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Construction of a Stable Attenuated *Shigella sonnei* Δ*virG* Vaccine Strain, WRSS1, and Protective Efficacy and Immunogenicity in the Guinea Pig Keratoconjunctivitis Model

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Construction of a stable *Shigella sonnei* vaccine has been complicated by the instability of the virulence phenotype caused by the spontaneous loss of the invasion plasmid. To select a suitable candidate for vaccine construction, 16 *S. sonnei* strains were screened for stability of the virulence phenotype. A stable strain, *S. sonnei* Mosely, was selected for further work. pΔvirG2, a deletion derivative of the *virG* gene in the *sacB* suicide vector pCVD442, was used to generate an *S. sonnei virG* deletion strain, WRSS1, which was invasive in HeLa cells but negative in the Sereny test. WRSS1 was found to be both immunogenic and protective in the guinea pig keratoconjunctivitis model.

During the recent Desert Storm operation, shigellosis was responsible for 26.2% of the diarrheal disease in U.S. military personnel, with *Shigella sonnei* being responsible for about 80% of the shigellosis cases. Of the troops affected by shigellosis, 60% were unable to perform their assigned duties (18). Civilian and military travelers to countries of shigella endemicity are also at risk for infection (6, 18). The development of a vaccine directed against *S. sonnei* is thus an important goal to ensure protection of troops deployed in areas where shigellosis is endemic. *S. sonnei* is also a major cause of illness in developed countries, particularly in institutional settings, such as day care centers and prisons, and in military field settings (5). The increasing occurrence of resistance and multiple-drug resistance among clinical *Shigella* isolates to commonly used antimicrobial agents such as ampicillin, tetracycline, trimethroprim-sulfamethoxazole, and nalidixic acid is particularly prevalent in developing countries and provides an additional impetus for the development of vaccines directed against *Shigella* species (3, 7, 21, 29, 34). Further emphasizing the need for vaccines against bacterial infection, the development of a successful *Haemophilus influenzae* vaccine has significantly decreased concern about antibiotic resistance in areas where the vaccine is being widely used (26).

Previous studies have shown that killed whole-cell vaccines administered parenterally or orally did not confer protective immunity in humans or in monkeys (12, 17, 37). Since natural infections confer serotype-specific protection in approximately 75% of cases of reinfection (10, 13), it was thought that live attenuated vaccines for oral administration would effectively produce protective immunity against shigellosis. However, noninvasive live vaccines that have been tested required multiple high doses and frequent boosting immunizations for efficacy and provided varying rates of protection, making them too impractical for continued use (24, 25). Therefore, current attempts to construct vaccines to be administered orally have centered on designing live vaccines which are attenuated by specific deletions that retain the invasiveness of the bacteria but affect intra- and intercellular dissemination. Genes affecting biochemical pathways, such as *aroA*, *aroD*, or *thyA* (1, 22, 39), or specific virulence genes, such as *virG* (*icsA*), whose expression is required for intra- and intercellular spread of shigellae (4, 20), have been targets for deletions in live attenuated *Shigella* vaccines. *Shigella* strains with *virG* deletions do not produce a positive Sereny reaction in guinea pigs or plaques in tissue culture monolayers, since both assays are indicators of invasion followed by intracellular multiplication and intra- and intercellular spread (28, 33). *Escherichia coli-Shigella flexneri* 2a hybrid vaccine strains with a deletion in the *virG* locus were attenuated and protective in the guinea pig keratoconjunctivitis model (2). CVD1203, a double mutant for *aroA* and *virG*, elicited a moderate immune response at a dose that produced only mild constitutional symptoms (14, 27). An *S. flexneri* 2a vaccine, SC602, a double mutant for *virG* and *iuc* (which eliminates the production of aerobactin and impairs growth in tissues) is immunogenic in humans at nonreactogenic doses and shows promising efficacy against challenge with wild-type *S. flexneri* 2a (8, 9, 14, 31, 32). Thus, the construction of an *S. sonnei* strain with a *virG* deletion seemed a logical choice for a suitable vaccine candidate.

Construction of a stable *S. sonnei* vaccine strain has been complicated by the instability of the virulence phenotype caused by spontaneous loss of the large invasion plasmid. It has been noted in previous studies that *S. sonnei* strains lose the form I invasion plasmid at a frequency ranging from 1 to 2% to about 50% (30), which is greater than that seen with other *Shigella* serotypes. Since the genes encoding the O antigen are located on the invasion plasmid in *S. sonnei*, in contrast to the chromosomal locations of the O antigen in other *Shigella* serotypes, loss of this plasmid in *S. sonnei* results in form II colonies, which are avirulent and rough. To select a suitable candidate for vaccine construction, 16 *S. sonnei* strains from the Walter Reed Army Institute of Research collection were tested for stability of virulence expression. A single form I colony from each strain was grown overnight in Luria-Bertani broth at 37°C, and appropriate dilutions were plated on tryptic

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TABLE 1. Stability of virulent *S. sonnei* strains

Strain	Form I (%)[a]	Stability[b]
Mosely	87.2 ± 4.0	S
Rudy	89.6 ± 9.2	S
VTi66	93.1 ± 2.8	S
53G	61.7 ± 17.5	U
13-40 (82)	78.0	U
212	74.8	U
5002-13	79.8	U
MBI	61.9 ± 13.7	U
Patty	71.2	U
W107	73.1 ± 10.6	U
LB	97.9 ± 1.4	S
1041	87.5	S
482-79	87.6 ± 3.7	S
EJ	98.6 ± 0.7	S
B120	55.5 ± 13.8	U
3175	51.7 ± 9.7	U

[a] The method used to calculate form I percentage is described in the text. Standard deviations of the mean are given when more than one experiment was done per strain.

[b] Stable strains (S), strains with >85% form I colonies after overnight incubation; unstable strains (U), strains with <85% form I colonies after overnight incubation.

soy agar plates the following day. After overnight incubation at 37°C, colonies were counted and scored for the percentage of form I and form II colonies present. At least two trials were done for most strains. Stable strains were defined as strains with greater than 85% form I colonies after overnight growth. With this criterion, 7 of the 16 strains were designated stable (Table 1). The percentage of form I colonies for individual strains ranged from 87 to 98% for the stable strains and from 52 to 80% for the unstable strains. Stable strains retain >85% stability even after several passages.

All stable strains were tested for antibiotic sensitivity, since strains sensitive to kanamycin or ampicillin could not be used with the suicide vector system and strains resistant to antibiotic(s) are not suitable for vaccine strains. *S. sonnei* Mosely, which was fully virulent in the Sereny test (Table 2) and was resistant only to tetracycline, was selected as the recipient strain in a filter mating experiment with donor strain SM10λpir (pΔvirG2). This strain was chosen over other stable strains because of its tetracycline resistance, which can be removed with fusaric acid (23). pΔvirG2 contains a 212-bp deletion within the *virG* structural gene cloned into the suicide vector pCVD442 as previously described (Fig. 1A and references 2 and 9). The deleted *virG* gene in pΔvirG2 contains stop codons in all three reading frames to prevent any expression beyond the deletion point. A schematic representation of the steps used in the construction of WRSS1, a *virG* deletion mutant of the *S. sonnei* Mosely strain, is shown in Fig. 1. A total of two crossover events, one on either side of the deletion, would result in the replacement of the wild-type *virG* allele on the invasion plasmid with the deleted version of pΔvirG2. Plasmid pΔvirG2 (Fig. 1A) was introduced from the donor strain SM10λpir(pΔvirG2) into the recipient strain *S. sonnei* Mosely (Fig. 1B) by an overnight filter mating and selection for ampicillin and tetracycline resistance as previously described (2). Individual colonies were selected and screened by PCR analysis. The first recombination event was monitored by the use of primers BA118 and BA76. These primers produce a 1.8-kb PCR product from the tetracycline-resistant, ampicillin-resistant strains, indicating that the insertion of the pΔvirG2 plasmid occurred in the *virG* gene (Fig. 1C). Neither the donor strain [SM10λpir(pΔvirG2)] nor the recipient strain (*S. sonnei* Mosely) contains both of these primer sequences, and thus these strains do not produce a PCR product (Fig. 1A and B). Primers BA114 and BA117 were also used to confirm the integration, since these primers yield two PCR products when integration has occurred, a 1,667-bp product corresponding to the wild-type gene and a 1,455-bp band corresponding to the 212-bp deleted gene (Fig. 1C and 2). A tetracycline-resistant, ampicillin-resistant strain with an insertion of pΔvirG2 into the *virG* gene was then put through sucrose selection as previously described (2). The presence of the *sacB* gene in pCVD442 inhibits growth on 5% sucrose. Therefore, growth on sucrose is used as a positive selection for the loss of vector sequences. Sucrose-resistant, tetracycline-resistant, ampicillin-sensitive colonies were tested for loss of suicide vector sequences, and the presence of the deleted *virG* gene was tested by PCR analysis as previously described (2). Figure 2 shows the result of PCR analysis of the second recombination event (Fig. 1D) with primers BA114 and BA117 and confirms that the *S. sonnei* recombinant strain contained a deleted *virG* gene. No product was obtained with primers BA118 and BA76, indicating the loss of vector sequences. The *S. sonnei* strain containing the deleted *virG* gene was plated on fusaric acid plates (21), and tetracycline-sensitive colonies were selected. PCR analysis as described above confirmed that the tetracycline-sensitive strain contained the deleted *virG* gene (Fig. 2). This strain was designated WRSS1.

Individual colonies of WRSS1 were first tested on colony immunoblots with monoclonal antibodies to IpaB and IpaC virulence proteins as previously described (38). All form I colonies of WRSS1 were found to express both proteins and agglutinated with *S. sonnei* antiserum. WRSS1 was invasive, as indicated by the HeLa cell invasion assay (11). WRSS1 consistently had >95% form I colonies after overnight growth, indicating that it had retained the stable phenotype of the parent strain. Unlike the parent strain, WRSS1 did not react with VirG-specific antiserum on Western blots (2, 38) and did not form plaques on epithelial cells in tissue culture (28), indicating the absence of intra- and intercellular spread, which is dependent on the expression of VirG.

The guinea pig keratoconjunctivitis test (Sereny reaction [33]) was used to test for attenuation of WRSS1 compared to that of the virulent parent strain. Four guinea pigs were inoculated with 4×10^8 CFU of the attenuated WRSS1 strain, and two guinea pigs were inoculated with 4×10^8 of the parent wild-type *S. sonnei* Mosely strain, and development of disease was monitored for 5 days. The following rating scheme for development of disease was used: 0, no disease or mild irritation; 1, mild conjunctivitis; 2, keratoconjunctivitis with no purulence; 3, fully developed keratoconjunctivitis with purulence (15, 16). Guinea pigs infected ocularly with 4×10^8 CFU of the

TABLE 2. Sereny test of vaccine strain WRSS1 and of WRSS1 complemented with wild-type *virG* gene

Strain	Genotype	No. of eyes inoculated	No. of eyes with indicated rating[a]:			
			0	1	2	3
S. sonnei Mosely	Wild type	4	0	0	0	4
WRSS1	Δ*virG*	8	8	0	0	0
WRSS1(pHS3188)[b]	*virG*$^+$	4	0	0	1	3
WRSS1(pHS3192)[b]	*virG*$^+$	4	0	0	4	0

[a] The rating system is described in the text.

[b] pHS3188 contains a 7.6-kb *Eco*RI fragment, with the entire wild-type *virG* gene ligated into pBR322 (4). pHS3192 contains a 6.1-kb *Eco*RI-*Sal*I fragment, with the entire *virG* gene from pHS3188 ligated into pBR322.

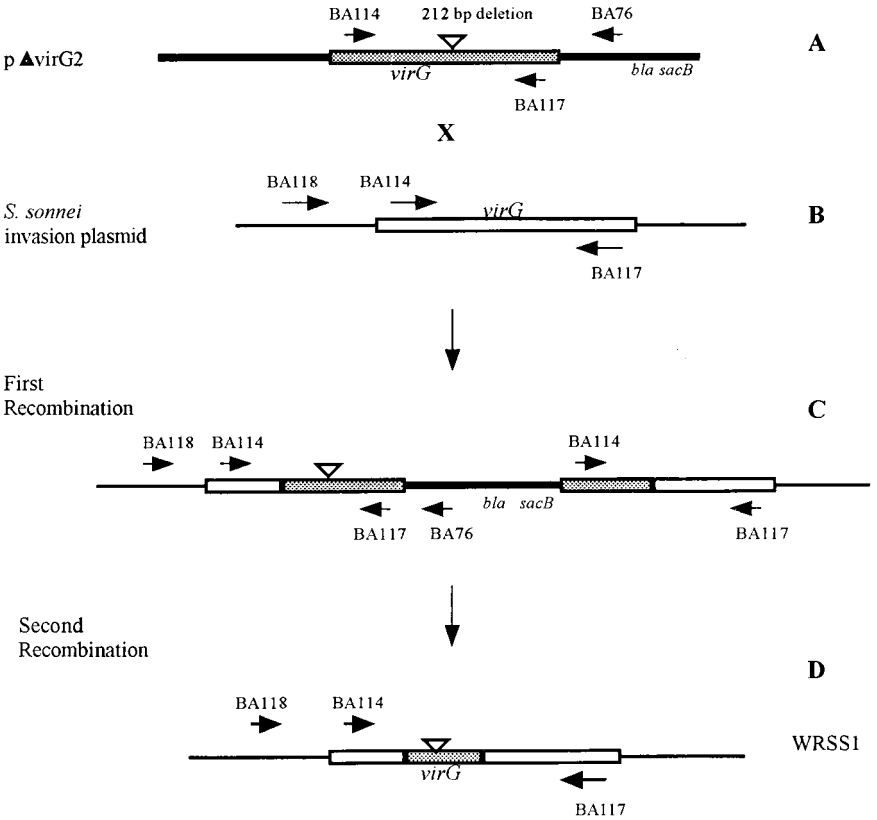

FIG. 1. Construction of strain WRSS1. (A) Plasmid pΔvirG contains a *virG* gene with a 212-bp deletion cloned into pCVD442. The deleted version is shaded to distinguish it from the wild-type gene. Primer BA76 is located in the oriR6K portion of pCVD442. (B) The *virG* gene located on the invasion plasmid of *S. sonnei* Mosely with the positions of the primers used to monitor recombinants. BA118 is located in the 5′ noncoding region of *virG*. (C) The product of the first recombination event. Primers BA118 and BA76 were used to monitor insertion of the pΔvirG plasmid into *virG*. (D) The product of the second recombination event generating WRSS1. Sequences homologous to primers BA114 and BA117 are present in panels A, B, C, and D. Sequences homologous to primer BA118 are present only in panels B, C, and D. Sequences homologous to primers BA76 are present only in panels A and C.

wild-type strain developed fully developed keratoconjunctivitis in both eyes within 2 to 3 days. In contrast, animals receiving WRSS1 showed no signs of irritation or disease over the observation period (a rating of 0 compared to a rating of 3 with the wild-type strain) (Table 2). In efficacy tests with larger groups of animals, to be discussed below, inoculation with WRSS1 resulted in the complete absence of irritation or disease (Table 3). WRSS1 complemented with either pHS3188 or pHS3192, which contain the entire wild-type *virG* gene (4), restored the Sereny reaction, indicating that the *virG* deletion in WRSS1 is responsible for the attenuation of its virulence (Table 2).

The protective efficacy and immunogenicity of WRSS1 were measured with the guinea pig keratoconjunctivitis model (15, 16). Ocular immunization with 3×10^8 to 4×10^8 CFU of WRSS1/eye on days 0 and 14 was followed by challenge with virulent *S. sonnei*. The immunizing inoculum was obtained from overnight growth plates (experiment 1) as previously described (2, 15, 16) or from rehydration of lyophilized product manufactured at the Walter Reed Army Institute of Research pilot vaccine production facility with Good Manufacturing Procedures (GMP) (experiment 2). Four weeks after the last immunization, both the immunized animals and the unimmunized control animals were challenged with 4×10^8 CFU of virulent *S. sonnei* 53G/eye. Animals were rated over a 5-day period as to time of development and severity of disease (Table 3) with the rating scheme described above. Percentage of protection was defined as follows: full, percentage of eyes with rating of 0; partial, percentage of eyes with rating of 1; combined, percentage of eyes with rating of 0 or 1 (sum of full and partial percentage). Results of both experiments are shown in Table 3. In animals immunized with WRSS1 grown from over-

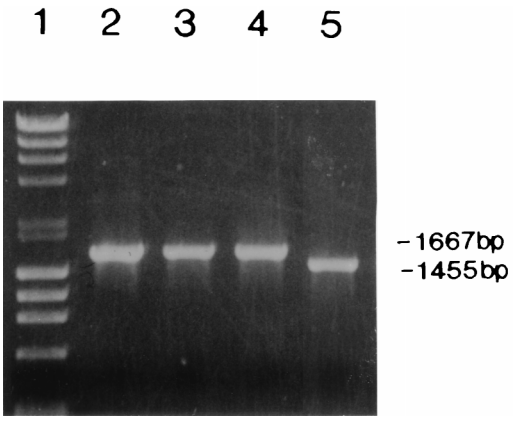

FIG. 2. PCR analysis of the *virG* gene in wild-type *Shigella* and vaccine strain WRSS1. PCR analysis of the size of *virG* was carried out with primers BA114 and BA117. Products were run on an ethidium-bromide-stained 0.8% agarose gel. Lane 1, molecular size markers; lane 2, *S. flexneri* 2a strain 2457T; lane 3, *S. flexneri* 5a strain M90T-W; lane 4, *S. sonnei* Mosely; lane 5, WRSS1. The sizes of the wild-type *virG* gene and the deleted gene found in WRSS1 are shown at the right.

TABLE 3. Protective efficacy of WRSS1 measured with the guinea pig keratoconjunctivitis model

Immunizing strain[a]	No. of eyes with indicated rating[b]							
	Postimmunization[c]				Postchallenge[d]			
	0	1	2	3	0	1	2	3
Expt 1								
WRSS1	16	0	0	0	11	5	0	0
None (nonimmunized)	NA[e]	NA	NA	NA	0	0	0	16
Expt 2								
WRSS1	16	0	0	0	10	4	2	0
None (nonimmunized)	NA	NA	NA	NA	0	0	1	15

[a] In each experiment, 16 eyes were inoculated with WRSS1 and 16 were left uninoculated.
[b] Ratings are described in the text.
[c] Ratings after the first and second immunizations were identical.
[d] Animals were challenged 4 weeks after the second immunization with virulent strain *S. sonnei* 53G.
[e] NA, not applicable.

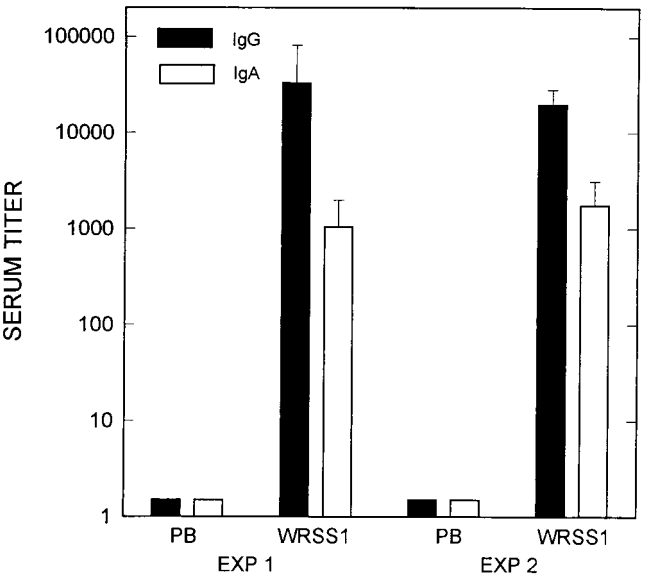

FIG. 3. Serum IgG and IgA titers against the *S. sonnei* O antigen 2 weeks after the boosting immunization of guinea pigs with WRSS1. The geometric mean titers from experiments 1 and 2 are shown. Background titers were determined from preimmunization bleeds (PB) and were <50 for both IgG and IgA. Standard errors of the mean are shown. Exp, experiment.

night plate cultures, 13 of 16 eyes showed no signs of disease (83% complete protection), while 3 eyes showed mild conjunctivitis (17% partial protection). When reconstituted lyophilized cultures were used, 10 of 16 eyes did not develop disease (63% complete protection), while 4 eyes developed mild disease (25% partial protection). In both cases, protection against challenge was significant by the Fisher exact test ($P < 0.001$), and there was no significant difference in the levels of protection conferred by the two formulations.

Immunogenicity of the vaccine was measured by determining levels of serum immunoglobulin G (IgG) and IgA antibodies specific for the *S. sonnei* O antigen. Two weeks after the last immunization, animals were bled and the serum antibody response against *Plesiomonas shigelloides* lipopolysaccharide (LPS), whose O antigen is immunologically identical to that of *S. sonnei* (35), was determined. Polyvinyl chloride microtiter plates were coated with 50 μl of *P. shigelloides* LPS at a concentration of 10 μg/ml in carbonate buffer (pH 9.6), and serum titers against *P. shigelloides* O antigen were measured by enzyme-linked immunosorbent assay as described previously (2, 16). Optical density was read at 405 and 570 nm. Endpoint titers were defined as the last dilution having an optical density at least 0.1 above that of the background well. Titers of prebleed sera used to obtain background titer values were <50 for both IgG and IgA. The geometric mean titers of experiments 1 and 2, shown in Fig. 3, demonstrate that both formulations of WRSS1 are immunogenic and produce comparable serum IgG and IgA titers against the O antigen.

In volunteer studies, EcSf2a-2, an *aroD* deletion *E. coli-S. flexneri* 2a hybrid vaccine strain, was too reactogenic at doses required for a good immune response (19, 36). A *virG* deletion derivative of this strain has been tested in guinea pigs and found to be immunogenic and protective, although higher doses were required (2). Since the guinea pig keratoconjunctivitis test measures both development of disease and inflammation, a major feature of shigellosis in humans, the degree of inflammation in the eye evoked by different *Shigella* strains may be an indication of possible reactogenicity in humans. Other *virG* deletion strains of *S. flexneri* 2a have produced some reactogenicity in guinea pig eyes; this was reflected in the human safety trials. CVD1203, containing deletions in *aroA* and *virG*, produced mild conjunctivitis in 4 of 16 animals and moderate conjunctivitis in 1 of 16 animals within 24 h, although symptoms disappeared by 48 h (27). This strain was reactogenic in humans at doses of 10^8 and 10^9 but was tolerated at 10^6, although some symptoms of malaise and headache occurred in 4 of 10 volunteers. The 10^6 dose produced moderate LPS-specific IgA antibody-secreting cells (13). Ocular immunization of guinea pigs with SC602, containing deletions in *virG* and *iuc*, resulted in mild irritation that cleared up within 24 h (14). In recent human trials of the *S. flexneri* 2a vaccine SC602, doses of 10^6 CFU or greater resulted in some reactogenicity (8, 14). However, a single dose of 10^4 CFU was well tolerated, immunogenic, and protective (8, 14). In contrast to SC602 and CVD1203, the same dose of WRSS1 produced no reaction at all in guinea pigs, indicating that WRSS1 may be safer in humans at doses that were reactogenic for either CVD1203 or SC602.

Tests have shown that WRSS1 is as stable as the parent strain, and this stability was maintained in the lot of WRSS1 produced under GMP conditions. The GMP product was also safe, immunogenic, and protective in guinea pigs. Phase 1 safety trials followed by phase II efficacy trials in North American volunteers are being planned for the GMP product. Data from these trials, along with the results from the SC602 phase I and phase II trials, will indicate whether *virG* deletion mutants of the most prevalent *Shigella* serotypes may be used to formulate multivalent *Shigella* vaccines.

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