The oral cholera vaccine Shanchol™ when stored at elevated temperatures maintains the safety and immunogenicity profile in Bangladeshi participants

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Informed consent was obtained from the study participants. The research was approved by the Ethical Review Committee of the International Centre of Diarrheal Disease Research, Dhaka, Bangladesh and IRB of the International Vaccine Institute. The study is registered in the Clinical Trials Data Bank (<http://clinicaltrials.gov>)

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Abstract

Background: The oral cholera vaccine (OCV), Shanchol™ has shown protective efficacy lasting up to 5 years, however, requirement for a cold chain limits its use in resource poor settings. The study was conducted to determine the safety and immunogenicity of Shanchol in adult participants in Bangladesh when stored at elevated temperatures.

Methods: The study was conducted in Mirpur, Dhaka. Four groups of healthy adult participants received two doses of Shanchol™, kept under standard storage temperature (Group A; 2-8°C) or at elevated temperatures (Group B; 25°C, Group C; 37°C, Group D; 42°C) for 14 days, respectively. Vaccine specific antibody responses were determined.

Findings: 145 participants were assigned to each group. Adverse events were mild not differing among groups. Vaccine stored at elevated temperatures remained stable with cumulative LPS content within admissible limits.

Vibriocidal antibody responses were observed in all groups after each dose of vaccine at day 7 and 21 compared to pre-immune levels ($P < 0.001$). Four-fold increases to *V. cholerae* O1 Ogawa were observed at day 7 and/or day 21 after vaccination in the standard temperature and the three elevated temperature groups, with responder rates of; 76% (95% CI LB; 70%), 80% (95% CI LB; 74%), 69% (95% CI LB; 63%), and 74% (95% CI LB; 68%) in Groups A, B, C, and D respectively ($P = 0.240$). Responses were also seen in all groups to *V. cholerae* O1 Inaba and *V. cholerae* O139 and in LPS specific IgA response to *V. cholerae* O1 antigens.

Interpretation: This is the first report to show that the OCV is stable at elevated temperatures, and the safety and immunogenicity profiles are not altered. This information will help formulate global policies for use of the vaccine at higher temperatures, resulting in easier distribution and vaccination costs and decrease logistical challenges to vaccine delivery.

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Key Words: ShancholTM; Oral cholera vaccine; Immune response; Thermal stability; Cold chain

1. Introduction

Over the past 30 years cholera has been spreading globally with an increasing trend, causing epidemics. Immunization against cholera is now recognized as a major intervention for control of the disease [1, 2]. Recently large studies have been carried out with an affordable oral, killed whole cell cholera vaccine (OCV), Shanchol, and have provided evidence of substantial protective efficacy [3-7] lasting for up to 5 years after immunization [3]. However, even though the vaccine has several important attributes which make it useful for delivery to age-groups, one year and above, the vaccine's label mandates for cold chain storage until delivery at 2-8°C, which can hinder its use in certain resource poor settings where cholera is prevalent. Globally cholera is seen in warm settings where ambient temperatures can exceed 40°C. Shanchol consists of heat and formalin inactivated whole cell components of *V. cholerae* O1 and O139; the major protective antigen, LPS, is heat stable [7]. Therefore, it should be possible to use the vaccine outside the cold chain of 2-8°C without changes to its stability, safety and immunogenicity profile.

Temperature stability studies of vaccines under the EPI (Expanded Programme for Immunization) have been carried out by national immunization programs as well as for hepatitis B vaccine [8, 9]. The recommended storage temperature of Shanchol, 2-8°C has been maintained in studies in India, Bangladesh and Haiti [10-13]. It is therefore very important to determine the effect of elevated storage temperatures on the stability, safety and immunogenicity profile of

Shanchol. The present paper reports on such a study conducted in adult participants in Bangladesh.

2. Methods

2.1. Field site and study population

The study was conducted in 580 healthy participants aged 18-45 years in Mirpur, Dhaka, Bangladesh [5, 14]. Participants consenting to the study were enrolled based on exclusion criteria: fever or gastrointestinal disorder in past 7 days, recent receipt of anti-diarrhoeal medication, known chronic illness or immunocompromised, pregnancy, or earlier receipt of OCV.

2.2. Ethical considerations

The protocol was approved by the IRB of the icddr,b. Written informed consent was obtained from all participants. The study was registered as ClinicalTrials.gov number, NCT01762930.

2.3. Sample size

The study was designed as a non-inferiority trial, whose primary aim was to exclude inferior immune responses in the higher temperature storage groups relative to the standard temperature storage group. Assuming a true immunogenicity of vaccine with standard temperature of 60% and immunological response at least 40% in recipients in different groups (25°C, 37°C and 42°C), to exclude a standard-experimental temperature difference of 5% (lower boundary of the 1-tailed 95% confidence interval) in immunogenicity with a power of 80%, the minimum number of participants required for each group was 132 and with 10% attrition, the number was 145 in each of the 4 groups A,B,C and D.

2.4. Study intervention: Oral cholera vaccine (Shanchol)

The study was a non-blinded open study. The investigators in the laboratory carrying out immunological assays were blinded as to the allocation using the OCV, Shanchol. Each dose of the Shanchol vaccine is remain in a 1.5 ml vial , contained heat killed and formalin killed whole cell bacteria consisting of 600 ELISA Units (EU) of lipopolysaccharide (LPS) of formalin-killed *V. cholerae* O1 Inaba, El Tor strain Phil 6973, 300 EU LPS of heat-killed *V. cholerae* O1 Ogawa classical strain Cairo 50; 300 EU LPS of formalin killed *V. cholerae* O1 Ogawa classical strain Cairo 50; 300 EU LPS of heat-killed *V. cholerae* O1 Inaba, classical strain Cairo 48; and 600 EU LPS of formalin killed *V. cholerae* O139 strain 4260B [13]. The Shanchol is suspended in a buffer known as the Oral Cholera Vaccine Diluent (OCVD) which is composed of sodium phosphate.

2.5. Vaccine allocation

The vaccine vials were labeled based on the temperature at which they had been incubated and grouped as A-D (Group-A; 2-8°C, Group-B; 25°C, Group-C; 37°C and Group-D; 42°C).

2.6. Study procedures

ShancholTM was incubated at 25°C, 37°C and 42°C for 14 days in incubators, calibrated to the specified temperatures. Following this, vaccines were stored at 2-8°C until used for the immunizations. To measure the LPS content after keeping at different temperatures, a set of 5 vaccine vials from each group was analyzed at Shantha Biotechnics Private Limited, Hyderabad, India. Two doses of vaccine were administered at a 14- day interval [13].

The study participants were monitored 14 days after each vaccination to assess the safety of the study agent. After each dose of vaccine, the recipients were observed for 30 minutes in the field clinic by a study physician. Trained study staff visited the participants' homes daily for the first 3 days post vaccination to record adverse events such as diarrhoea, vomiting, nausea and

other local and systemic reactions. For the remaining 11 days, all participants were instructed to visit the field clinic to record if they had any adverse events. The same schedule was maintained after the second dose. Diarrhoea was defined as 3 or more loose or watery stools within a 24 hour period. An adverse event is defined as an untoward medical event with an onset up to 14 days after receipt of a dose which may or may not be associated with the vaccine and serious adverse events as any untoward medical occurrence as has been carried out earlier [15].

Blood samples were obtained at day 0 prior to the first dose of vaccine and 7 days after each dose of vaccine at day 7 and at day 21. Acute responses to the vaccine were measured by serum vibriocidal antibodies and *V. cholerae* O1 Ogawa and Inaba LPS specific antibodies using serum specimens collected at the 3 time points [16-19].

2.7. *Method of vaccine LPS content measurement*

Shanchol vaccine is consist of inactivated strains of O1 Inaba Cairo 48, O1 Ogawa Cairo 50, O139 4260B and O1 Inaba El Tor Phil 6973. The LPS content of each strain in monovalent bulks and finished product (vaccine) is quantitatively measured by an inhibition ELISA. Strain specific purified LPS antigen is used as a coating reagent and the coated plates incubated for ~ 20 hours. Three fold dilutions of test samples and LPS standard are prepared in another 96 well plate. To these standard and sample preparations, strain specific primary antibodies are added and incubated to form Antigen-Antibody complexes. These Antigen-Antibody complexes suspension is transferred to the first coated plate to facilitate for binding of leftover primary antibodies to the LPS antigen. Strain specific primary antibodies bind to the specific LPS antigen to form complex. To this Ag-Ab complex, Horseradish peroxidase (HRP) conjugate binds and this reaction forms a color by the addition of tetramethylbenzidine (TMB) Substrate. Higher the concentration of the primary antibodies the stronger the color change. Color strength is

quantitatively measured by a Spectrometer. Absorbance values of these preparations are used for the calculation of LPS content in L.E.U/mL (LPS ELISA units per mL).

2.8. *Study outcomes*

The primary outcome of the study was to measure the safety and vibriocidal immune responses in Bangladeshi participants to Shanchol stored at elevated temperatures. Secondary outcomes were to measure the antibody responses to LPS.

2.9. *Data Analysis*

Data were double-entered into the data base using FoxPro Version 6. Descriptive statistics were generated using SPSS for Windows (Version 17) or Stata version 13. Overall rates of adverse reactions and seroconversion were compared by χ^2 test with Yates correction or by the Fisher exact test when mandated by small numbers. Seroconversion for the vibriocidal antibody response was defined as ≥ 4 fold rise in reciprocal serum titer and for the serum anti-LPS antibody response, a ≥ 2 fold rise between pre and post-vaccination levels. Titers were logarithmically transformed to measure the fold increases before statistical analyses. Graphs were generated using GraphPad Prism 5 software. The Wilcoxon signed Rank test and Mann Whitney U test were used where appropriate. We also performed Kruskal Wallis non parametric ANOVAs test for comparison of the four groups. Multiple linear regression models were used to adjust confounding variables, age and sex using Stata 13.

3. **Results**

3.1. *Study participants and adverse event monitoring*

Participants were divided into four groups based on vaccine temperature coding in groups A-D (Fig. 1). The male to female ratio and age distribution were comparable between the groups.

Mild adverse events were reported during the 28 days of follow-up after vaccination and there were no significant differences observed between the groups B-D with group A ($P=1.000$). All adverse events were tabulated regardless of whether they were judged causality related to dosing or not (Table 1). No severe adverse events were observed.

3.2. Vaccine antigen stability

The cumulative antigen (lipopolysaccharide) content of the constituent strains (collective value of LPS content of all the four strains) should not be less than 2100 LEU/SHD (Lipopolysaccharide ELISA Units/Single Human dose) according to the information in the package insert of Shanchol. For vaccines kept at 2-8°C, the cumulative antigen content was 2697 LEU/SHD. After storage at 25°C this was 2506 LEU/SHD, after storage at 37°C it was 2668 LEU/SHD, and after storage at 42°C it was 2524 LEU/SHD (Table 1).

3.3. Vibriocidal antibody response to *V. cholerae* O1 and O139

The baseline geometric mean titers (GMT) to *V. cholerae* O1 Ogawa serotype were 71, 55, 93, and 66 in Groups A-D, respectively ($P=0.028$), and there were over 7 fold increases of GMT in Group-A and in Group-B, and 6 fold in Group-C and in Group-D at day 21 ($P= 0.368$). In all groups, increases of vibriocidal responses of GMT were observed on days 7 and 21 compared to baseline ($P<0.001$). The cumulative responder frequency in Group A was 76% (95% CI Lower boundary (LB); 70%) and 80% for Group B (95% CI LB;74%), 69% (95% CI LB;63%) for Group C, and 74% (95% CI LB;68%) for Group D ($P=0.240$). Seroconversion rates in the groups in the three elevated temperatures (Groups B, C and D) were comparable with the Group A ($P=1.000$) (Fig. 2).

For the Inaba serotype, the baseline GMT was 98, 71, 84 and 68 in Groups A, B, C and D respectively ($P=0.306$). Over 8 fold rises of GMT were observed after receipt of two doses of vaccine in all groups ($P= 0.781$). Significant increases were observed on days 7 and 21 compared to baseline GMT at day 0 ($P<0.001$). Cumulative vaccine seroconversion rate to Inaba serotype in Group A was 76% (95% CI LB;74%) and in Groups B,C and D were 78% (95%CI LB;72%), 82% (95% CI LB;77%) and 77% (95% CI LB;71%) respectively ($P=0.659$). Comparable seroconversion rates were observed between the 4 groups ($P=1.000$) (Table 2). For the O139 serogroup, higher magnitude (GMT) was also seen to the O139 serogroup on days 7 and 21 compared to baseline ($P<0.001$). Comparable response rates were observed to O139 between the recommended temperature of storage and the other elevated temperatures ($P=.460$). (Table 3).

3.4. LPS- specific IgA antibody responses

We also assessed antibody responses against *V. cholerae* O1 LPS, prior to vaccination and post vaccination at day 7 and day 21.

The baseline GMT to O1 Ogawa LPS-specific IgA in Group A were 72, Group B;61, Group C; 73 and in Group D was 68 ($P= 0.334$). There were more than 3 fold increases of GMT in Group A and 4 fold increases observed in the other three groups at day 21 ($P= 0.039$). The GMT levels increased within 7 days of vaccination post each dose ($P<0.0001$). The cumulative response frequency in Group A was 62%, Group B ;75% ,Group C ;76% and Group D 74 % ($P= 0.034$).

Similarly a 76% LPS specific O1 Inaba response rate was seen in the standard temperature, Group A, and 70%, 81% and 84% in the other 3 elevated temperature groups ($P=0.028$). Comparable seroconversion rate was noted in both LPS specific Ogawa and in Inaba

in all 4 groups. Higher GMT were observed at day 7 after intake of the first dose than at day 21 after intake of second dose in all groups ($P<0.0001$) (Table 4).

4. Discussion

Information on the thermal stability of the oral cholera vaccine is needed for appropriate use of the vaccine in resource limited settings lacking cold chain management facilities. Such populations include some with endemic cholera, as well those affected by cholera after natural emergencies, such as typhoons, earthquakes, and tsunamis. Our results show that Shanchol has a good safety and immunogenic profile when stored under ambient temperature or even as high as 42°C for up to 14 days. Under these conditions the vaccine LPS antigenic content was also unaltered. Thus, the vaccine can be temporarily kept out of the cold during the vaccination phase of OCVs in epidemics or even during mass immunization programs in endemic countries. This will provide a margin of safety for storage and administration of this vaccine when cold chain facilities are limited. The immunogenicity of Shanchol when stored at higher temperatures is in line with previous results demonstrated in different settings, when vaccine was stored at 2-8°C as recommended by the manufacturer [10-13]. Reassuringly, no serious adverse events were incurred by the study participants after receipt of Shanchol under any of the tested thermal storage conditions.

In the study, the immune response in vaccine recipients were determined by vibriocidal antibody assay, since it is the standard measure of immunogenicity of OCVs [20]. It was encouraging to observe that the vibriocidal responses after intake of the first and second dose increased significantly compared to baseline pre-immune responses regardless of whether the vaccine was stored at 2-8°C or at the different elevated temperatures. Response rates both in

Ogawa and in Inaba serotype, vibriocidal antibodies were similar to that seen in other studies where vaccine was stored at recommended temperature in Bangladesh, Kolkata and Haiti [10, 12, 13]. The seroconversion rate for *V. cholerae* O139 vibriocidal antibodies was comparable in the different temperature groups (Groups A-D) and lower, as expected, than that seen in to the O1 serogroup vibriocidal antibodies.

In all the storage temperature groups, a robust vibriocidal antibody response to *V.cholerae* O1 was seen within 7 days of intake of a single dose of Shanchol which was significantly higher than the response seen at day 21 after intake of two doses. A significantly lower vibriocidal antibody response was seen after the second vaccination. This has been seen in previous studies in adults carried out in Bangladesh and India [11,13]. The reasons for this phenomenon have not been well explained, but clearly reflect the inability of the second dose to boost the response to the first dose in the Bangladeshi population, who already have a high level of preexisting immunity to cholera and for which the first dose is itself probably a booster dose [18, 21]. In non-primed vaccine recipients in Sweden and Haiti, however, the second dose elicits a higher response [10]. Moreover, in young children living in Bangladesh, a group that also lacks a high level of preexisting anti-cholera immunity, the second dose of the vaccine given within two weeks results in an increased response compared to that seen after the second dose [13]. Regardless, storage of the vaccine at elevated temperatures does not seem to change the immunogenicity profile of the vaccine.

The LPS specific IgA responses were comparable in all four storage-temperature groups. These results compare well with the results of the cumulative LPS content of the vaccine which remains above the acceptable level of 2100 LEU/SHD when stored at elevated temperatures [22].

261 This is the first study in which the impact of thermal storage conditions has been assessed
262 for Shanchol functionally, on both vaccine safety and immunogenicity. This is important,
263 because one of the manufacturing release assays for Shanchol, based only on an ELISA
264 assessment of LPS content, though specific for 01 and 0139 serogroup LPS, may not detect
265 thermal damage to the functional antigenicity of the killed whole cells. A few reports on thermal
266 stability of killed whole cell-based OCVs have been conducted earlier in animal models[23-
267 26]. [ENREF 22](#) In a study carried out with an OCV containing cholera toxin B subunit together
268 with killed whole cells (Dukoral™; Crucell Sweden AB) tested in rabbits, the response to LPS
269 remained unaltered by storage at ambient temperatures while the cholera toxin B response
270 decreased to 50%. However, Shanchol does not contain the cholera toxin B subunit.

271 There are certain limitations to this study. First, the study was conducted only in adults
272 and not in younger age groups. Since, as noted earlier, the pattern of immune responses to
273 Shanchol differs in young children, future studies should assess the impact on storage at elevated
274 temperatures in this age group. Secondly, the vaccine was stored at elevated temperatures for
275 only 14 days. We used this time frame based on the present recommendation of a 14 day interval
276 between the doses of this vaccine in its 2-dose regimen. We therefore felt that this was an
277 appropriate period of incubation of the vaccine at higher temperatures. However, it would be
278 desirable to test longer durations outside the cold chain to guide the longer term storage
279 conditions for this vaccine.

280 From 2011 onwards several large vaccination studies/campaigns have been conducted
281 with Shanchol where cold storage has been a major hindrance to transport, delivery and conduct
282 of the campaign [4, 5, 15, 27, 28]. We hope that with the encouraging results of the thermal
283 stability, safety and immunogenicity of the vaccine, the present recommendations of cold chain

management of Shanchol will be modified making the vaccine more readily accessible for use in the many humanitarian emergencies confronted by global and national public health agencies.

Interpretation

The results of this study show that the vaccine remains stable and retains its safety and immunogenicity profile when kept outside the cold chain at elevated temperatures for up to 14 days. This finding will help to formulate global policies for storage of the OCV at ambient temperatures in tropical settings and increase the availability of OCV to populations living in areas with limited cold chain capabilities.

Contributors

FQ, AS, JDC, AC contributed to the study design. AS, FQ, FK and AK contributed to the implementation and supervision of the study. AS, FQ and US analyzed the data. TR, US and NJ participated in the immunological part of the study. FC and AIK supported by supervision of the study. SM, SRK, MSD analyzed the antigenic content (lipopolysaccharide) of vaccine. All authors participated in the writing of the manuscript and had full access to the data in the study. All authors reviewed and approved the final version of the manuscript.

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FIGURE LEGEND

Figure 2.

Vibriocidal antibody titer using *V. cholerae* O1 Ogawa as target bacteria in different groups of vaccine recipients. The bars indicate geometric mean titers with 95% CI. *Indicates statistically significant differences in responses in vibriocidal antibody titers prior to immunization (D0) and after immunization (D7) or (D21). ^a Indicates statistically significant differences in responses in vibriocidal antibody titers between D7 and D21. CRF indicates cumulative responder frequency at day 7 and/or day 21. The Wilcoxon signed Rank test and Mann Whitney test were used for comparisons within groups and between two groups respectively. $P \leq 0.05$ considered statistically significant.

Table 1. Demographic characteristics and adverse events in study participants after intake of study agent

Characteristics		Group A (2-8°C) n= 143	Group B (25°C) n= 144	Group C (37°C) n= 144	Group D (42°C) n= 140
Demographic					
*Sex	Male (%)	27(18.6%)	40(27.6)	18(12.4%)	34(23.4%)
	Female (%)	118(81.4%)	105(72.4%)	127(87.6%)	111(76.6%)
*Age(years)	Mean(SD)	28.8(7.9)	31(8.3)	29.2(7.7)	31.3(8.0)
Adverse Events					
Diarrhoea (n)		1	0	0	2
Fever (n)		2	3	4	1
Nausea (n)		0	1	0	0
Gas (n)		0	1	0	0
Cough (n)		0	0	1	0
Common Cold (n)		0	0	0	1
**Any adverse events (n)		3	5	5	4
No adverse event		140	139	139	136
LPS content in L.E.U./SHD		2697	2506	2668	2524

No serious adverse events were reported within 28 days of follow-up period
*p=0.01, Multiple linear regression models were used to adjust confounding variables age and sex
** p=1.000, comparable between Group A and Groups B,C and D
The cumulative antigen (Lipopolysaccharide) content remains at an acceptable level (> 2100 LEU/SHD) in all temperature groups
L.E.U./SHD indicates Lipopolysaccharide ELISA Units/Single Human dose

Table 2: Vibriocidal antibody responses using *Vibrio cholerae* O1 Inaba as target bacteria

Vibriocidal antibody response	Group A (2-8°C) (n=143)			Group B (25°C) (n=144)			Group C (37°C) (n=144)			Group D (42°C) (n=140)		
	Day 0	Day 7	Day 21	Day 0	Day 7	Day 21	Day 0	Day 7	Day 21	Day 0	Day 7	Day 21
GMT ^a (95% CI)	98.5 (74-131)	1075 (900-1283)	856 (719-1018)	71.2 (54-93)	678 (553-830)	601.1 (504-715)	84.3 (64-110)	940.6 (786-1125)	818 (698-958)	68.2 (52-88)	672.4 (542-833)	565.5 (460-693)
Fold increase ^b (95% CI)		10.9 (7.8-15.2)	8.6 (6.2-12.1)		9.5 (6.7-13.3)	8.4 (6.1-11.6)		11.1 (8-15.3)	9.6 (7.1-13.2)		9.8 (7-13.7)	8.2 (5.9-11.4)
Responder frequency ^c n (%)		109 (76)	105 (73)		108 (75)	106 (74)		116 (81)	114 (79)		106 (76)	99 (70)
CRF% ^d , (95% CI)	76 (69-83)			78 (71-85)			82 (76-88)			77 (70-84)		
P ^e		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001

^a Geometric mean of titers (GMT).
^b Fold increase of GMT on day 7 and day 21 compared to pre-immune titers with 95 % Confidence Interval (CI)
^c Responder frequency indicates numbers and % of participants responding with ≥4 fold increase in titer at day7 and day 21 post immunization compared with pre-immune titers.
^d CRF indicates Cumulative Responder Frequencies with 95% CI .Proportion difference between Group A and Groups B,C and D were comparable (p=1.000). Proportion difference in different groups (lower boundary of one tailed) were 74%,72%,77% and 71% in Groups A, B,C and D respectively.
^e Statistically significant differences at day 7 and day 21 compared to day 0.

Table 3: Vibriocidal antibody responses using *Vibrio cholerae* O139 as target bacteria

Vibriocidal antibody response	Group A (2-8°C) (n=143)			Group B (25°C) (n=144)			Group C (37°C) (n=144)			Group D (42°C) (n=140)		
	Day 0	Day 7	Day 21	Day 0	Day 7	Day 21	Day 0	Day 7	Day 21	Day 0	Day 7	Day 21
GMT ^a (95% CI)	55.6 (42-72)	117.8 (92-150)	125.5 (100-157)	34.2 (26-44)	51.3 (41-63)	62.8 (50-77)	39.2 (31-49)	68.9 (57-82)	75.5 (64-88)	49.4 (37-64)	124.2 (101-152)	137.2 (113-165)
Fold increase ^b (95% CI)		2.1 (1.4-3)	2.2 (1.5-3.1)		1.4 (1.0-2.0)	1.8 (1.3-2.5)		1.7 (1.3-2.3)	1.9 (1.4-2.5)		2.5 (1.7-3.5)	2.7 (2.0-3.8)
Responder frequency ^c n (%)		42 (29)	45 (31)		27 (19)	37 (26)		34 (23)	41 (28)		43 (30)	49 (35)
CRF% ^d , (95% CI)	32 (24-39)			27 (20-34)			28 (21-35)			35 (27-43)		
P ^e		<0.001	<0.001		0.05	<0.001		<0.001	<0.001		<0.001	<0.001

^a Geometric mean of titers (GMT).

^b Fold increase of GMT on day 7 and day 21 compared to pre-immune titers with 95 % Confidence Interval (CI)

^c Responder frequency indicates numbers and % of participants responding with ≥4 fold increase in titer at day7 and day 21 post immunization compared with pre-immune titers.

^d CRF indicates Cumulative Responder Frequencies with 95% CI .Proportion difference between Group A and Groups B,C and D were comparable (p=1.000). Proportion difference in different groups (lower boundary of one tailed) were 26%,21%,22% and 27% in Gr A, B,C and in D respectively.

^e Statistically significant differences at day 7 and day 21 compared to day 0.

Table 4: Lipopolysaccharide (LPS) specific IgA antibody responses in study participants

Target bacteria	Response to LPS	Group A (2-8°C) (n=143)			Group B (25°C) (n=144)			Group C (37°C) (n=144)			Group D (42°C) (n=140)		
O1 Ogawa		Day 0	Day 7	Day 21	Day 0	Day 7	Day 21	Day 0	Day 7	Day 21	Day 0	Day 7	Day 21
	GMT ^a (95% CI)	72.4 (58-89)	207.5 (169-253)	159.1 (132-191)	60.8 (48-75)	246.2 (203-297)	177.4 (150-209)	72.9 (62-84)	258.2 (216-307)	200 (170-234)	67.7 (57-79)	245.8 (206-292)	186.6 (161-215)
	Fold increase ^b (95% CI)		2.8 (2.1-3.8)	2.1 (1.6-2.9)		4 (3.0-5.3)	2.9 (2.2-3.8)		3.5 (2.8-4.4)	2.7 (2.2-3.4)		3.6 (2.8-4.6)	2.7 (2.2-3.4)
	Responder frequency ^c <i>n</i> (%)		82 (57)	71 (50)		96 (67)	85 (59)		104 (72)	84 (58)		94 (67)	91 (65)
	CRF% ^d (95% CI)	62 (54-70)			75 (68-82)			76 (69-83)			74 (67-81)		
	<i>P</i> ^e		<0.0001	<0.0001		<0.0001	<0.0001		<0.0001	<0.0001		<0.0001	<0.0001
O1 Inaba	GMT ^a (95% CI)	41.4 (32-53)	185.8 (150-228)	133.1 (110-160)	74.5 (62-88)	296.6 (246-357)	201.5 (172-236)	54.3 (45-64)	247.6 (204-299)	179.6 (152-211)	34 (27-42)	197.9 (161-243)	141.6 (116-171)
	Fold increase ^b (95% CI)		4.4 (3.2-6.2)	3.2 (2.3-4.4)		3.9 (3.0-5.1)	2.7 (2.1-3.4)		4.5 (3.5-5.8)	3.3 (2.6-4.1)		5.8 (4.3-7.8)	4.1 (3.1-5.5)
	Responder frequency ^c <i>n</i> (%)		96 (67)	96 (67)		98 (68)	78 (54)		107 (74)	101 (70)		111 (79)	111 (79)
	CRF% ^d (95% CI)	76 (69-83)			70 (62-77)			81 (74-87)			84 (78-90)		
	<i>P</i> ^e		<0.0001	<0.0001		<0.0001	<0.0001		<0.0001	<0.0001		<0.0001	<0.0001

^a Geometric mean of titers (GMT).
^b Fold increase of GMT on day 7 and day 21 compared to pre-immune titers with 95 % Confidence Interval (CI)
^c Responder frequency indicates numbers and % of participants responding with ≥4 fold increase in titer at day7 and day 21 post immunization compared with pre-immune titers.
^d CRF indicates Cumulative Responder Frequencies with 95% CI . Proportion difference between Group A and Groups B,C and D were comparable (p=1.000).
^e Statistically significant differences at day 7 and day 21 compared to day 0.

Fig 1. CONSORT diagram for the study

Fig. 2: Vibriocidal antibody titer using *V. cholerae* O1 Ogawa as target bacteria in different groups of vaccine recipients. The bars indicate geometric mean titers with 95% CI. *Indicates statistically significant differences in responses in vibriocidal antibody titers prior to immunization (D0) and after immunization (D7) or (D21). ^α Indicates statistically significant differences in responses in vibriocidal antibody titers between D7 and D21. CRF indicates cumulative responder frequency at day 7 and/or day 21. The Wilcoxon signed Rank test and Mann Whitney test were used for comparisons within groups and between two groups respectively. $P \leq 0.05$ considered statistically significant.