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Evaluation of virulent and live *Shigella sonnei* vaccine candidates in a gnotobiotic piglet model☆

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ABSTRACT

Newborn gnotobiotic (GB) piglets given virulent *Shigella* orally develop many of the clinical symptoms and gastrointestinal (GI) manifestations that mimic human shigellosis. *Shigella sonnei* virulent strain Moseley, a mutant ShET2-1,2, lacking enterotoxin SenA and its paralog SenB, and vaccine candidates WRSS1 and WRSs3 were evaluated in this model for rates of diarrhea, colonization and other GI symptoms and pathology. Moseley-infected piglets developed diarrhea from 1 to 7 days, with the highest rates seen on days 2–4 after inoculation. In contrast, WRSs3-infected piglets did not have diarrhea over the entire experimental period. Compared to the Moseley group, lower diarrheal rates were observed in the double enterotoxin mutant and significantly lower in the WRSS1 group. Moseley infection also caused marked mucosal damage in the GI tissues at PID1 to PID8, and induced predominantly proinflammatory cytokine secretion. IL-8 and to a lesser extent IL-6 and IL-1β were observed early after inoculation and IL-12 secretion could be measured till late in infection. The ShET2-1,2 mutant, WRSS1 and WRSs3 also colonized the GI tract in a manner similar to Moseley; however, both vaccine candidates developed milder histopathological indices and cytokine responses. WRSs3-infected animals showed the least pathology. Furthermore, unlike the other strains, WRSs3 was rarely detected in organs outside the gastrointestinal tract. These results support the development of the GB piglet model as a sensitive *in vivo* oral model for the evaluation of virulence of different *Shigella* strains which could be applied to other oral vaccine candidates.

1. Introduction

Shigella causes diarrhea and bacillary dysentery, a human gastrointestinal (GI) disease that results in an acute inflammation of the distal colon and rectum with concomitant necrosis and erosion of the local mucosal epithelium. Symptoms range from fever, tenesmus, abdominal pain, and diarrhea to small volume excretion of stools with blood and mucus (dysentery) [1]. A key factor for bacterial virulence is the presence of a large, ~215 kb plasmid called the virulence or invasion plasmid [2].

Besides rhesus monkeys that succumb to shigellosis after oral infection, there are no well-characterized small animal models that mimic the path of a human infection [3–5]. Newborn gnotobiotic (GB) piglets, orally challenged with *Shigella dysenteriae* 1 strains were recently shown to develop clinical symptoms and gastrointestinal manifestations observed in human shigellosis [6–9]. *Shigella sonnei* causes 70% of shigellosis in the US and is frequently responsible for sporadic and epidemic enteritis in developed countries. Two new, live *S. sonnei* vaccine candidates, WRSs2 and WRSs3, that lack the ability to spread intercellularly due to loss of VirG(or IcsA) have entered clinical trials [10–12]. *In addition to VirG(IcsA), WRSs2* lacks the plasmid-based enterotoxin ShET2-1 or SenA and a putative paralog ShET2-2 or SenB, while WRSs3 also lacks MsbB2 whose function maximize lipid A endotoxicity [10–12]. In this study, the GB piglet model was used to assess virulence and compare reactogenicity between *S. sonnei* strain Moseley, a mutant lacking both ShET2-1 and ShET2-2 (ShET2-1,2), and the vaccine candidates WRSS1 and WRSs3 [13]. WRSS1 has been

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Table 1
Animal assignment in a representative GB piglet study with 5×10^9 CFU dose.

Inoculum	Strains	Total piglets	Number euthanized		
			PID1	PID3	PID8
Wild type	Moseley	16	4[a]	7	5
Double mutant	ShET2-1,2	14	3	4	7
Vaccine candidates	WRSS1	11[c]	4	4	3
	WRSs3	13	4	4	5
Placebo[b]		9	3	3	3

[a] At indicated times, piglets were euthanized for removal of tissues for analysis.
[b] The placebo control group were given PBS.
[c] Some animals (4 out of 20 animals inoculated with Moseley, 3 of 17 with ShET2-1,2 mutant, and 2 of total 13 in WRSS1) showed adverse clinical signs, such as labored breathing and decreased physical activities and milk-uptake, especially in 1–3 days post infection. These animals were euthanized according to IACUC guideline and excluded from the study groups shown in the table.

previously tested in clinical trials and lacks only VirG(IcsA) [14,15]. The results indicate that this model can be used to distinguish between virulent and vaccine candidates of *Shigella*.

2. Materials and methods

2.1. Bacterial strains

S. sonnei virulent strain Moseley was isolated in 1975 from an infected laboratory worker and was obtained from the culture collection maintained at WRAIR. Enterotoxin mutant strain (ShET2-1,2) as well as vaccine candidate strains WRSS1 and WRSs3 were derived from Moseley [10,13]. WRSS1 has a 212 bp deletion in the *virG(icsA)* gene while WRSs3 has deletions of *virG(icsA), senA, senB* and *msbB2* genes [10,13]. The ShET2-1,2 strain has deletions of only the *senA* and *senB* genes. *senA* and *senB* encode invasion plasmid-based enterotoxin ShET2-1 and its paralog ShET2-2, respectively.

2.2. Preparation of bacterial inoculums

The bacterial strains were grown on Tryptic Soy Agar (TSA) plates, and routinely propagated in Tryptic Soy Broth (TSB). The inoculum preparations were carried out essentially as previously described [8]. Briefly, bacterial cultures in mid-exponential growth phase were concentrated by centrifugation and resuspended in phosphate-buffered saline (PBS) to contain 5×10^9 CFU/3 ml inoculums [8].

2.3. Inoculation of GB piglets and collection of samples

GB piglets were orally administered 5×10^9 CFU of *Shigella* strains as previously described [8]. Piglets receiving 3.0 ml PBS were assigned to the placebo/control group (Table 1). Clinical manifestations as well as fecal consistency scores were scored as previously described [8]. Rectal swabs were collected daily for examination of bacterial shedding in the feces. Piglets were euthanized after oral inoculation on days 1, 3, and 8 ($n = 3$–7/time point) (Table 1). Gut tissues were collected for histopathology, immunohistochemistry (IHC), and electron microscopy as previously described [8].

2.4. Bacterial colonization, histopathology, and electron microscopy

Gut contents from individual segments of the intestinal tissue were collected as previously described [8]. A portion of the gut contents was used to determine bacterial counts and the rest diluted 5-fold in cold PBS and saved at −80 °C for cytokine ELISA assays [8]. Gut segments were prepared for histopathology and electron microscopic analysis as previously described [8]. Histological lesions were compared among the groups with regard to degree of erosion, hemorrhage, inflammatory infiltrate in the intestinal mucosa through lamina propria and other tissues. A generalized scoring was used as follows to evaluate pathology: 0, no significant damages in the gut mucosa, minimal inflammation, 1, mild, focal necrosis of the gut mucosa with mild inflammation, 2, moderate, multi-focal necrosis of the gut mucosa, mild erosion of the mucosa, moderate inflammation, 3, severe, multifocal to diffuse necrosis of the gut mucosa, moderate to severe ulceration of the mucosa, moderate inflammation. Histopathological lesions were scored by two pathologists by reading the tissue slides blinded.

2.5. Immunohistochemistry (IHC) for detection of S. sonnei in the gut tissues

Gut segments were subjected for IHC as described elsewhere [8]. The primary antibody was a rabbit polyclonal antibody specific for *Shigella species* (Novus Biologicals Inc., Littleton, CO). Negative control slides were stained without including the primary antibody.

2.6. Cytokine profiles in gut contents

Briefly, the 96-well plates were coated with antiporcine cytokine antibodies and incubated with gut supernatants [8]. The plates were incubated with biotinylated detection antibodies and antigen–antibody reactions detected by horseradish peroxidase linked to streptavidin. Antibodies were used to detect proinflammatory cytokines (IL-8, IL-6, IL-1b, and TNF-a), Th1-type cytokines (IL-12 and IFN-g) and Th2-type cytokines (IL-10). Cytokine concentrations were calculated by using the computer-generated standard curves (SOFTmax Pro; Molecular Devices Corp., Sunnyvale, CA, USA).

2.7. Statistical analysis

Bacterial counts in feces and gut contents were compared among the gut segments at each time-point, and along the different time-points within respective groups, by using Kruskal–Wallis nonparametric analysis of variance (ANOVA) followed by Mann–Whitney test. Diarrheal rates were expressed as percent piglets with fecal scores > 2 that were assessed at days 1 through 7 after inoculation. Fisher exact test was used to identify differences in diarrheal rates among the groups at the time-points, and among the time-points for each group. The values marked with different alphabets (*e.g.* A and B) are significantly different statistically. The data marked AB are not significantly different from the data marked with A or B. Fecal score data were also analyzed with the same method for the differences among the various groups and the time-points. Data were analyzed with SAS (SAS Institute, Cary, NC, USA), and a p-value of <0.05 was considered significant.

3. Results

3.1. Administration of Shigella strains to GB piglets

Groups of GB piglets as shown in Table 1 were orally administered the *Shigella* strains and various parameters of infection were noted.

3.2. Clinical symptoms of GB piglets

In dose standardization experiments, 5×10^9 CFU dose of Moseley was shown to reproducibly get most GB piglets sick with diarrhea. Similar to *S. dysenteriae* 1 infections with GB piglets [8], *S. sonnei*-infected animals developed serious symptoms such as watery diarrhea, anorexia, and dehydration within 24 h that peaked within 2–4 days after inoculation. Piglets with these symptoms had