**OUTBREAK OF DISEASE ASSOCIATED WITH
CRYPTOSPORIDIA ON A RED-LEGGED PARTRIDGE
(ALECTORIS RUFA) GAME FARM**

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Abstract

An outbreak of disease associated with cryptosporidia on a red-legged partridge (*Alectoris rufa*) game farm is described. Morbidity (diarrhoea and cough) was between 60 and 70% during the first weeks of life (4 to 25 days) and mortality was higher than 50%. The results of bacteriological and virological analyses were negative. Histological examination and antigenic diagnosis by ELISA revealed the presence of *Cryptosporidium* spp. in respiratory and intestinal tracts. The application of PCR-RFLP techniques and sequencing of a COWP gene fragment confirmed the existence of *C. meleagridis* in faecal samples. The results obtained suggest that avian cryptosporidiosis should be included among respiratory and enteric diseases routinely tested for in farmed birds.

Introduction

Species belonging to the genus *Cryptosporidium* are monoxenous protozoan parasites that infect a wide range of vertebrate hosts, including humans (Fayer, 2004).

Cryptosporidium spp. cause acute, self-limiting gastroenteritis in immunocompetent individuals, and chronic and potentially fatal infections in immunocompromised subjects worldwide (Cacciò *et al.*, 2005). In animals, cryptosporidiosis appears mainly to be a problem in neonatal ruminants, and causes considerable direct and indirect economic losses (de Graaf *et al.*, 1999).

Avian cryptosporidiosis is an emerging health problem in the poultry industry and is associated with respiratory/intestinal disease that can cause severe morbidity and mortality (de Graaf *et al.*, 1999). The birds can become infected by ingestion or inhalation and/or inspiration of oocysts present in the environment (litter, faeces, water, nesting materials, dust, etc.). As few as 100 oocysts can cause intestinal or respiratory infections (Sréter & Varga, 2000) and because of the limited availability of effective drugs, hygienic conditions and management strongly influence the incidence and persistence of avian cryptosporidiosis (Current, 1997; de Graaf *et al.*, 1999).

Although infections have been found in over 30 host species, including domestic, caged and wild birds, only three species of *Cryptosporidium* affecting avian hosts have been named on the basis of their morphological, biological and molecular differences (Xiao *et al.*, 2004). *Cryptosporidium meleagridis* (Slavin, 1955) may infect the intestinal tract, bursa of Fabricius (BF) and cloaca of turkeys and chickens, but infection has previously only been associated with illness, including diarrhoea and moderate mortality in turkeys. Oocysts measure $5.2 \times 4.6 \mu\text{m}$ (Sréter & Varga, 2000).

Cryptosporidium baileyi (Current *et al.*, 1986) may infect the respiratory tract (larynx, trachea, primary and secondary bronchi and air sacs), BF and cloaca mainly of chickens, turkeys and ducks. It has also been described in quails (Morgan *et al.*, 2001). *C. baileyi* is the most prevalent species of *Cryptosporidium* in poultry and the most commonly associated with disease in chickens. Oocysts of *C. baileyi* measure $6.2 \times 4.6 \mu\text{m}$ (Sréter & Varga, 2000).

A third species of avian *Cryptosporidium* was first reported by Pavlasek (1999, 2001) and recently re-described as *Cryptosporidium galli* (Ryan *et al.*, 2003). The oocysts are ellipsoidal (size range $8.0\text{--}8.0 \times 6.2\text{--}6.4 \mu\text{m}$) and the life cycle stages develop in the epithelial cells of the proventriculus in a variety of birds. Moreover, new genotypes of avian cryptosporidia have been found in different hosts, and may represent separate species (Xiao *et al.*, 2004).

In the present study, an outbreak of disease associated with cryptosporidia on a game farm of red-legged partridge (*Alectoris rufa*) is described.

Materials and Methods

Case history. The case occurred on a red-legged partridge game farm in Cataluña (northeast Spain) on which there were 100 breeding pairs. An outbreak of disease, characterized by diarrhoea and coughing, was detected in May 1999, coinciding with the first hatches of the season. From 4 to 5 days of age, the chicks produced a higher-than-usual number of depositions/caecal discharges of a pasty consistency, and on some occasions a watery halo was observed in normal faeces. In addition, approximately 10% of the animals had a dry cough and sometimes a nasal discharge. A few days after the

appearance of the clinical signs, the birds became apathetic, and then prostrate, with dropping wings, trembling and, following slight paralysis, they died in a lateral prone position. Morbidity varied between 60 to 70% between 4 and 25 days of age, with a mortality rate of 50%. From 30 days of age onwards, the surviving birds developed and behaved as normal after removal of between 2 to 3% of birds that remained affected. The situation occurred again with later hatches until July, causing considerable economic loss on the farm. In July the condition became more severe as the condition lasted for more than 30 days and caused greater mortality than before. Of the last 450 chicks that hatched, only 50 survived (89% mortality). During the outbreak (May to July), the birds were treated therapeutically and prophylactically in the drinking water with several drugs: ipronidazol (200 mg/l, for 5 days), sulfaquinoxaline (75 mg/l, two periods of three days separated by two rest days) and enrofloxacin (100 mg/l for 5 days). Additional biosecurity measures were introduced to physically separate the different hatches to avoid cross-contamination. Huts used for the youngest birds were thoroughly cleaned and disinfected using water with formaldehyde and salt. Daily movement of personnel was also reviewed in order to avoid cross-contamination. In addition, changes of shoes and clothing between huts were implemented. Special precautions were introduced to prevent access of wild birds (mainly sparrows) and rodents to the facilities by using appropriate nets and authorized traps.

Histopathology. Four affected partridges (two 11 days-old and two 25 days-old) were anaesthetized with thiopental sodium and killed by exsanguination. At autopsy, trachea, bronchi, lungs and intestinal organs (duodenum and caecum) were removed for bacteriological, viral and parasitological analyses, and the remaining parts of these organs were fixed in 10% neutral buffered formalin for histopathological examination.

After fixation, samples were embedded in paraffin, sectioned at 3 to 4 μm and stained with haematoxylin-eosin following routine histological methods.

Bacteriology. Samples of trachea, lung, duodenum and caecum were inoculated directly into TSA medium, modified brilliant green medium and Columbia III w/5% SB medium (ref. 254051, 254490 & 254097 respectively; BD Diagnostic, Diagnostic Systems, Sparks, MD, USA) and incubated at 37°C for 72 h. For isolation of *Mycoplasma* spp., samples of trachea and lung were initially inoculated into *Mycoplasma* broth base (PPLO, BD Diagnostics, Diagnostic Systems) supplemented with phenol red, glucose, horse serum, NAD, cysteine, thallium acetate and penicillin according to Frey *et al.* (1968) and incubated at 37°C in a atmosphere containing 10% CO₂ (BB-16, Heraeus, Munchen, Germany) for 7 days. Samples from the broth culture were then streaked on to *Mycoplasma* agar prepared as above but with 1.3% agar (ref. 214230; BD Diagnostics, Diagnostic Systems), maintained under identical conditions, and examined daily for 25 days.

Virology. For virus isolation, 9-days-old embryonated specified pathogen free (SPF) chicken eggs (Lohmann Gmb H, Cuxhaven, Germany) and primary fibroblast monolayer cultures were used according to Rovozzo & Burke (1973). Lung and intestine samples (duodenum and caecum) were suspended at a ratio of 1:10 (w/v) in phosphate buffered saline (PBS, pH 7.2) and mechanically homogenized (Stomacher[®] 400, Seward, Worthing, UK). The samples were then centrifuged at 3000 $\times$ g for 5 min. The supernatants were filtered through nitrocellulose filters (2 μm diameter pore) (Millipore Corporation, Bedford, USA) and 0.2 ml aliquots of filtrate were inoculated into each of three SPF eggs (via the allantoic cavity) and two Falcon[™] cell culture tubes

(BD Biosciences, San Jose, CA, USA) containing fibroblast monolayers, which were maintained for 4 days at 37°C.

Four days post inoculation, the eggs were opened aseptically and compared with the controls to detect any anomaly that indicated presence of a virus. Ten ml aliquots of allantoic fluid were collected and passaged twice following the same methodology. Likewise, at 4 days post inoculation, the fibroblast monolayers were examined microscopically (10×) to check for cytopathic effects or differences in development compared with control cultures. Two further passages were carried out with 5 ml aliquots of the fibroblast culture supernatants. In addition, the faeces were examined for rotavirus type A by an ELISA capture test, in accordance with the manufacturer's instructions (Ingezim Rotavirus Das 1.1.RT.K.2, Ingenasa, Madrid, Spain).

Parasitology. Faecal samples from the four necropsied birds were processed by a saturated sugar (density 1.27 g/ml) flotation technique and pools of homogenized samples from trachea, bronchi, lung, duodenum, caecum and faeces were used for antigenic diagnosis of *Cryptosporidium* by ELISA (Bio-X *Cryptosporidium parvum* ELISA kit, Bio-X diagnostics, Jemelle, Belgium), according to the manufacturer's instructions.

PCR-RFLP and sequencing analyses for *Cryptosporidium* spp. A modification of Boom's method (Boom *et al.*, 1990), as previously described by McLauchlin *et al.* (1999), was used to extract nucleic acids from faecal samples. Briefly, 200 µl of the sample were homogenized with zirconia beads using a Mini-Bead-Beater-8™ (BioSpec Products, Inc., Batlesville, Oklahoma, USA) in guanidinium thiocyanate solutions. Nucleic acids released from disrupted oocysts were purified with coarse activated silica

suspensions (Severn Biotech, Ltd., Kidderminster, UK), and stored at -20°C until use. A fragment of 341 bp from the *Cryptosporidium* oocyst wall protein (COWP) gene was amplified by a nested PCR procedure, with the PCOWPF/PCOWPR and PCOWPIF/PCOWPIR primers in the first and second phase respectively, as described elsewhere (Gómez-Couso *et al.*, 2004). DNA extracted from isolates of *Cryptosporidium parvum* and *C. meleagridis*, and water instead of DNA template were used as positive and negative controls, respectively. PCR products were visualized using 2% agarose gels stained with ethidium bromide. Restriction fragment length polymorphism (RFLP) analyses were carried out with *RsaI* and *AluI* enzymes (Stratagene, La Jolla, California, USA). The RFLP analyses were performed by digesting 10 µl of PCR product with 5 U of each restriction enzyme in 1× enzyme buffer (Stratagene), in a final volume of 30 µl, for 4 h at 37°C (Gómez-Couso *et al.*, 2004). Restriction fragments were analysed by electrophoresis in 3.2% agarose gels stained with ethidium bromide. PCR products were purified with Quantum Prep® PCR Kleen Spin Columns (Bio-Rad Laboratories, Hercules, California, USA) and sequenced with the CEQ™ DTCS- Quick Start Kit (Beckman Coulter, Inc., Fullerton, California, USA) in both directions, with inner primers of the nested PCR. Sequencing reactions were analysed with a Beckman Coulter CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Inc.). Sequence data was examined by the BioEdit Sequence Alignment Editor v7.0.1 (Copyright© 1997-2004 Tom Hall, Ibis Therapeutics, Carlsbad, California, USA), and Chromas v2.3 (Copyright© 1998-2004 Technelysium Pty Ltd, Tewantin Qld, Australia) programmes. The resulting sequences were compared with those of *Cryptosporidium* spp. deposited in the GenBank.

Results

The therapeutic and prophylactic administration of different drugs provided only slight improvement of clinical signs, and did not prevent the appearance of the disease in subsequent hatches, or reduce the rate of mortality.

Autopsy of dead 11-day-old partridges revealed macroscopic thickening of the small and large intestine walls and inflammation and brownish-yellow contents of the caecum. Similar intestinal contents were observed in the 25-day-old chicks, in addition to slight congestion of the lungs, although the other organs were of normal appearance.

Microscopic examination revealed numerous small, round parasitic structures, 4 to 6 μm in diameter, suggestive of *Cryptosporidium* spp., in the epithelial brush border of respiratory and intestinal samples from the four autopsied partridges (Figure 1). Occasionally epithelial hyperplasia with moderate heterophilic infiltration was also seen.

The bacteriological studies showed the presence only of *Escherichia coli* at intestinal level, which was possibly unrelated to the case given its sensitivity to the antibiotics tested. No *Mycoplasma* organisms were isolated nor were any viruses isolated in SPF eggs or fibroblast monolayers, after three consecutive blind passages. The tests for rotavirus type A by the ELISA capture technique were also negative.

Faecal samples analysed by the saturated sugar flotation technique did not reveal parasitic forms but the ELISA for the antigenic diagnosis of *Cryptosporidium* gave positive results in all the pools analysed (net optical densities > 0.150). Finally, the application of PCR-RFLP techniques (Figure 2) and sequencing of a COWP gene fragment confirmed the existence of *C. meleagridis* in faecal samples, with a sequence showing 100% similarity to the AF266266 sequence deposited in the GenBank.

Biosecurity procedures introduced before the next reproductive season gave good results and no other problems were recorded on this farm.

Discussion

For many years avian cryptosporidiosis has been considered to be an opportunistic disease. However, new data indicate that this parasitic disease is a serious problem in the poultry industry (de Graaf *et al.*, 1999). In chickens and other Galliformes, cryptosporidiosis is mostly manifested as a respiratory disease, although enteric disease has also been reported in turkeys and quails. Cryptosporidiosis is highly prevalent in chicken flocks (Sréter & Varga, 2000), and its pathogenicity increases when the infection coexists with immunosuppressive or respiratory agents, even when birds are vaccinated against other pathogens (de Graaf *et al.*, 1999).

The present report is the first description of an outbreak of disease associated with *C. meleagridis* in red-legged partridge reared on a game farm. The association was confirmed by the application of PCR-RFLP techniques and the sequencing of a COWP gene fragment from faecal samples, which provided a nucleotide sequence showing 100% similarity to *C. meleagridis*. The findings of the present study also extend the range of hosts of *C. meleagridis*, the potential aetiological agent responsible for high mortality during the first weeks of life of these birds. The pathogen may have contributed to the repeated disappearance of partridge flocks during their natural breeding period, as reported by rangers and gamekeepers, who have detected high mortality among recently hatched birds.

The observation of *Cryptosporidium*-like bodies in histological sections of the respiratory tract, in addition to the positive results obtained by ELISA with pools of

homogenized samples from trachea, bronchi and lung and the clinical symptoms of cough and nasal discharge observed in a small number of birds suggest a co-infection with *C. baileyi*. However, it could be possible that *C. meleagridis* spreads to the respiratory tract as may happen with *C. parvum* during the course of severe infections (Mascaró *et al.*, 1994; Hunter & Nichols, 2002).

Avian cryptosporidiosis can be spread via several different routes of transmission. Drinking water may serve as a primary route of distribution of oocysts among poultry flocks. Since avian *Cryptosporidium* species can infect a wide variety of birds, wild birds may play an important role in the epidemiology of cryptosporidiosis. Moreover, since some rodents also appear to be susceptible to *C. meleagridis* under experimental conditions, it is thus possible that mice, voles, and rats could also serve as carriers (see Sréter & Varga, 2000).

It is also important to consider that *C. meleagridis* may infect and cause cryptosporidiosis in farm personnel as shown with *C. parvum* and *C. hominis* (McLauchlin *et al.*, 2000). Moreover, infection with *C. meleagridis* may have a similar prevalence in humans to that of *C. parvum*, as found in Peru (Cama *et al.*, 2003).

In view of the detection of cryptosporidia in abnormal organs at necropsy and the detection by PCR-RFLP and sequence analyses of *C. meleagridis* in faecal samples, we believe that *Cryptosporidium* spp. could account for this outbreak of disease, although we cannot completely exclude the possibility other pathogens/non-infectious causes of morbidity and mortality. To do this it would be necessary to examine a greater number of samples and to carry out more extensive tests to rule out other possible causes. For example, electron microscopy on faecal samples for rotaviruses and other enteric viruses would have been helpful, especially since the ELISA used here only detects group A rotaviruses.

In view of the important economic losses associated with avian cryptosporidiosis and the findings associated with the outbreak described herein, we consider that tests for cryptosporidia should be included when investigating respiratory and enteric disease in farmed birds.

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Figure 1. *Intestinal section of red partridge (Alectoris rufa) stained with haematoxylin-eosin showing development stages of Cryptosporidium sp. in the brush border.*

Figure 2. PCR-RFLP of a 341 bp fragment of the *Cryptosporidium* oocyst wall protein gene using both *AluI* and *RsaI* restriction enzymes. Lanes M, 100 bp molecular ladders; lanes 1 and 2, *Cryptosporidium* isolates from red partridge (*Alectoris rufa*); lane 3, *C. parvum* control; lane 4, *C. meleagridis* control.