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RESEARCH PAPER

A phase I trial of WRSS1, a *Shigella sonnei* live oral vaccine in Bangladeshi adults and children

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ABSTRACT

Shigella sonnei live vaccine candidate, WRSS1, which was previously evaluated in US, Israeli and Thai volunteers, was administered orally to Bangladeshi adults and children to assess its safety, clinical tolerability and immunogenicity. In a randomized, placebo-controlled, dose-escalation, age-descending study, 39 adults (18–39 years) and 64 children (5–9 years) were enrolled. Each adult cohort (n = 13) received one dose of $3x10^4$, or three doses of 3×10^5 or 3×10^6 colony forming unit (CFU) of WRSS1 (n = 10) or placebo (n = 3). Each child cohort (n = 16) received one dose of $3x10^3$, or three doses of $3x10^4$, $3x10^5$, or 3×10^6 CFU WRSS1 (n = 12) or placebo (n = 4). WRSS1 elicited mostly mild and transient reactogenicity events in adults and children. In the 3×10^6 dose group, 50% of the adults shed the vaccine; no shedding was seen in children. At the highest dose, 100% of adults and 40% of children responded with a ≥ 4-fold increase of *S. sonnei* LPS-specific IgA antibody in lymphocyte supernatant (ALS). At the same dose, 63% of adults and 70% of children seroconverted with IgA to LPS, while in placebo, 33% of adults and 18% of children seroconverted. Both the vaccinees and placebos responded with fecal IgA to LPS, indicating persistent exposure to *Shigella* infections. In conclusion, WRSS1 was found safe up to 10^6 CFU dose and immunogenic in adults and children in Bangladesh. These data indicate that live, oral *Shigella* vaccine candidates, including WRSS1 can potentially be evaluated in toddlers and infants (<2 years of age), who comprise the target population in an endemic environment.

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Introduction

Diarrheal disease is the fourth leading cause of death in under 5 children, with 499,000 deaths in 2015.[1] Furthermore, the incidence of moderate-to-severe diarrhea in infants and children correlates with increased risk of mortality, stunting of physical growth and lowered cognitive abilities.[2] Accordingly, the development of vaccines against diarrhea remains a major global health focus, particularly for children in low-resource countries. Unfortunately, even licensed vaccines such as those for rotavirus and cholera elicit poor responses in this target group.[3,4] This suggests that even good vaccines may be poorly immunogenic unless strategies can be devised to improve the performance of orally-administered vaccines in children living in endemic countries.

Live attenuated or inactivated oral bacterial vaccines mimic natural infection, are administered needle-free, are cheaper to manufacture than subunit vaccines and are convenient for compliance rates if multiple doses are needed. Hence, oral vaccines, even with reduced efficacy, could be of great public health benefit in resource-poor countries. For example, licensed oral rotavirus vaccines, even with 50–60% efficacy, have significantly reduced global rates of diarrhea-related childhood hospitalization.[5-8]

Shigella was the second leading cause of diarrhea-related deaths in 2016 among all ages.[9] Among children aged 0–2 years, based on the multisite Malnutrition and Enteric Disease (MAL-ED) cohort, *Shigella* possessed the highest overall burden among ten pathogens accounting for 95.7% of attributable diarrhea.[10] Furthermore, the Global Enteric Multicenter Study attributed *Shigella* to be the cause of the second largest proportion of moderate-to-severe diarrhea in toddlers, and the largest contributor in 24–59 months old children.[2] Moreover, *Shigella* along with enteroaggregative *E coli, Campylobacter*, and *Giardia*, was substantially associated with sustained linear growth faltering during the first 2 years of life, and in some cases even for 5 years.[11] Historically *Shigella flexneri* has been the dominant *Shigella* serogroup in endemic populations, while *S. sonnei* predominates in high-resource countries. With improved living conditions and availability of clean water in low-resource countries including Bangladesh, *S. sonnei* is slowly replacing

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S. flexneri.[12-14] At the Dhaka Hospital of icddr,b, prevalence of *S. sonnei* increased from 39.3% in 2013 to 51.4% in 2016, whereas prevalence of *S. flexneri* remained the same (icddr,b surveillance data). Additionally, in 2016, of the *Shigella* isolates identified at the Dhaka Hospital of icddr,b, 68% were resistant to ciprofloxacin, 66% to cotrimoxazole and 50% to azithromycin, further emphasizing the need for a licensed *Shigella* vaccine. Such a vaccine, would be most effective in children <2 years of age, who are the target group.

Attenuated *Shigella* strains have been developed in the process of vaccine construction, which offers the opportunity to safely study immune responses to them in children living in low and middle income countries and to test strategies for improving these responses. A *S. sonnei* vaccine candidate WRSS1 has undergone sufficient clinical testing to be used to probe vaccination strategies in children. WRSS1 lacks the ability to spread from cell to cell due to loss of VirG (or IcsA).[15] VirG-based vaccine strains such as *S. flexneri* 2a SC602, *S. dysenteriae* 1 WRSd1, and WRSS1 have demonstrated safety at low doses, significant immunogenicity, and in some cases efficacy in naïve US volunteers.[16-18] WRSS1 was also found to be safe in Israeli and Thai adult volunteers.[19,20]

The current study was designed to evaluate the safety, clinical tolerability and immunogenicity of an oral vaccine, WRSS1 in Bangladesh, where *Shigella* is an important cause of moderate-to-severe diarrhea in children. This study was undertaken using WRSS1 as a tool to increase our understanding of how such a vaccine can be used in different age groups in an endemic area. In the future, data from this and other such studies will provide meaningful strategies for optimization of immune responses with live oral vaccines in toddlers and infants, who are the primary target population in Bangladesh.

Results

Study population

Among 252 screened participants, 39 adults and 64 children were enrolled based on inclusion and exclusion criteria (Supplementary Table 1). The CONSORT diagrams depicts screening, enrollment, allocation of vaccine/placebo, dose completion, and follow-up completion status of adults and children participants (Figure 1(a,b)). Demographic data of study participants by treatment groups are given in Supplementary Table 2.

Safety and clinical tolerability of WRSS1 in adults and children

In order to evaluate the safety of the WRSS1 vaccine, all participants were monitored for reactogenicity events, unexpected adverse events (AEs) and serious adverse events (SAEs) based on the definitions described in Materials and Methods. WRSS1 was generally well tolerated by Bangladeshi adults with reactogenicity symptoms occurring mostly after the first dosing in each dose group. Among 10 adults in each dose group, 4 (40%) in the 4-log, 5 (50%) in the 5-log and 8 (80%) in the 6-log dose groups experienced at least one reactogenicity event, showing a dose-dependent increase in the frequency of events. Five of 9 placebo recipients (56%) also experienced the same. The most common symptoms among vaccinees were headache, abdominal pain and bloating (Table 1). The majority of the symptoms were mild except for one moderate event each of abdominal pain (5-log dose group), and arthralgia and chills (6-log dose group), which resolved without sequelae within one to two days after the occurrence. Two participants in the 4-log and one in the 5-log dose group had loose stool for one day. One participant in the 6-log dose group experienced mild diarrhea for one day accompanied by mild abdominal cramps and mild headache. One adult each in 5-log and 6-log dose group had mild and transient fever, with the latter having co-existing symptoms of nausea, bloating, chills and arthralgia. The most common events in the placebo group were headache and nausea followed by bloating and abdominal pain (Table 1). There was one moderate event of abdominal cramps and one mild event of loose stool for one day among adult placebo recipients.

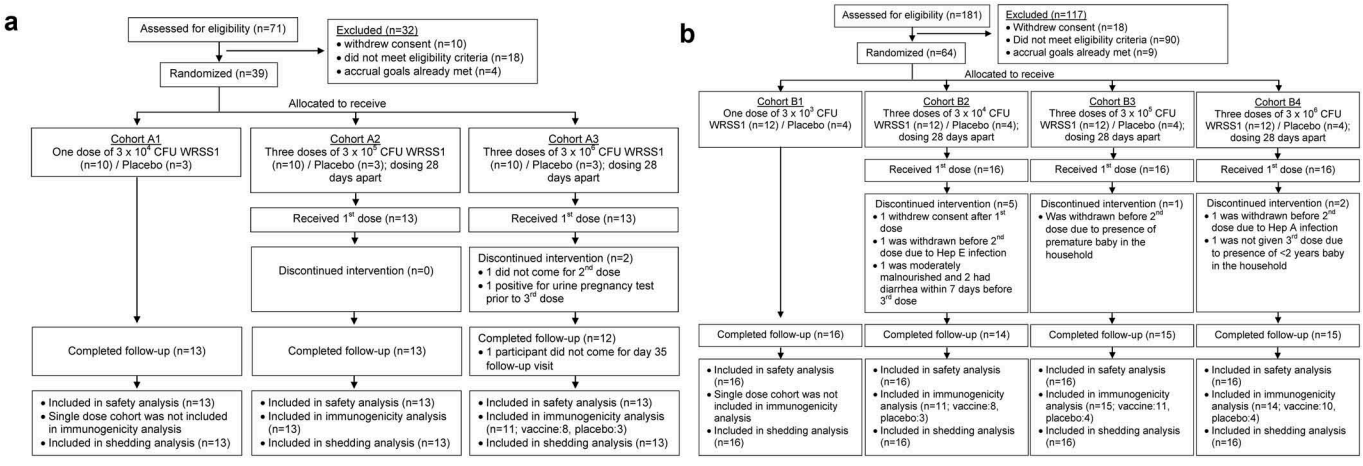

Figure 1. CONsolidated Standards of Reporting Trials (CONSORT) diagram showing screening, enrollment, allocation of vaccine/placebo, completion of intervention, and follow-up status of Bangladeshi (a) Adults and (b) Children participants. Each cohort of 13 adults (10 vaccinees and 3 placebo recipients) received either one dose of 3×10^4 (cohort A1) or three doses of 3×10^5 (cohort A2) or 3×10^6 (cohort A3) CFU of WRSS1 vaccine or placebo. Each cohort of 16 children (12 vaccinees and 4 placebos) received either one dose of 3×10^3 (cohort B1) or three doses of 3×10^4 (cohort B2), 3×10^5 (cohort B3) or 3×10^6 (cohort B4) CFU of vaccine or placebo.

Table 1. Reactogenicity events in Bangladeshi adults and children receiving WRSS1 or placebo.

	Adults (18–39 years)				Children (5–9 years)				
Reactogenicity Events	$3x10^4$ CFU dose group n = 10	$3x10^5$ CFU dose group n = 10	$3x10^6$ CFU dose group n = 10	Placebo n = 9	$3x10^3$ CFU dose group n = 12	$3x10^4$ CFU dose group n = 12	3×10^5 CFU dose group n = 12	$3x10^6$ CFU dose group n = 12	Placebo n = 16
Abdominal Pain	0	3[a] (30%)	2 (20%)	2 (22%)	1(8%)	1(8%)	0	3 (25%)	0
Abdominal Cramps	0	0	2 (20%)	1[a] (11%)	1(8%)	0	0	2 (17%)	0
Bloating	0	0	3 (30%)	2 (22%)	0	0	0	0	0
Diarrhea	0	0	1(10%)	0	0	1(8%)	0	0	0
Chills	0	0	2 [a] (20%)	0	0	0	0	0	0
Fever	0	1 (10%)	1 (10%)	0	0	3 (25%)	0	1 (8%)	1 (6%)
Headache	2 (20%)	0	6 (60%)	4 (44%)	0	0	0	1 (8%)	1 (6%)
Lightheadedness	0	0	0	0	0	0	0	1 (8%)	1 (6%)
Loose stools	2 (20%)	1 (10%)	0	1 (11%)	0	2 (17%)	3 (25%)	1 (8%)	0
Malaise	0	0	1 (10%)	0	0	0	0	0	0
Myalgia	0	0	0	0	0	0	0	1 (8%)	1 (6%)
Arthralgia	0	0	1[a] (10%)	0	0	0	0	1 (8%)	0
Nausea	0	0	2 (20%)	3 (33%)	1(8%)	0	0	1 (8%)	0
Vomiting	0	0	0	0	0	0	0	0	2 (13%)

Data are presented as number (%) of participants. [a]One subject had moderate symptoms, otherwise all events were mild.

In children, fewer reactogenicity events were seen compared to adults and all events were mild in nature. Among 12 participants in each dose group, the number (proportion) of participants experiencing a minimum of one reactogenicity event were 2 (17%) in the 3-log, 5 (42%) in the 4-log, 3 (25%) in the 5-log and 7 (58%) in the 6-log dose groups. Among placebo recipients, 19% (3 out of 16) experienced at least one reactogenicity event. The most common symptoms among vaccine recipients were loose stool, abdominal pain and fever (Table 1). In the 4-log dose group, two children had loose stool for one day, while one child developed mild diarrhea for two days accompanied by fever for one day after the 1st dose. Two other children in the 4-log dose group also had mild and transient fever after the 1st dose. Three children in the 5-log dose group had loose stool for one day with each having it after the 1st, 2nd and 3rd dose, respectively. In the 10^6 CFU dose group, one participant had loose stools for one day after the 3rd dose. Another child in the same dose group had mild fever for one day followed by transient abdominal pain. None of these children required antibiotic treatment. One placebo recipient had mild fever for 3 days after the 1st dose. Two children of the placebo group vomited, one after the 1st dose and another after the 2nd dose (Table 1). The proportion of child participants with abdominal pain was higher in the 6-log dose group ($p = 0.034$) while the percentage of participants with loose stool was greater ($p = 0.034$) in the 5-log dose group compared to placebo.

Besides reactogenicity, there were several unexpected AEs in both age groups, ranging from mild to moderate in adults and mild to severe in children, which were clinically judged as vaccine-unrelated (see Materials & Methods for vaccine unrelated AEs and SAEs). In adults, elevated levels of liver enzymes (alanine transaminase (ALT) and aspartate transaminase (AST)) were the most frequent unsolicited AEs, with epigastric pain, upper respiratory tract infection and back pain being the other common AEs. The most common vaccine-unrelated AEs across all cohorts of children were fever and upper respiratory tract infection.

Four SAEs were reported in one adult and three children, none of which were assessed as related to the vaccine. Incomplete abortion in a female adult was considered as SAE, which was eventually resolved after further medication. One child was hospitalized for 2 days due to suspected enteric fever, 21 days after the first dosing; stool culture did not show presence of either WRSS1 vaccine strain or other tested enteric pathogens (e.g. *Shigella, Salmonella, Escherichia coli, Vibrio, Campylobacter, Aeromonas, Plesiomonas, Yersinia*). Tonsillectomy was performed in another child 9 days after the third dosing. Among placebos, one child participant was hospitalized ten days after the first dose due to diarrhea and dehydration; enteroaggegative *E. coli* was found in the stool by culture that was confirmed by PCR. Although the child was released from the hospital just after 5 hours from admission, the event was considered as SAE upon discretion of internal protocol safety team.

Shedding of vaccine strain

Shedding of WRSS1 in stool specimens/rectal swab was assessed on day 0 (the day of first dosing) and on days 1, 2, 3, 7, 28 (all cohorts), and additionally on days 35, 56, 63 and 84 (multi-dose cohorts) (Figure 2) by culture and confirmed by PCR assay. One adult in the 3×10^4 CFU dose group, one in the 3×10^5 dose group, and five in the 3×10^6 dose group shed the vaccine for one day after the first dosing of the vaccine. No further shedding was seen after subsequent doses. There was no shedding of WRSS1 in children.

Immunogenicity

Only cohorts receiving three doses were included in the immunogenicity analyses (A2-A3 and B2-B4). Immunogenicity data are presented as responder frequency (proportion of participants with a ≥ 4-fold increase of antigen-specific antibody titers from prevaccination), fold rise (≥4-fold) of antibody titers in individual participants, and geometric mean (GM) of antibody titers with 95% confidence interval (CI).

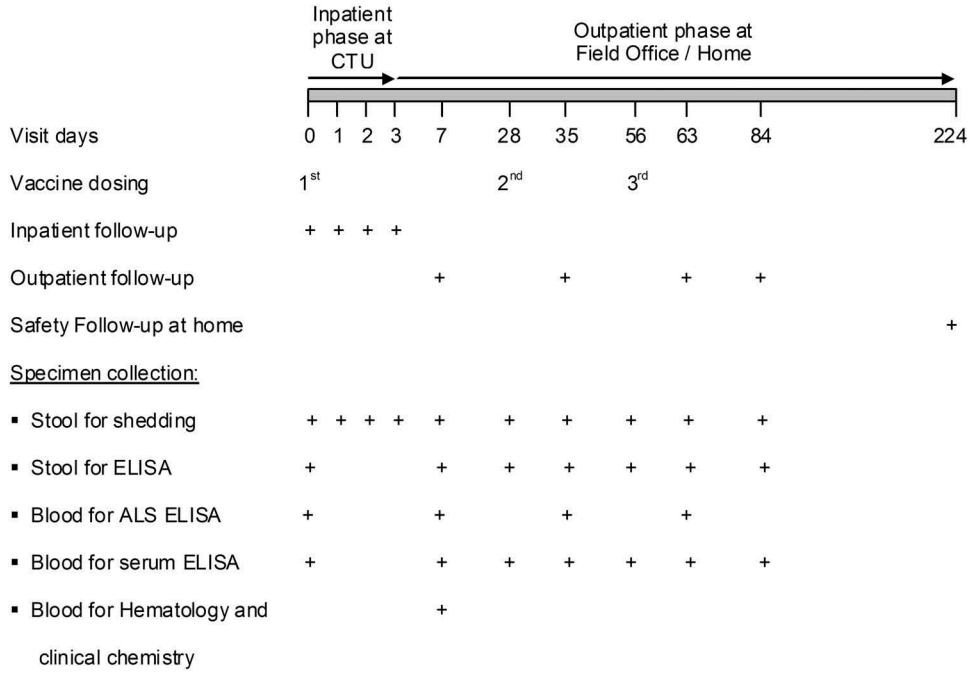

Figure 2. Outline for administration of vaccine/placebo, follow-up and specimen collection. Schedule for multi-dose cohorts (cohorts A2, A3, B2, B3 and B4) is given here. First vaccination and immediate inpatient safety evaluation for 72h was performed at the Clinical Trials Unit (CTU) of icddr,b. The second and third doses were given on an outpatient basis at the field office. Outpatient follow-up at day 7, 35, 63 and 84 were carried out at the field office. Long term safety follow-up took place at day 224 at the participants' home. cohorts A1 and B1were given single dose on day 0, followed-up on day 7 and 28, and long term safety follow-up was made on day 168.

(i) ALS antibody responses. LPS-specific IgA and IgG antibody in lymphocyte supernatant (ALS) was assessed before immunization and 7 days after each vaccination (Figure 2). In adults, the proportion of ALS IgA as well as IgG responders to LPS at any time after vaccination was 70% and 100% in the 5-log and the 6-log dose groups, respectively (Table 2), with the highest frequency obtained after the first vaccination (Supplementary Table 3) as expected in a primed population. Fold increase of antibody (post/pre-vaccination titers) in individual adult participants was as high as 310.7 for IgA and 1370.5 for IgG in the highest dose group (Supplementary Table 4). These responses were vaccine-specific since no placebo recipients had ≥4-fold increase in IgA or IgG ALS titers to LPS (Table 2).

In children, ALS antibody responses were notably weaker. In the 6-log dose group, the proportion of LPS-specific IgA and IgG responders after any vaccination were 40% and 50%, respectively (Table 2); multiple doses were required to achieve these responses (supplementary Table 3). At the individual level, the maximum fold increase of ALS IgA was 32.5 and that of ALS IgG was 28.3 (Supplementary Table 4). As in adults, these responses were vaccine specific in children, since placebos did not have ≥4 fold increase of LPS-specific ALS IgA and IgG (Table 2).

(ii) Serum antibody responses. LPS-specific IgA and IgG antibody titers were measured in serum before vaccination and on day 7 and 28 after each vaccination (Figure 2).In adults, LPS-specific serum IgA responder rates were 50% and 63%, respectively in the 5-log and the 6-log dose groups. Among placebo recipients, the responder frequency was 33% (Table 2). Highest responder frequencies in the adult vaccinees were obtained after the first vaccination (Supplementary Table 5). When comparisons of LPS-specific serum IgA titers (Figure 3) between vaccine and placebo recipients were made, significant increases were observed at day 63 (p = 0.007) and day 84 (p = 0.016) in the 5-log dose group and at day 63 (p = 0.016) in the 6-log dose group (Table 3).

In children, the responder frequency to serum LPS-specific IgA was dose dependent, and reached 70% in the highest dose group. However, 18% of the placebo recipients also responded to LPS (Table 2). In contrast to adult participants, responder frequencies in children were maximum after the 2nd or 3rd dose of the vaccine (Supplementary Table 5). After adjustment for age, sex and pre-existing titers, a significant increase in LPS-specific serum IgA titers was seen at day 7, 35, 56 and 63 in the highest dose group compared to placebo (Table 4). When serum IgA response in individual participants was considered, the magnitude of

Table 2. Proportion (%) of participants with ≥4-fold increase in *S.sonnei* LPS-specific antibody titers from baseline at any time post oral administration of WRSS1 vaccine or placebo in Bangladeshi adults and children.

		ALS		Serum		Stool
	Dose Group	IgA	IgG	IgA	IgG	IgA
Adult	3 x 10^5 CFU (n = 10)	70	70	50	0	30
	3 x 10^6 CFU (n = 8)	100	100	63	25	25
	Placebo (n = 6)	0	0	33	0	50
Children	3 x 10^4 CFU (n = 8)	0	0	25	0	25
	3 x 10^5 CFU (n = 11)	27	18	45	0	36
	3 x 10^6 CFU (n-10)	40	50	70	20	40
	Placebo (n = 11)	0	0	18	9	36

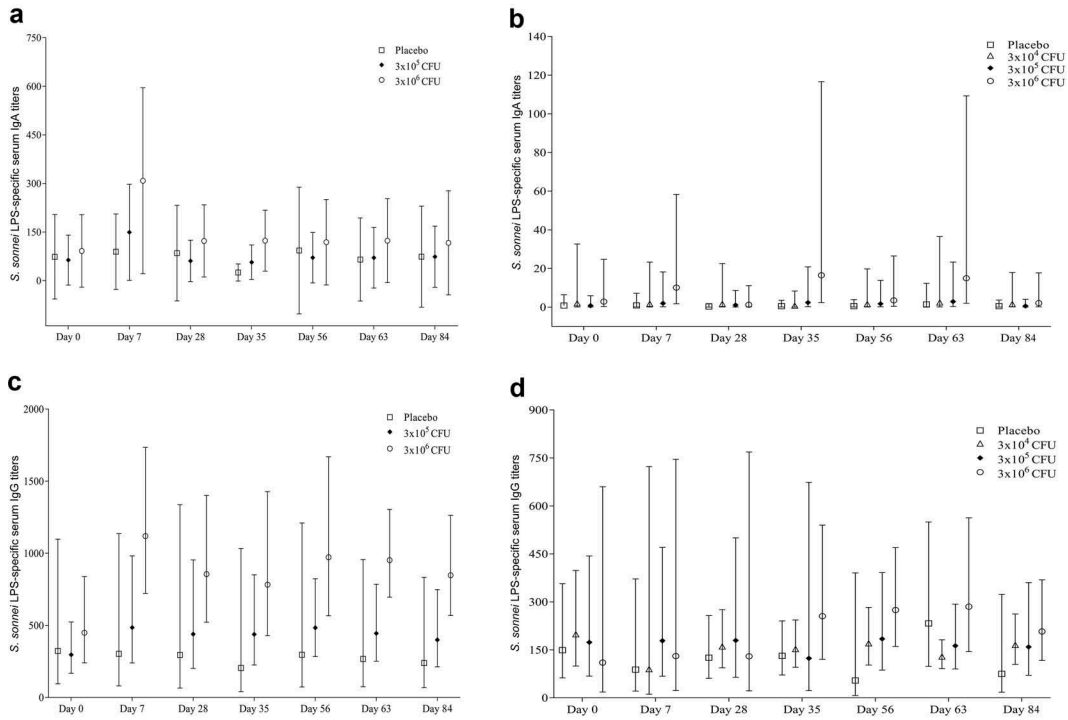

Figure 3. *S. sonnei* LPS-specific serum IgA and IgG antibody titers before and after administration of WRSS1vaccine or placebo in Bangladeshi adults (panels A, C) and children (panels B, D). Data are presented as Geometric mean with 95% confidence interval.

Table 3. Multivariate regression analysis of *S.sonnei* LPS-specific serum antibody responses in vaccine groups compared to placebo recipients among Bangladeshi adults.

			LPS-IgA		LPS-IgG	
		Placebo (n = 6)	$3x10^5$ CFU (n = 10)	$3x10^6$ CFU (n = 8)	$3x10^5$ CFU (n = 10)	$3x10^6$ CFU (n = 8)
Day 7	Unadjusted	Ref.	0.47 (−0.98, 1.92)	0.97 (−0.52, 2.46)	0.21 (−0.32, 0.73)	0.57 (0.10, 1.04)*
	Adjusted[a]	Ref.	1.15 (−0.13, 2.43)	1.32 (−0.17, 2.80)	0.44 (0.14, 0.73)**	0.73 (0.28, 1.17)**
Day 28	Unadjusted	Ref.	0.16 (−1.37, 1.70)	0.76 (−0.77, 2.29)	0.17 (−0.42, 0.76)	0.46 (−0.06, 1.00)
	Adjusted[a]	Ref.	0.75 (−0.60, 2.10)	0.99 (−0.40, 2.38)	0.43 (0.11, 0.75)*	0.66 (0.24, 1.08)**
Day 35	Unadjusted	Ref.	0.28 (−1.43, 2.00)	1.13 (−0.43, 2.69)	0.33 (−0.21, 0.87)	0.58 (0.05, 1.11)*
	Adjusted[a]	Ref.	0.44 (−1.14, 2.02)	0.91 (−0.72, 2.54)	0.44 (0.10, 0.77)*	0.79 (0.29, 1.28)**
Day 56	Unadjusted	Ref.	0.90 (−0.42, 2.22)	1.07 (−0.47, 2.61)	0.21 (−0.30, 0.69)	0.51 (0.004, 1.02)*
	Adjusted[a]	Ref.	1.16 (−0.15, 2.47)	0.93 (−0.62, 2.49)	0.37 (0.05, 0.69)*	0.62 (0.26, 0.97)**
Day 63	Unadjusted	Ref.	0.79 (−0.63, 2.20)	1.45 (0.21, 2.68)*	0.22 (−0.24, 0.69)	0.56 (0.13, 0.98)*
	Adjusted[a]	Ref.	1.43 (0.48, 2.39)**	1.34 (0.31, 2.37)*	0.43 (0.06, 0.80)*	0.63 (0.27, 1.00)**
Day 84	Unadjusted	Ref.	0.58 (−0.92, 2.09)	1.09 (−0.36, 2.54)	0.22 (−0.26, 0.70)	0.55 (0.12, 0.98)*
	Adjusted[a]	Ref.	1.33 (0.30, 2.36)*	1.00 (−0.31, 2.31)	0.34 (−0.01, 0.70)	0.60 (0.35, 0.86)***

Data are given as β-coefficient (Confidence Interval). [a]adjusted by age, sex and pre-existing titers. *p < 0.05, **p < 0.01, ***p < 0.001.

fold increase was much higher in children compared to adults (supplementary Table 6).

Both adults and children had high pre-existing *S. sonnei* LPS-specific serum IgG titers (Figure 3) reflecting prior exposure. Due to high baseline antibody titers, seroconversion of LPS-specific IgG antibodies was seen only in 25% of adults receiving the highest dose (Table 2). Despite poor responder rates in adults, WRSS1 showed significantly higher LPS-specific serum IgG titers compared to placebo (Table 3). In the 5-log dose group, significant difference were observed at day 7 (p = 0.008), day 28 (p = 0.014), day 35 (p = 0.017), day 56 (p = 0.027) and day 63 (p = 0.026) after controlling the post-vaccination titers for age, sex and pre-existing titers. In the highest dose group, LPS-specific serum IgG titers in vaccinees were significantly higher than placebo throughout the study period.

In children, 20% of the 6-log dose group and 9% of the placebo recipients showed IgG response to LPS at any time after vaccination (Table 2). After adjusting for preexisting titers, significant increases in antigen-specific serum IgG titers over placebo was seen in the children's 6-log dose group on day 56 and day 84 (Table 5).

(iii) Fecal IgA responses. LPS-specific IgA titers in stool were measured prior to vaccination and 7 and 28 days after each vaccination (Figure 2). Antigen-specific IgA responses in stool were normalized with total IgA. Among adults, responder frequencies for LPS-specific fecal IgA were 30% in the 5-log dose group and 25% in the 6-log dose group, while that in placebo

Table 4. Multivariate regression analysis of *S.sonnei* LPS-specific serum IgA response in Bangladeshi children receiving WRSS1 compared to placebo recipients.

			LPS-IgA		
		Placebo (n = 11)	$3x10^4$ CFU (n = 8)	$3x10^5$ CFU (n = 11)	$3x10^6$ CFU (n = 10)
Day 7	Unadjusted	Ref.	0.30 (−1.04, 1.64)	0.36 (−0.88, 1.60)	1.06 (−0.08, 2.20)
	Adjusted[a]	Ref.	0.27 (−0.60, 1.13)	0.49 (−0.35, 1.33)	1.34 (0.50, 2.17)**
Day 28	Unadjusted	Ref.	0.72 (−0.48, 1.91)	0.52 (−0.57, 1.61)	0.56 (−0.54, 1.69)
	Adjusted[a]	Ref.	0.69 (−0.10, 1.48)	0.63 (−0.02, 1.29)	0.75 (−0.19, 1.70)
Day 35	Unadjusted	Ref.	0.15 (−1.05, 1.35)	0.63 (−0.53, 1.79)	1.47 (0.37, 2.57)*
	Adjusted[a]	Ref.	0.15 (−0.78, 1.07)	0.70 (−0.27, 1.67)	1.52 (0.49, 2.56)**
Day 56	Unadjusted	Ref.	0.44 (−0.80, 1.68)	0.47 (−0.67, 1.62)	0.78 (−0.35, 1.91)
	Adjusted[a]	Ref.	0.39 (−0.43, 1.22)	0.56 (−0.05, 1.17)	0.94 (0.01, 1.88)*
Day 63	Unadjusted	Ref.	0.22 (−1.18, 1.63)	0.31 (−0.92, 1.53)	1.02 (−0.20, 2.23)
	Adjusted[a]	Ref.	0.15 (−1.12, 1.42)	0.38 (−0.65, 1.41)	1.14 (0.07, 2.20)*
Day 84	Unadjusted	Ref.	0.49 (−0.75, 1.73)	0.06 (−1.06, 1.17)	0.59 (−0.58, 1.76)
	Adjusted[a]	Ref.	0.45 (−0.33, 1.24)	0.16 (−0.29, 0.61)	0.72 (−0.25, 1.70)

Data are given as β-coefficient (Confidence Interval). [a]adjusted by age, sex and pre-existing titers. *p < 0.05, **p < 0.01.

Table 5. Multivariate regression analysis of *S.sonnei* LPS-specific serum IgG response in Bangladeshi children receiving WRSS1 compared to placebo recipients.

			LPS-IgG		
		Placebo (n = 11)	$3x10^4$ CFU (n = 8)	$3x10^5$ CFU (n = 11)	$3x10^6$ CFU (n = 10)
Day 7	Unadjusted	Ref.	0.01 (−0.97, 1.00)	0.30 (−0.42, 1.03)	0.17 (−0.74, 1.08)
	Adjusted[a]	Ref.	0.14 (−0.83, 1.11)	0.33 (−0.21, 0.87)	0.37 (−0.50, 1.24)
Day 28	Unadjusted	Ref.	0.11 (−0.30, 0.51)	0.16 (−0.35, 0.66)	0.02 (−0.71, 0.74)
	Adjusted[a]	Ref.	0.19 (−0.18, 0.57)	0.17 (−0.23, 0.56)	−0.10 (−0.83, 0.63)
Day 35	Unadjusted	Ref.	0.07 (−0.27, 0.41)	−0.03 (−0.73, 0.68)	0.29 (−0.10, 0.68)
	Adjusted[a]	Ref.	0.12 (−0.20, 0.45)	−0.01 (−0.67, 0.65)	0.32 (−0.05, 0.70)
Day 56	Unadjusted	Ref.	0.50 (−0.52, 1.52)	0.53 (−0.36, 1.43)	0.70 (−0.21, 1.62)
	Adjusted[a]	Ref.	0.59 (−0.33, 1.52)	0.56 (−0.22, 1.35)	0.99 (0.20, 1.79)*
Day 63	Unadjusted	Ref.	−0.26 (−0.74, 0.23)	−0.15 (−0.59, 0.28)	0.09 (−0.37, 0.55)
	Adjusted[a]	Ref.	−0.13 (−0.54, 0.28)	−0.15 (−0.58, 0.27)	0.19 (−0.17, 0.56)
Day 84	Unadjusted	Ref.	0.34 (−0.42, 1.11)	0.33 (−0.38, 1.03)	0.44 (−0.25, 1.14)
	Adjusted[a]	Ref.	0.40 (−0.26, 1.07)	0.36 (−0.21, 0.92)	0.69 (0.09, 1.28)*

Data are given as β-coefficient (Confidence Interval). [a]adjusted by age, sex and pre-existing titers. *p < 0.05.

recipients was 50% (Table 2). In children, responder frequencies for LPS-specific fecal IgA were 25%, 36% and 40% in the 4-log, 5-log and 6-log dose groups, respectively (Table 2). Among child placebo recipients, 36% responded to LPS. There were no significant differences in fecal IgA titers between vaccinees and placebo in both age groups even after adjusting for pre-existing titers.

Effects of shedding and reactogenicity on immune responses

To assess the effect of shedding of WRSS1 on immune response, LPS-specific antibody titers in ALS and serum were compared between shedders and non-shedders in the highest dose group of adults (Table 6). Multivariate regression analysis of antibody responses did not show any significant difference between shedders and non-shedders (data not shown). Furthermore, the effect of reactogenicity events on immune response profile was evaluated. No significant difference was found in the immunogenicity response between vaccinees with and without reactogenicity events in either adults or children (data not shown).

Table 6. Fold increase (post-/pre-vaccination)[a] of *S.sonnei* LPS-specific ALS and serum antibodies on day 7 among vaccine shedders and non-shedders in the 6-log dose group of adults.

		ALS		Serum	
Participant#	WRSS1 Shedder/Non-shedder	LPS-IgA	LPS-IgG	LPS-IgA	LPS-IgG
1	Non-shedder	63.7	19.4	-	-
2	Shedder	39.1	55.2	6.8	-
3	Non-shedder	-	-	-	-
4	Non-shedder	81.3	126.2	6.8	-
5	Shedder	15	64.3	-	-
6	Shedder	269.4	97.4	36.4	5.7
7	Non-shedder	310.7	1370.5	7.6	9.6
8	Non-shedder	244.6	179.5	1876	-
9	Shedder	109.9	170.9	-	6.9
10	Shedder	33.4	40.5	4.7	-

Data for each participant is given (intention to treat). [a] ≥ 4-fold increase was shown only; <4-fold increase in titers was indicated by '-'.

Discussion

This is the first Phase I trial of WRSS1, conducted in adults and children 5–9 years of age in Bangladesh, who represent different levels of immunologic experience in an endemic environment. Earlier trials with WRSS1 were conducted in naive US adults, Israeli soldiers and Thai adults, where a single dosing schedule was used.[17,19,20] In this study, up to three doses, spaced one month apart, were tested to maximize immune responses. The findings indicate that WRSS1 was safe and immunogenic in both age groups. Overall, children elicited lower mucosal immune responses than adults, but showed relatively higher systemic IgA responses, indicating that oral vaccine candidates can be evaluated in future studies, where infants and toddlers constitute the primary target.

WRSS1 up to the highest dose tested (10^6 CFU) demonstrated good safety profile in both adults and children in Bangladesh. In more naive US and Israeli adults, >30% of the participants receiving WRSS1 at doses above 10^4 CFU exhibited mild to severe constitutional symptoms including mild to moderate

diarrhea; the symptoms increased with increasing doses.[17,19] In contrast, only 10% of the Bangladeshi adults in the 6-log dose group and 8% of the children in the 4-log dose group had mild dirrahea; fever and other constitutional symptoms were mostly mild (in children, all symptomps were mild) and transient. A similar difference between naive adults, and endemic adults and children was seen for SC602 a *S. flexneri* 2a oral vaccine candidate.[21,22] These findings suggest that adults and children older than 5 years in endemic environments are immunologically more experienced than naïve non-exposed adults. This is also borne out by preexisting serum antibody titers to *Shigella* antigens in this population.

An important characteristic of live oral vaccines, especially against invasive microorganisms, is the association between vaccine shedding and immune responses, vaccine shedding being a marker for intestinal colonization. In US or Israeli adults, 50–90% of the volunteers receiving 3-log to 6-log doses of WRSS1 or SC602 shed the vaccine strain robustly for 5–7 days and mounted vigorous immune responses to LPS.[17,19,21] In Thailand, where *S. sonnei* is endemic, a single dose of 10^4 CFU of WRSS1 in adults led to much lower shedding and immune responses than observed in US and Israeli adults.[20] Even then, a moderate correlation between shedding and immune responses was demonstrated both post vaccination and post challenge. In Bangladesh, 50% of the adults at the highest dose shed WRSS1 for only one day post 24h vaccination, but no difference was seen in immunogenicity response between shedders and non-shedders. One could also argue that the vaccine shedding seen in Bangladeshi adults merely reflects the passage of the vaccine strain through the gut and is not an actual colonization of WRSS1. Still, 88% of the adults demonstrated *S. sonnei* LPS-specific ALS IgA and IgG responses after the first dose, indicating an anamnestic response in what would be considered primed individuals living in an endemic environment. Earlier studies have shown that primed adults mount higher immune responses to natural *Shigella* infection compared to naïve adults and children.[23-27] Antigen-specific memory B cells probably have a major role in this rapid response.[28] Although none of the Bangladeshi children shed the vaccine, they were still able to induce high systemic immunity. When SC602 was tested in Bangladeshi adults and children, LPS-specific serum IgA response was found only in the 4-log dose group which did not shed the vaccine.[22] These findings suggest that in an endemic population, with prior exposure to *Shigella*, robust colonization of oral live vaccines may not occur or necessarily influence the elicitation of immune responses.

In Bangladeshi children, where incidences of *Shigella* infection is presumably lower than in adults, multiple doses of WRSS1 was needed to increase the LPS-specific mucosal response though the magnitude of the response was lower than observed in Bangladeshi adults. Earlier studies by Mel *et al* have shown that multiple doses of a streptomycin-dependent live *Shigella* vaccine, administered at shorter intervals (separated by 3 days) to children living in an endemic environment helped achieve improved immunity.[29] In Bangladesh, the dosing of WRSS1 was planned to match the Expanded Program of Immunization (EPI) schedule. Even with the longer interval (28 days), fold increase in serum IgA in children was mostly seen after the 2nd and 3rd doses. Importantly, the systemic IgA response in children was better than that of adults. The generally lower responder frequency with respect to LPS-specific serum IgG in both adults and children may be partially explained by the presence of high pre-existing IgG titers resulting from previous and sustained exposure. This observation of high circulating IgG titers directed to *Shigella* antigens in endemic regions is well-known.[23,30,31] After adjusting for preexisting titers, significant increase in post-vaccination IgG response was seen in both adults and children, which indicates that previous exposures can blunt the response to an oral vaccine. High proportion of placebo recipients responding with fecal IgA to *S. Sonnei* LPS also indicated high prevalence of *S. sonnei* infections in the community. From the present results, it is apparent that in children, antigen-specific serum IgA antibody was a more sensitive biomarker of oral vaccination than ALS IgA or IgG and fecal IgA responses. Similar findings were reported in children and naive adults receiving oral ETEC vaccines.[32,33]

Lack of colonization and modest immunogenicity in endemic populations have been seen with other live oral vaccines such as polio, cholera, and rotavirus vaccines, that also require higher and repeated doses to be effective.[8,34-38] Reasons for colonization resistance in children are complex, multifactorial and poorly understood. These include breastfeeding, genetic risk factors (histo blood group antigens, variants within the HLA locus), malnutrition, micronutrient deficiencies, exposure to a wider variety of enteric organisms and higher pre-vaccination antibody titers.[39] Additionally, environmental enteropathy and the gut microbiome may play significant roles in colonization resistance.[39-46] Comparison of stool microbiota and stool metabolome between naive and primed subjects may help to elucidate the nature of these differences.

Although both SC602 and WRSS1 have now been tested in Bangladeshi adults and children, the immune responses elicited by WRSS1 are greater than previously seen with SC602. Administration of lower volumes of bicarbonate buffer in this study may have contributed to the improved immune response. A recent study has shown that much lower volumes of bicarbonate buffer than traditionally used can neutralize gastric acidity and be more optimal since *Shigella* is fairly acid resistant and the viability of *Shigella* is lower in bicarbonate solution.[47] Other means of improving the immune responses could be the use of an adjuvant such as the double mutant heat-labile toxin (dmLT) that was shown to increase the efficacy of ACE527, a live attenuated ETEC vaccine candidate.[48]Additional studies such as serum bactericidal activity and cytokine responses with WRSS1 cohort samples are ongoing, which may help to identify protective immune mechanisms in endemic populations.

Some of the limitations of this study are the lack of optimization of the dose, the number of doses, the dosing schedule and the bicarbonate volume to be ingested. Also, in order to be effective a *Shigella* vaccine must have more than one serotype. Currently a quadrivalent vaccine appears to be the goal, consisting of *S. flexneri* 2a, 3a, 6 and *S. sonnei* which are the prevalent circulating serotypes worldwide. It remains to be seen how such a mixture of serotypes will be combined to be effective in an endemic environment, realizing that there may be different levels of preexisting antibodies to the various *Shigella* serotypes. In addition, the real target of

such an oral vaccine are infants and toddlers less than 5 years of age, since children 5–9 years of age are apparently quite resistant to *Shigella*. Nonetheless, this study provides a starting point for future work in this area, and suggests that a live oral vaccine can be safely tested in a target population where it is most needed

Materials and methods

WRSS1 vaccine strain

WRSS1 was manufactured in 1997 under current good manufacturing practice at the Walter Reed Army Institute of Research (WRAIR) Pilot Bioproduction Facility.[15] Lyophilized vaccine vials (lot#0451) are removed periodically from −80° storage and tested for viability and stability of the Form I phenotype. Since its manufacture, WRSS1 has maintained its viability (~1–2 x10^{10} CFU/vial) and its stability with >90% Form I phenotype, i.e., round smooth-edged colonies indicating retention of the large virulence plasmid that encodes the genes for epithelial cell invasion as well as for LPS O-antigen synthesis. The lyophilized vaccine was shipped from WRAIR to icddr,b on dry ice where it was maintained at −65°C to −85°C.

Study design

This study was designed as a double-blinded, randomized, placebo-controlled, dose-escalating, age-descending study, starting out with healthy adults and sequentially moving into school-age children. The study was conducted between August 2013 and March 2016 at icddr,b, Dhaka, Bangladesh. Thirty nine healthy adults (18–39 years) and 64 healthy children (5–9 years) who met the eligibility criteria (Supplementary Table 1) were enrolled at the urban field site in Mirpur, a suburb of Dhaka. Some of the important inclusion criteria were (a) absence of obvious health problems as determined by clinical examination and medical history, (b) normal bowel habits (defined by <three grade 1 or 2 stools each day; ≥one grade 1 or 2 stools every 2 days) and (c) negative pregnancy test. Exclusion criteria included (a) significant medical or psychiatric abnormalities or any condition that might jeopardize the safety of study participants or interfere with the evaluation of the study objectives, (b) significant abnormalities on physical examination and in screening hematology and serum chemistry, (c) febrile illness within 48 hours prior to vaccination, (d) diarrhea within 7 days before vaccination, (e) receipt of antimicrobials within 7 days before vaccination, and (f) prior receipt of any *Shigella* vaccine. Written informed consent was obtained from adult participants and from guardians of children. Adults were randomized in 3 cohorts (cohorts A1-A3) and children in 4 cohorts (cohorts B1-B4). Each cohort of 13 adults (10 vaccinees and 3 placebo recipients) received either one dose of 3×10^4 (cohort A1) or three doses of 3×10^5 (cohort A2) or 3×10^6 (cohort A3) CFU of WRSS1 vaccine or placebo. Each cohort of 16 children (12 vaccinees and 4 placebos) received either one dose of 3×10^3 (cohort B1) or three doses of 3×10^4 (cohort B2), 3×10^5 (cohort B3) or 3×10^6 (cohort B4) CFU of vaccine or placebo. In each cohort, administration of the first dose and immediate inpatient safety evaluation for 72h were performed at the Clinical Trial Unit of icddr,b; the second and third vaccinations and all follow-up visits on days 7 and 28 for single-dose cohorts and days 7, 35, 63 and 84 for multi-dose cohorts were conducted at the field office (Figure 2). Long term safety follow-up took place at day 168 (single dose cohorts) and 224 (multi-dose cohorts) at the participants' home.

Administration of vaccine, placebo and bi-carbonate buffer

Lyophilized WRSS1 vaccine was reconstituted in 5 ml of sterile water and serially diluted in saline to the appropriate dose. One ml of the vaccine was mixed with 30 ml (adults) or 15 ml (children) of normal saline for ingestion. Inoculum concentration was verified before and within 2 hours after dosing by viable-cell counting of the diluted vaccine. The viability and stability of WRSS1 vaccine strain remained similar at pre- and post-vaccination time points. Placebo recipients received the same volume of normal saline. To neutralize gastric acidity, vaccination was preceded by an oral administration of 100 ml (adults) and 50 ml (children) of 0.15M bicarbonate buffer. The buffer volumes used here were lower than in previous trials with *S. flexneri* SC602[22] and WRSS1,[17,19,20] where 150 ml of bicarbonate in adults and 50–75 ml in children were used. Participants fasted for 90 minutes before and after ingestion of the vaccine/placebo.

Participants were randomized to receive vaccine or placebo using sequential, simple random sampling. The sequence was generated in SAS® (North Carolina, USA) and codes were assigned to participants using individual, sealed, and sequentially numbered envelopes.

Safety evaluation

For evaluation of safety, AEs including reactogenicity, and SAEs were assessed according to the following definitions. Adverse event (AE) is any unintended medical occurrence (new events or worsening of pre-existing conditions) in clinical trial participants during the conduct of the trial. AEs may or may not be associated with the study drug. Reactogenicity are those events that according to the current Investigator's Brochure and investigational plan/protocol are known to be caused by the study drug. Based on the prior studies, WRSS1 vaccine may result in symptoms similar to infection with *Shigella*, including loose stool, diarrhea, dysentery, nausea, vomiting, abdominal pain, abdominal cramps, bloating, constipation, fever, chills, headache, lightheadedness, generalized myalgia, malaise, decreased appetite, excess flatulence, reactive arthritis and arthralgia. In the current protocol, these symptoms were defined as reactogenicity events only when these occurred immediately after vaccination, i.e. up to 6 days after the first vaccination and 72h post second and third vaccination. If these events were experienced by the participants outside of the protocol-defined period, clinical judgment was applied to find out whether they were related to WRSS1. Events outside of the above list, irrespective of their occurrence time were also clinically judged to be related or unrelated to WRSS1. SAE is any AE that leads to (1) hospitalization or prolongation of existing hospitalization, (2) immediate risk of death (life-threatening), (3) persistent or significant incapacity or substantial disruption of the

ability to conduct normal life functions, (4) congenital abnormality or birth defect, (5) a medically important event that may jeopardize the participant or may require intervention to prevent one of the other outcomes listed above (e.g. intensive treatment at home for allergic bronchospasm; blood dyscrasia, or convulsions that do not result in hospitalization), (6) death.

AEs were monitored via focused medical interview and physical examination during the inpatient period, and on scheduled and unscheduled (need-based) follow-up visits in the field office. On days 4–6 and 72h post second and third vaccination at home, adult participants or guardians of the children recorded the frequency of defecation and stool grade in a simple memory aid. Other symptoms including temperature were documented by field staff during this period or when necessary. Laboratory evaluation including haematological and biochemical parameters was performed 7 days after the first vaccination and as suggested by the study clinicians. Participants with AE including SAE were provided appropriate care and treatment.

Assessment of fecal shedding

Stool samples were transferred using swab sticks to vials containing buffered glycerol saline within 3 hours of collection before transportation to the laboratory at icddr,b. In absence of stool, rectal swabs were collected in buffered glycerol saline. Stool or rectal swab samples were plated on Hektoen Enteric Agar and incubated at 37°C for 18 hours. Non-lactose-fermenting colonies were tested by agglutination with *S. sonnei* antiserum (Denka Seiken, Tokyo, Japan). Isolated *S. sonnei* was then tested for the *virG(icsA)* deletion in WRSS1 by PCR assay as described previously.[20]

Immunogenicity evaluation

Venous blood was collected from participants in BD Vacutainer SST tubes (Becton Dickinson, Franklin Lakes, NJ, USA) to separate serum by centrifugation. For ALS responses, blood was collected in BD sodium heparin containing tubes (Becton Dickinson). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, incubated in cell culture media at a concentration of $1x10^7$/mL at 37°C, 5% CO_2 for 72 h without any antigenic stimulation and the supernatant was collected and stored at −80°C. To extract protein-rich fraction from stool, extraction buffer containing soybean trypsin inhibitor (0.1 mg/ml) (Sigma, cat#T9003), EDTA (0.05M) (Sigma, cat#E5234), PMSF (0.002M) (Sigma, cat#P7626) and Tween 20 (0.05%) (Sigma, cat#P1379) in PBS (pH 7.2) was added to stool samples (4 ml buffer per gram of stool). After thorough mixing, the suspension was filtered using gauze fabric to remove large debris. The filtrate was finally centrifuged at 12,000g for 30 min and the supernatant was collected and stored at −80°C.

Endpoint antibody titers in serum, ALS, and stool extract were determined by enzyme-linked immunosorbent assay (ELISA) as described previously.[20] In brief, 96-well U-bottom polystyrene microtiter plates (medium binding; Thermo Scientific, Rochester, NY) were coated with 1 μg/well *S. sonnei* LPS. Non-specific antigen-binding sites were blocked with 2% casein filler (Sigma, cat#C5890) for ALS and serum specimens or 1% bovine serum albumin (Sigma, cat#A7906) in PBS for stool specimens. All samples were serially diluted (2-fold) and added into the wells in duplicate; only diluents were added as blank controls. Following incubation and washing, anti-human IgG (1:500) or IgA (1:500) conjugated with alkaline phosphatase (KPL, Gaithersburg, MD) were added for ALS and serum and subsequent color reaction was developed by adding para-nitrophenyl phosphate (pNPP) (Sigma, cat#71768) as substrate. For stool specimens, IgA conjugated with horseradish peroxidase (KPL) and 2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS)-peroxidase substrate system (KPL, cat#50-62-00) were applied. Microtiter plates were read at 450 nm in an automated ELISA reader (Thermo Scientific Multiskan EX, Thermo Fisher Scientific, Waltham, Massachusetts). Titers were calculated by interpolating the dilution of serum, ALS and stool specimens, which yielded an optical density (OD) ≥0.2. Pre-vaccine and post-vaccine sera were run in batches.

Total IgA level in stool was measured by biochemistry analyzer (Hitachi 902, Roche Diagnostics GmbH, Boehringer, Mannheim, Germany) and antigen-specific fecal IgA response was normalized to total IgA.

Ethics statement

The clinical trial was conducted under an Investigational New Drug application (IND 15335) to the United States Food and Drug Administration. Ethical clearance was obtained from the ethical review committee of icddr,b and the Western Institutional Review Board. The trial was carried out in accordance with the standards of the International Conference on Harmonization guidelines on Good Clinical Practices (ICH-GCP), and followed the ethical principles established in the Declaration of Helsinki. All adult participants and guardians of child participants provided written informed consent prior to enrollment. The trial was registered at www.clinicaltrials.gov as NCT01813071.

Statistical analysis

The statistical analyses were performed with Stata 13 (StataCorp, LP, College Station, Texas, USA) and Statistical package for the Social Science (SPSS) for Windows (version 20; Armonk, NY: IBM SPSS corp.; 2011). Figures with immunological data were prepared using the GraphPad Prism 7.0. For safety evaluation, intention-to-treat analyses were performed. Proportion of vaccine and placebo recipients with specific reactogenicity event was compared using chi-square test. For immunogenicity evaluation, per-protocol analyses were performed, which included participants completing all vaccination doses. When individual participant's immunological data were shown, all participants (intention-to-treat) were included in Tables. Normal distributions of the residuals in all the models were checked with normal k-density curve, probability of skewness and kurtosis and q-q plots. IgA and IgG titers in serum and stool was log-transformed to normalize data. Multivariate regression analysis was performed adjusting for age, sex and pre-existing titers to compare the means of serum and stool antibody

titers between vaccine and placebo recipients at each time point. Multivariate regression analysis was also used to evaluate the mean changes of serum, ALS and stool antibody titers between shedders and non-shedders as well as between participants with and without reactogenicity events. A p value <0.05 was regarded as significant.

Data availability

The data supporting the findings of this study are available from the corresponding author upon request.

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Disclosure of potential conflicts of interest

RR is the principal investigator. RR, KZ, NM, AB, RW, AF, MV conceived and designed the study. RR, PS, KZ, NHA, TW, NM, KT, AB, AS, FQ, RW, AF, MV participated in interpretation of results. AS and MV evaluated viability and stability of the vaccine candidate. RR and PS were responsible for the immunological analyses, and KZ and NHA for the clinical assessments and analyses of adverse events. RR and PS wrote the manuscript. All coauthors contributed to the critical review and revision of the manuscript and have approved the final version. The authors report no conflict of interests.

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