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OCCURRENCE AND RELEVANCE OF *MYCOPLASMA STURNI* IN FREE-RANGING CORVIDS IN GERMANY

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ABSTRACT: Several *Mycoplasma* spp. are well-known pathogens in poultry. In birds of prey, White Storks (*Ciconia ciconia*), and some waterfowl (Anatidae, Pelecanidae) species, mycoplasmas occur commonly and seem to be apathogenic or commensal and most likely belong to the physiologic microbial flora of the respiratory tract. In other bird species, such as Common Nightingales (*Luscinia megarhynchos*) and tits (Paridae), *Mycoplasma* spp. are absent in healthy birds. In corvids, the prevalence and role of *Mycoplasma* spp. in disease remains unclear. In previous studies, *Mycoplasma sturni* was detected in diseased corvids; however, those studies included only a limited sample size or preselected individuals. We collected tracheal swabs of 97 free-ranging Corvidae, including 68 randomly selected individuals from hunting bags and 29 birds that had been admitted to a veterinary clinic. Tracheal swabs were examined for *Mycoplasma* spp. using culture and genus-specific PCR. If *Mycoplasma* spp. were detected, the species were identified by sequencing the 16S ribosomal (r) RNA gene and 16–23S rRNA intergenic transcribed spacer region. Five of 68 (7%) of the hunted birds and nine of 29 (31%) of the birds admitted to the veterinary clinic were PCR positive. In 13 of 14 PCR-positive samples, mycoplasmas were cultured and *M. sturni* was the only mycoplasmal species identified. None of the positive corvids from the hunting bags had clinical signs, whereas five of nine birds admitted to the veterinary clinic showed apathy, lameness, injuries, or fractures, which may not be associated with mycoplasmal infections. These data support the notion that *M. sturni* is the *Mycoplasma* sp. most frequently found in corvids, though its prevalence and ability to cause disease may involve interaction with other aspects of bird health.

Key words: Apathogenic, commensal, Corvidae, *Corvus corone*, *Mycoplasma* spp., *Mycoplasma sturni*, song voice.

INTRODUCTION

Twenty-six avian *Mycoplasma* species have been described (Bradbury 2002; Suárez-Pérez et al. 2012). *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae*, *Mycoplasma meleagridis*, and *Mycoplasma iowae* are among the most relevant pathogens in domestic poultry. Combined, they cause high economic losses. However, they also affect nonpoultry species. Nolan et al. (1998) estimated that >100 million House Finches (*Carpodacus mexicanus*) died in an outbreak of disease caused by MG in the US because of increased predation as a result of severe *Mycoplasma* conjunctivitis and loss of eyesight. Besides House Finches, several other passerine species such as Evening Grosbeaks (*Coccothraustes vespertinus*) and Pine Grosbeaks (*Coccothraustes enucleator*) have been affected by MG infections (Ley et al. 1996; Mikaelian et al. 2001). Overall, little is known about the occurrence and pathogenicity of *Mycoplasma* spp. in free-ranging birds. The detection of *Mycoplasma* spp. in raptors with respiratory signs has been interpreted as a clinically relevant mycoplasmosis (Morishita et al. 1997). More recently, some *Mycoplasma* spp. have been detected in healthy captive and free-ranging birds of prey at a prevalence of almost 100% (Lierz et al. 2008). Similarly, 99% of healthy White Stork nestlings (*Ciconia ciconia*) were infected with *Mycoplasma* spp. (Hagen et al. 2004). The authors concluded that certain *Mycoplasma* species are commensal or apathogenic in certain free-ranging bird species. However, in other species such as Common Nightingales (*Luscinia megarhynchos*), Great Tits (*Parus major*), and Blue Tits (*Cyanistes caeruleus*), *Mycoplasma* spp. are absent in the respiratory tract (F.M.P.-R. unpubl. data).

These findings gave rise to a hypothesis that highly vocal species cannot tolerate latent infections of the respiratory tract. This is supported by a study demonstrating *Mycoplasma* spp. in parrots with chronic respiratory signs, while healthy parrots were negative for mycoplasmas in their respiratory microbial flora (Lierz and Hafez 2009). On the other hand, nutritional aspects influencing the regulation of the birds' microbial flora, their immune system, and therefore resistance to infectious diseases have been demonstrated (Klasing 1998). Whether this applies to mycoplasmas is unknown. Although some granivorous and insectivorous species (e.g., Common Nightingales and tits [Paridae]) are free of *Mycoplasma* spp. in their respiratory flora, in some omnivorous and carnivorous bird species *Mycoplasma* spp. are regularly detected. Hence, a second hypothesis proposes that omnivorous and carnivorous birds host mycoplasmas regularly.

We chose corvids to assess the applicability of these two hypotheses for the occurrence of mycoplasmas in birds. Corvids are songbirds, belonging to the order Passeriformes, suborder Passeri. Some species of Corvidae, such as Carrion Crows (*Corvus corone*), are especially interesting species for this study because they are songbirds and are omnivorous. As some corvids live near domestic poultry, they are potential vectors for several pathogens (Anderson et al. 1999; Benskin et al. 2009).

In corvids, *Mycoplasma gallopavonis*, *Mycoplasma gallinarum*, *Mycoplasma columborale,* and *Mycoplasma sturni* were isolated from birds with respiratory disease (Strugnell et al. 2011). In a study on free-ranging Indian Crows (*Corvus splendens*) in Malaysia, 10 of 39 tracheal swabs were positive for *Mycoplasma* spp. However, the species were not identified (Ganapathy et al. 2007). Other investigators studied preselected birds from rehabilitation centers with respiratory disease or prior contact with diseased individuals. In these studies, 81–100% of the corvids were positive for *M. sturni* (Ley et al. 1998; Wellehan et al. 2001). However, the prevalence of *Mycoplasma* spp. in healthy, free-ranging corvids in Europe has not been investigated.

Mycoplasma sturni was isolated and described in 1996 in a European Starling (*Sturnus vulgaris*) with bilateral conjunctivitis (Forsyth et al. 1996; Frasca et al. 1997). Since then, *M. sturni* has been isolated from Blue Jays (*Cyanocitta cristata*), Northern Mockingbirds (*Mimus polyglottos*) (Ley et al. 1998), American Crows (*Corvus brachyrhynchos*), and American Robins (*Turdus migratorius*) (Wellehan et al. 2001). All these birds showed clinical signs comparable with the European Starling (Forsyth et al. 1996) or showed no clinical signs (e.g., conjunctivitis or respiratory symptoms), but had prior contact with affected birds. In many cases, the affected birds developed clinical signs of conjunctivitis during their stay in a rehabilitation center. In a sample of carcasses of free-ranging birds in Scotland, 18 of 41 were positive for *M. sturni*. All of these birds, including Eurasian Blackbirds (*Turdus merula*), Rooks (*Corvus frugilegus*), Carrion Crows, Eurasian Magpies (*Pica pica*), and European Starlings, were immature and suffering from a range of infectious and noninfectious diseases. The significance of the detection of *M. sturni* in these birds with numerous diseases remained unclear (Pennycott et al. 2005). Therefore, data indicating prevalence and pathogenicity of mycoplasmas in free-ranging corvids remain incomplete.

We measured the occurrence of *Mycoplasma* species in randomly selected, free-ranging birds of the family Corvidae in Germany by sampling gun-shot birds without clinical signs of mycoplasmosis. We also evaluated the role of *Mycoplasma* spp. as a possible pathogen in corvids. Birds from hunting bags were randomly selected from the free-ranging population and thus were useful for prevalence assessment. For comparison, birds found injured or debilitated and presented to a veterinary clinic were included in the study. These birds were treated as a separate group, as they were preselected from the free-ranging population by their disease.

MATERIALS AND METHODS

Sampling

We included 97 corvids (91 Carrion Crows, four Eurasian Magpies, one Rook, one Eurasian Jay [*Garrulus glandarius*]) in the study performed between August 2012 and June 2014. Of the 97 birds, 68 were from hunting bags of the state Hesse, Germany. These birds were exclusively adult Carrion Crows and randomly singled out of the free-ranging population by hunting. The birds showed no clinical signs or altered behavior. Immediately after death, the birds were examined for potential gross lesions and tracheal swabs were collected.

The other 29 samples were collected from corvids admitted to the Clinic for Birds, Reptiles, Amphibians, and Fish of the Justus Liebig University of Giessen, Germany by private citizens after being found injured or debilitated. This included 23 Carrion Crows, four Eurasian Magpies, one Rook, and one Eurasian Jay. All presented birds were clinically examined. Eighteen birds were juvenile and presented with lameness (n=4), apathy (n=2), injuries (n=2), fractures (n=1), or were healthy fledglings (n=9). Eleven birds were adult and presented with fractures (n=5), apathy (n=2), or injuries (n=1), whereas three adult birds had no clinical signs. Sampling of all birds was performed no later than 48 h after admission and only if clinical signs for conjunctivitis or respiratory disease were absent. Birds with a history of antibiotic treatment were excluded.

Mycoplasma culture

Tracheal swabs were cultured using SP4 liquid and agar media as described by Bradbury (1998). The swab of each bird was immersed in the SP4 broth before it was removed and stored at –80 C until further investigation by PCR. The broth was diluted (10-fold dilution up to 10^{-2}) and an aliquot of 50 µL each was transferred onto agar media. Liquid and solid media were incubated at 37 C with 5% CO_2 in a humidified environment for up to 10 d. Recurrent subculturing of liquid broth on agar plates was performed routinely after 5 d. Daily, the broth was examined for color change and agar plates for colony growth. In case of mycoplasmal growth, several single-colony subcultures were performed. This process was repeated twice to ensure pure cultures. Each third single-colony subculture was stored at –80 C until further investigation by molecular biological methods.

PCR and sequencing

For DNA extraction, swabs were soaked and rubbed in 350 µL of phosphate-buffered saline (136.9 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.2). We used 100 µL of the liquid for DNA extraction using the DNeasy blood & tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For DNA extraction from single-colony subcultures, the fluid medium from culturing (2 mL) was centrifuged at 4,000 × G for 45 min. The remaining pellet was incubated with 180 µL of lysis buffer (ATL buffer, Qiagen) and 20 µL of proteinase K (Qiagen) for 2 h at 56 C. DNA was extracted as described earlier. The concentration of the DNA was measured via spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, Delaware, USA) and, if necessary, diluted to 10 ng/µL. Swabs and single-colony subcultures were screened via *Mycoplasma*-genus-specific PCR (target: 16S ribosomal [r] RNA gene sequence) for DNA of *Mycoplasma* spp. as described by van Kuppeveld et al. (1992) modified by Lierz et al. (2007). From all single-colony subcultures, an additional PCR was performed, targeting the 16–23S rRNA intergenic transcribed spacer region (ISR) sequence described by Ramírez et al. (2008). The PCR products were sequenced by LGC Genomics, Berlin, Germany.

Phylogenetic analysis

Sequences of PCR products were aligned with the 16S rRNA gene and 16–23S rRNA ISR sequence of *Mycoplasma* spp. using basic local alignment search tool (Altschul et al. 1990). The 16S rRNA gene sequences of the isolates, two other closely related *Mycoplasma* spp., and *M. mycoides* subsp. *mycoides* SC $PG1^T$ as outgroup were included in alignments constructed by Clustal V algorithm (Higgins et al. 1996). A bootstrapped maximum parsimony tree was generated using default search parameters and 1,000 bootstrap resamplings (MegAlign, Lasergene 9.0, GATC Biotech, Konstanz, Germany). In one case, no isolate was available, so the PCR product sequence of the *Mycoplasma*-genus-specific PCR (target: 16S rRNA gene sequence) was included.

Statistics

We compared prevalences of *Mycoplasma* spp. between free-ranging and clinically admitted birds using the chi-square test (Sachs 1992).

RESULTS

Mycoplasma spp. were detected by *Mycoplasma*-genus-specific PCR (target: 16S rRNA gene) in 14 of 97 (14%) of the sampled corvids (Table 1). In 13 of 14 samples, *Mycoplasma*

TABLE 1. *Mycoplasma sturni*-positive samples from Carrion Crows (*Corvus corone*) in Hesse, Germany, August 2012–June 2014. All samples were PCR positive for *Mycoplasma* 16–23S ribosomal RNA intergenic transcribed spacer region and all sequences matched *M. sturni*.

Sample no.	Age	Origin	Diagnosis	Culture
1974-2/2012	Adult	Luetzellinden	Hunting bag: no clinical signs for disease	Positive
1974-10/2012	Adult	Luetzellinden	Hunting bag: no clinical signs for disease	Positive
1974-11/2012	Adult	Luetzellinden	Hunting bag: no clinical signs for disease	Negative
1795-4/2013	Adult	Luetzellinden	Hunting bag: mild ectoparasite infestation	Positive
1795-5/2013	Adult	Luetzellinden	Hunting bag: mild ectoparasite infestation	Positive
1661/2012	Adult	Marburg	Clinic: wing fracture	Positive
1662/2012	Adult	Lich-Birklar	Clinic: wing and leg fracture	Positive
1109/2013	Juvenile	Allendorf	Clinic: lameness of both legs, mild ectoparasite infestation	Positive
229/2013	Juvenile	Giessen	Clinic: no clinical signs for disease	Positive
2181/2013	Juvenile	Giessen	Clinic: multiple skin lesions, died 12 h after admission	Positive
95/2014	Juvenile	Giessen	Clinic: no clinical signs for disease, died 12 h after admission	Positive
98/2014	Juvenile	Giessen	Clinic: multiple white feathers, multiple fractures, and bone deformations	Positive
99/2014	Juvenile	Giessen	Clinic: no clinical signs for disease	Positive
127/2014	Juvenile	Giessen	Clinic: no clinical signs for disease	Positive

spp. were cultured on SP4 agar media. The isolates grew rapidly on agar plates and acidified the liquid medium, indicating glucose metabolism. The sequence of the 16S rRNA gene of each single-colony subculture was 99% to 100% identical to *M. sturni*. Closely related *Mycoplasma* species were *M. columborale*, with up to 99% sequence similarity, and *Mycoplasma capricolum*, with 96% sequence similarity. From one sample, no isolate was obtained, although the *Mycoplasma*-genus-specific PCR (target: 16S rRNA gene) was positive and the PCR product was 99% identical to *M. sturni*. The sequenced ISR gene of these samples was 99% identical to the ISR of *M. sturni*. A closely related *Mycoplasma* species was *M. columborale*, with a sequence similarity of up to 90%. Figure 1 shows the phylogenetic tree of the 16S rRNA sequences of the 13 isolates and one PCR product as well as the closest related *Mycoplasma* spp. (*M. columborale* and *M. capricolum*). *Mycoplasma mycoides* subsp. *mycoides* SC PG1^T was used as an outgroup. Bootstrap confidence values >70% are shown.

Of the hunted birds, five of 68 (7%) were positive for *M. sturni*, whereas nine of 29 (31%) corvids admitted to the veterinary clinic were positive. Seven of these birds were juvenile Carrion Crows admitted to the clinic for lameness (n=1), injuries (n=1), apathy (n=1), or were healthy (n=4). Two positive birds were adult Carrion Crows showing fractures. The positive hunted birds were all adults. The occurrence of *M. sturni* in free-ranging birds randomly selected by hunting was significantly lower ($P<0.001$) than in the group of clinical cases.

DISCUSSION

We detected mycoplasmas in 14% of free-ranging, juvenile, and adult corvids without clinical signs of respiratory disease in Hesse, Germany. These samples were classified as *M. sturni* according to the results of phylogenetic analyses. As shown in the phylogenetic tree based on the 16S rRNA (Fig. 1), all isolates, and the one positive-tested sample in the *Mycoplasma*-genus-specific PCR, show the highest similarity to the 16S rRNA gene

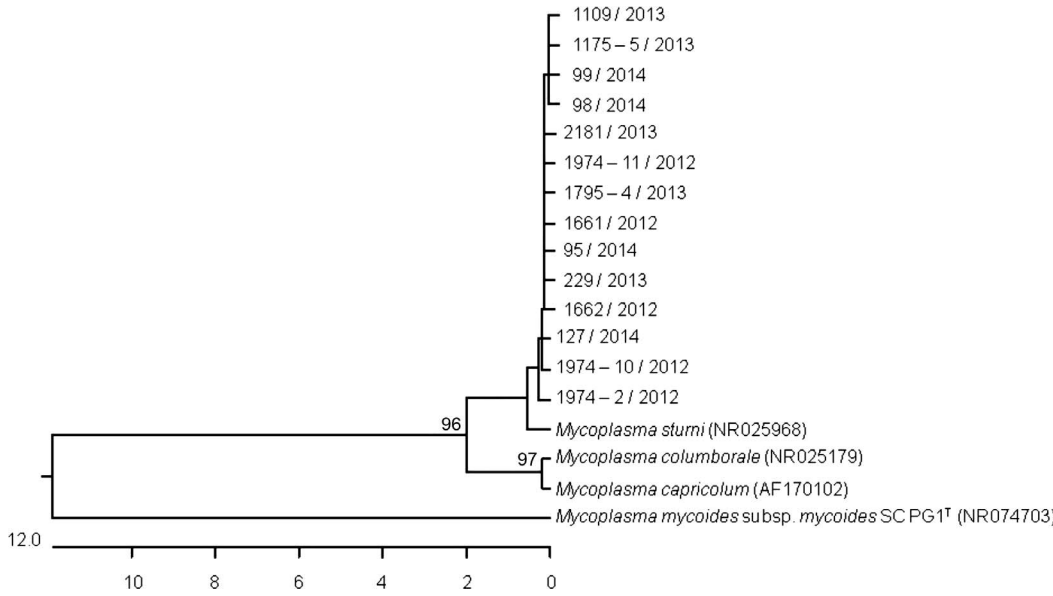

FIGURE 1. Phylogenetic tree of 16S ribosomal RNA sequences of 13 isolates from mycoplasmal culture of tracheal swabs from Carrion Crows (*Corvus corone*) collected in Hesse, Germany. Also included in the tree are one PCR product of a PCR-positive but culture-negative sample, and sequences from closely related *Mycoplasma* species. The sequence of *Mycoplasma mycoides* subsp. *mycoides* SC PG1^T was used as an outgroup. Numbers at nodes are bootstrap confidence values. Only values >70% are shown. Numbers on scale are nucleotide substitutions per 100 residues.

sequence of *M. sturni*. Additionally, the 16–23S rRNA gene sequences of these samples show a similarity of up to 100% to the ISR of *M. sturni*, respectively. However, the isolates from different birds show a high similarity.

However, the occurrence of *M. sturni* in the hunted birds was significantly lower than the birds admitted to the veterinary clinic. The discrepancy between admitted and hunted birds, both of which originated from a free-ranging population, clearly shows that the prevalence of *Mycoplasma* spp. in a bird species must be evaluated on randomly chosen individuals from a free-ranging population. The detection of *Mycoplasma* spp. in diseased birds or birds with prior contact to diseased individuals may not provide the real prevalence of a pathogen within a free-ranging population.

Mycoplasma sturni was previously detected in diseased corvids (Pennycott et al. 2005; Strugnell et al. 2011) and in healthy American Crows that had been in contact with infected birds. Although a pathogenic role for *M. sturni* is suspected (Wellehan et al. 2001), it remains unclear. Our findings of *M. sturni* in 7% of free-ranging, healthy individuals randomly taken from the free-ranging population leads to the conclusion that *M. sturni* is not an obligatory pathogen in Carrion Crows. These results coincide with results for other avian species. An *M. sturni* isolate from naturally infected California House Finches with severe conjunctivitis failed to cause disease in experimentally infected finches (Ley et al. 2010). Because most of the positive birds admitted to the veterinary clinic were juvenile, the influence of host age on susceptibility to *M. sturni* infection should be considered. Obligatory pathogenicity in crows can be excluded. Nevertheless, well-known pathogens such as MG and *M. synoviae* may not cause overt disease in chickens if environmental factors, such as other infections and overcrowding, are absent (Bradbury 2005).

We detected *M. sturni* in 31% of the birds presented as clinical cases. It is possible that *M. sturni* infection leads to other infectious

diseases by impairing the bird's immune response, as has been demonstrated for other *Mycoplasma* spp. (Bradbury 2005). Also, the probability of predation and other trauma and injury may be greater in birds infected with *Mycoplasma* (Wobeser 2006).

Birds of various species had clinical signs of a mycoplasmal infection during or after a stay at a rehabilitation center (Ley et al. 1998, 2010, 2012; Wellehan et al. 2001; Pennycott et al. 2005). The birds in this study were sampled $\leq$2 d after admittance; thus horizontal spread of *Mycoplasma* spp. within the clinic ward is unlikely. However, the higher percentage of infection with *M. sturni* in juvenile and emaciated or injured adult birds may be due to an immunocompromised status of the individual, promoting infection with *M. sturni*. A decreased immune response of wild birds caused by stressful conditions in human care is likely (Romero 2004).

Like White Storks and raptors, crows are omnivorous, whereas nightingales or tits selectively feed on seeds or insects. As *Mycoplasma* spp. prevalence in omnivores such as storks and raptors can be up to 100% (Hagen et al. 2004; Lierz et al. 2008), the 7% prevalence of *M. sturni* we found in free-ranging corvids is relatively low. Thus, our results do not suggest a diet-related influence on *Mycoplasma* prevalence in avian species.

Because crows phylogenetically belong to the songbirds, our results may support the theory that mycoplasmal infection reduces reproductive success in birds relying on their song for mating. Crows are not as specialized to vocal mating as nightingales or tits (Glutz von Blotzheim 1993). Hence, they might be intermediate between specialized singers and birds that do not rely on song, such as raptors and storks. In relation to prevalence of *Mycoplasma* spp., crows also occupy an intermediate position, supporting the theory that *Mycoplasma* spp. might affect reproductive success in some birds. Further studies are necessary to evaluate a correlation between evolutionary factors and the occurrence of *Mycoplasma* spp. as commensal bacteria in the respiratory tracts of songbirds.

The role of *Mycoplasma* spp. in diseases varies among bird species and we assume that species without commensal or apathogenic *Mycoplasma* spp. may develop clinical symptoms after infection. In bird species with *Mycoplasma* spp. in their commensal bacterial flora, it is essential to differentiate the *Mycoplasma* species to correctly interpret clinical signs shown by the bird. The detection of *Mycoplasma* spp. in clinically diseased birds from species commonly infected with *Mycoplasma* should be interpreted carefully. Detection of *Mycoplasma* should not automatically lead to the diagnosis of a mycoplasmosis.

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