NAVAL MEDICAL RESEARCH CENTER
INFECTIOUS DISEASE DIRECTORATE
ENTERIC DISEASES DEPARTMENT

Statistical Analysis Plan

"Dose-finding Study of Lyophilized *Shigella sonnei* 53G (Lot 1794) Challenge Strain"

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Table of Contents

1 Abbreviations and Definitions

AE	Adverse Event
ALS	Antibody Lymphocyte Supernatant
ANOVA	Analysis of Variance
AR	Attack Rate
ASC	Antibody Secreting Cells
B_M	B Memory Cells
CF	Colonization Factors
cfu	Colony forming units
CRF	Case Report Form
ELISPOT	Enzyme-lined Immunospot
ETEC	Enterotoxigenic *E. coli*
HBsAg	Hepatitis B Virus Antigen
HCV	Hepatitis C Virus
Hg	Mercury
HIV	Human Immunodeficiency Virus
ICF	Informed consent form
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LT	Heat Labile Enterotoxin
µg	Microgram
mL	Milliliter
mm	Millimeter
MNC	Mononuclear cells
OTC	Over the counter
PBMC	Peripheral Blood Mononuclear Cell
RPR	Rapid Plasma Reagin test
SAP	Statistical Analysis Plan
ST	Heat Stabile Enterotoxin
Tukey's HSD	Tukey's HSD (Honestly Significant Difference) test

2 Introduction

Diarrheal diseases continue to be a major public health problem throughout the world. In 1999, Kotloff et al. conducted a systematic literature review and estimated the global incidence of shigellosis, a major bacterial diarrheal pathogen, to be 164.7 million cases and approximately 1.1 million shigellosis-related deaths [1]. Shigella is comprised of four species (*S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei)* with *S. sonnei* being the most common species of Shigella-associated diarrhea in developed countries and *S. flexneri* being the most common species of Shigella-associated diarrhea in the developing world [2]. More recent data has estimated mortality rates to be lower; however epidemiological information on incidence rates of Shigella-mediated diarrhea from much of Asia and Africa are missing [3].

Shigella is a low-inoculum bacterium transmitted by the fecal-oral route with as few as 10 colony forming units (cfu) sufficient to cause disease in highly susceptible people [4]. Due to ease of transmission, multiple secondary cases from an initial case are common [4]. Infections caused by Shigella can range from mild watery diarrhea to severe disease characterized by fever, headaches, abdominal cramps, and dysentery (frequent small volume stools containing blood and mucus). Although Shigella-associated diarrhea typically is self-limiting, infection frequently is debilitating for 5-7 days if untreated [2, 5].

Gregory et al, have developed a challenge model of infection for *S. flexneri* in the *Aotus nancymaae* [6]. An oral dose of $1x10^{11}$ cfu of *Shigella flexneri* 2a strain 2457T induced infection in 75% of the monkeys. However, unlike the rhesus model; a previous infection with the challenge organism did not protect against infection when the animals were administered a second challenge dose 9 weeks after the first [6]. The limitations of the currently available animal models reinforce the need for a safe and reproducible human challenge model.

Two strains of Shigella, *S. flexneri* 2457T and *S. sonnei* 53G, have been most commonly used in human challenge models. As part of a study of a bi-valent Shigella vaccine, subjects from the United States were given 3 doses of vaccine or placebo followed by challenge with 500 cfu of *S. sonnei* 53G 1 month after the third dose of vaccine [7]. Of the 38 healthy control subjects, 20 (53%) developed diarrhea and fever. In a follow-up of the previously described study, 16 placebo recipients were challenged with 500 cfu of *S. sonnei* 53G 1 month after the third dose of placebo. Ten of the 16 subjects (62%) became ill [8]. A third study from the same group compiled results from previous studies to determine if there were a correlation between peak temperature of the subject and severity of the infection [9]. A total of 85 control subjects received 500 cfu of *S. sonnei* 53G and 40 (48%) developed fever and diarrhea [9]. The investigators noted that fever did correlate with more severe illness. However, the duration of symptoms were shorter in the group that developed the higher fevers. In a trial evaluating the cytokine response to Shigella infection, 11 subjects were administered 500 cfu of *S. sonnei* 53G and 6 (55%) became ill [10]. The summary of the above studies, all conducted in the United States, is that approximately 50% of subjects develop diarrhea and/or dysentery (range 40-60%) after ingesting 500 cfu of *S. sonnei* 53G.

A critical step to increase the likelihood of inducing an infection with an enteric organism is neutralization of stomach acid. All of the above studies used skim milk to neutralize stomach acidity. A finding of all the above studies was that the rate of infection plateaued at about 60% of subjects regardless of the dose of the challenge strain administered. In an attempt to increase the attack rate (AR); milk was substituted with sodium bicarbonate [11]. When subjects were administered 10^3 cfu of *S. flexneri* 2457T mixed with sodium bicarbonate, 10 of 14 subjects (71%) developed fever and 9 of 14 (64%) developed diarrhea and/or dysentery [11]. In a second study, subjects were administered 1.4×10^3 cfu of *S. flexneri* 2457T mixed with sodium

bicarbonate [12]. Of the 12 subjects, 10 (83%) developed fever along with diarrhea and/or dysentery. Lowering the dose to 1.4 x 10^2 cfu of *S. flexneri* 2457T lowered the infection rate to 43% (3/7 subjects). A third study confirmed the results of the previous work when 8 of 10 (80%) subjects administered 1.5 x 10^3 cfu of *S. flexneri* 2457T mixed with sodium bicarbonate developed diarrhea [13]. These studies thus demonstrated that substituting skim milk with sodium bicarbonate could markedly increase the rate of infection caused by *S. flexneri* 2457T. Additionally, the studies suggested a steep dose response curve between administration of 140 and 1400 cfu of the challenge strain.

While the human challenge studies of *Shigella* have produced important data, many concerns have been raised about these data including; lack of standard inoculum; lack of reproducibility; small sample size of study subjects; varying outcome definitions and minimal understanding of the immune response to infection [14]. Additionally, the method of preparation of the Shigella inoculum from a bacterial suspension with colony count estimated from a fixed optical density has been cited as a barrier to standardization and reproducibility [14]. This study will attempt to address these deficiencies and establish a well characterized model of infection with *S. sonnei* 53G.

3 Study Objectives

3.1 Primary Objectives

1. Establish a human challenge model of *S. sonnei* 53G infection using a lyophilized formulation of the challenge strain.
2. Identify a dose of lyophilized *S. sonnei* 53G that induces the primary outcome in approximately 60% of subjects with no adverse safety concerns.

The primary outcome measure for this study is shigellosis. Shigellosis is defined as the shedding of *S. sonnei* in the stool accompanied by moderate-severe diarrhea and/or dysentery along with moderate fever or one or more severe intestinal symptoms. During the inpatient phase of the study, all stools will be collected to weigh and assess for diarrhea. Only stools that either take the shape of the container and resembles thick gravy (grade 3), is an opaque liquid (grade 4), or resembles rice water (grade 5) will be considered for meeting the criteria of diarrhea. A diarrheal episode will be considered resolved when there are no loose stools for 48 hours. The components of the primary shigellosis endpoint are defined below:

Symptom	Severity	Parameter
Diarrhea	Moderate	4-5 loose or watery grade 3-5 stools or 400-800 grams of grade 3-5 stools per 24 hours.
	Severe	6 or more loose or watery grade 3-5 stools or >800 grams of grade 3-5 stools per 24 hours or requires medical intervention.
Dysentery	Present	A grade 3-5 stool with gross blood on at least 2 occasions and reportable constitutional symptoms.
Fever	Present	Oral temperature of ≥38.5°C

Intestinal symptoms (i.e. abdominal pain/abdominal cramps, gas, anorexia, nausea, vomiting)	Severe	Prevents daily activities and requires medical intervention

Percent of subjects with shigellosis. The number and percent of subjects with shigellosis will be determined based on the presence of moderate-severe diarrhea and/or dysentery along with moderate fever or one or more severe intestinal symptoms. The proportion meeting this endpoint will be compared first using an omnibus null hypothesis and secondly using pairwise Pearson's χ^2 or Fisher's exact test with appropriate alpha adjustments as needed. Initial estimates will be calculated (and comparisons made) with all subjects meeting the shigellosis endpoint. Subsequent analyses may stratify subjects meeting each of the individual composite endpoints.

Additional analyses will also be performed to further elucidate the clinical illness. These are outlined below.

Maximum 24-hour stool output. The maximum number of loose stools in a 24-hour period will be calculated by counting the frequency of loose stools in each 24 hour period from the stool log and identifying the highest output during that period. Similarly, the maximum volume of output in a 24 hour period will be estimated based on stool weight (assuming 1ml=1g) and calculated from the stool log. Both the maximum frequency and volume in a 24 hour period will be adjudicated by the adjudication board for each subject. By-cohort estimation of the mean (standard deviation) and/or median (interquartile range) will be calculated. Comparisons of the maximum number and volume of loose stools in a 24 hour period across cohorts will be made using a Kruskal-Wallis (and/or Mann-Whitney U) test across all of the study groups and appropriate post-hoc pairwise comparisons if the omnibus null hypothesis is rejected.

Percent of subjects with diarrhea (all severities). The number and percent of subjects with diarrhea (any severity and for each level of severity separately) will be determined based on the maximum 24-hour stool output. The proportion meeting these endpoints by loose stool weight and/or frequency will be compared first using an omnibus null hypothesis and secondly using pairwise Pearson's χ^2 or Fisher's exact test with appropriate alpha adjustments as needed. Initial estimates will be calculated (and comparisons made) with either frequency or volume. Subsequent analyses may stratify subjects meeting the definition by volume or by frequency. This will also be done for moderate to severe diarrhea endpoints separately.

Total weight of grade 3-5 stools passed per subject. The total weight (assuming 1ml=1g) of loose stools (grade 3-5) will be assessed by summing the weight of each loose stool by subject over the 120 hours of observation pre-treatment. Analysis will also be completed for the entire inpatient observation period. Comparisons of the total volume of loose stools across Cohorts will be made using a Kruskal-Wallis (and/or Mann-Whitney U) test across all of the study Cohorts and appropriate post-hoc pairwise comparisons if the omnibus null hypothesis is rejected.

Number of grade 3-5 stools per subject. The total number of loose stools will be assessed by summing the number of grade 3-5 stools by subject over the 120 hours of observation pre-treatment. Analysis will also be completed for the entire inpatient observation period. Comparisons of the total number of loose stools in a 24 hour period across Cohorts will be made using a Kruskal-Wallis (and/or Mann-Whitney U) test across all of the study Cohorts and appropriate post-hoc pairwise comparisons if the omnibus null hypothesis is rejected.

Percent of subjects with nausea, vomiting, anorexia, gas, or abdominal pain/cramps rated as moderate to severe. The number and percent of subjects with any adverse events that are related to the Shigella challenge will be calculated from the listing of adverse events that have occurred during the entire inpatient phase of the study. The proportion having each adverse event will be compared first using an omnibus null hypothesis and secondly using pairwise Pearson's χ^2 or Fisher's exact test with appropriate alpha adjustments as needed.

Mean/Median time to onset of diarrhea. Time to onset of diarrhea will be calculated by determining the number of hours it takes for a subject to produce the first grade 3-5 stool that meets the diarrhea definition after administration of the challenge strain (up to 120 hours post-challenge). Subjects that have diarrhea onset outside of the 120-hour window will be evaluated by the adjudication board to determine if their diarrhea is related to the challenge strain or if there are other factors involved. The time to diarrhea onset will be compared first under an omnibus null hypothesis for the time to event using the product–limit method and secondly using pairwise comparisons with appropriate alpha adjustments as needed. The time to meeting the shigellosis endpoint may not be able to be calculated as the onset time of intestinal symptoms is not typically recorded.

Mean/Median duration of diarrhea. The duration of diarrhea (in hours) will be assessed based on the number of hours from the first and last loose stool that are part of a diarrheal episode. The duration of diarrhea for subjects with no diarrhea will be handled in two separate analyses as follows: 1) included in the overall analysis with a value of '0' for the duration of the diarrheal episode; 2) excluded from the analysis. Comparisons of the diarrhea duration will be made using a Kruskal-Wallis (and/or Mann-Whitney U) test across all of the study Cohorts and appropriate post-hoc pairwise comparisons if the omnibus null hypothesis is rejected.

Shigella disease severity score post-challenge. Research is ongoing to develop a scale of Shigella disease severity following human challenge similar to recent efforts with enterotoxigenic *E. coli* ETEC [15]-[16]. Based on those efforts an overall disease severity score may be determined to assess a Shigella disease severity score for each subject. Comparisons of the disease scores across groups will be made using a Kruskal-Wallis (and/or Mann-Whitney U) test, if normality assumptions are not met, across all of the study Cohorts and appropriate post-hoc pairwise comparisons if the omnibus null hypothesis is rejected.

3.2 Secondary Objectives

The secondary outcome measures for this study are located below:

1. Immunogenicity
    a. Systemic Immunogenicity: Serum IgA, IgM and IgG response to Shigella antigens: *S. sonnei* LPS, *S. sonnei* Invaplex, IpaB, IpaC and IpaD.

Qualitative (responder rates) and quantitative assessments (log transformed values) will be analyzed. Median increases (fold rises) of antibody concentrations and seroconversion rates will be estimated along with their two-sided exact 95% confidence intervals. Geometric mean titers will also be determined and presented with their two-sided exact 95% confidence intervals. The number and percent of subjects seroconverting will be determined based on reciprocal endpoint titers for each assay based on the pre- and post-inoculation titers. The proportion meeting these endpoints will be compared first using an omnibus null hypothesis and secondly using pairwise Pearson's χ^2 or Fisher's exact test with appropriate alpha adjustments as needed. Seroconversion is defined as a ≥4-fold rise in reciprocal endpoint titers subsequent to inoculation; however, additional analyses may consider variations on this threshold of seroconversion. The magnitude of the serologic response will be determined by

assessing fold-change in reciprocal endpoint titers subsequent to inoculation as well as estimating maximum observed endpoint titers for each subject. All statistical analyses of endpoint titers will be performed on $\log_{10}$ – transformed titer values. Titers will be displayed graphically as $\log_{10}$ reciprocal endpoint titers or geometric mean titers. Nonparametric paired *t* tests (Wilcoxon paired signed rank test) will be used to compare individual post-inoculation to pre-inoculation $\log_{10}$ titers unless assumptions are fulfilled for paired t-test. Comparisons of maximum observed endpoint titers post-inoculation will be performed using the Kruskal-Wallis test or one-way ANOVA. Groups will initially be compared using an omnibus null hypothesis and secondly using pairwise comparisons with appropriate alpha adjustments as needed.

b. Mucosal immunogenicity: IgA and IgG Antibody Secreting Cells (ASC) and Antibody in Lymphocyte Supernatant (ALS) response to Shigella antigens: *S. sonnei* LPS, *S. sonnei* Invaplex, IpaB, IpaD and Fecal IgA response to Shigella antigens: *S. sonnei* LPS, *S. sonnei* Invaplex, IpaB, IpaC and IpaD

c. Salivary IgA response to Shigella antigens: *S. sonnei* LPS, *S. sonnei* Invaplex, IpaB, IpaD and Fecal IgA response to Shigella antigens: *S. sonnei* LPS, *S. sonnei* Invaplex, IpaB, IpaC and IpaD

ALS and fecal IgA responses will be measured and reported as outlined above for serologic responses. The number ASC will be presented as the number of antibody-specific secreting cells per 10^6 PBMC and a positive response will be defined as a value of > 10 ASC per 10^6 PBMCs following inoculation. The proportion meeting this endpoints will be compared first using an omnibus null hypothesis and secondly using pairwise Pearson's χ^2 or Fisher's exact test with appropriate alpha adjustments as needed. Additional analyses may consider variations on this threshold of a response. The maximum number of ASCs (cells per 10^6 PBMC) following inoculation will be compared using the Kruskal-Wallis test initially under an omnibus null hypothesis and secondly using pairwise comparisons with appropriate alpha adjustments as needed.

d. Cell Mediated Immunogenicity: Lymphocyte proliferation after *ex vivo stimulation* of PBMCs with Shigella-related antigens (IpaB, IpaC, IpaD, Invaplex, LPS, and appropriate controls). The phenotype of the immune response will be characterized as Th1 and Th2 based on the cytokines secreted during the proliferation assay using a multiplex assay format. Cytokines to be measured include, but not limited to; IL-2, IL-4, IL-5, IL-10, IL-12, IL-17, IL-18 and IFN-γ for Th1/Th2. These investigational assays are required to better understand the immune response to Shigella infections.

e. B Memory Cell assays: B memory cells (B_M) are long lived plasma cells that provide an anamnestic or recall response in the host upon reexposure to the same antigen. The response is usually of greater magnitude, is faster and better quality and has been correlative of protection in live Shigella vaccine studies [17, 18]. In this study IgG and IgA B_M cell responses will be measured as Enzyme-Linked ImmunoSpot (ELISPOT) assays with PBMCs to *S. sonnei* LPS, *S. sonnei* Invaplex, IpaB, IpaC and IpaD. PBMCs will be expanded *in vitro* with appropriate mitogens prior to measuring B_M responses to specific Shigella antigens.

f. Expanded Immunology and Systems Biology: Specific samples will be collected as part of this study to support future evaluations in systems biology. Cells and serum samples will be collected for use in a variety of omics-based work such as transcriptomic and cytokine analysis. No analyses are envisioned as part of this objective.

2. Fecal shedding of Shigella – Daily during the inpatient stay, a sample of stool will be screened for the presence of the *S. sonnei* strain. For quantitative cultures, 2-3 grams of stool will be placed into 4-6 ml of buffered glycerol saline and plated onto Hekoen enteric agar. Lactose negative colonies will be counted. Two lactose negative colonies will be tested with group D antiserum to determine the proportion that are *S. sonnei*. The total lactose-negative count will be multiplied by this proportion. If a volunteer is unable to pass a stool on a given day, a rectal swab will be taken and inoculated into Gram-negative broth.

   After collection of stools, the samples will be processed using study-specific procedures. The final number of colony forming units of the challenge strain per gram of stool will be calculated by multiplying the number of colonies on the dilution plate by the dilution factor and then by 10. Comparisons of the number of CFUs per gram of stool across groups will be made using a Kruskal-Wallis (and/or Mann-Whitney U) test across all of the study Cohorts and appropriate post-hoc pairwise comparisons if the omnibus null hypothesis is rejected.

4 Study Methods

4.1 General Study Design and Plan

An adaptive design will be used to determine the dose of *Shigella sonnei* 53G that induces the primary outcome in approximately 60% of subjects. Rather than a priori determining the dose in all study groups; subsequent doses are based on the response to previous doses. This approach has been shown to more rapidly identify the dose of interest which results in minimizing exposure of subjects to the risks of the study as well as maximizing utilization of time and resources. We have successfully used such a design in a study to determine the dose of norovirus required to induce infection in at least 50% of subjects administered the virus (unpublished data). By using the adaptive design, we will also will maximize the number of subjects receiving the dose of interest which will improve the power of the study.

An unexpected shock-like illness of unknown etiology occurred in one of six subjects at Johns Hopkins Center for Immunization Research at 10 hours after inoculation with *Shigella sonnei* strain 53G prepared from frozen stock. Therefore, for the initial dose cohort, enrollment will be limited to one subject per day for the first three days. If no unexpected symptoms occur within 24 hours of dosing the third subject, concurrent enrolment of the remaining subjects in the cohort may proceed.

In the first cohort, about 10 subjects (minimum 8) will be administered 500 cfu (range 400-600) of *S. sonnei* 53G. Depending on the AR of the starting inoculum, the dose for the second cohort will be adjusted. If the AR is less than 60%, the next cohort will receive a higher inoculum, not to exceed 1000 cfu, based on deliberations between the investigative team, the research monitor and the safety monitoring committee (SMC). ARs at or above 60% may result in verification of the attack rate at the same inoculum dose or a decrease in the inoculum to as

low as 100 cfu based on deliberations between the investigative team, the research monitor and the SMC. Using the process described above; the dosing for the third cohort will be based on the AR in the second cohort. The results from the three dose ranging cohorts will be reviewed to select the dose to be administered to the confirmatory cohort of 15 subjects.

If the targeted AR is not achieved (either too high an AR at the low inoculum or too low an AR at the high inoculum), the results will be evaluated to determine if the dose should be increased or decreased. An iterative process will be used to select the optimal dose with each step reviewed and approved by the SMC.

An interim safety report will compile findings from the preceding dose group with the PI's interpretation and plan for advancement. The research monitor, in collaboration with the SMC, must concur with the plan before proceeding. The decision to advance to the next dose level will be based solely on the observed safety profile and the disease attack rate at the tested dose level.

Screening will be done on days -45 to -2, with admission to the inpatient unit on day -1. Challenge will occur on day 0 and discharge is planned on day 8 but the exact date will be when the subject meets discharge criteria. Two to three additional subjects per cohort will be screened and deemed "alternates". The "alternates" will be admitted to the inpatient unit along with the "primary" cohort. Prior to dosing, the eligibility of subjects in the "primary" cohort will be re-confirmed. In the event a subject in the "primary" cohort is unavailable, or becomes ineligible, that subject will be replaced by an alternate. Any alternate not needed to become a member of the "primary" cohort will be discharged from the unit prior to administration of the challenge dose. Alternates not receiving any investigational product will be excluded from all analysis. Any subject who wishes to withdraw early from the inpatient portion of the study will be treated with antimicrobials and should have two negative stool cultures before they are discharged. These subjects will be requested to adhere to the same follow-up schedule as all other subjects.

During the inpatient stay and post challenge administration, subjects will be evaluated at least daily to assess for any symptoms or adverse events. Additional assessments will include at the minimum: daily measurement of vital signs (temperature, pulse, and blood pressure), daily history directed physical examinations, the collection and grading (for consistency) of each stool, and weighing of all loose or watery stools. Stool specimens or rectal swabs (if unable to produce a stool) will also be evaluated for grossly visible blood and tested for occult blood using hemoccult when visible blood is present. Additionally, up to one sample per day will be cultured for the presence of the challenge strain. All subjects will receive antibiotic therapy 5 days after challenge inoculation (unless meeting criteria for early treatment).

Subjects will be eligible for discharge from the inpatient unit on day 8 if they have completed their antibiotic treatment and passed 2 consecutive stools culture-negative for *S. sonnei* at least six hours apart. In the event stool cultures are still growing *S. sonnei*, subjects will remain in the inpatient unit until their stool is negative for shigella.

Following an evaluation and history directed physical examination, subjects will be discharged, with follow-up on study days 14 ± 2, 28 ± 2, and 56 ± 4 to provide additional stool and blood and saliva specimens for safety checks or immunology monitoring per the schedule of events. Subjects will be given an ice-pack and cooler for collection of a stool sample at home and instructed to bring the sample with them to the follow-up visit. At approximately study day 180, the subjects will be contacted by phone for supplemental surveillance to track the occurrence of any medically significant new chronic illnesses, pregnancy, or serious health event.

Dose escalation will occur in a stepwise fashion and will be dependent upon the assessment of safety parameters and by meeting criteria for advancement to the next dose. A SMC established as an advisory committee to the study sponsors for this study, along with the research monitor, will review safety data from enrollment through day 14 from each dose level prior to making a recommendation for advancement to the next dose level. The SMC would recommend one of the following alternatives: advancing to the next dose level, halting, additional subjects at the current dose, or decreasing the current dose. The next inpatient group will not be admitted until a minimum of 4 weeks have elapsed from the time of the preceding group's challenge to ensure adequate time for safety monitoring. Subjects participating in one dose level will not be permitted to participate in a subsequent dose level.

To minimize bias on study outcomes, an independent outcome adjudication committee, comprised of up to 3 individuals, independent of the study sponsors and investigative team, will review all study endpoints (diarrhea, fever, etc.) after study completion. The role of the committee will be to (1) review and confirm all primary endpoint cases; (2) review all protocol-specified entry criteria, adherence, and compliance issues to ascertain classification in the per-protocol and other study populations; and (3) provide guidance regarding secondary and other endpoint classifications to include agreement on objective criteria for classification of endpoints. Specific duties and responsibilities will be outlined by charter prior to the start of the study. The members of the adjudication committee may also serve as members of the SMC.

4.2 Inclusion-Exclusion Criteria and General Study Population

- Subjects are required to meet the following criteria in order to participate in the study:

1. Male or female between the ages of 18 through 49 years (inclusive)

2. General good health defined as (a) no significant medical illness, (b) no clinically significant physical examination findings and (c) no screening laboratory values significantly outside the normal limits of the testing laboratory within 45 days of challenge.

3. Demonstrate comprehension of the protocol procedures and knowledge of the study by passing a written examination (pass grade ≥70%) on day -1

4. Willing to sign an informed consent form (ICF)

5. Willingness to participate in an inpatient stay lasting up to 11 days and an outpatient follow-up lasting 6 months from challenge

6. Willing not to smoke during the inpatient stay

7. Available for all planned follow-up visits

8. Negative serum pregnancy test at screening and negative urine pregnancy test of the day of the admission to the inpatient phase for female subjects of childbearing potential. Females of childbearing potential must agree to use an effective method of birth control (birth control pills, injection hormonal contraceptive, implant hormonal contraceptive, hormonal patch, IUD, sterilization by hysterectomy or tubal ligation, spermicidal products and barrier methods such as cervical sponge, diaphragm or condom) within two months before challenge and through Day 180. Abstinence is acceptable. A woman is eligible if she is monogamous with a vasectomized partner.

9. Willing to not donate blood for up to 6 months after completion of the inpatient phase of the study.

10. Willing to refrain from participation in another investigational vaccine or drug trial at least until after completion of the 6 month follow-up safety call.

- Subjects that meet any of these criteria will not be eligible to participate in this study:

1. Presence of a significant medical condition (e.g. psychiatric conditions, alcohol or illicit drug abuse/dependency, or gastrointestinal disease, such as peptic ulcer, symptoms or evidence of active gastritis or gastroesophageal reflux disease, inflammatory bowel disease), or other laboratory abnormalities which in the opinion of the investigator precludes participation in the study.

2. Immunosuppressive illness or IgA deficiency

3. Positive serology results for HIV, HBsAg, HCV, or RPR (syphilis) antibodies.

4. Evidence of inflammatory arthritis on exam and/or HLA-B27 positive.

5. Family history of inflammatory arthritis.

6. Significant abnormalities in screening lab hematology or serum chemistry, as determined by PI.

7. Allergy to fluoroquinolones or trimethoprim-sulfamethoxazole

8. Fewer than 3 stools per week or more than 3 stools per day as the usual frequency.

9. History of diarrhea in the 2 weeks prior to planned inpatient phase

10. Use of antibiotics during the 7 days before receiving the challenge inoculum dosing

11. Use of prescription and/or OTC medications that contain Imodium, acetaminophen, aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs, during the 48 hours prior to investigational product administration

12. Travel within two years prior to dosing to countries where Shigella infection is endemic.

13. Use of any medication known to affect the immune function [e.g., oral steroids, parenteral steroids, or high-dose inhaled steroids (>800 µg/day of beclomethasone dipropionate or equivalent and others): nasal and topical steroids are allowed] within 30 days preceding receipt of the challenge inoculum or planned use during the active study period.

14. Serologic evidence of *Shigella sonnei* (titer > 1:2500)

15. A positive urine test for opiates.

16. A chronic disease (such as hypertension, hyperlipidemia or anxiety/depression) for which doses of prescription medications are not stable for at least the past 3 months.

17. Have immunocompromised household contacts.

18. A clinically significant abnormality on physical examination, including a systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg, or a resting pulse >100 beats/min or <55 beats/min (<50 beats/min for conditioned athletes).

19. Pregnant, nursing, or plan to become pregnant within 6 months of receipt of the study product.

20 In the 4 weeks following challenge, subject will be living with or having daily contact with elderly persons aged 70 years or more, diapered individuals, persons with disabilities, children <2 years old, or a woman known to be pregnant or nursing, or anyone with diminished immunity. This includes contact at home, school, day-care, nursing homes, or similar places.

21. Work in a health care setting, day care center, or as a food handler in the 4 weeks following the challenge with *S. sonnei*.

22. Use of any investigational drug or any investigational vaccine within 60 days preceding challenge, or planned use during the 6 months after receipt of the study agent.

23. Have received a licensed, live vaccine within 28 days or a licensed inactivated vaccine within 14 days of receiving the challenge inoculum.

24. Inability to comply with inpatient rules and regulations.

25. Has any other condition that, in the opinion of the Investigator, would jeopardize the safety or rights of a participant or would render the subject unable to comply with the protocol.

26. Received blood or blood products within the past six months.

5 Sample Size

The aim to select a *S. sonnei* strain 53G dose with a ≥ 60% attack rate can be accomplished in 10 subjects with a two-sided exact 95% confidence interval of 26 – 88%. Increasing the number of subjects by 15 for the selected inoculum/fasting regimen (total of 25 subjects at a given inoculum dose) will provide greater confidence (39 - 79%) that the target 60% attack rate will be achieved in future applications of the challenge model.

Follow-up studies evaluating tolerable immunogenic doses will be required with larger numbers of subjects in order to better define the safety profile. All subjects who receive *S. sonnei* 53G will be included in the safety analyses. Safety data, including AEs, physical exam assessments, and laboratory tests will be listed by subject. Adverse events will be coded with the number and proportion of subjects reporting a given event summarized by group. Immune response data will be summarized using similar methodology.

5.1 Purpose of the analyses

This analysis will focus on descriptive techniques to estimate rates, means, and their respective confidence intervals.

Rates of local and systemic solicited and unsolicited AEs will be tabulated by level of severity. All rates will be determined with two-sided exact 95% confidence intervals.

During each day of the inpatient period, subjects will be monitored for loose stools (not meeting the diarrhea definition), diarrhea, dysentery, nausea, vomiting, abdominal cramps, fever, headache, abdominal tenderness, abdominal distention or otherwise abnormal abdominal exam.

The planned statistical evaluation is based on the proportion of subjects meeting prospectively defined clinical, microbiological and immunological endpoints. The attack rate will be calculated for all study groups, using the standard definition of: (# with endpoint / # receiving inoculum) x 100%. Summary tables will also be created to detail quantitative and temporal features of the illness such as diarrhea stool frequency and volume, maximum temperature observed, and time to

illness and infection. Continuous variables will be analyzed using nonparametric statistics unless assumptions are fulfilled for parametric statistics.

All statistical tests will be interpreted in a two-tailed fashion using an *alpha* = 0.05.

5.2 General Considerationss

5.2.1 Safety Population

All subjects receiving investigational product will be included in the safety analysis. Adverse events will be listed individually and summarized by body system and preferred terms within a body system for each treatment group. Serious and/or unexpected AEs will also be discussed on a case-by-case basis.

5.2.2 Immunology Populations

Analyses will include both qualitative (responder rates) and quantitative results. All volunteers receiving investigational product will be included in the analysis.

5.3 Missing Data

No imputation is planned for missing data. Data will be assumed to be missing at random and missing data points will be excluded from analysis.

5.4 Outcome Adjudication

Prior to the closeout of the study, detailed data on stool output and other clinical outcomes will be entered into AdvantageEDC and monitored.

In order to obtain an unbiased determination of the study outcomes an independent adjudication board will be compiled tasked with adjudicating the primary endpoint (see SSP 019). All members of the board will be experts in diarrheal case identification and pathogen diagnosis. The adjudication board will review all protocol-specific entry criteria, adherence, and compliance issues to ascertain classification in the per-protocol and other study population, and provide guidance regarding secondary and other endpoint classifications to include agreement of objective criteria for classification of endpoints.

If there are any individuals selected to participate in the challenge phase of the study but did not receive the IP, they will be excluded from the information presented.

6 Safety Analysis

Safety data will be listed individually and summarized by body system and preferred terms within a body system for each treatment group. Serious and/or unexpected AEs will also be described on a case-by-case basis. For the tabulation of AEs by body system, a subject will be counted only once in a given body system. For example, a subject reporting nausea and diarrhea will be reported as one subject, but the symptoms will be listed as two separate AEs within the class. Therefore the total number of AEs reported within a body system may exceed the number of subjects within the body system reporting AEs.

The investigator is required to assign a relationship of each AE to the receipt of the investigational product. The investigator will use clinical judgement in conjunction with the assessment of a plausible biologic mechanism, a temporal relationship between the onset of the event in relation to the receipt of the investigational product, and identification of possible alternate etiologies including underlying disease, concurrent illness or concomitant

medications. The following guidelines are used by investigators to assess the relationship of an AE to study product administration:

Not related: There is not a reasonable possibility that the administration of the study product caused the event.

Related: There is a reasonable possibility that the study product caused the adverse event. Reasonable possibility means that there is evidence to suggest a causal relationship between the study product and the adverse event.

In addition, all AEs will be assessed for severity by the investigator. Inherent in this assessment is the medical and clinical considerations of all information surrounding the event including any medical intervention required. Each event will be assigned one of the following categories: mild, moderate, severe, or life-threatening. The criteria below may be used for any symptoms not included in the grading scale:

Mild	Grade 1	Does not interfere with routine activities Minimal level of discomfort
Moderate	Grade 2	Interferes with routine activities Moderate level of discomfort
Severe	Grade 3	Unable to perform routine activities Significant level of discomfort
Potentially life-threatening	Grade 4	Hospitalization of ER visit for potentially life-threatening event

The proportion of all subjects with each AE will be summarized with point estimate for percent and 95% confidence intervals calculated using exact asymptomatic estimates. Summary tables will be created which will describe the number and percentage of subjects who experience each adverse event. In addition, tables will be prepared to list each adverse event, the number of subjects experiencing an event at least once and the proportion of subjects with adverse event(s). Adverse events will be divided into defined severity grades (mild, moderate, severe, or potentially life-threatening). The tables will also divide the adverse events by severity and related or unrelated to the investigational product.

7 Immunology Analysis

Analyses will include both qualitative (responder rates) and quantitative outcomes.

Graphical displays of immune responses will include the following:

1) ALS response to *S. sonnei* LPS, *S. sonnei* Invaplex, IpaB, IpaC, IpaD
2) ASC response to *S. sonnei* LPS, *S. sonnei* Invaplex, IpaB, IpaC, IpaD
3) Serum IgG, IgM, and IgA responses to *S. sonnei* LPS, *S. sonnei* Invaplex, IpaB, IpaC, and IpaD
4) Fecal IgA response to *S. sonnei* LPS, *S. sonnei* Invaplex, IpaB, IpaC and IpaD
5) Salivary IgA response to Shigella antigens

Between-group comparisons will be examined with nonparametric tests (Kruskal-Wallis for continuous data and Fisher's exact test for categorical data) unless assumptions are

fulfilled for Analysis of Variance (ANOVA) or Pearson's χ^2. Nonparametric paired *t* tests (Wilcoxon paired signed rank test) will be used to compare individual post-vaccination to baseline response within each treatment group unless assumptions are fulfilled for paired t-test. Comparisons of ALS responses post-vaccination will be performed using the Kruskal-Wallis test. All statistical tests will be interpreted in a two-tailed fashion using $\alpha = 0.05$. Statistical analyses will be performed using SAS v9.x for Windows (The SAS Institute, Cary, NC).

Immunologic Responder Definitions

Serology: An immunological response is defined as a ≥ 4-fold rise in reciprocal serum ELISA antibody titers from Day 0. A 4-fold rise is calculated by dividing the post vaccination reciprocal endpoint titer by the day 0 reciprocal endpoint titer. All statistical analyses will be performed on $\log_{10}$ – transformed titer values. Titers will be displayed graphically as $\log_{10}$ reciprocal endpoint titers or geometric mean titers.

Antibody Secreting Cells (ASC): A positive responder is any subject who demonstrates ≥ 10 ASC per 10^6 mononuclear cells (MNC). If the resulting number of cells is ≥ 10 ASC per 10^6 mononuclear cells (MNC), the subject will be classified as a responder. Once a subject is defined as 'immunologic responder', that person is permanently categorized as a 'RESPONDER'. Between group comparisons will be made using the median.

Antibody in Lymphocyte Supernatants (ALS): A positive responder is any subject who demonstrates a ≥ 4-fold rise in reciprocal titers from Day 0. A 4-fold rise is calculated by dividing the post vaccination reciprocal endpoint titer by the day 0 reciprocal endpoint titer. Once a subject is defined as 'immunologic responder', that person is permanently categorized as a 'RESPONDER'. All statistical analyses will be performed on $\log_{10}$ – transformed titer values. Titers will be displayed graphically as $\log_{10}$ reciprocal endpoint titers or geometric mean titers.

Fecal IgA: Total IgA content in the fecal extract samples will be determined by a modified ELISA method using commercial purified total IgA standard. Specimens with IgA concentration < 10 µg/ml will be excluded from further analysis, since antibody titrations of specimens with such low IgA content give unreliable results. Subsequently, no comparison to post-immunization values will be performed. Specific antibody levels in the fecal extracts will be determined using similar ELISA methods described above. Fecal antibodies will be reported as adjusted end-point titers and calculated by dividing the endpoint titer by the IgA concentration of the sample. A ≥ 4-fold increase in the specific IgA per total IgA content between pre- and any post-vaccination specimens is considered a responder. All statistical analyses will be performed on $\log_{10}$ – transformed titer values.

In addition to the analyses detailed above, several post hoc analyses will be performed in an effort to further characterize the safety and immunogenicity of the study product.

8 Reporting Conventions

P-values ≥0.001 will be reported to 3 decimal places; p-values less than 0.001 will be reported as "<0.001". The mean, standard deviation, and any other statistics other than quantiles, will be reported to one decimal place greater than the original data. Quantiles, such as median, or minimum and maximum will use the same number of decimal places as the original data. Estimated parameters, not on the same scale as raw observations (e.g. regression coefficients) will be reported to 3 significant figures.

9 Technical Details

Statistical analyses will be performed using SAS v9.x for Windows (The SAS Institute, Cary, NC).

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11 Appendices

11.1 Tables to be considered

Summary of Analysis Populations
Summary of Subject Disposition
Summary of Demographics and Other Baseline Characteristics
Summary of Protocol Deviations
Summary of Medical History
Summary of Serum Antibody Titer
Summary of Fold Increases, Serum Antibody Titer
Summary of Mean Fold Increases, Serum Antibody Titer
Summary of Fecal Antibody Titer
Summary of Fold Increases, Fecal Antibody Titer
Summary of Mean Fold Increases, Fecal Antibody Titer
Summary of ALS Antibody Titer
Summary of Fold Increases, ALS Antibody Titer
Summary of Mean Fold Increases, ALS Antibody Titer
Overall Incidence of Adverse Events Following Injection by Severity – Safety Population
Incidence of Local Adverse Events Following Injection by Severity – Safety Population
Incidence of Local Adverse Events Following Injection by Severity and Related* to Study Treatment – Safety Population
Incidence of General Adverse Events Following Injection by Severity – Safety Population
Incidence of General Adverse Events Following Injection by Severity, and Related* to Study Treatment – Safety Population
Incidence of Unsolicited Adverse Events Following Injection by MedDRA© System Organ Class and Preferred Term – Safety Population
Incidence of Unsolicited Adverse Events Following Injection and Related* to Study Treatment by MedDRA© System Organ Class and Preferred Term – Safety Population
Individual Incidence of Most Common* Adverse Events** Following Injection by Severity, MedDRA© System Organ Class and Preferred Term – Safety Population
Incidence of Serious Adverse Events Following Injection by MedDRA© System Organ Class and Preferred Term – Safety Population
Incidence of Serious Adverse Events Following Injection Related* to Study Treatment by MedDRA© System Organ Class and Preferred Term – Safety Population
Incidence of Unsolicited Adverse Events Following Injection by MedDRA© System Organ Class, Preferred Term, and Severity* – Safety Population
Incidence of Most Common* Adverse Events** Following Injection Related*** to Study Treatment by MedDRA© System Organ Class and Preferred Term – Safety Population
Reporting of Deaths Following Injection – Safety Population
Reporting of Adverse Events Following Injection, Other Than Deaths, Resulting in Study Discontinuation – Safety Population
Reporting of Serious Adverse Events Following Injection – Safety Population
Summary of Observed Values and Changes from Baseline in Chemistry/Hematology Laboratory Evaluations – Safety Population (each parameter)
Summary of Vital Signs – Safety Population (each parameter)
Summary of Prior Medications – Safety Population
Summary of Concomitant Medications – Safety Population

Summary of Prior Non-Drug Therapies/Procedures – Safety Population
Summary of Concomitant Non-Drug Therapies/Procedures – Safety Population
Summary of Pregnancies – Safety Population

11.2 Sample data tables and figures

Sample data tables and figures are included below to guide through the analysis and data presentation. Final tables and figures may be modified to optimize data presentation.

Table 1: Demographic characteristics of study subjects

Characteristic	Participant (n =)	Screened (Not Enrolled)
Mean Age (sd)*	()	()
Age range	-	-
Gender (%)		
Male	()	()
Female	()	()
Race/Ethnicity		
African-American	()	()
Caucasian	()	()
Asian-American	()	()
Other	()	()

*Measured in mean (standard deviation)

Table 2: Baseline characteristics of study participants (by cohort)

Dose	500 cfu	1000 cfu	1000 cfu	1500 cfu	1500 cfu
N					
Age[a]					
Gender [N (%)]					
Male					
Female					
Race/Ethnicity [N (%)]					
African-American					
White					
Asian					
Other					

[a] Presented as mean (standard deviation)

Table 3: Attack rates following *S. sonnei* Challenge

	500 cfu	**1000 cfu**	**1000 cfu**	**1500 cfu**	**1500 cfu**
Diarrhea [N (%)]					
Mild					
Moderate					
Severe					
Median number (interquartile range) of loose stools					
Median volume (interquartile range) of loose stool					
Median (interquartile range) onset time to loose stool (hours)					
Median (range) Duration of diarrhea					
Colonization [N (%)]					
Median (range) shedding level (log_{10})					
I.V. Rehydration [N (%)]					
Early antibiotic treatment [N (%)]					

Table 4: Severity of Associated Symptoms

	500 cfu				1000 cfu				1000 cfu				1500 cfu				1500 cfu			
	None	**Mild**	**Moderate**	**Potentially Life-Threatening**	**None**	**Mild**	**Moderate**	**Potentially Life-Threatening**	**None**	**Mild**	**Moderate**	**Potentially Life-Threatening**	**None**	**Mild**	**Moderate**	**Potentially Life-Threatening**	**None**	**Mild**	**Moderate**	**Potentially Life-Threatening**
Vomiting																				
Headache																				
Nausea																				
Abdominal Pain/Cramps																				
Gas																				
Myalgia																				
Malaise																				
Anorexia																				
Arthralgia																				
Fever																				

Table 5: Attack rates (95% CI) outcomes following *S. sonnei* Challenge

	500 cfu	**1000 cfu**	**1000 cfu**	**1500 cfu**	**1500 cfu**	**All subjects**
All Diarrhea						
Mild Diarrhea						
Moderate/severe Diarrhea						
Moderate/severe cramps						
Moderate/severe nausea						
Moderate/severe vomiting						
Moderate/severe anorexia						

Immunology

Table 6: Immunological responses [N (%)] to after challenge based on pre-challenge titers

Cohort	Number of Volunteers (%)																													
	S. sonnei LPS						S. sonnei Invaplex						IpaB						IpaC						IpaD					
	ALS		ASC		Serum		ALS		ASC		Serum		ALS		ASC		Serum		ALS		ASC		Serum		ALS		ASC		Serum	
	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA
1																														
2																														
3																														
4																														
5																														

Table 7: Fecal IgA responses [N (%)] to after challenge based on pre-challenge titers					
Cohort	Number of Volunteers (%)				
	S. sonnei LPS	S. sonnei Invaplex	IpaB	IpaC	IpaD
500 cfu					
1000 cfu					
1000 cfu					
1500 cfu					
1500 cfu					

Table 8: Salivary IgA responses [N (%)] to after challenge based on pre-challenge titers					
Cohort	Number of Volunteers (%)				
	S. sonnei LPS	S. sonnei Invaplex	IpaB	IpaC	IpaD
500 cfu					
1000 cfu					
1000 cfu					
1500 cfu					
1500 cfu					