Diarrhea Caused by *Enterococcus villorum* in Piglets

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Abstract

Three of 10 piglets with watery diarrhea, aged 24, 21 and 22 days (cases 1, 2 and 3, respectively), were investigated in detail after euthanasia (as the remaining seven recovered without specific treatment). Enterococcal bacteria were isolated and multilocus sequence analysis showed 100% and 99% identity with the phenylalanyl tRNA synthase and RNA polymerase α subunit genes of strains of *Enterococcus villorum*, respectively. Histologically, severe epithelial desquamation, atrophy, and regeneration of ileal villi were observed in cases 1, 2 and 3, respectively. The number of bacteria was large in case 1, smaller in case 2, and sparse in case 3. These findings suggest that case 1 was at an earlier stage of enteropathy than case 2, and that case 3 was recovering. In case 1, the exfoliation of epithelial cells with many bacteria into the intestinal lumen was interpreted as a host reaction for eradicating marginally pathogenic enteroadherent bacteria. Folded smooth muscle cells and intact blood and lymphatic vessels in the atrophic lamina propria may have been linked to the rapid regeneration of villi.

Discipline: Animal health
Additional key words: Desquamative enteropathy, enteroadherent bacterium, mucin, immunohisto-chemistry

Introduction

Enterococcus is a genus of lactic acid bacteria of the phylum *Firmicutes*. Enterococcal bacteria are part of the normal intestinal flora of humans and animals, and some strains of *E. faecium* and *E. faecalis* are used as probiotic animal feed supplements (Benno 2011). However, they are also important nosocomial pathogens that can cause bacteremia, endocarditis and other infections in humans, and some strains are resistant to many antibiotics and possess virulence factors such as adhesins, invasins, pili and hemolysin (Franz et al. 2011).

E. durans, *E. hirae* and *E. villorum* are phylogenetically closely related, but can be differentiated by genomic methods and whole-cell protein analysis (Devriese et al. 2002). Diarrhea caused by *E. durans* has been reported in piglets, calves, foals and a pup (Tzipori et al. 1984, Collins et al. 1988, Rogers et al. 1992, Cheon & Chae 1996), and by *E. hirae* in piglets and kittens (Lapointe et al. 2000, Nicklas et al. 2010, Larsson et al. 2014). These organisms are adherent to the surface of villous enterocytes in the small intestine, but the pathogenesis of diarrhea is unknown (Rogers et al. 1992, Cheon & Chae 1996, Larsson et al. 2014). *E. villorum* has been identified in piglets with diarrhea, but without reference to clinical, macroscopic and microscopic findings (Vancanneyt et al. 2001). The current study reports on enterococcal enteropathy in three piglets, with particular emphasis on the pathogenesis of watery diarrhea. To our knowledge, this is the first report describing the histopathology of *E. villorum* infection.

Materials and methods

1. Animals

On a farm where approximately 800 sows were reared, watery diarrhea occurred in 10 piglets. These were crossbreeds of Large White/Landrace × Duroc from different sows and of different sexes. Porcine epidemic diarrhea (PED) was suspected, and three piglets aged 24, 21 and 22 days (cases 1, 2 and 3, respectively) were euthanized and examined to obtain a definitive diagnosis. Antibiotics had been included in the milk for suckling pigs on the farm. Although no treatment was given for mild diarrhea, the other seven piglets fully recovered within a week of the onset of the first case.

2. Virological and bacteriological examinations

Contents of the jejunum and ileum were examined for PED virus and transmissible gastroenteritis virus by polymerase chain reaction (PCR) and for group A rotavirus by immunochromatography. The contents were also cultured for bacterial identification, and cultures were tested using API 20 Strep (Nippon bioMérieux, Tokyo, Japan). In addition, multilocus sequence analysis (MLSA) of the RNA polymerase α subunit (*rpoA*) and phenylalanyl transfer RNA synthase (*pheS*) genes was performed as previously described (Naser et al. 2005), in order to identify bacterial species. The isolate was also tested for antibiotic susceptibility using the agar dilution and disc diffusion methods, as per guidelines (CLSI 2012) of the Clinical and Laboratory Standard Institute.

3. Histological and immunohistochemical examinations

To prevent postmortem changes (Wagner et al. 1979), immediately after euthanization, tissue samples from the small intestine were fixed in 10% buffered formalin. The samples were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (HE), Giemsa, trichrome, Gram and high iron diamine-alcian blue at pH 2.5 (HID-AB). Immunostaining was done by the streptavidin-biotin complex/horseradish peroxidase (SAB) method on histological sections using a commercially available kit (Nichirei, Tokyo, Japan). For the identification of *Enterococcus* sp., a rabbit polyclonal antibody raised against *E. faecium* (1:2,000, ViroStat, Portland, USA) was used as a primary antibody. The other primary antibodies were mouse monoclonal antibodies to α-smooth muscle actin (SMA; clone 1A4, 1:50, Dako A/S, Glostrup, Denmark) and desmin (clone D9, 1:10, Progen Biotechnik, Heidelberg, Germany) and rabbit polyclonal antibodies to CD3 (1: 50, Dako A/S), CD20 (prediluted, Spring Bioscience, Pleasanton, USA), CD31 (prediluted, Spring Bioscience), lymphatic vessel endothelial hyaluronan receptor (LYVE1; 1:200, Abcam, Cambridge, UK), immunoglobulin κ chain (prediluted, Fitzgerald Industries International, Acton, USA), and λ chain (prediluted, Fitzgerald Industries International). Antigen retrieval was performed by enzymatic digestion with 0.1% trypsin (*E. faecium*) at 37°C for 20 minutes, 0.05% pepsin at 37°C for 25 minutes (CD3) or microwave heating in 10 mM Tris buffer, pH 9.0 at 90°C for 9 minutes (CD31, LYVE1). As healthy controls, normal tissues from a 25-day-old piglet (Landrace × Landrace) were also examined in the same manner.

Results

1. Macroscopic findings

The small intestinal wall was slightly thickened in case 1. Similar thickening was also observed in the upper and middle parts of the ileum in cases 2 and 3, but the jejunum and other parts of the ileum in case 2 were thinner.

2. Virological and bacteriological findings

The viruses examined by PCR or immunochromatography were not detected in intestinal contents. Bacteriologically, a facultative anaerobic bacterium was isolated in cases 1 and 2, but no organisms were cultured in case 3. The isolate was presumptively identified as *Enterococcus* due to frequent diplococcal arrangement, positive Gram staining, and negative catalase activity. Testing using API 20 Strep indicated that the isolate was either *E. durans* or *E. hirae*. Because it was difficult to phenotypically differentiate between these two species, MLSA of the *rpoA* and *pheS* genes was performed by comparing the sequences to the National Center of Biotechnology Information (NCBI) database using BLAST. In analysis of the partial *rpoA* gene sequences, the isolate was most similar to *E. villorum* strains LMG 12287T, LMG 19179, LMG 19177 and LMG 12284 (99.0% sequence similarity), followed by *E. durans* strains IE14_1, IE18_5, IE12_1, HJ2 and the other species (≤ 96.0% sequence similarity). The partial *pheS* gene sequences of the isolate were most similar to those of *E. villorum* strains LMG 12287T, LMG 19177, LMG 17496, LMG 19179 (100% sequence similarity) and LMG 12284 (97%), but revealed lower identity with those of *E. durans* strains LMG 16193 and the other species (≤ 85% sequence similarity). In antibiotic susceptibility testing, the bacteria showed resistance to nalidixic acid, trimethoprim-sulfamethoxazole, oxytetracycline, doxycycline, erythromycin, chloramphenicol, fosfomycin and low levels of streptomycin and gentamicin, but not to ampicillin, ceftiofur, enrofloxacin, orbifloxacin, vancomycin and

high concentrations of streptomycin and gentamicin.

3. Histological and immunohistochemical findings

For histological examination, in the ileal villi of case 1, the lamina propria was shortened and thickened (Fig. 1A), occasionally with bodies of dense collagen fibers (Fig. 1B) that stained green with trichrome. Infiltration by a few neutrophils was detected, but hemorrhages were absent. The epithelium was either intact or torn at the tip of villi, frequently with edematous exudate within subepithelial spaces, and some villi were narrowed just above the top of the atrophic lamina propria. Similar histological features were also observed in the jejunum (Fig. 1C). The ileal villi were atrophic in case 2 (Fig. 1D), and were thickened but extended in case 3. Mitoses at the middle or lower part of the epithelium were conspicuous in case 2. Goblet cells did not predominate in cases 1 and 2 (Fig. 1E), whereas they were large in number in case 3 and in the control, and stained exclusively with HID in case 3 (Fig. 1F), but positively with AB at or near the crypt base in the control (Fig. 1G). Mitotic figures were scarcely seen in the villous lamina propria in all three cases.

There were great numbers of bacteria adherent or close to the ileal epithelial surface in case 1 (Fig. 1A), and small numbers in case 2. Bacteria were rarely found within the intestinal lumen in case 3. The organisms were Gram-positive, frequently occurred in pairs, and stained positively by immunohistochemistry for *Enterococcus* sp. (Fig. 2A). In neither the three cases nor the control were there any conspicuous differences in the number of CD3-positive or CD20-positive lymphocytes, and plasma cells stained for either λ or κ. The histology of the jejunum was relatively similar to that of the ileum in each case, but the number and staining pattern of goblet cells in all three cases resembled those in the control. In addition, enterococcal bacteria were rare in cases 1 and 2, and absent in case 3.

In cases 1 and 2, SMA- and desmin-positive smooth muscle cells showed a tendency to accumulate in the villous lamina propria (Fig. 2B), and CD31-positive vascular endothelial cells were mainly observed just beneath the epithelium (Fig. 2C). These features were less prominent in case 3. Lacteal vessels positive for CD31 and LYVE1 were also observed in all cases. In villi of the control, smooth muscle cells, blood capillaries and lymphatic vessels were longitudinally oriented (Fig. 2D).

Discussion

In the present study, the bacteria isolated from intestinal contents of piglets with diarrhea were identified as *E. villorum* by MLSA of the housekeeping genes *rpoA* and *pheS* (Naser et al. 2005). Both conventional phenotyping methods and 16S rRNA sequencing are unreliable and unable to properly identify enterococci (Tyrrell et al. 1997, Vancanneyt et al. 2001). Despite the close phylogenetic relationships between *E. villorum*, *E. durans* and *E. hirae* (Devriese et al. 2002), there may be differences in pathogenicity. *E. villorum* was first detected as a bacterium associated with diarrhea in piglets in 2001, and mortality attributed to it is reportedly negligible (Vancanneyt et al. 2001). This view was supported by the current study in which seven unexamined piglets recovered without any treatment. *E. durans* and *E. hirae* are capable of causing diarrhea in young animals of several species (Tzipori et al. 1984, Collins et al. 1988, Rogers et al. 1992, Cheon & Chae 1996, Lapointe et al. 2000, Nicklas et al. 2010, Larsson et al. 2014). No distinction was made between *E. hirae* and *E. villorum* in reports earlier than 2001, but it is probable that *E. hirae* induced enteropathy with ascending cholangitis and pancreatitis in a kitten (Lapointe et al. 2000) and septicemia in psittacine birds (Devriese et al. 1995). This species apparently causes severe endocarditis in humans (Poyart et al. 2002, Talarmin et al. 2011).

In the current case, the isolate was susceptible to vancomycin, but resistant to such antibiotics as nalidixic acid, trimethoprim-sulfamethoxazole, low levels of aminoglycosides (streptomycin and gentamicin), tetracyclines (oxytetracycline and doxycycline), a macrolide (erythromycin), and chloramphenicol. Two types of antimicrobial resistance, namely intrinsic and acquired, are present in enterococcal bacteria. Resistance to nalidixic acid, trimethoprim-sulfamethoxazole and tetracyclines was intrinsic in the bacteria described here. However, resistance to the latter antibiotics are the most common phenotypes of acquired resistance in enterococci from swine and from food (Cetinkaya et al. 2000, Novais et al. 2013). Moreover, tetracyclines and a macrolide (tylosin) are extensively used for promoting animal growth and disease prophylaxis in several countries (Chopra & Roberts 2001, Jackson et al. 2004). These data suggest the importance of *E. villorum* as a possible source of resistance genes for other enterobacteria (Novais et al. 2013), despite the fact that the present enterococci were only marginally pathogenic.

Case 1 was histologically characterized by desquamative villous epithelial cells with many bacteria on the surface, whereas the ileal villi were atrophic in case 2. These results suggest that case 1 was at an earlier stage of enteropathy than case 2. Case 3, in which regenerating villi were observed, was presumably recovering. In some villi of case 1, the surface epithelium appeared intact,
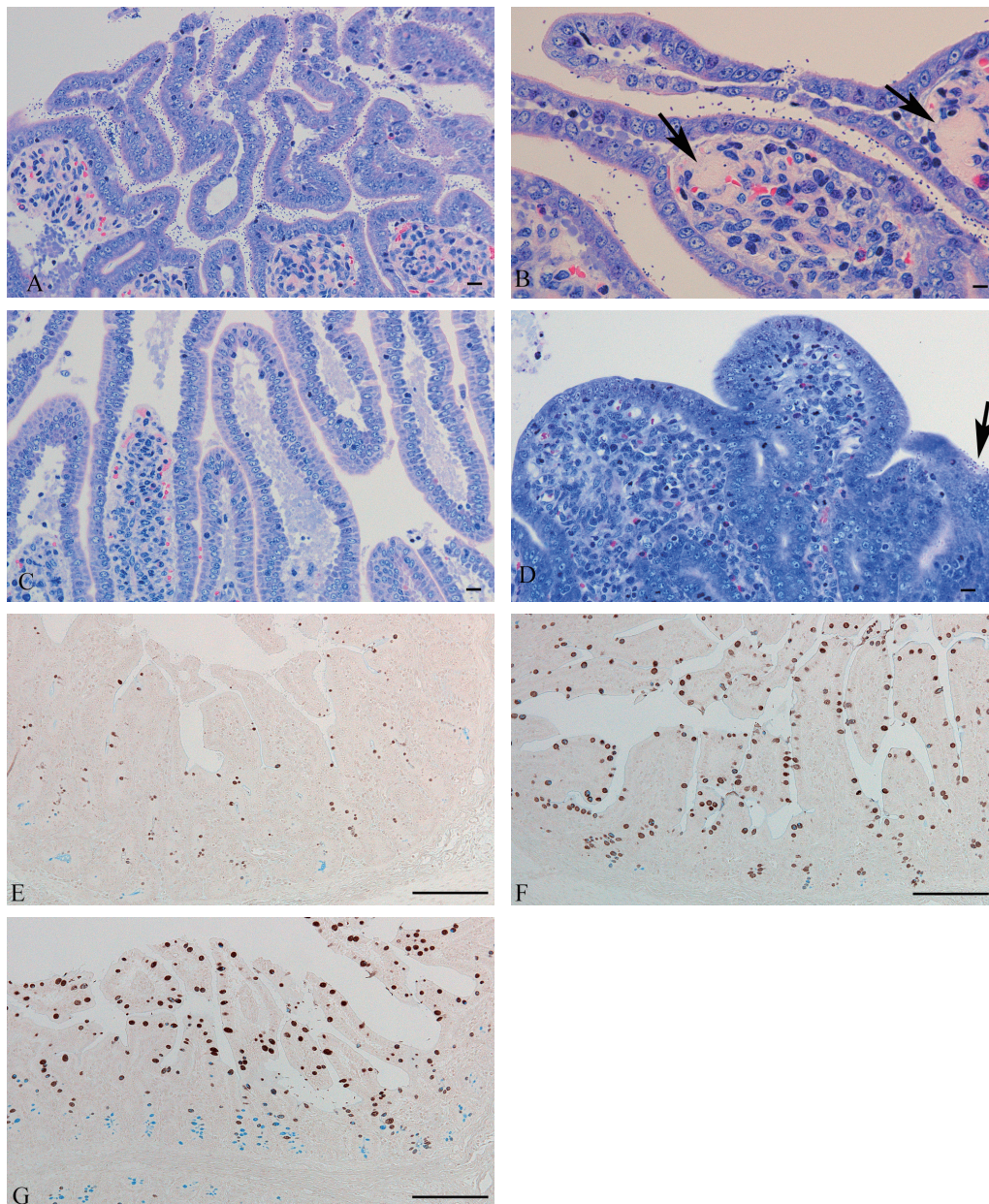
Fig. 1A. Case 1, ileum

The lamina propria is shortened and thickened, whereas intact epithelia with many bacteria on the surface appear to form lumen-like spaces. [Giemsa; Bar = 10 μm]

Fig. 1B. Case 1, ileum

There are collagenous bodies (arrows) at the tip of the atrophic lamina propria. Several enterococcal bacteria often arranged in pairs are observed in this field. [Giemsa; Bar = 5 μm]

Fig. 1C. Case 1, jejunum

Edematous fluid accumulates in cavities formed by atrophy of the lamina propria. Unlike in ischemic or postmortem changes, nearly all enterocytes are intact. [Giemsa; Bar = 10 μm]

Fig. 1D. Case 2, ileum

Compared with Fig. 1A, these atrophic villi are reminiscent of a histological image just after massive epithelial shedding. An arrow indicates residual enterococcal bacteria. [Giemsa; Bar = 10 μm]

Fig. 1E. Case 1, ileum

Epithelial cells with small amounts of HID-positive mucin are sparsely distributed. [HID-AB; Bar = 100 μm]

Fig. 1F. Case 3, ileum

HID-positive goblet cells predominate in regenerating villi. [HID-AB; Bar = 100 μm]

Fig. 1G. Control case, ileum

As in Fig. 1 F, goblet cells are abundant, but stain blue with AB at or near the crypt base. [HID-AB; Bar = 100 μm]

but the lamina propria became shortened and thickened with accumulations of SMA- and desmin-positive smooth muscle cells. As contraction of the villous smooth muscle can cause the villus to shorten in the normal small intestine (Frappier 2006), intensely contracted smooth muscle cells may have been folded toward the base of villi to form the accumulations seen in this case. Given the decreased numbers of bacteria in cases 2 and 3, the exfoliation of epithelial cells with bacteria into the intestinal lumen may be interpretable as a phenomenon beneficial to the host (Ashida et al. 2009), especially with regard to pathogens with no or little invasiveness. In case 1, the narrowed portion of desquamating villi might imply already accomplished or immediate sealing of neighboring cells after massive epithelial shedding. In addition, the preservation of smooth muscle cells, blood capillaries and lacteal vessels in the atrophic lamina propria implied their extension, possibly contributing to a rapid regeneration of villi. In the jejunum of cases 1 and 2, epithelial cell desquamation with a few bacteria may be helpful in clearing pathogens from the whole intestinal

lumen. In contrast, many highly pathogenic bacteria are able to enhance host cell adhesion to an extracellular matrix and colonize the intestinal epithelium efficiently (Kim et al. 2009).

There were no significant differences in the number of lymphocytes and plasma cells between the three cases and the control. However, mucin production was inactive in the ileum of cases 1 and 2. Considering the fact that mucins have been shown to block bacterial attachment to epithelial cell surfaces (Cone 1999), the small number of goblet cells and small amount of mucin production in each cell in cases 1 and 2 are important factors promoting bacterial adhesion. In contrast, goblet cells in case 3 were similar in number to those in the control, and characterized by sulfomucin production. In a study using hamsters infected with *Strongyloides venezuelensis*, increased numbers of goblet cells and highly sulfated mucins were helpful in detaching and expelling the worms (Ishikawa et al. 1999). Analogously, in case 3, abundant sulfomucin may have prevented subsequent bacterial adhesion, and was presumably helpful in
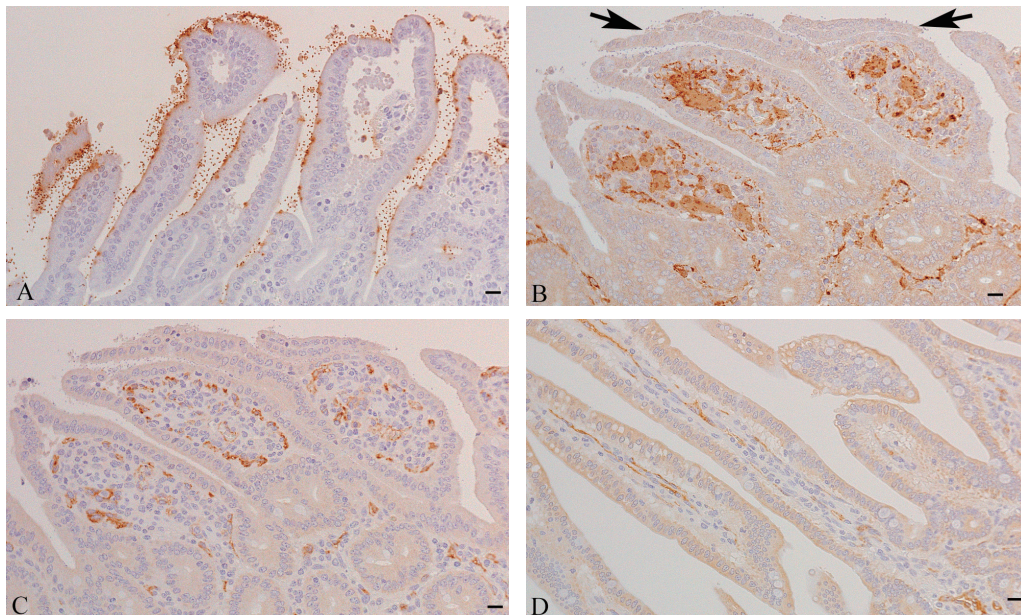

Fig. 2A. Case 1, ileum
　Enteroadherent bacteria, which are stained intensely with antibody to *Enterococcus* sp., are located chiefly at the upper part of villi. [SAB; Bar = 10 μm]
Fig. 2B. Case 1, ileum
　SMA-positive villous smooth muscle cells appear to have accumulated. Arrows indicate remaining villous epithelial cells. [SAB; Bar = 10 μm]
Fig. 2C. Case 1, ileum
　Blood capillaries composed of CD31-positive endothelial cells are arranged beneath the epithelium. [SAB; Bar = 10 μm]
Fig. 2D. Control case, ilem
　In intact villi, blood capillaries with CD31-positive endothelial lining are longitudinally oriented. [SAB; Bar = 10 μm]

protecting against various enteroadherent bacteria.

This is the first report to elucidate the pathogenesis of *E. villorum* in piglets. Rapid recovery from diarrhea was due to its slight pathogenicity, and could be explained histologically by the preservation of intestinal villous cores. Such a very mild infection may be rather helpful in making pigs resistant to other intestinal diseases.

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