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Clinical Report

Necrotic Enteritis Caused by *Clostridium perfringens* in Blue and Gold Macaws (*Ara ararauna*)

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Abstract: Clostridium perfringens types A and C, which are gram-positive, anaerobic, spore-forming bacteria, can cause necrotic enteritis in birds. Although *Clostridium perfringens* is considered a commensal organism in the avian intestinal tract, in association with severe stress, other infectious agents, or immunosuppressive conditions, it can cause disease outbreaks. This report describes a disease occurrence of necrotic enteritis caused by *C perfringens* in macaws (*Ara ararauna*). Two adult male blue and gold macaws maintained in a zoo exhibit were presented for postmortem examinations after histories of sudden death. Based on the gross examinations and microscopic evaluation of submitted tissue from both birds, the cause of death was determined to be necrotic enteritis. Microbiologic assays followed by polymerase chain reaction analyses identified the isolated strains as *C perfringens* type A, indicated by only being positive for the *cpa* gene that encodes the α-toxin. The birds were maintained in an exhibit in which patrons can interact with the animals within their environment. Thus, organisms, such as this pathogen, may present a danger for other birds because visitors could disperse the bacterium to other parts of the zoo.

Key words: endotoxemia, *Clostridium perfringens*, clostridiosis, microbiology, molecular assay, zoo, avian, psittacine birds, blue and gold macaw, *Ara ararauna*

Clinical Report

Two adult male blue and gold macaws (*Ara ararauna*) maintained in a zoo exhibit died without premonitory signs and were subsequently presented frozen for necropsy, to the Pathology Department of São Paulo State University (School of Agricultural and Veterinarian Sciences, Jaboticabal, Brazil). The birds lived with 26 other macaws in a walk-through zoo exhibit in which patrons can interact with the animals. The birds weighed 1.040 kg (bird 1) and 1.030 kg (bird 2). The birds died within 3 days of each other. There was no previous history of illness, and the macaws had no direct contact with other birds or domestic mammals. Although not referred for necropsy, one other bird from the same enclosure had died days before under similar circumstances as birds 1 and 2.

During the postmortem examination, specimens were obtained from a range of tissues, fixed in 10%-buffered formalin for 24 hours, dehydrated, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin for histopathologic analyses.[1] The slides were analyzed and photographed using a BX-51 light microscope (Olympus, Miami, FL, USA). In addition, several necrotic intestinal fragments were collected from each bird for bacterial culture. The fragments were collected in a glass tube with 10 mL of sterile, freshly prepared brain-heart infusion broth (Difco, Leeuwarden, the Netherlands) and incubated at 37°C for 24 hours under anaerobic conditions.

Total DNA was extracted from the bacterial culture by boiling[2] and screened for *Clostridium perfringens* by polymerase chain reaction (PCR)

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Table 1. Target gene, related toxin, oligonucleotide primer sequences and length of amplification fragment in *Clostridium perfringens* multiplex PCR. Modified from Bauns, 2004.[3]

Gene	Toxin	Oligonucleotide primers, 5′–3′	Fragment, approximate bp
cpa	α	AGT CTA CGC TTG GGA TGG AAT TTC CTG GGT TGT CCA TTT C	900
cpb	β	TCC TTT CTT GAG GGA GGA TAA ATG AAC CTC CTA TTT TGT ATC CCA	611
cpe	entero	GGG GAA CCC TCA GTA GTT TCA ACC AGC TGG ATT TGA GTT TAA TG	506
etx	ε	TGG GAA CTT CGA TAC AAG CAT TAA CTC ATC TCC CAT AAC TGC AC	396
iap	γ	AAA CGC ATT AAA GCT CAC ACC CTG CAT AAC CTG GAA TGG CT	293

Abbreviation: bp indicates base pairs.

assays to identify the *cpa* gene, present in all *C perfringens* strains. Positive samples underwent multiplex PCR to identify other possible genes: *cpb*, *etx*, *iap*, and *cpe*[3] (Table 1). The multiplex PCR assays were conducted in 20 μL total reaction volumes containing 60 ng of target DNA, 0.2 mM mixed deoxynucleotide triphosphates (Life Technologies, Carlsbad, CA, USA), 2.0 mM $MgCl_2$ (Life Technologies), 1.0 U Taq DNA polymerase (Life Technologies), 5.0 pmol of each primer (Síntese Biotecnologia, Belo Horizonte, MG, Brazil), 1× buffer (50 mM KCl, 200 mM Tris-HCl, pH 8.4), and sterile ultrapure water (Promega, Madison, WI, USA). The cycling conditions were an initial denaturation for 3 minutes at 94°C (201.2°F); 35 cycles of 30 seconds at 94°C (201.2 °F), 30 seconds at 55°C (131°F), and 40 seconds at 72°C (161.6°F), and a final extension for 10 minutes at 72°C (161.6°F). The PCR reactions were performed in a conventional thermocycler (Veriti Thermal Cycler, Applied Biosystems, Foster City, CA, USA). Reference strains for *C perfringens* types A, B, C, D, and E, obtained from Oswaldo Cruz Foundation (Fiocruz, Manguinhos, RJ, Brazil), were included as positive controls. The PCR products were separated by electrophoresis on 1% agarose gels stained with ethidium bromide (Life Technologies) and identified using a 1-kb DNA ladder.

During the postmortem examination, performed 12 days after death, bird 1 had ulcerated areas covered with yellow fibrillary material along the mucosa of the small intestine. Extraintestinal lesions involved reddening of the skin, heavy wet lungs, fluid in the pericardial sac, and splenic pallor. All lesions were consistent with bacteremia and endotoxemia. Similar to bird 1, bird 2 also had areas covered with yellow fibrillar material along the mucosa of the small intestine, which was interpreted as ulcers. In addition, the liver was attached to the air sac by fibrillar material (fibrin), and the gastric mucosa was hyperemic. Micro-

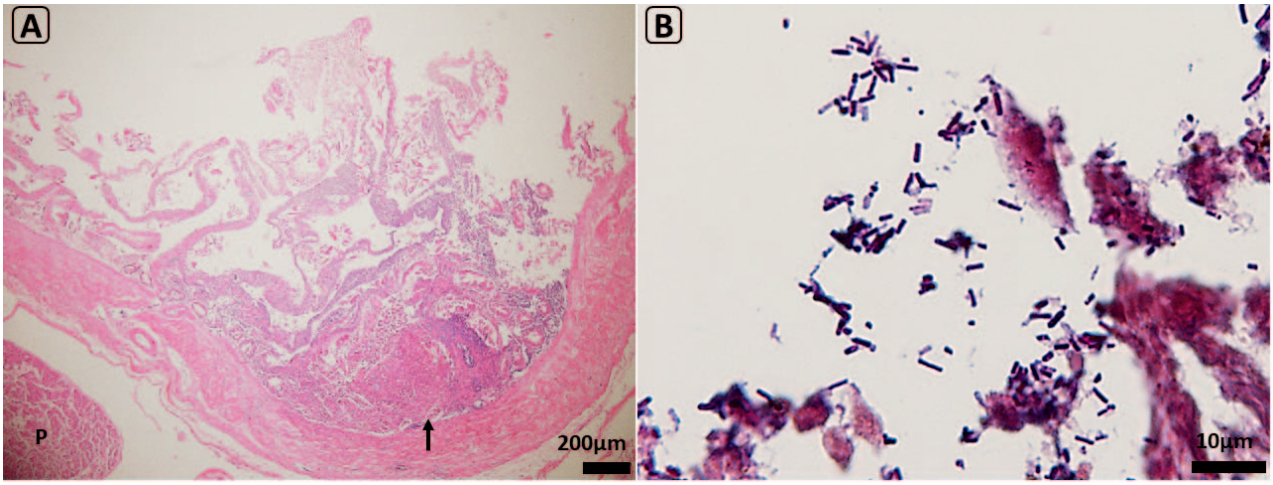

Figure 1. Photomicrographs of the intestines of an adult male blue and gold macaw (bird 1) bird 1, an adult male blue and gold macaw that was housed in a walk-through zoo exhibit. (A) Fibrinonecrotic enteritis with ulcerated areas and a loss of the characteristic villous architecture (arrow), P represents the pancreas (hematoxylin and eosin; bar = 200 μm); (B) Gram-positive, rod-shaped bacteria (*Clostridium perfringens*) in the necrotic lesions, area identified with arrow in (A) (gram stain; bar = 10 μm).

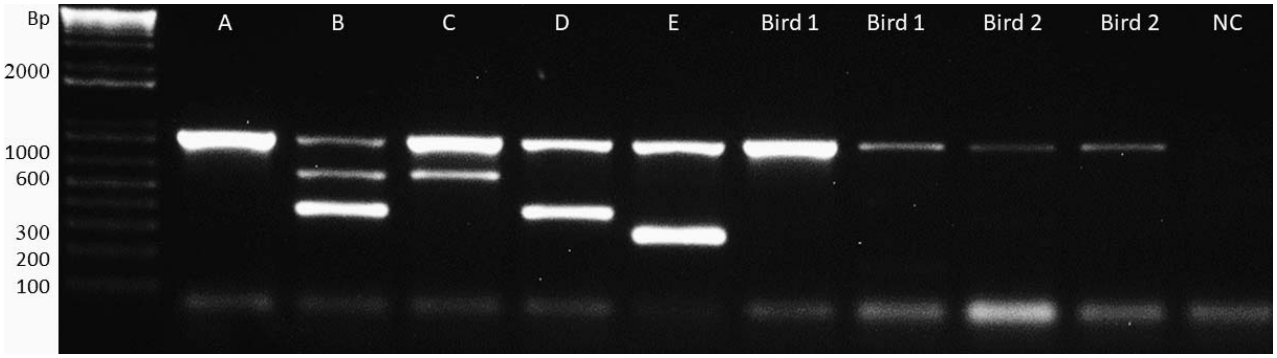

Figure 2. Agarose gel showing the polymerase chain reaction products resulting from amplification reactions for *Clostridium perfringens* based on the *cpa*, *cpb*, *etx*, *iap*, and *cpe* genes. Samples were taken from 2 adult male blue and gold macaws that were housed in a walk-through zoo exhibit. Column 1 base pair (bp): 1-kb DNA Ladder Plus (Invitrogen); columns A–E: positive controls for *C perfringens* types A, B, C, D, and E, respectively; and columns birds 1 and 2: positive results for the *cpa* gene for the 2 macaws. NC indicates negative control.

scopically, the intestinal tract of bird 1 had an ulcerated mucosa with an accumulation of cellular debris admixed with a large number of gram-positive rod bacteria (Fig 1). Extraintestinal lesions included fibrinous capsulitis in the spleen, parabronchial edema, glomerulopathy, and a congestion of blood vessels in the brain. Bird 2 presented with similar findings and severity of lesions. In addition, bird 2 had moderate multifocal splenic necrosis, accentuated renal tubular degeneration, and the presence of 3 parasitic cysts in the cardiac musculature that did not appear to be related to the clostridial infection. Neither of the birds presented with coccidiosis. Within 24 hours, the bacterial cultures from both birds confirmed the presence of *C perfringens*. These bacterial isolates were subsequently assayed with PCR technology, which found they were positive only for the *cpa* gene (Fig 2).

Discussion

Clostridium perfringens, a gram-positive, anaerobic, spore-forming bacteria, is the etiologic agent of necrotic enteritis.[4] Necrotic enteritis affecting chickens was first reported in 1961.[4] The *C perfringens* strains are classified into 5 toxinotypes (A, B, C, D, and E) based on the production of four major toxins (α, β, ε, and ι), which are the major virulence factors of the agent.[4,5] α-Toxin is expressed by all the toxinotypes (A–E), and the differentiation between types is based on the concomitant production of the other toxins (Table 2).[5] Toxinotypes A and C are described as being responsible for the development of necrotic enteritis in poultry.[4]

Table 2. *Clostridium perfringens* classification based on the production of 4 major toxins named α, β, ε, and ι.

C perfringens type	Toxin
A	α, entero[a]
B	α, β, and ε
C	α, β
D	α, ε
E	α, ι

[a] Variable: toxin responsible for the symptoms of *C perfringens* type A, not obligatory for classification.

In this case, based on multiplex PCR results, the *C perfringens* isolates were classified as type A because the samples were only positive for the *cpa* gene, which is responsible for producing the α-toxin. The fact that the *cpe* gene was not positive in these samples does not negate the type because the *cpe* is an expression gene and not obligatory for classification. The α-toxin is considered the major virulence factor in the pathogenesis of necrotic enteritis.[4] In poultry, certain factors can result in enteric dysbiosis and the proliferation of the organism, increasing toxin production and fatal endotoxemia induction.[6] Detecting the toxin types and subtypes is important because they help to better understand the infection epidemiology and may be helpful in developing preventive measures.[3] Isolating the agent from clinical cases without further typing of the strains for toxin production has little significance because *C perfringens* can be present in the healthy intestine.[7]

Previous studies indicate that *C perfringens* is a common, natural inhabitant of the poultry intestinal tract, although infections associated with *C perfringens* are also well recognized in domestic poultry.[7] In addition, the bacteria can be widespread in the environment such as in poultry feces,

soil, and open water.[4,8,9] The results from studies reporting on the incidence of *C perfringens* in the intestinal contents of broiler chickens have varied from 75% to 95%.[10–12] *Clostridium perfringens* has also been described in the droppings of wild birds near broiler-chicken houses, in which a high number of spores (4%–52% of the samples) have been observed.[13] The incidence of these bacterial enteropathogens in wild birds near broiler-chicken houses suggests that wild birds that gain entry to poultry grow-out houses have the potential to transmit these pathogens to the poultry[13] and vice-versa. Necrotic enteritis associated with *C perfringens* has been found sporadically in wild birds (eg, crows [*Corvus macrorhynchos*] in Japan).[14] Regarding psittacine birds, clostridial enteritis has already been described in captive and free-living birds, including lorikeets (*Trichoglossus* and *Eos* species), great-billed parrots (*Tanygnathus megalorynchos*), and blue and yellow macaw.[15–18]

The bacteria do not pose a danger when present in the intestines in small numbers because they are an expected part of healthy intestinal flora.[12] However, in the presence of cofactors such as some dietary ingredients, severe stress, other infectious agents, or immunosuppressive infections (eg, infectious bursal disease, chicken infectious anemia), it can cause disease outbreaks in many avian species,[6] especially birds with well-developed caeca. Intestinal damage caused by coccidial pathogens is the most important predisposing factor to clostridiosis in poultry.[4,7] Intestinal mucosal damage promoted by the parasite, along with increased mucus production, increased transit time, and a reduction in the local pH, allows for the establishment and proliferation of the bacterium.[19] Several studies have reported the presence of coccidial pathogens in poultry associated with necrotic enteritis outbreaks caused by *C perfringens*.[20,21]

An acute clinical infection in poultry is characterized by a sudden onset of high mortality. Macroscopically, the intestines are often friable and distended with gas, and the mucosa is often covered with a yellow-to-green diphtheritic membrane, frequently referred to as a *pseudomembrane*.[4] In addition to intestinal necrosis, the infection can also be associated with hepatitis or cholangiohepatitis.[22] In the subclinical form, damage to the intestinal mucosa leads to decreased digestion and absorption.[4]

Necrotic enteritis is a disease considered typical of juvenile poultry, rather than adults,[4] unlike the observations in this report, in which the disease was diagnosed in 2 adult birds. Nevertheless, the macroscopic and microscopic findings observed here are compatible with *C perfringens*–associated necrotic enteritis and enterotoxemia, and the lesions were similar to those previously described in macaws.[18]

The diagnosis of necrotic enteritis is usually based on typical gross and microscopic lesions, along with the isolation and identification of the causative agent. *Clostridium perfringens* can be isolated by anaerobically culturing the intestinal contents and intestinal mucosal scrapes. Immunodiagnosis (enzyme-linked immunosorbent assay) and molecular biology (PCR) techniques can also be applied to identify the bacterium in the samples.[4]

In this report, the source of the disease occurrence was not determined. These birds were maintained in an exhibit in which people were constantly walking through, which could represent a constant stress factor. In addition, the circulation of visitors could increase the likelihood of spreading *C perfringens* to other parts of the zoo, presenting a threat for wild free-living birds and for birds in other enclosures. Disease management should focus on controlling factors that expose birds to stress, infectious disease, and adverse dietary factors, such as feed changes or dietary inclusion of cereal grains (eg, wheat, barley, rye), increased animal protein, and decreased dietary fiber.[4] Neither of the birds presented with coccidiosis, which has been reported by other authors to increase the incidence of *C perfringens* necrotic enteritis.[16] Subclinical and mildly infected birds can serve as reservoirs and produce a large number of clostridia, and therefore, early detection and treatment are necessary to prevent seeding the environment.[4] The control of microorganisms can be very difficult in zoos and animal parks, in which the enclosures are established to provide more-natural environments with vegetation and substrate. Inadequate environmental hygiene can contribute to disease occurrence, especially for those species that feed on fruits, vegetables, or on the ground because the bacterium is widely distributed and highly resistant in soil.[4]

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