Salmonella Paratyphi A outer membrane vesicles displaying Vi polysaccharide as multivalent vaccine against enteric fever

G. Gasperini[1], R. Alfini[1], V. Arato[1], F. Mancini[1], M. G. Aruta[1], P. Kanvatirth[2], D. Pickard[2], F. Necchi[1], A. Saul[1], O. Rossi[1], F. Micoli[1*] and P. Mastroeni[2*]

1: GSK Vaccines institute for Global Health, Via Fiorentina 1, 53100, Siena (Italy)

2: University of Cambridge, Cambridge (UK)

* Contributed equally to the work

Corresponding authors:

Dr Pietro Mastroeni, pm274@cam.ac.uk, Department of Veterinary Medicine, Madingley Road CB3 0ES, Cambridge (UK)

Dr Francesca Micoli, francesca.x.micoli@gsk.com, GSK Vaccines institute for Global Health (GVGH), Via Fiorentina 1, 53100, Siena (Italy)

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Abstract

Typhoid and paratyphoid fever have a high incidence worldwide and coexist in many geographical areas, especially in Low-Middle Income Countries (LMIC) in South and South East Asia. There is extensive consensus on the urgent need for better and affordable vaccines against systemic *Salmonella* infections. Generalized Modules for Membrane Antigens (GMMA), outer membrane exosomes shed by *Salmonella* bacteria genetically manipulated to increase blebbing, resemble the bacterial surface where protective antigens are displayed in their native environment.

Here we engineered *S.* Paratyphi A using the pDC5-*viaB* plasmid to generate GMMA displaying the heterologous *S.* Typhi Vi antigen together with the homologous O:2 O-Antigen. The presence of both Vi and O:2 was confirmed by flow cytometry on bacterial cells and their amount was quantified on the resulting vesicles through a panel of analytical methods. When tested in mice, such GMMA induced a strong antibody response against both Vi and O:2 and these antibodies were functional in a serum bactericidal assay. Our approach yielded a bivalent vaccine candidate able to induce immune responses against different *Salmonella* serovars which could benefit LMIC residents and travellers.

Introduction

Salmonella enterica serovars Typhi (*S*. Typhi) and Paratyphi (*S*. Paratyphi) subtypes A, B and C cause enteric fevers, a major global-health concern. *S*. Typhi (Typhoid fever) causes an estimated 14.9M cases annually and 116,800 associated deaths with post antimicrobial relapses in up to 10% of patients and chronic carriage in up to 6% of treated individuals (1); *S*. Paratyphi causes an estimated 3M of paratyphoid fever and approximately 19,000 deaths annualy (1). These diseases coexist in many geographical areas, especially in Low-Middle Income Countries (LMIC). *S*. Typhi incidence is high in South and South East Asia as well as Africa; an increasing incidence of *S*. Paratyphi A has been reported over the past 2 decades in different parts of Asia, including Nepal (2), Cambodia (3), and China (4).

Current treatments for *S. enterica* infections are hampered by emergence of multi-drug-resistant strains (5-7, 8).

Vaccines are a powerful tool against systemic *Salmonella* infections. Several vaccines have been licensed for the prevention of typhoid fever; however, no vaccine is as yet available against paratyphoid fever (7, 9). The licensed *S.* Typhi Ty21a live typhoid vaccine is safe, but gives moderate protection after multiple dosing (10). Typhoid conjugate vaccines (TCV), in which *S*. Typhi Vi capsular polysaccharide is covalently linked to carrier proteins, offer several potential advantages over earlier generation vaccines, especially enhanced immunogenicity and ability to induce immune responses in infants (11). Similar strategies are currently being investigated for the development of a paratyphoid vaccine including live attenuated (LAV) and non-living vaccines (12). Some recent LAV candidates (*e.g.* the *aroC/ssaV*M01ZH09 and WT05 mutants) showed insufficient immunogenicity (13). LAV can cause lethal infections in immune-compromised hosts (14-17) and therefore, conjugate vaccines would represent a much safer alternative. Recently, an O-Antigen (OAg) glycoconjugate based on the

67 immunodominant O:2 factor has been proposed as a vaccine against *S*. Paratyphi A infections
68 (18, 19).
69 A bivalent formulation would probably be the wiser choice to induce antibody responses that
70 can potentially protect against both *S*. Typhi and *S.* Paratyphi A. Moreover, such vaccine
71 combination would increase the commercial attractiveness of the *S.* Paratyphi A component,
72 especially considering the disproportionate incidence of the two diseases.
73 Recently, General Modules for Membrane Antigens (GMMA) have been proposed as an
74 alternative delivery system for the OAg (20). GMMA are outer membrane vesicles (OMV)
75 naturally shed by Gram-negative bacteria specifically engineered to increase blebbing and
76 obtained through a simple and robust manufacturing process possibly leading to affordable
77 vaccines (21-23). GMMA contain mainly outer membrane proteins and lipopolysaccharides
78 (LPS) together with luminal periplasmic proteins, .GMMA are highly immunogenic and induce
79 T-cell-dependent, boostable, isotype-switched, highly functional IgG profiles (24). This is
80 crucial, given the importance of the quality of the antibody response in protection against
81 salmonelloses (25, 26). Compared to traditional glycoconjugate vaccines, GMMA have the
82 added value of combining multiple antigens in a single vaccine component, including
83 polysaccharides and proteins possibly contributing to clinical protection. Indeed, GMMA from
84 *S.* Typhimurium and *S.* Enteritidis are protective in animal models (24) and a *Shigella sonnei*
85 GMMA-based vaccine has been recently shown to be well tolerated and immunogenic in
86 healthy adults and endemic populations (27-29).
87 In this study we explored the possibility to induce functional immune responses against *S*.
88 Paratyphi A O:2 OAg and *S*. Typhi Vi polysaccharide antigen using GMMA from *S.* Paratyphi A
89 as a delivery system.
90

Results

Generation and characterization of OMV expressing Vi and OAg-specific antigen.

With the aim to engineer a *S.* Paratyphi A GMMA-producing strain that would display also the *S*. Typhi Vi antigen, *S.* Paratyphi A strain NVGH2041 (ParA O:2 Vi^-), lacking the *tolR* gene for increased outer membrane blebbing, was transformed with pDC5-*viaB*; this is a plasmid which contains the entire *viaB* locus from *S*. Typhi and therefore all genes needed for Vi production and anchoring to the membrane (30). Simultaneous surface exposure of both Vi and O:2 on the bacterial surface of the resulting strain, indicated as ParA O:2 Vi^+, was confirmed by flow cytometry using specific anti-O:2 and anti-Vi sera (Figure 1). ParA O:2 Vi^+ and O:2 Vi^- were both recognized by the anti-O:2 serum, indicating that the presence of Vi does not hinder the binding of antibodies to OAg. GMMA were produced from ParA O:2 Vi^+ and O:2 Vi^- strains and were fully characterized through a panel of analytical methods. Both sets of GMMA had a similar size (average size of 72 and 83 nm in diameter, Table 1) as determined by Dynamic Light Scattering (DLS), and a similar OAg/protien (w/w) ratio as determined by High-Performance Anion Exchange Chromatography-Pulsed Amperometric Detection (HPAEC-PAD). The amount of Vi (µg) in ParA O:2 Vi^+ GMMA was ~10 times lower compared to the amount of OAg. To determine whether such low Vi amount was due to heterologous expression of the *viaB* locus in *S.* Paratyphi A, *S*. Typhi BRD948 (Typhi O:9 Vi^+) and its isogenic □*tviB* mutant (Typhi O:9 Vi^-) were included as benchmarks in our analysis. Both *S*. Typhi strains display OAg containing the immunodominant O:9 factor but only the native BRD948 is Vi^+ due to the presence of the *viaB* locus in the chromosome. Similarly to what seen for ParA O:2 Vi^+, surface exposure of both Vi and O:9 was detected on the bacterial surface of *S*. Typhi O:9 Vi^+ (Figure 1). Naturally released OMV were produced from *S*. Typhi strains and compared to

GMMA obtained from *S*. Paratyphi A strains. OMV had more heterogeneous size compared to GMMA, with average size of 81 and 133 nm in diameter and higher polydispersion index (Table 1). The OAg/protein (w/w) ratio in *S*. Typhi O:9 Vi^- OMV was similar to that measured in *S*. Paratyphi A GMMA, while Typhi O:9 Vi^+ OMV showed a higher OAg/protein (w/w) ratio as compared to the other preparations (Table 1). Importantly, the Vi/protein (w/w) ratio in Typhi O:9 Vi^+ OMV was comparable to that of ParA O:2 Vi^+ GMMA (Table 1).

Immunogenicity of GMMA/OMV in a preclinical murine model.

To test the possibility of inducing immune responses against both Vi and OAg with OMV/GMMA vaccine candidates, 4 groups of six C57BL/6 mice were immunised subcutaneously with vesicles prepared from ParA O:2 Vi^+, ParA O:2 Vi^-, Typhi O:9 Vi^+ and Typhi O:9 Vi^-. All animals received a booster vaccination on day 28 and sera were collected from individual animals on day 42. Each mouse received a dose equivalent to 0.5 µg of Vi antigen; this dose also resulted in the administration of similar amounts of OAg (Table 2). The immunogenicity of GMMA/OMV was assessed by measuring total IgG against Vi (Figure 2A), O:2 (Figure 2B) and O:9 (Figure 2C). ParA O:2 Vi^+ and ParA O:2 Vi^- GMMA induced similar levels of anti-O:2 IgG confirming that display of Vi at the surface of the vesicles did not hinder their ability to induce anti-OAg IgG responses (Figure 2B). Immunization with ParA O:2 Vi^+ resulted in the induction of anti-Vi antibodies (Figure 2A) showing that the Vi antigen was delivered in immunogenic form when using Vi^+ vesicles. Interestingly, the anti-Vi response induced by the vesicles from the *S.* Paratyphi A, engineered to display Vi using episomal expression of the *viaB* locus, was comparable to that of OMV from the naturally Vi^+ serovar Typhi (Figure 2A). This indicated that it is possible to induce immune responses to Vi using vesicles produced from strains engineered for heterologous display of Vi. Moreover, *S.* Typhi O:9 Vi^+ and *S.* Typhi

O:9 Vi^- also induced similar level of anti-O:9 IgG (Figure 2C), once again confirming the lack of immune interference between Vi and OAg. Next, we tested the functional activity of resulting sera in a Serum Bactericidal Assay (SBA) using bacterial strains displaying either O:2, O:9 or Vi. Sera from mice immunized with ParA O:2 Vi^+ and ParA O:2 Vi^- GMMA showed similar bactericidal activity against the O:2-displaying *S.* Paratyphi A test strain (Figure 2E). Thus, display of Vi on the surface of *S*. Paratyphi A GMMA does not affect their ability to induce functional antibody responses capable of mediating bactericidal activity. We confirmed the ability of ParA O:2 Vi^+ GMMA antisera to exert SBA against a *C. freundii* sensu lato strain, displaying the Vi antigen, but not any other *Salmonella*-specific OAg determinants (Figure 2D). As previously observed in Enzyme-Linked Immunosorbent Assay (ELISA), the functional activity of anti-Vi antibodies induced by ParA O:2 Vi^+ GMMA was comparable to that induced by Typhi O:9 Vi^+ OMV (Figure 2D). This shows that vesicles from ParA O:2 Vi^+ and Typhi O:9 Vi^+, but not from their Vi^- counterparts, can induce functional antibodies able to activate complement deposition and exert Vi-specific SBA. Finally, sera from mice immunized with Typhi O:9 Vi^+ and Typhi O:9 Vi^- OMV also showed similar bactericidal activity against the O:9-displaying *S.* Enteritids test strain (Figure 2F).

Discussion

The possibility to deliver multiple antigens and to confer protection against multiple *Salmonella* serovars is becoming increasingly important in the light of the awareness of geographical coexistence of multiple *Salmonella* diseases such as typhoid and paratyphoid fever.

In the present study we explored the possibility to produce a vesicle-based bivalent vaccine candidate against enteric fever, based on GMMA delivering the Vi polysaccharide from *S*.

Typhi and the somatic O-Antigen from *S.* Paratyphi A. Different GMMA preparations were obtained at small scale and characterized, ensuring reproducibility of the main analytical characteristics. However, additional work will be needed for the process scale-up, including evaluation of lot-to-lot consistency. We show that a *S.* Paratyphi A GMMA engineered to display the Vi antigen from *S.* Typhi can induce both anti-Vi and anti-O:2 antibodies. This indicates that Vi does not render the underlying O-antigen inaccessible for recognition by the immune system. Furthermore, the immune responses induced by the O- and Vi-antigens are functional both against $O:2^{+}$ and Vi^{+} target strains in a serum bactericidal assay, further supporting their potential for broad protective activity.

A typhoid-paratyphoid vaccine would be a great asset for LMIC and travelers given that no paratyphoid vaccines are currently licensed. Glycoconjugates are a well-established bacterial vaccine approach and have been proposed as strategies against both *S.* Typhi and *S.* Paratyphi A (12, 20). More recently GMMA have been proposed as an alternative delivery system for OAg (20), particularly attractive when multicomponent preparations are required and when impoverished communities are the vaccine target population. Compared to traditional glycoconjugates, GMMA show similar or better immunogenicity and a simpler manufacturing process (31), representing a promising alternative for the development of affordable multicomponent vaccines against *Salmonella* serovars (24).

S. Typhi OMV, naturally displaying the Vi antigen, were included in this study as internal controls and compared to *S*. Paratyphi A GMMA engineered to display the heterologous Vi polysaccharide. No differences were observed either in the amount of Vi found on the resulting vesicles or in the immunogenicity and functional activity of anti-Vi antibodies elicited upon immunization. Moreover, *S*. Typhi OMV were able to induce both anti-Vi and anti-O:9 antibodies, similarly to what observed with ParA $O:2\ Vi^{+}$ GMMA inducing both anti-Vi and

anti-O:2 antibodies. These OMV therefore induced responses that would target both Vi^+ *S*. Typhi (anti-Vi and anti-O:9 antibodies) and Vi^- *S*. Typhi isolates, which occur in the field and are reported to be able to cause disease (32). Our previous work found that the Vi antigen is rapidly downregulated once the bacteria reach an intracellular location in the infected tissues with the majority of the bacterial population becoming Vi^- and no longer displaying a target for the immune response (33). Finally, these OMV would also target *S*. Enteritidis, which shares the O:9 antigen with *S*. Typhi.

In summary, our work shows that it is possible to deliver both O- and Vi-antigens using vesicle-based vaccine platforms, thus inducing strong and functional antibody responses against different polysaccharides. Moreover, the presence of protein antigens on *Salmonella* OMV/GMMA may represent an added value for GMMA vaccines compared to other polysaccharide-based formulations. In conclusion, bacterial outer membrane vesicles represent a flexible, affordable and highly immunogenic platform for the development of multivalent *Salmonella* vaccines.

Materials and Methods

Bacterial strains and growth conditions

Salmonella Paratyphi A NVGH308 (displaying the O:2 OAg (34)) is the isolate that has been engineered with a Δ*tolR* mutation to increase outer membrane blebbing (i.e. GMMA production), resulting in strain NVGH2041. Serovar Paratyphi A does not naturally produce the Vi antigen; heterologous display of Vi in *S.* Paratyphi A Δ*tolR* strain NVGH2041 was obtained through episomal expression of the *viaB* locus using the pDC5-*viaB* plasmid, a gift from Prof. Andreas Baumler, University of California Davis (30). These strains are referred to as ParA O:2 Vi^- and ParA O:2 Vi^+, respectively. Attenuated *S.* Typhi BRD948 (Ty2 Δ*aroC* Δ*aroD*

211 Δ*htrA*, naturally displaying the O:9 OAg and Vi antigen (35)) and *Salmonella* Typhi BRD948
212 Δ*tviB* (displaying O:9 but not Vi (36)) strains were used as *S.* Typhi OMV producing strain
213 (Typhi O:9 Vi^+ and Typhi O:9 Vi^-, respectively). All strains were grown at 30°C in liquid Luria-
214 Bertani (LB) medium in rotary shakers for 16 hours. For OMV/GMMA production, overnight
215 cultures were diluted in HTMC medium (15 g/L Glycerol, 30 g/L Yeast extract, 0.5 g/L $MgSO_4$,
216 5 g/L KH_2PO_4, 20 g/L K_2HPO_4) to 600 nm (OD600) optical density of 0.3 and grown at 30 °C for
217 8 hours with a liquid to air volume ratio of 1:5. A supplement of a mixture of aromatic amino
218 acids (Aro mix; 0.04 g/L phenylalanine, 0.04 g/L tryptophan, 0.01 g/L para-aminobenzoic acid
219 and 0.01 g dihydrobenzoic acid) and 0.04 g/L tyrosine was used for the *S.* Typhi strains (35).
220 ParA O:2 Vi^+ strain was grown in the presence of 100 μg/mL ampicillin to retain plasmid pDC5-
221 *viaB* expression.
222
223 **Flow Cytometry analysis**
224 To monitor the display of the O and Vi polysaccharide antigens on the surface of
225 OMV/GMMA-producing strains, bacteria were grown for 16 hours in liquid culture and
226 analysed by flow cytometry. Bacteria were pelleted at 4,000 *x g* for 5 minutes, washed with
227 Phosphate Buffered Saline (PBS), and fixed using Cytofix fixation buffer (BD Biosciences) for
228 30 minutes. Fixed bacteria were then blocked with PBS containing 3% (w/v) Bovine Serum
229 Albumin (BSA) for 15 minutes and incubated for 1 hour with rabbit polyclonal sera against
230 O:2, O:9 or Vi (Denka Saiken), diluted 1:500 in PBS + 1% (w/v) BSA. Rabbit polyclonal sera
231 against O:4 (Denka Seiken) was used as negative control. Samples were incubated with Alexa
232 Fluor 488 goat anti-rabbit IgG (Molecular Probes) diluted 1:500 in PBS+1% BSA for 30 minutes.
233 Flow cytometry analysis was performed using FACS Canto II flow cytometer (BD Biosciences).
234

OMV/GMMA production

OMV and GMMA were purified from the culture supernatant of each bacterial strain and characterized as previously described (23, 37). Bacteria were pelleted by centrifugation at 5,000 *x g* for 45 minutes. Cell-free supernatants were collected, filtered through 0.22 µm Stericups filter (Millipore) and ultra-centrifuged at 175,000 *x g* for 2 hours at 4°C using a SW32Ti rotor (Beckman Coulter). Pellets containing OMV/GMMA were resuspended in PBS, ultra-centrifuged at 175,000 *x g* for 2 hours, resuspended in PBS, filtered and stored at 4°C until use.

Analytical characterisation of GMMA/OMV

GMMA/OMV were characterised in terms of antigen composition and size. Micro BCA kit (Thermo Scientific) was used for GMMA/OMV total protein quantification using Bovine Serum Albumin (BSA) as a reference standard and following the manufacturer's instructions. The sugar monomers constituting the Vi and O-polysaccharide repeating units were quantified through HPAEC-PAD, as previously described (38, 39). Particle size distribution of GMMA/OMV was evaluated by DLS, as previously reported (23, 40).

Animal experiments

Female C57BL/6 mice were purchased from Envigo UK, and used when over 6 weeks of age (mean weight 20+3 g). The mice were housed in specific pathogen-free containment facilities and were allowed water and food *ad libitum*. Six mice per group were vaccinated subcutaneously at day 0 and 28 with either GMMA or OMV diluted in saline and normalised to contain approximately 5 µg of OAg per dose and 0.5 µg of Vi per dose (in case of Vi positive OMV/GMMA), as reported in Table 1. A separate control group of mice received saline as

control. Individual sera were collected at day -1 (pooled sera) and at day 42 (individual sera). All animal experiments were performed in accordance with good animal practice as defined by the relevant international (Directive of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes, Brussels 543/5) and local (University of Cambridge) animal welfare guidelines. This research has been regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

Assessment of anti-Vi and anti-OAg specific total IgG by ELISA

Anti-OAg and anti-Vi antigen specific IgG levels were measured two weeks after the second immunization (day 42) by ELISA as previously reported (41). Briefly, 96 well round-bottom Maxisorp microtitre plates (Nunc, Roskilde, Denmark) were coated with 100 µL/well of antigen overnight at 4°C. OAg purified from *S.* Paratyphi A (O:2) or *S.* Enteritidis (O:9), and Vi purified from *C. freundii* s.l. were used at 15 µg/mL and 2 µg/mL in carbonate, or at 1 µg/mL in phosphate buffer, respectively (38, 42). Plates were blocked with PBS + 5% fat-free milk (Sigma) for 2 hours at room temperature (RT) and afterwards washed 3 times with PBS + 0.05% Tween 20 (PBS-T). Serum samples were diluted 1:100 and 1:4000 in PBS-T supplemented with 0.1% BSA (diluent buffer) and both dilutions were assayed in triplicate. After incubation for 2 hours at RT, plates were washed three times with PBS-T, and incubated at 25°C for 1 hour with anti-mouse goat IgG-Alkaline Phosphatase (Sigma), diluted 1:6000, 1:8800, 1:2600 (for Vi, O:2 or O:9, respectively) in diluent buffer. After washing three times with PBS-T, plates were developed by adding the alkaline phosphatase substrate (Sigma, SIGMAFAST N2770) and read at 405 nm and 490 nm using ELx 800 reader (BioTek). ELISA units were expressed relative to a mouse antigen-specific antibody standard serum curve

283 composed by 10 standard points and 2 blank wells (run in duplicate on each plate), with the
284 best five-parameter fit determined by a modified Hill plot. One ELISA unit is defined as the
285 reciprocal of the dilution of the standard serum that gives an absorbance value equal to 1 in
286 this assay.
287
288 **Assessment of serum bactericidal activity by SBA**.
289 Individual mouse sera collected at day 42 were heat inactivacted (HI) at 56°C for 30 minutes
290 prior to being tested in a serum bactericidal assay based on luminescent readout against
291 *Salmonella* Paratyphi A NVGH308, *Salmonella* Enteritids CMCC3014 and Vi-positive
292 *Citrobacter freundii* sensu lato strain 3056 (43, 44). L-SBA was performed in 96-well round
293 bottom sterile plates (Corning). Dilutions of HI test sera were incubated for 3 hours in
294 presence of exogenous complement (baby rabbit complement, BRC) and bacteria as
295 previously described (43). Briefly, an adequate volume of reaction mixture containing the
296 target bacterial cells (around 100,000 CFU/mL), BRC (50% for *S.* Enteritidis, 20% for *S.*
297 Paratyphi A and 5% for *C. freundii* s.l.) and buffer (PBS) was added to SBA plates containing HI
298 sera dilutions and incubated for 3 hours at 37°C. At the end of the incubation, the plates were
299 centrifuged for 10 min at 4000×g, the supernatant discarded to remove ATP derived from
300 dead bacteria, and live bacterial pellets resuspended in PBS were transferred to a white
301 round-bottom 96-well plate (Greiner) and mixed 1:1 v:v with BacTiter-Glo Reagent (Promega).
302 The reaction was incubated for 5 min at room temperature (RT) in an orbital shaker, and the
303 luminescence signal was measured using a luminometer (Viktor). A 4-parameter non-linear
304 regression was applied to raw luminescence for all the sera dilutions tested as previously
305 described (45). The SBA titer is reported in IC50, defined as serum dilutions giving 50%
306 inhibition of the ATP level in the negative control well. Titers below the minimum measurable

level of luminescence were arbitrarily given an IC50=50, representing half of the first dilution of sera tested (that was 100). GraphPad Prism 7 software (GraphPad Software) was used for fitting and IC50 determination.

Statistical analysis - Unpaired, nonparametric t test (Mann-Whitney) was used to determine the statistically significant differences between gorups, using GraphPad Prism 7 software (GraphPad Software).

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Authors contribution

Conceived and designed the experiments: GG, AS, OR, FMi, PM. Performed the experiments: GG, RA, VA, FMa, MGA, PK, DP. Analysed the data: GG, RA, VA, FMa, MGA, PK, FN, AS, OR, FMi, PM. Contributed to the writing of the manuscript: GG, OR, FMi, PM. All authors had full access to the data and approved the final manuscript.

Conflict of interest

GG, RA, VA, FMa, MGA, FN, OR, FMi are employees of the GSK group of companies. AS was employed by the GSK group of companies at the time of the study, owns GSK shares and is listed as an inventor on patents owned by the GSK group of companies. This does not alter the authors' adherence to all Journal policies on data and material sharing.

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Figure Legends

Fig.1. Display of polysaccharide antigens on the bacterial surface. Flow Cytometry analysis of surface polysaccharides of ParA O:2 Vi^-, ParA O:2 Vi^+, *S*. Typhi O:9 Vi^+ and *S*. Typhi O:9 Vi^-. Flow cytometry was performed using rabbit anti-O:2, O:9 and Vi polyclonal serum, followed by AlexaFluor488-conjugated secondary antibodies. Bacteria stained with a rabbit anti-O:4 polyclonal serum were included as a negative control.

Fig.2. Immunogenic (ELISA) and functional (SBA) assessment of vaccines. Total anti-O:2, anti-O9 and anti-Vi ELISA IgG (top panels) and SBA titers (IC50) against *S*. Paratyphi A (O:2-positive), *S.* Enteritidis (O:9-positive) and *C. freundii* s.l. (Vi-positive) strains (bottom panels) are shown. Unpaired, nonparametric t test (Mann-Whitney) was used to determine the statistically significant differences between gorups (ns=not significant; * $p<0.033$; ** $p<0.002$).

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540 **Tables**

541 **Table 1:** GMMA and OMV analytical characterization.

Antigen	Vi/OAg* (w/w) ratio %	Vi/protein* (w/w) ratio %	OAg/protein* (w/w) ratio %	Z-average diameter ** (nm)	Poly-dispersion Index**
ParA Vi$^+$ GMMA	9.6	4.2	43	83	0.14
ParA Vi$^-$ GMMA	NA	NA	47	72	0.13
***S*. Typhi Vi$^+$ OMV**	10.1	5.0	50	133	0.41
***S*. Typhi Vi$^-$ OMV**	NA	NA	164	81	0.35

542 * = OAg content and Vi content were measured by HPAEC-PAD analysis, protein content by
543 micro BCA and reported ratios calculated. ** = GMMA diameter and polydispersion index
544 were calculated by DLS. NA = not applicable.

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546 **Table 2:** Mice immunogenicity study: antigens and doses.

Group	Protein dose (µg)	Vi dose (µg)	O:2 dose (µg)	O:9 dose (µg)
ParA Vi+ GMMA	12.0	0.5	5.2	0
ParA Vi- GMMA	11.0	0	5.2	0
***S*. Typhi Vi+ OMV**	9.9	0.5	0	4.9
***S*. Typhi Vi- OMV**	3.0	0	0	4.9

Saline	0	0	0	0

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