Development of an Experimental Model Allowing Discrimination Between Virulent and Avirulent Isolates of *Serpulina (Treponema) hyodysenteriae*

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ABSTRACT

Variation in virulence among different strains of *Serpulina hyodysenteriae* was studied by oral inoculation of specific pathogen free piglets and CD-1 mice. Piglets infected with serotype 2 reference strain B204 and an untypable field strain LHV-90-9-I had severe diarrhea tainted intermittently with mucus and fresh blood. The piglets inoculated with B169, B8044, B6933, and ACK300-8 reference strains representing serotypes 3, 5, 6, and 7 respectively developed moderate diarrhea. However, reference strains B234 and A-1 of serotypes 1 and 4, respectively, failed to cause any diarrhea. None of the *S. hyodysenteriae* strains caused diarrhea in mice. The results indicate a great variation in virulence among strains of different serotypes of *S. hyodysenteriae*. Mice were less susceptible to infection with *S. hyodysenteriae*.

RÉSUMÉ

L'inoculation orale de différentes souches de *Serpulina hyodysenteriae* à des porcelets exempts de pathogènes spécifiques et à des souris de lignée CD-1 a permis d'évaluer la variation du degré de virulence de ces souches. Les porcelets infectés avec la souche de référence du sérotype 2 (souche B204) ainsi qu'avec une souche sauvage non-typable ont présenté une diarrhée sévère parfois teintée de sang frais et de mucus. Une diarrhée modérée a été observée chez les porcelets inoculés avec les souches de référence B169, B8044, B6933, et ACK300-8 représentant respectivement les sérotypes 3, 5, 6, et 7. Par contre, les souches de référence B234 et A-1, représentant respectivement les sérotypes 1 et 4, n'ont pas induit de diarrhée chez les porcelets. Aucune des souches de *S. hyodysenteriae* n'a causé de diarrhée chez les souris. Les résultats démontrent qu'une grande variation existe dans la virulence des souches des différents sérotypes de *S. hyodysenteriae*. De plus, les souris étaient moins susceptibles que les porcs à l'infection par *S. hyodysenteriae*.

(Traduit par docteur Serge Messier)

INTRODUCTION

Serpulina hyodysenteriae is recognized as the causative agent of swine dysentery, a mucohemorrhagic enteritis confined to the large intestine (10). The enteropathogenicity of *S. hyodysenteriae* has been demonstrated by experimental infection in piglets and chickens (3,4). Attempts to induce the disease in guinea pigs, rabbits, and mice through parenteral inoculation of *S. hyodysenteriae* have failed (11). *S. hyodysenteriae* isolates have been classified into 9 serotypes (6,20,21). Factors implicated in the pathogenicity of *S. hyodysenteriae* are not well known. However, lipopolysaccharide and hemolysin have been considered as potential virulence determinants (9, 19,23,25,29). Lesions produced by experimental infection are characterized by a severe erosion of the epithelium and considerable elongation of the crypt (4). The murine model has been used for studying the pathogenesis of *S. hyodysenteriae* (16,28), but inconsistency of the disease in the mouse model often makes interpretation of results difficult. It is not known whether strains belonging to different serotypes are equally virulent. The present studies were designed to find out if there are variations in virulence among strains of *S. hyodysenteriae* in pigs and mice.

MATERIALS AND METHODS

BACTERIAL STRAINS

Serpulina hyodysenteriae reference strains serotype 1 (B234), 2 (B204), 3 (B169), 4 (A-1), 5 (B8044), 6 (B6933), and 7 (ACK300/8), were provided by Lynn Joens (University of Arizona) and an untypable local field strain (LHV 90-9-I) was isolated from a natural case of swine dysentery. All strains were subcultured on blood agar plates, collected and stored at −70°C, as described by Joens et al (16). Identification of the organism was based on hemolytic pattern on blood agar plates and the results of biochemical tests obtained using the Rapid ANA II system (1).

EXPERIMENTAL INFECTION

Piglets — Sixteen, 5 week-old specific pathogen free piglets were randomly divided into 8 groups with 2 pigs in each group and housed according to the guidelines of The Guide to the Care and Use of Experimental Animals. Each group was placed in an isolation room and fed a ration free of antibiotics or any other drugs. Feed was removed 24 h prior to the first challenge and withheld for

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TABLE I. Results of experimental infection of piglets with different strains of *S. hyodysenteriae*

Strain (serotype)		Clinical signs	Lesions[a]		Detection of	
		Diarrhea[b]	Macro	Micro	Antibody by ELISA	Antigen by IFAT
B234	(1)	−	−	−	+	+
B204	(2)	+	+	+	+	+
B169	(3)	+	+	+	+	+
A-1	(4)	−	−	−	+	+
B8044	(5)	+	+	+	+	+
B6933	(6)	+	+	+	+	+
ACK300/8	(7)	+	+	+	+	+
LHV 90-9-I	(UT)[c]	+	+	+	+	+

[a] Macro: Macroscopic. Micro: Microscopic
[b] Hemorrhagic diarrhea was observed only in pigs infected with B204 and LHV 90-9-I strains
[c] UT: Untypable

24 h postinoculation. All the piglets were infected with *S. hyodysenteriae* twice at 24 h-interval by intragastric intubation. Each piglet was dosed orally with 10 mL of bacterial suspension containing approximately 3.2×10^8 CFU/ mL. The infection was repeated 3 times with the use of progressively higher doses of *S. hyodysenteriae* in pigs inoculated with serotype 1 or 4 in order to ensure that the number of bacteria used to inoculate the pigs was not a limiting factor. Fecal samples were collected daily during a period of 7 d before infection and every week until 70 d postinoculation. At the end of the experiment, the animals were euthanized and tissues were collected for histopathological examination.

Mice — A total of 48, 4 week-old male outbred mice of CD-1 strain were divided into 8 groups each of 6 mice. They were housed in plastic cages on pine shavings, fed commercial mouse pellets and given water ad libitum. The mice were given 2 doses of bacteria 24 h apart by gastric intubation (14). Feed was removed 12 h prior to the first challenge and withheld for 36 h postinoculation. In each group, the mice were individually inoculated with 2 mL bacterial suspension containing approximately 2×10^8 CFU/ mL of different strains for 2 consecutive days. Fecal samples were collected daily until 15 d postinoculation.

OBSERVATION OF CLINICAL SYMPTOMS AND NECROPSY

Mice and pigs were observed daily for the presence of clinical signs (diarrhea tainted with mucus/blood, depression, or anorexia) and were necropsied on 15 and 70 d postinoculation (PI) respectively. Criteria used to determine infection were clinical signs, detection of *S. hyodysenteriae* in tissues and fecal materials, antibody response and macroscopic and microscopic lesions.

CULTURE ISOLATION

For isolation of *S. hyodysenteriae*, the cecal and colon contents were streaked on selective blood agar medium containing pig feces extract and antibiotics (2,18). Plates were incubated anaerobically at 42°C for 72 h. Colonies surrounded by a clear zone of hemolysis were confirmed to be *Serpulina* by dark-field microscopy and serological confirmation.

HYPERIMMUNE SERA

Hyperimmune sera for the various *S. hyodysenteriae* serotypes were produced in New Zealand white rabbits using formalinized whole bacterial cell suspensions adjusted to 1×10^8 cells/mL, as described by Amadou et al (5). Rabbits were bled by cardiac puncture under ether anesthesia and serum was collected.

HISTOLOGICAL EXAMINATION

Tissues for histopathologic evaluation consisted of jejunum (3 pieces), ileum (3 pieces), ileo-caecal valve (3 pieces), caecum (3 pieces), and colon (3 pieces) fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were stained by hematoxylin and eosin, Warthin-Starry, and hematoxylin-phloxin-safran (4).

ENZYME-LINKED IMMUNOSORBENT ASSAY

Serum antibodies against *S. hyodysenteriae* were detected by an enzyme linked immunosorbent assay (ELISA). ELISAs were performed according to the method of Joens (15) using flat-bottom microtiter plates (Nunc, Denmark) coated with 0.1 mL of the formalinized bacterial cell suspension standardized to an optical density of 1.0 at 540 nm. Optical density (OD) values of ≥0.256 were considered positive.

IMMUNOFLUORESCENCE ASSAY

Tissues from caecum and colon fixed in 10% formalin and embedded in paraffin were examined for the presence of *S. hyodysenteriae* by an indirect immunofluorescent antibody test (IFAT) (12,26). Deparaffinized tissue sections or fecal smears were covered with rabbit hyperimmune sera against *S. hyodysenteriae* of different serotypes and incubated at 37°C for 1 h. The sections were washed twice for 5 min each in PBS (0.01 M, pH 7.2) and treated with a fluorescein isothiocyanate-labelled antirabbit IgG. The controls included testing of: specific antiserum on paraffin-embedded sections and fecal smears from non-infected animals, normal rabbit serum on tissues and smears from infected animals, and nonspecific binding of the second antibody-conjugate by incubation of the tissue sections and smears with the conjugate. After staining, smears and tissue sections were examined on a Leitz Ortholux II under 340 nm magnification.

RESULTS

INFECTION OF PIGLETS WITH *Serpulina hyodysenteriae*

The piglets inoculated with B204 strain (serotype 2) and field strain LHV 90-9-I (untypable) had severe diarrhea tainted intermittently with mucus and fresh blood for 5 d. In contrast, piglets infected with strains B234 (serotypes 1) and A-1 (serotype 4) did not show any clinical signs of diarrhea and remained culture-negative throughout the period of observation (Table I). It was noted that none of the pigs inoculated with the higher doses showed any clinical signs, and they were culture-negative (data not shown). Piglets infected with serotypes 3, 5, 6, and 7 had moderate diarrhea without blood and mucus. The piglets were colonized by the organisms, which were detected in

the feces from d 7 PI until d 35 PI. At necropsy, all intestinal sites sampled yielded *S. hyodysenteriae* upon culture. The viable count of the organisms was maximum in the colon in 12 of the 16 infected pigs (results not shown). *S. hyodysenteriae* was detected by IFAT in all infected animals, including those that did not show any clinical signs (Table I). Organisms were detected in large numbers both on the luminal surface and in the crypts of the colons of infected pigs (Fig. 1). Macroscopic and microscopic lesions were localized mainly in the colon and caecum (Fig. 3).

INFECTION OF MICE WITH *Serpulina hyodysenteriae*

Mice infected with *S. hyodysenteriae* serotypes 1 to 7 and LHV 90-9-I did not show any clinical signs of disease. At necropsy, mice inoculated with *S. hyodysenteriae* did not have any gross lesions (Table II). However, microscopic lesions could be observed with different *S. hyodysenteriae* strains. The lesions were localized mainly in the caecum and consisted of dilated or damaged crypts containing numerous neutrophils associated with an inflammatory infiltrate in the surrounding lamina propria (Fig. 2). Warthin–Starry and hematoxylin-phloxin-saphran stains allowed visualization of spirochetes in close proximity to the surface epithelium and in necrotic or dilated crypts. The IFAT confirmed the localization and distribution of the microorganisms seen by phase microscopy (Table II; Fig. 2). Humoral antibodies were not detected in the serum obtained from any pigs or mice before exposure to infection. Inoculation of piglets and mice with *S. hyodysenteriae* provoked generally a good antibody response as detected by ELISA (Tables I and II).

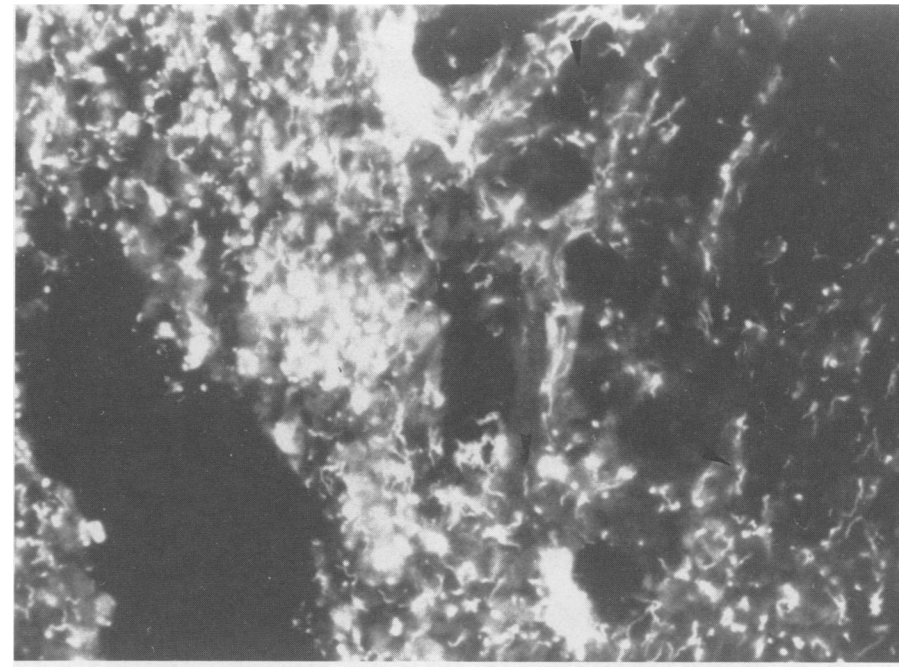

Figure 1. Colonic mucosa of piglet infected with *S. hyodysenteriae* strain B204 showing necrotic crypt containing numerous spirochetes and inflammatory cells. Arrows indicate helical bacteria resembling spirochetes by indirect immunofluorescent staining (×340).

Figure 2. Caecal mucosa of mouse infected with *S. hyodysenteriae* strain B204 showing necrotic crypt and goblet cells. Arrows indicate helical bacteria resembling spirochetes by indirect immunofluorescent staining (×340).

DISCUSSION

Serpulina hyodysenteriae is regarded as a primary pathogen (9). Currently, there is insufficient evidence to indicate whether or not all the serotypes are of comparable virulence. In this paper, variation in virulence among several strains of *S. hyodysenteriae* has been studied. The results indicate that swine dysentery can easily be reproduced in young piglets by oral inoculation of *S. hyodysenteriae*. It was also concluded that *S. hyodysenteriae* strains B234 and A-1 are only weakly virulent or avirulent and, therefore, should not be used in experimental infection of swine or in studies of virulence determination. Postmortem examination of pigs inoculated experimentally with strains B204 and LHV 90-9-I revealed lesions characteristic of swine dysentery, such as gross mucosal thickening and haemorrhage in the colon. Histological examination of the ileum, ileo-caecal

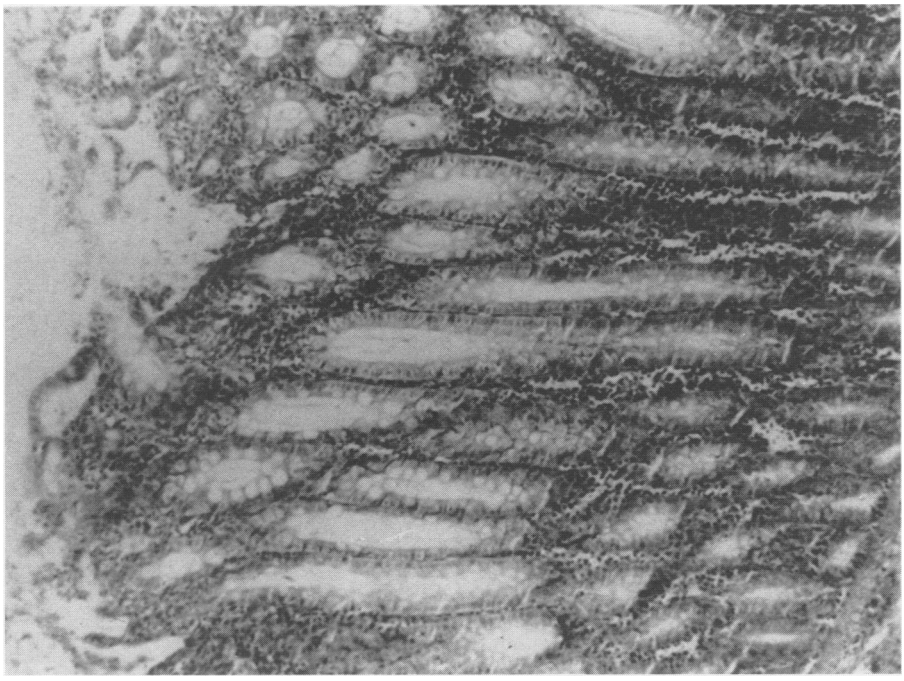

Figure 3. Colonic mucosa of piglet showing necrotic crypt containing numerous inflammatory cells. H&E stain (×340).

TABLE II. Results of experimental infection of mice with different strains of *S. hyodysenteriae*

Strain (serotype)		Clinical signs	Lesions[a]		Detection of	
		Diarrhea	Macro	Micro	Antibody by ELISA	Antigen by IFAT
B234	(1)	–	–	+	+	+
B204	(2)	–	–	+	+	+
B169	(3)	–	–	+	+	+
A-1	(4)	–	–	+	+	+
B8044	(5)	–	–	+	+	+
B6933	(6)	–	–	+	+	+
ACK300/8	(7)	–	–	+	+	+
LHV 90-9-I	(UT)[b]	–	–	+	+	+

[a] Macro: Macroscopic. Micro: Microscopic
[b] UT: Untypable

valve, caecum, and colon from infected pigs provided conclusive evidence of epithelial cell hyperplasia, which is also characteristic of swine dysentery (4,11,13). Lesions were detected in the colonic mucosa only in pigs in which *S. hyodysenteriae* infection was established. It is clear from these results that experimental challenge with 2 doses containing approximately 3×10^8 CFU/mL was able to initiate disease in the piglets. This was confirmed by recovery of the organisms from inoculated pigs and detection of the organisms in IFAT stained sections and fecal smears. Although fecal samples from pigs were culture-negative after day 35 PI, it was possible to isolate *Serpulina* from the colonic tissues at necropsy. The intraepithelial propagation of spirochetes may provide an explanation for the development of the carrier state without shedding *Serpulina* organisms (8). The results showed a strong correlation between IFAT, isolation from tissue, and histological methods for the detection of *S. hyodysenteriae*. The IFA test for detection of antigen has been used earlier and seems to be specific and sensitive (26). Antibody response of piglets was weak at d 14 PI, but became stronger up to d 70 PI. These results were in accordance with the observations of Chatfield et al (7).

The oral infection of pigs with live culture clearly allowed us to separate *S. hyodysenteriae* strains into highly virulent and less virulent groups. Recently, Hyatt et al (29) reported 2 mutant strains of *S. hyodysenteriae* of low virulence produced from virulent wild-type strains by homologous recombination. Results obtained in the present study show that strains B234 and A-1 proved to be less virulent or avirulent on oral inoculation in pigs. It is suggested that these strains could have undergone spontaneous mutation as a result of multiple passaging in the laboratory. Kinyon and others (17) reported that certain *Serpulina* isolates passaged in the laboratory 35 times or more became attenuated and were no longer enteropathogenic in pigs. A question remains as to why strain B204 remains highly virulent even after more than 100 laboratory passages carried out by Joens (14). We speculate that some strains may be more susceptible to mutation, thus resulting in loss of virulence factors.

To select a suitable mouse model, mice of CD-1 strain were used for experimental infection. In this model, the lesions were confined mainly to the caecum. The histopathological examination of caecal and colonic tissues from infected mice revealed characteristic lesions similar to those seen in swine dysentery. Also, the crypts of infected mice contained numerous spirochetes (Fig. 2). However, none of the mice showed any clinical signs or gross lesions of dysentery (Table II). Joens et al (16) reported that *S. hyodysenteriae* plus *Bacteroides vulgatus* induced cecal lesions in gnotobiotic mice, but that neither *S. hyodysenteriae* nor *B. vulgatus* alone induced gross lesions. However, others have reported varying susceptibilities of different mouse strains on infection with *S. hyodysenteriae* (22,24,27,28). They further suggested that differences in the intestinal microflora of the mice may affect susceptibility or colonization with *S. hyodysenteriae*.

The serological response in experimentally infected mice was essentially the same as that observed with experimentally and naturally infected piglets. Antibodies specific for *S. hyodysenteriae* were detected as early as 2 wk PI in experimental infection (data not shown). It would be useful to investigate the mechanisms of the immune response involved in the eventual elimination of *S. hyodysenteriae* from the gut of infected animals.

We suggest that studies involving identification of virulence factors should include *S. hyodysenteriae* strains B204 or LHV 90-9-I, which are highly virulent. Those involving the development of a potentially effective live vaccine should include strains B234 and A-1, which are less virulent or avirulent.

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