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

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

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

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

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

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

We describe herein the coinfection of *B. hyodysenteriae* and *Entamoeba polecki* leading to severe necrotizing lesions in colon and cecum in a diarrheic pig. A 4-mo-old, crossbred pig, from a fattening unit (site 3) on an indoor pig production farm, with ongoing problems of diarrhea died and was submitted to the Servei de Diagnòstic de Patologia Veterinària of the Veterinary Faculty of the Universitat Autònoma de Barcelona (Spain) for diagnostic purposes. The farm of origin was a 1,350 sow farm that was positive-stable for porcine respiratory and reproductive syndrome virus (PRRSV), seropositive to porcine circovirus 2 (PCV-2) and *Mycoplasma hyopneumoniae*, and negative to pseudorabies virus. Sows and piglets were vaccinated with a modified-live PRRSV vaccine. The affected pig belonged to a batch of 450 grower–finishers allocated to pens with a complete slatted floor; animals received a conventional finishing feed. Clinical problems were observed only in fatteners (14–16-wk-old), with ~10% of 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 PRRSV[11] and PCV-2[18] 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

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

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

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

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

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

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

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

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

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

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

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

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

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

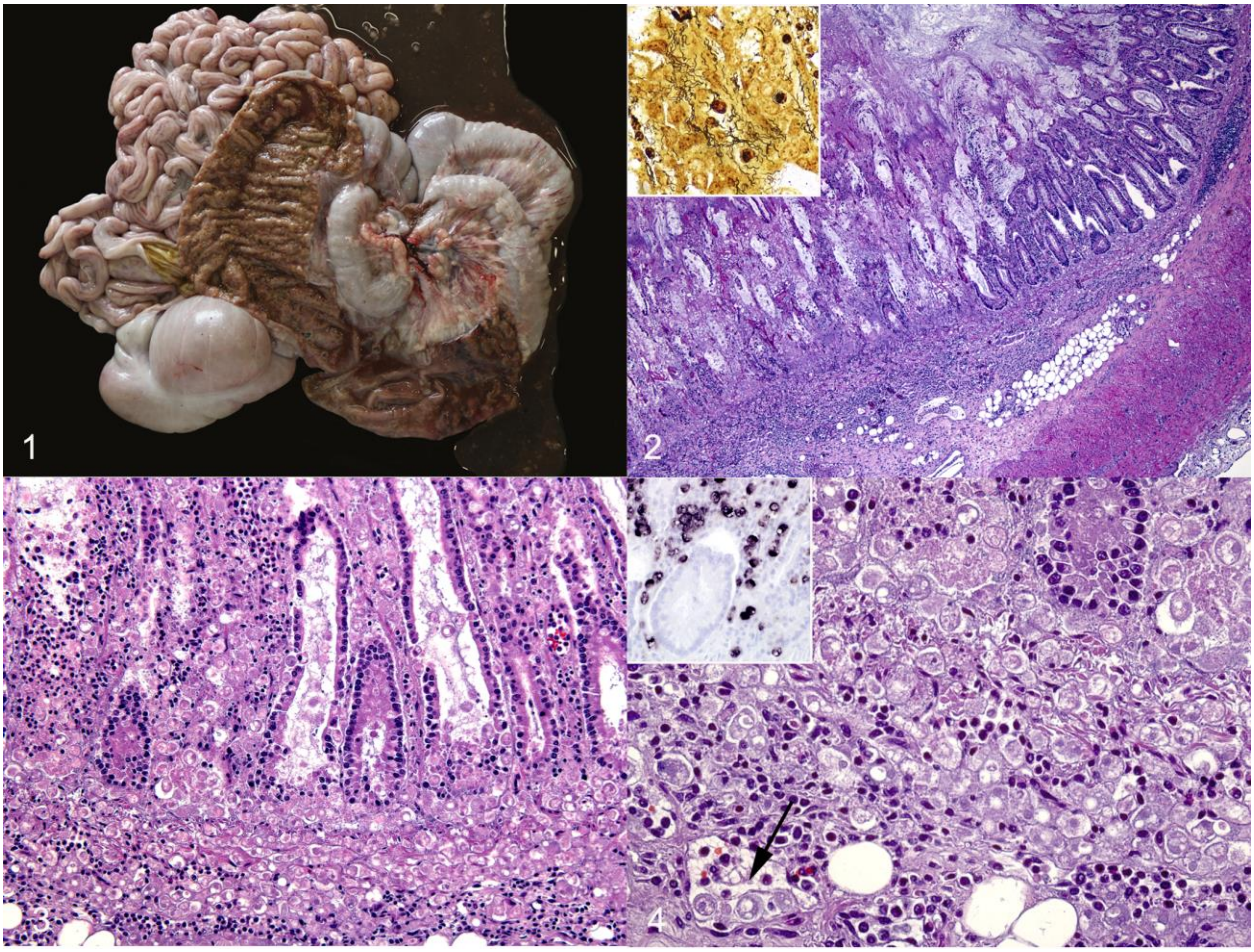
Protozoa	Targeted genomic region	Probe sequence (length in nucleotides)	Comments	Reference
Blastocystis sp.	18S rRNA	5'-ggatgttttcattaatcaagaacgaaagctaggggatc-3' (38 nt)	Cross-reactive with *Entamoeba* spp., *Eimeria* spp., *Sarcocystis* spp., *Cryptosporidium* spp., *Balantidium* spp., *Candida* spp.,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

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
Balantidium spp.	18S rRNA	462	fw: 5'-gataggggatcaaagacaatca-3' rv: 5'-acatataagggcatcacagacc-3'	95°C/3 min 40×: 95°C/15 s, 55°C/15 s, 72°C/25 s 72°C/1 min	This study
Blastocystis sp.	18S rRNA	479	fw: 5'-ggaggtagtgacaataaatc-3' rv: 5'-tgctttcgcacttgttcatc-3'	95°C/3 min 40×: 95°C/15 s, 55°C/15 s, 72°C/25 s 72°C/1 min	This study
Entamoeba spp.	18S rRNA	472	fw: 5'-attggagggcaagtctggtg-3' rv: 5'-gttaggactacgacggtatc-3'	95°C/3 min 40×: 95°C/15 s, 55°C/15 s, 72°C/25 s 72°C/1 min	This study
Trichomonas spp.	18S rRNA	250	fw: 5'-ggtaggctatcacgggtaac-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.

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



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