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# Safety and Colonization of Two Novel *virG*(*icsA*)-based Live *Shigella sonnei* Vaccine Strains in Rhesus Macaques (*Macaca mulatta*)

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***Shigella*** **are gram-negative bacterium that cause bacillary dysentery (shigellosis). Symptoms include diarrhea and discharge of bloody mucoid stools, accompanied by severe abdominal pain, nausea, vomiting, malaise, and fever. Persons traveling to regions with poor sanitation and crowded conditions become particularly susceptible to shigellosis. Currently a vaccine for** ***Shigella*** **has not been licensed in the United States, and the organism quickly becomes resistant to medications. During the past 10 y, several live attenuated oral** ***Shigella*** **vaccines, including the strain WRSS1, have been tested in humans with considerable success. These Phase I vaccines lack the gene for the protein VirG also known as IcsA, which enables the organism to disseminate in the host target tissue. However, 5% to 20% of the vaccinated volunteers developed mild fever and brief diarrhea, and the removal of additional virulence-associated genes from the vaccine strain may reduce or eliminate these side effects. We administered 2** ***Shigella sonnei*** **vaccines, WRSs2 and WRSs3, along with WRSS1 to compare their rates of colonization and clinical safety in groups of 5 rhesus macaques. The primate model provides the most physiologically relevant animal system to test the validity and efficacy of vaccine candidates. In this pilot study using a gastrointestinal model of infection, the vaccine candidates WRSs2 and WRSs3, which have additional deletions in the enterotoxin and LPS modification genes, provided better safety and comparable immunogenicity to those of WRSS1.**

**Abbreviations:** IcsA, intercellular spread protein A (VirG protein); Ipa, invasion plasmid antigen; LPS, lipopolysaccharide

Various species of *Shigella* cause bacillary dysentery, also known as shigellosis, in humans and other primates.[1,18,31,37,49] Symptoms include diarrhea, with various degrees of mucus and hematochezia, accompanied by severe abdominal pain, nausea, vomiting, malaise, and fever. *Shigella* is a gram-negative bacterium whose genomic sequence is very similar to that of *Escherichia coli* and is phylogenetically considered a pathotype of *E. coli*.[23] *Shigella* infections are spread by the fecal–oral route through the consumption of contaminated food and water or by mechanical vectors such as insects. Persons traveling in areas with poor sanitation and crowded conditions become particularly susceptible to such diseases.[2,39,48,56] In colonies of research macaques, *Shigella* infections are spread by the fecal–oral route, originating from addition of animals that are asymptomatic carriers, either from the wild or from other colonies.[1,55] Currently no *Shigella* vaccine has been licensed in the United States, although several are under development.[25,32,35,38,54] Antibiotics are used as treatment therapy, but many *Shigella* isolates are multidrug-resistant, increasing the need for a preventive vaccine for travelers, military personnel, and children in endemic areas.[16,34,41,44,57] Rhesus macaques represent an excellent model for studying *Shigella* because primates are the only known animal model that simulate natural human infection, including dysentery after oral challenge.[11-13,17,28,40,47,46] Other animal models, such as the mouse pulmonary model and the guinea pig ocular and intranasal models, mimic specific, isolated steps in *Shigella* pathogenesis.[14,19,26]

There are 4 major serogroups of *Shigella* and one or more serotypes within each serogroup. This classification is based on antigenic differences in the O-antigen polysaccharide component of the outer membrane-associated lipopolysaccharide (LPS). Estimates from the Centers for Disease Control (Atlanta, GA) indicate that approximately 400,000 cases of shigellosis occur in the US annually, with an estimated 165 million cases worldwide each year.[22] These occur predominantly in developing countries, where the population most affected is children younger than 5 y.[22]

Various in vitro cell culture models of infection, as well as studies in animal models including gastrointestinal infection in nonhuman primates, have contributed to the current understanding of *Shigella* pathogenesis.[4,42] *Shigella* organisms target the distal region of the colon and rectum, where the bacteria are captured by specialized M-cells located within the follicle-associated epithelium. The M-cells deliver bacterial antigens, which include bacterial LPS and invasion plasmid antigen (Ipa) proteins, to the underlying antigen-presenting macrophages and dendritic cells.[36] Virulent *Shigella* strains escape macrophages by a cytotoxic effect, causing release of the bacteria and inflammatory cytokines such as IL1β, TNFα, and IL18. The bacteria then invade adjacent intestinal epithelial cells at the basolateral side, through interaction between bacterial proteins and multiple signaling molecules within the host cell.[36,42]

*Shigella* are capable of orchestrating this uptake into nonphagocytic epithelial cells, a process termed invasion, through the se-

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cretion of proteins that are highly conserved among all virulent strains. These proteins are encoded by a large, 213-kb plasmid, referred to as the invasion plasmid or the virulence plasmid.[50-52] Each bacterium ultimately is engulfed within an endocytic vacuole. Subsequent lysis of the vacuole sets the bacterium free within the epithelial cell cytoplasm, where it replicates and moves to adjacent epithelial cells, with the help of the intercellular spread (Ics) A protein, also known as VirG.[3,24,45] The VirG(IcsA)-assisted intra- and intercellular spread of the bacteria within the epithelial tissue contributes significantly to the loss of epithelial cell integrity and accompanying tissue injury. *Shigella* strains with loss of the *virG*(*icsA*) gene are significantly attenuated in all animal models of virulence.[5] Several *Shigella* vaccine candidates that have undergone Phase 1 clinical trials are principally attenuated due to lack of the VirG(IcsA) protein, these include *S. flexneri 2a* strain SC602, *S. sonnei* strain WRSS1, and *S. dysenteriae 1* vaccine strain WRSd1.[5,10,15,21,53]

The *S. sonnei* first-generation vaccine candidate WRSS1 has been tested in several Phase I inpatient and outpatient trials.[15,21,33] WRSS1, like the previous *S. flexneri 2a* vaccine candidate, SC602, was shown to be safe in Phase I trials when orally administered at a dose of $10^3$ to $10^4$ CFU.[15,21,33] Both SC602 and WRSS1 colonized well as evidenced by fecal excretion of the vaccine strain for 5 to 7 d. The vigorous immune response seen with both these vaccine candidates was correlated with the robust colonization. However, 5% to 20% of the SC602- and WRSS1-immunized volunteers showed mild and transient symptoms of diarrhea and fever. In light of new knowledge about *Shigella* pathogenesis as well as data from other clinical trials, 2 new and presumably safer *S. sonnei* derivatives have been constructed (WRSs2 and WRSs3). In addition to loss of *virG*(*icsA*), WRSs2 has deletions in 2 genes, *senA* and *senB*, that are present on the virulence plasmid.[29,52] *senA* (also known as *shet2-1* and *ospD3*) encodes the enterotoxin ShET2-1, which has been shown to cause fluid accumulation in rabbit ileal loops.[7,8] *senB* (also known as *shet2-2* and *ospD2*) encodes a similarly sized protein as SenA and shows 40% identity at the amino acid level. The enterotoxic activity of ShET2-2 remains to be demonstrated.[20,29] Like WRSs2, WRSs3 lacks *virG(icsA)* and the 2 enterotoxin genes with the additional deletion of the virulence plasmid-based *msbB2* gene. Lack of the *msbB2* gene product has previously been shown to produce a less-toxic LPS molecule, which might help to reduce the symptoms of fever seen in WRSS1-immunized volunteers.[20,29] How these additional mutations, especially that of *msbB2*, will affect colonization of these strains is unclear, given that effective colonization is critical in the elicitation of immune response and protection. The primary goal of this pilot study was to compare the safety and colonization of 2 novel *S. sonnei* vaccine candidate strains (WRSs2 and WRSs3), with those of the clinically tested vaccine strain WRSS1, by using a gastrointestinal model of infection. The hypothesis tested was that the 2 novel *Shigella* vaccine strains would colonize the vaccinated animal and induce an immune response similar to that of WRSS1 but with less frequent and less severe clinical side effects.

## Materials and Methods

**Animal model.** Fifteen adult male, Indian strain, rhesus macaques were selected for this study from the combined Walter Reed Army Institute of Research and Naval Medical Research Center animal colony. All animals were in apparent good health, ages ranged from 8 to 21 y, and after randomized group assignment, the mean age of each group was 13.3 ± 0.1 y. Body weights 4 d prior to vaccination were between 8.1 and 14.8 kg. Nine animals had documented historical exposure and recovery from infectious agents such as *Plasmodium*, *Leishmania*, or dengue virus, or a combination of these. Baseline values were established for each animal's complete blood count and blood chemistry. All study animals were confirmed serologically negative for *Shigella sonnei* LPS antibodies by ELISA, and had *Shigella* species- negative fecal cultures, prior to selection for this study. Male animals were selected to avoid the possibility of menstrual discharge obscuring the characterization criteria of the stool samples, particularly visible blood and mucus in the stool.

The experimental protocol used for this study was approved by the appropriate institutional animal care and use committee (protocol IBO 01-07). The animal facility is AAALACI-accredited; all husbandry and experimental procedures were conducted according to US Department of Agriculture Animal Welfare Act regulations, the Public Health Service Policy on the Humane Care and Use of Laboratory Animals, and the *Guide for the Care and Use of Laboratory Animals*.[30] The study was conducted in a dedicated room of the institute, operated under Animal Biosafety Level 2 conditions and quarantine procedures for personal protective equipment and internal traffic patterns, to prevent possible spread of the live vaccine strain to personnel or other animals. Study animals were housed in standard squeeze-back metal cages with individual drop pans and perches. Water was purified by reverse osmosis and supplied ad libitum by an automatic watering system. A commercially formulated Old World primate biscuit diet (Lab Diet 5038, Purina Mills, St Louis, MO) was provided twice a day, with the amount based on historical requirements for normal weight maintenance in rhesus macaques. Standard environmental enrichment items were provided in accordance with institutional policy: any enrichment food items, such as fresh vegetables, were provided to all animals in all groups, on the same day. After completion of all study-related activities and at least 2 *Shigella*-negative fecal cultures, all animals were returned to the colony, for future use.

**Vaccine strains.** The strains used were developed at Walter Reed Army Institute of Research. WRSS1, a *virG*(*icsA*)-based *S. sonnei* vaccine contains a 212-bp deletion in the *virG*(*icsA*) gene which renders the strain positive in HeLa cell invasion assays but plaque-negative in cultured cells and negative for elicitation of keratoconjunctivitis in the eyes of guinea pigs (Sereny reaction).[14] WRSs2 has complete deletions of the *virG*(*icsA*), *senA*, and *senB* genes.[52] WRSs3 has all 3 deletions described for WRSs2 as well as a deletion in the virulence plasmid *msbB2* gene, which encodes an acyl transferase enzyme.[6,43] Prior to harvesting for this study, all 3 strains were determined to be stable in long-term storage and positive for invasiveness in cells of the HeLa epithelial cell line.

**Experimental design.** At all times, all procedures related to handling of the study animals were carried out by qualified veterinarians and animal technicians, who used appropriate personal protective equipment including all of the following items: single-use waterproof overall suit, double waterproof shoe covers, surgical mask, hair bonnet, plastic splash-guard face shield, and double sets of latex or nitrile gloves. These precautions are standard policy to prevent exposure of personnel to monkey B virus (CeHV1) and other zoonotic pathogens. Personnel handling the animals and biologic samples changed to clean sets of gloves and

table covers between each of the 3 study groups and changed all personal protective equipment in any case of visible contamination with animal fluids or feces.

**Group assignment.** The 15 rhesus macaques were assigned randomly to 3 groups, each with 5 animals, in a single-blind format, such that clinical observers did not know which strain of vaccine was administered to which group, and laboratory personnel did not know which animals were assigned to each group. Laboratory samples were identified only by the individual animal's identification number and letter combination.

For sedation on days requiring vaccination or blood sample collection, animals were fasted for approximately 12 h and allowed access to water ad libitum. Each animal was sedated by a single IM injection of ketamine (10 to 20 mg/kg of body weight) mixed with acepromazine (0.2 to 1 mg/kg of body weight) and allowed to become recumbent before removal from the cage for the procedures. As needed, as many as 2 additional partial doses of ketamine with acepromazine were given to maintain a safe level of sedation for handling. None of the experimental procedures would be expected to cause more than minimal and transient pain in a fully conscious animal or human. Study animals were all provided access to food 1 to 2 h after full recovery from sedation.

**Vaccination.** On the day of vaccination (day 0), each vaccine strain was freshly harvested from overnight growth on agar plates in water, and the culture suspension was diluted to obtain 1 to $2 \times 10^9$ CFU per ml. To maintain partial function of the gag–swallowing reflex, animals were lightly sedated by using the low end of the drug dose range. Each animal was intubated nasogastrically with a single-use, sterilized, human infant feeding tube, lubricated with sterile saline and a small amount of sterile water-soluble surgical lubricant. Sodium bicarbonate solution (20 ml) was administered by using the tube to neutralize gastric acidity. One minute later, 20 ml of the culture suspension (total dose, $2 \times 10^{10}$ CFU) was administered to each animal via the nasogastric tube, simulating administration of an orally administered vaccine for humans. As an approved alternative, orogastric intubation was used in 2 of the animals for which nasogastric tube placement was difficult.

**Observations.** The monkeys were observed twice daily for general activity level, appetite, consistency of stool samples passed into the drop pan of their cage, and the amount of blood or mucus observed in the feces (if any). Observations were recorded on a clinical scoring sheet for 7 consecutive days after vaccination. Animal activity was scored by laboratory animal technicians on a scale of 0 to 3 as follows: 0, active and responsive; 1, reduced activity; 2, immobile; 3, recumbent. Appetite was measured by counting biscuits left uneaten after each twice-daily feeding and comparing with the number normally eaten by that animal at each meal. Appetite data was not collected for the mornings of sedation procedures. Body weight and sedated rectal body temperature were recorded for each sedation episode, along with heart and respiratory rates.

**Clinical samples.** Blood samples were drawn on days 4, 7, 14, and 28 for complete blood count and serum chemistry assays, performed by the in-house clinical pathology department. The maximum volume of blood drawn for each animal was set at 37.8 ml per week. Stool samples were collected from the drop pan 2 times daily for the first 7 d and in the afternoons of days 10, 14, 18, 22, and 28. Portions of each of these samples were frozen for PCR analysis. For *Shigella* culture, the fresh stool samples were streaked onto Hektoen Enteric Agar plates, made from Difco dehydrated media (BD, Franklin Lakes, NJ). The plates were incubated overnight at 37 °C, and suspected *Shigella* colonies were tested by biochemical test media (BD BBL Enterotube, Becton Dickinson, Franklin Lakes, NJ) for characteristic *Shigella* reactions, or by slide agglutination with commercially available *S. sonnei* antiserum (group D, Becton Dickinson), or by colony immunoblot with anti-IpaB antibodies (2F1).[27]

**PCR analysis.** PCR analysis was conducted that targeted the *ipaH* gene, which is present both on the invasion plasmid and the bacterial genome. DNA was extracted from stool samples by mixing small amounts of stool (approximately 0.1 g, taken with a loop) in 250 µl $H_2O$. One volume of phenol:chloroform:isoamyl alcohol (24:24:1) was added to the mixture, which was vortexed for 20 s and centrifuged for 3 min. The same procedure was repeated on the upper phase. A 0.1-volume of 3 M sodium acetate (pH 5.2) was added to the separated upper phase, followed by 2 volumes of 100% ethanol. After 2 h at –80 °C, the mixture was centrifuged at 4 °C for 30 min, and the pellets were washed with 1 ml cold 70% ethanol. The pellets were allowed to dry at room temperature and were suspended in 20 µl $H_2O$. These solutions were used as DNA templates for the PCR reaction, with primers from the *ipaH* gene sequence (5´ GCT GGA AAA ACT CAG TGC CT 3´ and 5´ CCA GTC CGT AAA TTC ATT CT 3´; 2 picomoles / µl).[7] To evaluate the PCR yield and for normalization of bacterial presence in stool, a parallel PCR reaction was run with primers from conserved sequences of 23S RNA (23S RNA Forward and Reverse Primers, Clontech, CA).

**Proctoscopy.** Proctoscopy was performed during animal sedation on days 0, 2, and 4, by using a rigid scope and imaging through the side of a clear endotracheal tube. A new, sterile endotracheal tube was used for each group and was cleaned between animals. During all procedures, table covers and gloves were changed between groups. The proctoscope was flushed and cleaned with a chlorhexadine solution between groups. Mucosa of the rectum and distal colon was evaluated subjectively by the veterinarians present for hyperemic appearance, hemorrhage, mucus, and ulceration.

**Statistical analysis.** Although our intent was to conduct a pilot study, the neutrophil counts in peripheral blood were apparently different between the groups on days 14 and 28 after vaccination. These values were analyzed with NCSS software (NCSS, Kaysville, UT) using repeated analysis of variance followed by the Tukey–Kramer multiple comparisons procedure. A $P$ value of less than 0.05 was considered statistically significant.

## Results

**Colonization and hematologic response.** Evaluation of stool from vaccinated monkeys revealed that all 3 vaccine strains colonized the *Rhesus* gastrointestinal tract and were excreted to similar extents. A group mean for the last day after vaccination on which fecal samples were positive (by fecal culture or fecal PCR) is represented in Figure 1. Of the 15 animals, 14 had 3 or more fecal samples that were positive for *S. sonnei*, including at least 1 positive culture for each of these animals. The single exception was in the WRSs2 group.

Leukocyte (Figure 2) and neutrophil (Figure 3) counts varied among groups. At 2 and 4 wk after vaccination, macaques that received strain WRSs3 had the highest mean increase from baseline in peripheral blood total leukocytes; animals in this group also

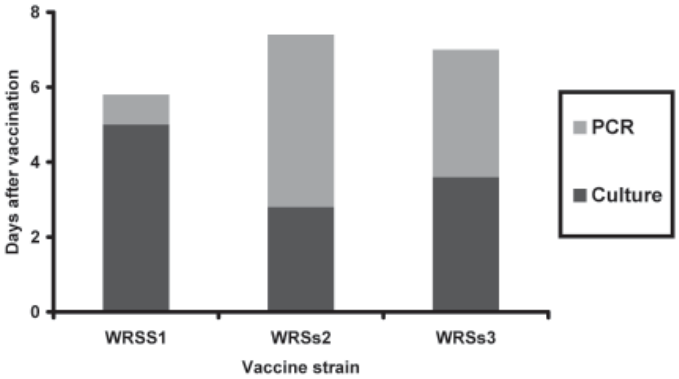


**Figure 1.** Mean number of days with *Shigella*-positive fecal samples after vaccination. Positive culture indicates excretion of viable *Shigella* bacteria; positive PCR indicates continued colonization of the lower gastrointestinal tract.

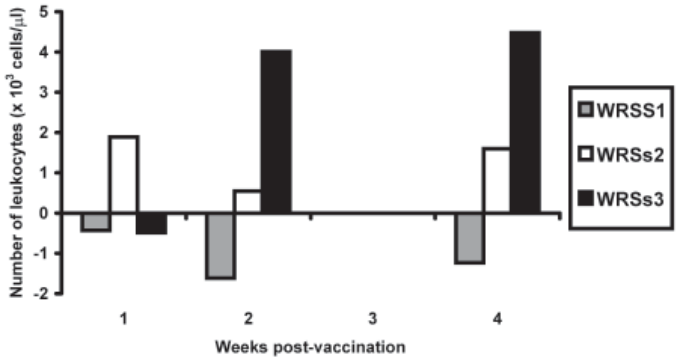


**Figure 2.** Mean peripheral blood leukocyte count change from baseline for each vaccine group. Any change from baseline identifies an immune-system–stimulating event.

had the most frequent occurrence of values above our laboratory's normal range for neutrophil cell counts in peripheral blood. Animals which received the strain WRSS1 had the most frequent occurance of values below our laboratory's normal range for this parameter. Comparing the mean neutropil count of the WRSs3 group to that of the WRSS1 group, on days 14 and 28, the WRSs3 group mean was higher and the bracketed *P* value was between 0.0005 and 0.0009, Comparing the mean neutrophil count on days 14 and 28, for the WRSs2 group, to that of the WRSS1 group, again the mean was higher for WRSs2, with a bracketed *P* value between 0.01 and 0.05. These data were analyzed using repeated analysis of variance followed by the Tukey–Kramer multiple comparisons procedure.

**Safety of vaccine in clinical use.** Animals were observed for side effects throughout the duration of the study. Clinical scores in 5 categories were recorded for the first 7 d after vaccination—overall, clinical abnormalities were minimal. Soft or loose stool was the most common finding in all groups and was highest for the WRSs2, followed by the WRSS1 and WRSs3 groups. Further, the animals that received strain WRSs3 had the lowest mean number of days with abnormal findings in all 5 clinical observation categories, and soft or loose stool was the only abnormality for any animal in this group. No animal in the study had abnormal stool after the fifth day after vaccination. Group means of days with abnormal findings during the first week after vaccination are displayed in Figure 4. The number of animals from each group with

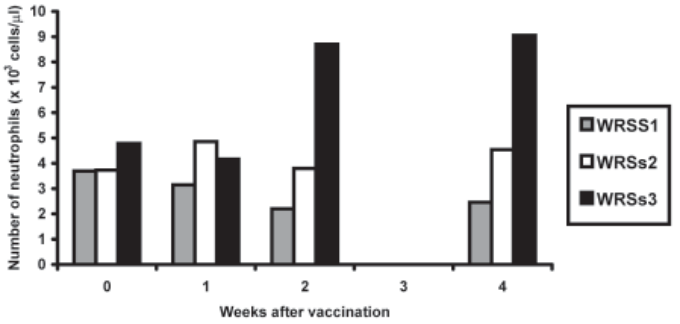


**Figure 3.** Mean peripheral blood neutrophil counts for each vaccine group before vaccination and in subsequent weeks. The normal range for the in-house clinical pathology laboratory is 2.2–to 5.6 × $10^3$ cells/μl.

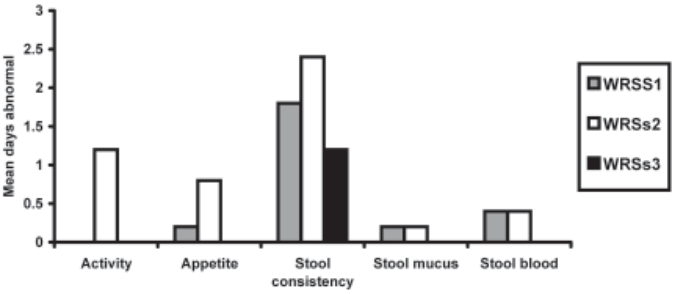


**Figure 4.** Mean number of days of abnormal clinical signs. The only abnormal clinical sign seen for the WRSs3 group was stool consistency.

abnormal signs in each clinical category through the first 5 d after vaccination is depicted in Figure 5. No animals in any group had abnormal signs after day 5.

Values observed for sedated body temperature, body weight change, and blood chemistry panels for all individual animals were within normal ranges, and intergroup variation was minimal. No animals exhibited fever, and mean weight gains and losses were less than 0.5 kg. No individual animal lost more than 0.9 kg in the 28 d after vaccination, a maximal decrease of 9.6%. The mean values for sedated body temperature and body weight of each group are shown in Figures 6 and 7.

**Proctoscopy.** Endoscopic exams of the rectum and distal colon on days 0, 2, and 4 revealed evidence of mild ulceration and hyperemic mucosa in several animals from each group. Because these lesions were observed most frequently on day 0, prior to vaccination, their significance is unknown, and they should not be attributed to vaccination.

## Discussion

**Colonization and hematologic response.** The goals of this study were to assess the ability of 2 novel *S. sonnei* vaccine strains to colonize the gastrointestinal tract after oral vaccination and to establish their clinical safety. A key question was whether the genetic attenuation of these 2 new vaccine strains weakened them to the extent that they could not survive transit through the gastrointestinal tract and successfully colonize the distal colon and rectum, steps required to induce an immune response from the vaccinated host. The results of our pilot study strongly indicate that all 3 vaccine strains successfully survived transit of the gastrointestinal tract, colonized the host colon, and induced a recog-

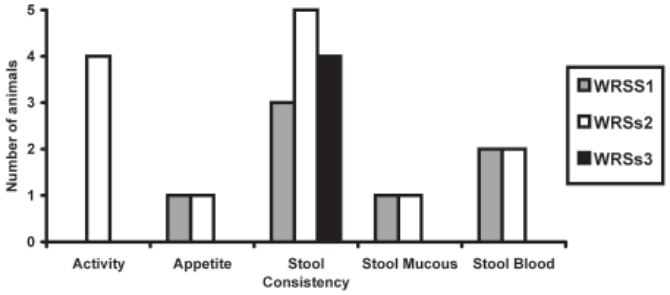


**Figure 5.** The number of animals in each group with abnormal signs during the first 5 d after vaccination. No abnormal signs were seen in any group after day 5.

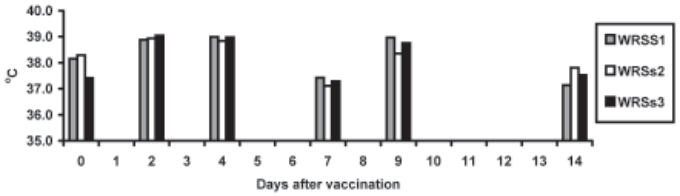


**Figure 6.** Mean body temperature of sedated animals; the day 0 value is before vaccination. The in-house normal range for body temperature in sedated macaques is 36.1 to 39.5 °C.

nizable immune response, with only minor clinical side effects. The 1 animal from the WRSs2 group that did not have *S. sonnei*-positive findings from fecal examinations exhibited a decreased appetite and scant feces during the period expected to have the highest excretion rate. The scarcity of fecal samples for testing may account for the lack of positive findings in this animal.

The peripheral blood leukocyte and neutrophil counts are indicators of an immunoreactive event. Previous studies have indicated that wild-type *Shigella* infection results in massive influx of neutrophils and monocytes to the infected portion of the intestinal mucosa.[6] Increased peripheral blood neutrophil counts occurred in the WRSs3 group: in all 5 animals on day 14, and 4 of the 5 animals on day 28. The peripheral neutrophilia may indicate ongoing immune stimulation from the vaccine, with no clinical abnormalities observed in any animals beyond the fifth day after vaccination. The duration of the significant neutrophilia in this group was unexpected; however the magnitude of the increase was approximately 2 times baseline, whereas clinically observable infections would be expected to cause 4- to 8-fold increases of the baseline value. The scope of this study did not present the opportunity to follow the neutrophilia beyond day 28. The laboratory results for the WRSs3 group contrast sharply with those of the WRSS1 group. Specifically, 3 of the 5 animals in the WRSS1 group maintained below-normal neutrophil counts at 14 and 28 d after vaccination, and none displayed above-normal neutrophil counts, despite complete resolution of all clinical signs within the first 5 d after vaccination. An abnormally low neutrophil count may indicate the sequestering of neutrophils in a region of ongoing infection; however, in immunocompetent animals neutrophilia is expected to occur within the first 2 wk after infection, especially with rapid resolution of clinical signs. This phenomenon deserves further investigation and should be correlated with results of proposed challenge trials for these vaccines in humans. Given the small numbers of animals in each group, these data may have

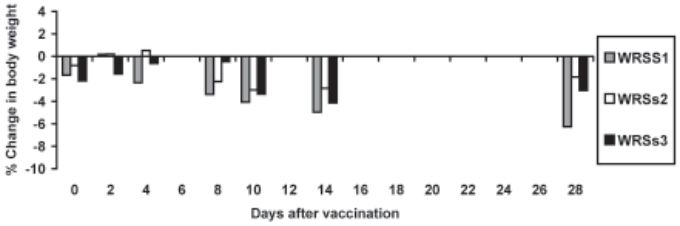


**Figure 7.** Mean percentage change in body weight compared to baseline weight. Baseline weight taken 4 days prior to vaccination

been influenced by historical exposure of some animals to other pathogens, including those for malaria, leishmaniasis, and dengue. One consideration in future pilot studies of this type would be to match previous exposure history in each group, instead of random group assignment.

**Safety of vaccine in clinical use.** The strongest side effects observed in a previous WRSS1 challenge study in humans[29,33] were fever and diarrhea during the first week after oral vaccination. Previous studies have shown that a dose of $2 \times 10^{10}$ CFU of virulent *S. sonnei* strain 53G administered in 20 ml of brain–heart infusion media can induce diarrheal illness in rhesus monkeys.[12]

Fever was not noted in any of the study animals, however temperature measurements were only made while the animals were sedated. Future studies of this type may consider methods to monitor the nonsedated temperature, such as telemetry implants, skin surface temperature, and auricular thermometers. In many species, fever is associated with a decreased activity level, which occurred only in animals assigned to the WRSs2 group. Although activity level in a singly housed cage is somewhat subjective, experienced technicians can be very accurate in assessing changes in those animals they are familiar with. Another indicator of fever and malaise, decreased appetite was observed transiently in the WRSs2 group and once in the WRSS1 group. It is notable that the WRSs3 group did not demonstrate any instances of decreased activity or appetite.

Diarrhea, including soft and loose stools, was observed in all 3 groups of this study, but no animal exhibited diarrhea that was persistent or severe. Frank blood and mucus were infrequent overall and were not seen in any animals that received the WRSs3 strain vaccine. No abnormal clinical signs were observed beyond day 5 after vaccination for any of the study animals.

On the basis of the data collected in this study, 2 novel vaccine strains, WRSs2 and WRSs3, successfully colonized the gastrointestinal tracts of macaques after oral vaccination, induced a recognizable immune response, and induced only mild and transient side effects. Both WRSs2 and WRSs3 are acceptable candidates for continued development as human *S. sonnei* vaccines. WRSs3 produced the most promising results, with the lowest frequency and severity of clinical side effects while maintaining colonization and excretion levels similar to those of the other 2 strains. The experimental design of this study—comparing the safety and immune response of novel vaccine strains with those of a known strain—is an effective refinement in animal testing of candidate vaccines, because it avoids the necessity of an animal challenge study.

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The material presented in this article has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation or publication. The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting true views of the WRAIR/NMRC, the Department of the Army, or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statues and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*.[30]

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