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Outbreak of microsporidiosis caused by *Enterocytozoon bieneusi* in falcons

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Abstract

Four falcons from a private collection of 137 falcons in Abu Dhabi (UAE) died suddenly in summer 2005. In order to screen for a possible disease among the remaining falcons in the aviary, all other birds were caught, examined and treated if necessary. Most of the falcons suffered from massive lice infestation and 74 falcons additionally from a heavy *Caryospora* sp. burden. Endoscopy revealed yellowish plaques on intestines, livers or kidneys in 70 birds (51.1% morbidity). Proliferative serositis was seen in 17 out of 24 necropsied birds with plaques on intestines, livers or kidneys, which did not resemble any known disease in falcons. However, apart from 20 falcons, which died within a 6-week period after the initial examinations due to advanced disease stages, all other falcons responded well to the treatment with dimetridazole (Emtryl®), indicating protozoal disease.

Immunohistochemistry confirmed the presence of microsporidial antigen. The final diagnosis of *Enterocytozoon* (*E.*) *bieneusi* genotype D was confirmed with materials from 6 birds by PCR and sequencing. To our knowledge this is the first report of microsporidiosis caused by *E. bieneusi* in raptors in general and in falcons in particular. However, it is still unclear for how long *E. bieneusi* was present in the falcon flock, and which role it played in the development of the disease. Predisposing factors such as high temperature and overcrowding in the aviary induced immune suppression causing massive lice infestation as well as coccidiosis, thus paving the way for invasion with microsporidial spores.

Keywords: *Enterocytozoon bieneusi*; Microsporidiosis; Zoonosis; Falcons; PCR

1. Introduction

Microsporidians are a diverse group of eukaryotic, single-cell organisms closely related to fungi (Thomarat et al., 2004). They are intracellular parasites of vertebrates and invertebrates (Wasson and Peper, 2000). Microsporidial infections with *Encephalitozoon (En.) cuniculi* are well known to occur in mammals like rabbits, dogs and foxes (Wasson and Peper, 2000) and were recently discovered in European brown hare (*Lepus europaeus*) with kidney lesions (DeBosschere et al., 2007). Other microsporidial agents like *En. hellem* infect avians like lovebirds (Novilla and Kwapien, 1977; Randall et al., 1986; Powell et al., 1989; Barton et al., 2003), ostriches (Snowden and Logan, 1999), budgerigars (Black et al., 1997), parrots (Pulparampil et al., 1998), lories (Suter et al., 1998), finches (Carlisle et al., 2002) and hummingbirds (Snowden et al., 2001), but have never been reported in falcons or other raptors.

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Enterocytozoon bieneusi was first detected 1985 in France in an immunosuppressed AIDS patient with diarrhoea and isolated from the jejunum (Desportes et al., 1985). Other risk groups are travellers especially to tropical countries (Lopez-Velez et al., 1999), people with suppressed immune state (Lores et al., 2002a) as well as transplant patients (Goetz et al., 2001). In humans this parasite is the microsporidian species most frequently found out of 14 known microsporidian species causing diarrhoea and systemic disease—followed by *En. intestinalis* (Reetz et al., 2002; Didier, 2005). A major symptom of microsporidian infection in humans is diarrhoea accompanied by slow weight loss (Canning, 1993).

E. bieneusi is known to be present in domestic animals (Lores et al., 2002b) like rabbits, goats, pigs and dogs (Breitenmoser et al., 1999; Del Aguila et al., 1999). Other mammals like cattle (Sulaiman et al., 2004), cats (Rinder et al., 2000; Santin et al., 2006) as well as wild mammals like raccoons, muskrats, beavers, foxes and otters (Sulaiman et al., 2003) can also become infected with *E. bieneusi*. The zoonotic interspecies potential was confirmed on a phylogenetic basis in rhesus macaques (Drosten et al., 2005). However, not all *E. bieneusi* isolates from other hosts, like cattle have a zoonotic potential (Sulaiman et al., 2004). Nevertheless, research on the phylogeny of *E. bieneusi* strains leads to the conclusion of the assumption of a possible much higher diversity than previously known (Sulaiman et al., 2003, 2004; Slodkowicz-Kowalska et al., 2007). The zoonotic potential of *E. bieneusi* from birds is not clear (Lobo et al., 2006). So far, *E. bieneusi* has been reported in 2 of 8 chickens examined in Germany (Reetz et al., 2002) and in 17 of 124 (13%) healthy pigeons examined in Spain (Haro et al., 2005). However, Lobo et al. (2006) recently detected *E. bieneusi* in 24 of 83 (28.9%) faecal droppings from birds in Portugal of the orders Columbiformes, Passeriformes and Psittaciformes.

2. Material and methods

2.1. Clinical investigations

Four falcons originating from a private collection died within 1 week. Since these falcons were kept together with 114 other falcons in the same large free-flight aviary (FFA), the possibility of a disease transmission was very high. Consequently, all birds in the FFA were caught, as well as 10 falcons of the adjacent new cage (NC) and 9 falcons of the adjacent long cage (LC) and presented at the Abu Dhabi Falcon Hospital. These 137 falcons belonged to 5 different species and breeds, respectively (Table 1). The FFA was massively overcrowded with a mixed population of falcons of different breeds and age, since 114 birds were kept in a cage designed for 30–40 falcons.

Beside urate analysis, radiography and endoscopy was carried out in all birds. Blood samples were taken and examined with the Alpha Wassermann analyser for biochemical parameters and manually for haematological parameters. Crop smears and faecal samples were screened for microbes and parasites. In addition, herpes virus PCR test (HSV 430/720 I&II Virus, Secace Biotechnologies, Caserta, Italy) and acid-fast bacilli (AFB) stain (Kit Quick-TB, Réactifs RAL, Martillac, France) were carried out. Furthermore, serological tests for the detection of antibodies against Avian Influenza virus and Infectious Bronchitis virus (Synbiotics), Avian Pneumovirus and *Mycoplasma gallisepticum* (Svanova Veterinary Diagnostics), *Chlamydophila* (Diagnostic Veterinaire), Avian Reovirus, Paramyxovirus I and *Chlamydia psittaci* (Cypress Diagnostics) were performed as per request of the owner to get full information about the health status of the falcons.

All remaining 133 falcons were treated with dimetridazole (Emtryl®, 50 mg/kg orally, daily for 10 days) and 70 falcons with endoscopic visible lesions received additional homeopathic treatment and repeated dimetridazole application (Müller, 2007). Due to heavy

Table 1
Clinical results of parasitological and endoscopic examination of 137 falcons

Species	Species	Cases examined	Cases with endoscopic visible lesions	Cases with coccidiosis
Falco rusticolus × *Falco cherrug*	Gyr–Saker falcon	69	36 (52.2%)	39
Falco rusticolus × *Falco peregrinus*	Gyr–Peregrine falcon	47	28 (59.6%)	22
Falco rusticolus	Gyr falcon	14	4 (28.6%)	9
Falco peregrinus	Peregrine falcon	6	2 (33.3%)	4
Falco biarmicus	Lanner falcon	1	0	0
Total		137	70 (52.6%)	74 (55.6%)

Caryospora sp. burden all 133 birds were also treated for coccidiosis with Baycox® (0.7 ml/kg, orally) given daily for 3 days. If *Caryospora* stages were still prevalent in the faeces after one week Baycox® treatment was repeated for another 2 days. Since most birds suffered also from massive mallophaga infestation, all birds received spray-treatment with Falcon Insect Liquidator™ (Vetafarm) containing 1.25 g/l permethrin, 6.25 g/l piperonyl butoxide and 20 mg/l methoprene. To improve the hygiene status the sand layer of all three aviaries was removed up to 20 cm depth and replaced by new sand in combination with proper cleaning and disinfection.

2.2. Pathology

In total 24 falcons (18 × FFA, 4 × LC, 2 × NC; Table 3) died within 6 weeks and were sent for necropsy to the Central Veterinary Research Laboratory, Dubai. Endoscopy was performed in 18 of these falcons from the FFA just before death. Necropsy was immediately done after arrival and samples were taken and processed for routine histopathology, parasitology and bacteriology. Suspicious formalin-fixed and paraffin-embedded organ samples were also stained with Ziehl–Neelsen (ZN), Periodic Acid-Schiff (PAS), Brown–Brenn gram staining, Masson-Trichrom, Warthin-Starry and Grocott. Parasitological examination included microscopy of unstained fresh direct smears taken from hepatic and intestinal lesions as well as examination of faecal samples after $NaCl/ZnCl_2$ flotation.

Immunohistochemistry (IHC) was established for the detection of microsporidia by light microscopy using the peroxidase–antiperoxidase (PAP) technique. Since cross-reactions are known between different microsporidian species (Weiss et al., 1992; Zierdt et al., 1993; Reetz et al., 2004) a commercially available polyvalent rabbit antiserum against *En. cuniculi* (Testmann, Uppsala, Sweden) was used. Briefly, slides of liver and intestine from 6 falcons (Table 3) were incubated with the polyvalent rabbit antiserum against *En. cuniculi* at a dilution of 1:400 overnight at 4 °C, followed by the immunolabelling using the streptavidin–biotin complex (ABC)-kit (Dako, Glostrup, Denmark) according to the producer's instructions, and with diaminobenzidine (DAB) as chromogen (Hsu et al., 1981).

2.3. Molecular analysis

DNA was extracted from frozen tissue samples (liver, intestines) of 6 falcons (Table 3) using High Pure PCR Template Preparation Kit (Roche) according to the manufacturers' instructions. Out of these 6 samples, 4 were from the free-flight cage and 1 each from the new and the long cage (Table 3). Two different PCRs were performed because of initially suspected amebiasis. The first being specific for *Entamoeba histolytica* using the EH1 forward and the EHD2 reverse primer and the second for *Entamoeba dispar* using the ED1 forward and the EHD2 reverse primer, both following the protocol of Gonin and Trudel (2003). Briefly, after an initial denaturation at 95 °C for 15 min, 40 cycles were run (denaturation at 94 °C for 30 s, annealing at 51 °C for 60 s, and extension at 72 °C for 40 s) and a final termination at 72 °C for 5 min. The amplified products were visualized by ethidium bromide staining in a 2% agarose gel electrophoresis. Parallel an alternative PCR following the protocol of Troll et al. (1997) and using the Eh5′ and Eh3′ primers was also performed.

Furthermore, a universal diagnostic PCR for microsporidia was carried out using the Mic3U and Mic421U primers (Kock et al., 1997) and 35 cycles of 95 °C for 1 min, 69 °C for 1 min, and 72 °C for 1 min with a final extension for 7 min. This PCR gives a 410–433 base pair (bp) fragment of the 18S rRNA gene depending on the microsporidian genus and species respectively. Amplification product was visualized by ethidium-bromide in a 2% agarose gel electrophoresis. Subsequently, a second, species specific, PCR was performed for confirmation of *E. bieneusi*. Here, a different 607 bp fragment of the 18S rRNA gene was amplified using the EBIEF1 and EBIER1 primers (Da Silva et al., 1996). The PCR was performed with 1 μl of whole cell DNA in 50 μl reaction volume and the following amplification program: 35 cycles; 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min. Finally, a nested PCR was carried out in order to determine the *E. bieneusi* genotype according to the protocol of Katzwinkel-Wladarsch et al. (1996) and using the MSP-1 + MSP-2B and the MSP-3 + MSP-4B primers. All amplification products were purified with the Amersham Pharmacia purification kit® (Vienna, Austria).

The amplified fragments were sequenced by direct sequencing from the PCR-product using the ABI PRISM® BigDye sequencing kit and a 310 ABI PRISM® automated sequencer (Applied Biosystems, Langen, Germany). Sequences were obtained from both strands and sequence data were processed with the GeneDoc sequence editor (Nicholas et al., 1997). Primer sites were excluded from the sequences for further analysis. The sequences were compared to sequences of various microsporidian taxa available at GenBank using BLAST search and a multiple sequence

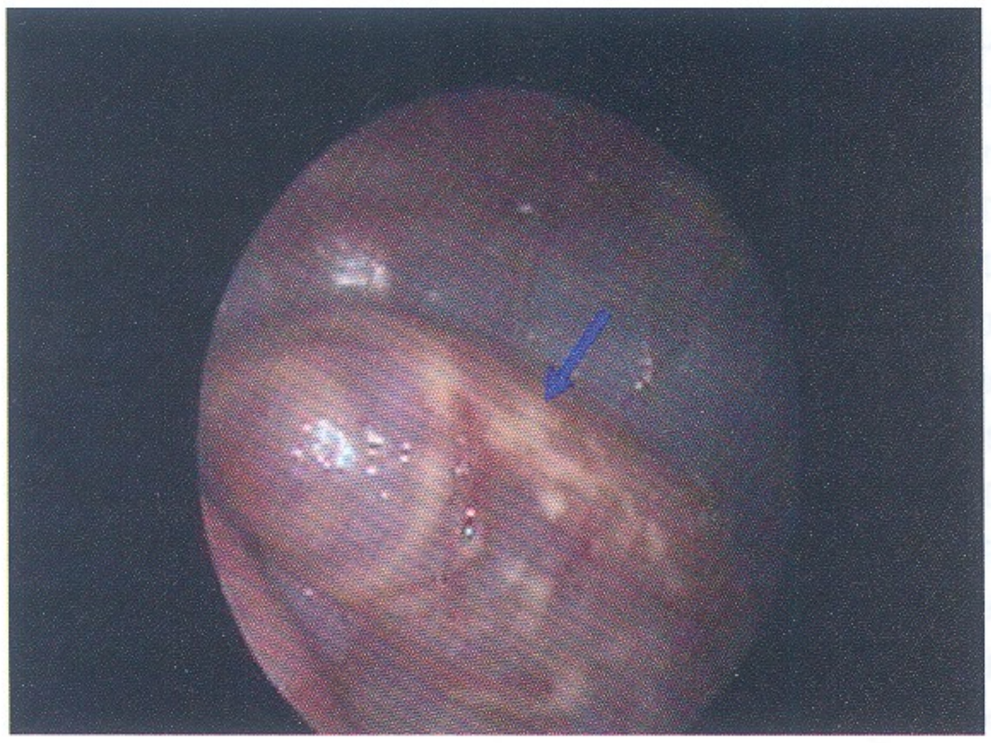

Fig. 1. Clinically apparent intestinal lesion (arrows) visible during endoscopic examination.

alignment was performed with the CLUSTAL X application (Thompson et al., 1997). The alignment was assessed by eye and revised manually. Sequence data were deposited at GenBank™ and are available under the following accession numbers: *E. bieneusi* partial 18S rDNA fragment (DQ793212) and *E. bieneusi* ITS1 (DQ793213).

3. Results

3.1. Clinical results

Out of the 133 examined falcons, 70 (52.6% morbidity) showed endoscopic visible lesions in intestines, liver and/or kidneys (Table 1; Fig. 1), which did not resemble tuberculosis, herpes virus infection or any other known disease. Seventy-four falcons suffered from a heavy *Caryospora* sp. burden. However, only 30 birds revealed endoscopic visible lesions and simultaneously coccidiosis. Most of the falcons suffered also from heavy mallophaga (*Laemobothrion* spp.) infestation, which significantly improved after spray-treatment, given to all birds. Other findings were aspergillosis (10%), bacterial infections (12.9%), hepatomegaly (14.3%) and nephromegaly (25.7%) as well as severe dehydration (22.9%). Haematological and biochemical parameters of all birds showed no significant changes. However, 60% of the falcons, which died from microsporidiosis had elevated white blood cell count (WBC) ranging from 11.4 to 16.4×10^9/l, whereas all surviving birds had WBC values below 10.2×10^9/l. Most falcons, which later succumbed to the disease revealed lower red blood cell count (RBC; $2.6–2.8 \times 10^{12}$/l; reference value $(3.2 \pm 0.3) \times 10^{12}$/l; Wernery et al., 2004) and had lower haemoglobin (Hb: 14.8–17.8 g/dl) and haematocrit (HCT: 40–53 l/l) values compared to surviving birds (Hb: 17.2–19.4 g/dl and PCV: 41–57 l/l, respectively).

The first cases of the disease with the highest numbers of sick birds were seen in the free-flight cage. Two falcons were transferred into the new cage and died after clinical examination; one of them was confirmed with intestinal and hepatic lesions at necropsy. No falcons were transferred into the long cage. However, one out of 4 dead falcons from the long cage revealed lesions at necropsy (Table 3), which were not visible during previous endoscopic examination. Another falcon from this group showed a tiny intestinal lesion in the endoscopic examination and died on the 8th day of treatment with dimetridazole. Differences in the species/breed distribution were observed regarding the presence of lesions in general and in different organs in particular (Table 2), with hybrid falcons being the most affected. Apart from 20 falcons, which died shortly after the initial examinations due to advanced disease stages, all other falcons responded well to the individual treatment plan, and recovered completely. Repeated endoscopic examinations showed a good response to the treatment until full recovery in surviving falcons. Falcons with smaller abscesses showed a continuous regression of the abscesses up to a full disappearance. The fully recovered falcons could be used for falconry again in the winter season of 2005/2006.

Seven falcons with large liver lesions died during treatment. However, the other 5 birds with minor liver lesions survived. No *Salmonella* spp., acid-fast rods or herpes virus were detected or isolated during clinical

Table 2
Endoscopic findings and relation to species/breed

Endoscopic findings	Total	Gyr–Saker	Gyr–Peregrine	Gyr	Peregrine
Intestinal lesions	26	11 (42.3%)	12 (46.5%)	2 (7.7%)	1 (3.9%)
Lesions in liver	12	5 (41.7%)	5 (41.7%)	1 (8.3%)	1 (8.3%)
Lesions in kidneys	20	12 (60.0%)	7 (35.0%)	1 (5.0%)	0 (0.0%)
Lesions in intestines and liver	1	0 (0.0%)	1 (100.0%)	0 (0.0%)	0 (0.0%)
Lesions in intestines and kidneys	5	3 (60.0%)	2 (40.0%)	0 (0.0%)	0 (0.0%)
Lesions in intestines, liver and kidneys	6	5 (83.3%)	1 (16.7%)	0 (0.0%)	0 (0.0%)

Table 3
Necropsy and molecular biology results from 24 dead falcons

No.	Location[a]	Necropsy on	Macroscopic visible lesions in	Other diagnosis	Coccidia stages in intestine	Caryospora oocysts in faeces	PCR-positive for *E. bieneusi*
1	FFA	05.06.05	Intestine, liver, kidney		+++	Negative	n.d.
2	FFA	08.06.05	Intestine, liver, kidney		Negative	Negative	Yes
3	FFA	08.06.05	Intestine, liver, kidney, pancreas		+	Negative	Yes
4	FFA	13.06.05	Intestine, liver, kidney,		+	Negative	n.d.
5	NC	19.06.05	Intestine, liver	Aspergillosis	Negative	Negative	Yes
6	FFA	19.06.05	Intestine, liver, kidney		Negative	Negative	Yes
7	FFA	20.06.05	Intestine, liver, kidney, pancreas	Coccidia	+	+	n.d.
8	FFA	26.06.05	Intestine, liver, kidney, spleen		++	Negative	n.d.
9	LC	26.06.05	No visible lesions	Aspergillosis, amyloidosis	++	Negative	n.d.
10	LC	26.06.05	No visible lesions	Amyloidosis	Negative	Negative	n.d.
11	NC	26.06.05	No visible lesions	Aspergillosis	Negative	Negative	n.d.
12	FFA	26.06.05	Intestine, liver, kidney		+++	Negative	n.d.
13	LC	02.07.05	No visible lesions	Gout	Negative	Negative	Yes
14	FFA	02.07.05	Intestine, liver, kidney		Negative	Negative	Yes
15	FFA	03.07.05	Intestine, liver, kidney	Coccidiosis	+	++	n.d.
16	FFA	05.07.05	Intestine, liver, pancreas	Aspergillosis, amyloidosis, coccidiosis	+++	++	n.d.
17	FFA	05.07.05	Intestine, liver	Coccidiosis	+++	++	n.d.
18	FFA	05.07.05	Intestine, liver	Coccidiosis	+++	++	n.d.
19	LC	05.07.05	Intestine	Bumble foot	+++	Negative	n.d.
20	FFA	09.07.05	Intestine	Gout	++	Negative	n.d.
21	FFA	09.07.05	Intestine, liver		+	Negative	n.d.
22	FFA	16.07.05	No visible lesions	Clostridia	Negative	+	n.d.
23	FFA	16.07.05	No visible lesions	Airsacculitis	Negative	Negative	n.d.
24	FFA	16.07.05	No visible lesions	Clostridiosis, coccidiosis	++	++	n.d.

[a] FFA = free-flight aviary, LC = long cage, NC = new cage, n.d. = not done.

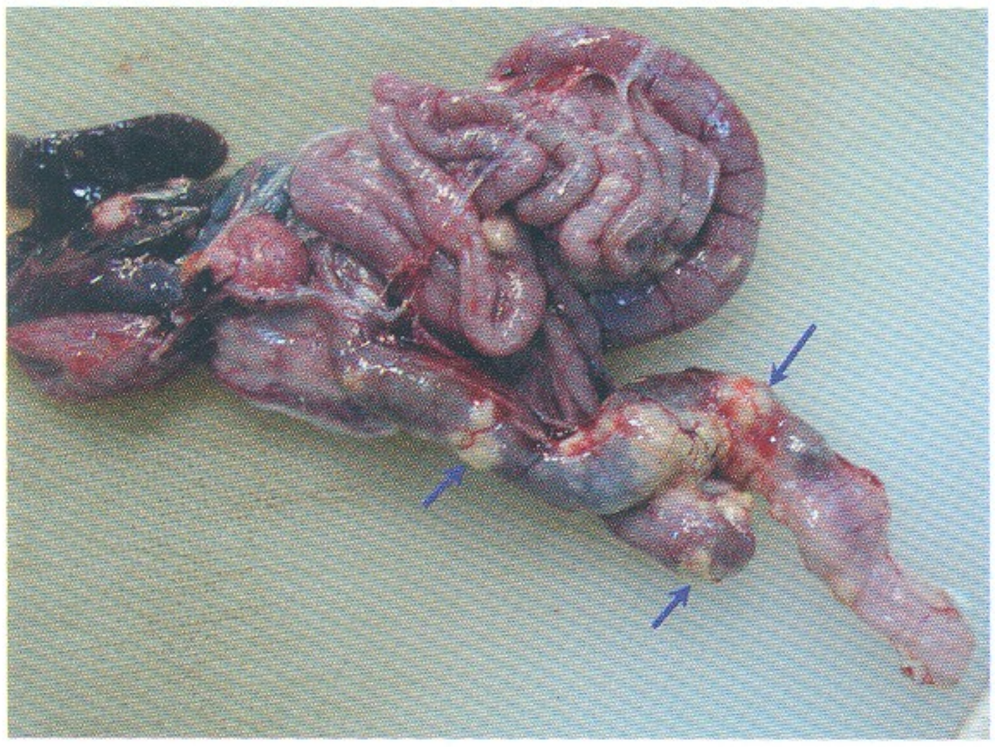

Fig. 2. Multiple yellowish plaques on the intestine (arrows) due proliferative serositis and diphtheroid enteritis and colitis.

examination. Additionally, none of the birds demonstrated antibodies against the above-mentioned viruses.

3.2. Pathology results

Twenty falcons died after the treatment began on top of four falcons, which had died before treatment started (24 total; 17.5% mortality; Table 3). Multiple yellowish plaques (3–5 mm in size; Fig. 2) occurred on the small intestine and colon of 17 birds. Several yellowish foci (1–5 mm in diameter) were also seen in the liver (15 out of the 24 cases; Fig. 3) and kidneys (10 out of the 24 cases). The pancreas was affected in 3 falcons and the spleen in another falcon, all four showing also lesions in intestine, liver and kidneys (Table 3).

Histologically, there was extensive thickening of the intestinal serosal surfaces characterized by proliferation of mesothelial cells and nonsuppurative inflammatory cell infiltrate (Fig. 4) involving all intestinal layers from the serosa to the mucosa. Histology of the liver lesions revealed large areas with foamy hepatocytes and bile

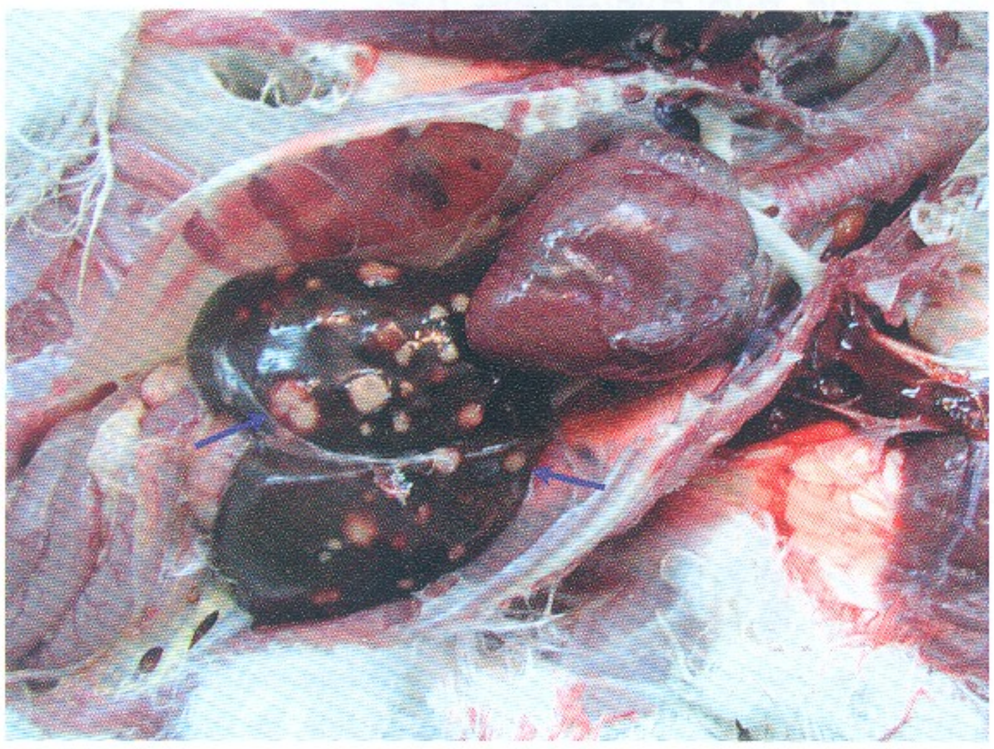

Fig. 3. Multiple yellowish prominent foci (arrows) in the liver.

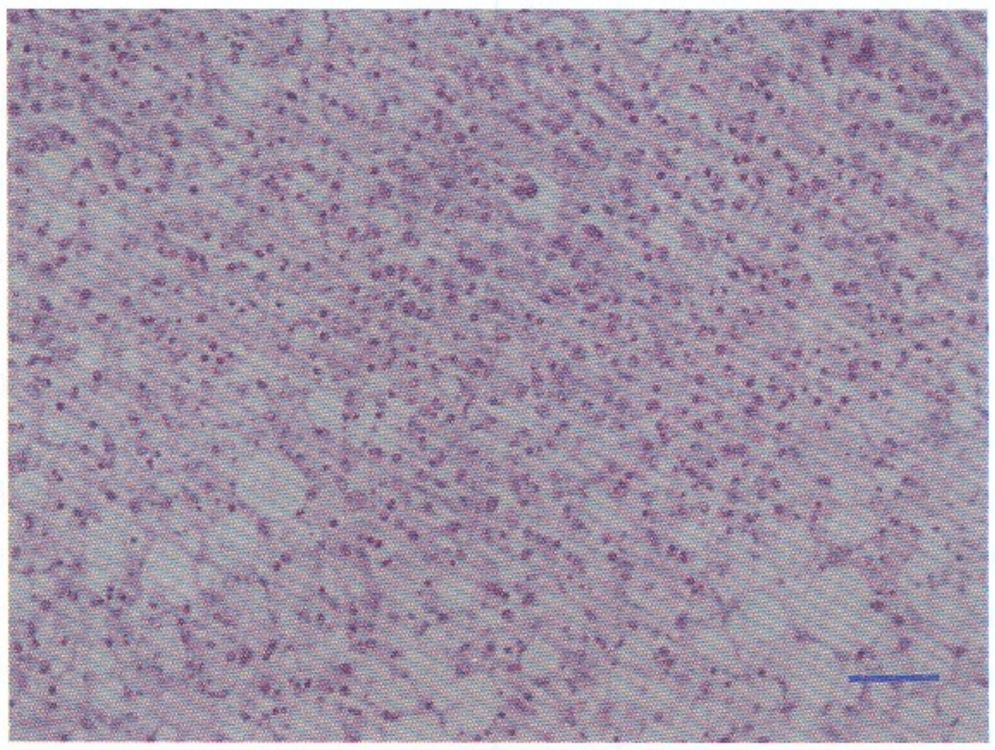

Fig. 4. Proliferative serositis of the small intestine with and numerous foamy cells (HE-staining; Bar = 60 μm).

duct proliferation, fresh necrosis and microabscesses in the adjacent areas (Fig. 5). Pancreas and kidneys showed multifocal severe diffuse degeneration with pyogranulomatous inflammation, with most renal tubuli containing protein cylinders. No acid-fast rods, fungi or parasites were seen in any case using special stains. However, immunohistochemistry was positive for microsporidian antigen in granulomatous lesions of liver (Fig. 6), kidney and intestine of all 6 tested falcons (Table 3). Microsporidian antigen was identified as brownish stained material in the cytoplasm of numerous cells in and around necrotic areas. Subsequent intensive retrospective histological analysis of haematoxylin–eosin (HE) stained slides revealed very few microsporidia-like organisms inside inflammatory cells (Fig. 7) in the first four cases, which did not receive any treatment.

From two out of the 24 necropsied falcons different *Salmonella* strains (*S. enteritidis* and *S. typhimurium*)

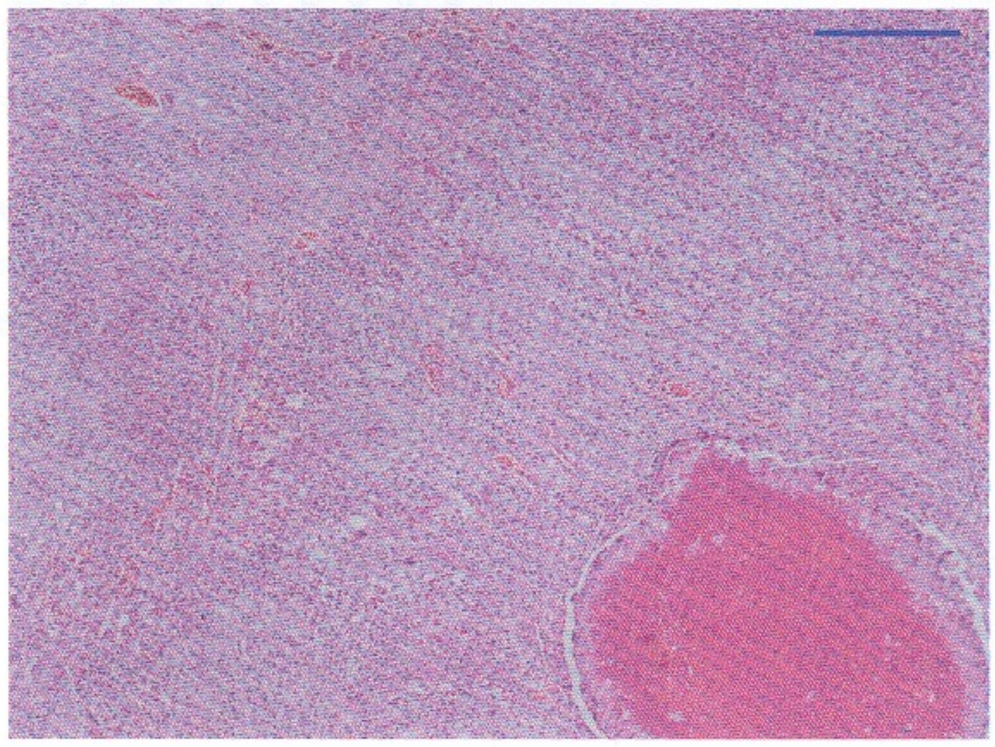

Fig. 5. Histology of the liver with large areas of foamy hepatocytes, bile duct proliferation and central necrotic abscess (HE-staining; Bar = 200 μm).

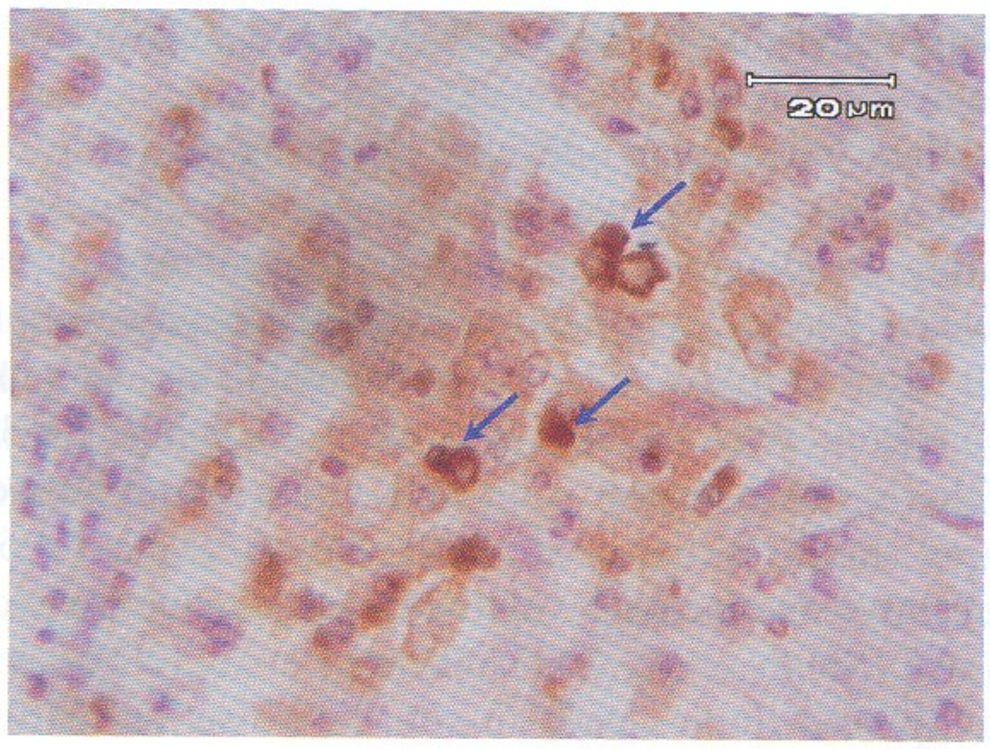

Fig. 6. Positive intracytoplasmatic reaction (arrows) for microsporidian antigen in granulomatous hepatic lesion (PAP technique).

were isolated from the intestine as well as *Clostridium perfringens* in 7 cases. No fungus, no acid-fast rods, nor any virus was detected or isolated. Coccidian oocysts of the genus *Caryospora* were detected in faecal samples in 7 of the 24 (29.2%) dead falcons (Table 3). However, histology revealed intestinal coccidian stages in 15 birds (62.5%), including 10 falcons (41.7%) with high numbers of coccidian stages. During clinical examination 7 of these 24 birds (29.2%) suffered from coccidiosis. All direct organ smears were negative for parasites by light microscopy.

3.3. Molecular results

All six falcon samples (Table 3) examined were negative in the *E. histolytica* and *E. dispar* specific PCRs, but positive in the microsporidia assay giving a 410 bp PCR product and were thus identified as *E. bieneusi*. The *E. bieneusi* specific PCR confirmed this finding showing the typical 607 bp fragment. Sequencing of the fragments revealed that all 6 samples were

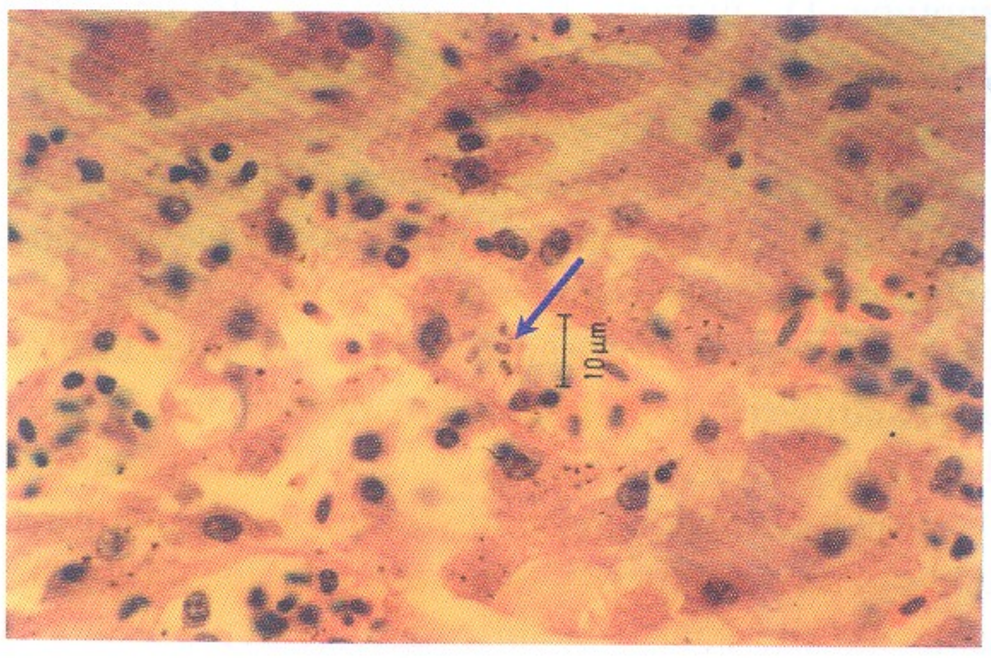

Fig. 7. Colony of oval shaped intracytoplasmatic microsporidial organisms (arrow) in hepatic lesion (HE-staining).

100% identical to each other in both fragments. As the 410 bp fragment of the universal primers and the 607 bp fragment of the *E. bieneusi* specific primers overlap by 118 bp they were aligned, together giving a fragment of 857 bp without primer sites. Comparison of this sequence to published sequences of other *E. bieneusi* strains revealed a 100% sequence identity to 4 of the 8 *E. bieneusi* strains of which sequences of the small ribosomal subunit DNA are available in GenBank, namely to the AF023245 strain isolated from a macaque and to the INDRE01, the AF024657 and the L16868 strain, all three isolated from AIDS patients (however, the sequence available of this strain is 296 bp shorter on the 5′end). The sequence difference to the other four *E. bieneusi* strains lies between 2 and 21 bp.

Sequencing of the internal transcribed spacer (ITS) regions showed that the isolates belong to genotype D showing a 100% sequence identity to one another and to the AF101200 strain (human) and the AF023245 strain (macaque). The sequence available in GenBank of the third genotype D isolate (pig) is slightly shorter and was thus removed from the alignment.

4. Discussion

Over a period of 6 weeks, more than 52% (70 out of 133 examined falcons) showed clinically apparent lesions in the intestine, liver and/or kidneys during endoscopic examination, which did not resemble any known falcon disease. The first and highest numbers of cases were seen in the free-flight cage. However, the disease spread also to the other cages. Most falcons showed also heavy lice infestation and 74 falcons (55.6%) suffered from a heavy *Caryospora* sp. burden, including 30 birds with endoscopic visible lesions and simultaneously coccidiosis. Beside this no other pathogens like *Salmonella* spp., acid-fast rods or herpes virus were detected or isolated during clinical examination from any bird. No significant changes were found in haematological and biochemical parameters of all birds. However, 60% of the falcons, which later succumbed to the disease showed elevated white blood cell counts (WBC) ranging from 11.4 to 16.4×10^9/l compared to normal values of $(9.64 \pm 3.96) \times 10^9$/l (Wernery et al., 2004). All surviving birds had WBC values below 10.2×10^9/l.

In 17 from 24 dead falcons sent for necropsy yellowish plaques were detected on the intestinal serosa. This proliferative serositis with diphtheroid enteritis and colitis involved all intestinal layers from the serosa to the mucosa. In similar lesions found in SIV-infected immunodeficient macaques (*Macaca*

mulatta) *E. bieneusi* was the cause of the proliferative serositis (Chalifoux et al., 2000). The authors suggest lymphatic spread of spore-infected macrophages from the gallbladder reservoir inducing the proliferative serositis. The liver was affected in 15 falcons, the kidney in 10 cases, the pancreas in 3 and the spleen was affected in one falcon. Histology revealed pyogranulomatous to necrotizing inflammation of the liver and other affected organs. Since *E. bieneusi* was found in bile duct epithelial cells of rhesus macaques (*M. mulatta*; Chalifoux et al., 1998; Mansfield et al., 1998) this might have been the source of the liver infections in our cases causing the described hepatic lesions.

Detection of causative agents was difficult in formalin-fixed, paraffin-embedded tissue, since very few parasites were observed in the first cases, only. The same has been observed for other microsporidial infections, like *En. cuniculi* infection in rabbits (Harcourt-Brown, 2007). The author found typical renal lesions in 13 cases by histology, although the organisms were visible in only one case. However, in our study immunohistochemistry with cross-reacting commercially available polyvalent rabbit antiserum against *En. cuniculi* (Reetz et al., 2004) revealed intracytoplasmatic microsporidian antigen inside the lesions in the liver, kidney and intestine of all tested animals. Since parasites were detected by histology only in the first (untreated) falcons, but microsporidial antigen was detected using IHC in all tested cases, microsporidia organisms were most probably destroyed during the treatment thus antigen was still detectable even so the parasites were not visible any more with conventional histology.

Focal severe pyogranulomatous to ulcerative enteritis was seen in the intestine of the falcons. However, little to no intestinal inflammation was found in 2-weeks-old budgerigar chicks (*Melopsittacus undulates*) with *En. hellem* infection (Black et al., 1997), but inflammation with macrophages and heterophils was seen around hepatic necrosis.

Routine haematoxylin-and-eosin stained parasites in tissue are easily overlooked even by experienced pathologists (McWilliam and Curry, 1990). Tissue Gram stains can be silver stains like Warthin-Starry stain (Field et al., 1993a, 1993b), modified chromotrope-based trichrome stain (Giang et al., 1993), Brown–Brenn or Brown-Hopps of paraffin-embedded tissue sections (Orenstein et al., 1992; Kotler et al., 1994; Black et al., 1997). However, in our cases no advantage for parasite detection was seen using special stains compared with conventional HE-staining. Detection of microsporidian stages in faecal samples is complicated due to the small size (1–2 μm) in some species (Canning, 1993) making reliable visualization by light microscopy difficult (Franzen and Müller, 1999). This might have been the reason in our case of failing to diagnose the spores of unstained direct smears. Faecal identification methods include simple salt flotation followed by cytospin and Giemsa staining (Van Gool et al., 1990), PAS stain (Canning, 1993), Weber's chromotrope stain and Gram chromotrope-based stains (Weber et al., 1992; Moura et al., 1996). Recently monoclonal antibody-based fluorescence assays (Singh et al., 2005; Sheoran et al., 2005) and multiplexed fluorescence in situ hybridisation (FISH) assay (Graczyk et al., 2007a) were developed. Other methods are transmission electron microscopy (TEM) (Franzen and Müller, 1999; Connolly et al., 1991; Carter et al., 1996) as well as in situ hybridization (ISH) on formalin-fixed, paraffin-embedded tissues (Carville et al., 1997; Mansfield et al., 1997; Chalifoux et al., 2000). Unfortunately neither TEM nor ISH were performed in our cases.

In recent years PCR has gained increasing importance for the detection of microsporidia in clinical samples and different protocols have been published (Franzen and Müller, 1999). In the present study identical strains of *E. bieneusi* genotype D were detected in all 6 analysed falcon samples. Genotype D has already been isolated from different hosts, including human, macaque and pig (Chalifoux et al., 2000; Dengjel et al., 2001; Buckholt et al., 2002). To the best of our knowledge this is the first identification of genotype D in a bird. Up to now only genotype J of *E. bieneusi* has been identified from avian hosts (chicken; Reetz et al., 2002). However, *E. bieneusi* has recently been detected in pigeons, but the genotype has not been identified (Haro et al., 2005). Interestingly, of the 4 strains (available at GenBank) to which our strain showed 100% identity in the 18S rDNA, two are also genotype D, namely the INDRE01 strain and the AF023245 strain (macaque). The other two strains had not been genotyped.

Since *E. bieneusi* has been detected in pigeons (Haro et al., 2005), disease transmission in our cases may have occurred through infected pigeons. Captive-bred pigeons, living at the same farm where the outbreak happened, were used as food for the falcons. Recently Slodkowicz-Kowalska et al. (2006) confirmed that waterfowl faecal droppings contained significant numbers of microsporidian spores. This might have been also the source of infection in our cases, since falcons are often trained on waterfowl. Contamination may also have occurred through droppings of free flying

feral pigeons or waterfowl, resting on the aviaries netting. Graczyk et al. (2007b) report that the *E. bieneusi* spores of infected urban pigeons' faeces can be aerosoled. This might lead to an airborne transmission of humans through inhalation of 4 times higher *E. bieneusi* spores concentrations than potentially viable. The spores can also contaminate water in areas with infected pigeons. This possibility of an air- and waterborne transmission route though infected pigeon faeces might also have led in our case to the transmission from the large pigeon flocks located closely to the falcon cages. Unfortunately, no other avian species were tested for this parasite with PCR methods. Transmission is also possible through contaminated chicken meat or water (Reetz et al., 2002). In our cases, it is noteworthy that apart from the infected falcons from the free-flight aviary, one falcon each from two other cages (new cage, long cage) was also positive for *E. bieneusi* in the PCR. This states clearly that a disease transmission from cage to cage had taken place either through contamination by staff members, by falcon movement or by fomites.

Since transmission of *E. bieneusi* spores from humans and rhesus macaques to gnotobiotic pigs was successful (Kondova et al., 1998), it cannot be fully ruled out that humans might be able to infect birds as well. The zoonotic potential of *E. bieneusi* has been analysed by Dengjel et al. (2001), but requires further investigation. It is known that travellers particularly from tropic countries (Lopez-Velez et al., 1999) can be infected with *E. bieneusi*. The staff working in our falcon cages often originates from tropical countries, which might have been a possible source of infection. Whether transmission from an infected human to the falcons might be possible is not known. Furthermore, one falcon with *E. bieneusi* infection did not show any histopathological lesions thus raising the question of possible carriers. Also Mansfield et al. (1998) found persistent infection of healthy rhesus macaques (*M. mulatta*) with *E. bieneusi* localized in the hepatobiliary tree. It is still unclear for how long *E. bieneusi* was present in the falcon flock, and which role it played in the development of the disease.

Most of the falcons showed massive lice infestation and 74 birds falcons suffered also from a heavy *Caryospora* sp. burden. However, only 30 birds showed also simultaneously endoscopic visible lesions. Coccidiosis caused by *Caryospora* sp. is mainly a problem of captive falcons (Heidenreich, 1995), since no coccidian were found in wild raptors (Krone, 1998). Our falcons suffered most likely from immune suppression caused by predisposing factors such as high temperature, overcrowding in the aviary and concurrent diseases like aspergillosis. The massive *Caryospora* burden may have led to a severe damage of the intestines (Forbes and Simpson, 1997) thus paving the way to an invasion of the microsporidial spores. Numerous studies have shown that coccidiosis can interact with a variety of other organisms or toxins including viruses, bacteria, helminths and mycotoxins (Kaldhusdal et al., 1995; Baba et al., 1997). In these cases, the interaction produced a more severe disease than one condition alone (Ruff, 1988; Williams, 2005).

Since microsporidiosis is a new avian disease, no treatment for *E. bieneusi* infections has been specified yet. For veterinary use, the nitroheterocyclic drug dimetridazole (1,2-dimethyl-5-nitroimidazole, Emtryl®) is established for treatment of certain protozoal infections like amebiosis (Raether and Haenel, 2003). Emtryl® used in our cases is known to be effective against amoebae and seemed to be effective against *E. bieneusi* as well due to regression of abscesses and high survival rate. *E. bieneusi* infections were successfully treated with albendazole in AIDS patients (Blanshard et al., 1992), inhibiting the development of *E. bieneusi* spores in the small intestine. However, albendazole was not effective in other AIDS patients with *E. bieneusi* infections, who responded well to the new broad-spectrum antiparasitic medicine nitazoxanide (Bicart-Sée et al., 2000), which is a nitroheterocyclic drug, similar to dimetridazole (Raether and Haenel, 2003). However, this drug is not approved for veterinary use in protozoal infections (Raether and Haenel, 2003). Molina et al. (2002) used oral fumagillin to successfully treat chronic *E. bieneusi* infections in humans. Another treatment approach in humans is the use of furazolidone, which reduced faecal excretion of *E. bieneusi* stages in clinical tests (Dionisio et al., 1995, 1997).

Microsporidiosis caused by *E. bieneusi* in falcons is a new parasitic disease, which needs to be taken into consideration from now on. It is still unclear for how long the infection was present in the falcon flock. Special faecal staining should be included in the routine examinations if there is frequent interaction or contact of falcons with free-living birds. To our knowledge this is the first report of microsporidiosis caused by *E. bieneusi* in raptors in general and in falcons in particular. The zoonotic potential of *E. bieneusi* (Mathis et al., 2005; Slodkowicz-Kowalska et al., 2006) is an issue that should be taken into consideration as falconers live in close contact with their falcons and might therefore be at risk of disease transmission from their birds. Human infection may arise through

inhalation, direct contact through oral mucosa and ingestion of the microsporidial spores (Haro et al., 2005; Graczyk et al., 2007b). Further research is required to identify other affected avian species as well as possible avian–avian, avian–human and human–avian transmission routes.

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