

## Pathogenicity of Porcine Intestinal Spirochetes in Gnotobiotic Pigs

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Twelve intestinal spirochete strains of porcine origin were characterized on the basis of their phenotypic properties, by multilocus enzyme electrophoresis, and by pathogenicity testing in gnotobiotic pigs. The spirochetes used included two strains of *Serpulina hyodysenteriae* (B204 and P18A), two strains of *Serpulina innocens* (B256 and 4/71), one strain from the proposed new genus and species "*Anguillina coli*" (P43/6/78), and seven non-*S. hyodysenteriae* strains recently isolated from United Kingdom pig herds with a history of nonspecific diarrhea and typhlocolitis. By multilocus enzyme electrophoresis, five of these were identified as *S. innocens*, one was identified as an unspecified *Serpulina* sp., and one was identified as "*A. coli*." *S. hyodysenteriae* B204 and P18A, "*A. coli*" P43/6/78 and 2/7, and three (22/7, P280/1, and 14/5) of the five *S. innocens* field isolates induced mucoid feces and typhlocolitis in gnotobiotic pigs. None of the other spirochetes produced clinical signs or large intestinal pathology in this model. The "*A. coli*" strains induced a more watery diarrhea, with lesions present more proximally in the large intestine, than did the other pathogenic spirochetes. *S. innocens* 22/7 was also tested for pathogenicity in hysterotomy-derived pigs that had previously been artificially colonized with a spirochete-free intestinal flora and shown to be susceptible to swine dysentery. Despite effective colonization, strain 22/7 did not produce any disease, nor was there any exacerbation of large intestinal pathology or clinical signs when pigs with an experimentally induced existing colitis caused by *Yersinia pseudotuberculosis* were superinfected with strain 22/7. Certain non-*S. hyodysenteriae* spirochetes are therefore capable of inducing disease in gnotobiotic pigs, but their role as primary or opportunistic pathogens in conventional pigs remains equivocal.

Porcine intestinal spirochetes form a diverse group of organisms that includes the strongly beta-hemolytic species *Serpulina hyodysenteriae*, the etiological agent of swine dysentery. Weakly beta-hemolytic intestinal spirochetes are commonly found in the feces of both normal and diarrheic pigs (16, 21). These were originally thought to be nonpathogenic and were assigned to the species *Treponema innocens* (19), now reclassified as *Serpulina innocens* (42, 43). Sample strains were found to be nonpathogenic in conventional pigs that were susceptible to swine dysentery (20), and it was assumed that this lack of pathogenicity applied to all weakly hemolytic strains.

It was reported subsequently, however, that this category of spirochetes is very diverse (23, 25) and that some weakly hemolytic strains may be enteropathogenic, albeit producing a clinical syndrome less severe than swine dysentery. These spirochetes have been implicated as causal agents in outbreaks of nonspecific colitis and diarrhea in growing pigs in Canada (9, 18, 41), the United States (1, 6), the United Kingdom (46, 48), Poland (3), and Australia (10). Similar spirochetes have been implicated in cases of diarrhea in humans (5, 8, 38) (intestinal spirochetosis [11]). The pathological significance of spirochete colonization in humans has been questioned (13), but in such cases, colonization may have been by *Brachyspira aalborgi* (14). *B. aalborgi* is genetically distinct from most spirochetes recovered from humans with diarrhea (22). The latter spirochetes are closely related to weakly hemolytic spirochetes that have been recovered from pigs with intestinal

spirochetosis and that belong to the proposed new genus "*Anguillina*" (22–24).

To date, there is limited experimental evidence to demonstrate a primary pathogenic role for weakly hemolytic spirochetes in pigs, and all such studies have utilized only conventional pigs (1, 20, 48). Although these animals have proved useful for the study of swine dysentery, they may be less suitable for the reproduction of disease caused by weakly hemolytic spirochetes. The reason for this is that the "normal" intestinal flora of conventional specific-pathogen-free pigs frequently includes a complement of indigenous weakly hemolytic spirochetes, the pathogenic potential of which is unknown. These spirochetes complicate the identification of any artificially inoculated spirochete in the feces, are likely to compete with any inoculated spirochete for the occupation of a similar ecological niche, and could induce immunity to infection. In addition, wide variations in fecal consistency and the histological appearance of large intestinal tissue have been observed in normal growing pigs from a variety of sources (34). It is therefore difficult, with this type of experimental animal, to determine the degree of colonic damage effected by putative pathogens for a disease in which the expected lesion may be quite subtle and the incidence of clinical signs may be low. Indeed, previous studies of spirochete enteropathogenicity with conventional pigs have been complicated by the presence in control animals of diarrhea or pasty feces, weakly hemolytic spirochetes, or both (20, 48).

In the present study, to eliminate these complicating factors and to investigate the pathogenicity of test strains in the presence and absence of an indigenous flora, two models were used. The first comprised gnotobiotic pigs infected with a nonpathogenic strain of *Bacteroides vulgatus* (12). The presence of this anaerobe is thought to facilitate colonization by

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the more fastidious spirochetes (49). The second model comprised hysterotomy-derived piglets artificially colonized with an enteric flora that was free from porcine enteric pathogens and spirochetes of any description. In addition, the possibility that the presence of a preexisting large intestinal lesion might predispose the animals to spirochete colonization and/or pathogenicity was investigated by inducing colitis with *Yersinia pseudotuberculosis* (37) and then superinfecting the animals with test spirochetes.

## MATERIALS AND METHODS

**Spirochetes.** Twelve porcine intestinal spirochete strains were tested for pathogenicity in gnotobiotic pigs. These included *S. hyodysenteriae* B204 and P18A, both of which have been shown to be pathogenic in conventional pigs (20). B256 (the type strain) and 4/71 are nonpathogens belonging to the species *S. innocens* (20, 43). Strain P43/6/78 is a weakly hemolytic porcine enteric pathogen (48) recently named the type strain of the proposed new genus and species "*Anguillina coli*" (22). Seven recent (1991) non-*S. hyodysenteriae* field isolates were also tested. Strains 1/5, 22/5, 16/7, 22/7, 2/7, and P280/1 were isolated from the large intestinal contents or mucosal scrapings of pigs in which porcine colitis syndrome (7, 30, 39, 46, 51) had been diagnosed on the basis of clinical signs, the absence of other relevant enteric pathogens, postmortem examination of at least one affected animal, and histological confirmation of colitis or typhlocolitis in that animal. Strain 14/5 was isolated from the feces of an asymptomatic pig from a herd in which porcine colitis syndrome had been diagnosed in the past but which was not clinically affected at the time that the sample was taken.

**Spirochete cultures.** Cultures used for inoculation had been passaged between 12 and 16 times on laboratory media, with the exception of strains B204 (passages 25 and 26), B256 (passages 22 and 23), and P43/6/78 (passage number unknown). Test strains were grown overnight at 37°C in an atmosphere of 95% nitrogen–5% carbon dioxide, with constant stirring, in brain heart infusion broth (Unipath Ltd., Basingstoke, United Kingdom) containing normal rabbit serum (5%; GIBCO), spectinomycin (400 mg/liter; Spectam; Upjohn), cysteine (1 g/liter; BDH Ltd., Poole, United Kingdom), and resazurin (0.01 g/liter).

**Phenotypic properties.** All spirochetes were tested for the strength of their beta-hemolysis reaction after 3 days of growth at 39°C by examining cultures on sheep blood agar (Oxoid blood agar base no. 2 supplemented with 5% defibrinated ovine blood). Indole production was assessed with overnight, flask-grown cultures of each strain: a 4-ml culture was shaken with 0.5 ml of ether, and then a few drops of Kovács reagent was added. All strains were tested with antiserum to *S. hyodysenteriae* P18A in a slide agglutination test which is specific for *S. hyodysenteriae* (4). The API ZYM (Bio-Merieux Ltd., Marcy l'Etoile, France) profiles of all spirochete strains were obtained from 3-day cultures on sheep blood agar with the recommended method. The five-digit scoring system devised by Hunter and Wood was used to compare strains (17). Finally, the number of axial flagella of test strains was determined by a direct examination of aliquots of cultures with a Philips transmission electron microscope (23).

**MEE.** Strains B204, P18A, B256, 4/71, and P43/6/78 were previously examined by the multilocus enzyme electrophoresis (MEE) technique (23). In the current study, the seven field isolates were examined in an identical manner (23). In brief, spirochetes in late log phase were harvested and sonicated, and the supernatants were collected. These lysates were subjected

to electrophoresis in horizontal starch gels, and the mobilities of the following 15 enzymes were analyzed: acid phosphatase, alcohol dehydrogenase, adenylate kinase, alkaline phosphatase, esterase, fructose-1,6-diphosphatase, glucose phosphate isomerase, guanine deaminase, glutamate dehydrogenase, hexokinase, mannose-6-phosphate isomerase, nucleoside phosphorylase, 1-leucyl glycylglycine peptidase, phosphoglucosyltransferase, and superoxide dismutase. Substrates and buffers were as previously described (23).

Mobility variants of the enzymes were interpreted as the products of different alleles at the corresponding locus. Results for the field isolates were compared with those previously obtained for 188 porcine intestinal spirochetes (23). Groups of one or more isolates with the same alleles at all loci were designated an electrophoretic type (ET). Genetic diversity ( $h$ ) at each enzyme locus was calculated as  $h = (1 - \sum p_i^2) / [n(n - 1)]$ , where  $p_i$  is the frequency of the  $i^{\text{th}}$  allele and  $n$  is the number of ETs. Genetic distance between ETs was calculated as the proportion of fixed loci at which dissimilar alleles occurred, and the clustering strategy of the unweighted pair-group method of arithmetic averages was used to create a phenogram (40).

**Pathogenicity testing in gnotobiotic pigs.** Forty-one gnotobiotic pigs (Large White/Landrace cross) were produced and maintained in plastic isolators as described previously (45). Pigs were fed tinned evaporated cows' milk diluted with water and a mineral supplement. All pigs except for the two pigs inoculated with strain P280/1, two of the four pigs inoculated with strain B256, and two controls were accidentally contaminated with *Staphylococcus* spp. A sample contaminated pig was colonized at a level of  $2 \times 10^6$  CFU of staphylococci per g of feces in conjunction with intentionally inoculated bacteria. All 41 experimental pigs were infected with overnight cultures of a nonpathogenic strain of *B. vulgatus* (12) by oral dosing at 27 to 30 days of age. The remainder were inoculated with test spirochete strains via a stomach tube at 24 h ( $5 \times 10^9$  organisms) and 48 h ( $1 \times 10^{10}$  organisms) after inoculation with *B. vulgatus*. Initially, all spirochete strains tested were used to inoculate two pigs each. If neither animal showed evidence of disease, two more pigs were inoculated with that strain in a subsequent experiment.

Fecal swabs or samples were taken daily from all pigs and cultured for the presence of *B. vulgatus* and spirochetes, and the appearance was noted. *B. vulgatus* was detected by incubation of sheep blood agar in an anaerobic cabinet (Don Whitley Scientific Ltd., Shipley, United Kingdom) for 24 to 48 h. Sample colonies were identified with the API 20A system (Bio-Merieux). Spirochetes were grown anaerobically at 39°C for up to 7 days on fastidious anaerobe agar (LabM) supplemented with 400 mg of spectinomycin per liter and identified by their morphological appearance under phase-contrast microscopy. Contaminating bacteria were detected by aerobic (37°C) and anaerobic (39°C) incubation of sheep blood agar for 24 and 48 h, respectively. Staphylococci were identified with the API Staph system (Bio-Merieux). The dry-matter content of samples from selected pigs was measured daily. Numbers of spirochetes per gram of feces were estimated at day 5 postinoculation (p.i.) and at postmortem examination (day 10 p.i.) by anaerobic incubation of successive dilutions of feces on spectinomycin-containing blood agar plates in a modification of the Miles-Misra technique (32).

Blood samples (EDTA treated and clotted) were taken immediately before slaughter from all pigs. Total and differential leukocyte counts were made. The pigs were killed by stunning and exsanguination, and histological samples were removed within 3 min of death for fixation in 10% Formol-

TABLE 1. ETs and phenotypic characterization of 12 strains of porcine intestinal spirochetes tested for pathogenicity in gnotobiotic pigs

Strain	ET	Hemolysis on sheep blood agar	Indole production	API ZYM profile <sup>a</sup>	Reaction in slide agglutination test with absorbed anti-P18A <sup>b</sup>	No. of axial flagella <sup>c</sup>	Identity <sup>d</sup>
P18A	13	Strong	+	14.0.4.10.1*	+	10	<i>S. hyodysenteriae</i>
B204	15	Strong	+	14.0.4.10.1*	+	8	<i>S. hyodysenteriae</i>
22/5	i	Weak	+	14.0.4.10.1*	-	12	<i>Serpulina</i> sp.
B256	38	Weak	-	14.0.4.11.1	-	10-14	<i>S. innocens</i>
22/7	ii	Weak	-	14.0.4.2.1*	-	15-16	<i>S. innocens</i>
16/7	iii	Weak	-	14.0.4.2.1*	-	14	<i>S. innocens</i>
P280/1	iv	Strong	-	14.0.4.2.1*	-	13	<i>S. innocens</i>
4/71	43	Weak	-	14.0.4.11.1	-	13	<i>S. innocens</i>
14/5	v	Weak	-	14.0.4.3.1	-	14-17	<i>S. innocens</i>
1/5	vi	Weak	-	14.0.4.3.1	-	13	<i>S. innocens</i>
P43/6/78	60	Weak	-	14.0.4.3.0	-	5	" <i>A. coli</i> "
2/7	vii	Weak	-	14.0.4.3.0	-	4	" <i>A. coli</i> "

<sup>a</sup> An asterisk indicates a lack of  $\alpha$ -galactosidase activity.

<sup>b</sup> See reference 4.

<sup>c</sup> Data were from the following references: B204, P18A, and B256, 15; 4/71, 19; and P43/6/78, 48.

<sup>d</sup> As defined in reference 23.

buffered saline. Tissue sections were taken from the terminal ileum (10 cm proximal to the ileocecal junction), cecum (blind end), proximal colon (first loop), midcolon (sigmoid flexure), and distal colon (final loop). Fecal samples were cultured for the presence of spirochetes, *B. vulgatus*, and contaminants.

**Histology and morphometry.** All sections were stained with hematoxylin and eosin and with periodic acid-Schiff-Alcian blue. The proximal colon sections were stained for spirochetes with Kerr's modification of the Warthin-Starry silver staining technique (44). The depth of longitudinally sectioned crypts in the large intestine was measured with an MOP 3 apparatus (Kontron, Echung/Munich, Germany). The mean of at least five readings for each section was calculated.

**Pathogenicity testing in "conventionalized" pigs.** Thirty five piglets were derived by hysterotomy and reared in gnotobiotic isolators until 3 weeks of age. At 10 days of age, all pigs were orally inoculated with an adult-type enteric flora (33) derived from the feces of a healthy adult bovine. Bovine flora was used to help ensure freedom from porcine intestinal spirochetes and porcine enteric pathogens. The inoculum consisted of a dilution of feces in prerduced culture medium and contained a variety of aerobic and anaerobic species. Salmonellae, *Yersinia* spp., or spirochetes of any description (including *S. hyodysenteriae*) could not be cultured by standard techniques. Pigs colonized in this way had a predominantly anaerobic fecal flora and were found to be susceptible to swine dysentery following inoculation of  $5 \times 10^9$  and  $1 \times 10^{10}$  *S. hyodysenteriae* organisms on two successive days.

Thirty pigs were conventionalized as described above and weaned onto solid food at 4 weeks of age, when they were randomly divided into four groups, 1 (6 pigs), 2 (8 pigs), 3 (8 pigs), and 4 (8 pigs), placed in four separate rooms. At 5 weeks of age, colitis was induced in pigs of groups 3 and 4 by inoculation of  $10^{10}$  CFU of *Y. pseudotuberculosis* NCTC 12718 from overnight cultures grown in Luria-Bertani broth at 28°C as described previously (37). Seven days later, each pig in groups 2 and 3 was inoculated with  $10^{10}$  spirochetes of strain 22/7 on two successive days. Strain 22/7 was chosen as a representative weakly beta-hemolytic, non-*S. hyodysenteriae* strain. Pigs were inspected daily, and the establishment of the inoculated spirochetes was monitored daily. All pigs were killed for postmortem examination 10 days after inoculation of the spirochetes. Fecal spirochete numbers, fecal dry-matter

content, peripheral leukocyte counts, and large intestinal crypt depth were measured as for the gnotobiotic pigs.

## RESULTS

**Phenotypic characterization of spirochetes.** The strength of beta-hemolysis, indole production, slide agglutination reaction, API ZYM profile, and number of axial flagella for each of the strains are given in Table 1. The two *S. hyodysenteriae* strains were strongly beta-hemolytic and indole positive and reacted in the slide agglutination test for *S. hyodysenteriae*. Of the non-*S. hyodysenteriae* strains, only P280/1 was strongly beta-hemolytic and only 22/5 was indole positive. None reacted in the *S. hyodysenteriae* slide agglutination test. As determined by the API ZYM test, strains B204, P18A, 16/7, 22/5, 22/7, and P280/1 did not possess  $\alpha$ -galactosidase activity. B204, P18A, and 22/7 showed a five-digit profile typical of swine dysentery strains (17). The remaining strains showed profiles typical of field strains not associated with swine dysentery. Most of the strains had eight or more axial flagella, the exceptions being P43/6/78 and 2/7, which had five and four flagella, respectively.

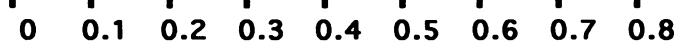
**Genetic characterization of spirochetes.** The relationships of the seven field isolates of non-*S. hyodysenteriae* spirochetes to a large collection of other porcine intestinal spirochetes (23) are demonstrated in the phenogram produced with data from the MEE analysis (Fig. 1). The ETs of the test strains and their identities are indicated in Table 1. Isolate 22/5 was a *Serpulina* sp. distinct from but related to *S. hyodysenteriae* and "*Serpulina intermedius*" (22); isolates 22/7, 16/7, P280/1, 14/5, and 1/5 were typical of *S. innocens*; and isolate 2/7 was located with the isolates of "*A. coli*" (23).

**Gnotobiotic pig experiments.** *B. vulgatus* was recovered from all pigs within 1 day of inoculation. Spirochetes were recovered from feces between 3 and 10 days p.i. Spirochetes were also recovered from the feces of all pigs at postmortem examination, and strains B204, P280/1, 22/5, 22/7, and P43/6/78 were also recovered from the ileal contents. In most cases, spirochete numbers per gram of feces increased between 5 and 10 days p.i., to levels comparable to those obtained from pigs inoculated with the *S. hyodysenteriae* strains (Table 2).

None of the inoculated pigs showed signs of dehydration, loss of appetite, or other indication of systemic illness. For strains P18A, B204, P280/1, 14/5, and 22/7, the feces of one or

# Genetic Distance

(Proportion of fixed allelic differences)



GROUP

E.T.

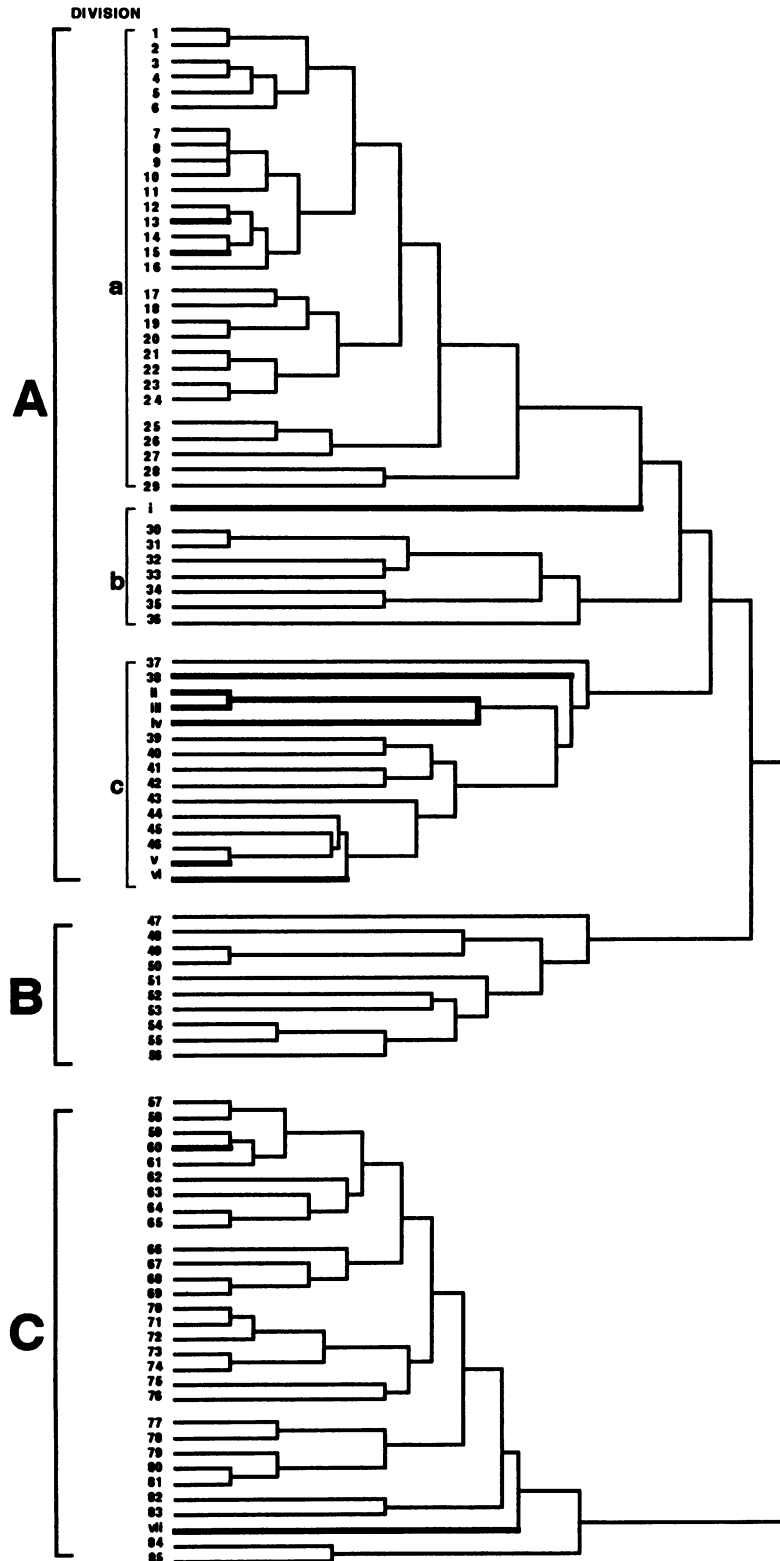


TABLE 2. Results of pathogenicity testing of 12 strains of porcine intestinal spirochetes in gnotobiotic pigs

Strain	No. of pigs tested	No. of pigs with clinical signs	Mean no. of spirochetes <sup>a</sup> at the following day p.i.:		Fecal DM <sup>b</sup> (%)	No. of pigs with colitis	Mean crypt depth (μm) in the following sample from pigs with (+) or without (-) clinical signs:							
			5	10			Cecum		Proximal colon		Midcolon		Distal colon	
							+	-	+	-	+	-	+	-
P18A	2	2	2 × 10 <sup>7</sup> (2)	2 × 10 <sup>9</sup> (2)	10	2	5,430		5,980		6,940		9,170	
B204	2	1	NT	4 × 10 <sup>7</sup> (2)	18	1	3,150	2,740	3,460	4,660	3,380	3,720	3,470	2,980
22/5	4	0	3 × 10 <sup>5</sup> (3)	2 × 10 <sup>7</sup> (2)	13	0		3,620		3,620		3,000		2,940
B256	4	0	NT	NT	22	0		3,590		3,240		3,330		2,790
22/7	2	1	4 × 10 <sup>6</sup> (2)	NT	12	1	5,680	2,860	6,380	2,730	6,210	2,500	7,870	2,240
16/7	4	0	1 × 10 <sup>5</sup> (2)	4 × 10 <sup>5</sup> (2)	19	0		3,720		3,500		3,250		3,090
P280/1	2	2	NT	NT	NT	2	5,360		4,670		5,350		5,380	
4/71	4	0	2 × 10 <sup>8</sup> (4)	1 × 10 <sup>8</sup> (4)	21	0		3,910		3,820		3,130		2,980
14/5	2	1	1 × 10 <sup>7</sup> (2)	NT	NT	1	3,970	3,170	4,180	2,890	5,270	2,970	6,790	2,690
1/5	4	0	1 × 10 <sup>8</sup> (1)	5 × 10 <sup>7</sup> (1)	12	0		3,470		3,450		3,070		3,000
P43/6/78	2	1	3 × 10 <sup>8</sup> (2)	8 × 10 <sup>8</sup> (2)	11	1	4,470	3,830	4,220	4,090	3,460	3,520	2,960	2,800
2/7	2	1	6 × 10 <sup>6</sup> (2)	3 × 10 <sup>8</sup> (2)	9	2	4,160	4,440	4,020	4,100	3,530	2,980	2,780	2,700
None (control)	7	0			17	0		3,490		3,270		3,100		3,230

<sup>a</sup> Per gram of feces (number of observations). NT, not tested.

<sup>b</sup> DM, mean fecal dry-matter content at postmortem examination.

both of the pigs changed to include large quantities of mucus, which contained large numbers of spirochetes. For strains 22/7 and 14/5, flecks of fresh blood were observed interspersed with the mucus and fecal material. The feces contained much mucus until the time of the postmortem examination (10 days p.i.). The clinical signs differed somewhat for strains P43/6/78 and 2/7, however. The feces of affected pigs inoculated with these strains appeared more liquid than the semisolid feces of the control pigs and sometimes contained small amounts of mucus and thinner, clear fluid interspersed with the fecal material. One pig from each of the pairs dosed with P43/6/78 and 2/7 passed large quantities of mucus, flecked with blood, over a single day (days 7 and 8 p.i., respectively), but the feces became relatively normal by the time of the postmortem examination. Pigs inoculated with strains B256, 4/71, 22/5, 16/7, and 1/5 showed no clinical signs.

The most consistent histological finding in pigs that were clinically affected was a thickening of the lamina propria in one or more of the large intestinal sections, associated with an increase in crypt depth. This finding was always accompanied by other evidence of inflammatory changes or epithelial damage. Other features included marked neutrophil infiltration of the lamina propria, hemorrhage or hyperemia, dilated crypts, and crypt abscesses. Piling up of epithelial cells at the inter-crypt extrusion zone was frequently found; erosion of the epithelium over small areas was sometimes found and was associated with subepithelial neutrophil accumulation and the apparent migration of neutrophils into the lumen (Fig. 2B and C). Large quantities of periodic acid-Schiff-Alcian blue-stained mucus were frequently visible in the lumen; this mucus contained trapped epithelial cell debris and neutrophils. The amount of goblet cell mucin was generally increased, although in the most severely affected areas, there was goblet cell

depletion. Silver-stained sections showed variable colonization of both the crypts and the luminal epithelium of the proximal colon. Pigs displaying clinical signs generally showed heavy colonization of the surface and/or crypts by spirochetes, but this was also the case with many of the pigs not showing clinical signs.

The morphometric studies indicated increased crypt depth in at least one of the sampled regions of the large intestine in all clinically affected pigs (Table 2). The most severely affected region was the distal colon, except for the pigs infected with strains P43/6/78 and 2/7, for which most pathological changes occurred in the cecum.

In all but one case, peripheral leukocyte counts in gnotobiotic pigs ranged from  $4.5 \times 10^9$  to  $11.3 \times 10^9$  cells per liter, with no significant differences between spirochete-inoculated and control pigs or between pigs displaying clinical signs and pigs not displaying clinical signs. The exception was one of the pigs infected with strain 14/5, which had shown marked clinical signs of blood and mucus in the feces and had a total leukocyte count of  $21.1 \times 10^9$  cells per liter, with 67% neutrophils. The mean fecal dry-matter content for each infected group is shown in Table 2. This content often varied greatly from day to day in individual pigs, including controls. When fecal samples contained large amounts of mucus, the value was generally below 10%. Similar values were also recorded on occasion for control pigs.

**Conventionalized pig experiments.** Spirochete 22/7 became established in all inoculated pigs within 3 days. Mean spirochete numbers at the postmortem examination were similar in group 2 (infected with spirochete 22/7 alone) and group 3 (infected with spirochete 22/7 and *Y. pseudotuberculosis*), approximately  $10^8$  spirochetes per g of feces. Spirochetes were not recovered from any uninoculated pigs. No clinical signs or

FIG. 1. Phenogram of genetic distances among 92 ETs of porcine intestinal spirochetes, clustered by the strategy of the unweighted pair-group method of arithmetic averages, modified from the method of Lee and Hampson (22) with permission. ETs containing the 12 spirochetes used to infect gnotobiotic pigs in this study are outlined in boldface type. ETs i to vii contain recent United Kingdom field isolates that have not been previously analyzed. ET 13, *S. hyodysenteriae* P18A; ET 15, *S. hyodysenteriae* B204; ET i, isolate 22/5; ET 38, *S. innocens* B256, ET ii, isolate 22/7; ET iii, isolate 16/7; ET iv, isolate P280/1; ET 43, *S. innocens* 4/71; ET v, isolate 14/5; ET vi, isolate 1/5; ET 60, "*A. coli*" P43/6/78; ET vii, isolate 2/7. Group A, *Serpulina* spp.; group B, group B spirochetes; group C, "*A. coli*"; division a, *S. hyodysenteriae*; division b, "*S. intermedius*"; division c, *S. innocens*.

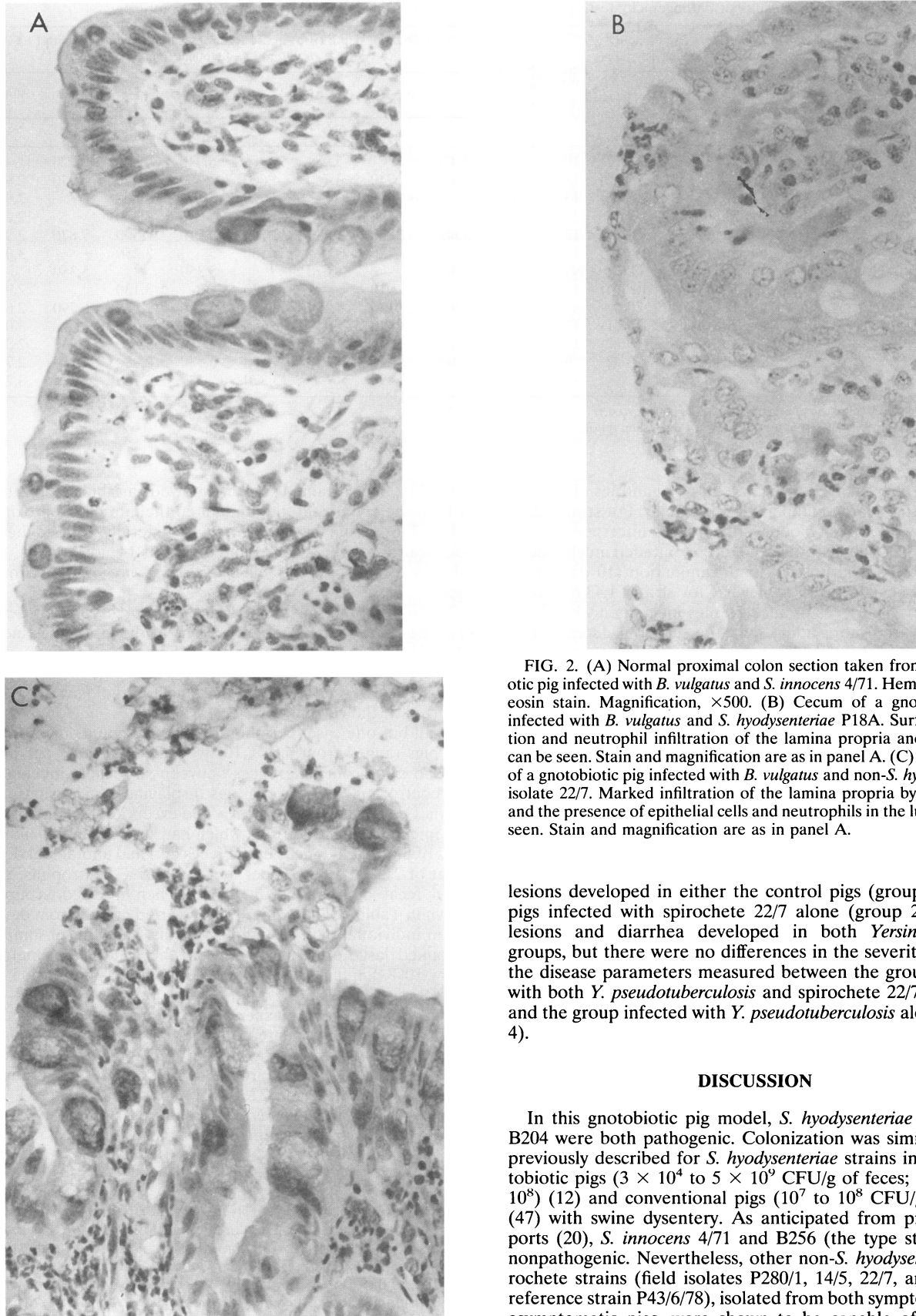


FIG. 2. (A) Normal proximal colon section taken from a gnotobiotic pig infected with *B. vulgatus* and *S. innocens* 4/71. Hematoxylin and eosin stain. Magnification,  $\times 500$ . (B) Cecum of a gnotobiotic pig infected with *B. vulgatus* and *S. hyodysenteriae* P18A. Surface congestion and neutrophil infiltration of the lamina propria and the lumen can be seen. Stain and magnification are as in panel A. (C) Distal colon of a gnotobiotic pig infected with *B. vulgatus* and non-*S. hyodysenteriae* isolate 22/7. Marked infiltration of the lamina propria by neutrophils and the presence of epithelial cells and neutrophils in the lumen can be seen. Stain and magnification are as in panel A.

lesions developed in either the control pigs (group 1) or the pigs infected with spirochete 22/7 alone (group 2). Colonic lesions and diarrhea developed in both *Yersinia*-infected groups, but there were no differences in the severity of any of the disease parameters measured between the group infected with both *Y. pseudotuberculosis* and spirochete 22/7 (group 3) and the group infected with *Y. pseudotuberculosis* alone (group 4).

## DISCUSSION

In this gnotobiotic pig model, *S. hyodysenteriae* P18A and B204 were both pathogenic. Colonization was similar to that previously described for *S. hyodysenteriae* strains in both gnotobiotic pigs ( $3 \times 10^4$  to  $5 \times 10^9$  CFU/g of feces; mean,  $5 \times 10^8$ ) (12) and conventional pigs ( $10^7$  to  $10^8$  CFU/g of feces) (47) with swine dysentery. As anticipated from previous reports (20), *S. innocens* 4/71 and B256 (the type strain) were nonpathogenic. Nevertheless, other non-*S. hyodysenteriae* spirochete strains (field isolates P280/1, 14/5, 22/7, and 2/7 and reference strain P43/6/78), isolated from both symptomatic and asymptomatic pigs, were shown to be capable of causing a

disease in gnotobiotic pigs which was similar to that produced by *S. hyodysenteriae* spirochete strains. According to the results of the MEE analysis, isolates 22/7, 16/7, P280/1, 14/5, and 1/5 were *S. innocens*, and three of these isolates were pathogenic. Overall, spirochetes that were pathogenic in this model included both indole-positive and indole-negative strains, strongly and weakly beta-hemolytic strains, and members of both genera *Serpulina* and "*Anguillina*." One of these putative pathogens, isolate 22/7, subsequently failed to cause clinical signs or significant lesions in pigs with a commensal enteric flora.

Weakly hemolytic strain P43/6/78, the type strain of the proposed new genus and species "*A. coli*" (23), has been described as causing diarrhea and colitis in experimentally inoculated conventional pigs (48). It was therefore of interest that this strain was also pathogenic in the gnotobiotic pigs in this study. Field isolate 2/7, which on morphological and genetic grounds was also shown to be a strain of "*A. coli*," caused changes similar to those caused by P43/6/78. This result suggests that members of this newly described group of spirochetes are potential pathogens. The transience of the clinical signs (possibly reflecting intermittent symptoms over a longer period) and the more watery nature of the feces of pigs colonized by these two strains may indicate a clinical syndrome different from that observed with other pathogens in this model. In addition, only the cecum was affected by these two strains, whereas the histological colitis produced by the other pathogens was always most marked in the more distal regions of the large intestine. These data indicate that the pathogenesis of the colitis caused by these two strains may be different from that of the disease produced by *S. hyodysenteriae* and the other potential pathogens identified in this model. The significance of "*A. coli*" in the field is still unclear, however. Not all the gnotobiotic pigs were affected by the two strains used here, and another "*A. coli*" strain, 3295, did not cause any clinical signs or pathological lesions in four gnotobiotic pigs despite colonization with up to  $10^{10}$  organisms/g of feces (36). Strain 3295 also failed to cause disease in experimentally infected conventional pigs (31).

Field isolates 22/7, P280/1, and 14/5 were also pathogenic in the gnotobiotic pig model. These isolates were considered to be *S. innocens* on the basis of their clustering by MEE analysis. The principal clinical sign observed was the passage of mucus in the feces, with accompanying fecal material which was usually of a normal consistency. The fecal dry-matter content of the gnotobiotic pigs was highly variable, even in the control animals. A better quantitative measure of the pathogenic effects of the spirochetes, in both the gnotobiotic and the conventionalized pigs, was the depth of the crypts in the various regions of the large intestine. This parameter was chosen as a broad measure of the size of the proliferative compartment and hence the level of epithelial cell turnover secondary to increased cell loss (2). Increased crypt depth was always associated with other evidence of inflammation, with or without tissue damage. This parameter was increased in all but one of the gnotobiotic pigs that displayed clinical signs and in the majority of the conventionalized pigs infected with *Y. pseudotuberculosis*, including all pigs with diarrhea.

Characterization of the test strains revealed no common factors that correlated with pathogenicity in this model. Past reports have suggested that indole production by weakly hemolytic spirochetes ("*S. intermedius*") (23) may correlate with pathogenicity (3), but indole-positive isolate 22/5 used in this study failed to produce lesions or clinical signs. This isolate was more closely related to *S. hyodysenteriae* than were any of the other field isolates. It has also been suggested that a lack of

$\alpha$ -galactosidase activity (as determined by API ZYM profiles) may be an indicator of pathogenicity (17). In this model, however, pathogenic strains included spirochetes with and without  $\alpha$ -galactosidase activity.

The hemolysin of *S. hyodysenteriae* has been suggested to be a possible virulence factor. With one reported exception (28), strongly beta-hemolytic strains are pathogenic for pigs (20), and purified hemolysin has produced epithelial cell damage when directly inoculated into porcine ileal and colonic loops (27). Extended laboratory passage, however, has produced strains that remain strongly beta-hemolytic but are no longer pathogenic for pigs (20). Whether the hemolysin of *S. hyodysenteriae* is an important virulence factor is left unclear by the results of these studies. The clinical syndrome was not noticeably worse in pigs inoculated with strongly beta-hemolytic strains, although disease was produced more consistently by these strains (two of two pigs for B204, P18A, and P280/1 and one of two pigs for all the other pathogens, except for 2/7). The hemolysin of the strongly beta-hemolytic strains may constitute a factor that renders the expression of pathogenicity (but not colonization) by the spirochete less dependent on individual host factors than appears to be the case with weakly beta-hemolytic strains.

The small number of strongly beta-hemolytic *S. hyodysenteriae* strains tested which produce clinical signs in gnotobiotic pigs have also proved pathogenic in susceptible conventional pigs (26, 50), but this correlation does not necessarily apply to the non-*S. hyodysenteriae* strains tested here. Strain 22/7, which caused mucoid diarrhea in gnotobiotic pigs, did not produce clinical signs in artificially colonized conventional pigs that were susceptible to swine dysentery. Similarly, strain P280/1, which has consistently caused disease in gnotobiotic pigs, has been shown not to cause disease in conventional specific-pathogen-free pigs from several sources despite use of a variety of experimental diets and up to  $10^{11}$  organisms at inoculation (29).

Factors other than those which cause symptoms in gnotobiotic pigs are involved in producing disease in conventional pigs. The clinical disease produced by *S. hyodysenteriae* strains in gnotobiotic pigs resembles some cases of swine dysentery in conventional pigs, for example, in the production of mucoid feces. It differs from it in that no systemic signs of illness, such as anorexia, weight loss, dehydration, or acidosis, are observed (despite restricted milk feeding being the only source of fluid available to the pigs). These facts indicate that factors which exacerbate swine dysentery (causing dehydration and mortality, etc.) are not sufficiently active in the absence of a cereal-based diet and normal intestinal flora to cause symptoms in gnotobiotic animals.

Conversely, these experiments showed that factors which allow the production of lesions and clinical signs by a non-*S. hyodysenteriae* spirochete in gnotobiotic pigs fail to do so in the presence of a diverse large intestinal bacterial population. This result is not associated with a failure to colonize, since in the case of both *S. hyodysenteriae* strains and non-*S. hyodysenteriae* 22/7, colonization appears to be similar in conventional and gnotobiotic pigs. This phenomenon also does not appear to be related to diet, since other experiments have indicated that milk-fed conventionalized pigs also do not develop clinical signs when inoculated with strain 22/7 (35). These discrepancies in the pathogenicity of certain spirochetes in conventional and gnotobiotic pigs have implications for the use of gnotobiotic pig models as a means of predicting the clinical significance of non-*S. hyodysenteriae* spirochete isolates.

It is possible that certain spirochete strains originally associated with clinical symptoms in the host may colonize existing



lesions in an opportunistic capacity. Superinfection with spirochete 22/7 of pigs with *Y. pseudotuberculosis*-induced colitis, however, did not increase colonization by the spirochete and failed to produce any significant exacerbation of the various disease parameters examined.

In conclusion, a number of weakly beta-hemolytic spirochete strains currently present in commercial pig herds are capable of producing large intestinal lesions and clinical signs in gnotobiotic pigs. This property appears to be unrelated to the production of indole, the presence or absence of  $\alpha$ -galactosidase activity, and the electrophoretic group into which these spirochetes fall. For at least one strain, pathogenicity in the gnotobiotic model does not necessarily indicate pathogenicity in conventional pigs. It is possible, however, that the pathogenic potential of such strains is fulfilled in conventional animals under certain circumstances. These circumstances are likely to be fairly specialized events, since in the case of strain 22/7 examined here, they appeared to be unrelated to diet, intestinal colonization by the spirochete, or the presence of preexisting large intestinal mucosal damage. The circumstances in which pathogenicity testing of intestinal spirochetes is performed should be defined more closely and, in particular, the definition of the enteropathogenicity of specific species should be reexamined.

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