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ORIGINAL PAPER

Drug Susceptibility of Non-tuberculous Strains of *Mycobacterium* Isolated from Birds from Poland

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Abstract

Mycobacterioses are a constant problem in backyard poultry, as well as pet birds. To date, no evidence of direct transmission of atypical bacilli between humans has been demonstrated, but it cannot be ruled out that sick animals can be a source of infection for people in their environment. The aim of the study was to identify mycobacteria isolated from birds with diagnosed mycobacteriosis and to determine the susceptibility of mycobacterial isolates from these animals to antituberculous drugs most commonly used in the treatment of mycobacterial infections in humans. For drug susceptibility tests, drugs such as isoniazid, rifampicin, streptomycin, ethambutol, ofloxacin, capreomycin, cycloserine and ethionamide were used. A high degree of drug resistance was demonstrated, particularly in *Mycobacterium avium*. Isolates of *Mycobacterium xenopi* showed a relatively good susceptibility to the drugs tested. The drug resistance of *Mycobacterium genavense* has not been determined, but this mycobacterium was identified in ten cases, which is the second most frequent occurrence in the cases studied.

Key words: avian mycobacteriosis, mycobacteriosis, *Mycobacterium avium*, *Mycobacterium xenopi*, *Mycobacterium genavense,* drug susceptibility tests

Introduction

The genus *Mycobacterium* consists of more than 180 species with validly published names (Parte 2014). Most of them are saprophytic species widely distributed in the environment, some of which are pathogenic to humans and animals, including birds (Tan et al. 2016). Tuberculosis caused by the *Mycobacterium tuberculosis* complex has been found in birds that had contact with humans. Although in most of these cases the source of infection was human, domestic birds not only can be infection vectors, but also can show symptoms typical of the open form of tuberculosis, which results in active mycobacteria release to the environment, sometimes for long time periods (Washko et al. 1998; Montali et al. 2001; Steinmetz et al. 2006; Peters et al. 2007; Ledwoń et al. 2008; Lanteri et al. 2011). In birds, mycobacteriosis caused by non-tuberculous mycobacteria (NTM) is definitely a more common problem than tuberculosis. Clinical manifestations of mycobacteriosis in birds include emaciation, depression and diarrhea along with a marked atrophy of the breast muscle. Tubercular nodules can be seen in the liver, spleen, intestine and bone marrow. Granulomatous lesions without calcification are a prominent feature. The disease is rare in the well-organized poultry sector due to improved farm practices, but occurs in backyard poultry, zoos and aviaries (Portaels et al. 1996; Shitaye et al. 2008; Dhama et al. 2011; Pfeiffer et al. 2017). Mycobacterioses also pose a constantly and increasingly growing threat to human health, especially in people with weakened immunity (Schitaye et al. 2008; Slany et al. 2016; Titmarsh 2017). The mycobacteria most frequently isolated from birds with mycobacteriosis are *Mycobacterium avium* and *Mycobacterium genavense*. These bacteria are also a frequent cause of disease in people infected with Human Immunodeficiency Virus (HIV) (Ristola et al. 1999) or those receiving immunosuppressive treatment after transplantation or other systemic diseases (Tortoli 2006; Lastours de et al. 2008; Tan et al. 2016). A study

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conducted by Garcia-Marcos et al. (2017) showed a correlation between the occurrence of mycobacteriosis in children and their exposure to chickens. But NTM can also cause disease in adult immunocompetent patients (Piersimoni and Scarparo 2006; Santos et al. 2014). Because backyard poultry, pet birds and synanthropic animals live in the immediate vicinity of humans, it is important to study strains with potential pathogenicity for humans, as well as to assess the risk posed by their antibiotic resistance.

Experimental

Materials and Methods

The 46 samples tested (Table I) were collected over ten years from 37 dead and nine live birds originating mainly from Warsaw (Poland) and nearby areas. In the necropsied birds, except for one case, when the cause of death was a mechanical injury, advanced mycobacteriosis was diagnosed as the main cause of death. From the necropsied animals, liver samples were collected for microbiological and molecular examination. Samples from living birds were taken from areas affected by lesions associated with the disease. In three peafowl with symptoms of dyspnoea, tracheal swabs were collected. In two falcons, samples from abscesses localized within the subcutaneous tissue of the legs were collected. Faecal samples were taken from canaries and finches. These birds came from breeding sites in which cases of canary death due to mycobacteriosis had been reported earlier. Microscopic examinations of individually collected faeces from these aviaries allowed identification of carriers. Moreover, the presence of leukocytosis with neutrophilia and monocytosis confirmed active infection in those birds.

Identification of mycobacteria. All samples were stained with TB Ziehl-Neelsen KIT (QCA, Spain). Decontamination of clinical and dissected material was carried out using Sputofluol® (Merck, Germany), in accordance with the manufacturer's instruction. An initial culture of samples was carried out for up to 10 weeks at 37°C on the Löwenstein-Jansen PACT medium (Becton Dickinson, USA) and then up to six weeks on Bactec MGIT 960 (Becton Dickinson, USA).

Table I
Microorganisms present in the materials taken from birds with recognized mycobacteriosis.

Avian species with diagnosed mycobacteriosis	No. of birds/AFB + samples	No. of cultured samples	Mycobacterium species (No. of isolates)
Ornamental chickens (*Gallus gallus*)	8/8	8	*Mycobacterium avium* (8)
Indian peafowl (*Pavo cristatus*)	5/3	5	*Mycobacterium avium* (5)
Pheasants (*Phasianus* spp.)	5/5	4 + 1*	*Mycobacterium avium* (5)
Turkey (*Melleagris gallopavo*)	1/1	1	*Mycobacterium avium* (1)
Red-fronted parakeet (*Cyanoramphus novaezelandiae*)	3/3	2 1	*Mycobacterium avium* (2) *Mycobacterium xenopi* (1)*
Barred parakeet (*Bolborhynchus lineola*)	1/1	1*	*Mycobacterium genavense* (1)
Fischer's lovebird *(Agapornis fischeri*)	1/1	1	*Mycobacterium xenopi* (1)
Budgerigar (*Melopsittacus undulatus*)	1/1	1*	*Mycobacterium genavense* (1)
Cockatiel (*Nymphicus hollandicus*)	1/1	0	*Mycobacterium genavense* (1)
Atlantic canary (*Serinus canaria*)	5/5	2* 1**	*Mycobacterium genavense* (5) *Mycobacterium xenopi* (1)
Goldfinch (*Carduelis carduelis*)	1/1	0	*Mycobacterim spp.* (1)
Bengalese finch (*Lonchura striata domestica*)	2/2	1* 1	*Mycobacterium genavense* (1) *Mycobacterium* spp. (1)
Common buzzard (*Buteo buteo*)	2/2	1 1*	*Mycobacterium avium* (1) *Mycobacterium genavense* (1)
Peregrine falcon (*Falco peregrinus*)	2/2	2	*Mycobacterium avium* (2)
Rock pigeon (*Columba livia*)	3/3***	3	*Mycobacterium avium* (3)
Common wood pigeon (*Columba palumbus*)	2/2	2*	*Mycobacterium avium* (2)
Rook (*Corvus frugilegus*)	2/2	2	*Mycobacterium avium* (2)
Total: 46	45/43	40	(46)**

* Lack of growth on media for drug resistance testing
** In one necropsied canary coinfection with *M. genavense* and *M. xenopi* were diagnosed
*** In one case of mycobacteriosis in a pigeon flock, *M. avium* was isolated from 19 individuals, but as all isolates were the same strain; therefore, they are considered as one case on the list

Positive cultures were investigated using the BD MGIT TBc Identification Test (TBc ID, Becton Dickinson, Sparks, MD), which is an immunochromatographic assay for a rapid discrimination between the *M. tuberculosis* complex and NTM. The TBc ID test is based on the detection of the antigen MPT64, one of the predominant proteins secreted by the *M. tuberculosis* complex strains during culture (Martin et al. 2011). The NTM samples were then tested using the GenoType Mycobacterium CM VER 2.0 test, a molecular genetic assay for the identification of clinically relevant mycobacterial species from mycobacterium contaminated material (Hain Lifescience GmbH, Germany).

Samples that did not grow on the Löwenstein-Jansen medium (Becton Dickinson, USA) were cultured onto the Middlebrook 7H9 (Becton Dickinson, USA) liquid medium supplemented with 2 mg/l Mycobactin J (Allied Monitor, Fayette, USA) (Coyle et al. 1992) for 20 weeks. Identification of samples from which no culture was obtained or were grown on the media with Mycobactin J was performed using SYBR® Green real-time PCR specific for *M. genavense*. DNA isolation from the liver tissue or bacterial cultures was performed using the GeneMATRIX Tissue & Bacterial DNA Purification Kit (Eurx®, Poland), while DNA isolation from faeces was made using the GeneMATRIX Stool DNA Purification Kit (Eurx®, Poland). The chosen forward primer MG 25-s(GAATCCGCTGCTGCTCTG) was located at nucleotides 378 to 395 of a *M. genavense* hypothetical 21 kDa protein gene (Chevrier, et al. 1999), while the backward primer MG25-as (TCAATGTAGTCCTGTCCGAAC) corresponded to nucleotides 313 to 291. Real-time PCR amplification was carried out in a total volume of 25 µl using SG qPCR Master Mix (Eurx®, Poland) according to the methodology described by the authors earlier (Ledwoń et al. 2009).

Drug susceptibility testing. Drug susceptibility tests were conducted using 34 mycobacterial isolates, including 31 *M. avium* subsp. *avium* and three *Mycobacterium xenopi*. Determination of susceptibility to drugs was performed by using the proportion method on the Löwenstein-Jensen and Middlebrook 7H9 media (Canetti et al. 1969; Zwolska et al. 1999; Augustynowicz-Kopeć et al. 2003). The susceptibility to isoniazid, rifampicin, streptomycin, ethambutol, ofloxacin, capreomycin, cycloserine and ethionamide was tested on the Löwenstein-Jansen medium. The susceptibility to clofazimine, rifabutin and sulfamethoxazole + trimethoprim was tested on the Middlebrook 7H9 medium. The concentrations of the antibiotics tested were as follows: streptomycin – 4.0, 8.0 µg/ml; isoniazid – 0.2, 0.4 µg/ml; rifampicin – 40.0, 80.0 µg/ml; ethambutol 2.0, 4.0 µg/ml; ofloxacin – 2.5, 5.0 µg/ml; capreomycin – 40.0, 80.0 µg/ml; cycloserine – 40.0, 80.0 µg/ml; ethionamide – 40.0, 80.0 µg/ml; clofazimine – 1.0, 2.0, 4.0 µg/ml; rifabutin – 0.5, 1.0, 2.0 µg/ml, and sulfamethoxazole + trimethoprim – 8.0, 16.0, and 32.0 µg/ml. Resistance was defined by the growth of ≥ 1% of a bacillary inoculum on the drug-containing medium compared to a drug-free control.

Results

Mycobacteriosis was found in 46 birds belonging to 17 bird species (Table I). Gallinaceous birds were diagnosed with only *M. avium* infections, while pet birds were also diagnosed with *M. genavense* and *M. xenopi* infections. One dead canary was found to be infected with both *M. genavense* and *M. xenopi*. In wild birds, *M. avium* predominated, although *M. genavense* was found in one buzzard. Positive Ziehl-Neelsen staining (AFB +) was observed in 43 cases analysed. The growth of bacterial colonies on the Löwenstein-Jensen and Bactec MGIT 960 media was noticed for 34 samples collected. 31 isolates of *M. avium* and three isolates of *M. xenopi* were cultured. Drug susceptibility was tested for 28 *M. avium* and three *M. xenopi* strains (Table II and Table III). *M. avium* strains from common pheasant (*Phasianus colchicus*) and wood pigeons (*Columba palumbus*) did not demonstrate any growth on the media used for the assessment of drug resistance. Ten samples were identified as *M. genavense*, five of which were cultured on the Middlebrook 7H9 medium with

Table II
Drug resistance of 28 strains of *M. avium* isolated from birds.

Drug*	SM	INH	RFP	EMB	CPM	OFLOX	CS	ETH	SXT	CFM	RIF
Susceptible	8	0	0	0	20	0	25	3	8	27	10
Moderately susceptible	0	0	0	0	0	0	0	0	10	0	0
Moderately resistant	0	0	0	0	0	0	0	0	6	0	8
Resistant	4	28	28	28	5	28	2	0	4	1	10
No data**	16	0	0	0	3	0	1	25	0	0	0

* SM – streptomycin, RFP – rifampicin, INH – isoniazid, EMB – ethambutol, CPM – capreomycin, OFLOX – ofloxacin, CS – cycloserine, ETH – ethionamide, SXT – sulfamethoxazole + trimethoprim, CFM – clofazimine, RIF – rifabutin

** Number of strains which did not demonstrate any growth on the media used for the assessment of drug resistance

Table III
Drug susceptibility of three *M. xenopi* strains isolated from pet birds.

Drug*	SM	INH	RFP	EMB	CPM	OFLOX	CS	ETH	SXT	CFM	RIF
Susceptible	1	0	2	2	3	3	2	3	3	3	3
Resistant	0	3	1	1	0	0	0	0	0	0	0
No data**	2	0	0	0	0	0	1	0	0	0	0

* SM – streptomycin, RFP – rifampicin, INH – isoniazid, EMB – ethambutol, CPM – capreomycin, OFLOX – ofloxacin, CS – cycloserine, ETH – ethionamide, SXT – sulfamethoxazole + trimethoprim, CFM – clofazimine, RIF – rifabutin
** Number of strains which did not demonstrate any growth on the media used for the assessment of drug susceptibility

Mycobactin J. Two samples were identified as *Mycobacterium* spp. These mycobacteria were also incapable of growing on the medium for susceptibility testing.

Discussion

M. avium and *M. genavense* have been the main causes of mycobacterioses described to date in birds (Tell et al. 2001; Shivaprasad and Palmieri 2012; Pfeffer et al. 2017). Our study confirmed the results published by other authors. Mycobacterioses caused by *M. xenopi* are very rarely diagnosed in birds (St-Jean et al. 2018). In our investigations, even though only three isolates were obtained, it should not be underestimated. *M. xenopi* has recently been isolated from water (Slozarek et al. 1993) which is also a source of infection for humans and animals. Birds that were diagnosed with this bacterial infection, however, came from various breeders, so it could not be concluded that they infected themselves with the same water intake.

M. genavense is very difficult to be cultured on the media, and its diagnostics are mainly based on molecular methods (Hoop et al. 1993; Portaels et al. 1996; Ledwoń et al. 2009; Palmieri et al. 2013). Therefore, the drug susceptibility tests used in this study may not be useful for testing of these mycobacteria. Strains of *M. avium* (Table II) demonstrated 100% resistance to isoniazid, rifampicin and ethambutol, while strains tested on ethionamide showed a good susceptibility to that antibiotic. These results differ from those described in a study by Parvandar et al. (2016) in which all *M. avium* strains isolated from pigeons for drug susceptibility tests were shown to be resistant to streptomycin, kanamycin, ethionamide and thiophene carboxylic acid hydrazide. Moreover, single isolates were susceptible to isoniazid, rifampicin and ethambutol (Parvandar et al. 2016). In the cases described by Stepień-Pyśniak et al. (2016), *M. avium* strains isolated from birds demonstrated resistance to isoniazid, rifampicin, ethambutol, ethionamide, capreomycin and ofloxacin, and were susceptible to streptomycin and cycloserine. *M. xenopi* isolated from birds (Table III) presented resistance similar to that of isolates originating from humans (van Ingen et al. 2010). These isolates were resistant to isoniazid, slightly less resistant to rifampicin and ethambutol, and fully susceptible to other antibiotics. All three species of mycobacteria identified from the cases of mycobacteriosis in birds are able to cause mycobacteriosis in humans, particularly in immunocompromised hosts (Coyle et al. 1992; Ristola et al. 1999; Bluth et al. 2009; Shah et al. 2016; Adzic-Vukicevic et al. 2018). Therefore, mycobacterioses in birds living in a direct proximity to humans should be taken seriously, since they can constitute a potential reservoir of infection with mycobacteria resistant to multiple antimycobacterial drugs.

Conflict of interest

Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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