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# Randomized placebo controlled human volunteer trial of a live oral cholera vaccine VA1.3 for safety and immune response

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ABSTRACT

A live oral cholera vaccine developed from a non-toxigenic *Vibrio cholerae* O1 El Tor strain VA1.3 was tested in a double-blind randomized placebo controlled study for safety and immunogenicity in 304 men aged between 16 and 50 years from Kolkata, India. A dose of $5 \times 10^9$ CFU ($n = 186$) or a placebo ($n = 116$) containing the diluent buffer was administered. The vaccine did not elicit adverse events except in two vaccine recipients with mild diarrhoea and vomiting. None excreted the vaccine strain. Vibriocidal antibody response developed in 105/186 (57%) and 5/116 (4%) in vaccine and placebo recipients, respectively. In a subgroup, anti-CT antibody rose (≥2-folds) in 23/30 (77%) and 6/19 (32%) in vaccine and placebo recipients, respectively. These studies demonstrate that VA1.3 at a dose of $5 \times 10^9$ is safe and immunogenic in adults from a cholera endemic region.



## 1. Introduction

Cholera is endemic in many countries, particularly in South and South East Asia. An effective cholera vaccine has substantial public health relevance for these countries. A single dose live oral cholera vaccine may also help in controlling cholera outbreaks. The only licensed live oral cholera vaccine is CVD 103-HgR that has been marketed is largely used for travelers [1]. Recently, its production has been discontinued. A killed oral cholera vaccine with added B-subunit of cholera toxin (CT) has also been licensed and marketed (Dukoral™ licensed by SBL Vaccine, Sweden). It is administered in two doses 1–2 weeks apart. While this vaccine was mainly used for travelers to cholera endemic regions, in recent years it has been successfully used in refugee camps in Uganda, Sudan and Aceh (Indonesia) to protect at risk populations. Recently, it has also been successfully used in an endemic area in Mozambique with high HIV prevalence [2]. A simpler version of a killed oral cholera vaccine without B-subunit has however been tested and implemented in Vietnam with reported success in reducing severe disease from cholera [3]. A reformulated version of this vaccine is undergoing field testing in India.

Genetically engineered live oral vaccines, incapable of elaborating the cholera toxin are generally derived from clinical non-toxigenic strains of *Vibrio cholerae* O1 that has the potential to cause mild to moderate levels of diarrhoea. Though the cause behind the expression of diarrhoea by these strains are not known, it was thought that this could be due to the presence of unknown diarrhoeagenic factor(s) in such strains. In an attempt to circumvent this problem, a new oral candidate cholera vaccine has been developed in India from a clinical non-toxigenic strain of *V. cholerae* biotype El Tor serotype Inaba, which is not only devoid of CTX prophage but also non-reactogenic as evidenced from the rabbit illeal loop assay. This strain was selected after screening more than 1000 clinical strains of *V. cholerae* O1 (4). This candidate vaccine strain contained *toxR* and *tcpA* genes that regulate CT and helps in colonization of *V. cholerae* in the human gut, respectively. Through a series of genetic manipulations (4), the cholera toxin B-subunit encoding gene (*ctxB*) of *V. cholerae* O1 was introduced into the cryptic hemolysin locus (*hlyA*) of the strain. But before doing that, *ctxB* was inserted immediate by downstream of the promoter, which is specific for the *ctx* operon in wild type *V. cholerae* strains. In addition, a gene encoding resistance to ampicillin was introduced, linked to the *ctxB* as the marker. The resulting strain, named Vaccine Attempt 1.3 (VA1.3), was found to be able to produce copious amounts of CTB. In the RITARD model, this strain was found to be non-reactogenic and provided full protection against the challenge

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doses of both *V. cholerae* O1, classical and El Tor biotypes [4]. The vaccine strain VA1.3 was susceptible to tetracycline and doxycline. The VA1.3 cholera vaccine is different from other candidate vaccine strains such as CVD 103-HgR 638 and Peru-15, by not having the intact hlyA.

In a randomized placebo controlled trial, we evaluated this candidate oral vaccine strain in 304 human volunteers in Kolkata, India, from 1999 to 2004 for safety and immune response. Kolkata is known to be a highly endemic zone for cholera.

## 2. Methods

### 2.1. Participants

The study was conducted in the Clinical Trials Unit jointly administered by the Society for Applied Studies and the National Institute of Cholera and Enteric Diseases (ICMR); it is situated at the Infectious Diseases Hospital in Kolkata. We recruited healthy adult men aged 16–55 years from February 1999 to December 2004. Medical history was recorded in a pretested form and a physician examined them for any overt or underlying illness. A routine blood test for hemoglobin and white blood cell count was done. They went through counseling before they were considered for participation. We obtained written informed consent from the volunteers prior to enrolment. Individuals with diarrhoea, vomiting, febrile illness, history of antibiotic use during the past one month or any chronic illness that may affect the study were excluded.

The Clinical Trials Unit is a completely independent and separate facility with independent entry and exit. It has space for 8–10 beds, a room for counseling, a room for examination. There is a separate and dedicated toilet facility. It has an attached clinical laboratory. This clinical facility was also subsequently used for a phase II study of a killed oral cholera vaccine [5].

### 2.2. Ethical consideration

An Expert Committee constituted by the Department of Biotechnology (DBT), Govt. of India reviewed the toxicology, safety, reactogenicity and immunogenicity studies in animals and approved it for further studies in human volunteers subject to statutory clearances. Drug Controller, Govt. of India (DCGI) approved the human volunteer studies protocol. Indian Council of Medical Research (ICMR) Ethical Review committee approved the trial protocol. Initially, a dose-ranging study was conducted in 16 participants in Kolkata before starting the vaccine trial.

### 2.3. Preparation of live oral vaccine

The vaccine strain (VA1.3) was grown in Luria–Bertani (LB) medium (Difco, Detroit, USA) supplemented with 50 μg/ml of ampicillin. The log phase culture was cryopreserved in 15 ml aliquots at −80 °C with 20% sterile glycerol (Merck, Germany). Before the vaccine trial, a vial of frozen VA1.3 seed was thawed on ice and 1.0 ml of the culture was serially diluted in sterile phosphate buffered saline (PBS) and plated in duplicate on LB agar containing 50 μg/ml of ampicillin. The plates were incubated for 16–18 h at 37 °C. Colony counts were made in plates showing 30–300 colonies and the live bacterial cells in the vaccine stock was quantified. Randomly selected colonies were also confirmed as *V. cholerae* O1 Inaba by slide agglutination test using commercial antisera (Denka Seiken, Tokyo, Japan). On the day of vaccination, the bacteria cells from the vaccine stock were washed thrice in sterile PBS and the bacterial pellet was suspended in 50 ml of cold sterile sodium bicarbonate buffer to give $5 \times 10^9$ colony forming units (CFU). We used the same buffer of the same amount as the placebo. Both looked identical and clear solutions. The optical density of the bacterial suspension in the buffer was determined and adjusted to make a stock suspension having a predicted number of CFU/ml. The vaccine dose was confirmed by standard dilution and plate count method on mock vaccine doses, which was made in parallel to those administrated to the volunteers.

#### 2.3.1. Vaccine administration

The vaccine formulation group prepared the vaccine and blinded the vaccine and the placebo according to the master randomization list. The vaccine and the placebo were administrated within an hour of preparation. The volunteers were admitted the night before and the vaccine or placebo was administered in the morning. They were randomized at a 3:2 ratio into vaccine and placebo groups, respectively. A master randomization list was prepared before starting the study by a person not directly involved in the study. The dose of vaccine was $5 \times 10^9$. The study participants were not allowed to eat or drink for an hour before and after the intake of the vaccine/placebo. Before the vaccination, 150 ml of sodium bicarbonate buffer was given to the volunteers. After 5 min, 50 ml of VA1.3 culture suspension in sodium bicarbonate buffer and 50 ml of sodium bicarbonate buffer alone were administered for the coded vaccine and placebo, respectively. An investigator inspected and confirmed that all the volunteers had taken the vaccine/placebo. The first 116 participants stayed in the unit for 5 days. The remaining ones stayed for 3 days. This change was made following recommendations from the Monitoring Committee of the Department of Biotechnology, Government of India, overseeing the project.

#### 2.3.2. Adverse events

Adverse event of interest was diarrhoea and vomiting. All stools were examined and graded. The stool consistency was ranked according to three grades: grade 1, firm or soft/mushy; grade 2, thick liquid; grade 3, watery. Three or more grade 2 stool or one or more grade 3 stool was treated as diarrhoea. The attending physician took decision on the severity of diarrhoea and graded them as mild with no dehydration, moderate with some dehydration and severe with marked dehydration. Other adverse events recorded routinely were vomiting, fever (axillary temperature ≥98 °F), abdominal pain or discomfort, or any other adverse event that may occur during the study period. The primary objectives of the study were to evaluate safety and immunogenicity of the vaccine. For safety the proportion of subjects with diarrhoeal adverse events during the study period and for immunogenicity, the proportion of subjects showing 4-fold or greater rise in serum vibriocidal antibody titre after 14 days of vaccine administration were considered. We evaluated all adverse events during 15 days of the study period in both vaccine and placebo recipients, and the excretion of the vaccine strain for 5 days for first 116 volunteers and for 3 days for the remaining ones after administration of the vaccine.

#### 2.3.3. Dose-ranging studies in human volunteers

Sixteen volunteers participated in a dose-ranging study. Three volunteers each received a dose of $4.80 \times 10^5$, $1.29 \times 10^7$, $1.50 \times 10^9$ and $2.49 \times 10^9$, respectively and four volunteers received a placebo. Volunteers were assigned according to a randomization list prepared for the dose-ranging protocol prior to starting the study. Except one volunteer who had mild vomiting (received a dose of $2.49 \times 10^9$ CFU), none of the volunteers had diarrhoea, vomiting, nausea, fever, abdominal pain, flatulence or any other adverse event. None of the volunteers shed the vaccine strain in the stool. Based on these dose-ranging studies, a dose of $5 \times 10^9$ was chosen for safety and immune response studies in volunteers.

### 2.4. Immune response

#### 2.4.1. Vibriocidal assay

Five millilitres of venous blood was collected from the volunteers for vibriocidal assay prior to (0 day) and 15 days subsequent to vaccination. Blood group was determined with blood drawn on 0 day. Vibriocidal assay was performed with the *V. cholerae* O1 Inaba (VA1.3) strain using sera collected during pre- and post-vaccine trial following the published methods [6]. Commercially prepared guinea pig serum was used as a complement in this study (Sigma, St. Louis, USA). The sera (100 μl) were added to 100 μl of PBS in the first well to give 2-fold dilution and the subsequent dilutions were made reciprocally up to 4800. A 4-fold or greater increase in titre between the 0 day and 15th day sera samples was used to signify seroconversion. Reference rabbit antiserum against O1 Inaba was included in each assay as a control. In addition, in every batch of the assay, serum obtained from a healthy volunteer who never had cholera and a high titre antiserum obtained from one of the volunteers in this study were included as negative and positive controls, respectively.

#### 2.4.2. Anti-CT assay

Enzyme-linked immunosorbent assay (ELISA) was used for the detection of response against CT antibody. Immunoglobulin G (IgG)-specific antibody response in the paired sera were determined against purified CT using micro titration plates (Nunc, Denmark). ELISA was made following the procedures of Nandy et al. [7] with slight modification. In brief, the micro titration plates were coated with 0.2 μg of purified CT (Sigma, St. Louis, USA) in PBS. Following 1 h incubation at 37 °C, wells were washed and blocked with 0.5% (w/v) bovine serum albumin (fraction V, Sigma). Next, wells were filled with 100 μl of the test serum serially double diluted in PBS containing 0.5% BSA; the initial dilution was 1:20 in all the samples. Following incubation and washing, 100 μl of appropriately diluted goat anti-human IgG peroxidase labeled conjugate (Sigma) was added to each well and colour was developed with the substrate solution *O*-phenylenedimine dihydrochloride (Sigma) and $H_2O_2$. Results were recorded by measuring absorbance at 490 nm using an ELISA reader (BioRad, Hercules, USA) and titre was expressed as the reciprocal of the highest dilution of antiserum that showed an $OD_{490}$ value ≥0.200 in the assay. Paired sera from a subsample of 49 volunteers (30 vaccine and 19 placebo recipients) were tested by the ELISA. The samples for anti-CT assay were chosen by a person not involved with the laboratory studies. A three digit random number was generated between 100 and 250 and using that as the serial number of the index case, sera of 50 consecutive ID numbers were used for choosing the samples. Sample from one participant was inadequate.

### 2.5. Excretion of vaccine strain

Stools for three consecutive days collected from the participants were examined, graded and weighed. Faecal excretion of vaccine strain was tested using conventional cultural and molecular methods. Stool specimens collected from the vaccine study participants were collected in sterile containers and transported to the laboratory and processed within 2 h of collection. For qualitative analysis, the stool specimens were inoculated in alkaline peptone water (pH 8.0) and incubated for 6–8 h, followed by streaking on thiosulfate citrate bile salts sucrose (TCBS) agar (Eiken, Tokyo, Japan). For quantification of the vaccine strain, 1 g of the stool specimen was serially diluted in sterile PBS and plated on to TCBS agar plates. Typical sucrose fermenting colonies were tested using biochemical tests and were serologically confirmed using antiserum specific for *V. cholerae* O1 Inaba. PCR assay was performed using previously published method [8] with all the isolated colonies targeting *ctxA* and *ctxB* genes for molecular confirmation of the vaccine strain.

### 2.6. Safety measures

A physician clinically monitored the volunteers at least twice daily. The study participants used a specially designed commode and the faeces were passed into disposable biohazard bags. Faeces were decontaminated by autoclaving before disposal. Doxycycline 300 mg once daily for 3 days was given to all the participants on day 7 to clear the vaccine strain.

### 2.7. Sample size

Assuming 50% seroconversion (i.e., vibriocidal antibody titre rise of ≥4-folds after 2 weeks) as a worthwhile immune response after a single dose of live oral cholera vaccine VA1.3, a sample size of 180 in the vaccine group will give a 95% confidence limits of 43–57.5%. With a 3:2 randomization plan we need a total of 300 volunteers to be enrolled in the study. This gives us a lower limit of 95% confidence of about 43%, which is what was reported in military recruits in Bangkok (low socio-economic group) after administration of the licensed live oral vaccine CVD 103-HgR [9,10].

### 2.8. Study organization

This vaccine was developed in three research laboratories of the Government of India and was not driven by the industry. The Department of Biotechnology of the Ministry of Science and Technology, Government of India was the lead agency which constituted several committees for overseeing the progress of the work. After completing study on 25–30 volunteers the report was reviewed by these committees before new volunteers could be recruited.

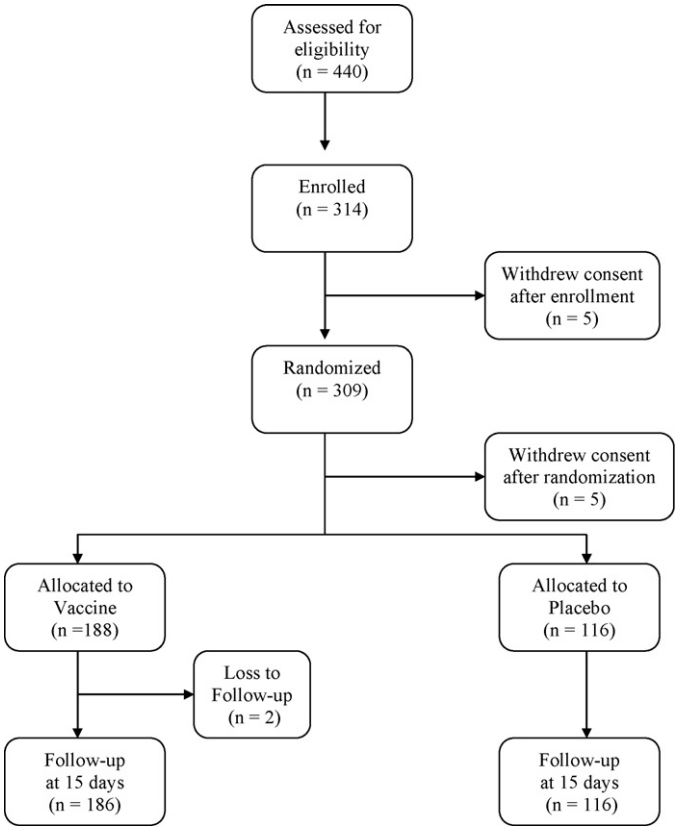


**Fig. 1.** Flow chart for the recruitment of volunteers in the study.

**Table 1**
Admission features of the volunteers in Kolkata ($n = 304$).

| | Vaccine group ($n = 188$) | Placebo group ($n = 116$) |
|---|---|---|
| Age[a] | | |
| Median (quartiles) | 22.5 (20–28.5) | 22.5 (20–28) |
| 16–25 years | 128 (68%) | 76 (66%) |
| 26–35 years | 40 (21%) | 28 (24%) |
| 36–45 years | 18 (10%) | 11 (9%) |
| >45 years | 02 (1%) | 01 (1%) |
| BMI | | |
| Median (quartiles) | 19.18 (17.49, 20.94) | 18.87 (17.35, 20.67) |
| Hemoglobin, g/dl | | |
| Mean (SD) | 12.84 (0.978) | 13.00 (0.978) |
| Range | (10.01–16.01) | (10.56–15.01) |
| Education | | |
| Years in school/college | | |
| 11–17 years | 34 | 14 |
| 6–10 years | 101 | 62 |
| 0–5 years | 53 | 40 |
| Blood groups | | |
| Group O | 56 (30%) | 36 (31%) |
| Others | 132 | 80 |

[a] Vast majority are young men in the age group of 16–25 years.

The process took time and the study extended over several years.

### 2.9. Analysis

Data entry, editing and analysis were done using statistical programmes EpiInfo Version 6 (CDC, Atlanta) and Stata Version 7.0 (Stata Corporation, 4905 Lakeway Drive, College Station, TX 77845, USA).

## 3. Results

### 3.1. Participants

Enrolment profile of the participants for the study and immune response studies is shown in Fig. 1. A total of 186 participants in the vaccine group and 116 in the placebo group completed the study.

The features of the participants are given in Table 1. Vast majority of them were young men in the age group of 16–25 years and came from low socio-economic status. Nearly 30% of them belonged to blood group 'O'. BMI levels were generally low indicating a less than ideal level of nutrition. The same is true for the hemoglobin level.

### 3.2. Adverse events

Adverse events were rare (Table 2). Two participants after receiving the vaccine had mild diarrhoea and did not require any oral or IV hydration fluids. One of them also vomited once and the other volunteer had mild vomiting but no diarrhoea.

**Table 2**
Adverse events among volunteers following one dose of vaccine or placebo.

| Events | Vaccine ($n = 186$) | Placebo ($n = 116$) |
|---|---|---|
| Diarrhoea | 2[a] | 0 |
| Vomiting | 2[b] | 0 |
| Fever | 0 | 0 |
| Abdominal pain | 0 | 0 |
| Any other adverse events | 0 | 0 |
| Total | 3 | 0 |

[a] Mild with no dehydration, did not require oral or I.V. fluids.
[b] One or two times in total in each volunteer; no treatment required.

**Table 3**
Serum vibriocidal antibody titres to *Vibrio cholerae* O1 at baseline and day 15.

| GMT[a] | Vaccine ($n = 186$) | Placebo ($n = 116$) | $p$ |
|---|---|---|---|
| Baseline | 54.16 | 51.32 | 0.79 |
| Day 15 | 316.1 | 53.5 | 0.001 |
| GMF[b] rise | 5.84 | 1.04 | 0.001 |

[a] GMT: geometric mean reciprocal titre.
[b] GMF: geometric mean reciprocal titre: folds rise over baseline.

### 3.3. Immune response

#### 3.3.1. Vibriocidal antibody titre

Serum vibriocidal antibody titres to *V. cholerae* O1 at baseline and on day 15 are shown in Table 3. Geometric mean titres at baseline were similar between the vaccine and placebo groups. The geometric mean titre on day 15 in the placebo group was similar to the baseline. In the vaccine group the mean titre was nearly 6-folds higher on day 15 compared to the baseline. The proportion of participants in the vaccine or placebo group with 4-fold rise and 8-fold or higher rise are shown in Table 4. The proportion of the participants in the vaccine group who seroconverted (i.e., with 4-fold or higher rise in titre) was 57% (95% CI: 49–64%) compared to only 5% (95% CI: 1.4–9.8%) in the placebo group. Rate ratio for seroconversion between vaccine and placebo groups was 13.1 (95% CI: 5.5–31.2%). Two volunteers among the placebo recipients had an 8-fold and three had 4-fold rise in the titre. As the volunteers come

**Table 4**
Rise in vibriocidal antibody titre between baseline and day 15 of vaccine or placebo dose.

| Number with[a] | Vaccine ($n = 186$) | Placebo ($n = 116$) |
|---|---|---|
| No rise | 55 (29.6%) | 93 (80.2%) |
| 2-fold rise | 26 (14%) | 18 (15.5%) |
| 4-fold rise | 21 (11.3%) | 3 (2.6%) |
| 8-fold rise | 23 (12.4%) | 2 (1.7%) |
| ≥16-fold rise | 61 (33%) | 0 |
| Number of subjects who seroconverted (%)[a] | 105 (56.5%) | 5 (4.3%) |
| 95% confidence interval[b] | 49.0–63.7% | 1.4–9.8% |

[a] Number of subjects with ≥4-fold rise in titres from baseline on day 15.
[b] Binomial, exact.

**Table 5**
Rise in vibriocidal antibody titre between baseline and day 15 of vaccine or placebo dose in volunteers with blood group O.

| Number with[a] | Vaccine ($n$ = 56) | Placebo ($n$ = 36) |
|---|---|---|
| No rise | 19 | 26 |
| 2-fold rise | 6 | 8 |
| 4-fold rise | 5 | 0 |
| 8-fold rise | 5 | 1 |
| ≥16-fold rise | 22 | 0 |
| Number who seroconverted (%)[a] | 32 (51%) | 1 (2.8%) |
| 95% confidence interval[b] | 43.2–70.3% | 0.07–14.5% |

[a] Number (%) of subjects with ≥4-fold rise in titre from baseline to day 15.
[b] Binomial, exact.

from a highly endemic region for cholera, it is not unlikely for some of them acquiring in apparent infection with cholera and have a booster immune response. In the subgroup of participants belonging to blood group 'O' (Table 5), the proportion with 4-fold or higher rise in vibriocidal antibody titre was 51% (95% CI: 43–70%) in the vaccine group and 3% (95% CI: 0.07–15%) in the placebo group. The rate ratio for seroconversion between vaccine and placebo group for blood group O participants was 19.7 (95% CI: 2.8–137.5%). Representative sera samples were sent to the International Centre for Diarrhoeal Diseases Research, Bangladesh (ICDDR,B) and the fold increase in the vibriocidal titres were highly reproducible (data not shown).

### 3.3.2. Anti-CT antibody titre

Anti-CT antibodies were measured at baseline and 15 days after a single dose of vaccine or placebo in 49 volunteers (Table 6). Thirty of them received the vaccine and 19 received placebo. The linear trend for proportionate rise in anti-CT titre is significant (chi squared for linear trend = 7.3, $p$ = 0.007 with 1 degree of freedom) with higher proportions in the vaccine group having 2-fold and 4-fold rise. The proportion with ≥2-fold rise in anti-CT titre in the vaccine group was 76.7% compared to 31.6% in the placebo group (RR = 2.32, 95% CI: 1.22–4.84, $p$ = 0.002). Geometric mean fold rise was significantly higher in the vaccine group ($p$ = 0.011).

### 3.4. Faecal excretion of the vaccine strain

The vaccine strain was not excreted in the stool in any of the participants.

## 4. Discussion

In a highly endemic region of cholera, the seroconversion rate (i.e., ≥4-fold rise in vibriocidal titre) was impressive after a single dose of the live oral candidate vaccine VA1.3. The cholera vaccine CVD 103-HgR was the first licensed live oral vaccine for cholera. It has been extensively studied both in developed and developing country volunteers for immunogenicity and safety [1,9,10]. This vaccine however has been withdrawn from the market partly because of indifferent results on protection in a large filed trial in Indonesia [11]. In recent years a live oral cholera vaccine Peru-15 has been extensively tested in human volunteers, both in developed countries and in places where cholera is endemic and was found to be safe and immunogenic both in adults and in children [12–14]. We understand that this vaccine is now awaiting field trials for protective efficacy. Preliminary studies of another live oral cholera vaccine developed in Cuba, *V. cholerae* 638, has undergone human volunteer studies in Cuba, and found to be immunogenic but showed mild reactogenicity [15,16]. This vaccine, to our knowledge, has not yet been tested in cholera endemic regions. We compared our results with those on volunteers from endemic areas who received either a single dose of a licensed live oral cholera vaccine (CVD 103-HgR) or a candidate live oral vaccine Peru-15 (Table 7). The vaccine CVD 103-HgR gave a seroconversion rate from 20% to 48% among adult Thai volunteers of low socio-economic status [9,10]. The seroconversion rate after a single dose of candidate vaccine Peru-15 was 75% (95% CI: 59–87%) in adult volunteers from a highly endemic region (Bangladesh). Of the two licensed oral cholera vaccines, one is an inactivated vaccine currently recommended by WHO (Dukoral™, licensed by SBL vaccine, Sweden). It consists four batches of heat or formalin-killed whole-cell *V. cholerae* O1 of both serotypes and biotypes (classical and El Tor), with added recombinant cholera toxin B-subunit. Initially it was successfully field tested in Bangladesh and was also successfully used for mass vaccination in refugee camps in Uganda, in Darfour (Sudan) and in Aceh (Indonesia) to protect at risk populations from potential cholera outbreaks. As stated earlier this vaccine was also used in an endemic area in Beria (Mozambique) and demonstrated a protective efficacy of 89% against severe diarrhoea with dehydration. An important observation of public health importance is that this study area has a high HIV prevalence [2]. A killed whole-cell bivalent vaccine (*V. cholerae* O1 and O139) without B-subunit has been licensed in Vietnam in 1997 following a technology transfer from Swedish scientists. The vaccine is safe, does not require a buffer to administer, easy to produce and is inexpensive. With another technology transfer the vaccine was produced in India that complies with WHO guidelines on killed oral cholera vaccine. In a phase II trial in Kolkata India [5] it was found to be immunogenic and very recently data from a phase III trial with 2 years follow-up with this vaccine have been reported (D. Sur et al., Abstract: The 13th International Conference on Emerging Infectious

**Table 6**
Rise in anti-CT antibodies on day 15 after a single oral dose of vaccine (in a subgroup).

| Rise in anti-CT | Vaccine group* ($n$ = 30) | Placebo group* ($n$ = 19) |
|---|---|---|
| No rise | 6 (20%) | 13 (68.4%) |
| 2-fold rise | 13 (43%) | 3 (15.8%) |
| 4-fold rise | 11 (37%) | 3 (15.8%) |
| ≥2-fold rise | 24 (80%) | 6 (32%) |
| **GMT** | **Vaccine group+ ($n$ = 30)** | **Placebo group+ ($n$ = 19)** |
| Baseline | 3789 | 5308 |
| Day 15 | 7072 | 6614 |
| GMF rise | 1.87 | 1.24, $p$ = 0.011 |

GMT: geometric mean reciprocal titre.
GMF rise: geometric mean reciprocal titre: folds rise over baseline.
* Chi squared for linear trend for proportionate rise in anti-CT titre = 8.3, one degree of freedom, $p$ = 0.004.
+ Rate ratio for seroconversion (i.e., ≥2-fold rise) = 2.27 (1.22–4.17), $p$ = 0.002.

**Table 7**
Vibriocidal response (≥4-fold rise) to live oral cholera vaccines in human volunteers from literature.

| Vaccine type | Study site | Dose | Age | With ≥4-fold % rise ($n$)[f] |
|---|---|---|---|---|
| CVD 103-HgR[a] | Thai (LSEL)[e] | $5.18 \times 10^8$ | Adult | 20% ($n$ = 95) |
| | | Placebo | Adult | 7% ($n$ = 104) |
| CVD 103-HgR[b] | Thai (LSEL) | $4.8 \times 10^8$ | Adult | 33% ($n$ = 39) |
| | | $6.37 \times 10^9$ | Adult | 43% ($n$ = 40) |
| | | $1.27 \times 10^{10}$ | Adult | 48% ($n$ = 40) |
| Live oral Peru-15[c] | Bangladesh | $2 \times 10^8$ | Adult | 75% ($n$ = 40) |
| | | Placebo | Adult | 10% ($n$ = 30) |
| Live oral VA1.3[d] | India | $5 \times 10^9$ | Adult | 57% ($n$ = 186) |
| | | Placebo | Adult | 4% ($n$ = 116) |

[a] Ref. [9].
[b] Ref. [10].
[c] Ref. [13].
[d] Present study.
[e] LSEL: low socio-economic level.
[f] $n$: number of participants.

Diseases in the Pacific Rim, April 6–9, 2009, Kolkata, India, p. 139). After 2 years of follow-up the overall protective efficacy was 68%. This vaccine is being marketed in India. The killed whole-cell oral cholera vaccines developed so far need to be given in two doses. In the present study, the live oral cholera vaccine VA1.3 was found to be immunogenic and largely free of adverse events in human volunteers after a single oral dose of $5 \times 10^9$ in an endemic area.

It is of interest to note that these volunteers coming from a low socio-economic group and living in a highly endemic city for cholera did not excrete the vaccine strain. VA1.3 has an intact *tcpA* gene which is directly correlated with colonization ability of the vaccine strain. Moreover, in animal model study, we have shown that the vaccine strain has the ability of colonization in higher numbers. In some of the live oral cholera vaccine studies in adults from endemic region it was shown that the faecal shedding of the vaccine strains is generally low [10].

The serum vibriocidal antibody titre as a marker of protection has been found over four decades of epidemiological studies and vaccine efficacy trials, as a consistent and robust correlate for protection in endemic areas, independent of age groups. Epidemiological studies were conducted in the 60s and 70s by Mosley and colleagues in connection with a parenteral vaccine trial [17–19], in the 80s by Glass et al. [20], and in the 90s by Clemens et al. in connection with a field trial of an oral killed whole-cell vaccine [21]. Based on the strong association, a strong biologic gradient, the temporal relationship and the immunological plausibility, there is a general consensus that high vibriocidal antibody is a good marker of protection. Experimental studies using live oral cholera vaccine in volunteers also demonstrated that, the degree of stimulation of serum vibriocidal antibody following ingestion of live oral cholera vaccine is the best correlate of the antibacterial immunity in the intestine [22,23]. While this correlation is high, vibriocidal antibody titre is considered an incomplete and surrogate marker for other immune responses that are more directly related to protection [24]. A better definition of the specific mechanisms of immunity to cholera is yet to be determined.

One additional finding of considerable interest is the ability of this vaccine to induce good anti-CT antibody response in these volunteers. We speculate that this added immunological characteristic may further enhance the protection in field studies. It is to be noted that *ctxB* gene was incorporated in the construct of VA1.3 and is a good producer of cholera toxin-B [4]. Classical and El Tor CTs are immunologically distinct and this difference has an influence on the epidemiology of cholera that led the classical strains to be completely replaced by the El Tor strains with the advent of the seventh pandemic in 1961. In recent years, El Tor strains that produce classical cholera toxin have replaced the prototype El Tor seventh pandemic strains of *V. cholerae* in Bangladesh [25], India [26] and other countries in Asia and Africa [27]. In Kolkata, typical El Tor CT producers are replaced by the El Tor variant strains that produce classical CT since 1994–1995 [26]. Use of effective cholera vaccine is now encouraged by the WHO during large cholera outbreaks. Considering these events, the development of VA1.3 as a vaccine strain is suited to the events that have occurred in the epidemiology of cholera since, this oral vaccine recombinant strain has *ctxB* of the classical biotype inserted into the hemolysin locus and therefore would be ideally suited against the current El Tor strains of *V. cholerae* which produce classical cholera toxin. We should however note that in population studies anti-CT antibody titre is not well correlated with protection from disease.

It has been suggested that an attenuated cholera vaccine strain could acquire *ctx*A gene from a wild type *V. cholerae* in the intestine or in the environment. We contend that even if such a transfer were to occur the adverse consequences if any would be minimal; for such a transfer to occur there must already be a large number of toxigenic *V. cholerae* present in the intestine representing simultaneous co-infection or, toxigenic *V. cholerae* present in the environment to serve as a source of *ctx*A. The impact of one more toxigenic strain of *V. cholerae* in the environment where a single cholera patient can excrete up to 10 l of watery stool containing $10^7$–$10^8$ toxigenic *V. cholerae* per ml per day would be insignificant. To reduce this remote possibility further, *Rec*A gene has been deleted in the vaccine strain Peru-15. However CVD-103 which is not devoid of *Rec*A gene, has been tested in a variety of settings on a large number of people and has an excellent safety record. It has amply demonstrated that the consequences of the presence of the *Rec*A gene neither compromised its safety nor led to any adverse consequences arising from re-acquirement of *ctx*A.

Like all microbes *V. cholerae* can indeed evolve and acquire new attributes the emergence of *V. cholerae* O139 being a case in point. It is perhaps worthwhile to note that the rare theoretical possibilities with minimal practical consequence such as the reacquisition of the *ctx*A by an attenuated cholera vaccine strain are not significant compared to the normal evolutionary process acting on a wild type pathogen.

Multiple studies in cholera endemic regions have shown that individuals with blood group O are more susceptible to severe cholera due to classical and El Tor *V. cholerae* O1 as well as *V. cholerae* O139 [28–32]. However, two studies have shown that there is no difference in the risk of infection (asymptomatic or mild illness) with *V. cholerae* among persons with blood group O antigens from those with other blood groups [32,33]. Vibriocidal antibody response to the live oral cholera vaccine VA1.3 in blood group O individuals was comparable to the overall response. Its relevance to protection from severe disease due to cholera can only be evaluated in a field intervention trial.

Live oral cholera vaccine VA1.3 was found to be very safe. Untoward events after immunization were virtually absent. In human volunteers from a highly endemic region the vaccine strain was not excreted in the stool after oral immunization. The rise in vibriocidal titre, the surrogate marker of protection, in volunteers from a highly endemic area is comparable to the other live oral cholera vaccines tried in endemic regions. Good anti-CT antibody response is a relevant feature for a candidate vaccine for cholera. We speculate that insertion of *ctxB* of the classical biotype in the hemolysin locus of VA1.3 may be an added advantage. The findings of this study suggest further studies of the candidate vaccine VA1.3 including a field intervention trial for protective efficacy.

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