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Impact of conjugation chemistry on the immunogenicity of *S.* Typhimurium conjugate vaccines

G. Stefanetti, S. Rondini, L. Lanzilao, A. Saul, C.A. MacLennan, F. Micoli*

Novartis Vaccines Institute for Global Health, Via Fiorentina 1, 53100 Siena, Italy

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ABSTRACT

Salmonella Typhimurium is major cause of invasive nontyphoidal *Salmonella* disease in Africa. Conjugation of *S.* Typhimurium O-antigen to an appropriate carrier protein constitutes a possible strategy for the development of a vaccine against this disease, for which no vaccines are currently available. The conjugation chemistry used is one of the parameters that can affect the immunogenicity of glycoconjugate vaccines. Herein different glycoconjugates were synthesized to investigate the impact of this variable on the immunogenicity of *S.* Typhimurium conjugate vaccines in mice, all with CRM_{197} as carrier protein. Random derivatization along the O-antigen chain was compared with site-directed activation of the terminal KDO sugar residue of the core oligosaccharide. In particular, two different random approaches were used, based on the oxidation of the polysaccharide, which differently impact the structure and conformation of the O-antigen chain. For the selective conjugation methods, linkers of two different lengths were compared.

When tested in mice, all conjugates induced anti-O-antigen IgG antibodies with serum bactericidal activity. Similar anti-O-antigen antibody levels were elicited independent of the chemistry used and a higher degree of saccharide derivatization did not impact negatively on the anti-O-antigen IgG response. Bactericidal activity of serum antibodies induced by selective conjugates was similar independent of the length of the spacer used. Random conjugates elicited antibodies with greater bactericidal activity than selective ones, and an inverse correlation was found between degree of O-antigen modification and antibody functional activity.

1. Introduction

In many parts of Africa, nontyphoidal *Salmonellae* (NTS) are the leading cause of bacteremia. Incidence of disease caused by different serovars varies depending upon the country, but *S.* Typhimurium is the overall major cause of invasive NTS (iNTS) disease [1,2]. iNTS disease was recently estimated at 2.58 million cases per year with a 20% case-fatality rate leading to 517,000 deaths [3]. Young children [4,5], children with HIV infection [6], malaria [7], anemia and malnutrition [8], and HIV infected adults [9,10] are particularly affected. Antibiotics are widely used to treat iNTS disease, but the increasing frequency of multidrug-resistant clinical isolates is concerning and hampers the effectiveness of this treatment in man [11]. Until improved sanitary conditions and widespread provision of clean drinking water can be guaranteed, vaccination constitutes the most promising strategy for the control of iNTS disease in developing countries. No vaccines are currently available to prevent iNTS disease in man.

Surface polysaccharides from bacteria have been used for many years in vaccine applications, being both essential virulence factors and targets for protective antibodies. Covalent conjugation to an appropriate carrier protein is an important mean of increasing the immunogenicity of polysaccharides [12–15]. Glycoconjugate vaccines elicit T cell-dependent immunogenicity against the saccharide. With the involvement of T cells, immunological memory is induced, and affinity maturation and isotype switching from IgM to IgG occur. Unlike pure polysaccharides, glycoconjugate vaccines are effective in young infants.

Antibodies directed against the O-antigen (OAg) of NTS mediate killing [16–18] and confer protection against infection in animal models [19,20]. Therefore, OAg glycoconjugates have been proposed as a vaccine strategy against *Salmonella* for use in man [21].

The synthesis of glycoconjugate vaccines requires a covalent linkage between the saccharide and the carrier protein. Many conjugation methods have been proposed, all following two main approaches: random chemical activation along the polysaccharide

* Corresponding author. Tel.: +39 0577 539087; fax: +39 0577 243540.
E-mail address: francesca.micoli@novartis.com (F. Micoli).

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chain, followed by conjugation to the carrier protein, and coupling to the protein through selective activation of the terminal reducing unit of the saccharide chain [14,15,22,23]. The choice of conjugation strategy can affect the efficiency of conjugation, saccharide to protein ratio and glycoconjugate structure and size, with consequent impact on immunogenicity [15]. Spacer molecules are often introduced between the saccharide and protein to reduce steric hindrance and facilitate conjugation.

Here we investigate different conjugation strategies for linking *S.* Typhimurium OAg to CRM_{197} [23] and compare the impact of these chemistries on the immunogenicity of the resulting conjugates in mice.

2. Materials and methods

SI Materials and Methods feature additional information.

2.1. OAg purification and characterization

S. Typhimurium OAg was purified as previously described [24], following fermentation of the animal-derived isolate, 2192, obtained from the University of Calgary, or of the laboratory strain LT2, obtained from the Novartis Master Culture Collection. OAg preparations were characterized by protein content <1% (by micro BCA), nucleic acid content <0.5% (by A_{260}) and endotoxin level <0.1 UI/μg (by LAL). Full characterization of the OAg chains from these two strains have been previously reported [25]. In particular, 2192 OAg, used for the synthesis of the conjugates tested in mice, was 24% glucosylated and 100% O-acetylated on C-2 abequose (Abe). It showed an average molecular weight (MW) distribution of 20.5 kDa, determined from the molar ratio of rhamnose (Rha; sugar of the OAg chain) to N-acetyl glucosamine (GlcNAc; core sugar), sugar composition analysis by HPAEC-PAD and considering the level of O-acetylation by NMR analysis. OAg chains showed the presence of NH_2 groups (NH_2 to GlcNAc molar ratio % of 37.6), as detected by TNBS colorimetric method [26,27], probably as pyrophosphoethanolamine residues in the core region (Fig. S1).

2.2. Synthesis of OAg-CRM_{197} glycoconjugates

OAg-oxNaIO$_4$-CRM$_{197}$: random activation of the OAg chain with NaIO$_4$ and conjugation to CRM$_{197}$. OAg (10 mg/mL in AcONa 100 mM pH 5) was stirred for 2 h in the dark with 3.75 mM $NaIO_4$. The mixture was desalted using a HiPrep™ 26/10 desalting column 53 mL, prepacked with Sephadex™ G-25 Superfine [GE Healthcare], and the pool, eluted at the void volume of the column, was dried. The activated OAg was designated OAg-oxNaIO$_4$. For conjugation to CRM_{197}, OAg-oxNaIO$_4$ was added to CRM_{197} in NaH_2PO_4 100 mM pH 7.2 to give a final concentration of 10 and 5 mg/mL, respectively. $NaBH_3CN$ was added immediately after (OAg-oxNaIO$_4$:$NaBH_3CN$ = 1:1 w/w), and the reaction mixture stirred overnight at 37 °C. After this time, $NaBH_4$ (OAg-oxNaIO$_4$:$NaBH_4$ = 1:1 w/w) was added and the mixture was stirred at 37 °C for 2 h. The conjugate was designated OAg-oxNaIO$_4$-CRM_{197}.

OAg-oxTEMPO-CRM$_{197}$: random activation of the OAg chain with TEMPO and conjugation to CRM$_{197}$. OAg (3 mg/mL, corresponding to [CH_2OH] of 7.69 mM) and $NaHCO_3$ (molar ratio $NaHCO_3/CH_2OH$ = 30), were added to a stirred solution of TEMPO (molar ratio TEMPO/CH_2OH = 0.05) in DMF. The reaction was cooled to 0 °C and TCC (molar ratio TCC/CH_2OH = 1.6) was added. The activated sugar was recovered from the reaction mixture by precipitation with EtOH (85 v/v% in the final mixture) after 2 h of stirring at 0 °C. The pellet was washed twice with 100% EtOH (1.5 volumes with respect to the reaction mixture volume) and lyophilized. The activated OAg was designated OAg-oxTEMPO2h. The same procedure was used for the synthesis of OAg-oxTEMPO12h, increasing the reaction time to 12 h. OAg-oxTEMPO2 h and OAg-oxTEMPO12h were conjugated to CRM_{197}, using the same conditions for OAg-oxNaIO$_4$. The two corresponding conjugates were designated OAg-oxTEMPO2h-CRM_{197} and OAg-oxTEMPO12h-CRM_{197}, respectively.

OAg-ADH-SIDEA-CRM$_{197}$: selective activation of the terminal KDO with ADH, followed by reaction with SIDEA and conjugation to CRM$_{197}$. The synthesis of this conjugate was performed as previously described [28] and detailed in SI.

OAg-NH$_2$-SIDEA-CRM$_{197}$: selective activation of the terminal KDO with NH$_4$OAc, followed by reaction with SIDEA and conjugation to CRM$_{197}$. OAg was solubilized in 500 mM NH_4OAc pH 7.0 at a concentration of 40 mg/mL. $NaBH_3CN$ was added immediately ($NaBH_3CN$:OAg = 2:5 w/w). The solution was mixed at 30 °C for 5 days. The reaction mixture was desalted on a G-25 column and the OAg-NH_2 was dried. The following steps of conjugation were performed as for OAg-ADH-SIDEA-CRM_{197} and the resulting conjugate was designed OAg-NH_2-SIDEA-CRM_{197}.

All conjugates were purified by hydrophobic interaction chromatography (HIC) on a Phenyl HP column [GE Healthcare], loading 500 μg of protein for mL of resin in 50 mM NaH_2PO_4 3 M NaCl pH 7.2. The purified conjugate was eluted in water and the collected fractions were dialyzed against 10 mM NaH_2PO_4 pH 7.2.

2.3. Conjugates characterization

Total saccharide was quantified by phenol sulfuric assay [29], protein content by micro BCA (using BSA as standard and following manufacturer's instructions [Thermo Scientifics]) and the ratio of saccharide to protein calculated. OAg-CRM_{197} conjugates profiles were compared with free CRM_{197} by HPLC-SEC and SDS-PAGE (see SI).

Methods used for the characterization of derivatized OAg intermediates are described in SI.

2.4. Immunogenicity study in mice and serological analysis

Seven groups of eight 5-week old female C57BL/6 mice were purchased from Charles River Laboratory and maintained at Novartis Vaccines Animal Care. Mice received three subcutaneous immunizations at 14 days-interval with 200 μL/dose of 1 μg of conjugated OAg. Mice were bled before the first immunization (day 0) and two weeks after each immunization. All animal protocols were approved by the local animal ethical committee (approval N. AEC201018) and by the Italian Minister of Health in accordance with Italian law.

Serum IgG, IgM and IgA levels against both OAg and CRM_{197} were measured by ELISA (see SI) [28,30]; day 42 sera were additionally assessed for serum bactericidal activity (SBA) and binding capacity (flow cytometry) of two invasive clinical isolates (see SI). Statistical analysis of ELISA results was conducted using Kruskal–Wallis test, with Dunn's post hoc analysis ($\alpha = 0.05$).

3. Results

3.1. OAg oxidation with NaIO$_4$ and reductive amination with CRM$_{197}$

$NaIO_4$-based oxidation affects vicinal diols to generate two aldehyde groups, opening the sugar ring. In the case of *S.* Typhimurium OAg, this reactivity can involve Rha and glucose (Glc) residues (Fig. 1a). The resulting aldehyde groups can then react with the amine group on lysine residues of the carrier protein to form a covalent C=N linkage, which is subsequently reduced to a stable

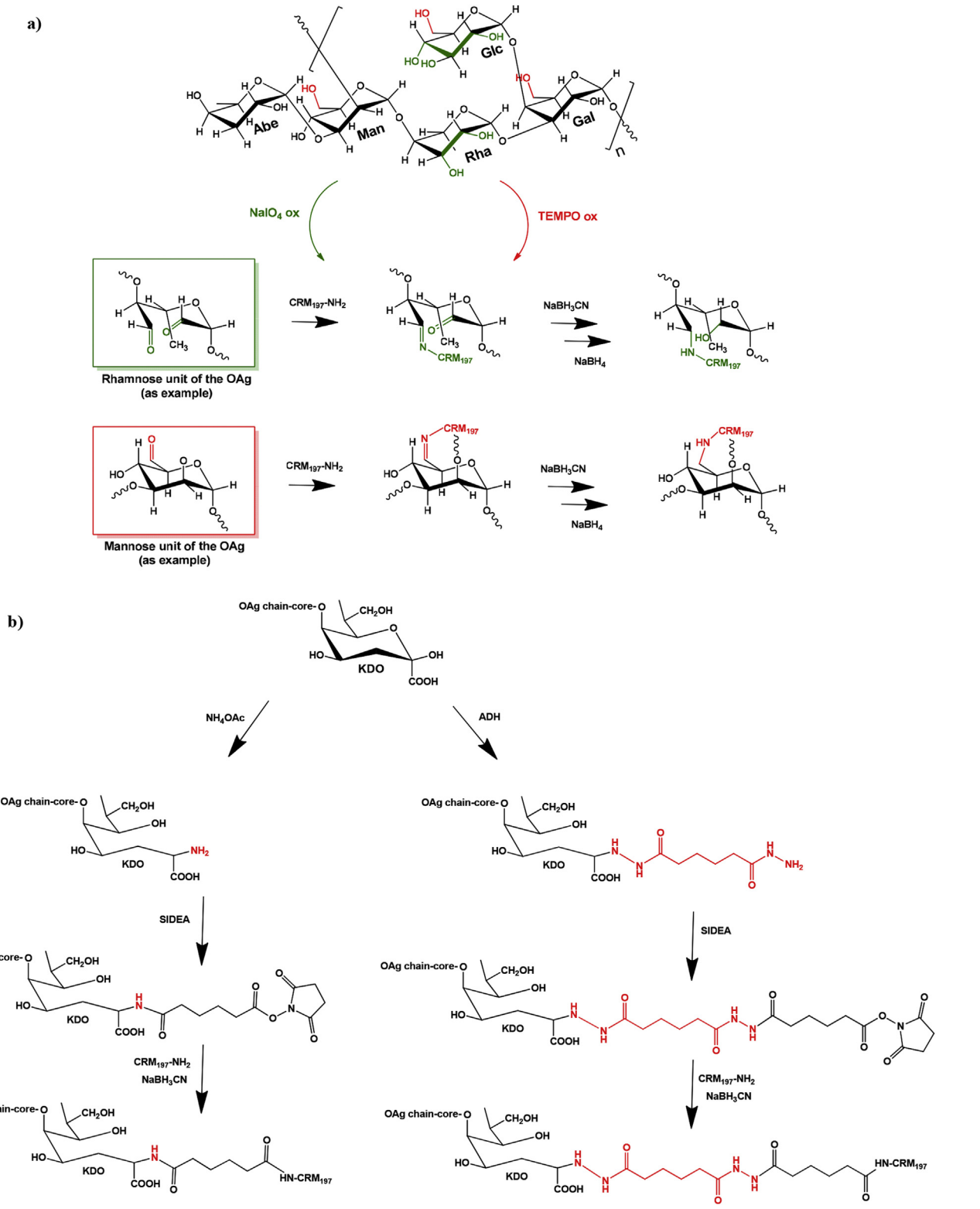

Fig. 1. (a) Structure of *S.* Typhimurium OAg chain and random derivatization by $NaIO_4$ and TEMPO oxidation. For 2192 OAg, repeating units number (n) = 28, as calculated from the molar ratio of Rha to GlcNAc by HPAEC-PAD analysis. The $NaIO_4$-based oxidation affects vicinal diols generating two aldehyde groups and opening the sugar ring. For *S.* Typhimurium OAg, this can involve Rha and Glc residues. TEMPO oxidation targets primary alcohol groups, which in *S.* Typhimurium OAg are present in the sugar moieties of Man, Gal and Glc, with one per monosaccharide. In both cases, the resulting aldehyde groups can then react with the lysine residues of the carrier protein to form a covalent C=N linkage, which is subsequently reduced to a stable C—N bond with $NaBH_3CN$. A further step of reduction with $NaBH_4$ was introduced to quench unreacted C=O groups. (b) OAg selective derivatization of the reducing end unit KDO on the core oligosaccharide. Reductive amination of KDO with NH_4OAc or ADH, followed by SIDEA addition and conjugation to CRM_{197}. The two chemistries differ for the spacer length.

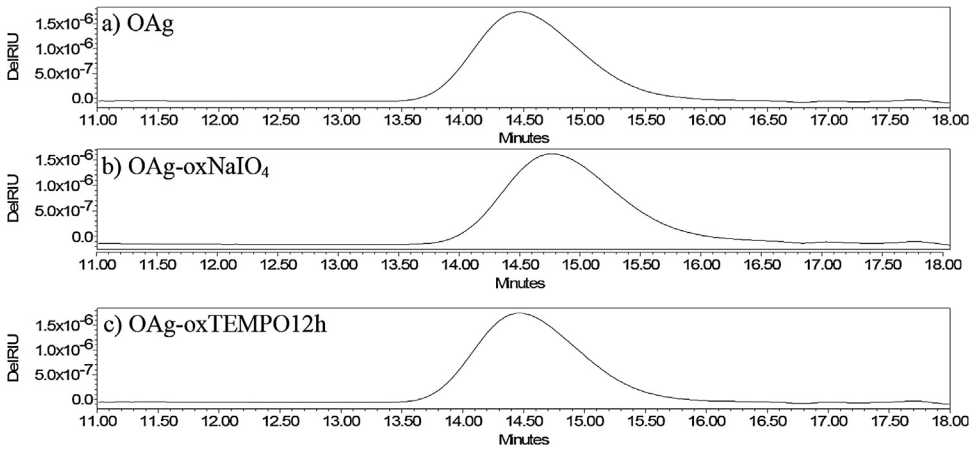

Fig. 2. HPLC-SEC profiles of (a) underivatized 2192 OAg (average MW of 20.5 kDa) in comparison with (b) OAg-oxNaIO$_4$ and (c) OAg-oxTEMPO12h. TSKgel 3000 PWXL, 0.5 mL/min, 100 mM NaCl 100 mM NaH_2PO_4 5% CH_3CN pH 7.2; V_{tot} 23.29 min; V_0 11.20 min; RI detection.

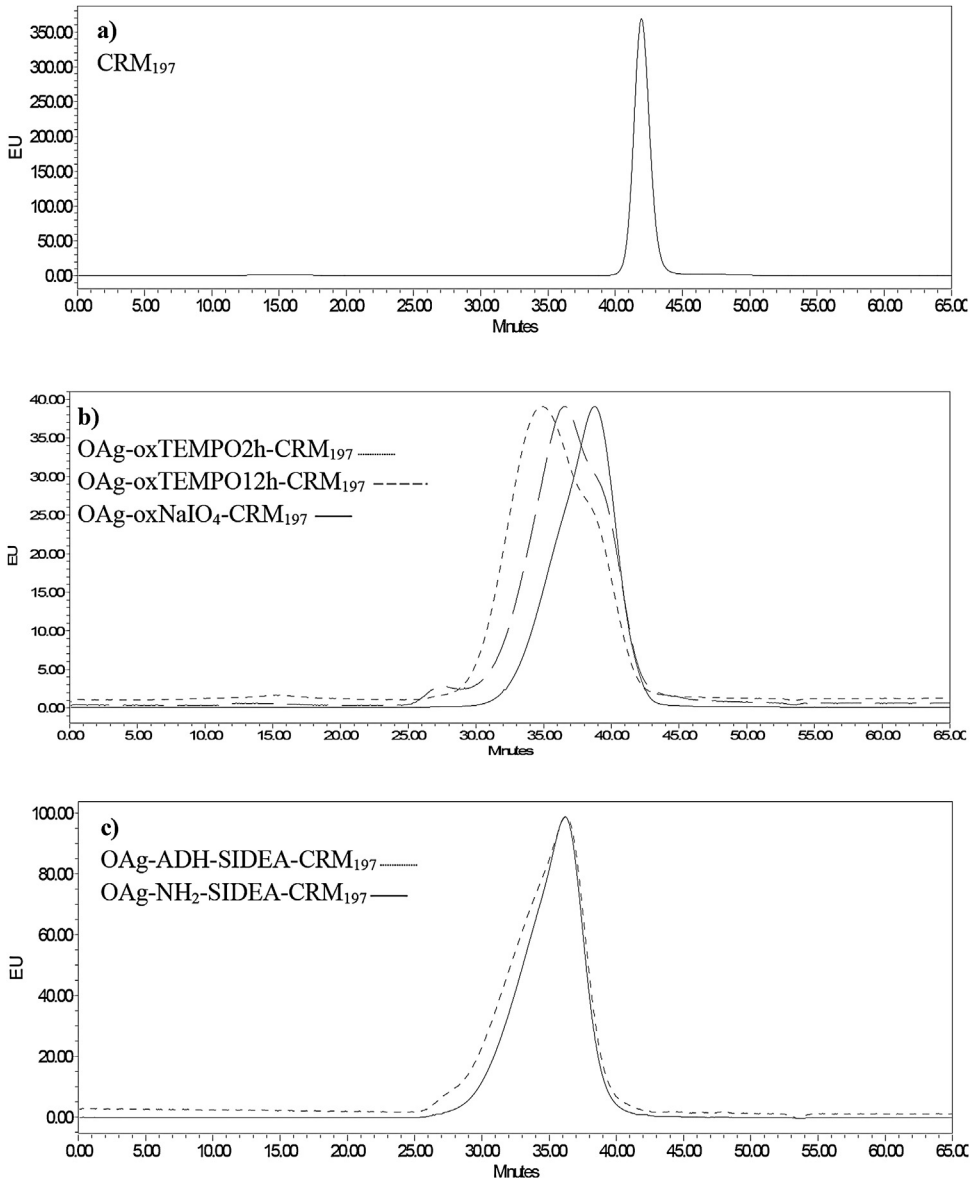

Fig. 3. HPLC-SEC (fluorescence emission profiles) of (a) free CRM_{197}; (b) random conjugates: OAg-oxTEMPO2h-CRM_{197} (dotted line), OAg-oxTEMPO12h-CRM_{197} (dashed line) and OAg-oxNaIO$_4$-CRM_{197} (solid line); (c) selective conjugates: OAg-ADH-SIDEA-CRM_{197} (dotted line) and OAg-NH_2-SIDEA-CRM_{197} (solid line). TSKgel 6000 PW + 5000 PW, 0.5 mL/min, 100 mM NaCl 100 mM NaH_2PO_4 5% CH_3CN pH 7.2; V_{tot} 49.004 min; V_0 24.382 min.

C—N bond with $NaBH_3CN$. A further reduction step with $NaBH_4$ was introduced to quench unreacted C=O groups (see SI).

The reaction conditions applied to 2192 OAg were derived from an optimization performed with the LT2 *S.* Typhimurium laboratory strain (see SI). The HPLC-SEC profile of the oxidized OAg in comparison with the underivatized OAg (average MW of 20.5 kDa) showed a shift of the main peak to a slightly lower MW (Fig. 2a and b). By micro BCA, 14% of OAg repeating units were found to be derivatized (calculated as number of oxidized monomers/total OAg repeating units × 100). HPAEC-PAD analysis showed that 14% of the Rha and 6.4% of the Glc residues were oxidized, with 15.5% of total repeating units modified. All CRM_{197} in the conjugation mixture became linked to OAg, while 36% of OAg was conjugated. HPLC-SEC analysis demonstrated a shift for the conjugate to a higher MW compared with free protein (Fig. 3b and a) and was used for estimating conjugate MW distribution (Table 1).

3.2. OAg oxidation with TEMPO and reductive amination with CRM_{197}

Oxidation of 2192 OAg with TEMPO allowed random formation of aldehyde groups along the chain without opening the sugar rings, as oxidation with $NaIO_4$ does. TEMPO oxidation targets primary alcohol groups. These are present in Man, Gal and Glc residues of *S.* Typhimurium OAg, with one per monosaccharide. The resulting aldehyde groups can then react with the lysine residues on the carrier protein by reductive amination as for derivatization with $NaIO_4$ (Fig. 1a).

Oxidation of 2192 OAg with TEMPO was followed over time and the % of OAg monomers oxidized increased from 15% after 2 h to 36% after 12 h, as detected by micro BCA. HPLC-SEC analysis showed that OAg MW distribution remained unchanged after oxidation, even when the reaction was performed for longer times (Fig. 2c and a), in contrast to what was obtained with $NaIO_4$ (Fig. 2b).

OAg-oxTEMPO with an average percentage number of oxidized repeating units of 36% and 15% were conjugated to CRM_{197}, to investigate the impact of the degree of OAg derivatization on the immunogenicity of the corresponding conjugates.

The same conditions for the conjugation and purification of OAg-oxNaIO$_4$ were applied and in both cases all CRM_{197} in the reaction mixtures was conjugated, with 19–28% of OAg conjugated (Fig. 3b). Conjugates obtained using less derivatized OAg (both after treatment with $NaIO_4$ or TEMPO) were characterized by a higher OAg to protein ratio with respect to the conjugate obtained from more oxidized OAg which was able to couple to more CRM_{197} molecules (Table 1).

3.3. Selective conjugation chemistries

The terminal KDO residue of the core oligosaccharide was used for selective linking of OAg to CRM_{197} without modifying the OAg chain. To generate one conjugate vaccine, reductive amination with ADH was followed by reaction with SIDEA and conjugation to CRM_{197} [28]. A similar chemistry was evaluated where the first step of reductive amination was conducted with NH_4OAc, allowing the synthesis of a conjugate with a linker about half the length of ADH-SIDEA (Fig. 1b).

After testing the reactivity of OAg-KDO with NH_4OAc under different conditions (see SI), in order to synthesize the corresponding conjugate, the reaction was performed at pH 7.0 for 5 days resulting in the activation of 90% of OAg chains. Use of the longer ADH linker with the hydrazide functionality allowed the reaction to proceed, with activation close to 100% after only 2 h at pH 4.5. In the following step where the OAg derivatives were reacted with SIDEA, >90% of total NH_2 groups were coupled to SIDEA, for both OAg-NH_2 and OAg-ADH. The analysis of the corresponding conjugation mixtures

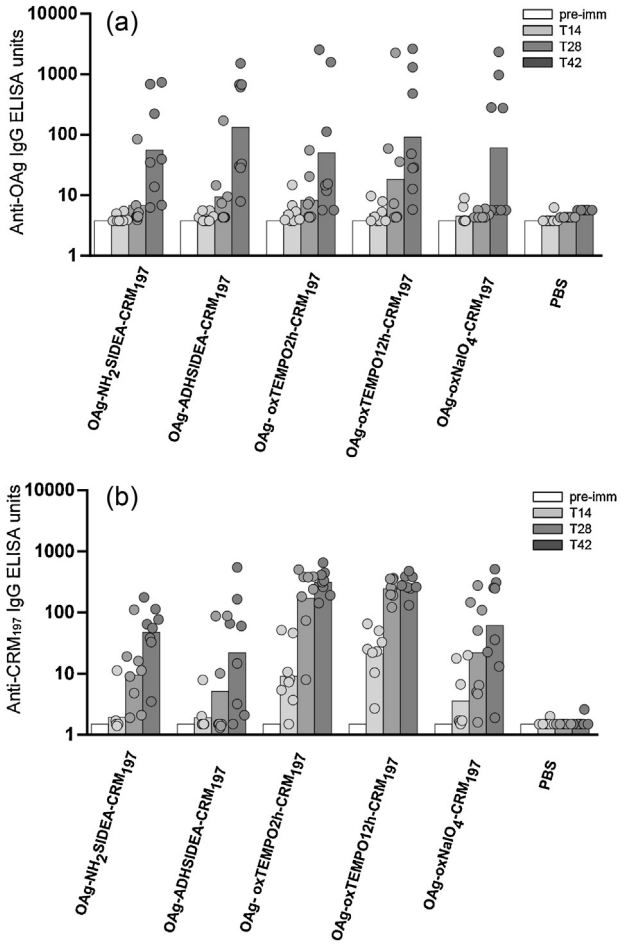

Fig. 4. (a) Anti-OAg IgG ELISA units against 2192 OAg coating agent. (b) Anti-CRM_{197} IgG ELISA units against CRM_{197} coating agent. Individual animals are represented by the dots; horizontal bars represent Geometric Mean Units.

by HPLC-SEC, confirmed conjugate formation without residual free protein, while the amount of conjugated OAg was close to 15% in both cases. The resulting conjugates were very similar in terms of OAg to CRM_{197} ratio (4–5 OAg chains linked per protein) and molecular size, measured as distribution coefficient Kd by HPLC-SEC; even if OAg-NH_2-SIDEA-CRM_{197} showed a slightly broader population (Table 1, Fig. 3c). Selective conjugates contained higher OAg to protein ratios than random conjugates (Table 1).

3.4. Immunogenicity study in mice of OAg-CRM_{197} comparing different conjugation chemistries

The synthesized conjugates were tested in mice, with the following main objectives: to compare the immunogenicity of random versus selective conjugates; to analyze the impact of linker chain length on the immunogenicity of selective conjugates; to evaluate whether the degree of random modification of the OAg chain impacts on immunogenicity.

After three doses, all the conjugates generated anti-OAg IgG levels that were not statistically different (Fig. 4a). Antibody subclass analysis showed that IgG1 was the predominant subclass for all conjugates and no IgA was detected in any serum (Fig. S2). Anti-OAg IgM were detected only at day 42 for OAg-oxTEMPO conjugates (Fig. S3). After two doses, anti-CRM_{197} IgG responses obtained with OAg-oxTEMPO-CRM_{197} conjugates were higher than for the other groups, likely the result of the higher proportion of carrier protein present in these vaccines compared with the

Table 1
OAg-CRM_{197} conjugates generated using different chemistry.

Conjugate	% Repeating units oxidized*	OAg/CRM_{197} (w/w) in purified conjugate	OAg/CRM_{197} (mol/mol) in purified conjugate	Kd purified conjugate (HPLC-SEC)	Peak width (min) at half length (HPLC-SEC)
OAg-oxNaIO$_4$-CRM_{197}	14	0.72	2.05	0.57	4.96
OAg-oxTEMPO2h-CRM_{197}	15	0.56	1.6	0.44	7.59
OAg-oxTEMPO12h-CRM_{197}	36	0.38	1.08	0.50	6.67
OAg-NH_2-SIDEA-CRM_{197}	na	1.42	4.05	0.50	5.75
OAg-ADH-SIDEA-CRM_{197}	na	1.74	4.96	0.49	4.75

Kd values were calculated on a TSKgel 6000 PW + 5000 PW, 0.5 mL/min, 100 mM NaCl 100 mM NaH_2PO_4 5% CH_3CN pH 7.2; V_{tot} 49.004 min; V_0 24.382 min; Kd of free CRM_{197}: 0.72; Kd of free 2192 OAg: 0.67. na: not applicable.
* % repeating units oxidized calculated by micro BCA.

others (Table 1). After three doses, differences were significant only between OAg-oxTEMPO2h-CRM_{197}, and both OAg-NH_2-SIDEA-CRM_{197} and OAg-ADH-SIDEA-CRM_{197} ($p = 0.0025$) (Fig. 4b).

Sera collected at day 42 were pooled and tested for SBA against *S.* Typhimurium D23580, an invasive Malawian clinical isolate [31]. All conjugates induced bactericidal antibodies with complete killing achieved with as little as 0.1 anti-OAg IgG ELISA units/mL (Fig. 5a). Bactericidal activity of sera from mice immunized with selective OAg-KDO conjugates was similar, regardless of the length of the spacer used, while all the random conjugates induced sera with greater bacterial growth inhibition per anti-OAg IgG ELISA unit than the selective conjugates. There was a trend for less bactericidal activity with increasing degree of OAg chain derivatization of the random conjugates: the least derivatized OAg-oxTEMPO2h-CRM_{197} conjugate produced sera with the highest bactericidal activity.

To evaluate possible differences in cell-surface binding, pooled sera at day 42 were tested by FACS against two *S.* Typhimurium invasive clinical isolates D23580 and Ke238. As shown in Fig. 5b, all sera could bind both strains, and greater antibody binding was found with random conjugates-sera.

4. Discussion

There is increasing awareness of the significance of NTS as a major public health concern in the developing world [1,32,33]. While responsible for gastroenteritis in high-income countries, NTS is a common cause of fatal invasive disease in Africa. Currently no vaccines are available against this disease and glycoconjugation is a promising approach for vaccine development [34].

The conjugation chemistry used to synthesize a glycoconjugate vaccine can impact on its immunogenicity [15]. Here *S.* Typhimurium OAg-CRM_{197} conjugates obtained by random derivatization along the sugar chain were compared with conjugates obtained by one-site linkage at the terminus of the core region. For the random approach, a milder oxidation by TEMPO was compared to oxidation with $NaIO_4$ which opens the sugar units with corresponding likely greater impact on OAg epitopes and conformation. Regarding the selective approach, two different lengths of the spacer present between the sugar and the protein were compared.

From a process perspective, all conjugation methods resulted in no residual free protein, which is the most expensive component

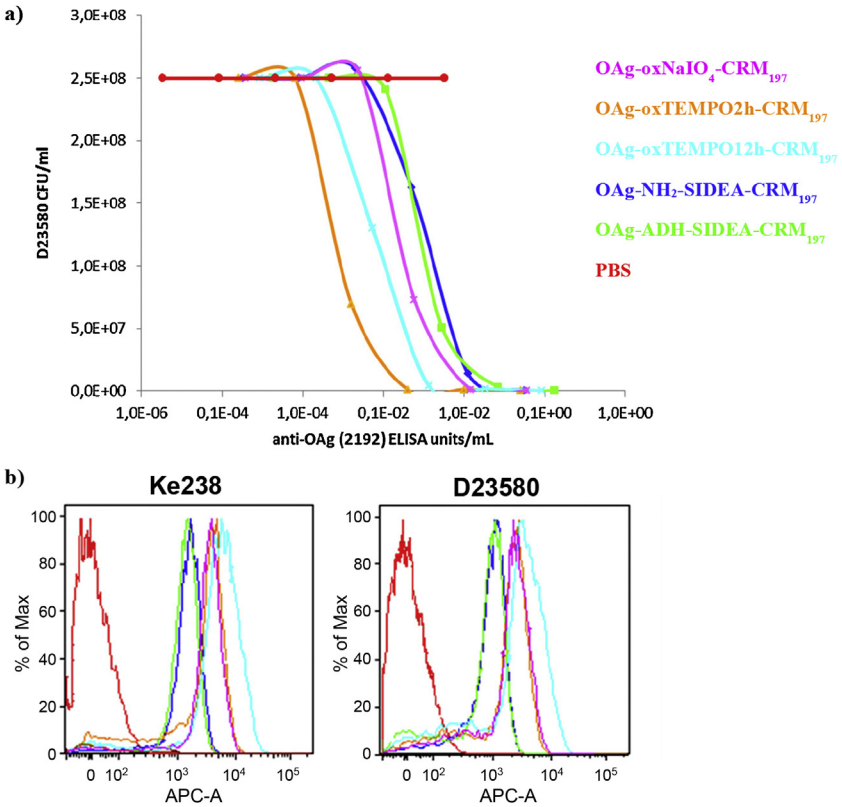

Fig. 5. (a) SBA analysis of pooled sera at day 42: CFU/mL of *S.* Typhimurium D23580 strain against anti-OAg IgG ELISA units/mL. (b) Flow cytometry analysis of surface staining of *S.* Typhimurium Ke238 and D23580. Bacteria were stained with day 42 pooled mice sera raised against the different conjugates.

of the vaccine. The carrier protein did not need to be derivatized for both type of chemistries, but the production of random conjugates required one step less compared with the selective ones. Moreover, random approaches resulted in higher OAg conjugation yields (about twice as much as with selective approach) with advantages in terms of total vaccine costs. However, use of selective chemistry can add benefits in terms of production consistency [35–37].

Selective and random conjugates induced a similar anti-OAg IgG response and no differences were found between selective conjugates synthesized with different linker lengths. Anti-OAg IgM were detected only in mice immunized with TEMPO conjugates after three doses.

Random conjugates induced antibodies with greater bactericidal activity per anti-OAg IgG ELISA unit compared with selective conjugates, confirming that the modification along the sugar chain did not negatively affect conjugate immunogenicity, even though it could impact on OAg epitope integrity and conformation. However, there was an inverse correlation between degree of derivatization and bactericidal activity of the antibodies induced among the random conjugates. FACS analysis confirmed that the higher degree of random derivatization did not negatively impact on the ability of the corresponding conjugates to induce antibodies able to recognize the two invasive *S.* Typhimurium strains tested.

The difference in the bactericidal activity could be related to the different OAg to protein ratio of the various conjugates (lower for random ones), or to the different structures of the conjugates themselves: a sun-structure for the selective conjugates with no points of direct linkage between the OAg polysaccharide and the protein, versus a cross-linked heterogeneous structure of the random conjugates. This second configuration may lead to more CRM_{197}-OAg glycopeptides after processing in the B-cells. According to a recent study, T cell populations can recognize carbohydrate epitopes on glycopeptides derived from antigen-presenting cell processing of Group B Streptococcus conjugate vaccines and high-density presentation of carbohydrate epitopes could have an important role in determining the success of a conjugate vaccine [38]. Different chemistries could also impact on the presentation of the sugar and carrier epitopes to the immune system. Furthermore, the presence of the linker in the selective but not in the random conjugates could be an additional factor affecting antibody functional activity [28,39].

In the context of NTS OAg-based glycoconjugate vaccines, there are only a few studies that have investigated to date the influence of conjugation chemistry on immunogenicity, and contrasting findings have been obtained [19,20,28]. This emphasizes the complexity of the immune response to glycoconjugates which is influenced by different strongly-interconnected conjugation parameters [15].

This study highlights the importance of conjugation chemistry in the design of *S.* Typhimurium OAg-based glycoconjugate vaccines. A better knowledge of the fine structure of such candidate vaccines, together with the design and testing of other well-defined conjugate vaccines, would contribute to further understanding of the relationship between conjugate structure and immunogenicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.08.056.

References

[1] Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. Lancet 2012;379:2489–99.
[2] Reddy EA, Shaw AV, Crump JA. Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. Lancet Infect Dis 2010;10:417–32.
[3] Ao T. 8th International Conference on Typhoid Fever and Other Invasive Salmonelloses – Draft Estimated Global Burden of Invasive non-typhoidal Salmonelloses; 2013. p. 31–2.
[4] MacLennan CA, Gondwe EN, Msefula CL, Kingsley RA, Thomson NR, White SA, et al. The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of Salmonella in African children. J Clin Invest 2008;118:1553–62.
[5] Feasey NA, Archer BN, Heyderman RS, Sooka A, Dennis B, Gordon MA, et al. Typhoid fever and invasive nontyphoid salmonellosis, Malawi and South Africa. Emerg Infect Dis 2010;16:1448–51.
[6] Bachou H, Tylleskar T, Kaddu-Mulindwa DH, Tumwine JK. Bacteraemia among severely malnourished children infected and uninfected with the human immunodeficiency virus-1 in Kampala, Uganda. BMC Infect Dis 2006;6:160.
[7] Mabey DC, Brown A, Greenwood BM. Plasmodium falciparum malaria and Salmonella infections in Gambian children. J Infect Dis 1987;155:1319–21.
[8] Calis JC, Phiri KS, Faragher EB, Brabin BJ, Bates I, Cuevas LE, et al. Severe anemia in Malawian children. N Engl J Med 2008;358:888–99.
[9] Gilks CF, Brindle RJ, Otieno LS, Simani PM, Newnham RS, Bhatt SM, et al. Life-threatening bacteraemia in HIV-1 seropositive adults admitted to hospital in Nairobi, Kenya. Lancet 1990;336:545–9.
[10] Gordon MA, Banda HT, Gondwe M, Gordon SB, Boeree MJ, Walsh AL, et al. Non-typhoidal salmonella bacteraemia among HIV-infected Malawian adults: high mortality and frequent recrudescence. AIDS 2002;16:1633–41.
[11] Gordon MA, Graham SM, Walsh AL, Wilson L, Phiri A, Molyneux E, et al. Epidemics of invasive *Salmonella enterica* serovar enteritidis and *S. enterica Serovar typhimurium* infection associated with multidrug resistance among adults and children in Malawi. Clin Infect Dis 2008;46:963–9.
[12] Avery OT, Goebel WF. Chemo-immunological studies on conjugated carbohydrate-proteins: II. Immunological specificity of synthetic sugar–proteins antigens. J Exp Med 1929;50:533–50.
[13] Avery OT, Goebel WF. Chemo-immunological studies on conjugated carbohydrate-proteins: V. The immunological specificity of an antigen prepared by combining the capsular polysaccharide of type III pneumococcus with foreign protein. J Exp Med 1931;54:437–47.
[14] Jones C. Vaccines based on the cell surface carbohydrates of pathogenic bacteria. An Acad Bras Cienc 2005;77:293–324.
[15] Costantino P, Rappuoli R, Berti F. The design of semi-synthetic and synthetic glycoconjugate vaccines. Expert Opin Drug Discov 2011;6:1045–66.
[16] Rondini S, Lanzilao L, Necchi F, O'Shaughnessy CM, Micoli F, Saul A, et al. Invasive African *Salmonella Typhimurium* induces bactericidal antibodies against O-antigens. Microb Pathog 2013;63:19–23.
[17] Trebicka E, Jacob S, Pirzai W, Hurley BP, Cherayil BJ. Role of antilipopolysaccharide antibodies in serum bactericidal activity against Salmonella enterica serovar Typhimurium in healthy adults and children in the United States. Clin Vaccine Immunol 2013;20:1491–8.
[18] MacLennan CA, Tennant SM. Comparing the roles of antibodies to nontyphoidal Salmonella enterica in high- and low-income countries and implications for vaccine development. Clin Vaccine Immunol 2013;20:1487–90.
[19] Watson DC, Robbins JB, Szu SC. Protection of mice against Salmonella Typhimurium with an O-specific polysaccharide-protein conjugate vaccine. Infect Immun 1992;60:4679–86.
[20] Simon R, Tennant SM, Wang JY, Schmidlein PJ, Lees A, Ernst RK, et al. Salmonella enterica serovar enteritidis core O polysaccharide conjugated to H:g,m flagellin as a candidate vaccine for protection against invasive infection with *S. enteritidis*. Infect Immun 2011;79:4240–9.
[21] Konadu EY, Lin FY, Ho VA, Thuy NT, Van BP, Thanh TC, et al. Phase 1 and phase 2 studies of Salmonella enterica serovar paratyphi A O-specific polysaccharide-tetanus toxoid conjugates in adults, teenagers, and 2- to 4-year-old children in Vietnam. Infect Immun 2000;68:1529–34.
[22] Frasch CE. Preparation of bacterial polysaccharide–protein conjugates: analytical and manufacturing challenges. Vaccine 2009;27:6468–70.
[23] Broker M, Costantino P, DeTora L, McIntosh ED, Rappuoli R. Biochemical and biological characteristics of cross-reacting material 197 CRM197, a non-toxic mutant of diphtheria toxin: use as a conjugation protein in vaccines and other potential clinical applications. Biologicals 2011;39:195–204.
[24] Micoli F, Rondini S, Gavini M, Pisoni I, Lanzilao L, Colucci AM, et al. A scalable method for O-antigen purification applied to various Salmonella serovars. Anal Biochem 2013;434:136–45.
[25] Micoli F, Ravenscroft N, Cescutti P, Stefanetti G, Londero S, Rondini S, et al. Structural analysis of O-polysaccharide chains extracted from different Salmonella Typhimurium strains. Carbohydr Res 2014;385:1–8.
[26] Satake K. The spectrophotometric determination of amine, amino acid and peptide with 2,4,6-trinitrobenzene 1-sulfonic acid. J Biochem 1960;47:654–60.
[27] Palmer DW, Peters Jr T. Automated determination of free amino groups in serum and plasma using 2,4,6-trinitrobenzene sulfonate. Clin Chem 1969;15:891–901.

[28] Micoli F, Rondini S, Gavini M, Lanzilao L, Medaglini D, Saul A, et al. O:2-CRM(197) conjugates against *Salmonella paratyphi* A. PLoS ONE 2012;7:e47039.
[29] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350.
[30] Rondini S, Micoli F, Lanzilao L, Hale C, Saul AJ, Martin LB. Evaluation of the immunogenicity and biological activity of the *Citrobacter freundii* Vi-CRM197 conjugate as a vaccine for Salmonella enterica serovar Typhi. Clin Vaccine Immunol 2011;18:460–8.
[31] Kingsley RA, Msefula CL, Thomson NR, Kariuki S, Holt KE, Gordon MA, et al. Epidemic multiple drug resistant Salmonella Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. Genome Res 2009;19:2279–87.
[32] MacLennan CA, Levine MM. Invasive nontyphoidal Salmonella disease in Africa: current status. Expert Rev Anti Infect Ther 2013;11:443–6.
[33] Okoro CK, Kingsley RA, Connor TR, Harris SR, Parry CM, Al-Mashhadani MN, et al. Intracontinental spread of human invasive Salmonella Typhimurium pathovariants in sub-Saharan Africa. Nat Genet 2012;44:1215–21.
[34] Simon R, Levine MM. Glycoconjugate vaccine strategies for protection against invasive Salmonella infections. Hum Vaccine Immunother 2012;8:494–8.
[35] Beuvery EC, vd KA, Kanhai V, Leussink AB. Physicochemical and immunological characterization of meningococcal group A polysaccharide-tetanus toxoid conjugates prepared by two methods. Vaccine 1983;1:31–6.
[36] Carmenate T, Canaan L, Alvarez A, Delgado M, Gonzalez S, Menendez T, et al. Effect of conjugation methodology on the immunogenicity and protective efficacy of meningococcal group C polysaccharide-P64k protein conjugates. FEMS Immunol Med Microbiol 2004;40:193–9.
[37] Seppala I, Makela O. Antigenicity of dextran-protein conjugates in mice. Effect of molecular weight of the carbohydrate and comparison of two modes of coupling. J Immunol 1989;143:1259–64.
[38] Avci FY, Li X, Tsuji M, Kasper DL. A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design. Nat Med 2011;17:1602–9.
[39] Konadu E, Shiloach J, Bryla DA, Robbins JB, Szu SC. Synthesis, characterization, and immunological properties in mice of conjugates composed of detoxified lipopolysaccharide of Salmonella paratyphi A bound to tetanus toxoid with emphasis on the role of O acetyls. Infect Immun 1996;64:2709–15.