Phase 1 Study of the Safety, Tolerability, and Immunogenicity of Oral Doses of CVD 1208S-122, a Prototype Attenuated *Shigella flexneri* 2a Live Vector Expressing Enterotoxigenic *Escherichia coli* antigens

Protocol Shigella CVD 31000

Sponsored by:
University of Maryland, Baltimore (UMB)

Funded by:
National Institute of Allergy and Infectious Diseases (NIAID)
Division of Microbiology and Infectious Diseases (DMID)

DMID Funding Mechanism: U01-AI143493

Principal Investigator: Wilbur H. Chen, MD, MS

Laboratory Co-Principal Investigator: Eileen M. Barry, PhD

Version Number: 11.0

06 December 2023

Statement of Compliance

The study will be carried out in accordance with Good Clinical Practice (GCP) as required by the following:

- United States Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46, 21 CFR Part 50, 21 CFR Part 54, 21 CFR Part 56, and 21 CFR Part 312)
- International Conference on Harmonization (ICH) E6; 62 Federal Register 25691 (1997)
- The Health Insurance Portability and Accountability Act (HIPAA) Privacy Rule-Final Modification (45 CFR Parts 160 and 164)
- The local laws and regulations, which prevail over the University of Maryland, Baltimore
- NIH Clinical Terms of Award

Compliance with these standards provides public assurance that the rights, safety, and well-being of study subjects are protected, consistent with the principles that have their origin in the Declaration of Helsinki.

All key personnel (all individuals responsible for the design and conduct of this trial) have completed Human Subjects Protection Training.

INVESTIGATOR SIGNATURE PAGE

The signature below constitutes the approval of this protocol and the attachments; it provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable US federal regulations and ICH guidelines.

I will provide copies of the protocol and all pertinent information to all individuals responsible to me who assist in the conduct of this study. I will discuss this material with them to ensure that they are fully informed regarding the product and the conduct of the study.
I agree that all information pertaining to the study, including protocols, electronic case report forms (eCRFs), and verbal and written consent information will be kept strictly confidential. Distribution of such information or information on the conduct, progress, or results of the study will be restricted to the clinical personnel involved with the conduct of the study, members of the Institutional Review Board/Independent Ethics Committee (IRB/IEC), and/or regulatory authorities.

Principal Investigator: _____

Name / Title (Print)

Signature: _____

Date: _____

Table of Contents

Page

STATEMENT OF COMPLIANCE ..... **I**

INVESTIGATOR SIGNATURE PAGE ..... **II**

LIST OF ABBREVIATIONS ..... **V**

PROTOCOL SUMMARY ..... **VI**

1 KEY PERSONNEL **11**

2 BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE **12**

2.1 Background Information **12**
2.1.1 Live, Attenuated *Shigella* vector 13
2.1.2 Pre-clinical studies of *Shigella* vector expressing ETEC antigens 13
2.1.3 Clinical studies of CVD 1208S-122 15
2.2 Rationale **15**
2.3 Potential Risks and Benefits **16**
2.3.1 Potential Risks 16
2.3.2 Known Potential Benefits 18

3 STUDY DESIGN **18**

4 STUDY POPULATION **19**

4.1 Selection of the Study Population **19**
4.2 Inclusion/Exclusion Criteria **19**
4.3 Enrollment and Randomization Procedures **22**
4.4 Withdrawal from Study **23**
4.5 Discontinuation from further vaccination (Cohort 4) **24**

5 STUDY PRODUCT **24**

5.1 Study Vaccine, CVD 1208S-122 ..... **24**
5.1.1 Preparation of Vaccine 24
5.1.2 Preparation of Placebo 25
5.2 Study Product Accountability **25**
5.3 Blinding of the Study Product **25**

6 STUDY PROCEDURES/EVALUATIONS **26**

6.1 Study Procedures **26**
6.2 Laboratory Evaluations **30**
6.2.1 Laboratory Evaluations/Assays 30
6.2.2 Special Assays or Procedures 31
6.2.3 Specimen Collection, Preparation, Handling and Shipping 35

7 STUDY SCHEDULE **35**

7.1 Screening **35**

7.2 For Cohorts 1 – 3, Inpatient Containment Period 37
7.2.1 Acclimatization (Days -2 and -1, one-two days prior to vaccination,) 37
7.2.2 Enrollment/Vaccination Day (Day 1) 38
7.2.3 Post-Vaccination Observation Period (Day 1 through discharge) 38
7.2.4 Discharge (~Day 5) 38
7.3 For Cohort 4, Vaccination Visits for Dose #1 and Dose #2 (Day 1 and Day 29 ±3) 39
7.4 Outpatient Clinic Visits 39
7.4.1 For Cohorts 1 – 3, Day 8 (+ 1), Day 29 (± 3), Day 43 (± 3), Day 181 (± 7) 39
7.4.2 For Cohort 4, Day 3 (+ 1), Day 8 (+ 1), Day 31 (+ 1), Day 36 (+ 1), Day 57 (± 3), Day 210 (± 7) 40
7.5 Unscheduled Visit(s) or Early Termination Visits, if applicable 40
7.6 COVID-19 related considerations 41

8 SAFETY ASSESSMENT AND REPORTING 41

8.1 Definition of Adverse Event (AE) 41
8.1.1 Grading of Severity of an AE 42
8.2 Relationship to Study Product 42
8.3 Definition of Serious Adverse Event (SAE) 43
8.4 Reporting Procedures 43
8.4.1 SAE Detection and Reporting 43
8.4.2 Regulatory Reporting 44
8.4.3 Reporting of Pregnancy 44
8.5 Halting Rules 44
8.6 Safety Oversight 45

9 CLINICAL MONITORING STRUCTURE 45

9.1 Site Monitoring Plan 46

10 STATISTICAL CONSIDERATIONS 46

10.1 Study Outcome Measures 46
10.2 Sample Size Considerations 46
10.3 Statistical Analysis Plan 47
10.4 General Statistical Principles 49
10.5 Go/No-Go Milestones 50

11 ACCESS TO SOURCE DATA/DOCUMENTS 50

12 QUALITY CONTROL AND QUALITY ASSURANCE 51

13 ETHICS/PROTECTION OF HUMAN SUBJECTS 52

13.1 Ethical Standard 52
13.2 Institutional Review Board 52
13.3 Informed Consent Process 52

13.4 Exclusion of Women, Minorities, and Children (Special Populations) 53
13.5 Subject Confidentiality 53
13.6 Future Use of Stored Specimens 53
14 DATA HANDLING AND RECORD KEEPING 53
15 PUBLICATION POLICY 54
16 LITERATURE REFERENCES 55
APPENDIX A: STUDY SCHEDULE OF EVENTS 58
APPENDIX B: ACCEPTABLE CLINICAL LABORATORY VALUES FOR ELIGIBILITY 60
APPENDIX C: GRADING SCALES FOR CLINICAL SAFETY LABORATORY TOXICITY 61
APPENDIX D: REACTOGENICITY & CLINICAL TOXICITY GRADING.....62

List of Abbreviations

AE	Adverse Event
AESI	AE of Special Interest
ALT	Alanine aminotransferase
ALS	Antibody in Lymphocyte Supernatant
ANC	Absolute Neutrophil Count
ASC	Antibody Secreting Cell
BGS	Buffered Glycerol Saline
BRC	Baby Rabbit Complement
CFA/I	Colonization Factor Antigen 1
CFR	Code of Federal Regulations
cfu	Colony Forming Units
CIOMS	Council for International Organizations of Medical Sciences
CRF	Case Report Form
CVD	Center for Vaccine Development and Global Health
DMID	Division of Microbiology and Infectious Diseases, NIAID, NIH, DHHS
DSMB	Data and Safety Monitoring Board
ETEC	Enterotoxigenic *Escherichia coli*
FA	Full Analysis (population)
FDA	Food and Drug Administration
FWA	Federal-Wide Assurance
GCP	Good Clinical Practice
GN	Gram-negative
Hg	Hemoglobin
HLA	Human Leukocyte Antigen
ICF	Informed Consent Form
ICH	International Conference on Harmonization
IEC	Independent or Institutional Ethics Committee
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IND	Investigational New Drug
IRB	Institutional Review Board
ISM	Independent Safety Monitor
JAMA	Journal of the American Medical Association
LT	Heat-Labile Enterotoxin
LThA2B	B and A2 subunits of human heat-labile enterotoxin
MOP	Manual of Procedures
N	Number (typically refers to subjects)
NEJM	New England Journal of Medicine
NIAID	National Institute of Allergy and Infectious Diseases, NIH, DHHS
NIH	National Institutes of Health
OHRP	Office for Human Research Protections
OPKA	Opsonophagocytic Killing Antibody

List of Abbreviations

PBMC	Peripheral Blood Mononuclear Cell
PI	Principal Investigator
PP	Per Protocol (population)
RBC	Red Blood Cell
SAE	Serious Adverse Event
SBA	Serum Bactericidal Antibody
SMC	Safety Monitoring Committee
SOP	Standard Operating Procedure
ST	Heat-Stable Enterotoxin
UMB	University of Maryland, Baltimore
UMMC	University of Maryland Medical Center
UMSOM	University of Maryland School of Medicine
WBC	White Blood Cell
WHO	World Health Organization

Protocol Summary

Title: Phase 1 Study of the Safety, Tolerability, and Immunogenicity of Oral Doses of CVD 1208S-122, a Prototype Attenuated *Shigella flexneri* 2a Live Vector Expressing Enterotoxigenic *Escherichia coli* antigens

Population: A total of 54 healthy adults age 18 – 49 years from the Baltimore-Washington metropolitan area will be enrolled

Number of Sites: Single site

Study Duration: 2.5 years

Subject Duration: 6 – 7 months, not inclusive of the screening period of up to 60 days

Study Products: Vaccine: **CVD 1208S-122** is a prototype *Shigella* - Enterotoxigenic *Escherichia coli* (ETEC) live vector vaccine strain, consisting of an attenuated *S. flexneri* strain CVD 1208S harboring deletion mutations in *guaBA*, *sen* and *set* and that has chromosomally integrated genes which express colonization factor antigen I (CFA/I) and the B subunit of ETEC heat-labile enterotoxin.

Placebo: Sodium bicarbonate buffer solution with corn starch USP.

Objectives:

Primary Objectives:

1. To evaluate the safety and clinical tolerability of oral doses of CVD 1208S-122, ranging from 10^8 cfu to 10^{10} cfu organisms, with particular attention to the occurrence of objective signs of diarrhea, dysentery or fever
2. To assess the degree of fecal shedding of CVD 1208S-122 and the genetic stability of the excreted live vector isolates by genetic characterization of the vaccine organisms recovered in stool cultures

Secondary Objectives:

1. To measure the mucosal (α4β7-positive IgA antibody secreting cell [ASC], antibody in lymphocyte supernatant [ALS], fecal IgA antibody) and systemic (serum IgG and IgA antibody) immune responses to ETEC antigens (CFA/I and LThA2B) and *S. flexneri* 2a antigens (LPS and Ipa) following oral immunization with CVD 1208S-122
2. To evaluate the ETEC and *Shigella* functional antibody responses following oral immunization with CVD 1208S-122
3. To assess transmissibility of CVD 1208S-122 by determining whether the vaccine strain is acquired (as detected by stool culture) by placebo recipients living in close proximity on the Research Isolation Ward, thus approximating household contact conditions (to be assessed in Cohorts 1 – 3)

Exploratory Objectives:

1. To measure the circulating B memory cell responses to ETEC antigens (CFA/I and LThA2B) and *S. flexneri* 2a antigens (LPS and Ipa) following oral immunization with CVD 1208S-122
2. To collect, separate and store (at -70°C or colder) peripheral blood mononuclear cells (PBMCs) so that subsequently the immune responses to CVD 1208S-122 can be further characterized in greater detail including the measurement of T memory and effector cells, homing markers and cytokine production

Primary Endpoints:

To evaluate the safety and clinical tolerability of oral doses of CVD 1208S-122, with particular attention to the occurrence of objective signs of diarrhea, dysentery or fever.

- The number, proportion, and severity of fever, diarrhea, or dysentery (hemoccult testing will only be performed during the inpatient days) within 7 days of vaccination, for all those receiving vaccine and for each of the vaccine dosages evaluated
- The number, proportion, and severity of solicited local and systemic adverse reactions (diarrhea, dysentery, fever, nausea, vomiting, abdominal discomfort, tenesmus, myalgia, arthralgia, and anorexia) within 7 days of vaccination for all those receiving vaccine and for each of the vaccine dosages evaluated
- The number, proportion, severity, and relatedness of non-serious unsolicited adverse reactions within 28 days of vaccination for all those receiving vaccine and for each of the vaccine dosages evaluated
- The number, proportion, and severity of clinical safety laboratory adverse events from the time of each study vaccination through approximately 7 days after each study vaccination.
- The occurrence of SUSARs in the study
- The occurrence of all SAEs, regardless of the assessment of relatedness, from the time of the first vaccination through approximately 6 months after the last study vaccination

To assess the degree of fecal shedding of CVD 1208S-122 and the genetic stability of the excreted live vector isolates by genetic characterization of the vaccine organisms recovered in stool cultures, to be determined with inpatient cohorts 1 – 3.

- The geometric mean number (and interquartile range) of vaccine organisms, per day and at the peak of shedding, expressed as cfu/mL for each dosage group
- The number and proportion of sequential days of fecal shedding of *Shigella* organisms which are documented to contain the genes expressing ETEC antigens for each dosage group. Genetically stable organisms will be defined as being PCR positive for *Shigella* (*ipaH* or *virG*), CFA/I (*cfaB*), and LTB (*eltB*)

[*The endpoints for additional study objectives are described in Section 10.3.*]

Brief Description of Study Design:

This will be a phase 1, double-blind, placebo-controlled, dose-escalating, single-center study, involving three vaccine dosage escalation cohorts (10^8, 10^9, and 10^{10} cfu vaccine organisms). Each of the three dose-escalation cohorts will consist of 8 study participants who will be randomly allocated to receive either vaccine (n=6) or placebo (n=2), as a single, oral dose. A Safety Monitoring Committee (SMC) will review the available safety data for Cohorts 1 – 3 through 7 days post-vaccination before proceeding to the enrollment of the next cohort. The fourth cohort will be an adaptive design cohort consisting of 30 study participants to be randomly allocated to receive either two doses of vaccine (n=12), one dose of vaccine (n=12), or two doses of placebo (n=6), with the dosage selection based on the highest well-tolerated dose in the dose-escalation cohorts (1 – 3), as determined by the SMC.

Each of the dose-escalation cohorts (1 – 3) will receive the oral dose of blinded study product while in the inpatient setting. During the following subsequent 96 hours (4 days), participants will remain on the inpatient research isolation ward to be closely monitored, and each stool will be collected by study staff. The evaluation and monitoring of participants enrolled in the fourth cohort will be conducted entirely in the outpatient setting. (**Table 1**)

Table 1: Overview of the Study Cohorts

Cohort	Setting	Dose (cfu)	No. Subjects	Dose #1	Dose #2	Vaccination Days
1	Inpatient	10^8 -	6 2	Vaccine Placebo	- -	1
2	Inpatient	10^9 -	6 2	Vaccine Placebo	- -	1
3	Inpatient	10^{10} -	6 2	Vaccine Placebo	- -	1
4	Outpatient	10^8	12	Vaccine	Vaccine	1 & 29*
		10^8	12	Vaccine	Placebo	1 & (29)*
		- (*highest, well-tolerated dose among cohorts 1 – 3, determined by SMC*)	6	Placebo	Placebo	(1) & (29)*
		Total	N = 54			

***** In Cohort D, the vaccine will be administered as a single dose (Day 1) or as a two-dose (Days 1 and 29) regimen. In order to maintain the blinding of study products, a placebo will be administered when indicated by the parentheses.

SMC, Safety Monitoring Committee

1 KEY PERSONNEL

Principal Investigator
Wilbur H. Chen, MD, MS
Professor of Medicine
Center for Vaccine Development and Global Health
Phone: 410-706-5328
Email: wchen@som.umaryland.edu

Laboratory Co-Principal Investigator
Eileen M. Barry, PhD
Professor of Medicine and Microbiology & Immunology
Center for Vaccine Development and Global Health
Phone: 410-706-3702
Email: embarry@som.umaryland.edu

Immunology Co-Investigators
Marcela F. Pasetti, PhD
Professor of Pediatrics and Microbiology & Immunology
Center for Vaccine Development and Global Health
Phone: 410-706-2341
Email: mpasetti@som.umaryland.edu

Marcelo B. Sztein, MD
Professor of Pediatrics, Medicine, and Microbiology & Immunology
Center for Vaccine Development and Global Health
Phone: 410-706-2345
Email: msztein@som.umaryland.edu

Microbiology Co-Investigator
Sharon M. Tennant, PhD
Associate Professor of Medicine and Microbiology & Immunology
Center for Vaccine Development and Global Health
Phone: 410-706-5336
Email: stennant@som.umaryland.edu

Research Institution
Center for Vaccine Development and Global Health
University of Maryland School of Medicine
685 W. Baltimore Street, Suite 480
Baltimore, MD 21201, U.S.A.

2 BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

2.1 Background Information

Diarrheal disease is a significant cause of morbidity and mortality worldwide; it is the second leading cause of death in children under the age of 5[1], with approximately 800,000 deaths annually. In 1999, the global burden of *Shigella* was estimated to include 163 million cases and >600,000 deaths each year with the greatest burden in children under five years of age in developing countries.[2] The Global Enteric Multicenter Study (GEMS), which investigated the cause of moderate-to-severe diarrhea (MSD) in children under 5 years of age in 7 sites in Africa and Asia identified *Shigella* among the top 4 pathogens.[1] GEMS follow-on studies using molecular diagnostic methods revealed an increased attributable burden due to *Shigella* and confirmed that it was the number one pathogen associated with MSD in the two older age strata (12 – 23 and 24 – 59 months old) and fourth most important pathogen in the youngest age group (0 – 11 months) across all sites.[3,4] Moreover, *Shigella* was disproportionately associated with severe outcomes.

In industrialized countries shigellosis, mostly caused by the *S. sonnei* species, persists both as sporadic cases,[5] and as a problem in certain sub-populations, such as children in daycare and other settings with suboptimal personal hygiene.[6] *Shigella* also causes significant disease in travelers to endemic regions including military personnel.[7,8] The increasing resistance of *Shigella* isolates to multiple antibiotics reduces therapeutic options.[6,9] The World Health Organization (WHO) has named *Shigella* a priority for vaccine development and implementation,[10] but despite intense efforts, no licensed vaccine to prevent *Shigella* diarrheal illness is currently available.

The GEMS also identified ETEC as an important diarrheal pathogen in young children of developing countries. Similarly, ETEC is known as the leading cause of diarrhea in travelers to endemic regions, including military populations.[11,12] Following ingestion of contaminated food or water, ETEC attach to the small intestine by fimbriae (also named colonization factor antigens or coli surface antigens, CFAs or CSs), organelles emanating from the bacterial surface. Thereupon, the bacteria elaborate heat stable toxin (ST) and/or heat labile toxin (LT), which are responsible for secretion of fluid into the intestinal lumen and subsequent watery diarrhea.

Earlier vaccine studies support the protective capacity of immune responses targeting fimbriae (to block colonization) and LT toxins (toxin-neutralizing antibodies) from ETEC.[13] ST consists of small peptides (18 or 19 amino acids). Neutralizing anti-ST has not been observed in nature.[14] In order to induce neutralizing anti-LT antibodies, the B subunit must form a pentamer by inclusion of the LTA2 subunit.[15] LThA2B allows for the expression of B subunits of human LT (as opposed to porcine LT), which can form pentamers when stabilized by the A2 domain and which lack the enzymatically-active and toxigenic A1 subunit domain. The most common fimbriae expressed on clinically important isolates include CFA/I, CS1, CS2, CS3, CS4, CS5, and CS6.[16,17]

There are currently no licensed vaccines for ETEC. Both *Shigella* and ETEC are classified as category B agents of biodefense concern, making them priorities for development of therapeutics and vaccines. A multitude of strategies have been pursued to develop vaccines against *Shigella* including live attenuated, killed whole cell, subunit, and conjugates.[18-20] Decades of literature

including field trials and volunteer challenge studies support the protective efficacy of live attenuated vaccines, which present the entire antigenic repertoire of the pathogen and which are delivered via the most immunologically relevant route, i.e., oral.[21-23] Protective immunity against *Shigella* is directed against the LPS O-antigen and is serogroup-specific and often serotype-specific.

2.1.1 Live, Attenuated *Shigella* vector

From wild-type *S. flexneri* 2a strain 2457T, a prototype vaccine candidate, designated strain CVD 1208, containing deletion mutations in *guaBA*, *set*, and *sen* was constructed. The *guaBA* operon encodes two enzymes involved in the synthesis of guanine nucleotides—thereby deletion of this operon creates a guanine auxotroph incapable of survival without a source of exogenous guanine. The mutations in the *set* and *sen* genes, encoding *Shigella* enterotoxins ShET1 and ShET2 respectively, are further attenuations intended to render the organism incapable of diarrheal reactogenicity. A phase 1 trial of oral doses of 10^7, 10^8, and 10^9 cfu with the attenuated strain CVD 1208 demonstrated good tolerability and strong immunogenicity.[24]

Strain CVD 1208 was subsequently reconstructed on animal-free media (peptone soy medium, Hy soy) to address general regulatory concerns about the possible contamination of vaccine products constructed on animal-containing media with prions, causing Bovine Spongiform Encephalopathy (BSE). This new construct was designated strain CVD 1208S. A phase 1 clinical trial confirmed that oral doses of 10^8 and 10^9 cfu of strain CVD 1208S was similarly well-tolerated and immunogenic compared to CVD 1208.[25] A subsequent phase 1 clinical trial of oral doses of 10^7 and 10^8 cfu of strain CVD 1208S with a current Good Manufacturing Practice (cGMP) pilot lot of vaccine (Shigella 28000) grown in Hy soy broth (manufactured at the Walter Reed Army Institute of Research Pilot Bioproduction Facility [WRAIR PBF]) also demonstrated minimal reactogenicity and good immunogenicity[26], thus encouraging continued clinical development of this prototype strain as a viable oral attenuated vaccine.

In order to construct a rational prototype *Shigella*-ETEC vaccine strain, two ETEC genes were added into the chromosome of the parent vaccine strain CVD 1208S. The integration of genes encoding CFA/I fimbriae and LT antigen, LThA2B, into the chromosome resulted in strain CVD 1208S::PmLpp-CFA/I-LTA2B (renamed strain CVD 1208S-122). Western blot analysis demonstrated high level expression of CfaB, the structural subunit of CFA/I. However, electron microscopy analysis of cGMP lot material of CVD 1208S-122 did not show full-length CFA/I fimbriae on the surface of the bacteria. Instead, the CfaB subunit monomers are expressed at high levels in the periplasm and also on the bacterial surface but not in the native morphology. Additional information can be found in IND section 1.11.3.

2.1.2 Pre-clinical studies of *Shigella* vector expressing ETEC antigens

Immunization of guinea pigs with two doses of CVD 1208S-122 induced robust responses to the *Shigella* live vector, as well as CFA/I, and to a lesser extent the LTB antigen (**Figure 1**). The lead chromosomally-encoded gene construct, CVD 1208S-122, was compared to the multi-copy plasmid-bearing construct, strain CVD 1208S(pCFA/I-LTB), that was previously tested in

volunteers. All vaccinated animals responded with robust and comparable serum and mucosal anti-*S. flexneri* 2a LPS IgG and IgA titers following two doses; there was no significant difference in the geometric mean titers between any vaccine groups. Consistent with this strong response, all vaccinated animals were protected against keratoconjunctivitis following Sereny test challenge with wild-type *S. flexneri* 2a strain 2457T two weeks after the second immunization. All five unimmunized control animals succumbed to infection, reaching a clinical score of 4 by 48 – 72 h post-inoculation. All of the vaccinated animals responded with strong serum and mucosal antibodies to CFA/I. Responses to the LTB antigen were variable; 75% (3 of 4) animals immunized with the plasmid-bearing construct responded to LTB and 50% (4 of 8) animals immunized with CVD 1208S-122 responded with anti-LTB IgG titers above pre-immune levels.[27]

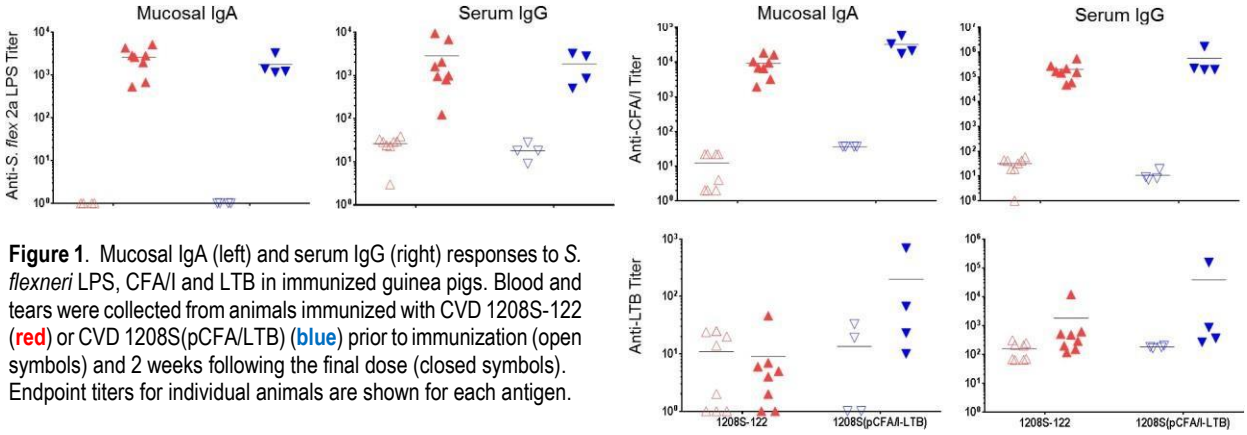

Figure 1. Mucosal IgA (left) and serum IgG (right) responses to *S. flexneri* LPS, CFA/I and LTB in immunized guinea pigs. Blood and tears were collected from animals immunized with CVD 1208S-122 (**red**) or CVD 1208S(pCFA/LTB) (**blue**) prior to immunization (open symbols) and 2 weeks following the final dose (closed symbols). Endpoint titers for individual animals are shown for each antigen.

The functional activity of elicited antibodies was assessed with *Shigella* serum bactericidal assays (SBA), ETEC hemagglutination inhibition, and cell binding inhibition assays for ETEC.[28] All animals vaccinated with CVD 1208S-122 had SBA titers >1200 (an endpoint titer that exhibits 50% killing) against wild-type *Shigella* (**Figure 2A**). These serum samples were also able to inhibit hemagglutination (HAI) of human type A red blood cells by wild-type ETEC strain H10407 (which expresses CFA/I). This measurement is considered a proxy for inhibition of ETEC binding to the human host.[29,30] Additional studies confirmed the capacity of immune sera from immunized animals to inhibit wild-type ETEC binding to human intestinal cell lines *in vitro* (**Figure 2B**).

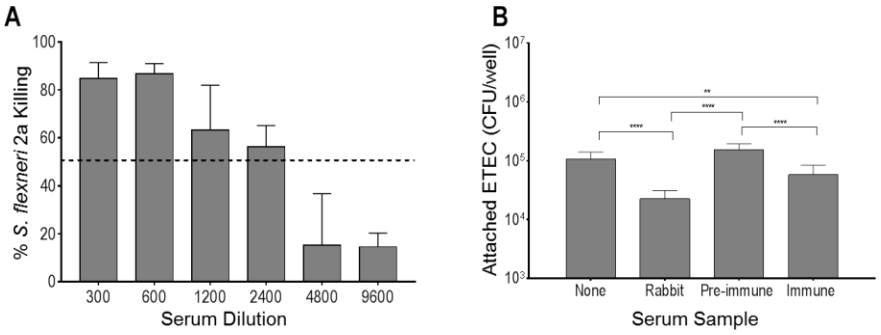

Figure 2. Functional activity of immune sera. A) Mean bactericidal activities against WT *Shigella* at increasing dilutions. B) cfu of attached WT ETEC strain H10407 following incubation with no sera, rabbit polyclonal anti-CFA/I, pre-immune guinea pig sera, or pooled sera from animals immunized with CVD 2108S-122 (immune sera). ***p<0.001, **p<0.01 2-way ANOVA with Sidak and Tukey's tests for multiple comparisons.

2.1.3 Clinical studies of CVD 1208S-122

No human clinical studies have evaluated CVD 1208S-122.

2.2 Rationale

This will be a phase 1, randomized, double-blind, placebo-controlled, dose-escalating, single-center study of CVD 1208S-122. We hypothesize that the chromosomal integration of foreign genes for CFA/I and LThA2B into the parent attenuated vector (CVD-1208S) will be stable and not introduce further reactogenicity. It is possible that the metabolic burden of the expression of these foreign genes could further attenuate the live organism and diminish stability or immunogenicity. The proposed starting dose of 10^8 cfu of CVD 1208S-122 will be evaluated in the first dose-escalation cohort (Cohort 1). Cohort 2 will evaluate a target dose of 10^9 cfu of CVD 1208S-122. Cohort 3 will evaluate a target dose of 10^{10} cfu of CVD 1208S-122. Each of these three dose-escalation cohorts will consist of a total of 8 participants who will be randomly allocated to receive a single oral dose of a blinded study product such that six participants will receive vaccine and two participants will receive placebo.

A fourth cohort will be included to further evaluate the highest well-tolerated dose of the vaccine and will evaluate the safety and immunogenicity of one or two doses of vaccine. The dose range was selected because the safety and immunogenicity at these doses was demonstrated with 10^7 - 10^9 cfu of the parent strain (CVD 1208S) and 10^7 – 10^{10} cfu of the plasmid-expression-based *Shigella*-ETEC strain (CVD 1208S(pCFA/I-LThA2B)) in prior clinical studies.[25,26,31]

In order to characterize the fecal shedding and genetic stability of the ingested organism, Cohorts 1 – 3 will be administered the assigned dose of blinded study product while in the inpatient setting at the Research Isolation Ward. Reactogenicity will be assessed for 96 hours (4 days) following administration of the product. Participants will be discharged after 96 hours of observation if they are free from objective signs of adverse reactions, including fever, diarrhea,

and dysentery for at least 12 hours. Twice daily stool cultures will be performed (one in the AM and one in the PM) to characterize the pattern of fecal shedding, assess the genetic stability of the excreted live vector vaccine organisms, determine whether the vaccine is transmitted to placebo recipients living in close proximity on the ward, and document the duration of vaccine excretion. Blood and stool specimens will also be collected to measure the immune response to the oral doses of vaccine. Since the assumption will be for the fourth cohort to be selected based on the elicitation of minimal reactogenicity in the dose-escalation cohorts, this cohort of the study will be conducted on an outpatient basis. Nonetheless, to ensure the assessment of safety, reactogenicity information will be collected from the participants during the first week post-vaccination.

2.3 Potential Risks and Benefits

2.3.1 Potential Risks

Shigellosis and Diarrheal Disease. Although engineered to be attenuated, since CVD-1208S-122 is a live vaccine, subjects could develop shigellosis with symptoms such as fever, headache, diarrhea, dysentery, abdominal cramps, nausea, and vomiting. If a subject develops unacceptable reactogenicity (as described above), is uncomfortable, and/or does not appear to be recovering spontaneously, he/she will be offered early antibiotic therapy. The potential exists for virulent *S. flexneri* strains to cause dehydrating diarrheal disease. Under the controlled management in this study, even the dehydrating effects of heavy purging can be prevented by early recognition of dehydration, by giving oral rehydration solutions preventively after each loose stool (Grade 3 – 5), and by appropriate therapy with oral and/or intravenous fluids. If intravenous fluids are required, blood chemistries will be monitored. As a precaution, subjects who have poor venous access will be excluded from participating in the trial.

Reactive Arthritis. Reactive inflammatory arthritis, alone[32] or as part of a constellation of arthritis, conjunctivitis or iritis (uveitis), and urethritis[33] is a rare post-infectious complication that occurs mostly in adults who have had an infection with a wild-type *S. flexneri* serotype. Arthritis usually begins acutely, 2 to 4 weeks after the intestinal illness. Joint symptoms become chronic in about 10% of cases, ranging from mild arthralgia to severe polyarthritis. Persons with the human leukocyte antigen type B27 (HLA-B27) histocompatibility antigen are predisposed, accounting for approximately half of the cases.[34] The risk to the remaining 92 – 99% of the HLA B27-negative population is thus extremely low. As such, subjects who carry HLA-B27 will be excluded from participating in the study. Uveitis can be a component of the reactive arthritis syndrome (which has many causes), but to our knowledge has never been associated with *Shigella* human challenge infections. Uveitis in association with reactive inflammatory arthritis can cause pain, eye redness, and visual disturbance, but often completely resolves within 3 months.

Bacteremia and/or Localized Infection. Available information suggests that even during infection with virulent *Shigella* spp., bacteremia and localized infection are rare in persons of good nutritional state; i.e., typically only observed in developing country settings and in association with poor nutritional status. Among adults of developing countries, *Shigella* bacteremia has only been documented among older persons with underlying chronic medical conditions or among the immunocompromised, therefore, this theoretic risk is prevented through our eligibility criteria and not specifically discussed in the consent form.

Transmission and Secondary Spread of the Live Organism. Wild-type *Shigella* are capable of efficient person-to-person transmission in settings where there is lack of sanitation, safe water, and hygiene. This risk has been minimized for several reasons:

a) Previous studies with either wild-type or vaccine strains of *S. flexneri* at the CVD have not detected symptomatic transmission events. We acknowledge there was an observation of two recipients of an early attenuated *Shigella* strain (strain CVD 1204) at 10^7 cfu and one placebo recipient which demonstrated positive stool cultures in enrichment broth while on ciprofloxacin therapy; this is believed to be the result of a laboratory error and not nosocomial transmission of the vaccine strain. In this study, there will be the performance of daily stool cultures to systematically identify whether any transmission events occur while inpatient.
b) The vaccine strain has a stringent growth requirement (guanine auxotrophy) rendering it capable of only limited survival in the environment. This inability to persist in the environment further minimizes the risk of transmission of the organism.
c) A lapse in observing hygiene is the ultimate culprit for transmission events in industrialized country settings. Therefore, study participants will be repeatedly educated during the course of the study about the precautions needed to prevent spread of the vaccine strain to close contacts. Hand washing will be emphasized to all the participants. Stools will be treated with bleach for five minutes prior to flushing. In Cohort 4 (outpatients), training on proper toileting and handing of waste will be provided. Those who have close contact with vulnerable persons who can develop more severe or even fatal infections with wild-type *Shigella* strains (children, immunocompromised, or older adults), healthcare workers, or who are occupational food-handlers will not be eligible to participate.

Pregnancy. This *Shigella*-ETEC vaccine has not been evaluated in pregnancy, so pregnant women may not participate in this first in human study. Women of child-bearing potential must agree to use effective methods of birth control for at least 4 weeks after vaccination.

Special Circumstance.

COVID-19. At the time of this writing, there is a pandemic caused by the SARS-CoV-2 virus and causing COVID-19 illness. As long as the threat of COVID-19 continues to represent a U.S. Public Health Emergency, a number of steps will be undertaken to

protect research staff and study participants from COVID-19. The eligibility criteria will include a question regarding the presence of symptomatic COVID-19-like illness, wherein a positive response regarding symptomatic illness will exclude the participant from eligibility to be vaccinated. Also, the incorporation of SARS-CoV-2 testing will be a required element of the procedures to be performed prior to inpatient admission (Cohorts 1-3), wherein a negative molecular diagnostic test result will be required for final eligibility for vaccination. This testing is intended to further reduce the risk of transmission of COVID-19 on the inpatient setting from an asymptomatic or pre-symptomatic infected participant.

Furthermore, at all points during the conduct of the study, a number of non-pharmacologic interventions will be required. The observance of universal masking, physical distancing, hand hygiene, administrative changes (e.g., decreasing the density of study personnel), and other control or mitigation measures will be implemented by research staff. All study participants will be required to wear a face mask and instructed to perform hand washing or hand sanitation during the inpatient period (Cohorts 1-3) and for all scheduled outpatient visits. Lastly, COVID-19 vaccination must have been completed at least 14 days prior to scheduled first vaccination. A valid COVID-19 vaccine is one that has been authorized by the U.S. FDA or has received WHO pre-qualification.

When the COVID-19 pandemic resolves and the U.S. Public Health Emergency has ended, the study team will re-evaluate the local conditions for consideration of removing these additional precautionary measures.

2.3.2 Known Potential Benefits

This is a healthy volunteer study which does not provide any guarantee of benefit. The benefit is largely the scientific knowledge to be gained from the study.

3 STUDY DESIGN

This will be a phase 1, double-blind, placebo-controlled, dose-escalating, first-in-human, single-center study involving healthy adults (age 18 – 49 years). This study will involve three vaccine dose-escalation cohorts and a fourth adaptive design cohort. Participants will be screened for eligibility up to 60 days prior to enrollment. Randomization will be performed by the assigned unblinded biostatistician and will occur upon enrollment, after a final confirmation of eligibility. Study product administration will be performed by prospectively assigned unblinded study staff members, who will not have any role in the subsequent follow-up assessments of the participants. Following completion of each dose-escalation cohort (7 days post-vaccination), there will be an independent Safety Monitoring Committee (SMC) which will review the cumulative safety data and will provide their recommendation for whether the study should proceed to the subsequent cohort.

The three dose-escalation cohorts will evaluate doses of 10^8, 10^9, and 10^{10} cfu vaccine organisms. Each of the three dose-escalation cohorts will be randomly allocated to receive either vaccine (n=6) or placebo (n=2). In Cohorts 1 – 3, eligible participants will be administered a single oral dose of blinded study product while residing in a Research Isolation Ward (inpatient setting). All participants will be observed for at least 4 days (96 hours) following product administration while inpatient. During the inpatient observation period, protocol-specified clinical evaluation procedures will be implemented, including management of diarrhea, procedures for fluid replacement, and criteria for antibiotic therapy.

Subjects will be discharged after 96 hours of observation if they are free of fever, diarrhea, and dysentery for at least 12 hours—i.e., subjects have an oral temperature <100.4°F, 12 hours have passed since the last loose stool meeting the definition of diarrhea, and 12 hours have passed since the last loose stool containing gross blood. After discharge and through 7-days following vaccination, the subject will record onto a standardized memory aid the occurrence of solicited symptoms, graded by severity, oral temperature, record the character of all stools passed as either formed or loose (defined as stools which take the shape of the container) or containing visible blood.

Subsequent scheduled outpatient follow-up visits will be completed for the continued collection of safety information and blood and stool specimens. Subjects will return to the CVD outpatient clinic on Day 8 and research staff will collect and review the information in the memory aid. Additional scheduled outpatient clinic visits will also occur on Days 29, 43, and 181.

The adaptive design cohort (Cohort 4) will consist of 30 participants that will receive either two doses of vaccine, one dose of vaccine, or placebo; the dosage selection for Cohort 4 was based on the highest well-tolerated dose in the dose-escalation cohorts. Cohort 4 is planned to be conducted entirely in the outpatient setting with several in-clinic follow-up visits. Blinded oral doses of study product will be administered on Days 1 and 29. A stool specimen and safety information will be collected 2 days following each vaccination (Days 3 and 31). Another stool specimen and a blood sample will be collected, and a complete review of the memory aid will be performed 1 week following vaccination (Days 8 and 36). Additional scheduled outpatient follow-up visits will also occur on Days 57 and 210. A complete schedule of events in provided in ***Appendix A: Study Schedule of Events***.

4 STUDY POPULATION

4.1 Selection of the Study Population

Healthy subjects, ages 18 – 49 years will be recruited on the basis of satisfying eligibility criteria during screening. Up to 54 subjects will be enrolled, meaning randomized and receiving the first dose of blinded study product.

4.2 Inclusion/Exclusion Criteria

Inclusion Criteria:

1. Male or female, 18 – 49 years of age
2. Written informed consent provided
3. Determined to be in good health* based on medical history and review of concomitant medications

   *Good health as defined by an absence of an active chronic medical condition which requires daily prescription medication(s). Participants may be eligible if the medical condition only requires infrequent as needed (PRN) medication and if the investigator determines that the condition does not pose a risk to participant safety or the assessment of reactogenicity and immunogenicity. Any chronic medical condition which does not require a daily prescription medication but might pose a risk to a participant with rapid dehydration (i.e., rapid intravascular volume changes) would be ineligible to participate.

4. Documented acceptable results from screening laboratory work (defined in **Appendix B**), including:
   - Complete blood count (CBC) with differential for total white blood cell count (WBC), absolute neutrophil count (ANC), hemoglobin (Hg), platelet count
   - Creatinine, alanine aminotransferase (ALT), total bilirubin
   - Serum Immunoglobulin A (IgA) level
   - Human immunodeficiency virus (HIV) antibody, Hepatitis B surface antigen (HBsAg), Hepatitis C virus antibody (HCV)
   - HLA-B27 histocompatibility testing
   - Serum Beta human chorionic gonadotropin (β-HCG) test, if the participant is a woman of child-bearing potential
5. A passing score (≥70%) on a Comprehension Assessment Tool
6. Agrees not to participate in another interventional clinical trial during the study period (*participation in non-interventional studies, such as surveys, is acceptable*)
7. Females of child-bearing potential[†] agree to use an acceptable form of birth control[‡] from enrollment and through at least 4 weeks after vaccination

   [†]Females of child-bearing potential, defined as having not been sterilized via tubal ligation, bilateral oophorectomy, salpingectomy, hysterectomy, or successful Essure® placement (permanent, non-surgical, non-hormonal sterilization) with documented radiological confirmation test at least 90 days after the procedure, and still menstruating or <1 year has passed since the last menses, if menopausal.

   [‡]Acceptable birth control includes barrier methods such as condoms or diaphragms/cervical cap with spermicide; effective intrauterine devices; NuvaRing®; and licensed hormonal methods such as implants, injectables or oral contraceptives ("the pill") or alternatively, monogamous relationship with a vasectomized partner who has been vasectomized for 180 days or more prior to the subject receiving the study vaccination, abstinence from sexual intercourse with a male partner, or sexual relationships with non-male partners.

8. Available for up to a 6-day inpatient stay
9. For Participants of Cohorts 1-3 during the time when a U.S. Public Health Emergency for COVID-19 exists, SARS-CoV-2 testing must be performed prior to admission to the inpatient ward and must document a negative test result prior to vaccination.

Exclusion Criteria:

1. A positive pregnancy test at screening or within 24 h prior to study product dosing
2. A female who is breastfeeding
3. Poor venous access, as defined by inability to obtain venous blood, for screening labs, after 3 venipuncture attempts
4. Abnormal vital signs, defined as:
   - Systolic BP >150 mmHg or Diastolic BP >90 mmHg
   - Resting heart rate >100 bpm
   - Oral temperature ≥38.0°C
5. Having received prior vaccines for or have had prior infection with ETEC, LT, cholera, or *Shigella*, within the past 3 years
6. History of diarrhea during travel to a developing country within the past 3 years
7. History of chronic gastrointestinal illness, including severe dyspepsia, lactose intolerance, or another significant gastrointestinal tract disease (e.g., irritable bowel syndrome, inflammatory bowel syndrome, gastric ulcer disease)
8. Regular use (≥once weekly) of laxatives, anti-diarrheal agents, anti-constipation agents, or antacid therapies
9. History of major gastrointestinal surgery (uncomplicated laparoscopic appendectomy or cholecystectomy >1-year prior is permitted)
10. Abnormal bowel habits, as defined by <3 stools per week or >2 stools per day in the past 6 months
11. Use of systemic antibacterials[§] within the past 2 weeks

    [§]use of topical (skin), otic, or ophthalmic antibiotics is acceptable, if those doses are not expected to result in significant systemic absorption levels
12. Use of oral, parenteral or high-dose inhaled steroids within 30 days

    (high-dose oral steroids is defined as prednisone ≥ 20 mg total daily dose, or equivalent dose of other glucocorticoids; high-dose inhaled steroids is defined as >800 µg/day of beclomethasone dipropionate or equivalent)
13. Use of any medication which might affect immune function[#] within 30 days[**]

    [#]examples include anti-cancer drugs, immunomodulating monoclonal antibody therapeutics, and rheumatologic therapies

    [**]Due to the prolonged half-life of some immunosuppressive medications (e.g., monoclonal antibody biologic response modifiers), the restricted use period may need to be extended to 3 or 6 months, based on the anticipated duration of activity with longer half-lives. This is to be interpreted by the investigator.
14. Diagnosis of schizophrenia or other major psychiatric disease
15. Alcohol or drug abuse within last 5 years
16. Presence of immunosuppression, which could be due to active neoplastic disease or a

history of any hematologic malignancy (excluding resolved non-melanoma skin cancers), radiation therapy, or primary or secondary immunodeficiencies

17. History of allergy to quinolone (e.g., ciprofloxacin), sulfa drugs (e.g., trimethoprim-sulfamethoxazole), or soy or corn products
18. Known history of seizure disorder (remote history of a childhood seizure disorder which has completely resolved is acceptable)
19. Occupation involving the handling of ETEC, cholera, or *Shigella* bacteria
20. Occupation as a healthcare worker, in food handling industry or care of very young children (<2 years old), elderly (≥70 years), or immunocompromised; this exclusion also applies to individuals that have close contact with young children (<2 years old), elderly (≥70 years), or immunocompromised, for example by virtue of living in the same household with such individuals
21. During the time when a U.S. Public Health Emergency for COVID-19 exists, within 14 days prior to the time of vaccination, the presence of 2 or more of any of the following symptoms: fever (≥38°C, chills, myalgia, headache, sore throat, or new olfactory and taste disorder
22. During the time when a U.S. Public Health Emergency for COVID-19 exists, within 14 days prior to the time of vaccination, the presence of 1 or more of any of the following respiratory symptoms: cough which cannot otherwise be explained, shortness of breath, or difficulty breathing
23. During the time when a U.S. Public Health Emergency for COVID-19 exists, has not completed a COVID-19 vaccine within 14 days prior to the time of vaccination
24. Any other criteria which, in the investigator's opinion, would compromise the safety of the study, the ability of a subject to participate, or the results of the study

4.3 Enrollment and Randomization Procedures

Per International Conference on Harmonisation (ICH) guideline E6: Good Clinical Practice (GCP), screening records will be kept at the clinical site to document the reason why an individual was screened, but failed trial entry criteria. The reasons why individuals failed screening will be recorded in the data system.

Once consented to and upon confirmation of eligibility for this trial, the subjects will be enrolled and randomized. Subjects will be randomized at a 3:1 ratio (vaccine to placebo) for Cohorts 1 – 3. In Cohort 4, randomization will be a 2:2:1 ratio for 2-dose vaccine vs. 1-dose vaccine vs. placebo. The randomization code will be prepared by a statistician at CVD. A designated individual at the site will be provided with a code list for emergency unblinding purposes, which will be kept in a secure place.

Subjects who sign the informed consent form and are enrolled and randomized but have not received study vaccine may be replaced by back-up eligible participants. Subjects who sign the

informed consent form, are randomized and vaccinated, and subsequently withdraw, are withdrawn, terminated from this trial, or are lost to follow-up will not be replaced.

4.4 Withdrawal from Study

Reasons for Withdrawal or Termination. Participation in the study is strictly voluntary. Participants have the right to withdraw from the study at any time and for any reason, without penalty. The Principal Investigator (PI) and/or designee may, at his discretion, withdraw a subject from continuing in the study if it is considered to be in the participant's best interest, or if the participant is not willing or able to comply with the study requirements. The reason for withdrawal will be documented.

Handling of Participant Withdrawals or Termination. Every effort will be made to undertake protocol-specified safety follow-up procedures to capture adverse effects (AEs), serious adverse events (SAEs), and unanticipated problems (UPs). In the event of withdrawal from the study, reasonable efforts should be made to conduct the following procedures:

- A review of the diary card/memory aid, if still in use prior to withdrawal
- Update any ongoing AE/SAEs that remain ongoing at time of subject's last visit prior to withdrawal
- Query about AEs, SAEs and concomitant medications, if the interval between the subject's last visit and the time of withdrawal is within the protocol-defined reporting period
- Physical examination
- Blood specimen collection for safety testing, if withdrawal occurs before Visit 28
- Update contact information

Premature Termination or Suspension of Study. This study may be temporarily suspended or prematurely terminated if there is sufficient reasonable cause. Written notification, documenting the reason for study suspension or termination, will be provided by the suspending or terminating party to the investigator. If the study is prematurely terminated or suspended, the PI will promptly inform the IRB and will provide the reason(s) for the termination or suspension. Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Demonstration of insufficient immunogenicity that would warrant halting the study
- Insufficient compliance to protocol requirements
- Data that are not sufficiently complete and/or evaluable
- Determination of futility

Study may resume once concerns about safety, protocol compliance, data quality is addressed and satisfy the sponsor, IRB and/or FDA.

4.5 Discontinuation from further vaccination (Cohort 4)

Participants that are planned to receive two sequential doses of blinded study product (Cohort 4) may be discontinued from further vaccination to avoid exposure of subjects to unreasonable risk. If a participant experiences a grade 3 or higher AE which is deemed to be related to study product or an SAE that is deemed to be related to study product, then the second dose of study product will be discontinued. Also, antimicrobials will be administered immediately if a subject meets the criteria for discontinuation from further vaccination. Antimicrobials will consist of a 3-day course of ciprofloxacin (primary) or trimethoprim-sulfamethoxazole (secondary). The investigator may also discontinue a participant from second dose administration for other significant and unexpected reasons; whereupon the investigator may also promptly treat the participant with a 3-day course of antimicrobial therapy. The reason for discontinuation must be clearly documented by the investigator. Any participant that is discontinued from further vaccinations will be asked to remain in the study to complete all immunologic and safety follow up visits, except the participant will not complete the 7-day memory aid related to the second dose of vaccine.

5 STUDY PRODUCT

5.1 Study Vaccine, CVD 1208S-122

This prototype monovalent, live, attenuated, vaccine strain consists of *S. flexneri* 2a into which deletion mutations have been introduced into the *guaBA*, *set*, and *sen* genes. The *guaBA* operon encodes two enzymes involved in the biosynthesis of guanine nucleotides. The *set* and *sen* genes encode *Shigella* enterotoxins ShET1 and ShET2, respectively. The CFA/I operon of ETEC, with the genes encoding the A2 and B subunits of LTh toxin (also of ETEC), were inserted into the chromosome of this live, attenuated vector. Thus, the resulting strain CVD 1208S-122 bears the O-antigen of *S. flexneri* and expresses both CFA/I and LT of ETEC.

5.1.1 Preparation of Vaccine

Vials of the study product, CVD 1208S-122, will be removed from the freezer and allowed to thaw at room temperature in a biosafety cabinet (BSC). After thawing, while within the BSC, the vial contents will be diluted with sterile Phosphate Buffered Saline (PBS) to the desired concentration. Dilutions of vaccine will be kept on wet ice, until just prior to administration. Once the vial has been thawed, the contents should be administered to subjects within 2 hours of thawing. A bicarbonate buffer solution will be prepared with each vaccine dose (see below). Subjects will be required to fast for at least 90 minutes prior to vaccination and for 90 minutes after dosing. Approximately 1 – 2 minutes prior to vaccination, subjects will drink 120 mL of the bicarbonate solution. Then, the designated dosage of vaccine will be administered orally to the subject, delivered in 30 mL of bicarbonate buffer. If a subject vomits after receiving the vaccine,

the subject will not be re-dosed, but will remain in the inpatient unit for observation as if they had received the intended dose.

To prepare the sodium bicarbonate buffer solution, combine 2 g USP-grade white crystalline powder sodium bicarbonate ($NaHCO_3$) to 150 mL sterile non-bacteriostatic water.

5.1.2 Preparation of Placebo

The placebo will have the appearance of, and be administered in a similar manner to, that of the active vaccine. The placebo will consist of corn starch USP mixed in 30 mL of sodium bicarbonate buffer, as described above. Subjects will be required to fast for at least 90 minutes prior to vaccination and for 90 minutes after dosing. The bicarbonate buffer solution will be prepared as described. Approximately 1 – 2 minutes prior to placebo vaccination, subjects will drink 120 mL of the bicarbonate solution. Then, the 30 mL volume of bicarbonate buffer with corn starch USP will be administered orally. If a subject vomits after receiving the vaccine, the subject will not be re-dosed, but will remain in the inpatient unit for observation.

5.2 Study Product Accountability

The study product will be stored in the University of Maryland Medical Center (UMMC) Investigational Drug Service (IDS) pharmacy or the CVD Clinical Microbiology Lab. The FDA requires accounting for the disposition of all investigational products. The Investigator is responsible for ensuring that an accurate record of product disposition is maintained, and product is dispensed only by authorized personnel, as required by applicable regulations and guidelines. Records of product disposition, as required by federal law, consist of the date received, date administered, quantity administered, and the subject number to whom the study product was administered. The investigational pharmacist will be responsible for maintaining accurate records of the shipment and accountability of the study product. The dispensing of the study product (thawing, dilutions, and dosing of volunteers) will be performed by CVD research staff; the process and procedures are documented and maintained at CVD. The pharmacy records (either IDS pharmacy and/or CVD) will be made available for inspection by external monitors and by the relevant regulatory agencies (e.g., FDA) at any time.

5.3 Blinding of the Study Product

A prospectively assigned vaccinator and checker will be designated as unblinded personnel and will not have any role in the management of illness or assessment of AEs in study participants. Unblinded personnel will be responsible for the preparation of the study products and for the blinded labeling of the study products. The designated vaccinator will be responsible for maintaining the randomization key and will secure these study documents in a locked cabinet.

6 STUDY PROCEDURES/EVALUATIONS

6.1 Study Procedures

Informed Consent. All aspects of the protocol will be reviewed with prospective subjects. Subjects who satisfy all inclusion/exclusion criteria will provide informed, written consent to participate in the trial. To evaluate comprehension of the study and to document that informed consent has been elicited, all subjects must pass a written examination before vaccination (minimum passing grade is 70%). Staff will review incorrect answers with the subject. A subject who scores below 70% may take the test a second time but will be excluded if she/he scores <70% again.

Medical Screening. After signing the consent form, receipt of Notification of Privacy Practices (NPP) will be verified and HIPAA authorization forms will be completed. Subjects will undergo a clinical evaluation to ensure that they are in good mental and physical health, which will include medical history, vital signs, and a brief physical examination (auscultation of heart and lungs and oral cavity, abdominal, and lymph node examinations). This screening will also include a review of concomitant medications, relevant travel history, normal stool characteristics, and other aspects needed to document eligibility.

Blood will be drawn for selected laboratory measures including a CBC with differential (WBC, ANC, hemoglobin, platelets), blood chemistries (creatinine, ALT, total bilirubin), serum IgA, serological tests for HIV, hepatitis C, hepatitis B surface antigen, and HLA-B27 typing. Serum pregnancy test in females must be negative during screening and a urine pregnancy test must be negative within 24 h prior to vaccination.

Subjects who are excluded based on screening results will be counseled by the clinical staff and referred for medical care, as appropriate. If elevated blood pressures are detected after enrollment, the investigator will make a determination as to whether continued participation would be unsafe or would interfere with the study evaluations.

Acclimatization (Cohorts 1 – 3). Study participants will begin their inpatient stay one day prior to vaccination for acclimatization, when each participant will be educated and familiarized with the protocol-required procedures (e.g., stool handling), hygiene practices, and the *Policies and Procedures* to be followed while on the inpatient unit. During the acclimatization period, person-to-person interactions, mood, and other behaviors will be monitored to assess for any conduct or attitudes which might not be appropriate for an inpatient study (i.e., combativeness, anti-social behavior, anger outbursts, destruction of property, etc.). Evidence that a subject has demonstrated behavior which might pose a safety risk to themselves, other subjects, or staff could be cause for ineligibility for vaccination and the remainder of the inpatient stay. Refusal to comply with protocol-required procedures, adherence to hygiene practices, or repetitively breaking the *Policies and Procedures* may also constitute ineligibility. Any subject who is deemed ineligible will be discharged, prior to vaccination.

During the time when a U.S. Public Health Emergency for COVID-19 exists, a SARS-CoV-2 molecular diagnostic test will be performed on each participant prior to admission to the inpatient unit. A negative test result and the absence of COVID-19 symptoms will be required for final eligibility for vaccination.

Blinded Vaccination. The vaccine and placebo will be administered by the oral route for ingestion. Two grams of $NaHCO_3$ (sodium bicarbonate) will be dissolved in 150 mL of sterile water, to make sodium bicarbonate buffer solution. 120-mL of the bicarbonate buffer solution will be aliquoted for the participant to drink, to neutralize gastric acidity, prior to drinking the vaccine. One to two minutes later, the participants will ingest 1 mL of the appropriate assigned vaccine dose, suspended in the remaining 30 mL of the buffer solution, or 1 mL of placebo suspended in the remaining 30 mL of buffer solution to which corn starch USP has been added to match the turbidity of the vaccine inoculum. Subjects will not eat, drink, or smoke for 90 minutes pre- and post-inoculation.

To maintain the blind, a member of the investigative team who will not be involved in the clinical evaluation of the subjects will be responsible for preparing and labeling the vaccine and placebo with a randomized number. An unblinded checker will be present to verify that the correct randomization scheme has been followed.

Inpatient Observation (Cohorts 1 – 3). Subsequent to vaccination, participants will remain on the ward for at least 4 days (96 h). Vital signs and oral temperature will be measured at least every 8 ± 1 h by staff nurses who remain on the ward 24 h/day. Participants will be interviewed daily by a study physician to determine the occurrence of illness signs and symptoms (e.g., anorexia, malaise, abdominal cramps, headache); these data will be recorded and graded for severity. A focused physical examination may be performed at the discretion of the physician according to the nature of a participant's complaint. All stools will be collected by research staff and there will be a requirement for at least two stool samples per day, one every morning and one every evening. If a participant is unable to produce a stool within the 12-hour period, a rectal swab will be performed.

Measurement of Diarrhea and Dysentery (Cohorts 1 – 3). Since diarrhea and/or dysentery are key measures of the safety of the vaccine, all participants will be expected to collect every stool that is passed from the time of vaccination until discharge. Participants will be instructed to use a plastic stool collection basin, commonly called a "hat." All stools will be graded for consistency by the study staff. The grading of stool consistency is as follows:

- Grade 1 – well formed (normal stool, does not take the shape of the container)
- Grade 2 – soft (normal stool, does not take the shape of the container)
- Grade 3 – thick liquid (diarrhea, readily takes the shape of the container)
- Grade 4 – opaque, watery liquid (diarrhea
- Grade 5 – rice water (clear, watery diarrhea)

Any Grade 3 or higher (looser) stool is considered a stool having diarrheal consistency and must be weighed to estimate the volume of fluid loss (assume ~1 g diarrheal stool = 1 mL of fluid

lost). Gross blood or suspicion of blood in any stools must be evaluated with a hemoccult test, for confirmation of dysentery.

Similarly, any episodes of vomiting should be collected in a provided stool "hat" or plastic "kidney" basin. If a vomiting or diarrhea episode is not able to be collected in a basin (e.g., the subject has an "accident" while sleeping or before they are able to reach a toilet), then the volume of the output will be estimated.

Management of Fluid Losses (Cohorts 1 – 3). Subjects who develop diarrheal stools (Grade 3 or higher) during the inpatient observation will be required to ingest standard World Health Organization (WHO) Oral Rehydration Solution (ORS) at 1.5 times the stool volume. Vomitus will be replaced with ORS in equal amounts, 1:1 ratio. At the discretion of the investigator, additional ORS may be administered. If a subject develops severe watery diarrhea or persistent vomiting and cannot maintain full hydration by oral means, IV fluid replacement will be administered.

For the duration of diarrhea, the subject will be requested to provide a urine specimen from every void that they experience; the specimen will be tested for specific gravity. In the event that IV fluids are required, serum electrolytes (Sodium, Potassium, Chloride, Bicarbonate), BUN, and creatinine will be measured.

A physician investigator shall always be available by telephone or beeper. Nurses will notify the on-call physician if any of the following occurs in a subject who is experiencing diarrhea and/or vomiting:

- Syncope
- Complaint of dizziness or light-headedness or established orthostatic hypotension
- Urine specific gravity >1.025
- >500 mL behind in ORS replacement
- Vomiting ≥500 mL once or total volume within the past 4 hours
- High fever ≥39°C (≥102.2°F)
- Severe headache, severe malaise, or severe abdominal pain
- Subject has a complaint for which he/she requests treatment
- Any other clinical situation that concerns the nurse

Frequency of Vital Signs Assessment (Cohorts 1 – 3). Vital signs (blood pressure, pulse, and oral temperature) will be measured approximately every 8 h, unless more frequent monitoring is needed. Once a subject has passed a diarrheal stool (Grade 3 or higher), vital signs will be measured every 4 h, until either the subject passes a Grade 1 or 2 stool, or 24 h have passed since the last grade 3 – 5 stools, whichever comes first. Vital signs will also be measured every 4 h when a subject has a fever ≥39°C (≥102.2°F), until the subject has 2 consecutive temperatures of ≤38°C (100.4°F). Any subject that complains of dizziness or light-headedness upon standing will have orthostatic blood pressures assessed. Orthostatic hypotension is defined as a drop in systolic BP >20 mmHg or in diastolic BP >10 mmHg.

Fluid Therapy/Hydration (Cohorts 1 – 3). ORS (Jianas Bros ORS) will be offered as the primary means of hydration and will be prepared according to the manufacturer's package insert. Unused ORS should be discarded 24 h after preparation.

Intravenous fluids (Lactated Ringers solution) will be administered to subjects with diarrhea who meet any of the following criteria:

- Orthostatic hypotension
- Urine specific gravity >1.025, or determined as necessary by the Investigator
- No urine output for ≥8 h
- >1000 mL deficit in ORS replacement
- At the investigator's discretion, based on clinical evaluation or on a subject's difficulty in keeping up with ongoing diarrheal losses by oral rehydration alone

Intravenous therapy will continue until the above criteria are no longer satisfied, the subject is able to take fluid by mouth, and a study physician determines that it is no longer required.

Indications for Antibiotics. Antimicrobials will be administered immediately if a subject meets the criteria for moderate diarrhea or dysentery (defined in ***Appendix D***). Antimicrobials will consist of a 3-day course of ciprofloxacin (primary) or trimethoprim-sulfamethoxazole (secondary). In rare and unexpected instances, the study investigator will be allowed to administer antimicrobials for alternate reasons—e.g., if in his/her judgment it is necessary to ensure the safety of the subject. Otherwise, antibiotic therapy will be initiated for any individual that demonstrates continued shedding of vaccine organisms on the Day 8 visit (one-week post-vaccination), to be started as soon as the participant is able to return back to clinic for this unscheduled visit.

Indications for Other Concomitant Medications. Any concomitant prescription or over-the-counter medications will be evaluated for continuation during the inpatient setting. Any such medication will need to be discussed with and approved by the Investigator prior to admission. The supply of medication will be the responsibility of the subject and will be handed over to the research staff upon admission; daily administration will be recorded. Any prescription or over-the-counter medications that were not declared and are discovered during the inpatient stay will constitute a violation of the ward *Policies and Procedures*.

Other medications may be administered during the study period as follows:

- Smoking is not allowed on the ward, but subjects will be able to request a nicotine patch.
- Antipyretics and analgesics (i.e., ibuprofen, acetaminophen, aspirin, or similar non-steroidal agents) may be prescribed for severe headaches, other pains, or fevers (e.g., sustained temperatures of ≥39°C).
- At the investigator's discretion (e.g., upon review of serum electrolyte results during severe diarrhea), oral potassium may be administered for repletion of electrolyte losses.
- Other medications, which are deemed necessary for the safety and welfare of the subject

Any medications prescribed must be ordered and signed by the Investigator and each administration must be recorded.

Stool Microbiology. For Cohorts 1 – 3, stool cultures will be performed to characterize the pattern of fecal shedding, to determine whether the vaccine is transmitted to placebo recipients living in close proximity on the ward, to assess the genetic stability of the excreted live vector vaccine organisms, and to document the duration of vaccine excretion. All stools will be collected, graded, and weighed while participants are on the Research Isolation Ward; quantitative cultures will be performed on the first stool sample of each 12-h period; qualitative cultures will be performed on all additional stool samples. Qualitative cultures will be performed on rectal swabs. A stool specimen or rectal swab will also be collected on the Day 8 clinic visit.

For Cohort 4, a stool specimen will be collected for qualitative culture on the day of (prior to) vaccination (Days 1 and 29), 2 days after vaccination (Days 3 and 32), and 7 days after vaccination (Days 8 and 36). In addition, quantitative stool culture will be performed on the stools collected on Days 3 and 32.

Shed *Shigella* organisms will be genetically characterized to ensure that the vaccine organism remains stable.

Memory Aids. During the 7 days after each dose of vaccination, the documentation of reactogenicity will be completed. For Cohorts 1 – 3, the PI (or designee) will complete the reactogenicity case report form for those days the participant is on the inpatient unit; this is typically performed during the daily physician rounds. For Cohorts 1 – 3, upon discharge and until the Day 8 clinic visit, the participant will complete a paper memory aid, to document reactogenicity. For Cohort 4, The participant will complete a paper memory aid for the 7-day post-vaccination period. On the Day 8 clinic visit (for Cohort 4, both the Day 8 and Day 36 clinic visits), the memory aid document will be reviewed by study staff and the reactogenicity case report form will be completed by the study staff, based on the information on the memory aid and a discussion with the participant regarding the responses on the memory aid.

6.2 Laboratory Evaluations

6.2.1 Laboratory Evaluations/Assays

Screening Laboratory. The following clinical laboratory assessments will be performed as part of the screening for eligibility (***Appendix B***):

- CBC with differential for WBC, ANC, Hg, platelet count
- Creatinine, ALT, total bilirubin
- HBsAg, HCV, HIV
- Serum β-HCG (if woman of child-bearing potential)
- Serum IgA level
- HLA-B27 histocompatibility testing

During the time when a U.S. Public Health Emergency for COVID-19 exists, a SARS-CoV-2 molecular diagnostic test will be performed for Cohort 1-3 participants.

Safety Laboratory. The following clinical laboratory assessments will be performed at baseline (Day 1, prior to vaccination) and at the Day 8, 29, and 36 visits (see ***Appendix C*** for Toxicity):

- CBC with differential for WBC, ANC, Hg, platelet count
- Creatinine, ALT, total bilirubin
- Sodium, potassium, aspartate aminotransferase (AST), and alkaline phosphatase may be added clinical laboratory measures if indicated by the Investigator's judgement, in order to further investigate reactogenicity or adverse effects

The following tubes are be used for the evaluation of clinical laboratories:

- 4 mL EDTA for CBC with differential
- 8.5 mL tiger top for chemistries, serum IgA, HBsAg, HCV, and β-HCG
- 4 mL EDTA for HLA-B27 test (Monday - Thursday shipments only)
- 3.5 mL tiger top for HIV test

6.2.2 Special Assays or Procedures

Serum ELISA. Serum antibody responses will be measured against the *Shigella* live vector (anti-*S. flexneri* 2a LPS and T3SS component IpaB) and to each ETEC antigen (CFA/I and LTB). Standardized ELISAs for immunoglobulin G (IgG) and IgA will be used to quantify antibody responses to all antigens.[24,25] ELISA titers will be calculated as the inverse serum dilution that produces an absorbance value (450 nm) of 0.2 above background.

Shigella Serum Bactericidal Antibody (SBA) and Opsonophagocytic Killing Antibody (OPKA). The functional activity of immune sera will be analyzed using the serum bactericidal antibody (SBA) assay and opsonophagocytic killing antibody (OPKA) assay.[28] For the SBA assay, heat-inactivated serum are serially diluted 2-fold with *S. flexneri* 2a strain 2457T and baby rabbit complement (BRC). For the OPKA assay, heat-inactivated serum samples are serially diluted 2-fold with *S. flexneri* 2a strain 2457T, BRC, and dimethylformamide-differentiated HL-60 cells (ATCC CCL-240). After incubation for 1 h (for SBA) or 45 min (for OPKA) at 37°C, viable colony counts are determined by plating 10 µL of reaction mixture on Trypticase Soy Agar (TSA) plates. Negative-control wells containing only bacteria (no serum), bacteria plus BRC, bacteria plus HL-60 cells (for OPKA) and a positive-control serum with known high bactericidal activity will be included in each assay. SBA and OPKA titers are calculated from the reciprocal of the serum dilution that produces 50% bacterial killing.

ETEC Mannose-resistant Hemagglutination Assay of Human Group A Erythrocytes. The functional ability of immune serum to inhibit hemagglutination of human type A red blood cells (RBC) by wild-type ETEC strain H10407 will be tested.[37,38] Serial dilutions of immune serum (100 µL) are mixed with a standardized suspension of ETEC strain H10407 and incubated at 37°C for 1 h. The bacteria-serum mixtures are then added to U-bottom 96-well plates. Equal volumes of 0.1 M D-mannose solution and standardized human type

A RBC solution are added to each well. The plates are incubated for 2 h at 4°C. Hemagglutination inhibition titer is calculated from the highest dilution which results in the loss of the pelleting of RBC at the bottom of the well. Each serum sample is tested in duplicate.

Serum Inhibition of ETEC Adhesion to HT-29 cells. The immune serum will be tested for the ability to inhibit wild-type ETEC H10407 binding to HT-29 cells.[39] Bacteria are grown overnight in 2 mL of LB, diluted 1:100 into fresh medium on the morning of the experiment, and grown for an additional 90 min to mid-logarithmic growth phase. Serial dilutions of immune serum (100 µL) are mixed with a standardized suspension of ETEC strain H10407 and incubated at 37°C for 1 h. The bacteria-serum mixtures are then added to triplicate wells of a 48-well tissue culture plate containing confluent monolayers of HT-29 cells. After 1 h, the monolayers are washed three times with tissue culture medium and then treated with 0.1% Triton X-100 in PBS for 5 min. Triton X-100 lysates containing total cell-associated bacteria are then diluted 1:10 in PBS and plated onto agar plates to enumerate attached bacteria. Inhibition of bacterial adherence (IC_{50}) is defined as the highest dilution of immune serum needed to inhibit 50% recovery of organisms, compared to an irrelevant control antibody.

LT Neutralizing Antibody Assays. Serum will be used to determine toxin neutralization using an *in vitro* neutralization assay employing Y-1 adrenal cells as substrate, following neutral red uptake.[40] Titers (ED_{50}s) will be calculated as the reciprocal of the serum dilution which resulted in a 50% reduction in toxin activity.

Alternatively, LT neutralization can be measured by inhibition of intracellular cAMP accumulation in CaCo2 or T84 cells.

Antibody-Secreting Cells (ASC). Plasmablasts recognizing *S. flexneri* LPS and IpaB and ETEC CFA/I and LT will be measured using ELISPOT. In brief, purified peripheral blood mononuclear cells (PBMCs) resuspended in complete medium (2×10^5 cells/well in quadruplicate) will be incubated in flat-bottom, 96-well nitrocellulose plates (MAHA S4510, Millipore) previously coated with target antigens, overnight at 37°C, 5% CO_2. Optimal coating concentrations have been determined in preliminary experiments. Antibodies produced by antigen-specific plasmablasts will be detected by horse radish peroxidase-labeled anti-human IgG and IgA antibodies (HRP, Jackson Immunoresearch, West Grove, PA), followed by 3-amino-9-ethylcarbazole substrate (AEC, Calbiochem). The frequency of spot-forming cells (sfc) from 4 wells will be determined and reported as ASC/10^6 PBMC. A positive response will be defined as ≥8 ASC/10^6 PBMC.

Antibodies in Lymphocyte Supernatants (ALS). To measure the antibodies in lymphocyte supernatants (ALS), isolated PBMCs at 1×10^7 viable lymphocytes per mL, will be re-suspended and incubated at 37°C, 5% CO_2 for 72 h without antigenic stimulation. Supernatant fluid will be collected and tested in an ELISA assay to measure antigen-specific IgA and IgG antibodies released by the PBMCs. Briefly, ALS samples will be added to flat-bottom 96-well ELISA plates that have been previously coated with *S.*

flexneri LPS and IpaB and ETEC CFA/I and LT. Antigen-specific antibodies will be revealed by adding goat anti-human IgA conjugated to HRP, followed by AEC substrate. Endpoint titers will be calculated as the reciprocal dilution giving rise to an absorbance value of 0.4 above the background at 450 nm. A positive response will be defined as a ≥4-fold increase in ALS titer over baseline.

ASC Homing Studies (only for Cohort 4). To measure the homing potential of these plasmablasts, PBMC will enumerated by flow cytometry based on their expression of molecules that are expressed in naïve (B_n) and memory B (B_M) cells (e.g., CD19, CD27) and that direct these cells to home to distinct sites (e.g., integrin α4β7, CD62L).[41] Several of these populations (e.g., B_n [$CD19^+$ $CD27^-$], B_M [$CD19^+$ $CD27^+$] expressing integrin α4β7, but not CD62L, etc.) will be sorted and their ability to secrete specific anti-*S. flexneri* LPS and IpaB and ETEC CFA/I and LT antibodies will be determined by ELISPOT as described above. Sorting will be performed using a MoFlow Astrios EQ flow cytometer/cell sorter state-of-the-art system available in the CVD Flow Cytometry Core Laboratory.

B Memory Cells (B_M). B_M cells are responsible for mounting rapid anamnestic antibody responses (recall responses) upon re-exposure to microbial antigens and thus are considered an indicator of long-term protection induced by vaccines or natural infection. BM responses will be measured using a dual color ELISPOT assay method, allowing for the simultaneous measurement of IgA and IgG sfc.[42-45] Briefly, PBMC will be seeded in duplicate wells of plates coated with Shigella or ETEC antigens or total IgG or IgA. Plates will then be incubated with anti-human IgG-alkaline phosphatase and IgA-horseradish peroxidase (Jackson ImmunoResearch). IgA sfc are visualized with nitro-blue tetrazolium chloride (NBT) substrate (Vector laboratories) as blue spots, washed, and incubated with AEC substrate to visualize IgG sfc as red spots. Spots (sfc) will be enumerated using an automated ELISPOT reader (Immunospot 3B, Cellular Technologies Ltd) with aid of the Immunospot software version 5.0 (Cellular Technologies Ltd). Total and antigen-specific B_M SFC are calculated as sfc/10^6 cells. Isotype-specific frequencies of B_M against specific antigens are expressed as the percentages of specific B_M per total IgA or IgG (specific B_M sfc per 10^6/total Ig sfc per $10^6 \times 100$).

Qualitative/Quantitative Stool Culture. For each stool culture specimen, a sterile cotton swab will be inserted into the stool (sampling nearest the most loose or liquid parts of stool or areas with gross blood) and then placed into a tube containing buffered glycerol saline (BGS) transport medium; short-term storage at 4°C (2°C-8°C). If a rectal swab is obtained, then the swab will be placed into a tube containing Gram-negative enrichment broth (GN, Fisher) with short-term storage at 4°C (2°C-8°C). For all stool specimens collected during the inpatient period and one specimen per outpatient day that requires stool culture, the CVD Microbiology Lab will plate the stool specimen onto *Salmonella-Shigella* agar (SS, Fisher) and MacConkey agar (MAC, Fisher) plates and GN broth for incubation overnight at 37°C. Hemoccult testing is to be repeated from stools that appear to contain gross blood; this procedure will have already been performed by the clinical research team on the inpatient unit and is a confirmation test. After overnight incubation, the GN broth tubes

will be sub-cultured onto SS agar and incubated overnight at 37°C. After incubation, SS and MAC plates are examined for single pure lactose-negative colonies (colorless and translucent) and are picked onto Triple Sugar Iron (TSI, Remel) agar slants and Motility Indole Ornithine medium (MIO, BD-Difco) for overnight incubation at 37°C. Those colonies that are non-motile and produce typical TSI reactions for *Shigella* (alkaline slant over acid but without gas) may be identified by commercial API 20E and must be confirmed by agglutination. Positive agglutination of a *Shigella* colony with one or more specific antisera (Denka Seiken available from Hardy Diagnostics as MastAssure) will identify a *S. flexneri* 2a strain.

To quantify the level of excretion of the strain (quantitative counts), 10-fold dilutions will be made from ~1 g of each stool; from each dilution, 100 µL will be spread on MAC agar and incubated overnight at 37°C. From the dilution plates, lactose negative colonies are counted; plates containing 30 – 300 colonies yield the most accurate counts. Up to 10 suspicious colonies will be agglutinated with antisera to calculate the quantitative counts of the *Shigella* strain. The quantitative count is calculated by multiplying the number of suspicious colonies by the ratio of confirmed positive isolates out of the 10 isolates tested.

Genetic Stability of Vaccine Organisms. In selected recovered isolates, the confirmation of the presence of the genes encoding both CFA/I and LThA2B will be performed in the laboratory of Dr. Eileen Barry. Colonies identified as *Shigella* will be tested by PCR for the presence of the genes encoding CFA/I and LTB. Briefly, 10 – 20 *Shigella*-positive colonies from each volunteer at each time point post-vaccination will be tested. Direct colony PCR or extracted DNA will be used as the template for PCR using primer pairs specific for *Shigella* (targeting the *ipaH* or *virG* genes), CFA/I (targeting *cfaB* gene) or LTB (targeting *eltB* gene). These data are expected to confirm the proportion of *Shigella* isolates shed that maintain ETEC antigen-encoding genes over time in the human host. A subset of colonies from different volunteers will be selected for expansion, genomic DNA isolation, and sequencing of the genomic region encoding the CFA/I and LTB antigens. These studies will confirm the chromosomal integrity and location of transgene insertion, which will be an exploratory endpoint.

An additional subset of colonies from multiple volunteers will be expanded to confirm maintenance of *Shigella* and ETEC antigen-specific phenotypes. Isolates will be tested for expression of CFA/I by western blot and positive hemagglutination of human type A RBC. LTB expression will be confirmed by western blot analysis of periplasmic fractions. The invasion capacity of *Shigella* in intestinal HT-29 cells will be quantified and lack of intracellular replication confirmed.

Fecal IgA. Fecal supernatants will be tested for total and antigen-specific (*Shigella* LPS, IpA, CFA/I, and LT) IgA ELISA as previously described.[46] Total and antigen-specific IgA concentrations are calculated by interpolation of the regression-corrected absorbance values produced by serially diluted stool samples and extrapolation from a standard curve of known concentrations of human IgA (Calbiochem). In order to adjust for individual variations in the IgA content of stools, the ratio of specific IgA to total IgA antibody are

calculated, and a positive response is defined as a ≥4-fold increase of the ratio over the baseline ratio. The threshold of detection for each assay will be determined for each antigen and any baseline values below this level of detection will be designated the value of that limit of detection for the calculation of fold increases post-vaccination.

6.2.3 Specimen Collection, Preparation, Handling and Shipping

Clinical laboratory specimens will be shipped to the contract CLIA-approved clinical laboratory for analysis. Research study specimens are intended to be stored for potential future-use research at CVD's long-term storage facility. Instructions for research specimen preparation, handling, and storage are described in the ***Manual of Procedures*** (***MOP***). At this time, there is no planned shipment of research study specimens.

7 STUDY SCHEDULE

Complete study schedule details listed by type of visit are described below. Refer also to ***Appendix A: Study Schedule of Events***.

7.1 Screening

Potential participants may be screened for eligibility up to 60 days prior to enrollment. Interested potential participants, responding to advertisements, campus flyers, or notification through existing databases, will be instructed to call CVD to receive further information about the study. CVD research staff will receive these inquiries and read through an IRB-approved telephone script which describes basic information on the study topic, duration and number of visits, procedures, and eligibility criteria. No personal health information is solicited and afterwards interested potential participants may be given an appointment to attend an Orientation Session for formal screening.

At the Orientation Session, the consent process continues. CVD research staff will provide a detailed description of all aspects of the study, including the rationale and background, the public health significance, the procedures and schedule of visits, and a detailed discussion of the risks and the presence or absence of benefit to them, as appropriate. This Orientation Session is commonly one hour long. The prospective subject is given a copy the study consent form (without the signature page) and is encouraged to discuss his/her participation with family members or advisors before agreeing to sign the informed consent form and proceeding with any screening procedures.

After the informed consent form is signed, the subject is interviewed one-on-one by a member of the study team to discuss the study. A brief written examination is administered to assess the volunteer's comprehension of the study (i.e., Comprehension Assessment Tool). If this quiz is passed (≥70% correct answers), the research staff complete the medical history and concomitant medication forms and draw blood for eligibility testing. Study subjects are also asked to sign a verification of Notification of Privacy Practices receipt and Health Insurance Portability and Accountability Act (HIPAA) authorization form. The subject also meets the PI, or designee, and

has a physical examination to complete the eligibility screening. If the subject has passed all the eligibility criteria, he/she is then invited to proceed in the study and is given an appointment for the next visit. The screening procedures may be completed in a single visit, but it is acceptable to complete all the screening procedures on multiple visits, as long as the data of initiation of screening does not exceed 60 days prior to vaccination.

It is recognized that volunteer studies must be carried out in an environment where no coercion is applied, and where volunteers can be adequately informed of the purpose, nature, procedures, risks and hazards of the study. To assess and document comprehension of the material presented, each subject must pass a written quiz that covers aspects of the study including the purpose, procedures, risks, benefits and pertinent microbiology with a score 70% or higher. Incorrect answers will be reviewed with the subject. The quiz may be retaken once, after a review of the consent form. The Comprehension Assessment Tool is dated and signed by the subject and by a research staff member and made part of the permanent record.

Another important feature of our consent process is the repeated demonstration of both initiative and reliability by the prospective subject. There are multiple opportunities for the subject to decline to proceed further in the process. This deliberate education and screening process contributes to the informed nature of the subject's consent. The process also increases the likelihood that the subject will be committed to completing the entire inpatient containment portion of this study. Prospective subjects will be carefully screened to ensure that they are in good physical and mental health.

The screening criteria also are intended to rule out those with histories of major gastrointestinal surgery, functional gut problems, and persons with IgA deficiencies.

The screening procedures include:

- Signed informed consent
- Administration of the study Comprehension Assessment Tool
- Obtaining vital signs (oral temperature, blood pressure, pulse, height, and weight)
- Collection of medical history
- Collection of concomitant medication history
- Collection of a detailed travel history, including any episodes of diarrhea
- Perform physical examination, to be performed by a study clinician
- Obtain the following screening studies (~16 mL blood); the acceptable values for the screening tests are defined in ***Appendix B***:
    - Complete Blood Count (CBC) with differential and platelet count for the evaluation of WBC, ANC, Hemoglobin, and Platelets
    - Creatinine, ALT, and Total Bilirubin
    - Serum IgA
    - HLA-B27 histocompatibility
    - Pregnancy test (if female; serum β-HCG)
    - HIV antibody
    - Hepatitis B surface antigen
    - Hepatitis C antibody

Note: Since intravenous fluids are a part of the planned management of severe diarrhea, if more than 3 attempts at venipuncture are required for obtaining screening labs, then we will consider that person ineligible on the basis of poor venous access.

7.2 For Cohorts 1 – 3, Inpatient Containment Period

Study participants that are determined to be eligible will be scheduled for admission to the inpatient containment unit (research isolation ward). The anticipated duration of the inpatient stay is 6 days.

7.2.1 SARS-CoV-2 PCR test (within 72 hours before admission to the inpatient unit)

During the time when a U.S. Public Health Emergency for COVID-19 exists, study participants will be instructed to come to the research clinic to perform a SARS-CoV-2 PCR test. This is a procedure that is consistent with current hospital policy (UMMC PolicyStat 11947187). It is anticipated that should the testing policy change, the protocol would be revised to be consistent with the hospital policy. A nasal swab is to be performed for the PCR test; no additional procedures are planned to be performed at this visit. This test result must be negative for eligibility to enter the inpatient unit.

7.2.2 Acclimatization (Day -1, one-day prior to vaccination,)

Study participants will start their inpatient stay with 1 day for acclimatization, during which we educate and familiarize each subject with the protocol-required procedures (e.g., stool handling), hygiene practices, and the “Rules and Procedures” to be followed while on the inpatient unit. In addition, during the acclimatization period, we monitor behavior, person-to-person interactions, mood, etc. to assess each study participant for any behavior or attitudes which might not be appropriate for an inpatient containment study (i.e., combativeness, anti-social behavior, anger outbursts, destruction of property, etc.). Any evidence that a subject may demonstrate behavior which might pose a safety risk to themselves, other subjects, or staff could be cause for ineligibility for vaccination and the remainder of the inpatient stay. Refusal to comply with protocol-required procedures, adherence to hygiene practices, or repetitive breaking of the “Rules and Procedures” could also constitute ineligibility. This observation during the acclimatization period may be considered an imperfect and rather subjective method, but we have not identified any other good alternate objective measures that substitute for this direct observation procedure. Any subject who is deemed ineligible will be discharged, prior to vaccination, and eligible back-up participants will be used. All back-up study participants that are not vaccinated will be discharged from the inpatient ward upon the completion of the target number of vaccinations.

7.2.3 Enrollment/Vaccination Day (Day 1)

On the morning of vaccination, the enrollment and randomization process will be completed prior to oral ingestion of the blinded study product and a final eligibility confirmation will be completed. Additionally, the following lab tests will be completed prior to vaccination:

- Baseline vitals (oral temperature, pulse, and blood pressure) will be recorded
- Baseline Clinical Safety Laboratory tests (a 12.5 mL blood sample, collected prior to vaccination) which will include:
    - CBC with differential and platelet count for the evaluation of WBC, ANC, Hemoglobin, and Platelets
    - Creatinine, ALT, and total bilirubin
- Baseline Research Blood tests (approximately 125 mL blood volume will be obtained)

After fasting for a minimum of 90 minutes, participants will drink 120 mL of sodium bicarbonate solution (~1.3% $NaHCO_3$); approximately 1 – 2 minutes later, subjects will ingest the blinded study product. Subjects will have nothing by mouth, except water, for 90 minutes before and after ingestion of the blinded study product.

7.2.4 Post-Vaccination Observation Period (Day 1 through discharge)

For the 96 hours (4 days) following vaccination, study participants will be observed on the Research Isolation Ward and the following procedures will be performed:

- Vital signs will be measured at least 3 times daily (approximately every 8 h)
- All stools will be graded for consistency (Grade 1 – 5) and any diarrheal stool (grade 3 or higher) will be weighed; a 1:1 weight per volume ratio will be assumed for conversion of diarrheal stools
- All vomitus will be weighed
- For each day post-vaccination, the maximum temperature, total diarrheal stool volume, number of diarrheal stools, total vomitus volume, and number of vomiting episodes will be calculated, as part of the reactogenicity assessment.
- For each day, the maximum of an adverse symptom over that day will be recorded. The documentation of the previous day's maximum adverse symptom (prior 24 h) will include an attribution—either to the study drug or an alternate etiology (unrelated to study). The anticipated subjective adverse symptoms include abdominal discomfort (pain/cramping), nausea, myalgia/arthralgia, anorexia, malaise, and headache. These adverse symptoms will be graded according to the scales shown in ***Appendix D***.

7.2.5 Discharge (~Day 5)

Participant must feel in good (baseline) health and have no fever or diarrhea for at least 12 h prior to discharge. Should there be excessive reactogenicity (e.g., severe diarrhea or

ongoing diarrhea within 12 h of discharge), then the participant will be initiated on a 3-day course of antibiotic therapy. Subjects may be discharged during their 3-day course of antibiotics as long as they are symptom-free for at least 12 h, unless the investigator deems discharge not to be in the best interest of the participant's welfare.

Prior to discharge, participants will be provided a digital thermometer for the assessment of daily oral temperatures; participants will be encouraged to take their temperature at the same time each day. Participants will also be provided a Memory Aid document and will be instructed on how to record the daily temperature and adverse events onto the document.

7.3 For Cohort 4, Vaccination Visits for Dose #1 and Dose #2 (Day 1 and Day 29 ±3)

On the morning of vaccination, subjects will report to the clinic and baseline vitals will be recorded (oral temperature, pulse, and blood pressure) and a final eligibility confirmation will be completed prior to oral ingestion of the first dose of blinded study product. The enrollment and randomization process will be completed, prior to vaccination.

Baseline Clinical Safety Laboratory tests (12.5 mL) will be obtained prior to vaccination to include:

- CBC with differential and platelet count for the evaluation of WBC, ANC, Hemoglobin, and Platelets
- Creatinine, ALT, and total bilirubin

Baseline Research Blood tests (approximately 125 mL) will be obtained prior to vaccination.

Baseline Stool will be collected prior to vaccination. A stool collection kit and instructions will have been provided prior to the scheduled visit.

After confirmation of at least 90 minutes of fasting, eligible subjects will drink 120 mL of sodium bicarbonate solution (~1.3% $NaHCO_3$); approximately 1 – 2 minutes later, subjects will ingest the blinded study product. Subjects will have nothing by mouth, except water, for 90 minutes before and after ingestion of the blinded study product.

Prior to the completion of the first vaccination day visit, participants will be provided a digital thermometer for the assessment of daily oral temperatures; participants will be encouraged to take their temperature at the same time each day. Prior to the completion of both vaccination day visits, participants will be provided a Memory Aid document and will be instructed on how to record the daily temperature and adverse events onto the document.

7.4 Outpatient Clinic Visits

7.4.1 For Cohorts 1 – 3, Day 8 (+ 1), Day 29 (± 3), Day 43 (± 3), Day 181 (± 7)

Participants will be scheduled for outpatient visits at approximately 1 week, 4 weeks, 6 weeks and 6 months after vaccination. On visit Day 8, the Memory Aid document will be

collected and the information with be reviewed with the participant. During the visits on Day 8 and Day 29, the emergence of adverse events and any changes to medical history and concomitant medications will be assessed. The occurrence of any serious adverse events will be assessed through Day 181. Blood will be drawn for safety labs (12.5 mL) on Day 8 and for research (approximately 37.5 – 100 mL) on Days 8, 29, 43, and 181 (target volumes according to Appendix A). Stool specimens are to be collected on each scheduled outpatient clinic visit. A stool collection kit and instructions will have been provided prior to the scheduled visit.

7.4.2 For Cohort 4, Day 3 (+ 1), Day 8 (+ 1), Day 31 (+ 1), Day 36 (+ 1), Day 57 (± 3), Day 210 (± 7)

Outpatient follow-up visits will be scheduled 2 days and one-week post-vaccination for each dose. (The timing of Days 31 and 36, and the allowable windows associated with those visits, are intended to be relative to the timing of dose #2.) Additional scheduled clinic visits are to be completed at 4 weeks and 6 months after second dose of vaccination. On visits for Day 8 and Day 36, the Memory Aid document will be collected, and the information will be reviewed with the participant.
During all visits through Day 57 (or 28 days following the last dose of vaccine), the emergence of adverse events (AEs) and any changes to medical history and concomitant medications will be assessed. The occurrence of any serious adverse events (SAEs) will be assessed through Day 181. Blood will be drawn for safety labs (12.5 mL) on Days 8 and 36 and for research (approximately 50 – 100 mL) on Days 8, 36, 57, and 210 (target volumes according to Appendix A). Stool specimens are to be collected on each scheduled outpatient clinic visit. A stool collection kit and instructions will have been provided prior to the scheduled visit.

7.5 Unscheduled Visit(s) or Early Termination Visits, if applicable

Subjects who experience any serious or severe adverse effects or who experience an event of concern can be scheduled for an outpatient visit for further evaluation. If an unscheduled visit occurs, a member of the clinical study team (PI, sub-investigator, nurse coordinator, or clinical nurse) will interview and evaluate the subject to determine the cause of the visit and provide care as needed.

Symptomatic participants who demonstrate shedding of vaccine organisms on the Day 8 visit will be contacted for the initiation of antibiotic therapy and the collection of a stool specimen; this unscheduled visit will be arranged for the earliest convenient time for that participant. Asymptomatic participants who demonstrate shedding of vaccine organisms on the Day 8 visit will not be initiated on antibiotic therapy but will have an unscheduled visit arranged for the earliest convenient time for that participant for the collection of a stool specimen. Additional outpatient clinic visits, scheduled every other day, will be continued for the collection of stool specimens until there are two negative stool culture days in a row.

7.6 COVID-19 related considerations

The investigators will monitor the situation related to the COVID-19 pandemic to ensure that potential risks to study participants and staff are mitigated. The following strategies will be implemented:

- The conduct of the study will be in accordance with state and local travel limitations/restrictions.
- Study staff will take appropriate precautions to protect study participants
- Safety assessments may be performed by phone call, when appropriate
- If travel restrictions or COVID-19 related illness impacts the conduct of the study, specific measures will be taken to mitigate risk to study staff and participants and monitor protocol deviations due to COVID-19 illness and/or COVID-19 control measures.
- The investigators will attempt to comply with the FDA Guidance on Conduct of Clinical Trials of Medical Products during COVID-19 Public Health Emergency. https://www.fda.gov/media/136238/download
- compliance with hospital (UMMC) policies, when applicable

8 SAFETY ASSESSMENT AND REPORTING

8.1 Definition of Adverse Event (AE)

An AE is any untoward medical occurrence in a participant after administration of the blinded study product regardless of its causal relationship to the study treatment. An AE can therefore be any unfavorable and unintended sign (including abnormal laboratory findings), symptoms, physical examinations, or disease temporally associated with the use of the study product, whether or not related to the study product. This definition includes exacerbations of pre-existing conditions. Stable, pre-existing conditions which do not change in nature or severity during the study are not considered AEs; however, these should be reported as part of the medical history.

Solicited AEs are pre-specified AEs that could potentially be in association with the blinded study product. Typically, a solicited AE is assumed to be associated with the study product, but investigators may assign causality to an alternate etiology, if clearly present.

- The solicited AEs are diarrhea, dysentery*, fever, nausea, vomiting, abdominal discomfort, tenesmus (a feeling on inadequate defecation), myalgia (muscle aches), arthralgia (joint aches), and anorexia (decreased appetite) (see ***Appendix D***).

  *Dysentery can only be reliably assessed during the inpatient stay (Cohorts 1 – 3), whereupon every stool will be evaluated with hemoccult testing.

Unsolicited AEs are any AEs reported spontaneously by the participant, observed by the study personnel during study visits or those identified during review of medical records or source documents. Investigators may attempt to assign causality of unsolicited AEs to either the study product or an alternate etiology.

Adverse events of special interest (AESI) which may occur will be recorded and given special attention for review of ascertainment of relationship with vaccination. Some example AESIs include any occurrence of reactive arthritis, uveitis, urethritis, bacteremia, and new diagnoses of auto-immune or immune-mediated conditions post-vaccination.

8.1.1 Grading of Severity of an AE

All AEs will be assessed by the clinician using a protocol-defined grading system (***Appendix D***). For events not included in the protocol-defined grading system or in the DAIDS toxicity table or the FDA toxicity table, the following guidelines will be used to quantify severity:

- Mild: events which require minimal or no treatment and do not interfere with the participant's daily activities.
- Moderate: events which result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning.
- Severe: events which interrupt a participant's usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually incapacitating.
- Life-threatening: any adverse drug experience that, in the view of the investigator, places the participant at immediate risk of death as a result of the reaction as it occurred; this does not include a reaction that, had it occurred in a more severe form, might have caused death.

Changes in the severity of an AE should be documented to allow an assessment of the duration of the event at each level of intensity to be performed. AEs characterized as intermittent require documentation of onset and duration of each episode.

8.2 Relationship to Study Product

Relationship (causality or attribution) of all AEs to the study product or to an alternate etiology (unrelated to the study) is part of the documentation process, but it is not a factor in determining what is (or is not) reported in the study. If there is any doubt as to whether a clinical observation is an AE, the event should be reported. All AEs must have their relationship assessed using one of two terms: "related to study drug" or "not related." To help assess whether an AE is related to a study product or not, the following guidelines are to be used:

- Related to study product – There is a reasonable possibility that the study drug caused the adverse event (perhaps due to the timing of onset of the symptoms). "Reasonable possibility" means that there is evidence to suggest a causal relationship between the study product and the AE and no reasonable alternate etiology can be determined.
- Not Related – The event is not related to the study product because of a reasonable alternate etiology. That is, an alternate etiology can be identified and is likely and/or feasible.

8.3 Definition of Serious Adverse Event (SAE)

An SAE is any AE that results in any of the following outcomes:

1. Death
2. A life-threatening event. Life-threatening events mean that the study participant was, in the opinion of the site PI or Sponsor, at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.
3. Requires inpatient hospitalization or prolongation of existing hospitalization
4. Results in persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
5. Congenital abnormality or birth defect
6. An important medical event that may not result in one of the above outcomes but may jeopardize the health of the study participant and/or requires medical or surgical intervention to prevent one of the outcomes listed in the above definition of SAE. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

8.4 Reporting Procedures

The assessment of the safety of the blinded study product will be through the detection and documentation of AEs, both solicited AEs (reactogenicity) and unsolicited AEs, and/or clinically significant laboratory abnormalities, through 28 days after the last dose of vaccination. The occurrence of SAEs will be reported for the interval between the last day of vaccination and 180 days after (Days 29-181 for Cohorts 1 – 3; Days 57-210 for Cohort 4).

8.4.1 SAE Detection and Reporting

All SAEs will be:

- Recorded in the research record
- Reported to the local IRB, per local IRB guidelines
- Reviewed and evaluated by a study clinician and the PI
- Followed through resolution by a study clinician

All deaths and immediately life-threatening events, whether related or unrelated, will be reported to the study sponsor within 24 h of site awareness. Other SAEs regardless of relationship, will be reported to the study sponsor within 24 h of site awareness

All SAEs will also be reported to DMID within 24 hours of awareness. The DMID Phamacovigilance Group (PVG) will forward the SAE report to the designated DMID Medical Monitor for review. The DMID Medical Monitor may contact the PI for any questions or concerns regarding the event. The PI holds the responsibility of the assessment of the SAE and reporting requirements to the FDA and DMID's role is consultative.

DMID Pharmacovigilance Group
Clinical Research Operations and Management Support (CROMS)
6500 Rock Spring Dr., Suite 650
Bethesda, MD 20817
SAE Hot Line: 1-800-537-9979 (US)
SAE Fax: 800-275-7619 (US)
SAE Email: PVG@dmidcroms.com

8.4.2 Regulatory Reporting

A suspected unexpected serious adverse reaction (SUSAR) is a suspected adverse reaction that is both serious and unexpected. As specified in 21 CFR 312.32, SUSARs will be reported by the Sponsor of the Investigational New Drug Application (IND) to the FDA and to all participating PIs in an IND safety report as soon as possible, no later than 15 calendar days after the Sponsor becomes aware of the suspected adverse reaction (21 CFR 312.32(c)(1)).

Unexpected fatal or life-threatening suspected adverse reactions represent especially important safety information and will be reported to FDA as soon as possible but no later than 7 calendar days following the sponsor's initial receipt of the information.

8.4.3 Reporting of Pregnancy

Pregnancies occurring in study subjects will be reported on the Pregnancy Report form. No further study vaccinations will be administered to pregnant subjects, but with the subject's permission all protocol-required venous blood samples will be obtained and the subject will continue to be followed for safety for the duration of this trial. Efforts will be made to follow all pregnancies reported over the course of this trial through pregnancy outcome, pending the subject's permission.

8.5 Halting Rules

Further enrollments and study vaccinations will be halted for an ad hoc Safety Monitoring Committee (SMC) review if any of the following events occur:

- One or more participants experience an SAE related to the product
- Two or more participants experience the same or similar Grade 3 AE considered related to the study product
- Three or more participants experience the same moderate (Grade 2) safety laboratory AE.

The study will not continue until the SMC has made the determination that the halt may be lifted. The lifting of a halt may require changes to the protocol and/or Informed Consent Form and is upon the advisement of the SMC.

Should any subject be experiencing a higher than grade 2 AE at the time of the scheduled second dose of blinded vaccine, then the second dose will be deferred or cancelled. This decision can be made upon investigator judgement and with discussion with the study participant.

8.6 Safety Oversight

An independent SMC will perform the oversight of safety for this study. The SMC voting members will consist of 4 scientists that are not involved in the conduct of the study. The primary responsibility of the SMC is to monitor participant safety. The SMC will take study-specific data, as well as relevant background information about the disease, test agent, and target population under study into consideration. The SMC will review the cumulative safety data shortly after completion of each inpatient phase, prior to initiation of enrollment for the subsequent study cohort and will approve or reject advancement to the next cohort. The SMC will be authorized to suspend the study, recommend amendments to the protocol, and/or request further information for their review. One of the non-voting members of the SMC will be designated as the Independent Safety Monitor (ISM); this person will be local to CVD but will not be a part of the study team. The ISM will be responsible for conducting a comprehensive review, should there be a halt in the study or at the request of the SMC.

Should there be a halt in the study, no further study product dosing will be performed until the halt is lifted; all enrolled participants will continue to be followed for safety. For participants that are in the Research Isolation Ward at the time of a halt, the management of diarrhea or other symptoms will continue, until resolution. An SMC Charter will be reviewed and approved by the SMC members prior to the initiation of the trial and will include the scheduled frequency/timing of SMC meetings, types of data for review, halting rules, and roles/responsibilities.

The SMC will also recommend the dose to be evaluated in the fourth cohort. The intent will be for the fourth cohort to evaluate the highest dose that is well-tolerated, that is, the highest dose for which there is an absence of significant reactogenicity. Nonetheless, the SMC may also consider data from immunological outcome measures as part of the target dose selection for the fourth cohort. The immunological outcome data, which may be available at the time of SMC review, may consist of serum IgG and IgA ELISA, ASC, and ALS responses. Functional antibody assay data and cellular-mediated immune responses may not be available at the time of the SMC review.

9 CLINICAL MONITORING STRUCTURE

Site monitoring is conducted to ensure that the rights and well-being of human subjects are protected, that the reported trial data are accurate, complete, and verifiable, and that the conduct of the trial is in compliance with the currently approved protocol/amendment(s), with GCP, and with applicable regulatory requirement(s). External monitoring will be performed at standard intervals to oversee the conduct of and reporting of data from this study. The site monitoring will include ensuring appropriate clinical investigator supervision of study site staff and third-party contractors.

9.1 Site Monitoring Plan

Independent internal audits will be conducted, as described in the CVD Quality Management Plan. All clinical trials conducted at CVD are monitored for quality assurance and quality control under the CVD-Wide Quality Management (QMP) Plan. The results of the internal audit are reviewed with the PI, coordinator, and Clinical Research Manager; Corrective Action Plans are formulated in response to any issue. The CVD's 19-page QMP (Version 10.0, 04 February 2022) has been reviewed against the minimum requirements set forth in current standards, such as the DMID CQMP Policy (Version 6.0, 13 July 2016).

10 STATISTICAL CONSIDERATIONS

10.1 Study Outcome Measures

The safety of CVD 1208S-122 will be measured by reactogenicity and AEs, within the time periods described. The immune response to CVD 1208S-122 will be summarized according to the outcome measures described. The genetic stability of the attenuated organism will be evaluated by carefully documenting shed organisms recovered from stool cultures and examining these isolates for the genes that have been inserted into the chromosome.

10.2 Sample Size Considerations

This is a phase 1 first-in-human study, so the primary purpose of statistical comparisons is to screen out potential solicited reactogenicity events that need further clinical evaluation. Therefore, the statistical analyses are not considered formal statistical hypothesis tests and it is acknowledged that there will be inflated Type I errors (i.e., inflated false statistical significances) from performing multiple unadjusted comparisons. No formal sample size calculations were performed to guide the sample size of this phase 1 study. Based on the result of the observed highest tolerated dose (Table 2), a total of 18 to 30 participants will receive active vaccine and this study design allows for at least 87% chance of observing an AE that has an 11% chance of occurrence. This study is not designed to evaluate for rare AEs.

Table 2: Sample size

Highest tolerated dose	Number of participants receiving active vaccine	Number of participants receiving placebo	Among the participants who receive active vaccine, probability of observing at least one participant with an adverse effect that has an 11% chance of occurrence
10^8 cfu	6+12= 18	2+4=6	0.877
10^9 cfu	6+6+12 = 24	2+2+4=8	0.939
10^{10} cfu	6+6+6+12 = 30	2+2+2+4=10	0.970

10.3 Statistical Analysis Plan

Primary Objective 1. To evaluate the safety and clinical tolerability of oral doses of CVD 1208S-122, ranging from 10^8 cfu to 10^{10} cfu organisms, with particular attention to the occurrence of objective signs of diarrhea, dysentery or fever.

Endpoints:

- The number, proportion, and severity of fever, diarrhea, or dysentery (hemoccult testing will only be performed during the inpatient days) within 7 days of vaccination, for all those receiving vaccine and for each of the vaccine dosages evaluated
- The number, proportion, and severity of solicited local and systemic adverse reactions within 7 days of vaccination for all those receiving vaccine and for each of the vaccine dosages evaluated
- The number, proportion, severity, and relatedness of non-serious unsolicited adverse reactions within 28 days of vaccination for all those receiving vaccine and for each of the vaccine dosages evaluated
- The number, proportion, and severity of clinical safety laboratory adverse events from the time of each study vaccination through approximately 7 days after each study vaccination.
- The occurrence of SUSARs in the study
- The occurrence of all SAEs, regardless of the assessment of relatedness, from the time of the first vaccination through approximately 6 months after the last study vaccination

Primary Objective 2. To assess the degree of fecal shedding of CVD 1208S-122 and the genetic stability of the excreted live vector isolates by genetic characterization of the vaccine organisms recovered in stool cultures, to be determined with inpatient Cohorts 1 – 3.

Endpoints:

- The geometric mean number (and interquartile range) of vaccine organisms expressed as cfu/mL for each dosage group, per day, and at the peak of shedding
- The number and proportion of sequential days of fecal shedding of *Shigella* organisms which are documented to contain the genes expressing ETEC antigens for each dosage group. Genetically stable organisms will be defined as being PCR positive for *Shigella* (*ipaH* or *virG*), CFA/I (*cfaB*), and LTB (*eltB*)

Secondary Objective 1. To measure the mucosal (α4β7-positive IgA antibody secreting cell [ASC], antibody in lymphocyte supernatant [ALS], fecal IgA antibody) and systemic (serum IgG and IgA antibody) immune responses to ETEC antigens (CFA/I and LThA2B) and *Shigella flexneri* 2a antigens (LPS and Ipa) following oral immunization with CVD 1208S-122.

Endpoints:

- The number and proportion of 4-fold rises; GMT; mean fold-rises (compared to baseline); median titer; and peak post-vaccination responses in serum anti-LPS, anti-Ipa,

anti-CFA/I, and anti-LT IgA, IgG, or both antibodies post-vaccination, for each dosage group
- The number and proportion of participants with a positive response (≥8 sfc/10^6 cells); mean (and geometric mean) count in anti-LPS, anti-Ipa, anti-CFA/I, and anti-LT ASC IgA and IgG, for each dosage group
- The number and proportion of participants with a 4-fold rise; mean (and geometric mean) titers in anti-LPS, anti-Ipa, anti-CFA/I, and anti-LT ALS IgA and IgG, for each dosage group
- The number and proportion of 4-fold rises; GMT; mean fold-rises (compared to baseline); median titer; and peak post-vaccination responses in adjusted antigen-specific fecal IgA ratios, for each dosage group
- The number and proportion of participants with anti-LPS, anti-Ipa, anti-CFA/I, and anti-LT α4β7-positive IgA ASC, for each dosage group (*ASC mucosal homing studies will only be performed on participants of Cohort 4*)

Secondary Objective 2. To evaluate the ETEC and *Shigella* functional antibody responses following oral immunization CVD 1208S-122.

Endpoints:
- The number and proportion of 4-fold rises; GMT; mean fold-rises (compared to baseline); median titer; and peak post-vaccination responses in *Shigella* SBA and OPKA titer, for each dosage group
- The number and proportion of 4-fold rises; GMT; mean fold-rises (compared to baseline); median titer; and peak post-vaccination responses in ETEC mannose-resistant Hemagglutination Assay (MRHA) titer, for each dosage group
- The number and proportion of 4-fold rises; GMT; mean fold-rises (compared to baseline); median titer; and peak post-vaccination responses in ETEC IC_{50} titer (for adhesion to HT-29 cells), for each dosage group
- The number and proportion of 4-fold rises; GMT; mean fold-rises (compared to baseline); median titer; and peak post-vaccination responses in LT neutralizing titer, for each dosage group and timepoint

Secondary Objective 3. To assess transmissibility of CVD 1208S-122 by determining whether the vaccine strain is acquired (as detected by stool culture) by placebo recipients living in close proximity on the Research Isolation Ward, thus approximating household contact conditions (*to be assessed in Cohorts 1 – 3*).

Endpoint:
- The number and proportion of transmission events, defined as any time during the inpatient period a placebo recipient has a detectable vaccine strain in a stool sample (as long as it is not considered a contaminant during handling or in the lab), for each dosage group

Exploratory Objective 1. To measure the circulating B memory cell responses to ETEC antigens (CFA/I and LThA2B) and *Shigella flexneri* 2a antigens (LPS and Ipa) following oral immunization with one dose of CVD 1208S-122

Endpoint:

- The number and proportion of participants with a positive response (≥8 sfc/10^6 cells); mean (and geometric mean) count in anti-LPS, anti-Ipa, anti-CFA/I, and anti-LT IgA and IgG memory B cells, for each dosage group

Exploratory Objective 2. To collect, separate and store peripheral blood mononuclear cells (PBMCs) so that subsequently the immune responses to CVD 1208S-122 can be further characterized in greater detail including the measurement of T memory and effector cells, homing markers and cytokine production

Endpoint:

- Not applicable

10.4 General Statistical Principles

The distributions of all measures will be examined and described in terms of numbers, means, standard deviations, median, interquartile ranges, minima and maxima for continuous variables (such as geometric mean titers); counts and proportions for categorical variables (such as the presence of the genes encoding CFA/I and LTB, antibody response [Y/N]), will be determined for each group separately. Continuous variables of interest that are not normally distributed will be transformed if needed. To compare each vaccine dose group with the placebo group, t-test or Mann-Whitney U test will be used for continuous variables as appropriate; a Chi-squared test or Fisher's exact will be used for categorical variables as appropriate. All analyses will be performed using Stata/SE version 15.

All participants in the enrolled population who are randomized and receive a blinded vaccination will be included in the Full Analysis (FA) Population. All safety, fecal shedding, and genetic stability analysis will be performed using this FA population. Treatment groups for the safety analysis will be assigned according to the actual treatment received at enrollment.

All participants in the FA population and that have no major protocol violations that are determined to potentially interfere with the immunogenicity assessment of the vaccine and who have at least one immunogenicity assessment will be included in the Per Protocol (PP) Population. The PP population will serve as the primary analysis population for the immunogenicity endpoints. The specific criteria for exclusion of participants from the PP population will be established before breaking the blind and will be based on the blinded review of protocol violations.

In general, all missing data will be treated as missing completely at random and no imputation will be performed except for the safety endpoints, as described below. If some safety data are available for a participant in the FA population, but respective secondary endpoint-related data

are missing, then the participant will be included in the safety analysis and data will be treated as follows for immediate AEs, unsolicited AEs and SAEs.

- If Severity is missing for any AE, then it will be considered as an AE of maximum severity (Grade 3) "Severe", unless it is captured as an SAE
- If "Relationship" is missing, then it will be considered as "Related" to the vaccine administered
- If, for Start date, the day of event/condition is missing due to any adverse event, then it will be imputed as the date of last visit
- If the Stop date of an AE is missing, then it will be treated as ongoing

For solicited AEs, the following assumptions will be made:

- If dates are missing, but symptoms are reported, then the best estimated day post-vaccination will be used to calculate the date of the symptom
- If a symptom is not reported at any time through Day 7, then no imputation for missing data will be performed; the data will be summarized as “not reported” on the tables

10.5 Go/No-Go Milestones

A set of criteria are to be defined for the safety and tolerability of each of the dose-escalation cohorts, in order to proceed to the next higher dosage cohort and/or to decide on the target dose in Cohort 4. These criteria are to be reviewed and accepted by the independent SMC as described in Section 7.6.

Any dose of vaccine which results in more than one episode of a Grade 2 or higher diarrhea or dysentery may be considered too reactogenic for dose-escalation. Any dose of vaccine which results in more than one episode of a Grade 2 or higher solicited AE of the same type may also be considered too reactogenic for dose-escalation, unless the SMC recommends otherwise.

11 ACCESS TO SOURCE DATA/DOCUMENTS

Source data are all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for validation of the clinical data. Examples of these original documents and data records include, but are not limited to, hospital records, clinical and office charts, laboratory notes, memoranda, subjects’ memory aid or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, microfiches, photographic negatives, microfilm or magnetic media, x-rays, and subject files and records kept at the pharmacy, at the laboratories, and medico-technical departments involved in the clinical trial. All information on the Case Report Form (CRF) will be traceable to these source documents, which are generally maintained in the subject’s study file. The source documents will include a copy of the signed Informed Consent/HIPAA authorization. The source document data collection forms for

Screening, Inpatient Pre-Challenge to Day of Challenge, Inpatient Post-Challenge, Flow Sheet of Stool and Emesis Record, Outpatient Visits and AEs will also serve as Case Report Form (CRF) data collection instruments.

The Investigator is responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported. Source documents are maintained for recording data for each subject enrolled in this clinical study. Study subjects' data collected on the CRF during the trial will only be identified by subject number. If, as an exception, it is necessary for safety or regulatory reasons to identify the patient, both the Sponsor and the Investigator are bound to keep this information confidential.

All the information required by the protocol should be provided; any omissions require explanation. Each source document and corresponding CRF should be completed and available for monitoring and/or collection within a timely manner so that the monitor may check the entries for completeness, accuracy and legibility, ensure the CRF is signed by the Investigator, and transmit the data to the Sponsor.

All source documents and CRFs should be completed in a neat, legible manner to ensure accurate interpretation of data. Black or blue ink is required to ensure clarity of reproduced copies. When making a change or correction, cross out the original entry with a single line and initial and date the change. DO NOT ERASE, OVERWRITE OR USE CORRECTION FLUID OR TAPE ON THE ORIGINAL.

The Investigator or designee must enter the information required by the protocol onto the CRF provided. The Sponsor's clinical site monitor will review the CRF for completeness and accuracy and instruct site personnel to make any required corrections or additions.

12 QUALITY CONTROL AND QUALITY ASSURANCE

All clinical trials conducted by CVD are internally monitored for quality assurance and control under the CVD-Wide Quality Management (QMP) Plan. The purpose of the QMP is to describe Quality Control (QC) and Quality Assurance (QA) procedures designed to ensure that the site and Investigator comply with applicable regulations, adhere to the IRB-approved protocol, generate quality data, protect data integrity, and safeguard the safety and well-being of study participants. The plan has the authority to enforce and correct deficiencies in clinical and laboratory conduct of a trial. A Protocol-Specific Quality Assurance Plan is also developed for each study. This plan, composed of QC and QA, is developed in collaboration with the PI, clinical study coordinator, Regulatory Affairs Specialist, and the QM Coordinator. The QC process involves day-to-day logic and edits checks of source documents, case report forms, and laboratory documents. The QA process involves periodic retrospective audits of study records and prospective reviews of clinical operations and assurance that all research required training has been completed as applicable (e.g. GCP, HIPAA, CITI, Lab Safety, Biological Specimen/Shipping). These audits also involve the review of the regulatory file, consent forms/process, eligibility, vaccine accountability and storage, specimen collection, processing,

storage, and shipping. All observations are reviewed with the PI, coordinator, and Clinical Research Manager; Corrective Action Plans are formulated in response to any issue.

13 ETHICS/PROTECTION OF HUMAN SUBJECTS

13.1 Ethical Standard

The Investigator will ensure that this study is conducted in full conformity with the principles set forth in The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research of the US National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research (April 18, 1979) and codified in 45 CFR Part 46 and/or the ICH E6; 62 Federal Regulations 25691 (1997).

13.2 Institutional Review Board

The local institution must provide for the review and approval of this protocol and the associated informed consent documents by an appropriate ethics review committee or Institutional Review Board (IRB). Any amendments to the protocol or consent materials must also be approved before they are placed into use unless it is in the best interest of the subjects' safety to implement changes prior to approval. In both the United States and in other countries, only institutions holding a current US Federal-Wide Assurance, issued by the Office for Human Research Protections (OHRP), may participate.

Refer to: http://www.hhs.gov/ohrp/assurances/

Prior to enrollment of subjects into this clinical study, the protocol and the informed consent form(s) will be reviewed and approved by the appropriate IRB. Any amendments to the protocol or consent materials will also be reviewed and approved by the appropriate IRB. The responsible official for the IRB will sign the IRB letter of approval of the protocol prior to the start of this clinical study. Should amendments to the protocol be required, the amendments will be submitted to the IRB; an IRB letter of approval of the amendment must be obtained prior to acting upon the amendment in the protocol.

13.3 Informed Consent Process

Informed consent is a process that is initiated prior to an individual agreeing to participate in the study and continues throughout the individual's participation in the study. Extensive discussion of risks and possible benefits of this therapy will be provided to the participant. Consent forms describing, in detail, the study, study procedures and risks are given to the potential participant and written documentation of informed consent is required prior to starting any study procedure. Consent forms will be IRB-approved. Participants will be asked to read and review the document. Upon reviewing the document, the Investigator will explain the research study to the participant and answer any questions that may arise. The participants will sign the informed consent document prior to the start of any procedures specific to the study. The participant

should have the opportunity to discuss the study with their surrogates or think about it prior to agreeing to participate. The participant may withdraw consent at any time throughout the course of the trial. A copy of the informed consent document will be given to the participant for their records. The rights and welfare of the participant will be protected by emphasizing that the quality of their medical care will not be adversely affected if they decline to participate in this study.

13.4 Exclusion of Women, Minorities, and Children (Special Populations)

This clinical study will include women, children of 18 years of age and older, and all minorities who meet the inclusion/exclusion criteria, regardless of religion, sex, or ethnic background.

13.5 Subject Confidentiality

Participant confidentiality is strictly held in trust by the investigators, their staff, and the Sponsor. This confidentiality is extended to cover testing of biological samples and other testing, in addition to clinical information relating to the participant.

The study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor.

The study monitor or other representatives authorized by the sponsor may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site will permit access to such records.

Participant identity data will be contained in paper study records which will be kept in a locked file cabinet and in a secure electronic database, accessible only to authorized users at each clinical site. The study database will be user-restricted and password-protected. Participants will not be identified by name. The study database will identify participants by a coded study Volunteer ID number assigned by clinical site personnel.

13.6 Future Use of Stored Specimens

It is intended that any remaining specimens at the closure of the study will be stored at CVD indefinitely. Participants must provide a written request to the PI to have their specimens destroyed. This is described in the consent form.

14 DATA HANDLING AND RECORD KEEPING

The site's PI is responsible to ensure the accuracy, completeness, legibility, and timeliness of the data reported.

Source data may be collected on data collection forms that will be generated by the site or entered directly into the case report forms by study personnel or received by direct data transfer.

Source data collected on data collection forms will be entered into the REDCap data management system, managed by the Clinical and Translational Research Informatics Center (CTRIC), University of Maryland School of Medicine. REDCap is a secure web-based application for building an electronic database that provides a 21 CFR Part 11, FISMA, and HIPAA-compliant environment. Quality control audits of all key safety, laboratory, and clinical data in the database will be made after data entry has been completed. Coexisting medical conditions, AEs and other medical events will be coded using MedDRA dictionary. Concomitant medications will be coded using the WHO-DD dictionary. When the database has been declared to be complete and accurate, the database will be locked. Any changes to the database after that time will only be by joint written agreement of the study team.

All data collection forms will be completed in a neat, legible manner to ensure accurate interpretation of data. Black or blue ink is required to ensure clarity of reproduced copies. When making a change or correction, research staff members will cross out the original entry with a single line and initial and date the change. They will not erase, overwrite, or use correction fluid or tape on the original.

Data reported in REDCap are derived from the data collection forms and should be consistent with the data collection forms or the discrepancies should be explained.

The sponsor and/or its designee will provide guidance to the site PI and other study personnel on making corrections to the data collection forms and eCRF.

15 PUBLICATION POLICY

All investigators funded by the NIH must submit, or have submitted for them, an electronic version of their final, peer-reviewed manuscripts, upon acceptance for publication, to the National Library of Medicine's PubMed Central (http://www.ncbi.nlm.nih.gov/pmc/). The NIH Public Access Policy ensures the public has access to the published results of NIH-funded research. It requires investigators to submit final peer-reviewed journal manuscripts that arise from NIH funds to the digital archive PubMed Central upon acceptance for publication. Further, the policy stipulates that these papers must be accessible to the public on PubMed Central no later than 12 months after the official date of publication.

Refer to:

- NIH Public Access Policy, http://publicaccess.nih.gov/
- NIH Office of Extramural Research (OER) Grants and Funding, http://grants.nih.gov/grants/oer.htm

Following completion of this clinical trial, the PI will publish the results of this research in a scientific journal. The International Committee of Medical Journal Editors (ICMJE) member journals have adopted a trials-registration policy as a condition for publication. This policy requires that all clinical trials be registered in a public trials registry, such as ClinicalTrials.gov (http://clinicaltrials.gov/), which is sponsored by the National Library of Medicine. Other biomedical journals are considering adopting similar policies.

16 LITERATURE REFERENCES

1. Kotloff KL, Nataro JP, Blackwelder WC, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet*. Jul 20 2013;382(9888):209-22. doi:10.1016/S0140-6736(13)60844-2
2. Kotloff KL, Winickoff JP, Ivanoff B, et al. Global burden of Shigella infections: implications for vaccine development and implementation of control strategies. *Bull World Health Organ*. 1999;77(8):651-66.
3. Livio S, Strockbine NA, Panchalingam S, et al. Shigella isolates from the global enteric multicenter study inform vaccine development. *Clin Infect Dis*. Oct 2014;59(7):933-41. doi:10.1093/cid/ciu468
4. Liu J, Platts-Mills JA, Juma J, et al. Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: a reanalysis of the GEMS case-control study. *Lancet*. Sep 24 2016;388(10051):1291-301. doi:10.1016/S0140-6736(16)31529-X
5. Haley CC, Ong KL, Hedberg K, et al. Risk factors for sporadic shigellosis, FoodNet 2005. *Foodborne Pathog Dis*. Jul 2010;7(7):741-7. doi:10.1089/fpd.2009.0448
6. Arvelo W, Hinkle CJ, Nguyen TA, et al. Transmission risk factors and treatment of pediatric shigellosis during a large daycare center-associated outbreak of multidrug resistant Shigella sonnei: implications for the management of shigellosis outbreaks among children. *Pediatr Infect Dis J*. Nov 2009;28(11):976-80. doi:10.1097/INF.0b013e3181a76eab
7. Kasper MR, Lescano AG, Lucas C, et al. Diarrhea outbreak during U.S. military training in El Salvador. *PLoS One*. 2012;7(7):e40404. doi:10.1371/journal.pone.0040404
8. Castelli F, Pezzoli C, Tomasoni L. Epidemiology of travelers' diarrhea. *J Travel Med*. Dec 2001;8(Suppl 2):S26-30.
9. Marcoleta A, Toro C, Prado V, et al. [Antibiotic susceptibility patterns among Shigella sonnei, isolated during three different periods in Region Metropolitana, Chile]. *Rev Chilena Infectol*. Dec 2013;30(6):616-21. Patrones de susceptibilidad antimicrobiana de cepas de Shigella sonnei aisladas durante tres periodos diferentes en la Region Metropolitana, Chile. doi:10.4067/S0716-10182013000600007
10. Giersing BK, Porter CK, Kotloff K, Neels P, Cravioto A, MacLennan CA. How can controlled human infection models accelerate clinical development and policy pathways for vaccines against Shigella? *Vaccine*. Aug 7 2019;37(34):4778-4783. doi:10.1016/j.vaccine.2019.03.036
11. Hyams KC, Bourgeois AL, Merrell BR, et al. Diarrheal disease during Operation Desert Shield. *N Engl J Med*. Nov 14 1991;325(20):1423-8. doi:10.1056/NEJM199111143252006
12. Sanders JW, Putnam SD, Gould P, et al. Diarrheal illness among deployed U.S. military personnel during Operation Bright Star 2001--Egypt. *Diagn Microbiol Infect Dis*. Jun 2005;52(2):85-90. doi:10.1016/j.diagmicrobio.2005.02.005
13. Walker RI, Steele D, Aguado T, Ad Hoc ETEC. Analysis of strategies to successfully vaccinate infants in developing countries against enterotoxigenic E. coli (ETEC) disease. *Vaccine*. Mar 30 2007;25(14):2545-66. doi:10.1016/j.vaccine.2006.12.028
14. Taxt A, Aasland R, Sommerfelt H, Nataro J, Puntervoll P. Heat-stable enterotoxin of enterotoxigenic Escherichia coli as a vaccine target. *Infect Immun*. May 2010;78(5):1824-31. doi:10.1128/IAI.01397-09
15. Dickinson BL, Clements JD. Dissociation of Escherichia coli heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. *Infect Immun*. May 1995;63(5):1617-23.

16. Cassels FJ, Wolf MK. Colonization factors of diarrheagenic E. coli and their intestinal receptors. *J Ind Microbiol*. Sep 1995;15(3):214-26.
17. Isidean SD, Riddle MS, Savarino SJ, Porter CK. A systematic review of ETEC epidemiology focusing on colonization factor and toxin expression. *Vaccine*. Aug 26 2011;29(37):6167-78. doi:10.1016/j.vaccine.2011.06.084
18. Barry EM, Pasetti MF, Sztein MB, Fasano A, Kotloff KL, Levine MM. Progress and pitfalls in Shigella vaccine research. *Nat Rev Gastroenterol Hepatol*. Apr 2013;10(4):245-55. doi:10.1038/nrgastro.2013.12
19. Levine MM, Kotloff KL, Barry EM, Pasetti MF, Sztein MB. Clinical trials of Shigella vaccines: two steps forward and one step back on a long, hard road. *Nat Rev Microbiol*. Jul 2007;5(7):540-53. doi:10.1038/nrmicro1662
20. Venkatesan MM, Ranallo RT. Live-attenuated Shigella vaccines. *Expert Rev Vaccines*. Oct 2006;5(5):669-86. doi:10.1586/14760584.5.5.669
21. Mel DM, Terzin AL, Vuksic L. Studies on vaccination against bacillary dysentery. 3. Effective oral immunization against Shigella flexneri 2a in a field trial. *Bull World Health Organ*. 1965;32(5):647-55.
22. Mel D, Gangarosa EJ, Radovanovic ML, Arsic BL, Litvinjenko S. Studies on vaccination against bacillary dysentery. 6. Protection of children by oral immunization with streptomycin-dependent Shigella strains. *Bull World Health Organ*. 1971;45(4):457-64.
23. Coster TS, Hoge CW, VanDeVerg LL, et al. Vaccination against shigellosis with attenuated Shigella flexneri 2a strain SC602. *Infect Immun*. Jul 1999;67(7):3437-43.
24. Kotloff KL, Pasetti MF, Barry EM, et al. Deletion in the Shigella enterotoxin genes further attenuates Shigella flexneri 2a bearing guanine auxotrophy in a phase 1 trial of CVD 1204 and CVD 1208. *J Infect Dis*. Nov 15 2004;190(10):1745-54. doi:10.1086/424680
25. Kotloff KL, Simon JK, Pasetti MF, et al. Safety and immunogenicity of CVD 1208S, a live, oral DeltaguaBA Deltasen Deltaset Shigella flexneri 2a vaccine grown on animal-free media. *Hum Vaccin*. Nov-Dec 2007;3(6):268-75.
26. Kotloff KL, Pasetti MF, Shirley D-A, et al. Phase 1 and 2 Trials of CVD 1208S, a live, oral ΔguaBA,Δsen,Δset *Shigella flexneri* 2a vaccine. presented at: Oral Abstract presentation, Vaccines for Enteric Diseases; 2013; Bangkok, Thailand.
27. Barry EM, Wu T, DeLaine BC, et al. Preclinical Evaluation of Combined *Shigella*-ETEC Vaccine Candidates. 2016:
28. Shimanovich AA, Buskirk AD, Heine SJ, et al. Functional and Antigen-Specific Serum Antibody Levels as Correlates of Protection against Shigellosis in a Controlled Human Challenge Study. *Clin Vaccine Immunol*. Feb 2017;24(2)doi:10.1128/CVI.00412-16
29. Anantha RP, McVeigh AL, Lee LH, et al. Evolutionary and functional relationships of colonization factor antigen i and other class 5 adhesive fimbriae of enterotoxigenic Escherichia coli. *Infect Immun*. Dec 2004;72(12):7190-201. doi:10.1128/IAI.72.12.7190-7201.2004
30. Baker KK, Levine MM, Morison J, Phillips A, Barry EM. CfaE tip mutations in enterotoxigenic Escherichia coli CFA/I fimbriae define critical human intestinal binding sites. *Cell Microbiol*. May 2009;11(5):742-54. doi:10.1111/j.1462-5822.2009.01287.x
31. Barry EM, Levine MM. A tale of two bacterial enteropathogens and one multivalent vaccine. *Cell Microbiol*. Nov 2019;21(11):e13067. doi:10.1111/cmi.13067
32. Sieper J, Braun J, Wu P, Hauer R, Laitko S. The possible role of Shigella in sporadic enteric reactive arthritis. *Br J Rheumatol*. Jul 1993;32(7):582-5.
33. Finch M, Rodey G, Lawrence D, Blake P. Epidemic Reiter's syndrome following an outbreak of shigellosis. *Eur J Epidemiol*. Mar 1986;2(1):26-30.

34. Keat A. Reiter's syndrome and reactive arthritis in perspective. *N Engl J Med*. Dec 29 1983;309(26):1606-15. doi:10.1056/NEJM198312293092604
35. Boyd MA, Tennant SM, Saague VA, et al. Serum bactericidal assays to evaluate typhoidal and nontyphoidal Salmonella vaccines. *Clin Vaccine Immunol*. May 2014;21(5):712-21. doi:10.1128/CVI.00115-14
36. Sears KT, Tennant SM, Reymann MK, et al. Bioactive Immune Components of Anti-Diarrheagenic Enterotoxigenic Escherichia coli Hyperimmune Bovine Colostrum Products. *Clin Vaccine Immunol*. Aug 2017;24(8)doi:10.1128/CVI.00186-16
37. Sakellaris H, Munson GP, Scott JR. A conserved residue in the tip proteins of CS1 and CFA/I pili of enterotoxigenic Escherichia coli that is essential for adherence. *Proc Natl Acad Sci U S A*. Oct 26 1999;96(22):12828-32.
38. Giuntini S, Stoppato M, Sedic M, et al. Identification and Characterization of Human Monoclonal Antibodies for Immunoprophylaxis Against Enterotoxigenic Escherichia coli Infection. *Infect Immun*. Jun 4 2018;doi:10.1128/IAI.00355-18
39. Kumar P, Kuhlmann FM, Bhullar K, et al. Dynamic Interactions of a Conserved Enterotoxigenic Escherichia coli Adhesin with Intestinal Mucins Govern Epithelium Engagement and Toxin Delivery. *Infect Immun*. Dec 2016;84(12):3608-3617. doi:10.1128/IAI.00692-16
40. Glenn GM, Villar CP, Flyer DC, et al. Safety and immunogenicity of an enterotoxigenic Escherichia coli vaccine patch containing heat-labile toxin: use of skin pretreatment to disrupt the stratum corneum. *Infect Immun*. May 2007;75(5):2163-70. doi:10.1128/IAI.01740-06
41. Toapanta FR, Simon JK, Barry EM, et al. Gut-Homing Conventional Plasmablasts and CD27(-) Plasmablasts Elicited after a Short Time of Exposure to an Oral Live-Attenuated Shigella Vaccine Candidate in Humans. *Front Immunol*. 2014;5:374. doi:10.3389/fimmu.2014.00374
42. Wahid R, Simon JK, Picking WL, Kotloff KL, Levine MM, Sztein MB. Shigella antigen-specific B memory cells are associated with decreased disease severity in subjects challenged with wild-type Shigella flexneri 2a. *Clin Immunol*. Jul 2013;148(1):35-43. doi:10.1016/j.clim.2013.03.009
43. Simon JK, Wahid R, Maciel M, Jr., et al. Antigen-specific B memory cell responses to lipopolysaccharide (LPS) and invasion plasmid antigen (Ipa) B elicited in volunteers vaccinated with live-attenuated Shigella flexneri 2a vaccine candidates. *Vaccine*. Jan 22 2009;27(4):565-72. doi:10.1016/j.vaccine.2008.10.081
44. Simon JK, Maciel M, Jr., Weld ED, et al. Antigen-specific IgA B memory cell responses to Shigella antigens elicited in volunteers immunized with live attenuated Shigella flexneri 2a oral vaccine candidates. *Clin Immunol*. May 2011;139(2):185-92. doi:10.1016/j.clim.2011.02.003
45. Alam MM, Aktar A, Afrin S, et al. Antigen-specific memory B-cell responses to enterotoxigenic Escherichia coli infection in Bangladeshi adults. *PLoS Negl Trop Dis*. Apr 2014;8(4):e2822. doi:10.1371/journal.pntd.0002822
46. El-Kamary SS, Cohen MB, Bourgeois AL, et al. Safety and immunogenicity of a single oral dose of recombinant double mutant heat-labile toxin derived from enterotoxigenic Escherichia coli. *Clin Vaccine Immunol*. Nov 2013;20(11):1764-70. doi:10.1128/CVI.00464-13

APPENDIX A: STUDY SCHEDULE OF EVENTS

Schedule of Events, Cohorts 1 – 3													
Study Period	Screen		Inpatient Period						Outpatient Follow-Up				
Study Day	-60 to -2	-3*	-1	1	2	3	4	5	8	29	43	181	ET
Informed Consent	X												
Screening Procedures[1]	X	*											
Admission & Acclimatization			X										
Pregnancy Test (females only)				X									
Vaccination				**X**									
Clinical Safety Labs[2]				12.5					12.5				
Reactogenicity Assessments				X	X	X	X	X					
Adverse Event Monitoring				X	X	X	X	X	X	X	X	X	X
Research Blood for Serology (mL)			(x)	10						10	10	10	10
Research Blood for ASC/ALS Assay (mL)			(x)	25					25				
Research Blood for PBMC (mL)			(x)	90						90	90	90	90
Stool Grading (while inpatient only)				X	X	X	X	X					
Stool Culture, for fecal shedding				X	X	X	X	X	X				X
Stool IgA				X					X	X	X	X	
Total daily blood volume (mL)	16			137.5					37.5	100	100	100	
Cumulative blood volume (mL)	16			153.5					191	291	391	491	

Schedule of Events, Cohort 4										
Study Period	Screen	Outpatient								
Study Day	-60 to -2	1	3	8	29	31	36	57	210	ET
Informed Consent	X									
Screening Procedures[1]	X									
Pregnancy Test (females only)		X			X					
Vaccination		**X**			**X**					
Clinical Safety Labs[2]		12.5		12.5	12.5		12.5			
Memory Aid (**d**isperse or **c**ollect)		d		c	d		c			
Adverse Event Monitoring		X	X	X	X	X	X	X	X	X
Research Blood for Serology (mL)		10			10			10	10	10
Research Blood for ASC/ALS Assay plus homing (mL)		25		50	25		50			
Research Blood for PBMC (mL)		90			50			90	90	90
Stool for Culture		X	X	X	X	X	X			
Stool IgA		X			X		X	X	X	
Total daily blood volume (mL)	16	137.5		62.5	97.5		62.5	100	100	
Cumulative blood volume (mL)	16	153.5		216	313.5		376	476	576	

ET = early termination

(x) = research blood for serology, ASC/ALS, and PBMC may be collected on Day -1 or Day 1, as long as it is prior to vaccination

1 - Screening includes clinical laboratory testing:

- Serology: HIV Ab, HBsAg, HCV Ab
- Other labs: HLA B27, hematology (WBC, ANC, hemoglobin, platelet count), chemistry (creatinine, ALT, total bilirubin), serum IgA, and β-HCG for women of Childbearing Potential (CBP)

2 - Clinical Safety Labs include: hematology (WBC, ANC, hemoglobin, platelet count), and chemistry (creatinine, ALT, total bilirubin)

* A nasal swab for a SARS-CoV-2 test is to be performed within 72 hours prior to admission to the inpatient unit, for Cohorts 1-3 only, and as per current hospital policy

Note: all research blood volumes indicated are intended to be approximate target volumes; therefore, the collection of volumes which are not exactly equal to the volumes indicated in the protocol do not necessarily result in protocol deviations

APPENDIX B: ACCEPTABLE CLINICAL LABORATORY VALUES FOR ELIGIBILITY

Analyte	Unit	Reference Range*	Acceptable Values for Screening Labs:
White Blood Cell count (WBC)	$10^3/\mu L$	3.5 – 11.5	3.15 – 12.6 (2.8 – 12.6 for African Americans)
Absolute Neutrophil Count (ANC)	$10^3/\mu L$	1560 – 8100	1400 – 8910 (1200 – 8910 for African Americans)
Hemoglobin (Females)	g/dL	12.0 – 16.0	10.8 – 17.6
Hemoglobin (Males)	g/dL	13.5 – 17.5	12.2 – 19.2
Platelet count	$10^3/\mu L$	140 – 400	126 – 440
Creatinine (Females)	mg/dL	0.6 – 1.2	≤1.3
Creatinine (Males)	mg/dL	0.7 – 1.3	≤1.4
Alanine aminotransferase (ALT)	U/L	7 - 45	≤54
Bilirubin, total	mg/dL	0.2 – 1.2	<1.5
Serum IgA (Females)	mg/dL	87 - 352	≥87
Serum IgA (Males)	mg/dL	–90 - 386	≥90
Urine pregnancy test	n/a	n/a	Negative
Serum β-HCG (Females)	n/a	n/a	Negative
HLA-B27	n/a	n/a	Negative
Hepatitis B surface antigen	n/a	n/a	Negative
Hepatitis C virus ELISA	n/a	n/a	Negative
HIV	n/a	n/a	Negative
SARS-CoV-2[#]	n/a	n/a	Negative

*The reference ranges provided are from Garcia Labs, published 10 October 2022.

[#]A SARS-CoV-2 molecular diagnostic test

APPENDIX C: GRADING SCALES FOR CLINICAL SAFETY LABORATORY TOXICITY

Test	Mild Grade 1	Moderate Grade 2	Severe Grade 3
WBC, increase (xULN)	>1.1 – 1.5	1.6 – 2.0	≥2.1
WBC, decrease* (xLLN)	<1.1 – 1.5	1.6 – 2.0	≥2.1
ANC, increase (xULN)	>1.1 – 1.5	1.6 – 2.0	≥2.1
ANC decrease* (xLLN)	<1.1 – 1.5	1.6 – 2.0	≥2.1
Hemoglobin, increase (xULN)	>1.1 – 1.5	1.6 – 2.0	≥2.1
Hemoglobin, decrease* (xLLN)	<1.1 – 1.5	1.6 – 2.0	≥2.1
Platelet, increase (xULN)	>1.1 – 1.5	1.6 – 2.0	≥2.1
Platelet, decrease* (xLLN)	<1.1 – 1.5	1.6 – 2.0	≥2.1
Creatinine* (xULN)	>1.1 – 1.5	1.6 – 2.0	≥2.1
ALT (xULN)	>1.2 – 2.0	2.1 – 4.0	≥4.1
Total Bilirubin* (xULN)	>1.2 – 1.5	1.6 – 2.0	≥2.1

*if the baseline value is outside of the reference range, then the relative changes to the baseline will be evaluated for toxicity

ULN, upper limit of normal

LLN, lower limit of normal

APPENDIX D: REACTOGENICITY & CLINICAL TOXICITY GRADING

Objective Reactogenicity	Mild	Moderate	Severe	Potentially Life-Threatening
	Grade 1	Grade 2	Grade 3	Grade 4
Diarrhea (Number of loose stools with Grade 3 – 5 consistency, as defined below)	2 or more loose stools of ≥200 mL within 48 h *or* a single loose stool of ≥300 mL	Cumulative loose stools of ≥ 1 L over 96 h *or* loose stools of ≥400 mL in 24 h *or* 4 – 5 loose stools in 24 h	Cumulative loose stools of ≥1 L in 48 h *or* loose stools of ≥800 mL in 24 h *or* >5 loose stools in 24 h	Requires hospitalization
Dysentery (Number of heme-positive loose stools)	1	2 – 3	≥4	
Vomiting (Number of episodes/24 h)	1	2 – 3	≥4	
Fever (°C (°F))	38.1 – 38.4 (100.5 – 101.1)	38.5 – 38.9 (101.2 – 102.1)	≥39 (≥102.2)	
Tachycardia (bpm)	101 – 115	116 – 125	>125	
Systolic Hypotension (mmHg)	85 – 94	80 – 84	<80	
Diastolic Hypotension (mmHg)	45-59	35-44	<35	

Subjective Reactogenicity and Unsolicited AEs, including abdominal pain/cramping, tenesmus, malaise, nausea, anorexia, arthralgia, and myalgia	No interference with activity	Some interference with activity	Prevents daily activity	Requires hospitalization

Stool Consistency Grading Scale				
Normal stool		*Loose or diarrheal stool*		
Grade 1	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 5**
Well formed; does not take the shape of the container	Soft; does not easily take the shape of the container	Thick liquid stool; easily takes the shape of the container	Opaque, watery diarrheal stool	Clear, watery or "rice water" diarrheal stool