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Pathogen specific carbohydrate antigen microarrays: a chip for detection of *Salmonella* O-antigen specific antibodies

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Abstract A *Salmonella* O-antigen microarray was developed by covalent coupling of oligosaccharide antigens specific for serogroups *Salmonella enterica* sv. Paratyphi (group A), Typhimurium (group B) and Enteritidis (group D). Antibodies were correctly detected in sera from patients with culture verified salmonellosis. High serogroup-specificity was seen with the disaccharide antigens. With the larger antigens, containing the backbone sequence Manα1–2Rhaα1–2Gal (MRG), common backbone-specific antibodies (O-antigen 12) were also detected. This is "proof of principle" that pathogen-specific carbohydrate antigen microarrays constitute a novel technology for rapid and specific serological diagnosis in either individual patients or larger sero-epidemiological and vaccine studies.

Keywords Glycanarray · Polysaccharide · Oligosaccharide · Antibody · Salmonellosis

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Introduction

Bacteria of the genus *Salmonella* (*S.*) causes a variety of diseases in both animal and man, commonly referred to as salmonellosis. In man, severe disease and bacteremia are associated primarily with three serovars (sv.) of *Salmonella enterica* sv. (Cholerasuis, Parathyphi A and Typhi) while most of the other *Salmonella* species primarily are associated with gastroenteritis. The severity of the disease depends on the virulence of the particular strain, as well as the general condition of the host. Infections in humans are mainly due to the consumption of contaminated food products. Worldwide, there are about 1.3 billion cases of human salmonellosis each year, with three million associated deaths [1].

The costs of *Salmonella* infections both in humans and livestock in the food industry are substantial. Therefore, many countries have introduced national *Salmonella* control programs. Within EU countries, there is an active legislative effort to control especially *Salmonella* in the food industry but also other bacterial, viral and parasitic zoonotic agents. By the adoption of the new Zoonoses Monitoring Directive 2003/99/EC, the control programs have recently been further strengthened. To implement control programs precise, rapid and cost-effective diagnostic tests are a prerequisite. Most bacterial pathogens carry both protein/peptide and carbohydrate surface antigens. Carbohydrate antigens are often much more specific than protein/peptide antigens and are therefore frequently used for sub-typing bacterial genus down to the subspecies (spp.) level [2, 3].

Current diagnostic laboratory tests for identification of *Salmonella* include both bacteriological and serological methods. Commercial agglutination antibody tests are available to identify *Salmonella* to the spp. level. In

addition to verifying disease in the individual human patient (as with the controversial 'Widal-test' [4, 5]), serological tests are particularly well suited for large sero-epidemiological surveillance studies of livestock in the food industry. The most commonly used serological technique is the enzyme-linked immuno-sorbent assay (ELISA) [6, 7]. In ELISA as well as in all serological techniques, the most crucial parameter is the purity and specificity of the selected antigen and the reproducibility of its preparation [8].

Today, more modern, reproducible and efficient ways to prepare carbohydrate antigens for use in serological tests have been developed. Hence, either total organic synthesis or specific degradations of the native polysaccharides to give chemically defined poly- or oligosaccharide fragments with high specific antigenic activity is feasible [9, 10]. The availability of defined carbohydrate antigens has also spurred the development of new, highly sensitive and miniaturized techniques for detecting antigen–antibody reactions. One such technique is the "microarray" technique, in which defined antigen molecules (each one representing a specific pathogen) are covalently bound as microdots onto a miniature chip (e.g. a small glass or silica plate) [11]. Binding of antigen-specific antibodies to a given antigen molecule on the chip may then be detected by a variety of techniques. One commonly used technique is exposure to a labeled secondary antibody, specific for the antibodies of the species under investigation. This secondary antibody carries a "label" (e.g. a fluorescing moiety), which upon illumination with ultra-violet (UV) light, is detected either by the naked eye or by an instrument. Other, similar array formats have also recently been conceived [12, 13], including peptide and protein arrays [14].

Here, we demonstrate a novel *Salmonella* antigen microarray using a number of well-defined synthetic or lipopolysaccharide (LPS)-derived carbohydrate antigens representing *S. enterica* sv. Parathyphi (group A; O-antigens 2, 12), Typhimurium (group B; O-antigens 4, 12) and Enteritidis (group D; O-antigens 9,12). This O-antigen specific microarray correctly detected antibodies in sera from culture-verified *Salmonella* serogroup BO (O4,12) and DO (O9,12) patients. Sera collected from healthy non-vaccinated individuals serving as controls gave no or minimal response.

Results

Preparation of *Salmonella* O-antigen microarrays

In previous studies, oligosaccharides corresponding to the O-antigen determinants of the *S. enterica* sv. Parathyphi (group A; O-antigens 2,12), Typhimurium (group B; O-antigens 4,12) and Enteritidis (group D; O-antigens 9,12) were used [15, 16]. From these studies, synthetic di-, tri-, and tetrasaccharide glycosides (*p*-aminophenyl aglycon, *p*AP) corresponding to the immuno-dominant portions of *Salmonella* O-antigens of serogroups AO, BO, and DO were available [17–19] (Table 1). Compounds **1**–**3** were used as controls and two dodecasaccharides (three repeating units of the native O-polysaccharide chain with a free reducing terminal Rha residue) of the *S. enterica* sv. Typhimurium and Enteritidis, respectively [20, 21] were also available from earlier studies. These latter oligosaccharides were conjugated to glass plates via a new bi-functional spacer, 2-aminoethyl-(*N*-methyl)-hydroxylamine, [22] (Fig. 1b). The complete O-antigen polysaccharide (including a portion of the core oligosaccharide) obtained from *S. enterica* sv. Typhimurium LPS by mild acid hydrolysis [23], was also conjugated similarly. (Fig. 1a) [22].

Table 1 Synthetic and PS O-antigenic oligosaccharides from *Salmonella*, AO, BO, DO and E_2O serogroups

No.	Abbreviation	Structure	Serogroup	Factor
1	M	Manα-Sp1	Contr.	–
2	GN1	GlcNAcβ-Sp_1	Contr.	–
3	GN2	GlcNAcβ-Sp_3	Contr.	–
4	G	GalαO-Sp_3	–	–
5	RG	Rhaα1-3Galα-Sp_3	–	–
6	MRG	Manα1-4Rhaα1-3Galα-Sp_3	E_2	O12
7	TMRG	Tyvα1-3Manα1-4Rhaα1-3Galα-Sp_3	D_1–D_3	O9, O12
8	TM	Tyvα1-3Manα Sp_3	D_1–D_3	O9
9	$(G[A]MR)_3$	(-3Galα1-2[Abeα1-3]Manα1-4Rhaα1)$_3$-Sp_2	B	O4, O12
10	PMRG	Parα1-3Manα1-4Rhaα1-3Galα-Sp_3	A	O2, O12
11	PM	Parα1-3ManαO-Sp_3	A	O2
12	A-LPS	LPS-*typhimurium*-conjugate-Sp_2	B	O4, O12
13	AM	Abeα1-3ManαO-Sp_3	B	O4
14	$(G[T]MR)_3$	(-3Galα1-2[Tyvα1-3]Manα1-4Rhaα1)$_3$-Sp_2	D_1–D_3	O9, O12
15	AMRG	Abeα1-3Manα1-4Rhaα1-3Galα-Sp_3	B	O4, O12

Sp_1 2-Aminoethyl;
Sp_2 2-aminoethyl-(*N*-methyl)-hydroxylamine;
Sp_3 O*p*AP=*p*-aminophenyl

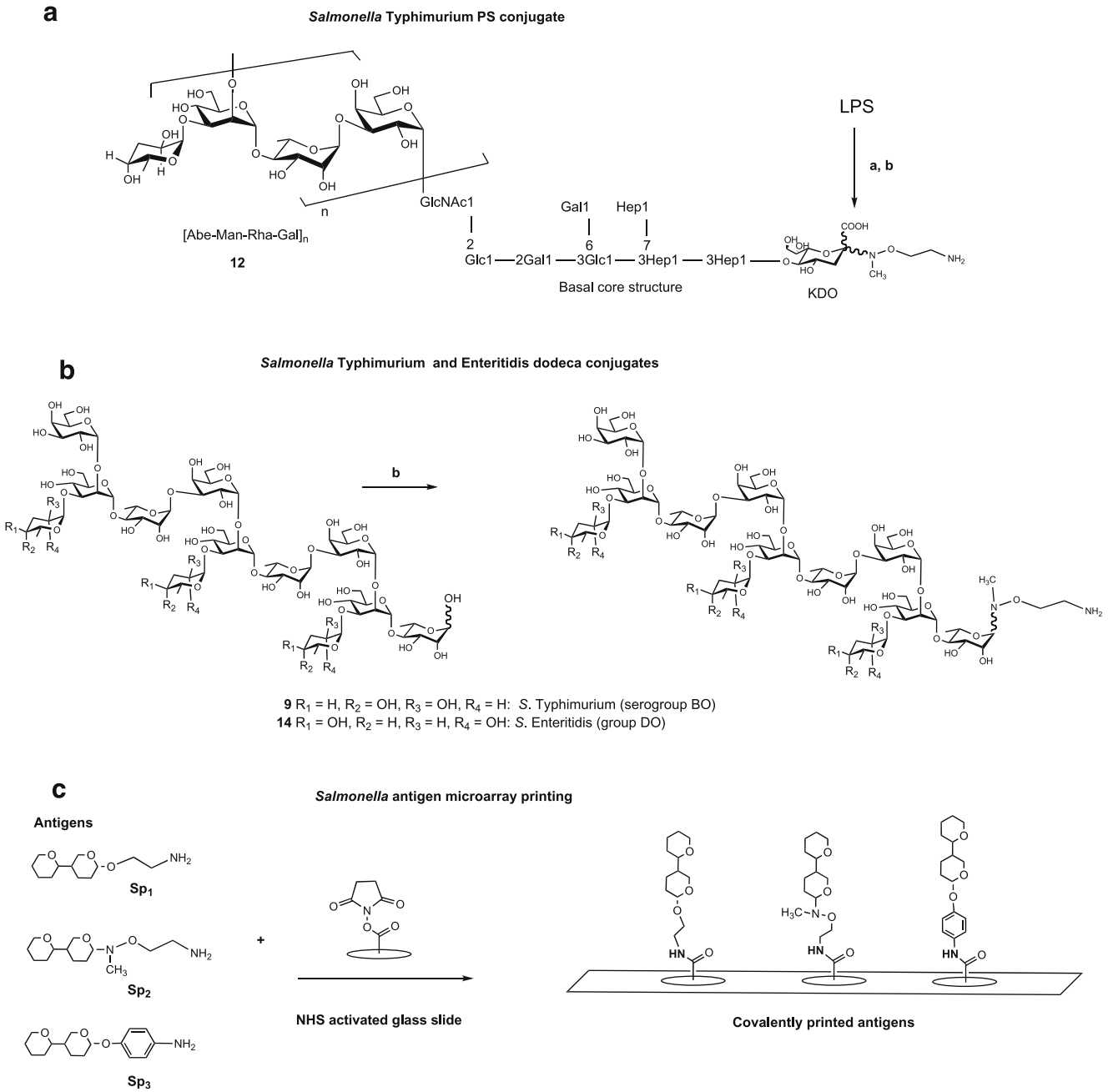

Fig. 1 Structures of the core region and O-antigen polysaccharides from *S. enterica* sv. Parathyphi (group A; O-antigens 2,12), Typhimurium [group B; O-antigens 4,(5),12] and Enteritidis (group D; O-antigens 9,12). **a** Preparation of *Salmonella* Typhimurium PS spacer conjugate. Common phosphate groups at the basal core structure are not indicated in the structure; **b** conjugation of dodecasaccharides of serogroups BO and DO with a bifunctional spacer. **c** Covalent printing of amino-derivatized antigens onto NHS-activated glass slides. **a** De-lipidation with acetic acid, 90°C, 1 h and size-exclusion chromatography; **b** reducing end PS and bi-functional spacer (50 eqv), 24 h, 37°C and purification with size-exclusion chromatography (see "Experimental" for details)

The amino-functionalized antigens were deposited and covalently immobilized onto glass plates using an amino-reactive (*N*-hydroxysuccinimide, NHS, ester groups) glass microarray surface using a standard DNA array printer as previously described [11] (Fig. 1c). All compounds were printed in replicates of ten and each in a twofold dilution series. To achieve maximum immobilization of the less reactive *p*AP compounds, a starting concentration of 500 μM was used whereas all other amino-compounds were printed at 100 μM starting concentration. Both the *p*AP and the alkylamines were imprinted at saturated conditions which was confirmed by the comparable binding of the GlcNAc-specific *Griffonia simplicifolia* (GS-II) lectin to imprinted compounds **2** and **3** (Fig. 2a).

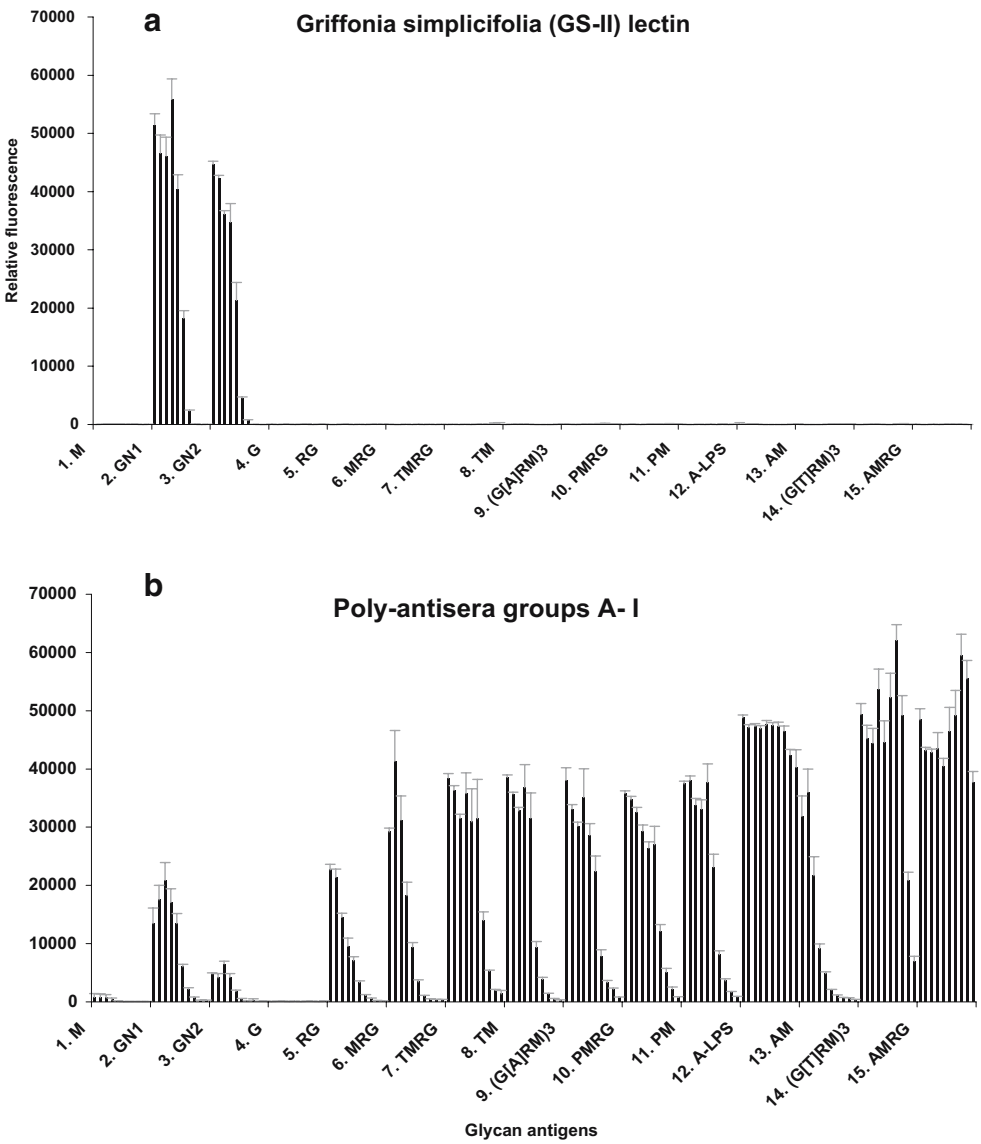

Fig. 2 Antibody and lectin binding to printed *Salmonella* O-antigen microarrays. Each antigen was printed in ten series of a twofold dilution of respective antigens (from left to right for each data set); **a** *G. simplicifolia* lectin–biotin conjugate. Detection was obtained with Streptavidin–AlexaFlour-488 conjugate; **b** Rabbit poly-antisera of *Salmonella* groups A–I. Detection of antiserum was obtained with a secondary goat anti-rabbit-IgG–AlexaFlour-488 conjugate

Test of *Salmonella* O-antigen microarrays using commercial polyclonal rabbit agglutination sera

First, a pool of rabbit antisera (BD, Sparks, MD, USA) directed against *Salmonella* serogroups AO–IO and the capsular Vi polysaccharide were tested with the microarrays. Bound IgG antibodies were detected using an AlexaFlour-488-labeled anti-rabbit-IgG antibody. As expected, strong signals from all AO, BO and DO specific printed antigens were detected. Only weak (GlcNAc derivates 2 and 3) or no signals (Man and Gal derivates 1 and 4, respectively) were seen with other antigens (Fig. 2b).

Subsequently, rabbit antisera (BD, Sparks, MD, USA) corresponding to individual serogroups A–I were tested (Fig. 3). The group A-specific antiserum bound, as expected, strongly to the paratosyl-containing compounds **10** and **11** related to the O2 epitope of group AO. Binding to the polysaccharide of group BO was also seen. The group BO-specific antiserum bound strongly to the abequosyl-containing compounds (**9**, **12**, **13**, **15**) related to the immuno-dominant part of the O4 epitope. Weak reactivity with compounds (**7**) related to the group DO antigens was also seen, most likely caused by a remaining O-antigen 12 reactivity of this antiserum. The group DO-specific antiserum showed, as expected, the strongest binding to the tyvelosyl-containing compounds **7**, **8** and **14** although a weak reactivity was also seen with the group AO-related disaccharide Par1-3Man (**11**).

Overall, the rabbit polyclonal group-specific antisera showed a rather specific binding to the defined O-antigens, showing the highest specificity for the respective disaccharides with 3,6-dideoxy sugar residues earlier reported to

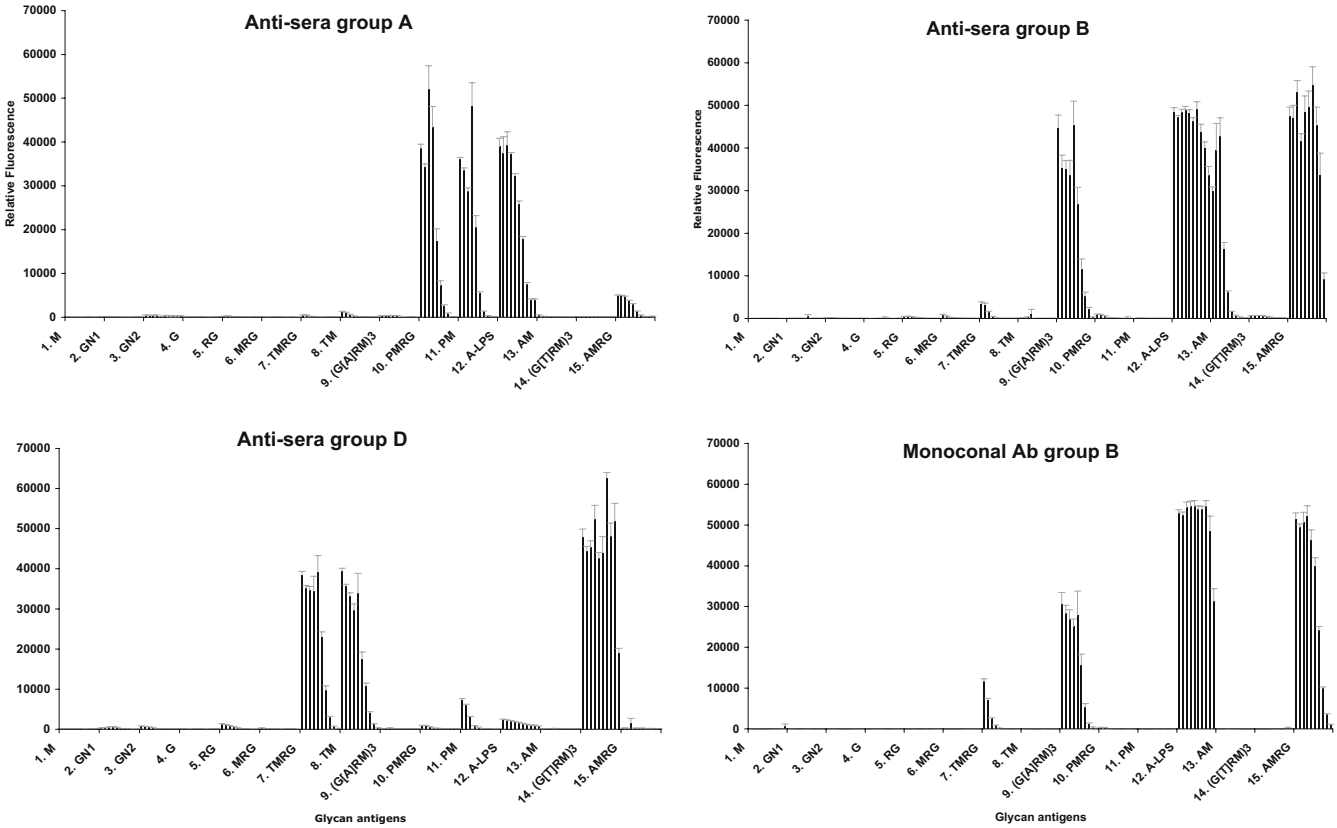

Fig. 3 Binding of rabbit poly-antiserum *Salmonella* serogroups AO, BO and DO and a rabbit anti-*Salmonella* serogroup BO Mab. Secondary detection was obtained with a goat anti-rabbit-IgG–AlexaFlour-488 conjugate

be immuno-dominant for respective serogroup [15]. Other group-specific antisera (C_1, E, F, G, H, I and Vi) did not show any binding (data not shown).

Next, we evaluated a *S.* Typhimurium BO-specific monoclonal antibody (Mab) preparation. This Mab reacted strongly with the larger *Salmonella* O4 and O12 containing antigens (**9**, **12**, **15**). Weak cross reactivity towards structure **7** was also observed (Fig. 3). This Mab obviously recognizes only larger O-antigen 4,12 epitopes as it did not interact with the disaccharide structure (**13**). This finding is also in accordance with earlier findings that mouse antibodies generally react only with larger saccharide antigen epitopes while larger mammals, including man, produce antibodies recognizing also smaller saccharide antigen epitopes [24].

Determination of optimal antigen concentration and serum dilutions for analysis of human sera

The antigen print concentration for maximum sensitivity was established from dilution series of printed antigens and found to be to be >100 μM for compounds with the less reactive amine of the pAP spacer and >25 μM for the aliphatic amine spacers. Next, serum dilution experiments with human sera were performed. Here, a serum dilution of 1:100 gave maximum differences between the three disaccharide antigens TM (**8**), PM (**11**) and AM (**13**) corresponding to serogroups DO (O-antigen 9), AO (O-antigen 2) and BO (O-antigen 4), respectively (see Fig. 4 for an example). The larger antigens containing the "backbone" sequence MRG in general showed higher sensitivity but were less discriminative. Hence, we conclude that the disaccharide carbohydrate antigens showed the highest serogroup discrimination whereas the larger oligosaccharide fragments and/or the complete O-polysaccharide chain, showed lower specificity but higher sensitivity, and may therefore be useful as screening antigens for salmonellosis.

Test of the *Salmonella* microarrays against human patient and control sera from healthy non-salmonellosis patients

Sera from ten patients having culture verified salmonellosis (five from infections with *S. enterica* sv. Enteritidis serogroup DO and five with *S. enterica* sv. Typhimurium serogroup BO) and sera from five healthy controls were compared against the three synthetic disaccharides **8**, **11** and **13** (Fig. 5). The control O2 specific antigen PM (**11**) gave only background signals. All five patients with *S. enterica* sv. Enteritidis (serogroup DO) showed significantly

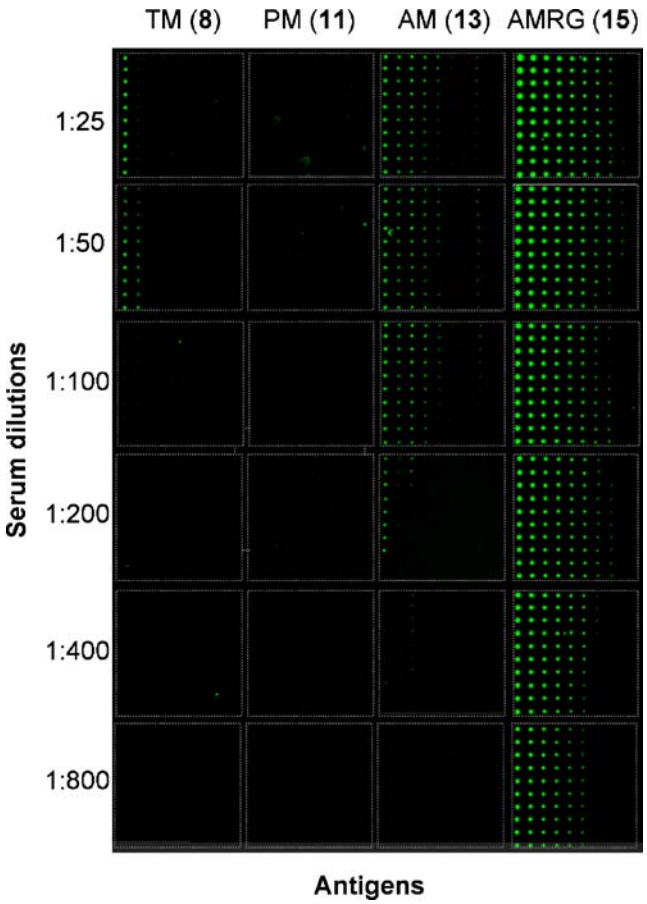

Fig. 4 Optimization of antibody binding to disaccharide antigens with human serum. Each of the antigens [8, 11, 13, 15] were printed in ten replicates (*rows*) at ten twofold dilution concentrations (*columns*) and bound antibodies from a dilution series of human antisera (shown is a patient with a bacteriologically verified serogroup BO infection) were detected with a goat anti-human-IgG,M,A–biotin followed by streptavidin–AlexaF488 conjugate

elevated levels (>tenfold) of antibodies against the O-antigen 9 (TM, **8**), and the five patients with *S. enterica* sv. Typhimurium, showed a corresponding reactivity towards the O-antigen 4 (AM, **13**). Four out of five at the time healthy non-vaccinated individuals did not show any significant increased antibody levels against any of the three disaccharide antigens. However, one individual had a slightly elevated signal towards group BO antigen disaccharide (Fig. 5c). We do not know the disease history of these healthy controls and we cannot rule out that the control that exhibited somewhat elevated anti-BO titers might have experienced an earlier clinical or subclinical *Salmonella* serogroup BO infection. The signal difference between the larger oligosaccharides of serogroup BO (**9**) and DO (**15**), respectively, was not as prominent as for the corresponding disaccharide antigens. However, signals were detected a lower concentrations for the larger oligosaccharide antigens and this was even more prominent with the complete PS antigen (data not shown). This is as expected and in accordance with the polyclonal antibody response which display a clonal disparity where some antibody clones recognizes a variety of different sized epitopes i.e. the disaccharide antigens exclude some clones from binding and as the size of the antigen structure increases more clones will be able to bind. Hence, this was also evidenced by the Mab (reported on above) which only bound to a tetra-saccharide sized antigen and not to the disaccharide antigen.

Discussion

A large collaborative effort towards developing a pathogen polysaccharide microarray has been initiated under the auspicious of the Consortium for Functional Glycomics (CFG). We describe here how the new technique of pathogen specific carbohydrate antigen microarrays can be used in this context using *Salmonella* antigens as an example. The chemical structures of the *Salmonella* LPS (O-antigens) have been thoroughly investigated (for a review see [25]). Currently used ELISA diagnostic tests most often use rather crude capsular polysaccharide (CPS) or LPS antigen preparations for direct coating of the plastic wells. Such preparations are inherently heterogeneous and batch-to-batch variation may be substantial, since chemical characterization is rarely performed [3, 6]. Product quality control relies instead primarily on costly, extensive and time-consuming functional tests.

It is conceivable that new formats, such as the microarray, can be made not only much cheaper and more rapid, but also more specific than currently used methods e.g. ELISA, since it can (because of its miniature format) more readily use chemically defined, covalently linked antigens. The microarray technique uses very small amounts of antigen and sample, and is well suited for automatization. However, the most important feature of the microarray technique is that antibodies directed against a large number of selected pathogens can be detected simultaneously on a single chip using very small sample volumes. A complete serological panorama in an individual can be established rapidly. This work demonstrates suitable chemistry and protocols for use of microarrays in this context. First, the covalent attachment of carbohydrate antigens via a bifunctional spacer onto a glass or silica chip proved to be very efficient. Secondly, the sensitivity and specificity of defined and/or synthetic fragments of the repeating O-antigen polysaccharide from *Salmonella* of serogroups AO, BO and DO using human sera from patients diagnosed with salmonellosis was assessed. As the dideoxyhexoses paratose, abequose and tyvelose earlier have been shown to be the immuno-dominant sugars for O-antigen specificities 2, 4, and 9, respectively, we tested a selected panel of such

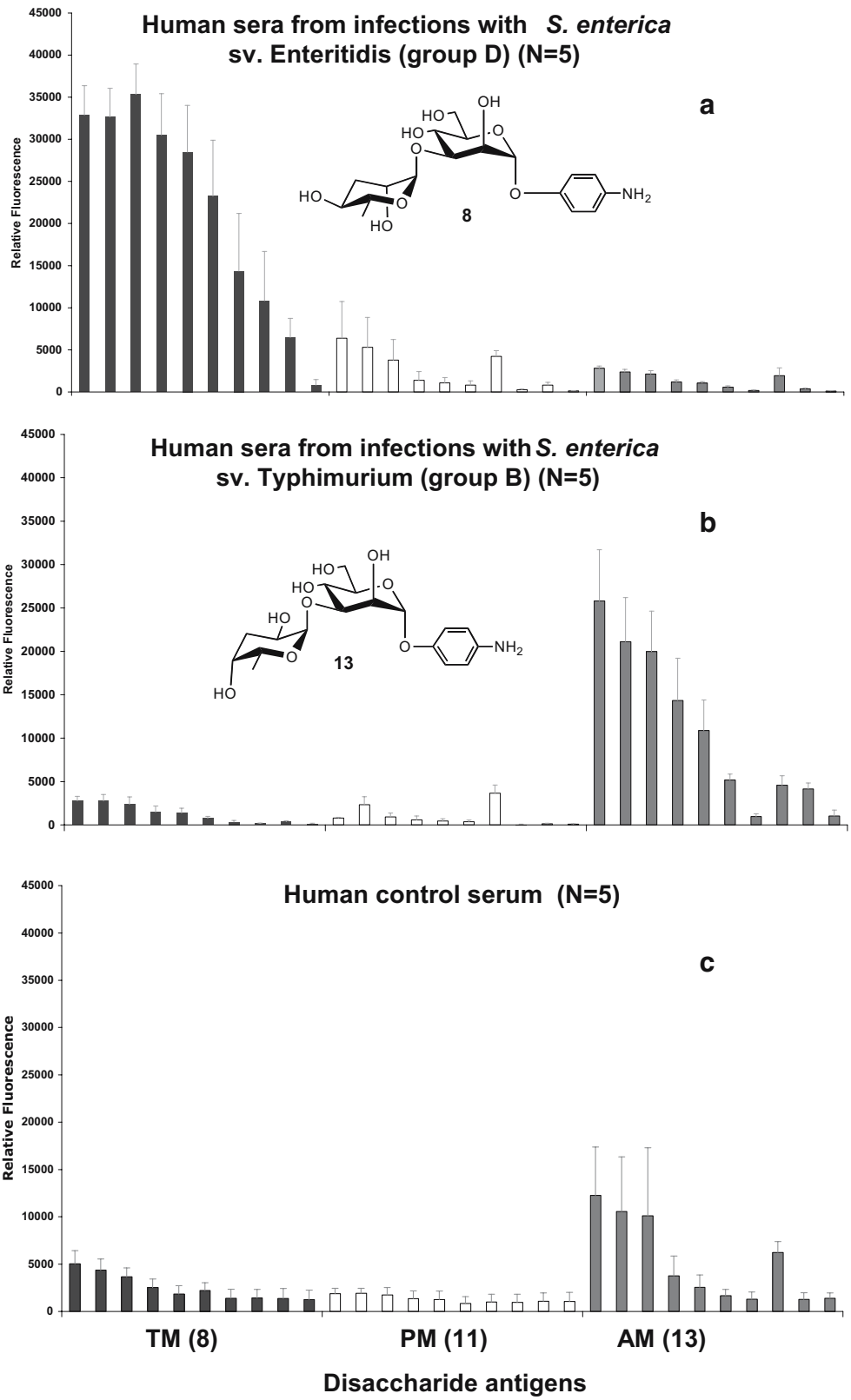

Fig. 5 Microarray analysis of sera from human salmonellosis patients and control sera from healthy non-salmonellosis patients. Sera were analyzed at a serum dilution of 1/100 with a twofold dilution series of printed disaccharide antigen concentrations (500–0.5 μM) from serogroups AO, BO and DO. *Each bar* represents the average mean of five individual patient sera analyzed separately. **a** Sera from infections with *S. enterica* sv. Enteritidis (group D); **b** sera with *S.enterica* sv. Typhimurium (group B); **c** sera from controls

well-defined, synthetic saccharide derivatives of different sizes. Analysis of commercial rabbit agglutination sera showed a strong and relative specific binding to the expected antigens (Fig. 3). The larger oligosaccharides (tetrasaccharides ≤) showed stronger binding, which was also demonstrated previously in competitive inhibition assays [16]. A host infected with *Salmonella* generates a polyclonal antibody response against the O-polysaccharide

chain. Therefore, it was not surprising that rabbit typing sera, as well as patient sera, gave higher titers against the larger saccharides. The immuno-dominant O-factor disaccharides showed lower binding signals but, as expected, much higher differentiation between the serogroups. This demonstrates the importance of well-defined antigens with optimal structure in the establishment of new serological tools. We conclude from our current results that *Salmonella* O-antigen specific microarrays may be a more rapid, precise and low-cost alternative in the serodiagnosis of both human and livestock *Salmonella* infections.

Most importantly, we would like to point out the possibility of using carbohydrate antigen arrays in conjunction with peptide/protein antigens in microarrays for sero-epidemiological surveillance, evaluation of vaccine trials, and livestock herd immunity studies. We are currently in the process of expanding this effort to include a wide variety of bacterial polysaccharide antigens into this technology platform for these purposes including Haemophilus, influenzae, a number of the most relevant pneumococcal CPS and also a variety of mycobacterial glycolipid antigens including M. tuberculosis.

Experimental

General methods

Compound **1**, **2** [26], **3**–**8**, **10**, **11**, **13**, **15** [15–19], dodecasaccharides **9** and **14** [20, 21] and the bi-functional 2-aminoethyl-(*N*-methyl)-hydroxylamine spacer [22] was prepared as described previously. Plant lectin *G. simplicifolia* (GS-II) was from Vector Laboratories. *Salmonella* Typhimurium LPS and all other chemicals were purchased from Sigma-Aldrich (St Louis, MO). Human serum was obtained from healthy volunteers at the General Clinical Research Center, Scripps Hospital, La Jolla, CA. All chemical reactions were monitored by Thin layer chromatography (TLC), using Silica Gel 60F pre-coated TLC plates (EMD Chemicals Inc.). After development with appropriate eluants, the spots were visualized by UV light and/or by either dipping in 5% sulfuric acid in ethanol or by spraying with a solution of ninhydrin (0.5 M in dimethylsulfoxide, DMSO), in both cases followed by heating. Mass spectrometry (MS) profiles were recorded with an LC MSD TOF (Agilent Technologies) using dihydroxybenzoic acid as matrix. Water was purified by NanoPure Infinity Ultrapure water system (Barnstead/Thermolyne) and degassed by vacuum treatment before use. Data images were analysed and the intensity of fluorescence in spots corresponding to the antibodies bound to the individual glycans was quantified using a ScanArray 5000 (Perkin Elmer, Boston, MA) confocal scanner and image analyses were carried out using ImaGene image analysis software (BioDiscovery Inc, El Segundo, CA). Signal to background was typically >50:1 and no background subtractions were performed. Data were plotted using Microsoft Excel software.

Synthesis of compounds **9** and **14**

Free reducing dodecasaccharides (-3Galα1-2[Tyvα1-3]Manα1-4Rhaα1)$_3$ or (-3Galα1-2[Abeα1-3]Manα1-4Rhaα1)$_3$ (1 mg, 0.55 μmol) and the bi-functional spacer 2-aminoethyl-(*N*-methyl)-hydroxylamine (3.8 mg, 42 μmol) were each dissolved in aqueous acetate buffer (0.1 M, pH 4.5, 100 ul), and incubated at 37°C for 24 h. TLC (ethyl acetate: acetic acid:methanol:water, 6:3:3:2, by volume) and MS analysis showed >90% conversion of starting material to compounds **9** and **14**, respectively. To desalt and remove excess spacer, the reaction mixture was purified on a Carbograph column (0.5 ml) [27]. Derivatized glycans were eluted with acetonitrile (30%) and lyophilized to give white fluffy powders (~1 mg each). Lyophilized structures were used for printing without further purifications. ESI-TOF high-accuracy MS *m/z* calculated for (M+H) 1,891.7668; found 1,891.7698.

Synthesis of compound **12**

S. Typhimurium LPS (6 mg) was dissolved in 1 M aq. acetic acid and incubated at 90°C for 1 h. The mixture was then cooled and extracted with chloroform:methanol (2:1, 1.5 volumes). The water layer was lyophilized and further redissolved in water (0.3 ml) and applied onto a size-eclusion chromatography column (Sephadex G15, 0.8× 120 cm) equilibrated and eluted with water:n-BuOH (95:5). Fractions containing the lipid-free polysaccharide were pooled and lyophilized. The residue (1 mg, 0.55 μmol) was dissolved in aqueous acetate buffer (0.1 M, pH 4.5, 100 ul), containing the spacer 2-aminoethyl-(*N*-methyl)-hydroxylamine (3.8 mg, 42 μmol) and incubated at 37°C for 24 h. The reaction mixture was purified on a size-eclusion chromatography column (Sephadex G15, 0.8× 120 cm) equilibrated and eluted with water:n-BuOH (95:5). The lyophilized conjugate (12) was used for printing without further purification.

Microarray printing of antigens

The antigen arrays were created by robotic contact printing of ~0.6 nl of NH_2-spacered antigens in print buffer (300 mM phosphate, 0.005% Tween 20, pH 8.5) onto NHS-activated glass slides [11]. Each antigen was printed at ten different concentrations in twofold dilutions (from 500 or 100 μM), and each dilution was deposited ten times,

creating a 10×10 subgrid for each compound. Post-printing humidification of the slides followed array fabrication immediately at 80% humidity for 30 min. The remaining NHS groups were blocked by immersing the slides in blocking buffer (50 mM ethanolamine in 50 mM borate buffer, pH 9.2) for 1 h. Slides were rinsed in water, dried under a stream of nitrogen, and stored in desiccators at RT before use.

GS-II Lectin analysis

Biotinylated GS-II (10 μg/ml) diluted into phosphated saline buffer (PBS) containing BSA (3%) and Tween-20 (0.05%; 1 ml) was applied onto the slide and incubated in a humidified chamber at 20–22°C for 1 h with gentle shaking. Washing in PBS (+Tween-20, 0.01%, 10 dips) and PBS (10 dips) was followed by immediate incubation with labeled streptavidin–Alexa 488 1/100 (1 mg/ml) in 1 ml PBS (+3% BSA, 0.05% Tween-20) for 30 min, which was followed by washing with PBS (+Tween-20, 0.01%, 10 dips), PBS (10) dips and de-ionized water (2×3 dips), finally the slides were dried in a stream of air, followed by immediate analysis using a confocal slide array laser scanner.

Group specific antisera and antibody analysis

Rabbit *Salmonella* O-grouping kit containing group A, B, C_1, C_2, D, E, F, G, H, I and Vi antisera (#240995, BD, Sparks, MD), or the mouse group B monoclonal Ab (US Biological, Swampscott, MA, USA, L4070250, 1 mg/ml) were each diluted (1:10) into phosphated saline buffer (PBS) containing BSA (3%) and Tween-20 (0.05%) (0.5 ml). The samples (0.5 ml) were applied onto the array slide surface and incubated in a humidified chamber with gentle shaking for 1 h. The slides were washed (10 dips) in PBS (+Tween-20, 0.01%) and PBS (10 dips) followed by immediate incubation with labeled secondary antibodies (goat anti rabbit-IgG–Alexa 488 or goat anti-mouse-IgG–Alexa 488) diluted into PBS containing BSA (3%) and Tween-20 (0.05%, 0.5 ml). Slides were washed (10 dips) in PBS (+Tween-20, 0.01%), PBS (10 dips), de-ionized water (2×3 dips) and dried in a stream of air followed by immediate analysis using a confocal slide array laser scanner.

Human serum sample analysis

Serum samples from healthy humans or patients with salmonellosis were diluted (1:5–800) with incubation buffer (PBS, 0.05% Tween-20, 3% BSA) and applied onto the microarray glass slides (1 ml) After 1 h incubation at 20–22°C, the slides were washed in PBS (+Tween-20, 0.01%, 10 dips) and PBS (10 dips). The slide was further incubated with biotinylated goat anti-human IgG/A/M mAb diluted (1/200) in incubation buffer (1 ml, PBS, 0.05% Tween-20). After 30 min incubation at 20–22°C, the slides were washed in PBS (+Tween-20, 0.01%, 10 dips) and PBS (10 dips). The slides were further incubated with streptavidin–Alexa 488 conjugate diluted (1:200) with incubation buffer (1 ml, PBS, 0.05% Tween-20) for 30 min at 20–22°C The slides were washed in PBS (+Tween-20, 0.01%, 10 dips), PBS (10 dips) and rinsed with de-ionized water followed drying in a stream of air followed by immediate analysis using a confocal slide array laser scanner.

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