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Occurrence of mycoplasmas in semen samples of birds of prey

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Running title: *Mycoplasma* in raptor semen

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Abstract

Mycoplasmas are well known pathogens in a variety of animals. In poultry it is known that some species can be transmitted by semen and infect the uterus of females. As the prevalence of mycoplasmas in birds of prey is very high and artificial insemination is a commonly used technique for reproduction, the possibility of transmission *Mycoplasma* spp. by contaminated semen in birds of prey was investigated. Isolation of mycoplasmas was possible in five of 32 (15.6%) semen samples of different bird of prey species. Two additional semen samples were positive for mycoplasma DNA using a *Mycoplasma* genus-specific PCR. The isolation of mycoplasmas from a testicular sample indicates the testis as the possible source of contamination. Sequencing of large parts (>90%) of the 16S rRNA gene of the isolated mycoplasmas suggests that all isolates belong to the same species. Alignment of the sequenced products with the 16S rRNA gene of *Mycoplasma* species in GenBank demonstrated a similarity of 97% to *M. verecundum*, but serological testing by immunobinding assay failed to identify it as such. It is recommended that the semen of donor birds of prey is examined for mycoplasmas before its use in artificial insemination.

Introduction

The reproduction of birds of prey in captivity was established in the late 1970s as the bird of prey population decreased significantly worldwide. Today, birds of prey continue to be bred for conservation projects but also reared for falconry. One well-adapted reproduction technique in birds of prey is artificial insemination. It allows the fertilization of human imprinted females (increases the number of eggs/female) as well as cross breeding of different bird of prey species that will not usually mate under natural circumstances (Heidenreich, 1997; Lierz, 2008).

Bird of prey semen donors are still rare because the management of such birds is time consuming. Therefore the semen, especially if obtained from a bird with high genetic or commercial value, is used to inseminate several females. If the semen is contaminated with a pathogen then several females may be infected, compared with only a single female in a natural pairing. Thus artificial insemination increases the risk of infecting the breeding females with pathogens that are shed in the semen and it is known that certain mycoplasmas have this property.

In turkeys, mycoplasma contamination of semen is well recognised. Hatching eggs can be infected by *M. iowae* through uterine contamination after transmission by contaminated semen during artificial insemination (Shah-Majid & Rosendal, 1986; Kempf *et al.*, 1989; Baxter-Jones, 1991). Vertical transmission of *M. meleagridis* also occurs through an infection of the uterus, when female turkeys are infected with contaminated semen (Kumar & Pomeroy, 1969; Matzer & Yamamoto, 1970). The infection of *M. meleagridis* can persist from hatching to sexual maturity leading to contaminated semen of these birds (Yamamoto & Ortmayer, 1967).

Considering the high prevalence of mycoplasmas in birds of prey (Morishita *et al.*, 1997; Lierz *et al.*, 2000; 2002; 2008a,b) and the detection of mycoplasmas in a raptorial egg

(Lierz *et al.*, 2007), it is important to know if mycoplasmas are present in the semen. Such investigations are absent within the available literature.

Materials and Methods

Semen and testis samples. The samples originated from the collection of three different bird of prey breeders (one each in South-, East- and North Germany). The birds were maintained according to standard breeding practices as described by Heidenreich (1997).

Semen samples of 32 imprinted male raptors were collected: 12 Gyr/Saker hybrid falcons (*Falco rusticolus*/*F. cherrug*), eight Gyrfalcons (*F. rusticolus*), five Peregrine falcons (*F. peregrinus*), two Red-naped Shahins (*F. pelegrinoides*), two Golden eagles (*Aquila chryseatos*), one Northern goshawk (*Accipiter gentilis*), one White-tailed Sea-eagle (*Haliaeetus albicilla*) and one Steppe eagle (*Aquila nepalensis*). A second semen sample was obtained from one gyrfalcon a year later (No. 50587). The birds were regularly used as semen donors and well accustomed to the procedure. The semen was collected immediately after defecation was observed in order to reduce urine or faecal contamination of the sample. Each bird was restrained in dorsal recumbency with the pelvic limbs held cranial. Gentle digital massage was initiated on the lateral aspects of the cloaca with a gradual increase in pressure on the ampullae of the vas deferens (Heidenreich, 1997; Lierz, 2008). Ejaculated semen was collected directly from the papillae of the vas deferens using a haematocrit tube without anticoagulant (No. 564, Assistant, Heiland, Hamburg, Germany). Half was placed into mycoplasma broth warmed to 37°C and immediately used for culture. The other half was soaked by a culturette swab (Bakteriette®, Copan-Italia, EM-TE-Vertrieb, Hamburg, Germany) and stored at – 80°C for later investigations by PCR. In addition the testis of a Gyr/Saker hybrid falcon that died accidentally during the breeding season was collected. The testis was briefly immersed in 70% ethanol and flamed to avoid

possible contamination of mycoplasmas from the air sacs before placing into mycoplasma broth or a sterile tube for PCR.

Cultures, identification and PCR. Semen and testis samples were cultured for mycoplasmas immediately after sampling using liquid and agar media produced according to Bradbury (1998) without adding thallium acetate. Mycoplasma colonies were investigated by an immunobinding assay (IBA) according to Kotani & McGarrity (1985), using antiserum against the reference strains of the following avian *Mycoplasma* spp.: *M. anseris*, *M. buteonis*, *M. corogypsi*, *M. falconis*, *M. gallisepticum*, *M. gypis*, *M. iowae*, *M. lipofaciens*, *M. meleagridis*, *M. synoviae*. Following the observations described below, the isolates were also tested for *M. verecundum*.

Each bird of prey sample was also subjected to a *Mycoplasma*-genus specific PCR as described by Lierz *et al.* (2007). Positive samples were further evaluated by using *Mycoplasma*-species-specific PCRs for the detection of *M. buteonis*, *M. corogypsi*, *M. falconis* and *M. gypis* as described by Lierz *et al.* (2008a) as these species are regularly detected in birds of prey (Lierz *et al.*, 2000; 2002; 2008a,b)

DNA sequencing. Each mycoplasma isolate was subjected to a DNA sequence analysis of its 16S rRNA gene. As a first step a single colony subculture (three times) of each was produced and tested again with the above mentioned antisera by the IBA. DNA extraction of the pure culture as well as the PCR of the 16S rRNA gene was performed according to Tanner *et al.* (2000) and Blackwood *et al.* (2003), using the 27F (forward) and 1492R (reverse) primers. After purification with the Invisorb Spin DNA Extraction Kit (Invitek, Berlin, Germany) the PCR products were sequenced in both directions (Agowa GmbH, Berlin, Germany) using the same forward and reverse primers. The sequences were compared to the 16S rRNA gene sequences of *Mycoplasma* spp. provided by GenBank as well as to each other, using the software programs Blast (NCBI, USA) (Altschul *et al.*, 1990) and Clustal W (Higgins *et al.*, 1996).

Results

Isolation, identification and PCR. Table 1 shows the individual birds with a positive culture. Mycoplasmas were cultured from five of 32 (15.6%) semen samples while 27 (84.4%) samples were negative. The positive samples included the semen sample of the gyrfalcon (No. 50587), whose second semen sample taken a year later was also culturally positive. Mycoplasmas were cultured from the testicular sample. None of the isolates could be identified with the IBA.

The *Mycoplasma* genus-specific PCR amplified a product from seven of 32 (21.9%) semen samples and also from the testis (Table 1). The species-specific PCRs were negative in all these cases.

Sequencing. The 16S rRNA genes of isolates from two Gyrfalcons (No. 50587 and ARNO), one Gyr/Saker hybrid falcon (SF12), one Golden Eagle (SF9), the testicular sample (Z61) and the second semen sample of bird No 50587 were sequenced. Subculture of the isolate from one Gyr/Saker hybrid falcon (4K13) failed. Table 1 indicates the size of the sequenced products and the GenBank Accession numbers of the sequences although as the sequences of both samples from bird No. 50587 were identical, only one sequence is provided.

The sequences varied in size due to the different sequencing quality, but all included more than 90% of the 16S rRNA gene. They showed a 99% (change of four bases within the aligned sequences) to 100% homology when compared to each other. Using the GenBank 16S rRNA gene sequences for comparison, the closest relative, with 97% homology, was *M. verecundum* (GenBank Accession no. AF 412989).

Discussion

Artificial insemination is a commonly used technique in birds of prey but investigations into the risk of this technique for pathogen transmission and semen quality are lacking. In humans it was shown, that semen quality is negatively affected by the presence of mycoplasmas (Gdoura *et al.*, 2007) and in turkeys, the infection of females by *Mycoplasma meleagridis*-contaminated semen was shown (Kumar & Pomeroy, 1969; Matzer & Yamamoto, 1970). In the present study, seven of 32 (21.9%) semen samples were positive for mycoplasma DNA and five of these samples yielded mycoplasma isolates. Considering the high prevalence of mycoplasmas in birds of prey in earlier studies (Lierz *et al.*, 2002; 2008a,b) the number of positive samples in this study seems low. However, only certain *Mycoplasma* species might be capable of shedding in semen. This is supported by the sequencing results, which demonstrated that all the isolates appear to belong to the same species since the obtained sequences showed a very high homology. Furthermore, the situation might be similar to *M. meleagridis* in turkeys where not all males transmit the pathogen in the semen (Rott *et al.*, 1989). However, the repeated isolation of apparently the same *Mycoplasma* sp. from one Gyrfalcon in two consecutive years is a first indication that once positive, the males may shed mycoplasmas in semen for longer periods.

The route of seminal contamination in raptors is presently unknown. It is possible that the testis is the primary source of infection, as apparently the same *Mycoplasma* sp. was isolated from both semen and testis. However, the testis was excluded as a source of semen contamination for turkeys (Gerlach *et al.*, 1968). As it is not known if mycoplasmas are able to cross blood/tissue barrier in the testis, haematogenous contamination of the sample cannot be excluded. As only one testicular sample was examined and as contamination of semen may also occur in the cloaca, further investigations are necessary, including histopathological examination of the testis.

Mycoplasma contamination of semen, most likely with the same *Mycoplasma* species, was detected in the *Falconidae* and *Accipitridae* of different collections, and therefore appears to be a regular finding. Studies describing reference parameters for high quality bird of prey semen are lacking so it is unclear if the mycoplasma contamination found in this study had a negative effect on the semen quality. Records of the fertilization success of the different males investigated were not available impeding interpretation of the importance of our findings. Further studies are indicated but as any bacterial contamination of semen should be considered abnormal it is recommended, pending further studies, that semen contaminated with mycoplasmas should not be used for artificial insemination.

As already stated, all isolates obtained in this study appear to belong to the same *Mycoplasma* species and according to analysis of the 16S rRNA gene sequence, this species is closely related to *M. verecundum*. However, the antigenic difference revealed by the IBA between the isolates and *M. verecundum*, suggests that the isolates may represent a new species. This study appears to be the first demonstration of mycoplasmas in semen samples of birds of prey.

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Table 1: Distribution of *Mycoplasma* isolates from semen and testicular samples originating from birds of prey

Species	<i>Mycoplasma</i> genus-specific PCR ^{a,b}	<i>Mycoplasma</i> culture ^c	Name of isolate / size of sequenced 16S rRNA gene product / GenBank Accession No
Golden eagle (<i>Aquila chryseatos</i>) ^d	Positive	Positive	SF9 / 1373bp / EU544228
Gyr/falcon (<i>Falco rusticolus</i>) ^d	Positive	Positive	50587 / 1343bp / EU544229
Gyr/falcon (<i>Falco rusticolus</i>) ^d	Positive	Positive	ARNO / 1369bp / EU544230
Gyr/Saker Falcon Hybrid (<i>F. rusticolus</i> * <i>F. cherrug</i>) ^d	Positive	Positive	SF12 / 1369bp / EU544226
Gyr/Saker Falcon Hybrid (<i>F. rusticolus</i> * <i>F. cherrug</i>) ^d	Positive	Positive	4K13 / Subculture failed
Golden eagle (<i>Aquila chryseatos</i>) ^d	Positive	Negative	---
White-tailed Sea eagle (<i>Haliaeetus albicilla</i>) ^d	Positive	Negative	---
Gyr/Saker Falcon Hybrid (<i>F. rusticolus</i> * <i>F. cherrug</i>) ^e	Positive	Positive	Z61 / 1339bp / EU544227

^a*Mycoplasma* genus-specific PCR according to Lierz *et al.* (2007)

^b*Mycoplasma* species-specific PCRs for the detection of the 16S rRNA gene of *M. falconis*, *M. gypis*, *M. corogypsi*, *M. buteonis* (Lierz *et al.*, 2008a) were also used and negative in all cases.

^c*Mycoplasma* isolates were subjected to an immunobinding assay using antisera against *M. anseris*, *M. buteonis*, *M. corogypsi*, *M. falconis*, *M. gallisepticum*, *M. gypis*, *M. iowae*, *M. lipofaciens*, *M. meleagridis*, *M. synoviae* and *M. verecundum* and all gave negative results.

^dSemen sample

^eTestis