The Organic Acid Profiles in the Feces of Pigs at *Brachyspira hyodysenteriae*- or *B. pilosicoli*-Positive Farms

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ABSTRACT. We observed a significant difference in the organic acid profile of diarrheal feces between pigs infected with and free from pathogenic spirochetes. Diarrhea and loose feces were collected from growing pigs, held at 15 different commercial farms. A total of 106 samples were measured for organic acid concentration by HPLC and were checked for the presence of *B. hyodysenteriae* and *B. pilosicoli* by PCR. *B. hyodysenteriae* was detected in 3 samples collected from one farm. *B. pilosicoli* was detected in 5 samples collected from another farm. Lower concentrations of iso-butyrate and iso-valerate were likely associated with development of pathogenic spirochete infection.

KEY WORDS: organic acid, pathogenic spirochetes, swine.

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Pigs are often infected by two species of pathogenic spirochete, *Brachyspira hyodysenteriae* and *B. pilosicoli*. The former causes swine dysentery (SD), which is an economically important diarrheal disease in the swine industry. SD-infected pigs show severe mucohemorrhagic colitis that leads to diarrhea containing blood and mucus and subsequent severe dehydration and growth loss [12]. The latter also causes a diarrheal disease called porcine intestinal spirochetosis. Pigs infected with *B. pilosicoli* have milder colitis and diarrhea than those infected with *B. hyodysenteriae* [12]. However, *B. pilosicoli* colonizes a broad range of vertebrates, including swine, humans, dogs, and birds [2, 8, 12, 15, 17], which makes disease control difficult. Interestingly, there are three non-pathogenic *Brachyspira* spp. [12, 15], and their distribution and function have not been thoroughly studied. Since a germ-free pig challenged with *B. hyodysenteriae* did not develop SD [11], the etiology of SD cannot be explained by *B. hyodysenteriae* infection alone [12]. Durmic *et al.* [3] suggested that some intestinal bacteria, such as *Bacteroides* spp., may be involved in the development of SD. In this context, these authors also suggested that diet-related changes in the large intestinal environment may result in development of SD [3, 4, 13]. However, further studies are needed to demonstrate this hypothesis. In this study, we observed significant differences in the organic acid profiles of diarrheal feces of pigs infected with and free from pathogenic spirochetes.

B. hyodysenteriae ATCC27164 and *B. pilosicoli* ATCC51139 were used as standard strains. They were cultured on tryptic soy agar (Difco Laboratories, Detroit, MI, U.S.A.) plates supplemented with 5% (v/v) defibrinated

horse blood, spectinomycin (400 µg/mL), vancomycin (25 µg/mL), and colistin sulfate (25 µg/mL) [10]. The plates were placed in an anaerobic jar and cultured at 37°C. Anaerobiosis was maintained using a GasPak Plus system (BBL, Cockeysville, MD, U.S.A.). Colonies were individually transferred to a Tryptic soy broth medium (Difco Laboratories) supplemented with glucose (0.5% w/v), sodium carbonate (0.2% w/v), L-cystein-HCl (0.05% w/v), yeast extract (1.0% w/v, Nacalai Tesque, Kyoto, Japan), clarified porcine fecal aqueous extract (5% v/v), and defibrinated horse blood (5% v/v) for culture at 37°C for 3 to 5 days according to the method of Kunkle *et al.* [9]. A fecal aqueous extract was prepared from freshly collected adult sow feces that contained neither antimicrobials nor dietary prebiotics or probiotics. The diet for this sow has been reported previously [19]. Feces were mixed with 4 weights of distilled water and homogenized in a blender. After removal of coarse materials by squeezing through 4 layers of surgical gauze, the liquid was centrifuged at 35,000 × g. The resultant supernatant was autoclaved at 121°C for 20 min.

Diarrhea and loose feces were collected just after defecation from growing pigs held at 15 different commercial pig farms. The feces were transported at 4°C to Kyoto Prefectural University. A portion of the feces was subjected to determination of water content by drying at 80°C for 48 hr. Feces with more than 69% moisture was regarded as diarrheal feces, as described previously [18]. In all, 106 fecal samples were selected as diarrhea or loose stools and subjected to further analyses.

Bacterial DNA was extracted from 0.2 g of feces according to the method of Godon *et al.* [6]. DNA was also extracted from pure cultures of two pathogenic *Brachyspira* by the method of van Hoek *et al.* [21]. Full-growth cultures of *B. hyodysenteriae* and *B. pilosicoli* were centrifuged at 25,000 × g at 4°C for 10 min. Cell pellets were mixed with

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approximately 0.08% (v/v) chelex solution and frozen at -80°C for 20 min. After thawing at ambient temperature, 0.2 vol. of a proteinase K solution (10 mg/mL) was added. Tubes were kept at 56°C for 4 hr and then at 96°C for 10 min. They were then centrifuged at $25,000 \times g$ at 4°C for 10 min to collect the supernatant. The 16S rDNA gene of *Brachyspira* spp. was amplified using the genus-specific primers Br-F (5'-GAGTGACAGTAGATAATGTAAG-3') and Br-R (5'-GGCACTCCTATTTAAATAG AAGC-3') (Table 1). The reaction mixture was constructed with recombinant *Taq* DNA polymerase (Toyobo, Osaka, Japan) and its antibody for hot-start PCR (Anti-taq high; Toyobo). Each 50 μL PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.2 mM each deoxynucleoside triphosphate (dNTP), 5U recombinant *Taq* DNA polymerase, 2U anti-taq high, 0.4 μM of each primer, and 50 ng template DNA. The samples were amplified in a thermal cycler (TaKaRa PCR Thermal Cycler MP, TaKaRa, Kyoto, Japan) using the following program: initial denaturation at 95°C for 4 min; 35 thermal cycles of 95°C for 45 sec, 58°C for 45 sec, and 72°C for 60 sec; and final elongation at 72°C for 7 min. The *B. hyodysenteriae* *tylA* gene was amplified using the primers reported by Fellstrom *et al.* [5]. The PCR conditions were the same as above except for the annealing temperature, which was 56°C . Two *B. pilosicoli* 16S rDNA gene-specific primers were constructed, Bp-F (5'-GTAGAGGAAAGTTTTT CGCTTC-3') and Bp-R (5'-GGCACTCCTATTTAAATAGAAAGC-3') (Table 1). The PCR conditions were the same as above except for the

annealing temperature, which was 54°C . The size of the PCR products was confirmed by analyzing 5 μL samples using 1% agarose gel (w/v) electrophoresis and ethidium bromide staining. The partial sequence (ca. 500 bp) of the amplified product was determined for each primer set on 2 fecal samples and a pure culture of *B. hyodysenteriae* and *B. pilosicoli* after TA cloning to check the validity of the PCR conditions used. TA-cloning subsequent sequencing was conducted as described previously [7].

The organic acid concentration was determined by ion-exclusion HPLC, as described previously [20]. The organic acid concentrations were analyzed by one-way ANOVA or the Kruskal-Wallis test to compare the values taken from feces with pathogenic spirochetes with those from feces free from pathogenic spirochetes. Scheffe's post hoc comparison was used for multiple comparisons when needed. All data were analyzed using StatLight 2000 [16]. Differences among means were considered significant at $p < 0.05$.

Genus-specific PCR detection for *Brachyspira* spp. was positive for 21 samples. The *B. hyodysenteriae* *tylA* gene was detected in only 3 of the 21 *Brachyspira* spp.-positive feces. *B. pilosicoli* 16S rDNA was detected in 5 of the 21 *Brachyspira* spp.-positive feces (Table 2). There were no feces in which both pathogenic *Brachyspira* were simultaneously present. Only one farm was *B. hyodysenteriae*-positive, and only one farm was *B. pilosicoli*-positive.

The correlations between the fecal organic acid concentrations and the presence of pathogenic *Brachyspira* are shown in Table 2. The concentration of iso-butyrate was

Table 1. Primers used in this study

Gene	Primer pairs	References
<i>Brachyspira</i> 16S rDNA ^{a)}	Forward	5'-GAGTGACAGTAGATA ATGTAAG-3'
	Reverse	5'-GGCACTCCTATTTAA ATAGAAGC-3'
<i>B. hyodysenteriae</i> <i>tylA</i>	Forward	5'-GCAGATCTAAAGCACAGGAT-3'
	Reverse	5'-GCCTTTTGAAACATCACCTC-3'
<i>B. pilosicoli</i> 16S rDNA	Forward	5'-GTAGAGGAAAGTTTTTCGCTTC-3'
	Reverse	5'-GGCACTCCTATTTAAATAGAA GC-3'

a) Genus-specific primers.

Table 2. Organic acid concentrations in the feces of pigs infected with *Brachyspira* spp.^{a)}

PCR detection of <i>Brachyspira</i>	Number of fecal samples	Organic acid concentration (mmol/kg wet feces)								
		Succinate	Lactate	Formate	Acetate	Propionate	iso-Butyrate	n-Butyrate	iso-Valerate	n-Valerate
Not detected	85	0.2 $\pm$ 0.8	5.4 $\pm$ 13.6	1.0 $\pm$ 0.7	94.8 $\pm$ 23.7	46.1 $\pm$ 21.3	4.2 $\pm$ 2.4a	27.3 $\pm$ 18.0	4.9 $\pm$ 3.1b	10.0 $\pm$ 6.9
<i>B. hyodysenteriae</i>	3	0.0 $\pm$ 0.0	6.9 $\pm$ 5.7	0.9 $\pm$ 0.1	95.0 $\pm$ 12.6	37.7 $\pm$ 1.1	2.6 $\pm$ 1.6b	13.5 $\pm$ 3.0	3.8 $\pm$ 1.7bc	5.5 $\pm$ 2.1
<i>B. pilosicoli</i>	5	0.3 $\pm$ 0.3	3.4 $\pm$ 3.7	0.8 $\pm$ 0.3	99.6 $\pm$ 11.6	40.9 $\pm$ 15.7	2.1 $\pm$ 0.6b	19.7 $\pm$ 7.5	1.6 $\pm$ 1.0c	5.8 $\pm$ 2.7
Non-pathogenic <i>Brachyspira</i> spp.	13 ^{b)}	0.1 $\pm$ 0.2	2.9 $\pm$ 4.0	1.2 $\pm$ 0.8	110.8 $\pm$ 14.5	49.6 $\pm$ 17.2	7.0 $\pm$ 2.9a	28.9 $\pm$ 14.8	8.1 $\pm$ 3.4a	11.1 $\pm$ 6.3
One-way ANOVA or Kruskal-Wallis test p value		N.C.	0.44	0.83	0.12	0.78	<0.001	0.32	<0.001	0.32

a) Values are the means $\pm$ SD.

The means in a column without a common letter differ significantly (abc; $p < 0.05$).

b) The number of feces carrying *B. hyodysenteriae* or *B. pilosicoli* was subtracted from the number of feces carrying *Brachyspira* spp. as determined by *Brachyspira* genus-specific PCR.

Table 3. Organic acid concentration in the pig feces collected from farms contaminated with pathogenic *Brachyspira*^{a)}

Pathogenic <i>Brachyspira</i>	Number of farms	Number of feces analyzed	Organic acid concentration (mmol/kg wet feces)								
			Succinate	Lactate	Formate	Acetate	Propionate	iso-Butyrate	n-Butyrate	iso-Valerate	n-Valerate
Not detected	13	87	0.2±0.8	4.8±12.8	1.1±0.8	98.8±22.6a	48.0±21.1a	4.8±2.6a	29.1±17.6a	5.7±3.3a	10.6±7.0a
<i>B. hyodysenteriae</i> –positive farm	1	8	0.1±0.2	10.6±13.2	0.7±0.3	80.8±27.8a	33.4±12.7a	1.8±1.1b	13.4±9.7b	2.5±1.4b	6.3±4.8b
<i>B. pilosicoli</i> –positive farm	1	11	0.2±0.2	7.9±17.2	0.8±0.3	87.5±22.9a	37.2±13.2a	2.4±1.2b	15.5±8.5b	2.3±1.7b	5.4±2.5b
One-way ANOVA or ruskal-Wallis test p value			0.31	0.88	0.47	0.047	0.049	<0.001	0.002	<0.001	0.02

a) Values are the means ± SD.

The means in a column without a common letter differ significantly (ab; p<0.05).

significantly lower in the feces containing the pathogenic *Brachyspira* than in those free from *Brachyspira* spp. The concentration of iso-valerate was also lower in the feces containing the pathogenic *Brachyspira* than in those free from *Brachyspira* spp. On the other hand, the feces with non-pathogenic *Brachyspira* detected a higher concentration of these acids than those free from *Brachyspira* spp. (Table 2). Other organic acids were not affected, with or without pathogenic spirochete infection. Comparison of the organic acid concentrations of the feces sampled from the farm with 3 pigs infected with *B. hyodysenteriae* with those of the farms free from pathogenic *Brachyspira* (Table 3) showed that the feces taken from the infected farm contained lower iso-butyrate and iso-valerate concentrations than those of the non-infected farms. The same tendency was observed for the feces taken from the farm in which 5 pigs were infected with *B. pilosicoli*. The concentrations of n-butyrate and n-valerate were also lower in the feces from the pathogenic *Brachyspira*-positive farms than in those from the non-infected farms.

The large-intestinal environment of pigs reflects the development of SD [3, 4, 13]. Rapidly fermentable carbohydrates, such as guar gum, are linked to clinical expression of SD [13], whereas a high animal-protein diet is protective against SD [14]. In this study, lower concentrations of iso-butyrate and iso-valerate were associated with development of pathogenic spirochete infections. Interestingly, the feces collected from the two farms where pigs were infected with pathogenic *Brachyspira* showed lower iso-butyrate and iso-valerate concentrations than those collected from non-infected farms. iso-Butyrate and iso-valerate are organic acids that are produced by deamination of branched chain amino acids, such as valine and leucine [1]. A lower concentration of these iso-acids, therefore, suggests lower activity of bacterial deamination and/or a smaller supply of branched chain amino acids. In this context, protection against SD by feeding a high animal-protein diet [14] seems interesting. It is noteworthy that the farm in which 3 pigs were infected with *B. hyodysenteriae* used a diet consisting of waste bred (50%) and a commercial swine formula feed (50%). It appears that this diet was richer in rapidly fermentable carbohydrates than the commercial formula feed only. The pigs on this farm were fed this particular diet

from weaning to finishing. The present observation suggests the importance of the animal-protein concentration in the swine diet, in which low protein and highly fermentable carbohydrates may support colonization by *B. hyodysenteriae*. This point requires further elucidation. On this farm, SD has been observed for a long time, particularly in mid-summer, and *B. hyodysenteriae* has also been detected. Consistent detection can be related to the particular diet formulation used on this farm. The other farm contaminated by *B. pilosicoli* had once suffered from SD caused by *B. hyodysenteriae*. Since a typical formula feed with no modifications was used on this farm, it was difficult to determine why there were lower concentrations of iso-acids. This farm was free from *B. hyodysenteriae* by the start of sampling in this study, and non-pathogenic *Brachyspira* spp. and/or weakly pathogenic *Brachyspira* (*B. pilosicoli*) apparently replaced *B. hyodysenteriae*. The reasons and mechanisms for this replacement are unknown. However, Moxley and Duhamel [12] suggested in a review article that colonization by non-pathogenic *Brachyspira* would be effective for prevention of colonization by pathogenic *Brachyspira*. The possibility of competitive exclusion by non-pathogenic *Brachyspira* should be studied in detail in further studies because such evidence can develop an alternative treatment for preventing infection and colonization by *B. hyodysenteriae*.

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