The Journal of Veterinary Medical Science

NOTE

Pathology

Swine multifocal ulcerative colitis and crypt abscesses associated with *Entamoeba polecki* subtype 3 and *Salmonella* Typhimurium

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J. Vet. Med. Sci.
82(4): 463–466, 2020
doi: 10.1292/jvms.19-0682

Received: 20 December 2019
Accepted: 10 February 2020
Advanced Epub:
26 February 2020

ABSTRACT. Piglets aged approximately 50 days exhibited diarrhea and wasting. Multiple white foci were detected in the colon of a dead piglet; histopathological findings revealed multifocal ulcers and crypt abscesses with *Entamoeba* trophozoites and gram-negative bacilli in the piglet. These pathogens were identified as *Entamoeba polecki* subtype 3 and *Salmonella enterica* serovar Typhimurium, respectively. Numerous *E. polecki* subtype 3 trophozoites were located on the edge of the ulcerative and necrotic lesions in the lamina propria. Crypt abscesses were associated with *S.* Typhimurium. These results suggest that *E. polecki* subtype 3 caused multifocal ulcerative colitis accompanied by crypt abscesses with *S.* Typhimurium in the piglet. This study is the first report of colitis with *E. polecki* subtype 3 and *S.* Typhimurium coinfection.

KEY WORDS: diarrhea, *Entamoeba polecki* subtype 3, multifocal ulcerative colitis, post-weaning pig, *Salmonella enterica* serovar Typhimurium

The genus *Entamoeba* is an intestinal protist found in humans and other animals [19]. *E. histolytica* is a common protozoan source of human amebiasis [19] and causes colitis and liver abscesses [17]. *E. dispar* is considered to be a major protozoan owing to its high prevalence in humans, although it is non-pathogenic [15].

E. polecki and *E. suis* are associated with chronic diarrhea in pig amebiasis [9–11]. *E. polecki* has four subtypes (ST 1–4), of which ST 1 and 3 are identified in pigs [12]. *E. polecki* is thought to be non-pathogenic in cases of single infection [8]; however, it has been reported as a coinfection with some other pathogens [3, 8]. Although coinfections of *E. polecki* ST 3 with *Lawsonia intracellularis* and *Salmonella* spp. have been previously reported, the *Salmonella* serovars have not been identified [8] and coinfection of *E. polecki* ST 3 with *Salmonella* Typhimurium has not been reported to date. Furthermore, the pathogenic roles of this protozoan in coinfected diarrhea have yet to be elucidated.

E. polecki has a nucleus and vacuoles, but no mitochondria in the cytoplasm [11, 13]. Engulfed bacteria are also found in *E. polecki* [7, 11–13] and *E. histolytica* has bacteria only within its vacuoles; in contrast, *E. dispar* commonly has bacteria in the free cytoplasm [18]. Although the presence of bacteria in these protozoans (within vacuoles or free in the cytoplasm) could be related to pathogenesis [18], the effect of different bacterial locations in these protozoans on their pathogenicity remains unknown. Moreover,

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little information about *E. polecki* ST3 is available. An *E. polecki* transmission electron microscope (TEM) study identified engulfed bacteria in vacuoles in *E. polecki* ST 1; however, there was no mention of free bacteria in the cytoplasm [11, 13]. To date, there have been no reports of bacteria in the cytoplasm of *E. polecki* ST 3, although only one TEM study has been conducted [12]. In addition, the previous study did not address the morphological characteristics of *E. polecki* ST 3 [12].

S. Typhimurium is the most common serovar of pork-related salmonellosis in humans [16]. Multiple antimicrobial resistances associated with *S.* Typhimurium (related to pork consumption) could become a serious human health hazard [1]. In pigs, salmonellosis is commonly caused by *S.* Typhimurium and *S.* Choleraesuis [4].

This study was conducted to elucidate the effect of *Entamoeba* spp. trophozoites in lesions and describes an *E. polecki* ST 3 and *S.* Typhimurium coinfection. To the best of our knowledge, this is the first report of multifocal colitis caused by coinfection with *E. polecki* ST 3 and *S.* Typhimurium.

The study site consisted of a farrow to finish farm housing approximately 800 sows, located in Hiroshima Prefecture on Honshu Island, Japan. Twenty-five of 100 piglets aged approximately 50 days exhibited diarrhea and wasting in January 2016. The 25 diseased piglets were placed in an isolation pen on January 22, and penicillin was administered for 3 days. On January 25 and 26, ceftiofur was also administered to the diseased piglets. One piglet died and was subjected to necropsy on January 26.

For the isolation of *Salmonella* species, the intestinal homogenate was pre-enriched in Hajna tetrathionate broth (Eiken Chemical Co., Ltd., Tokyo, Japan) and incubated at 37°C for 24 hr. A loopful of each isolate was subcultured on deoxycholate hydrogen sulfide lactose agar at 37°C for 24 hr. The isolates were identified as *Salmonella* spp. based on the Rapid ID 32 E Microbial Identification Kit (bioMérieux Japan Ltd., Tokyo, Japan). Serovar identification was performed using antisera (Denka Seiken, Tokyo, Japan). Antimicrobial susceptibility tests were performed to determine susceptibility using BD BBL Sensi-Discs (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) for 12 antibiotics: ampicillin, amoxicillin, penicillin, ceftiofur, kanamycin, gentamicin, streptomycin, oxytetracycline, sulfamethoxazole and trimethoprim mixture, chloramphenicol, colistin, and enrofloxacin.

For histopathological analysis, formalin-fixed (in 10% neutral-buffered formalin), paraffin-embedded (FFPE) tissue samples were sectioned at approximately 3 μm and stained using hematoxylin and eosin (HE), Gram stain (Brown Hopps method), Periodic Acid Schiff (PAS), Warthin-starry, and Immunohistochemistry staining using the *Salmonella* O4 (Denka Seiken Co.) and *Lawsonia intracellularis* antisera [11].

To confirm the presence of engulfed bacteria and the ultrastructural characteristics of *E. polecki* ST 3, the colon was subjected to TEM observation. The TEM procedure was conducted based on previous report [13].

FFPE samples were also used for molecular identification of protozoa. DNA was purified from three intestinal FFPE samples using the QIAamp DNA FFPE Tissue Kit (QIAGEN GmbH, Hilden, Germany). Polymerase chain reaction (PCR) was performed on the purified DNA to identify *Entamoeba* spp. (*E. histolytica*, *E. dispar*, *E. suis*, and *E. polecki*) and subtypes of *E. polecki* (ST 1–ST 4). These molecular identification procedures were also based on a previous report [8].

The intestinal isolates were identified as *S.* Typhimurium. Although the *S.* Typhimurium isolates exhibited resistance to penicillin, kanamycin, streptomycin, and oxytetracycline, they were susceptible to ceftiofur, which was administrated in the present cases.

Multiple white foci were observed in the pig colon (Fig. 1a). Crypt abscesses or ulcers were histopathologically confirmed in these foci. The ulcers were composed of inflammatory cells (primarily neutrophils) and cell debris and pseudomembranes were also detected in the upper section of the necrotic lesion (Fig. 1b). Many PAS-positive *Entamoeba* trophozoites were detected in the bottom section of the ulcers (Fig. 1c–f). Several gram-negative bacterial masses were also observed in the upper area. Most bacteria did not react with the *Salmonella* O4 antiserum. Diffuse necrosis of the lamina propria was also present with a high number of *Entamoeba* trophozoites. Few gram-negative bacteria were detected in the cytoplasm of *Entamoeba* trophozoites (Fig. 1g). Moreover, *Entamoeba* trophozoites were found in the lamina propria under the degenerated epithelium accompanied by gram-negative bacteria. *Salmonella* O4 antigen-positive bacteria and inflammatory cells were observed mainly in the crypt abscesses (Fig. 1h), and some of these were detected surrounding the degenerated epithelium and in the lamina propria by immunohistochemical staining (Fig. 1i). Numerous bacteria around the trophozoites did not react with the *Salmonella* O4 antiserum. *L. intracellularis* antigen was not detected in the intestinal specimens by immunohistochemistry.

Nuclei, bacteria, and vacuoles were observed in the cytoplasm of the trophozoites in the TEM study (Fig. 1j). A few bacteria were found in the *Entamoeba* trophozoites, evenly distributed in and out of the vacuoles. In addition, a large amount of debris was observed in the vacuoles.

A PCR product was successfully amplified from a colon sample and confirmed (100% identity) as *E. polecki* ST 3 (accession number: LC067574) by sequencing.

Our investigation indicated that the multifocal ulcerative colitis and crypt abscesses were mainly associated with *E. polecki* ST 3 and *S.* Typhimurium, respectively. To our knowledge, an *E. polecki* ST 3 and *S.* Typhimurium coinfection has never been reported.

Our report demonstrates that the pathological amebiasis characteristics in the pig were due to *E. polecki* ST 3. In human amebiasis, *E. histolytica* is known as a highly pathogenic protozoan [6, 19]. *E. histolytica* damages the mucosa epithelium and lamina propria owing to secreted molecules such as cysteine proteinase [5]. Infiltration of neutrophils and other cells also necrotize the lamina propria [5]. It is unclear whether or not *E. polecki* and *E. suis* are implicated in these pathogenic mechanisms. A high number of *E. polecki* ST 3 cells were observed at the bottom section of the necrotic layer, indicating that *E. polecki* ST 3 induced the enlarged necrotic layer. *E. histolytica* enhances pathogenicity owing to the presence of non-pathogenic *Escherichia coli* [2, 14]. A high number of bacteria were observed at the upper section of the necrosis area, although *E. polecki* ST 3 was located below

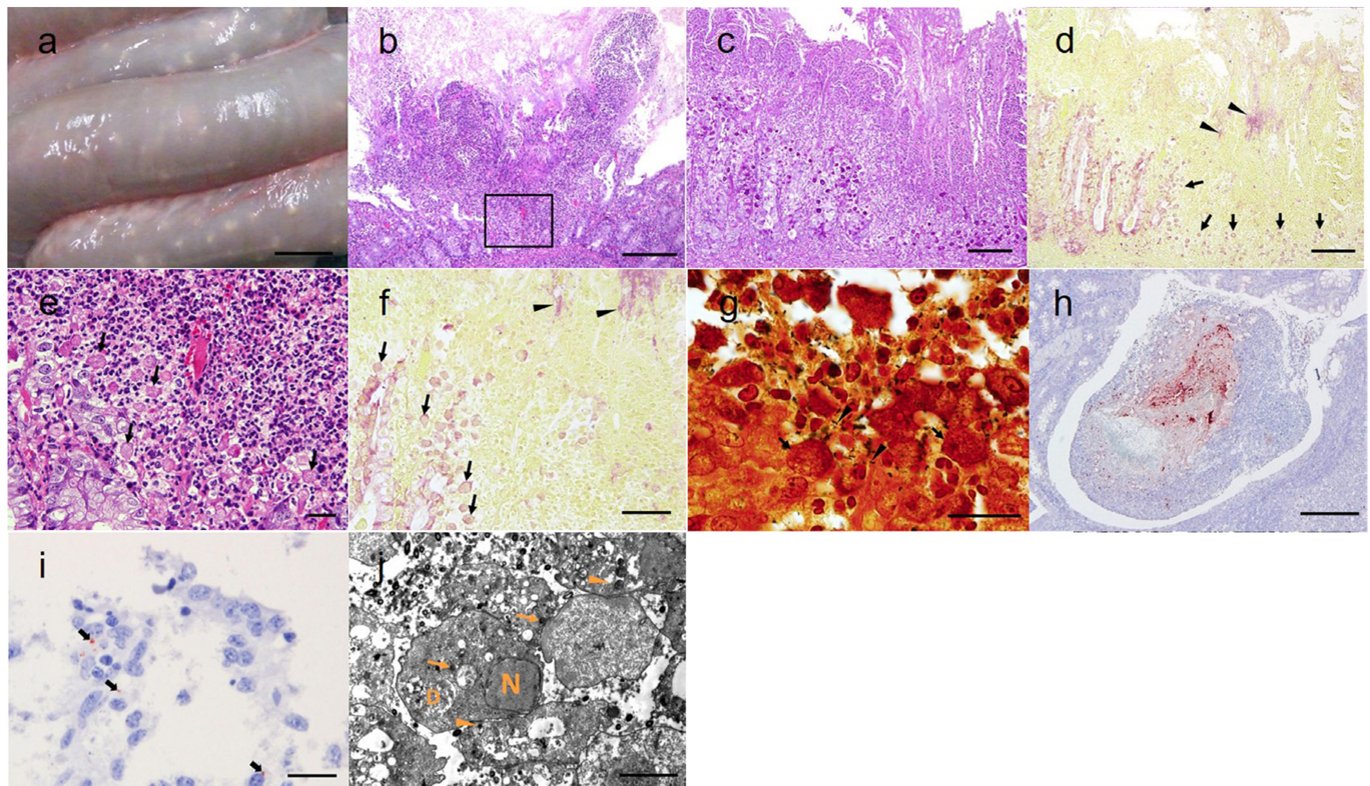

Fig. 1. (a) Gross findings in the colon. Many multifocal white foci were found throughout the whole colon; bar=1 cm. (b) Pseudomembrane and ulcer in the colon. The ulcer is composed of inflammatory cells and cell debris. The square inlay is enlarged in Fig. 1e. HE stain; bar=200 μm. (c) Many PAS-positive *Entamoeba* trophozoites (red) were detected in the bottom region of the ulcers PAS stain; bar=100 μm. (d) Serial section of Fig. 1c. Gram-negative bacterial masses (arrowheads) were observed in the upper right section. Most of the gram-negative bacteria did not react with the *Salmonella* O4 antiserum.Numerous *Entamoeba* trophozoites (arrows) were detected in the bottom region of the ulcers. Gram stain (Brown Hopps method); bar=100 μm. (e) Bottom field shown in Fig. 1b. Numerous *Entamoeba* trophozoites (arrows) were present in the lower section of the ulcer. Crypt structure was maintained below *Entamoeba* trophozoites. HE stain; bar=20 μm. (f) Serial section of Fig. 1c. Ulcerative colitis. *Entamoeba* trophozoites (arrows) were present in the bottom section of the necrotic region, while the bacterial masses (arrowheads) were observed in the upper right section. Gram stain (Brown Hopps method); bar=50 μm. (g) Mucosal epithelium of the colon. The epithelium was degenerated and *Entamoeba* trophozoites (arrows) were observed in the lamina propria. Many bacterial cells (arrowheads) were observed surrounding the degenerated epithelium. Most gram-negative bacteria did not react with the *Salmonella* O4 antiserum. Warthin-Starry staining; bar=20 μm. (h) A crypt abscess in the colon. *Salmonella* O4 antigen-positive bacteria (red) were observed in the abscess. Immunohistochemistry; bar=200 μm. (i) Mucosal epithelium of the colon. *Salmonella* O4 antigen-positive bacteria (arrows) and leukocytes were observed surrounding the sloughed epithelium. Immunohistochemistry; bar=20 μm. (j) *Entamoeba* trophozoites observed by TEM. Nucleus (N), debris (D) in vacuoles, and bacteria were found in the cytoplasm. *Entamoeba* trophozoites engulfed debris rather than bacteria. Bacteria were evenly dispersed in vacuoles (arrowheads) and free in the cytoplasm (arrows); bar=5 μm.

them. These results suggest the presence of several bacteria related to the pathogenicity of *E. polecki* ST 3, indicating that *E. polecki* ST 3-induced necrosis could be related to the presence of several bacteria.

E. polecki ST 3 cells engulfed a few bacteria in this study. The amount of engulfed bacteria can correlate with the pathogenicity of *E. histolytica* and *E. dispar* [18]. Although little information is available regarding the pathogenicity of *E. polecki* ST 3 [12], the present findings suggest that *E. polecki* ST 3 acquires high pathogenicity with a small number of bacteria. *E. histolytica* is highly pathogenic and is the main cause of human amebiasis [19]; in contrast, *E. dispar*, commonly found in humans, is thought to have low pathogenicity [15]. *E. histolytica* has been shown to have several bacteria within vacuoles and few in the cytoplasm [18]. In contrast, in *E. dispar*, some bacteria were mainly found free in the cytoplasm [18]. In this study, we observed a few engulfed bacteria within vacuoles, but also in the cytoplasm. Some debris were commonly found in the vacuoles, suggesting that *E. polecki* ST 3 prefers debris to bacteria. Our results indicate that *E. polecki* ST 3 has different characteristics compared with other *Entamoeba* spp.; in particular, the amount of engulfed debris in the vacuoles appears to be related to *E. polecki* ST 3 pathogenicity. Coincubation of *E. histolytica* with *E. coli* or *Shigella dysenteriae* enhances adhesion and cytotoxicity [5]. Sulfur and iron derived from *Desulfovibrio desulfuricans* and *E. coli* accelerated the culturing of *E. polecki* ST 1 [20]. Although the detailed mechanisms of pathogenicity in *E. polecki* ST 3 remain unknown, it is possible that several bacteria observed at the upper section of the necrotized lesion are related to sulfur and iron generation. Further studies are needed to elucidate the association between pathogenicity and

these fast-acting factors.

Bacterial examination showed that the detected *S.* Typhimurium isolate, which necrotized the epithelium, was sensitive to ceftiofur, which was administered to the piglets in the present case. Most of the past case reports also mentioned that antibiotic therapy had failed [7, 10]. Ceftiofur is a second-choice antibiotic in Japan; thus, early administration was hindered in the present case. Had the antibiotic susceptibility to first choice antibiotics been known earlier, amebiasis could have been prevented, as epithelium degeneration would not have become severe. Adequate hygiene management (especially focused on early detection and treatment) should be defined for farms where *E. polecki* ST 3 is present.

Our results indicate that *E. polecki* ST 3 has the ability to cause severe necrotizing ulcerative colitis accompanied by crypt abscesses with *S.* Typhimurium. Histopathological and molecular examination is needed to detect the underlying cause of amebiasis in pig diarrheal cases. As the number of cases of swine amebiasis is increasing in Japan, additional critical prevention methods and *in vitro* studies for detecting pathogenic mechanisms are needed.

ACKNOWLEDGMENTS. We wish to thank Mr. M. Kobayashi and Ms. M. Shimada for histopathological assistance; Mr. S. Honda and Dr. N. Tanimura for useful suggestions; and Mrs. R. Sekiguchi and Mrs. N. Asama for assistance with the molecular examinations.

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