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1 **Coinfection with *Entamoeba polecki* and *Brachyspira hyodysenteriae* in a pig with severe**
2 **diarrhea**

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4 Maria Cuvertoret-Sanz, Christiane Weissenbacher-Lang, Madeleine Lunardi, René Brunthaler,
5 Mònica Coma, Herbert Weissenböck, Joaquim Segalés¹

6
7 Servei de Diagnòstic de Patologia Veterinària (SDPV), Departament de Sanitat i d'Anatomia
8 Animals, Universitat Autònoma de Barcelona (Cuvertoret-Sanz, Segalés), UAB, Centre de
9 Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la Universitat Autònoma de
10 Barcelona (Segalés), Barcelona, Spain; Institute of Pathology and Forensic Veterinary Medicine,
11 University of Veterinary Medicine Vienna, Vienna, Austria (Weissenbacher-Lang, Lunardi,
12 Brunthaler, Weissenböck); Cooperativa Plana de Vic, Barcelona, Spain (Coma).

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14 ¹Corresponding author: Joaquim Segalés, UAB, Centre de Recerca en Sanitat Animal (CReSA),
15 IRTA-UAB, Campus de la Universitat Autònoma de Barcelona, Spain. joaquim.segales@uab.cat

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17 Running head: *Entamoeba* and *Brachyspira* coinfection in a diarrheic pig

19 **Abstract.** Enteric disease in pigs is usually of multifactorial etiology, including infectious and
20 non-infectious factors. In many cases of endemic diarrhea in weaner-to-finisher pigs, the
21 combination of 2 or more microorganisms leads to aggravation of intestinal lesions and,
22 consequently, clinical signs. We autopsied a 4-mo-old fattening pig with diarrhea and diagnosed
23 severe fibrinonecrotizing typhlocolitis. Numerous spiral-shaped bacteria and amoeba-like PAS-
24 positive protozoa were observed in the cecal and colonic mucosa and submucosa. *Brachyspira*
25 *hyodysenteriae* was detected by PCR from colonic content. By in situ hybridization, large
26 numbers of *Entamoeba polecki* were found within the lamina propria and submucosa; moderate
27 numbers of *Blastocystis* and scattered trichomonads were present in intestinal content. In
28 addition, *Entamoeba polecki*, *Balantidium* spp., *Blastocystis* spp., and *Trichomonas* spp. were
29 also detected by PCR.

30

31 **Key words:** *Brachyspira hyodysenteriae*; diarrhea; *Entamoeba polecki*; fibrinohemorrhagic
32 necrotizing colitis; pigs; swine dysentery.

33

34 Enteric disorders in swine are usually of multifactorial origin, including combinations of
35 microorganisms plus the concurrence of different non-infectious risk factors.⁴ Coinfection with 2
36 or more agents often causes enhanced mucosal inflammation. In addition, damage to the
37 intestinal epithelial barrier may allow the uncontrolled proliferation of other organisms that
38 would be harmless under healthy conditions.

39 Swine dysentery (SD) is one of the most severe enteric diseases of pigs. *Brachyspira*
40 *hyodysenteriae*, its etiologic agent, is a beta-hemolytic spirochete able to cause significant large
41 intestinal lesions, without the need for other coinfecting agents.⁵ However, several coinfecting
42 agents have been described in pigs affected by SD, including *Campylobacter* spp.,
43 *Fusobacterium necrophorum*, and *Bacteroides vulgatus*.^{2,6,20} Protozoan overload is also a usual
44 finding in enteric lesions, including in cases of SD.^{1,16,19}

45 We describe herein the coinfection of *B. hyodysenteriae* and *Entamoeba polecki* leading
46 to severe necrotizing lesions in colon and cecum in a diarrheic pig. A 4-mo-old, crossbred pig,
47 from a fattening unit (site 3) on an indoor pig production farm, with ongoing problems of
48 diarrhea died and was submitted to the Servei de Diagnòstic de Patologia Veterinària of the
49 Veterinary Faculty of the Universitat Autònoma de Barcelona (Spain) for diagnostic purposes.
50 The farm of origin was a 1,350 sow farm that was positive-stable for porcine respiratory and
51 reproductive syndrome virus (PRRSV), seropositive to porcine circovirus 2 (PCV-2) and
52 *Mycoplasma hyopneumoniae*, and negative to pseudorabies virus. Sows and piglets were
53 vaccinated with a modified-live PRRSV vaccine. The affected pig belonged to a batch of 450
54 grower–finishers allocated to pens with a complete slatted floor; animals received a conventional
55 finishing feed. Clinical problems were observed only in fatteners (14–16-wk-old), with ~10% of
56 animals with mucoid-red diarrhea starting 1 mo after entering the fattening unit. The field

57 veterinarian established a differential diagnostic list including porcine proliferative enteropathy,
58 SD, and colibacillosis.

59 At autopsy, the pig was emaciated and pale. Severe diffuse fibrinonecrotizing
60 hemorrhagic typhlocolitis was observed, with abundant mucous exudate and bloody fluid in the
61 lumen (Fig. 1). Nasal turbinates had severe bilateral atrophy. No other lesions were observed
62 grossly.

63 Samples of lung, spleen, liver, kidney, mandibular and superficial inguinal lymph nodes,
64 skeletal muscle, heart, stomach, ileum, colon, cecum, and brain were collected and fixed by
65 immersion in 10% buffered formalin at room temperature for 48 h before routine processing;
66 slides were stained with hematoxylin and eosin. Immunohistochemical staining to detect
67 PRRSV¹¹ and PCV-2¹⁸ was performed on lung and lymphoid tissues (tonsil, lymph nodes, and
68 spleen) as part of the investigation. In both cases, samples were negative.

69 Histologically, severe diffuse necrosis of the apical two-thirds of the colon and cecal
70 mucosa, or complete mucosal necrosis, was observed in the colon and cecum (Fig. 2). Necrotic
71 and sloughed epithelial cells were present in the lumen admixed with abundant mucus,
72 degenerate neutrophils, fibrin, and myriad rod- and spiral-shaped bacteria. The latter finding was
73 confirmed by Warthin–Starry stain, in which numerous spiral-shaped bacteria were observed
74 within the crypt lumina (Fig. 2 inset). Numerous protozoan structures suggestive of amoeba
75 trophozoites were observed free in the necrotic debris, the lamina propria, submucosa, and within
76 lymphatic vessels (Figs. 3, 4). These structures were round, 10–15 µm diameter, with a single
77 nucleus and intracytoplasmic vacuoles. Amoeba-like structures were periodic acid–Schiff (PAS)-
78 positive and Grocott-negative. No fungal structures were observed in the Grocott stain. Scattered
79 *Balantidium coli* were also seen throughout the intestinal lumen. Based on these results, further

80 microbiologic and molecular investigations were pursued to identify the lesion-associated
81 bacteria and protozoa.

82 Routine bacterial cultures were attempted for *Escherichia coli* (blood agar and
83 MacConkey agar) and *Salmonella* spp. (brain-heart infusion– and Rappaport-Vassiliadis–
84 enriched broths) on samples of ileum and colon, which yielded growth of non-hemolytic *E. coli*
85 colonies and no growth of *Salmonella* spp. DNA was extracted from 200 mg of intestinal content
86 (QIAamp DNA stool mini kit, Qiagen, Vienna, Austria). *B. hyodysenteriae*, *B. pilosicoli*, and
87 *Lawsonia intracellularis* DNA were tested by specific PCR methods^{8,9} on samples of colon
88 contents. *B. hyodysenteriae* was detected in colon, but no PCR products for *B. pilosicoli* or *L.*
89 *intracellularis* were obtained by PCR.

90 In situ hybridization (ISH) was used to probe for several protozoa (Table 1) on paraffin-
91 embedded intestinal tissue (colon) based on a previously described protocol.³ Briefly, proteolysis
92 with proteinase K (2.5 µg/mL; Roche, Basel, Switzerland) in Tris-buffered saline was carried out
93 for 30 min at 37°C. For hybridization, slides were incubated overnight at 40°C with
94 hybridization mixture and a final probe concentration of 20 ng/mL for the labeling of
95 *Blastocystis* spp. and trichomonads, and 10 ng/ml for *Entamoeba* spp. (Microsynth, Balgach,
96 Switzerland). Digoxigenin-labeled hybrids were labeled with anti-digoxigenin–alkaline
97 phosphatase Fab fragments (1:200; Roche) for 1 h at room temperature. The detection reaction
98 was carried out using the color substrates 5-bromo-4-chloro-3-inodyl phosphate and 4-nitro blue
99 tetrazolium chloride (Roche). Slides were evaluated by light microscopy using semiquantitative
100 scoring. ISH yielded positive signals for all 3 tested protozoa. Large numbers of *Entamoeba*
101 were predominantly present within the lamina propria and submucosa (Fig. 4, inset), whereas

102 moderate numbers of *Blastocystis* were exclusively located in superficial necrotic debris and
103 intestinal contents. Scattered trichomonads were confined to crypt lumina.

104 To support the ISH results, PCR to detect *Balantidium* spp., *Blastocystis* spp., *Entamoeba*
105 spp., and *Trichomonas* spp. was used (Table 2). The PCR reaction master mixture consisted of
106 12.5 µL of KAPA2G Fast HotStart ready mix with dye (Sigma-Aldrich, Vienna, Austria), 0.4
107 µM of each primer, 2 µL of template DNA, and distilled water to a total volume of 25 µL per
108 reaction. An aliquot of 10 µL of each PCR product was analyzed by gel electrophoresis using 2%
109 Tris acetate–EDTA–agarose gel. The agarose gel was stained (GelRed nucleic acid gel stain;
110 VWR, Vienna, Austria), and bands were detected (BioSens gel imaging system software;
111 GenXpress, Wiener Neudorf, Austria). PCR products of the expected sizes (Table 2) were
112 evaluated positively. Finally, PCR products were extracted (MinElute PCR purification kit;
113 Qiagen) and were submitted for Sanger DNA sequencing (Microsynth). Nucleotide sequences
114 were analyzed using a BLAST search of the GenBank database.

115 The intestinal content was PCR-positive for *Entamoeba* spp., *Balantidium* spp.,
116 *Blastocystis* spp., and *Trichomonas* spp. Sanger DNA sequencing of the *Trichomonas* spp. PCR
117 product had 100% identity to the 18S rRNA gene sequence in GenBank (accession JF742057), a
118 sequence of porcine origin with 96–97% similarity to *Trichomitus batrachorum*.¹⁶ Furthermore,
119 the PCR products had 100% identity to the 18S rRNA gene sequences of *Balantidium coli*
120 (accession GQ903678), *Blastocystis* spp. subtype 5 (accession KF410605), and *E. polecki*
121 (accession MG747668).

122 To our knowledge, *B. hyodysenteriae* coinfection with *E. polecki* associated with
123 fibrinonecrotizing typhlocolitis has not been described previously in a domestic pig. Although
124 other protozoa were found by PCR (*Balantidium coli*, *Trichomonas* spp., and *Blastocystis* spp.),

125 no tissue damage was associated with the presence of trophozoites of these agents. This was in
126 contrast with *E. polecki*, in which trophozoites were in mucosa, submucosa, and lymphatic
127 vessels of the colon wall, and were associated with severe fibrinonecrotizing inflammation.
128 Importantly, *B. hyodysenteriae* can also produce necrotizing lesions in large intestine by itself. It
129 is possible that the severity of macroscopic and microscopic lesions observed was the result of
130 the interaction between *B. hyodysenteriae* and *E. polecki*. *E. polecki* was found in the lamina
131 propria and submucosa only in areas of erosion and ulceration, suggesting that it can be an
132 opportunistic pathogen secondary to ulceration. However, the high number of trophozoites may
133 also indicate that *E. polecki*, under certain circumstances, is able to multiply extensively in
134 tissues and cause severe local damage. Immune suppression may be a contributing factor;
135 however, no significant lesions were observed in lymphoid organs, and 2 well-known
136 immunomodulating viruses—PCV-2 and PRRSV—were not found by immunohistochemistry in
137 the affected animal.

138 The proliferation of *E. polecki* may be explained by intestinal dysbiosis caused by *B.*
139 *hyodysenteriae* infection. Changes in the intestinal nutrient content caused by inflammatory
140 exudates, mucus, and blood can induce alterations in proportions of microorganisms in the
141 intestinal lumen,^{1,4,7,8,17,19} including amoebae.¹² Loss of epithelial barrier integrity secondary to
142 *B. hyodysenteriae* infection may have allowed invasion of *E. polecki* into the lamina propria,
143 submucosa, and even invasion of lymphatic vessels.

144 To date, the pathogenicity of *E. polecki* in domestic pigs has not been fully studied. There
145 are few reports of amoebiasis in the large intestine of pigs. *E. polecki* subtype 3 and *E. suis* have
146 been detected in pigs from Japan with colonic ulcerative and hemorrhagic lesions,^{13,14}
147 respectively. In the case of *E. suis*, the presence of *Brachyspira* spp. was ruled out. Interestingly,

148 *E. polecki* has been identified in the small intestine of a pig with proliferative ileitis caused by *L.*
149 *intracellularis*, and was it suggested that coinfection exacerbated the lesions.¹² Amoebae were
150 found in the injured ileum, but not the colon in that case.

151 *Balantidium* spp., *Blastocystis* spp., and *Trichomonas* spp. were also detected in colonic
152 feces. These protozoa are considered normal intestinal commensals in porcine intestine, and they
153 do not typically cause disease or intestinal lesions. However, host immunosuppression, intestinal
154 dysbiosis, or disruption of the epithelial layer can predispose to overgrowth of these intestinal
155 commensals.^{1,17,19} The 2 latter possibilities may account for the proliferation of these protozoa,
156 given that the farm used antimicrobials to control the problem (potential dysbiosis associated),
157 and SD is a well-known cause of disruption of the mucosa epithelium. In any case, only *B. coli*
158 was observed microscopically, and was limited to the colon lumen.

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218 **Table 1.** In situ hybridization protocols applied to detect genomes of different protozoa.

Protozoa	Targeted genomic region	Probe sequence (length in nucleotides)	Comments	Reference
<i>Blastocystis</i> sp.	18S rRNA	5'-ggatgtttcattaatcaagaacgaaagctaggggatc-3' (38 nt)	Cross-reactive with <i>Entamoeba</i> spp., <i>Eimeria</i> spp., <i>Sarcocystis</i> spp., <i>Cryptosporidium</i> spp., <i>Balantidium</i> spp., <i>Candida</i> spp., <i>Aspergillus</i> spp.	10
<i>Entamoeba</i> spp.	18S rRNA	5'-gatcatgaatttcacctctccc-3' (23 nt)	None	17
Trichomonadida	18S rRNA	5'-ttcgggtcgtagttccccagagccaagaact-3' (33 nt)	None	15

219

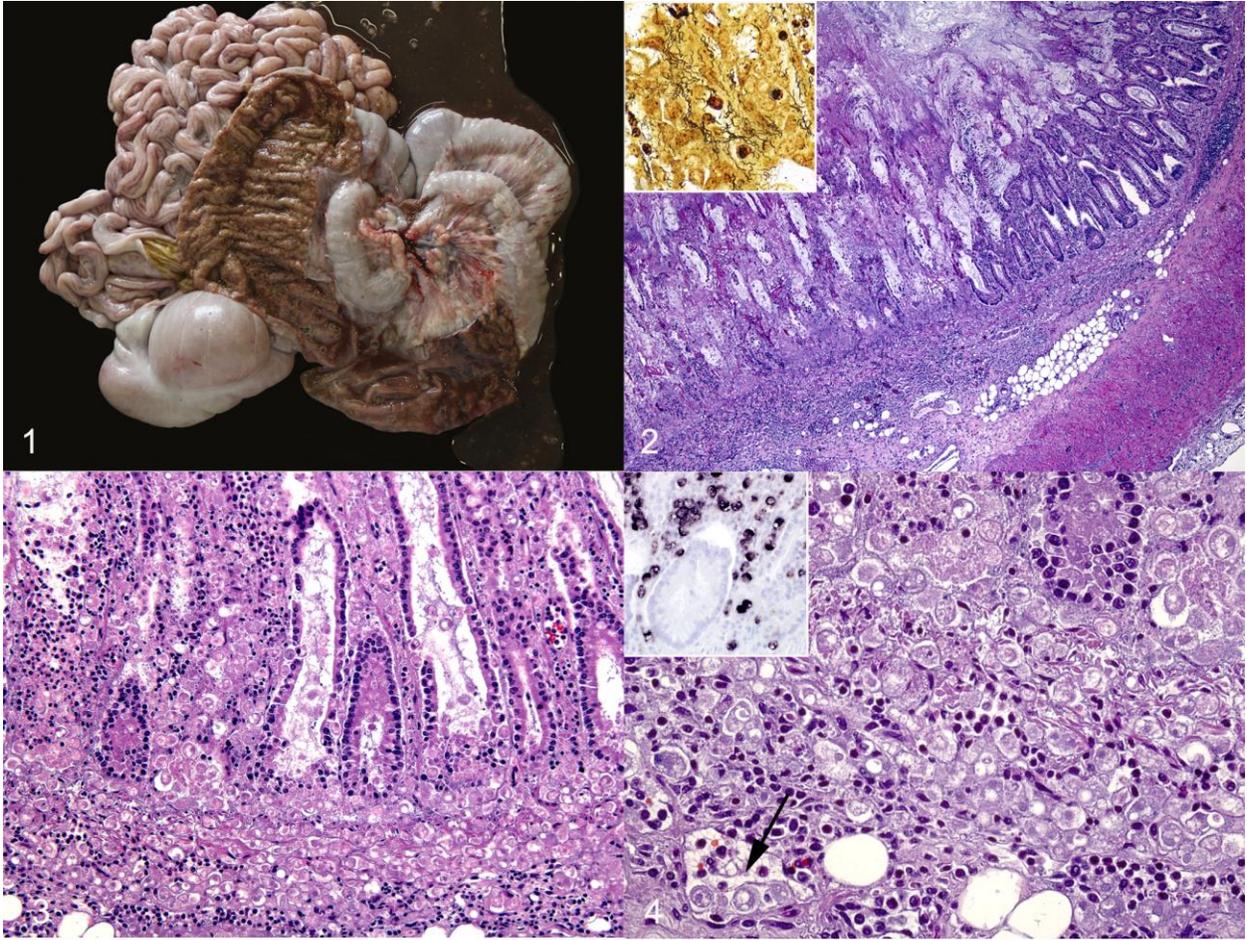
220

221 **Table 2.** Primer sequences and temperature conditions of the PCR methods used to detect different protozoan genomes.

Protozoan	Targeted genomic region	PCR amplicon (bp)	Primer sequences	PCR temperature conditions	Reference
<i>Balantidium</i> spp.	18S rRNA	462	fw: 5'-gataggggatcaaagacaatca-3' rv: 5'-acataaaggcatcacagacc-3'	95°C/3 min 40×: 95°C/15 s, 55°C/15 s, 72°C/25 s 72°C/1 min	This study
<i>Blastocystis</i> sp.	18S rRNA	479	fw: 5'-ggaggtagtacaataaatc-3' rv: 5'-tgcttcgcacttgttcac-3'	95°C/3 min 40×: 95°C/15 s, 55°C/15 s, 72°C/25 s 72°C/1 min	This study
<i>Entamoeba</i> spp.	18S rRNA	472	fw: 5'-attggaggcaagtctggtg-3' rv: 5'-gtaggactacgacggtac-3'	95°C/3 min 40×: 95°C/15 s, 55°C/15 s, 72°C/25 s 72°C/1 min	This study
<i>Trichomonas</i> spp.	18S rRNA	250	fw: 5'-gtaggctatcacggtaac-3' rv: 5'-actygcagagctggaattac-3'	95°C/3 min 40×: 95°C/15 s, 58°C/15 s, 72°C/25 s 72°C/1 min	Adapted protocol based on ref. 16

222 fw = forward primer; rv = reverse primer.

223 **Figures 1–4.** Macroscopic and microscopic lesions of colon and cecum of a pig coinfecting with
224 *Brachyspira hyodysenteriae* and *Entamoeba polecki*. **Figure 1.** Diffuse severe subacute
225 fibrinonecrotizing typhlocolitis with abundant liquid and hemorrhagic content. **Figure 2.**
226 Apical-to-transmural necrosis of the mucosa with abundant production of mucus. Inset:
227 numerous Warthin–Starry-positive, spiral-shaped bacteria are present in the mucosa. **Figure**
228 **3.** Presence of numerous amoebic structures in crypt lumina and expanding the lamina propria
229 and submucosa. **Figure 4.** Amoebae are round bodies with a single nucleus and a few
230 intracytoplasmic vacuoles, observed in mucosa, submucosa, and in the lumen of lymphatic
231 vessels (arrow). Inset: in situ hybridization with an *Entamoeba*-specific probe shows clear
232 labeling of the protozoal structures in the lamina propria.
233



234