Supplementary Information for

Comparative immunogenicity and efficacy of equivalent outer membrane vesicle and glycoconjugate vaccines against nontyphoidal *Salmonella*

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Supplementary Information Text

Materials and methods

Bacterial strains

S. Typhimurium 2189 was obtained from University of Calgary, Canada (*Salmonella* Genetic Stock Centre, SGSC) and belonged to the *Salmonella* reference collection A (SARA) (1). *S.* Enteritidis 618 was obtained from Quotient Bioresearch Limited, UK, and was isolated by the European Antimicrobial Susceptibility Surveillance in Animals (EASSA), coordinated by the European Animal Health Study Centre, Brussels (CEESA) (2). We previously selected these as potential sources of OAg for NTS OAg-conjugate vaccines (3). From these two strains, mutants were generated for GMMA production by replacing the *tolR* gene with the kanamycin resistance gene *aph*, as previously described (4). *S.* Typhimurium D23580 and *S.* Enteritidis D24954, endemic clinical isolates from Malawi (5, 6), obtained from the Malawi-Liverpool-Wellcome Trust Clinical Research Programme, Blantyre, Malawi, and the laboratory strain *S.* Enteritidis CMCC4314 (corresponding to ATCC4931), obtained from the Novartis Master Culture Collection (NMCC), were used for bactericidal assays and the mouse infection study. *Salmonella* strains employed in the SBA studies and challenge studies were different from those used to synthesise the vaccines. The strains were those used to establish the SBA (conducted at GVGH, Siena) and challenge model (conducted at WTSI, Cambridge). While the same *S.* Typhimurium strains was used in both systems, different *S.* Enteritidis strains were used for SBA and animal challenge studies.

Immunogenicity studies

Two studies were conducted to compare the immunogenicity of *S.* Typhimurium and *S.* Enteritidis GMMA and glycoconjugates in mice. The first study was performed at Toscana Life Science Animal Care Facility and was approved by the Italian Ministry of Health (Approval number AEC201309). The second study was performed at the Wellcome Trust Sanger Institute, under Project Licence PPL 80/2596 of the United Kingdom Animals (Scientific Procedures) Act 1986.

In both studies, five weeks old female C57BL/6 mice were immunised subcutaneously with 200 µL of vaccine at day 0 and 28. Sera were collected at days -1, 14, 27 and 42. In the first study, 8 mice per group were injected with either monovalent formulations of GMMA or conjugates, both with Alhydrogel (0.7 mg/mL Al^{3+} for GMMA and 2 mg/mL Al^{3+} for OAg-CRM_{197}). Different OAg doses were tested. Formulations at 1 µg OAg/dose were also tested in the absence of Alhydrogel.

Individual mouse sera, collected at each time point, were tested for anti-OAg IgG antibodies response by enzyme-linked immunosorbent assay (ELISA) (3).

Determination of OAg-specific serum IgG subclasses (IgG1, IgG2a, IgG2b, IgG3) and IgM was performed by ELISA, using the kit Mouse Monoclonal Antibody Isotyping Reagents (Sigma-Aldrich). Round bottom Maxisorp microtitre plates (Nunc, Roskilde, Denmark) were coated with *S.* Typhimurium and *S.* Enteritidis OAg (at a concentration of 5 µg/mL and 15 µg/mL respectively in carbonate buffer) overnight at 4°C. Coating was then removed and plates blocked using PBS with 0.05% Tween 20 (Sigma) and 5% fat-free milk (Merck) for 2 hours at room temperature (RT). Serum samples were added and titrated in two-fold serial dilutions in PBS-0.05% Tween 20-0.1% bovine serum albumin (BSA) (diluent buffer) in 100 µL/well. After incubation for 2 hours at RT, plates were washed with PBS-0.05% Tween 20, and incubated for 30 minutes RT with isotyping secondary antibodies (Sigma, ISO2-1KT), diluted 1:1000 in diluent buffer. After washing, plates were incubated for 30 minutes RT with the tertiary alkaline phosphatase-conjugate antibody anti-goat IgG, diluted 1:30000 in diluent buffer, and developed by adding the alkaline phosphatase substrate (Sigma, SIGMAFAST™ N2770). Plates were read

at 405 nm using ELx 800 reader (BioTek). Antibody titres were expressed as the reciprocal of the highest dilution with an optical density (OD) value ≥ 0.2 for anti-*S.* Enteritidis IgG subclasses or ≥ 0.5 for IgM and anti-*S.* Typhimurium IgG subclasses.
For each group, equal volumes of sera collected at day 42 from each mouse were pooled and tested for serum bactericidal activity (SBA) against *S.* Typhimurium and *S.* Enteritidis (3). Bactericidal activity was determined as the serum dilution needed to obtain a 50% reduction in colony forming units (CFU) after 3 hours incubation at 37 °C, in relation to the maximum growth of bacteria obtained in the test. To evaluate possible nonspecific inhibitory effects of baby rabbit complement (BRC) or mouse serum, bacteria were also incubated with SBA buffer and active BRC as a negative control, and with pooled sera from the control placebo group diluted at the same dilutions of the test sera (starting from 1:100) with active BRC. For all negative controls, no killing was observed at any of the concentrations tested.
In the second study, 12 mice per group were immunized with either bivalent formulations of GMMA or conjugate at 1 μg OAg/dose for each antigen together with Alhydrogel. As part of this study, the ability of GMMA and conjugate vaccines to reduce bacterial numbers in the tissues was evaluated 17 days following second immunisation, with 6 mice from each group challenged intraperitoneally with 104 CFU of *S.* Typhimurium D23580 (5), and 6 mice with 104 CFU of *S.* Enteritidis D24954 (7). 24 hours post- challenge, mice were sacrificed by Schedule 1 method and spleens and livers collected for bacterial load determination. In both studies, a negative control group of mice received Alhydrogel.
As in the first study, individual mouse sera were collected at each time point and tested for anti-OAg IgG antibodies by ELISA (3). A high-throughput method using bacterial ATP measurement as survival readout (L-SBA) was used to evaluate SBA of single sera at day 42 from the second study (8). Previous work has shown the equivalence between serum titres calculated with the standard CFU-counting method and the luminescence-based method.

Statistics
Statistical and graphical analysis was performed using GraphPad Prism 6 software. The non-parametric Mann-Whitney test (two-tailed) and Kruskal-Wallis analysis with Dunn's test for post hoc analysis were used to compare two or multiple groups, respectively.

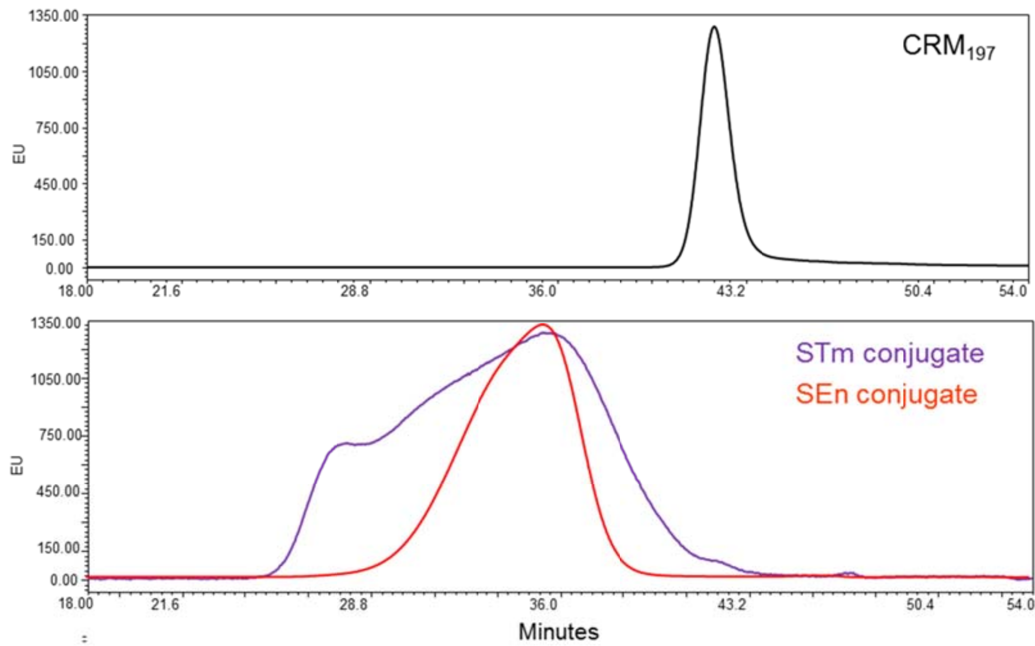

Fig. S1. HPLC-SEC profiles (fluorescence emission detection) of OAg-CRM_{197} conjugates compared with unconjugated CRM_{197} (80 µL injected at 100 µg/mL protein concentration).

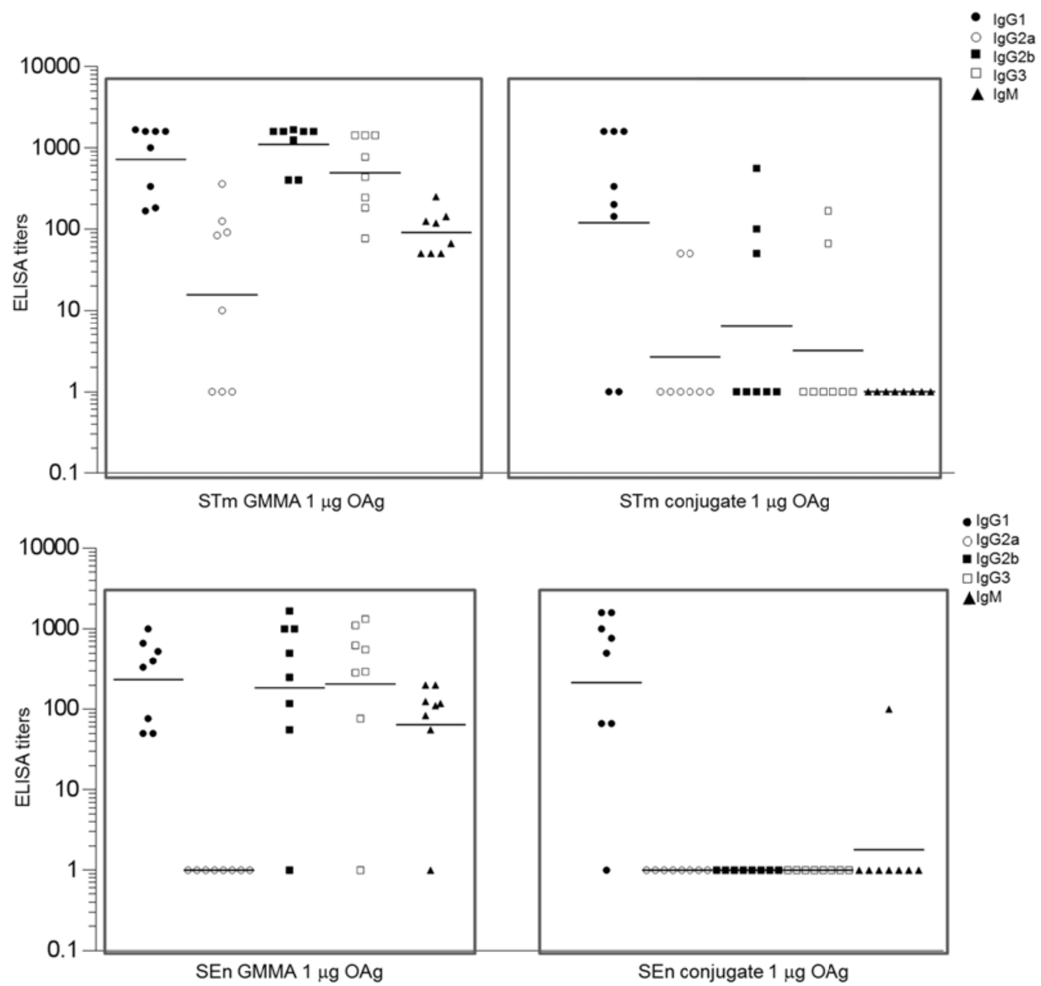

Fig. S2. IgM and IgG subclasses analysis of day 42 sera from mice immunized with GMMA or conjugate at 1 μg OAg dose with Alhydrogel. Summary graphs of individual antibody levels (dots) and geometric means (horizontal lines).

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