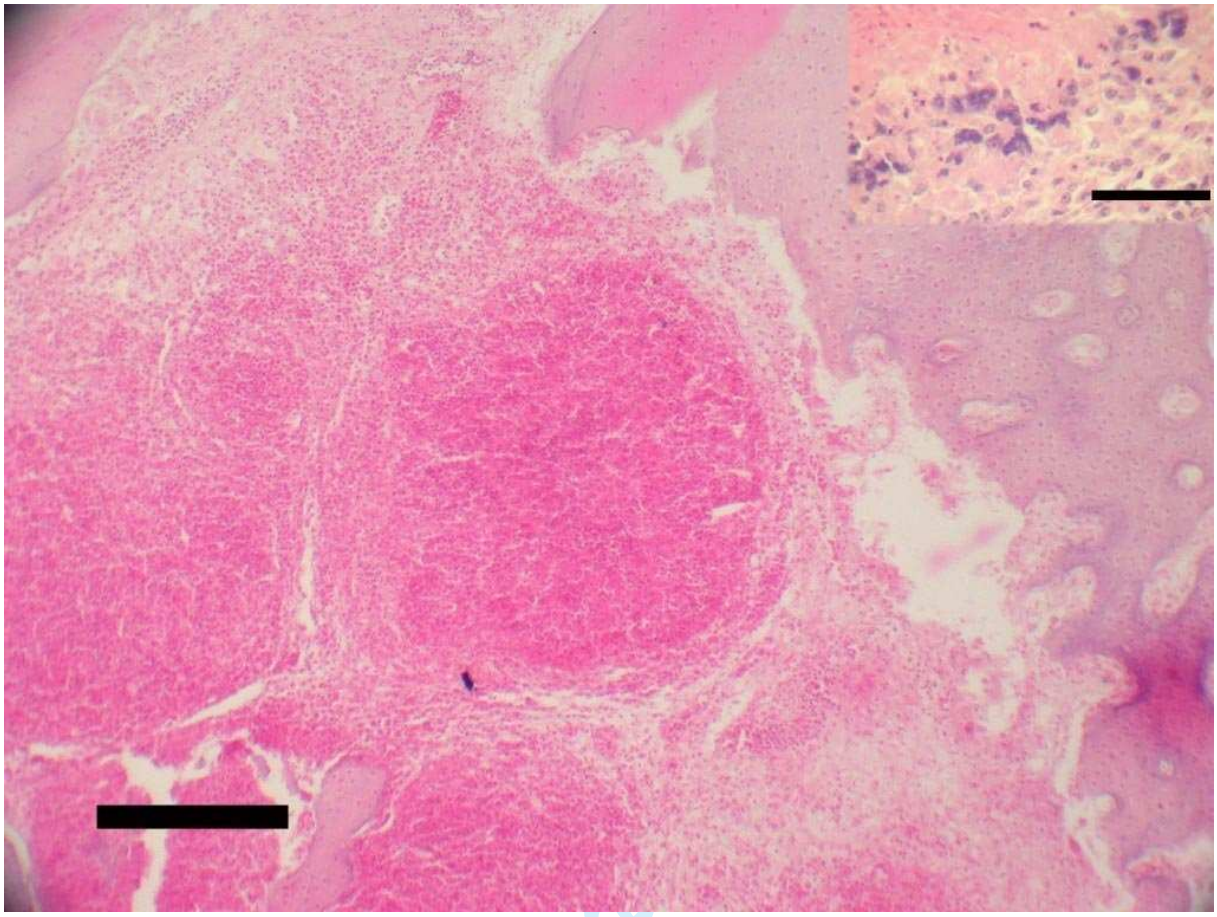

Fig. 2 Radial bone tuberculosis due to *Mycobacterium bovis* infection- nonencapsulated, granulomatous inflammation. H&E. Bar 400um. Inset: Multinucleated giant cells. H&E. Bar 100um.