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**Disseminated chlorellosis in a dog**

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**Running title:** Canine chlorellosis

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18

19 **Abstract.** An adult dog with ataxia and a lingual mass, previously diagnosed as  
20 protothecosis, was euthanized. At postmortem examination, the lingual mass,  
21 regions of the lungs and hilar lymph nodes, liver, mesenteric and sublumbar  
22 lymph nodes, and the spinal meninges had pronounced green discoloration.  
23 Histologically, pyogranulomatous inflammation and algal organisms were found  
24 in the tongue, spinal meninges, hilar and mesenteric lymph nodes, liver, and  
25 lung. The algae had PAS-positive cell walls and cytoplasmic granules.  
26 Ultrastructurally, the algae had a well-defined cell wall, stacks of grana and  
27 thylakoid membrane, and dense bodies, typical of starch granules. The  
28 organisms were identified as *Chlorella*, a green alga, based on the results of  
29 histochemistry and electron microscopy. This is the first report of disseminated  
30 *Chlorella* infection and the first report in a companion animal.

31

32 **Key words:** Algae; canine diseases; *Chlorella*; dog; meningitis.

33

34 A 9-year-old neutered female Golden Retriever dog developed progressive  
35 paraparesis and hind-limb ataxia of 1 week's duration. Six months previously, a  
36 mass on the tongue was diagnosed as granulomatous glossitis with algal  
37 organisms, suspected to be *Prototheca*. The dog also had recent weight loss,  
38 dysuria, halitosis, and cough, with rapid deterioration during the week preceding  
39 this clinical episode.

40 A similar transient hind-limb ataxia that developed 3.5 years previously was  
41 attributed to coccidioidomycosis, based on a positive serum titer by agar gel  
42 immunodiffusion. Thereafter, fluconazole had been administered through the  
43 recent illness. Nevertheless, the dog remained seropositive for *Coccidioides*  
44 *immitis*, with a titer of 1:16 at 3 months before presentation. Serum biochemical  
45 abnormalities included hypoalbuminemia (2.4 g/dL; reference range, 2.5-4.0  
46 g/dL) and hyperglobulinemia (4.6 g/dL; reference range, 2.1-4.5 g/dL).

47

48 On physical examination, the dog was thin with a crouched hind limb posture,  
49 requiring assistance to walk. It exhibited discomfort in response to palpation over  
50 the lumbosacral spine. The tail was flaccid and hypesthetic; anal tone was  
51 reduced; the urinary bladder was distended with urine, but easily expressed. An  
52 irregularly shaped, raised, green mass was in the dorsal aspect of the base of  
53 the tongue.

54

55 Using magnetic resonance imaging (MRI), an extradural mass surrounded and  
56 compressed the L4-L6 segments of the spinal cord. The mass was iso- to hyper-  
57 intense on T2-weighted images, with heterogeneous enhancement of extradural  
58 tissue by gadolinium-enhanced post-contrast T1-weighted imaging. Similar  
59 enhancement occurred in the adjacent lumbar epaxial musculature and in the  
60 lamina and pedicle of the fourth and fifth lumbar vertebrae.

61 The dog was treated with fluconazole, cephalexin, and prednisone. Although its  
62 condition improved slightly over the next 4 days, the owner requested euthanasia  
63 7 days after presentation.

64

65 Grossly, an irregularly shaped, dark green, ulcerated mass elevated the dorsal  
66 surface of the base of the tongue (Fig. 1) and extended into underlying lingual  
67 tissue. The lumbar epaxial musculature was infiltrated by soft, poorly demarcated  
68 greenish exudate that was contiguous with similar exudate in the vertebral canal.  
69 The exudate was loosely adhered to the dura mater along 4 vertebral segments.  
70 Cross sections of spinal cord demonstrated involvement of external and internal  
71 surfaces of the dura mater (Fig. 2). Sublumbar, mesenteric, and hilar lymph  
72 nodes were enlarged and green. Slight patchy green discoloration was also  
73 present on visceral pleura and in pulmonary parenchyma as well as on the  
74 hepatic capsule and in hepatic parenchyma.

75

76 Samples of liver, lung, hilar and sublumbar lymph nodes, tongue, and lumbar  
77 spinal cord were fixed in 10% neutral buffered formalin, paraffin embedded, and  
78 stained with hematoxylin and eosin (HE) for light microscopic examination.  
79 Histochemistry on tongue and spinal cord sections included periodic acid-Schiff,  
80 Gomori methenamine silver-HE, Brown and Hopps tissue Gram stain, and  
81 Gridley fungal stain.

82

83 Histologically, sections of the affected spinal cord had intense pyogranulomatous  
84 inflammation with myriad organisms in the subarachnoid space and on the  
85 exterior surface of the dura mater. The inflammation extended only slightly into  
86 the perivascular spaces of the spinal cord and was composed mainly of  
87 neutrophils and macrophages with fewer lymphocytes and plasma cells.  
88 Individual organisms were most often free in the exudate, but some were in the  
89 cytoplasm of macrophages and multinucleated giant cells. The organisms were  
90 round with a well-defined, narrow cell wall. Single large sporangia (Fig. 3), 7-25  
91  $\mu\text{m}$  in diameter, were mixed equally with compartmentalized organisms of equal  
92 or slightly larger diameter that contained a variable number of morula-like  
93 sporangiospores (Fig. 3). Eukaryotic nuclei were frequently observed in the  
94 organisms. Spinal nerve roots that ran through the inflamed segment of  
95 meninges contained swollen axons or empty axon sheaths, consistent with  
96 Wallerian degeneration. Organisms, particularly sporangiospores, had numerous  
97 strongly periodic acid-Schiff (PAS)-positive cytoplasmic granules that were PAS-  
98 negative after diastase treatment. The cell walls were also PAS-positive, but this  
99 feature was more easily observed in less granular sporangia (Fig. 4). Organisms  
100 stained poorly with Gram's stain, but the cell walls were weakly positive with  
101 Gomori methenamine silver-HE (Fig. 3). Similar microscopic lesions were found  
102 in the mesenteric lymph node, liver, lung and tongue. No evidence of  
103 coccidioidomycosis was found on histologic examination.

104

105 Meningeal samples were fixed in formalin then divided into 1-mm cubes,  
106 transferred to 2% glutaraldehyde-2% paraformaldehyde in 0.1 M cacodylate  
107 buffer, post-fixed in 1% osmium tetroxide and embedded in Epon-Spurr's  
108 medium. Thin sections were stained with uranyl acetate and lead citrate, and  
109 examined with a JEOL 1400 electron microscope.

110

111 Ultrastructurally, the organisms were identified as algae. Individual  
112 sporangiospores had well-defined cell walls that were closely associated with a  
113 cell membrane (Fig. 5 and Fig. 6). The outer wall of the sporangium had similar  
114 structure, but was more wrinkled, probably an artifact of fixation. The cytoplasm  
115 of individual organisms had numerous polyhedral electron-dense starch granules  
116 with adjacent stacks of membranes (Fig. 6), consistent with the ordered grana  
117 and less ordered thylakoid membranes of chloroplasts. The ultrastructural  
118 features were those of a photosynthetic alga, with characteristics of *Chlorella*.  
119 Confirmatory isolation was not attempted.

120

121 Green algal infection of animals was first reported as the cause of green hepatitis  
122 and lymphadenitis in a slaughtered lamb.<sup>3</sup> Since the initial report, *Chlorella* sp.  
123 infections have been reported rarely in various herbivorous species, fish, and a  
124 human.<sup>3,7-9,16,18,19,22</sup> Many animal cases were incidentally detected during meat  
125 inspection at abattoirs.<sup>18,22</sup> Collectively, lymph nodes, liver, lung, skin, and  
126 intestines were involved.<sup>3,8,9,12,16,18,19,22</sup> Chlorellosis has not been reported in  
127 dogs before and never as a cause of dysfunction of the central nervous system.

128

129 *Chlorella* is a chlorophyll-containing green alga closely related to *Prototheca*,  
130 which is thought to be its achlorophyllous mutant.<sup>1,6,15</sup> *Chlorella* is unique among  
131 algae in that a single large chloroplast dominates its cytoplasm.<sup>21</sup> Unlike  
132 *Prototheca*, *Chlorella* spp. contain numerous starch granules that are strongly  
133 PAS-positive.<sup>1,3,7-10,12,15,16,18,19,22</sup> The granules become PAS-negative following  
134 diastase digestion,<sup>11</sup> and under polarized light are birefringent in unstained or  
135 HE-stained sections.<sup>1</sup> *Prototheca* lacks chloroplasts or starch granules,  
136 although smaller related organelles, protoplasts, are present. Therefore, only the  
137 cell wall of *Prototheca* is PAS-positive.<sup>1</sup>

138

139 The life cycles of *Chlorella* and *Prototheca* are similar.<sup>1,15</sup> Both produce hyaline  
140 cells (sporangia) that mature to produce asexually 2-20 sporangiospores  
141 (endospores). After endosporulation, the outer sporangial wall ruptures,  
142 releasing the sporangiospores to repeat the cycle.

143

144 Light microscopy demonstrates similarities between *Chlorella* and *Prototheca* in  
145 shape, size, wall and endosporulating reproductive mode. Because chlorophyll  
146 dissolves during fixation and embedding, the green color is lost with light  
147 microscopy.<sup>1</sup> Wet mounts or Giemsa-stained smears are needed in order to  
148 observe the bright green pigment granules of *Chlorella*.<sup>22</sup> The finding of  
149 chloroplasts with electron microscopy supports a diagnosis of algal infection.

150

151 The dog in this report had lesions in the tongue, lymph nodes, lung, liver, spinal  
152 meninges and epaxial muscle. This disseminated infection is more extensive  
153 than that previously reported for chlorellosis in any species. Lymph nodes, lung  
154 and liver have been target organs of infection in sheep and cattle.<sup>3, 9,16,18,22</sup> The  
155 primary portal of entry in cases of disseminated chlorellosis is often thought to be  
156 the gastrointestinal tract.<sup>12,16,22</sup> A single portion of jejunum obtained from the dog  
157 of this report was not affected; it is possible that additional sections may have  
158 revealed algae. Secondary hematogenous dissemination and lymphatic  
159 drainage were thought to account for the localized visceral lesions in previous  
160 cases.<sup>12,16,22</sup>

161

162 Algae were detected in the tongue of this dog 6 months before the onset of  
163 neurological signs. This protracted course is not unusual.<sup>13</sup> Dogs with  
164 disseminated protothecosis often have a chronic history of intermittent bloody  
165 diarrhea 1-10 months before ocular or neurological signs develop.<sup>5,6,13,20</sup> It is  
166 possible that the lingual lesions were the primary portal of entry, leading to  
167 secondary hematogenous and lymphatic dissemination. Alternatively, another  
168 primary lesion on the surface of the tongue could have allowed secondary  
169 invasion of *Chlorella*. An analogous situation occurred in the single human  
170 *Chlorella* infection, in which a surgical wound was exposed to contaminated river  
171 water.<sup>8</sup>

172

173 It is speculated that defective cell-mediated immunity is responsible for some  
174 cases of disseminated protothecosis in dogs.<sup>2,4,14,17,20</sup> Immunosuppressive  
175 medications and neoplasia are often cited as predisposing factors. Evidence of  
176 heavy environmental exposure is also frequently present.<sup>9,12,16</sup> Both *Chlorella*  
177 and *Prototheca* are ubiquitous, with *Prototheca* spp. isolated from diverse  
178 environmental sources including tree sap, fresh and marine water, potato skins,  
179 fingernails, sludges and animal feces.<sup>9,12,13,15,20</sup> *Chlorella* is considered an  
180 opportunistic pathogen, often requiring immunosuppression, wound inoculation,  
181 or heavy exposure to organisms for infection to occur.<sup>3,12</sup> Cases of mammalian  
182 chlorellosis are much less frequent than protothecosis.

183

184 Sheep with chlorellosis had prior access to stagnant water that was covered by  
185 mats of bright green algae.<sup>9</sup> However, the dog of this report was kept mainly  
186 indoors and had no known contact with stagnant water. Undetected primary  
187 immunodeficiency or immunosuppression secondary to chronic *Coccidioides*  
188 *immitis* infection are potential contributing factors for the infection in this dog.  
189 Immunosuppression in humans can predispose to coccidioidomycosis,<sup>4</sup> but no  
190 evidence of active coccidioidomycosis was found at postmortem in this dog. The  
191 failure of dogs with disseminated protothecosis to respond to treatment<sup>2</sup> may  
192 reflect a delay in commencement of therapy and/or underlying immunodeficiency.

193

194 This case demonstrates the potential of *Chlorella* organisms to infect dogs and  
195 cause widely disseminated disease. Although rare, disseminated chlorellosis

196 should be considered, particularly when lesions have a green discoloration.  
197 *Chlorella* should be differentiated from *Prototheca* by cytochemical or  
198 ultrastructural means.

199

200 **Acknowledgements.** The authors gratefully thank Ms. Cheryl Jensen and Mr.  
201 Howard Wilson for assistance with electron microscopy and preparation of  
202 illustrations, respectively. The patience of technicians in the VMDL  
203 histopathology laboratory in performing histochemistry is also appreciated.

204

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266 **Figure Legends**

267

268 **Fig. 1.** Tongue; dog. An irregular, focally ulcerated, slightly raised green mass is  
269 at the base of the tongue. Bar = 1 cm.

270 **Fig. 2.** Lumbar spinal cord; dog. Exudate with green tint is present inside and  
271 outside the dura mater, extending into adjacent soft tissues. Prolonged formalin  
272 fixation has reduced the green color. Bar = 0.5 cm.

273 **Fig 3.** Meningeal exudate; dog. Algal organisms are in pyogranulomatous  
274 exudate. Numerous sporangia (arrowheads) and morula-like endosporulating  
275 organisms (arrows) have well-defined cell walls. Gomori methenamine silver-HE  
276 stain. Bar = 30  $\mu\text{m}$ .

277 **Fig 4.** Meningeal exudate; dog. Sporangiospores (arrows) and, to a lesser  
278 extent, sporangia (arrowheads) contain many PAS-positive granules. PAS  
279 reaction. Bar = 30  $\mu\text{m}$ .

280 **Fig 5.** Meningeal exudate; dog. A sporulated alga, surrounded by host  
281 macrophages (m), contains electron-dense, angular starch granules. Lead citrate  
282 and uranyl acetate. Bar = 2  $\mu\text{m}$ .

283 **Fig 6.** Algal sporangiospore, meningeal exudate; dog. The cell wall (W) is  
284 present peripherally. Stacks of grana and thylakoid membrane (T) occur between  
285 starch granules (S) within the chloroplast. Lead citrate and uranyl acetate. Bar =  
286 0.2  $\mu\text{m}$

287

