Original Article

Characterization of Citrobacter sp. line 328 as source of Vi for Vi-CRM_{197} glycoconjugate vaccine against Salmonella Typhi

Simona Rondini, Francesca Micoli, Luisa Lanzilao, Ivan Pisoni, Vito Di Cioccio, Allan J. Saul, Laura B. Martin

Novartis Vaccines Institute for Global Health (NVGH), Siena, Italy

Abstract

Introduction: *Salmonella enterica* serovar Typhi is the causative agent of typhoid fever with over 22 million cases and over 200,000 deaths reported annually. A vaccine is much needed for use in young children and the Novartis Vaccines Institute for Global Health (NVGH) is developing a conjugate vaccine which targets *S.* Typhi Vi capsular polysaccharide.

Methodology: Here we describe a method suitable for industrial scale production of the Vi antigen based on expression by a *Citrobacter* line. We optimized the production of Vi by selecting a suitable *Citrobacter* strain (*Citrobacter* 328) that yields high and stable expression of Vi in chemically defined medium under industrial-scale fermentation conditions.

Results: Vi-CRM_{197} made using Vi from *Citrobacter* 328 elicited high anti-Vi antibody levels in mice and rabbits.

Conclusions: *Citrobacter* 328 is a suitable strain for production of Vi for conjugate anti-Typhi vaccines. Being a BSL-1 organism, which grows in defined medium and stably produces high yields of Vi, it offers excellent potential for the safe production of inexpensive vaccines for populations at risk of typhoid fever.

Key words: *S.* Typhi; Vi; *Citrobacter*; conjugate vaccine; Vi-CRM_{197}

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Introduction

Salmonella enterica serovar Typhi (*S.* Typhi) causes typhoid fever, a systemic disease that remains a major public health problem, predominantly in children in developing countries. The global burden of typhoid fever is uncertain, but estimates range from 17 to 22 million cases per year with 216,000 to 600,000 associated annual deaths [1,2]. *S.* Typhi expresses a virulence capsule antigen of polysaccharide (Vi) which allows the bacteria to modulate host responses during infection by evading innate immune surveillance [3,4]. Vi consists of repeating ([alpha]1-4),2-deoxy-2-N-acetyl galacturonic acid moieties, variably O-acetylated at C3 position, and is a target of protective immunity against typhoid fever [5].

A parenteral vaccine based on administration of Vi polysaccharide is licensed in more than 90 countries and extensive data document its safety, efficacy and practicality [6]. Due to poor immunogenicity of Vi polysaccharide vaccine in infants and the need for repetitive boosts to maintain protection even in adults [5,7], Vi has been conjugated to carrier proteins such as tetanus toxoid (Pedatyph) and the nontoxic recombinant exotoxin A of *Pseudomonas aeruginosa* (Vi-rEPA) [8]. Although no efficacy data are available for Pedatyph, a licensed vaccine only in India [9], Vi-rEPA showed a field efficacy of 89% in children two to five years of age for 46 months [10,11] and the generation of anti-Vi antibody at protective levels in infants in Vietnam [12]. Until proper sanitary conditions and clean drinking water are guaranteed in developing countries, vaccination against typhoid fever remains the most cost-effective defense for susceptible populations and is recommended by the World Health Organization (WHO) [13]. While the available data suggest that conjugate Vi vaccines will be more effective than unconjugated Vi, the affordability of a conjugate vaccine will be a critical factor for its implementation in endemic and impoverished populations. Therefore, all production steps, from antigen source through downstream processing, must be optimized to obtain a robust, inexpensive process that can be easily transferred to manufacturers.

Currently, Vi polysaccharide is obtained from the fermentation of *S.* Typhi (Ty-2 strain), which is a

biosafety level 3 (BSL-3) organism and requires high level of safety standards and risk management. *Citrobacter freundii* has been reported to produce a Vi capsular polysaccharide indistinguishable from *S.* Typhi Vi [14]. Thus the use of *Citrobacter* as a source of Vi presents several advantages in terms of safety and manufacturing costs and has been proposed for use in vaccines against *S.* Typhi [15]. However, the phenomenon of Vi-switching in *C. freundii* [16] potentially limits its use as a source of Vi. Vi-switching has been described for *C. freundii* strain WR7004, also known as *C. freundii* ballerup, which undergoes frequent transition between Vi-expressing and Vi-non expressing clones [17] and displays two types of colonies visualized on agar [16].

C. freundii strain WR7011 with stable Vi expression was generated from the parent strain WR7004 by nitrosoguanidine mutagenesis [17]. WR7011 has been used as the Vi source in studies assessing immunogenicity of Vi-vaccine conjugates [15, 18-21]. The random mutagenesis that generated WR7011 affected other metabolic pathways, rendering the organism unable to grow in defined media [15], thus limiting its utilization for large-scale production of Vi.

We have identified a wild type *Citrobacter* line 328 that showed both homogeneity and stability of Vi expression, and whose Vi was released into the culture supernatant when grown in chemically defined medium. Growth conditions and fermentation protocols were optimized and scaled to obtain Vi at high yield. Both fermentation and downstream processing were scalable for large-scale Vi manufacture.

Purified Vi was conjugated to the well-characterized diphtheria toxin mutant CRM_{197}, which was used as the carrier protein. This 58.4 kDa protein is a licensed carrier for childhood vaccines and has been shown to be safe and effective in numerous clinical trials [22-25]. We also evaluated antibody responses of Vi-CRM_{197} conjugate (in text defined as Vi(328)-CRM_{197}) elicited in sera of mice and rabbits, compared with a previously synthesized CRM_{197}-conjugate with Vi derived from WR7011 (in text defined as Vi(WR7011)-CRM_{197}). The industrial suitability of Vi production from *Citrobacter* 328 and the immunogenicity of its conjugate vaccine supports the development of Vi-CRM_{197} as a new affordable anti-typhoid vaccine.

Methodology

Antigens

Vi polysaccharides were either purified at the Novartis Vaccines Institute for Global Health (NVGH), or generously provided (source: *Citrobacter freundii* WR7011) by Dr. Shousun Szu (Laboratory of Developmental and Molecular Immunity, National Institute of Child Health and Human Development, National Institutes of Health [NIH]).

The carrier protein, CRM_{197}, was obtained from Novartis Vaccines and Diagnostics (NV&D), Siena, Italy.

Vi(WR7011) obtained from the NIH contained 1% nucleic acid (A260), 0.3% protein (micro BCA), and O-acetylation level of 68% according to ^{1}H NMR. Molecular size was estimated by HPLC-SEC running the sample on TSK gel 6000 PW in series with TSK gel 5000 PW, finding a Kd value of 0.45 [21]. Vi(328) purified by NVGH contained <1% (A260) nucleic acid, 0.3% protein (micro BCA), and O-acetylation level close to 100% (according to ^{1}H NMR). Molecular size was estimated by HPLC-SEC running the sample on TSK gel 6000 PW in series with TSK gel 5000 PW, finding a Kd value of 0.33 [26].

Citrobacter *strains and characterization*

Citrobacter strain 5396/38 (ATCC 10053, WR7010) and wild type *Citrobacter freundii* strains (as classified by the source) were obtained from the Novartis Master Culture Collection (NMCC, Emeryville, CA, USA) and provided with an NVGH strain number; *Citrobacter* 7851 (WR7004, ballerup) was obtained from the Wellcome Trust Sanger Institute (Hinxton, UK). WR7011 was obtained from the United States Public Health Service. Bacteria were screened for Vi expression, using anti-Vi polyclonal rabbit sera slide agglutination test (anti-*Salmonella* Vi, BioStat Sifin) to detect the presence of Vi capsular polysaccharide on the bacterial surface. A positive control (*Citrobacter* WR7011 strain) and a negative control (*S.* Typhimurium LT2 strain) were used. Among the wild type *Citrobacter freundii* strains obtained from NMCC, *Citrobacter* strain NVGH328 (subsequently referred to as *Citrobacter* 328), is a previously undescribed clinical isolate. On this strain biochemical profiling was performed by the Quality Control Department of Novartis Vaccines and Diagnostics using the MiniApi test (BioMérieux, Marcy l'Etiole, France) with ID32E strips, and at GenIbet Biopharmaceuticals (CITY, Portugal) using a Biolog GN2 MicroplateSystem (Biolog, Inc., Hayward, CA, USA). An initial antibiogram, to

determine *Citrobacter* 328 susceptibility to commonly used antibiotics, was performed at the time of submission to the NMCC and again prior to use at NVGH by the Molecular Biology Department, Università degli Studi (Siena, Italy).

Vi-competitive ELISA

A Vi-competitive ELISA method was developed to evaluate whether the strains also shed Vi polysaccharide into the culture medium. Each strain was inoculated into 5 ml of LB-medium using a sterile needle and starting from the original cryotubes. The bacteria were incubated overnight at 37°C under 210 rpm of agitation. Culture supernatants were collected by centrifugation at 16000 g, filter sterilized (0.22 µm) and stored at 4°C prior to use. The Vi competitive ELISA is based on the inhibition provided by Vi-containing supernatants to the binding of an anti-Vi standard serum to Vi-coated ELISA plates. The assay is based on our standard Vi ELISA [15] with some modifications. Nunc MaxiSorp ELISA plates were coated with Vi polysaccharide (obtained from NIH, purified from *Citrobacter freundii* WR7011 strain) at 1µg/ml in 0.05 M carbonate coating buffer (1.59 g/l Na_2CO_3, 2.93 g/l $NaHCO_3$, pH 9.6) overnight. The ELISA plates were washed and blocked with 5% fat free milk powder in PBS containing 0.05% Tween 20 (PBST) for one hour at room temperature. After three washes with PBST, wells were co-incubated with 100 µl of anti-Vi standard serum and Vi-containing supernatants. Unknown samples were tested in triplicate and a reference standard curve (defined below) was run in duplicate. Both anti-Vi standard serum and unknown samples were diluted in PBST containing 0.1% BSA. ELISA plates containing the test samples were incubated for two hours at room temperature and then washed with PBST. Incubation with secondary antibody (anti-mouse IgG phosphatase conjugated, Sigma A3438) diluted 1:10,000 and used at 100 µl/well was performed for one hour at room temperature. After washes with PBST, wells were incubated with 100 µl of phosphatase substrate and 4-nitrophenol phosphate disodium salt hexahydrate (Sigma, N.9389-50TAB 5 mg), in 1 M diethanolamine buffer (97ml/l diethanolamine, 0.1 g/l $MgCl_2$-$6H_2O$, pH 9.6). After incubation for one hour at room temperature, absorbance was read using an ELISA plate reader (ELx405, Biotek, Winooski, VT, USA) at 405 nm and 490 nm wavelengths. The reference standard curve for the competitive ELISA was created by incubating the anti-Vi standard serum with defined Vi concentrations to have a ten-point curve, made by dilution of Vi in PBST with 0.1% BSA starting from 0.39 µg/ml (final concentration in well) to 0.0198 ng/ml in three-fold dilutions. An additional two wells, containing only standard serum and no Vi, were considered positive controls (no inhibition expected). To the 90 µl/well of anti-Vi standard serum, 10 µl of either the Vi serial dilutions (to generate the standard curve) or the *Citrobacter* culture supernatants (diluted to give a final 1:1000 in well dilution) were added. Vi quantification in culture supernatants was achieved by interpolating Vi competitive ELISA absorbance values with a reference standard curve, created by spiking anti-Vi standard serum with defined Vi concentrations (three-fold Vi dilutions from 0.39 µg/ml to 0.0198 ng/ml). Hill Plot analysis calculated the following standard curve parameters: A = 0.7656; B = 1.0709; C = 0.0029; D = -0.6714; R2 = 0.9974.

The initial screening of new *Citrobacter* isolates and the evaluation of homogeneity of expression in 50 randomly picked colonies was done using Vi-competitive ELISA as a qualitative tool: Vi was not quantified and its presence was evaluated in terms of reduction in absorbance values. Negative control was anti-Vi serum co-incubated with culture medium. WR7011 culture supernatant was used as a positive internal control and run in parallel with all other strains.

Growth conditions and homogeneity/stability of Vi expression in Citrobacter *328*

Citrobacter 328 was grown under different conditions using both complex (Mod-LB, Glut-medium) and chemically defined media [15]. The following complex media were used:

1. Modified Luria Bertani (Mod-LB): 5 g yeast extract (Difco), 10 g ultrafiltered yeast extract (PTK), 8 g NaCl, 0.5% glycerol (no components of animal origin were used)

2. Glutamine medium (Glut-medium): 1 g/l glutamine, 10 g/l yeast extract, 0.07 M Na_2HPO_4, 0.03 M NaH_2PO_4, 0.01 M $MgSO_4$•$7H_2O$, with either 1% glucose or 0.5% glycerol.

The chemically defined (Chem. Def.) medium used was as follows: 13.3 g/l KH_2PO_4; 4 g/l $(NH_4)_2HPO_4$; 1.7 g/l citric acid monohydrate; 1 M $MgSO_4$ $7H_2O$; 0.1 M thiamine hydrochloride; 5 ml/l PTM4 Trace Salts. PTM4 Trace solution is composed of 2 g/l CuSO4•5H2O; 0.08 g/l sodium iodide; 3g/l $MnSO_4$•H_2O; 0.2 g/l Na_2MoO_4•$2H_2O$; 0.02 g/l boric acid; 0.5 g/l $CoCl_2$•$6H_2O$; 0.5 g/l $CaSO_4$•$2H_2O$; 7 g/l $ZnCl_2$; 22 g/l $FeSO_4$•$7H_2O$ (to aid in dissolution, 1 ml H_2SO_4 is added); and 0.2 g/ml d-Biotin in 2 N

NH_4OH. The carbon source used was either glucose or glycerol.

Homogeneity of Vi expression in *Citrobacter* 328 was evaluated by picking 50 colonies at random and performing competitive-ELISA. *Citrobacter* 328 was also evaluated for the stability of Vi expression over multiple generations. Starting from a single colony, cultures were grown in chemically defined medium and repeatedly plated for a total of 39 generations. Each time a single colony was picked, grown in liquid culture, and plated again. From each plate, two random colonies were picked and inoculated in chemically defined medium to quantify their Vi production by Vi-competitive ELISA. The concentration of viable bacteria in the starting culture and at each time point was estimated by plating aliquots and counting colonies. The number of elapsed generations were calculated as [log(C2/C1)]/log2 where C1 and C2 are the start and end bacterial concentrations.

Fermentation of Citrobacter *328 and scale-up*

To optimize growth conditions and Vi production, *Citrobacter* 328 was grown in a 7 L bioreactor (EZ-Control, Applikon, Schiedam, The Netherlands). Initial fermentation trials used 4 L chemically defined medium with glucose as the carbon source. Subsequent runs were conducted by replacing glucose with glycerol. Two different fermentation strategies were used: growth under glycerol limiting conditions (fed-batch fermentation) and a batch fermentation where all the glycerol (2-3%) was present in the medium from the beginning of the run. Subsequently, the fermentation protocol (using the batch fermentation) was scaled-up in four steps with a 5, 20, 30 and 50 liter scale, using B-Braun Sartorius Ltd bioreactors (Aubagne Cedec, France). The runs at 5 and 20 liter scale preparations were performed at Novartis Vaccines and Diagnostics (NV&D), Technical Development (TD) Department; the 30 and 50 liter scale runs were performed at GenIbet Biopharmaceutical (Portugal). A bacterial master cell bank was used to generate the working cell bank. The media used were chemically defined and contained no compounds of animal origin. Each fermentation run started from shake flask cultures prepared from the working cell bank. Initially, the bacteria were grown overnight in the same medium as that used for fermentation and they were diluted to obtain an inoculum with an OD between 0.001 and 0.3 (measured at 600 nm). The pH was controlled (AppliSens pH electrode, Applikon, Schiedam, The Netherlands) at 6 units by automatic addition of 14% NH_4OH. The temperature was maintained at 37°C with a heating blanket and a closed loop water circulation. The air sparging flow rate was set 1 L/min per culture volume and the dissolved oxygen (DO) was controlled at 30% air saturation (calibrated polarographic electrode: Oxygen sensor low drift, Applikon, Schiedam, The Netherlands). DO was maintained at 30% saturation by automatic variation of the agitation rate controlled in the range 500-1600 rpm (stirrer motor P100, Applikon). Foam was controlled by adding 0.25 ml/L polypropylenglicole (PPG) directly in culture medium at the time of media preparation. When needed, glucose concentration was measured by glucose oxidase method using glucose analyzer (GM8 Micro-Stat analyzer, Analox Instruments, Hammersmith, United Kingdom).

At 5 and 20 liter scale preparations, the fermentor was inoculated with *Citrobacter* 328 at OD = 0.02 (1:50 fermentation volume) and left to grow until reaching the desired final OD value. The fermentation runs were controlled as previously described, with an additional automatic variation of air flow (range: 0.5-4.7 L/minute) to maintain DO at not less than 30% saturation. Stir range was 100-1500 rpm and a slight overpressure of 0.5 bar was applied. At 30 liter scale run, bacteria were inoculated at OD = 0.004. All parameters were controlled as above, with an air flow range of 30-45 L/minute, a stir range between 200 and 800 rpm, and an overpressure up to 1 bar. At 50 liter scale preparation, bacteria were inoculated at OD = 0.004. All parameters were controlled as above, with an air flow range of 50 L/minute, a stir range between 300 - 800 rpm and an average overpressure of 0.06 bar (max reached: 0.4 bar).

Fermentation broth was harvested either by centrifugation, or microfiltration (0.22 μm pores) with Sartocon Slice 0.1 m^2. *Citrobacter* 328 expression of Vi was checked by slide agglutination, and Vi concentration in fermentation medium was measured using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) [21].

Purification and quantification of Vi and synthesis of Vi-CRM_{197} conjugates

Vi was purified from the culture media using a new procedure that is mild, robust and scalable, and does not require toxic reagents (such as phenol) [26]. Compared with Vi obtained from NIH, Vi purified at NVGH by this new protocol had a higher level of *O*-acetylation [21,26].

Figure 1. Vi expression in *Citrobacter* 328
The initial screening of new *Citrobacter* isolates for Vi production was performed using Vi-competitive ELISA on 5 ml of overnight culture supernatants collected by centrifugation, which were sterile filtered prior to use. All strains gave a similar final OD of 4-5. Negative control was anti-Vi reference serum co-incubated with culture medium. WR7011 culture supernatant was used as a positive internal control and run in parallel with all other strains (ten strains are shown as examples).

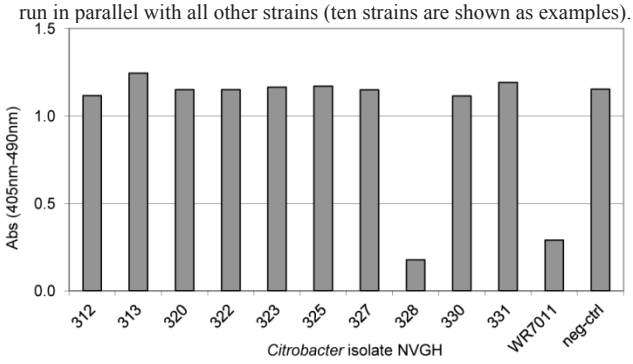
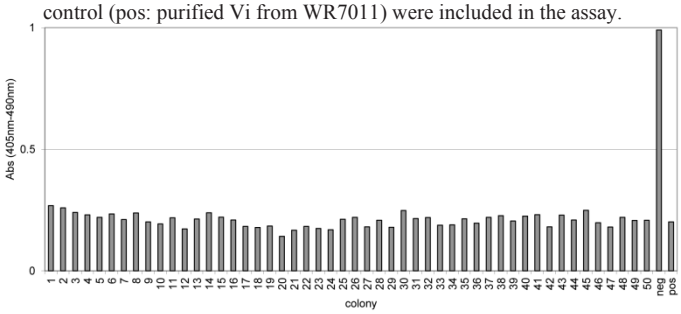


Figure 2. Homogeneity of Vi-expression in *Citrobacter* 328 colonies
Homogeneity of Vi expression was evaluated in 50 randomly picked *Citrobacter* 328 colonies using Vi-competitive ELISA (Vi positive cultures give a reduced signal relative to the negative control). Colonies were grown in 5 ml chemically defined medium with 0.5% glycerol, to OD values between 2 and 3. All colony cultures were normalized to a final OD of 2. Bacterial supernatants were centrifuged and sterile filtered. Negative control (neg: culture medium) and positive control (pos: purified Vi from WR7011) were included in the assay.



Vi-CRM_{197} conjugates were synthesized according to a method based on that reported by Kossaczka [18]. Briefly, CRM_{197} was derivatized with ADH in MES buffer pH 6.0. Then Vi was activated with EDAC and linked to the derivatized protein. Conjugates were prepared using a ratio Vi to CRM_{ADH} 1:1 in weight. Conjugates were synthesized using Vi from WR7011 [Vi(WR7011)-CRM_{197} conjugate] and from *Citrobacter* 328 [Vi(328)-CRM_{197}]. Vi(WR7011)-CRM_{197} was purified by gel filtration, using a Sephacryl S1000 column, while Vi(328)-CRM_{197} was purified by tangential flow filtration (300 K membrane). Conjugates were characterized by SDS-PAGE (3-8% gels stained with Coomassie) and by HPLC-SEC coupled with UV and fluorescence detectors. Polysaccharide concentration, both in unconjugated and Vi-CRM_{197} samples, was estimated by HPAEC-PAD. Vi-CRM_{197} protein content was measured by micro BCA (Thermo Scientific, CITY, COUNTRY), using BSA as standard reference. Vi(WR7011)-CRM_{197} and Vi(328)-CRM_{197} had 0.86 and 0.99 saccharide/protein ratio, respectively.

Immunization studies

For evaluation of immunogenicity, unconjugated Vi from *Citrobacter* 328 (referred as Vi(328)), Vi(328)-CRM_{197} and Vi(WR7011)-CRM_{197} conjugates were used to vaccinate CD1 mice and New Zealand white rabbits. Sera were collected and analyzed by ELISA as previously described [15]. The Spearman rank correlation was used to evaluate antibody dose-response relationships. The Student's t-test was used on log-transformed data to compare each of the Vi-CRM_{197} conjugates with Vi(328), and the Vi-CRM_{197} conjugates with each other at 8 µg/dose. All experimental protocols involving animals were approved by the Italian Animal Ethical Committee.

Results

Anti-Vi slide agglutination test

The *Citrobacter* strains 5396/38 (ATCC 10053, WR7010) and 7851 (WR7004, ballerup), when plated onto LB agar, showed the presence of two colony morphologies: one type slightly creamy-yellow and opaque, and the second type white and transparent. Mixed colony morphology was previously reported, correlating with a switch in Vi expression [16,27]. In contrast, WR7011 showed only the first morphology. Positive anti-Vi agglutination tests correlated with the first colony morphology, while colonies of the second morphology were consistently negative. These results were in agreement with those previously reported for the WR7004 strain, demonstrating the Vi-switch phenomenon, and the constitutive Vi expression of the WR7011 strain. We also confirmed that a single Vi positive colony from *Citrobacter* WR7004, when plated again, would produce a mixed population of Vi-positive and Vi-negative clones [17]. *Citrobacter* 328 was the only strain that produced a strong agglutination pattern and a single Vi-positive colony type. The other *Citrobacter* strains tested produced a single colony type, negative for Vi-expression.

Vi-competitive ELISA

Inhibition of the absorbance signal indicated the presence of Vi in the competitive ELISA. The only *Citrobacter* strain that provided a strong inhibitory signal was *Citrobacter* 328, which gave an inhibition greater than WR7011 (Figure 1). No inhibition provided by culture medium was observed for any of the other strains tested. Based on the ability to express

Table 1: Stability of Vi expression of *Citrobacter* 328 over 40 generations

Criteria	Plate A		Plate B		Plate C		Plate D		Plate E	
Generations	0		16		25		34		39	
Colony	A1	A2	B3	B4	C5	C6	D7	D8	E9	E10
Morphology	creamy-yellow and opaque									
Culture OD [a]	4.6	4.4	5.0	4.6	5.0	4.7	4.6	4.7	4.6	4.6
Vi, µg/ml	74.3	81.3	74.0	96.1	85.9	79.2	99.0	105.6	97.9	85.7
Anti-Vi agglutination	+	+	+	+	+	+	+	+	+	+

[a] OD: Optical Density

and release high levels of Vi antigen, *Citrobacter* 328 was selected for further evaluation.

Growth conditions in shake flasks

Citrobacter 328 grew well in both complex (Mod-LB, Glut-medium) and chemically defined medium in shake flasks. Mod-LB medium supported a similar growth with either 0.5% glucose or glycerol (OD = 4 and 4.8, respectively). Glut-medium with 0.5% glycerol supported the highest growth (OD = 8). In chemically defined medium *Citrobacter* 328 grew to OD = 3.8 with 0.5% glycerol and OD = 2.7 with 0.5% glucose. The correlation between OD and colony forming units (CFU) was determined to be $2x10^9$ CFU/OD/ml as measured by plate dilutions. Considering the manufacturing advantages of using a chemically defined medium instead of complex media, all further experiments were done with chemically defined medium.

Homogeneity/Stability of Vi expression in Citrobacter *328*

Citrobacter 328 stably released Vi capsular polysaccharide into the culture media at similar levels for all of 50 colonies tested (Fig. 2). *Citrobacter* 328 strain was also evaluated for the stability of Vi expression over multiple generations (Table 1). All colonies tested grew to similar OD values, produced comparable levels of Vi (74-105 µg/ml), and were positive for agglutination assay. Additionally, all showed the same colony morphology, indicative of Vi-positive colonies (approximately 100 colonies were screened at each time point).

Culture identification and characterization

Citrobacter 328 was classified by the Biolog test as *C. freundii*; alternatively, the MiniApi test identified the strain as *C. youngae*. Given the known difficulties in distinguishing *Citrobacter* genus using the commercially available identification tests, *Citrobacter* 328 was classified within the *Citrobacter freundii* complex (*Citrobacter freundii sensu lato*). *Citrobacter* 328 showed a broad sensitivity to most commonly used antibiotics.

Fermentation

Initial fermentation trials were done in chemically defined medium with glucose as the carbon source. Although different strategies were tried (batch fermentation, pulse-feed glucose addition, additional feeds of citrate), the maximum OD was not greater than 8, suggesting possible catabolite growth suppression effect (Figure 3A). Although initial glucose concentration in medium (2.6%) slowly decreased during bacteria growth, duplication time (Td) of *Citrobacter* 328 steadily increased up to stationary growth (Td = 2h to OD = 3). The addition of more glucose at the end of fermentation did not restore bacterial growth. At the end of fermentation, Vi in the culture medium was measured by HPAEC-PAD and by Vi-competitive ELISA; both assays detected 60-65 µg/ml of Vi (7.8 µg/ml/OD).

Subsequent fermentations were performed using glycerol as the main carbon source in chemically defined medium and no growth suppression occurred under this condition. The first fermentation strategy using glycerol was fed-batch fermentation (Figure 3B). Initially, bacteria grew exponentially until all glycerol in the medium was exhausted (starting glycerol concentration: 0.18%). After exhaustion of the carbon source, glycerol was pumped into the bioreactor at 1.5 g/L/h, which produced linear

Figure 3: Typical fermentation of *Citrobacter* 328 using glucose or glycerol as carbon source
A. Typical 4 L fed-batch fermentation run using glycerol as carbon source and relative Vi production. OD of initial inoculum: 0.001; air flow: 3-10 L/min; initial glycerol concentration: 0.18%; feeds: glycerol was pumped in at 1.5 g/L/h at 15 h post-inoculum (OD=12); final OD: 35. A 7 L-Applikon bioreactor was employed.
B. Typical 4 L fermentation run in chemically defined medium with glucose as carbon source. OD of initial inoculum: 0.3; air flow: 3 L/min; initial glucose concentration: 2.6%; feeds: 2% glucose 25th h post-inoculation; final OD: 8. A 7 L-Applikon bioreactor was employed.

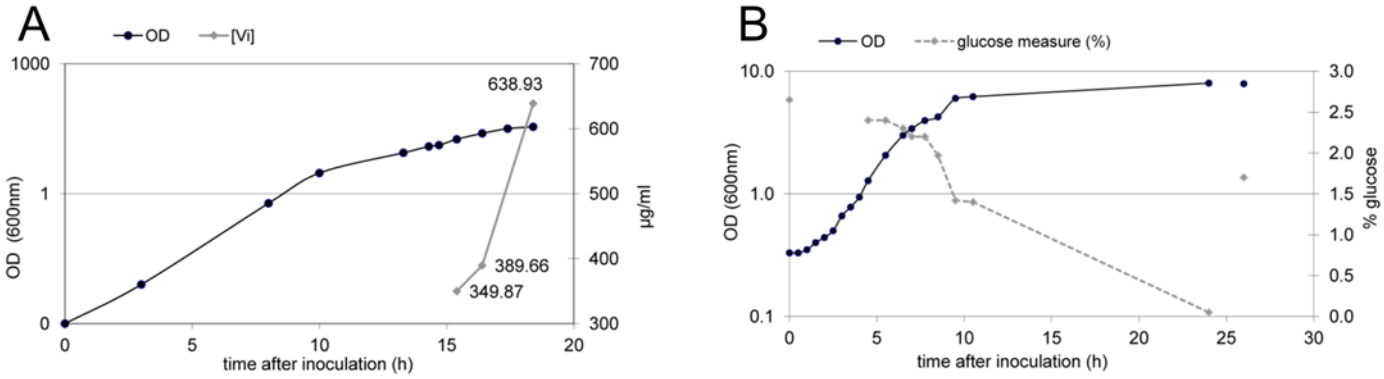


Figure 4. Growth curve comparison during scale up from 4 to 30 L
Air flow range: 0.5-4.7 L/minute (5 and 20 L run), 30-45 L/minute (30 L run); stirrer range: 100-1500 rpm (5 and 20 L run), 200-800 (30 L run); overpressure: 0.5 bar (5 and 20 L run), 1 bar (30 L run). At 5 L-scale, Td: 1.3 h and Vi: 16.1 µg/ml/OD; at 20 L-scale, Td: 1.3 h and Vi: 13.6 µg/ml/OD; at 30 L-scale, Td: 1 h and Vi: 10.8 µg/ml/OD; at 50 L-scale, Td: 1.2 h and Vi: 15.2 µg/ml/OD

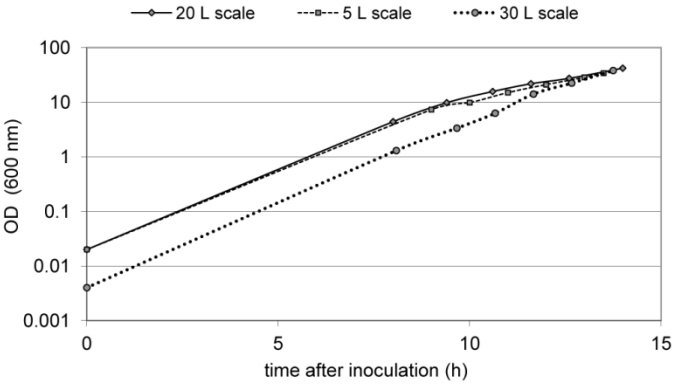
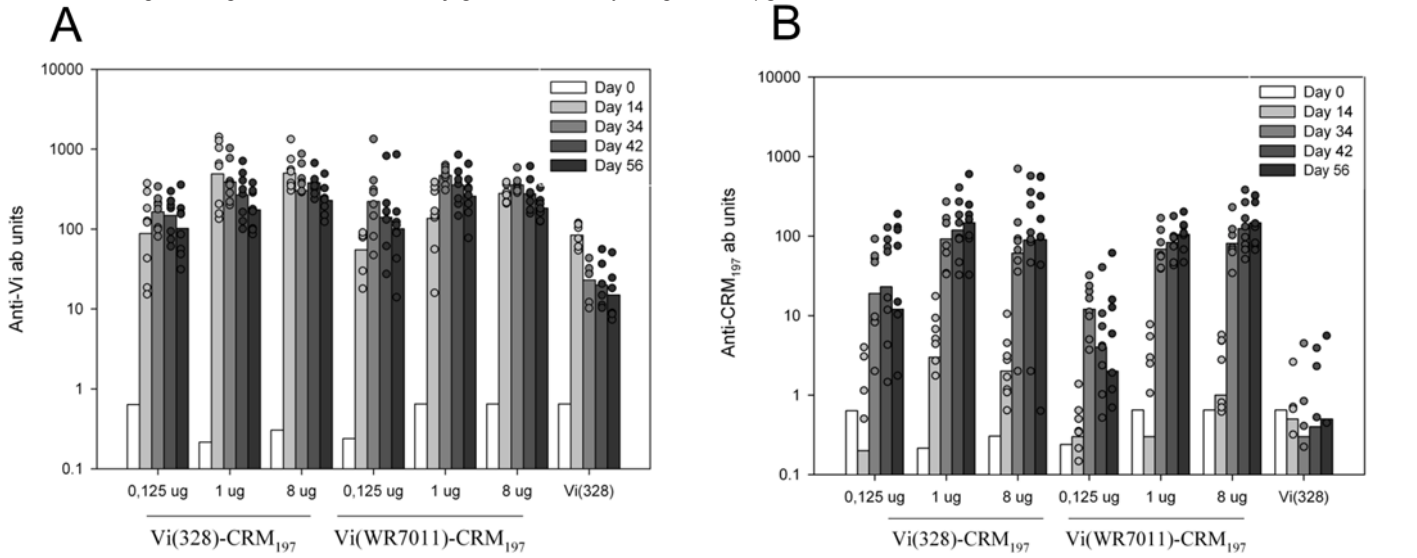


Figure 5. Immunization of mice with Vi(328)-CRM$_{197}$ and Vi(WR7011)-CRM$_{197}$
A: anti-Vi antibody response
B: anti-CRM$_{197}$ antibody response. Vaccinations were given by two subcutaneous injections (200 µl) at day 0 and day 14. Eight mice per group were immunized with increasing doses of conjugates (from 0.125 to 8 µg of Vi/injection). An additional group of 8 mice received 8 µg of Vi (328)/injection. Blood was collected at days 0 (pre-immune serum), 14, 34, 42 and 56. Individual mouse ELISA units are represented by the scatter plots; bars represent the group geometric mean. Day 34 sample was considered the definitive bleed for comparison of antibody responses among groups and for statistical analysis. Spearman rank correlation for anti-Vi antibodies in Vi(328)-CRM$_{197}$ group: $\rho = 0.5$ and $p = 0.009$. Spearman rank correlation for anti-Vi antibodies in Vi(WR7011)-CRM$_{197}$ group: $\rho = 0.2$ and $p = 0.3$. Spearman rank correlation for anti-CRM$_{197}$ antibodies in Vi(328)-CRM$_{197}$ group: $\rho = 0.5$ and $p = 0.01$; in Vi(WR7011)-CRM$_{197}$ group: $\rho = 0.8$, $p < 0.001$. Student *t* test to compare anti-Vi antibodies between each Vi-CRM$_{197}$ conjugate and unconjugated Vi (328) at 8 µg/dose: $p < 0.001$ in both comparisons; $p = 0.7$ when the two conjugates were directly compared at 8 µg/dose.



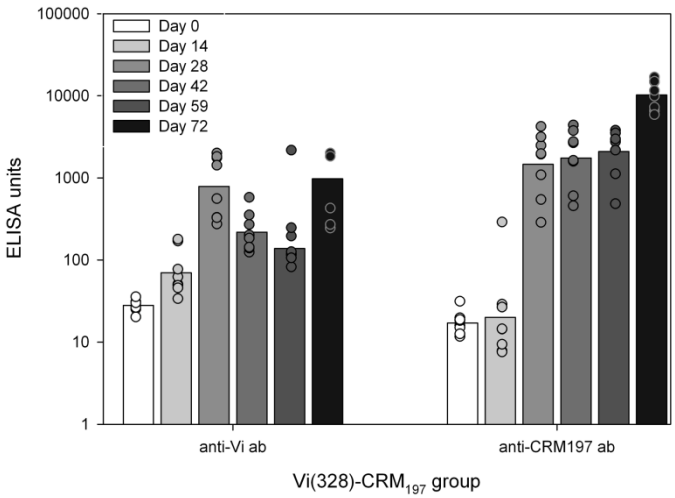
bacterial growth under glycerol limiting conditions. As shown in Figure 3B, glycerol was completely consumed when bacteria reached an OD of 12 (after about 15 hours). Growth was maintained for the next 4 hours by glycerol constant feed until OD = 35. Td during the exponential phase (up to OD = 12) was approximately 1 hour; it increased to 2.6 hours under glycerol-limiting conditions. Vi accumulated in culture medium proportional to OD. Approximately 640 µg/ml of Vi was detected at the end of fermentation (18.2 µg/ml/OD).

To further simplify the fermentation protocol, a batch fermentation was developed, where all the glycerol required to reach at least a final OD of 35 (3% glycerol) was present from the beginning of fermentation. As seen in Figure 4, the 4 L-fermentation was stopped at OD = 34, after 13.5 hours after inoculation, with an overall duplication time of 1.3 hours. Vi release into the medium at harvesting time was approximately 550 µg/ml (16.1 µg/ml/OD). The process was scaled to a 30 L fermentation run (Figure 4) in Sartorius B-Braun bioreactors. Although higher OD values could have been reached (OD~60), a final OD = 35 was chosen for harvest to standardize the subsequent purification process. Scale-up from 4 to 30 L showed comparable Td (from 1.3 h to 1.2 h) and a small decrease of Vi yield (from 16.1 to 15.2 µg/ml/OD). Engineering and current good manufacturing practices runs were also performed at 50 L-scale resulting in similar profiles.

Immunization experiments

To compare the immunogenicity of the new Vi(328)-CRM_{197} vaccine with previously synthesized Vi(WR7011)-CRM_{197}, mice were immunized twice with increasing doses of conjugate and compared with an unconjugated Vi(328) as a control group (Figure 5 A-B). Anti-Vi antibodies were detected even at the lowest dose tested (0.125 µg) in both conjugate groups, but only anti-Vi antibodies from mice receiving Vi(328)-CRM_{197} showed a dose-response relationship. Dose-response was also found for anti-CRM_{197} antibodies in both groups immunized with the conjugate vaccines. At 8 µg/dose, both groups immunized with conjugate vaccines showed significantly higher anti-Vi antibody response compared with the group immunized with unconjugated Vi(328), but no significant difference was found by direct comparison of the two conjugates. Also in the rabbit model, sera from the Vi(328)-CRM_{197} immunized group contained significantly higher anti-Vi antibodies compared with the unconjugated Vi group. Anti-Vi levels reached a peak at day 28 and a booster response was observed at day 72, following a delayed third immunization. Similarly, anti-CRM_{197} antibodies were boosted after the last immunization (Fig. 6).

Figure 6. Anti-Vi and anti-CRM_{197} antibodies in rabbit sera
Two groups of 8 New Zealand White rabbits received 25 µg of Vi (328)-CRM_{197}/injection (first group), and 25 µg of unconjugated Vi (328)/injection (second group, not shown). Both groups were immunized intramuscularly (500 µl injection volume) on days 1, 14 and 63, and bled prior to immunization and on days 14, 28, 42, 59 and 72. Individual rabbit ELISA units are represented by the scatter plots; bars represent the group geometric mean. Student *t* test for anti-Vi antibodies between Vi(328)-CRM_{197} and unconjugated Vi groups on day 28: $p < 0.001$.



Discussion

Typhoid fever is endemic in many developing countries and remains a substantial public health problem despite ongoing progress in water and sanitation coverage. Treatment costs are high, up to $820 per case per hospitalized patient in India [13], and due to the spread of multidrug resistant strains, therapy is often ineffective [28]. School-age children and young adults were originally thought to be the main risk groups, but increasing evidence shows high incidence rates in younger children in endemic countries [10,29,30]. The need to provide protection also to children younger than two years of age has stimulated the development of new conjugate vaccines, such as Pedatyph or Vi-rEPA [8,9,12], as better alternatives to unconjugated vaccines.

However, if public health professionals evaluate the opportunity of introducing a new vaccine in terms of reduced mortality and morbidity rates, national health and finance ministries must estimate cost savings through the implementation of proposed vaccines. To be adopted into the health system, a new vaccine must be effective and every step of

development must be carefully planned to ensure low cost and feasibility of manufacture. This is especially true for vaccines needed in developing countries, where local manufacturers may be responsible for cGMP production.

Under this guiding principle, NVGH undertook the development of a Vi-CRM_{197} conjugate vaccine, where Vi is produced by a *Citrobacter* strain. We chose *Citrobacter* rather than *S.* Typhi for its safety profile, and the reduced handling costs. Although it has been reported that *Citrobacter* can express a Vi polysaccharide indistinguishable from *S.* Typhi, its use for large-scale production has been impaired by unstable Vi expression. In our previous report, we used an engineered *Citrobacter* strain (WR7011) as the Vi source. WR7011 constitutively expressed Vi; however, its growth requirements render it not optimal for large-scale fermentation.

We identified a wild type *Citrobacter* strain 328 with high levels of Vi expression and stability in chemically defined media, making it a good source of Vi for manufacture. The fermentation process has been optimized and scaled to 50 L obtaining a Vi yield of 13.1 µg/ml/OD, which is more than twice what was previously reported for fermentation of *S.* Typhi Ty2 [31]. Considering a 50% overall yield of the purification process, we estimate that from one single 50 L fermentation run, resulting in 700 µg/ml of crude Vi, about 17.5 g of purified Vi can be obtained [26]. With a Vi-CRM_{197} conjugation yield of 70%, about 490,000 vaccine doses (at 25 µg per dose) can be obtained, as confirmed by cGMP manufacture.

C. youngae and *C. freundii* have similar phenotypic and genotypic characteristics, are both classified as part of the *C freundii* complex [32], and are not definitively distinguishable using commercial identification systems. Although some *C. freundii* strains have been shown to produce a Vi capsule, these reports predate the revision of the classification of the *C. freundii* complex, so it is not known which members of this complex are likely to express Vi. It is also unknown how prevalent the Vi capsule is in *C. freundii sensu lato* clinical isolates, and whether the presence of Vi in *Citrobacter* 328 is either an isolated event, or a more generalized and uninvestigated phenomenon. SPI-7, which encodes Vi, may have acted as conjugative transposon not only in *Salmonella* serogroups such as *S.* Typhi, *S.* Dublin and *S.* Paratyphi C [14], but also in other *Citrobacter* species beyond *C. freundii* [33].

Vi O-acetylation and its molecular size are factors that influence immunogenicity [20]. Vi provided by the NIH from *Citrobacter* WR7011 and Vi purified by NVGH from *Citrobacter* 328 differ both in molecular size (Kd of 0.36 and 0.33 respectively by gel permeation chromatography on TSK gel 6000 PW in series with TSK gel 5000 PW) and O-acetylation level (68% O-acetylation for Vi from WR7011 and close to 100% for Vi from *Citrobacter* 328 purified at NVGH). Immunogenicity studies showed that the antibody response is significantly reduced when O-acetylation reaches 30%, and it is completely abolished in de-acetylated Vi samples [20,34].

We previously showed that the Vi conjugates prepared from *Citrobacter* WR7011 induced responses in mice that were similar to the responses generated by *S.* Typhi derived Vi-rEPA conjugates [21]. In this study, we further show that despite differences in size and O-acetylation levels, the conjugates derived from the WR7011 and *Citrobacter* 328 Vi give similar responses. In the earlier immunogenicity studies, the *S.* Typhi Vi and WR7011 Vi both had similar O-acetylation levels of about 70%. These observations suggest that the difference between 70 and 100% O-aceylation do not grossly change the immune properties. However, when Vi is extracted from *S.* Typhi using the purification process developed for *Citrobacter* 328 [Micoli, unpublished observations], we find O-acetylation levels close to 100%. Although our immunogenicity results showed no difference between Vi(328)-CRM_{197} and Vi(WR7011)-CRM_{197}, we consider it preferable to use a Vi preparation with the highest O-acetylation levels, as the starting material for a conjugate vaccine.

Preclinical rabbit repeat dose toxicology studies have confirmed the safety of administration of Vi from *Citrobacter* 328, and Phase 1/2 clinical trials have been conducted in European adults demonstrating the safety and immunogenicity of Vi-CRM_{197} conjugate vaccine [35]. Additional Phase 2 trials in populations of endemic areas are currently ongoing. In conclusion, *Citrobacter* 328 is a suitable strain for production of Vi, offering excellent potential for safe manufacturing of inexpensive vaccines for populations at risk of typhoid fever.

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Corresponding author

Simona Rondini
Novartis Vaccines Institute for Global Health
via Fiorentina 1
53100 Siena, Italy
Telephone: +39-0577-243846;
Fax: +39-0577-243540
Email: simona.rondini@novartis.com

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