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Investigating the candidacy of a lipoteichoic acid-based glycoconjugate as a vaccine to combat *Clostridium difficile* infection

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Abstract A lipoteichoic acid has recently been shown to be conserved in the majority of strains from *Clostridium difficile* and as such is being considered as a possible vaccine antigen. In this study we examine the candidacy of the conserved lipoteichoic acid by demonstrating that it is possible to elicit antibodies against *C. difficile* strains following immunisation of rabbits and mice with glycoconjugates elaborating the conserved lipoteichoic acid antigen. The present study describes a conjugation strategy that utilises an amino functionality, present at approximately 33 % substitution of the *N*-acetyl-glucosamine residues within the LTA polymer repeating unit, as the attachment point for conjugation. A maleimide-thiol linker strategy with the maleimide linker on the carboxyl residues of the carrier protein and the thiol linker on the carbohydrate was employed. Immunisation derived antisera from rabbits and mice, recognised all strains of *C. difficile* vegetative cells examined, despite an immune response to the linkers also being observed. These sera recognised live cells in an immunofluorescence assay and were also able to recognise the spore form of the bacterium. This study has illustrated that the LTA polymer is a highly conserved surface polymer of *C. difficile* that is easily accessible to the immune system and as such merits consideration as a vaccine antigen to combat *C. difficile* infection.

Keywords *Clostridium difficile* · Conjugate vaccine · Lipoteichoic acid

Introduction

Clostridium difficile is a Gram-positive bacterium that is widely recognized as one of the most common causes of hospital acquired infection, especially following antibiotic treatment [1]. Furthermore, there is growing evidence that the threat of community acquired *C. difficile* disease is increasing [2]. In the last 20 years the number of hospital stays associated with *C. difficile* in the US has tripled and close to 10 % of these *C. difficile* infections result in the death of the patient (compared to 2 % for all inpatient hospitalisations) [3]. The fact that *C. difficile* can persist indefinitely in a robust sporulated form, which is easily transferred from person to person in the hospital environment and which is recalcitrant to many standard cleaning regimens, only serves to exacerbate this problem [4]. In addition to the development of new antimicrobial agents to treat this serious infection, several vaccine approaches are also being considered to combat *C. difficile* associated disease (CDAD). The majority of these vaccine approaches are focused on the generation or provision of antibodies to neutralize the toxins which are the key virulence factors produced by the bacterium [5–8]. These toxins are responsible for the severe pathology observed in CDAD which includes severe diarrhea, abdominal pain and the more serious complications such as pseudomembranous colitis and toxic megacolon. It is important to realize that while antibody mediated neutralization of toxin activity will alleviate clinical symptoms; this anti-toxin approach will not target the pathogen itself and thus will not eradicate the bacterium. Recurrent *C. difficile* infection is now recognized as a serious

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problem and as such it is critical that in addition to neutralization of toxins, steps are taken to ensure that the organism is cleared from the intestine. Other approaches to facilitate this process include the development of new antibiotics [9], the provision of non-toxigenic *C. difficile* spores to patients to provide competition for colonization by pathogenic *C. difficile* [10], or fecal replacement therapy to restore a balanced host gut microbiome and prevent *C. difficile* colonization [11]. The latter while considered as a treatment of last resort has demonstrated considerable success in fulminant infections.

We and others are focusing our efforts on targeting cell-surface carbohydrates as vaccines. Glycoconjugate vaccines are relative newcomers as vaccine strategies but have been tremendously successful in the eradication of a number of important bacterial pathogens. The Hib glycoconjugate vaccine to prevent *Haemophilus influenza* type B respiratory infections was the trailblazer following its introduction in the late 1980's [12] and has been followed by successful glycoconjugate approaches to combat *Neisseria meningitidis* serotypes A,C,W-135 and Y [13] and 13 serotypes of *Streptococcus pneumoniae* [12]. All these approaches are based upon the identification of a conserved surface carbohydrate structure for the targeted serotype. Poxton *et al.* were the first to investigate lipocarbohydrate antigens of *C. difficile* and demonstrate that these antigens were immunologically conserved amongst isolates and exhibited cross reactivity with only *C. sordellii* and *C. bifermentans* [14, 15]. More recently, Ganeshapillai *et al.* identified two carbohydrate polymers of *C. difficile* that they termed PS-I and PS-II. PS-I was found to be elaborated only by a small sub-population of *C. difficile* strains, whereas PS-II appears to be conserved across the majority of *C. difficile* strains [16]. Several groups have looked to exploit these structures *via* the production of glycoconjugates based on the native polymer [17] or on synthetic oligosaccharides [18–21]. We recently added to the repertoire of carbohydrate polymers that *C. difficile* elaborates with the identification and structural characterization of a lipoteichoic acid (LTA) like polymer [22]. The LTA we identified in all strains of *C. difficile* examined consisted of the repeat unit [−6)-α-D-Glc*p*NAc-(1–3)-[−P-6]-α-D-Glc*p*NAc-(1–2)-D-GroA] where GroA is glyceric acid. The repeating units were linked by a phosphodiester bridge between C-6 of the two GlcNAc residues (6-P-6). A minor component consisted of Glc*p*N-(1–3) instead of Glc*p*NAc-(1–3) in the repeat unit. Through a 6–6 phosphodiester bridge this polymer was linked to −6)-β-D-Glc*p*-(1–6)-β-D-Glc*p*-(1–6)-β-D-Glc*p*-(1–1)-Gro, with glycerol (Gro) substituted by fatty acids. A recent study by Martin *et al.* has described the synthesis of a disaccharide of the repeating unit and by utilizing this molecule in a glycan array confirmed that antibodies to LTA were circulating in patients with *C. difficile* infection [23]. In this study we describe our work in preparing glycoconjugates of the LTA and the determination of the cross-reactivity of the sera generated following immunization of mice and rabbits with these glycoconjugates against a library of *C. difficile* strains.

Materials and methods

Growth of bacteria and preparation of purified lipoteichoic acid (LTA) and PS-II

C. difficile strain 630 was grown in brain heart infusion (BHI) broth supplemented with 0.5 g L^{-1} cysteine-HCl, 5 mg L^{-1} hemin, 1 mg L^{-1} vitamin K, 5 g L^{-1} yeast extract, 1.2 g L^{-1} NaCl (BHIS) under anaerobic conditions and harvested at late logarithmic phase (OD_{600} 1.5–2.0). The bacterial cells were harvested (8200 ×*g*, 4 °C, 20 min), killed with the addition of phenol to 4 % and washed with 10 mM phosphate buffered saline, pH 7.4. To isolate the LTA and PS-II, the cells were first extracted in boiling water for 30 min and the resulting solution separated by low-speed centrifugation and the supernatant was dialyzed against tap water and lyophilized. Contaminating proteins and nucleic acids were removed from a 5 mg/mL aqueous solution of the lyophilized material by precipitation with 15 % trichloroacetic acid O/N at 4 °C, low-speed centrifugation followed by dialysis of the supernatant against water. The water-soluble material was separated by anion exchange chromatography on a HiTrap Q column using a H_2O-1 M NaCl gradient to give PS-II.

The remaining cells were subjected to extraction with 45 % phenol (68 °C, 30 min). The water phase was separated from the phenol phase and cell debris by centrifugation. The phenol phase and cell debris was then re-extracted with more water and treated as per above. The two water phases were combined and dialyzed against tap water until phenol-free, then lyophilized. The dried sample was dissolved in water to give a 1–2 % solution (w/v) and treated with deoxyribonuclease I (DNase) (0.01 mg/mL) and ribonuclease (RNase) (0.01 mg/mL) for 3 h at 37 °C, then treated with proteinase K (0.01 mg/mL) for 3 h. The sample was then dialysed against tap water for 17 h and lyophilized. The resulting crude LTA containing sample was purified by anion exchange chromatography as above.

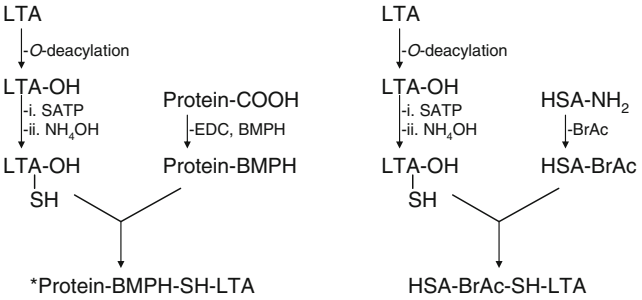
Immunisations of rabbits with killed whole cells of *C. difficile*

C. difficile strains 630 and R20291 were initially grown under anaerobic conditions overnight on BHI plates. Growth from these plates was used to inoculate 100 mL of BHI broth to a starting OD of 0.1 and flasks were incubated anaerobically until an OD_{600} of 0.6 was reached, cells were collected by centrifugation and resuspended in 3 % formalin in PBS and left at 4 °C overnight. Cells were recovered by centrifugation and washed × 3 with 50 mL of PBS to remove formalin. For strain 630, a New Zealand white rabbit (1.5–2 kg) was immunised with 2 × 0.25 mL subcutaneous injections containing 2×10^9 bacterial cells mixed 1:1 with incomplete Freund's adjuvant (IFA) and boosted three times (D28, 56 and D77) with an identical antigen preparation. For strain R20291 two

other New Zealand white rabbits (1.5–2 kg) rabbit were immunised with 2×0.25 mL subcutaneous injections containing 2×10^9 bacterial cells mixed 1:1 in incomplete Freund's adjuvant (IFA) and boosted two times (D28, D56) with the identical antigen preparation. Rabbits were bled by heart puncture 14 days after the last injection.

Preparation of conjugates from purified LTA

The glycoconjugates were prepared as described below and as illustrated in the following scheme:-



*For the BMPH activation either HSA or ExoA were the carrier proteins. In some cases LTA was thiolated without prior *O*-deacylation.

O-deacylation

Purified LTA was treated with 14 % NH_4OH in 10 % MeOH at 50 °C for 3 h. The solution was rotary evaporated to dryness, redissolved in water, and gel purified on a Sephadex G-25 column (Amersham), eluting with water. The product fraction was collected and lyophilised to prepare *O*-deacylated LTA (LTA-OH).

Attachment of linker molecule

LTA or LTA-OH (4 mg/mL) was dissolved in 200 mM sodium phosphate at pH 7.5 and a 3× molar equivalent of *N*-succinimidyl-*S*-acetylthiopropionate (SATP, Pierce) dissolved in 100 μL of DMSO (BDH Chemicals) was added. The reaction was left at 22 °C for 2 h in the dark. The sample was then purified down a Sephadex G-25 column, eluting with water and the product peak was lyophilised. The product was monitored by NMR as detailed below.

Recombinant expression of Exoprotein A (ExoA)

A genetically inactivated *P. aeruginosa* exotoxin A protein (ExoA) with an upstream OmpA leader peptide [24] was constructed in a pET28a-TEV vector and expressed in *E. coli* BL21-CodonPlus(DE3)-RIL (Agilent Technologies, Santa Clara, CA, USA). Protein was produced by diluting a fresh overnight culture in LB medium containing 25 ug mL^{-1} kanamycin and 30 μg mL^{-1} chloramphenicol. The culture was grown at 37 °C and 200 rpm to an OD_{600} of 0.6 upon which time the temperature was reduced to 30 °C and the culture was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Cells were harvested 3 h later by centrifugation at 10,000 g for 10 min. Cell pellets were re-suspended in lysis buffer (20 mM Tris-Cl, pH 8.0) containing Complete® protease inhibitor mixture, EDTA-free (Roche Applied Science, Laval, QC, Canada) and 10 μg mL^{-1} each of RNaseA and DNaseI (Roche Applied Science). Cell lysis was performed *via* two passes through an Emulsiflex C5 (Avestin, Ottawa, ON, Canada) at 20,000 psi. Lysates were then centrifuged at 100,000 g for 50 min at 4 °C. Protein was then purified by ion exchange. Supernatant was loaded onto a Macro-Prep High Q Support Strong Anion Exchange column (Bio-Rad Laboratories, Hercules, CA, USA). The column was washed with 5 volumes of wash buffer (20 mM Tris-Cl, pH 8.0) before a gradient of 0–1 M NaCl in 20 mM Tris-Cl, pH 8.0 was applied over 20 volumes followed by a high salt wash at 1 M NaCl in 20 mM Tris-Cl, pH 8.0 over 5 volumes. Fractions containing ExoA as determined by SDS-PAGE were further purified by gel filtration. Fractions were concentrated by centrifugal filtration (Millipore, Billerica, MA, USA) and equilibrated in a buffer comprising of 20 mM Tris-Cl, 150 mM NaCl, pH 8.0 before being loaded onto a Hiprep 16/60 Sephacryl S-200 High Resolution column (Bio-Rad Laboratories) using 20 mM Tris-Cl, 150 mM NaCl, pH 8.0 as an elution buffer. Fractions containing ExoA were confirmed by SDS-PAGE and protein yield was determined by Bradford assay (Bio-Rad Laboratories).

Activation of protein carrier

In order to conjugate the protein carriers HSA or ExoA to the thiol-tagged LTA it was necessary to modify the carboxyl groups on the carrier protein (15 mg) by treatment with an 600 × molar excess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce) and a 80 × molar excess of *N*-(β-maleimidopropionic acid) hydrazide trifluoroacetic acid salt, (BMPH, Pierce) dissolved in 3 mL of 100 mM 2-(*N*-morpholino) ethanesulfonic acid, (MES, Aldrich) at pH 5.2 at 4 °C for 16 h. The sample was purified on a Sephadex G-25 column, eluting with 100 mM sodium phosphate pH 6.8. The product peak was concentrated to approximately 0.5 mL using an Amicon ultra-15 10 kDa MMCF spin column and stored at 4 °C. The activated proteins were characterised by MALDI-MS as described below. In an alternative activation approach the amino-groups of the lysine amino acids of HSA were bromo-acetylated. This was achieved by dissolving the HSA protein (15 mg) in 4 mL of 100 mM sodium phosphate at pH 8 and adding a 200 × molar excess of

bromoacetic acid *N*-hydroxysuccinimide ester (Sigma) in 200 μl of DMSO (BDH Chemicals). The reaction was left for 17 h at 4 °C then purified, concentrating the sample 4 × with 10 mL of 100 mM sodium phosphate at pH 6.8 in an Amicon ultra-15 30 kDa MMCF spin column and stored at 4 °C. The activated protein was characterised by MALDI-TOF MS as described below.

Conjugation reaction

The thiol protecting groups of the activated carbohydrates were de-protected at 5 mg/mL in 100 mM sodium phosphate pH 6.8 containing 100 mM hydroxylamine hydrochloride (JT Baker) at 22–24 °C for 1.5 h under nitrogen. The sample was purified on a Sephadex G-25 column, eluting with 100 mM sodium phosphate pH 6.8. For all conjugations the eluted de-protected carbohydrates were collected directly into the activated proteins. The mixtures were left to react at 22–24 °C for 3 h in the dark under nitrogen while rocking. The mixtures were left for a further 16 h at 4 °C and concentrated to ~1 mL as above. The concentrates were washed and concentrated a further four times using Dulbecco's PBS (Gibco) containing 10 mM sodium citrate (Sigma). The final concentrates were stored at 4 °C. The glycoconjugates were characterised by MALDI-MS as described below.

For ELISA examination of whole cell generated polyclonal sera recognition of LTA and PS-II the purified carbohydrate antigens were oxidized (5 mg/mL in 25 mM periodate in 50 mM sodium acetate at 5 mg/mL at room temperature for 30 min) and conjugated at 4 mg/mL to BSA (1 mg/mL) *via* reductive amination in sodium phosphate buffer for 20 h at 37 °C. The conjugates were purified on 30 kDa MMCF spin columns as described above and characterised by MALDI and sugar analyses (data not shown).

Analytical methods

Sugars were determined as their alditol acetate derivatives by GLC-MS as described previously [25].

Mass spectrometry and nuclear magnetic resonance spectroscopy

Capillary electrophoresis electrospray mass spectrometry (CE-ES-MS) and NMR spectroscopy were performed as described previously [26]. Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectra were obtained using a Voyager DE-STR mass spectrometer (Applied BioSystems, Foster City, CA, U.S.A.). The instrument was operated in positive, linear ion mode under delayed extraction conditions (200 ns) using an accelerating voltage of 25 000 V. Each spectrum is the average of approximately 100 laser shots. The matrix used was 3,5-dimethoxy-4-hydroxy cinnamic acid (sinapinic acid), prepared at a concentration of 10 μg/μL in 30 % acetonitrile and 0.1 % formic acid (v/v). These solutions were spotted directly on the MALDI target in a 1:3 ratio with matrix.

Conjugate immunisations

Conjugate 1: HSA-BMPH-SH-de-O-LTA

Three New Zealand white rabbits (1.5–2 kg) were immunised subcutaneously with the HSA-BMPH-SH-de-*O*-LTA conjugate. Each rabbit received 50 μg of HSA-BMPH-SH-de-*O*-LTA conjugate (RCDV1-3) as 2×0.5 mL per immunisation with incomplete Freund's adjuvant for the prime immunisation and boosts. The rabbits were boosted on days 28 and 56; sera were recovered following trial bleed on day 42 and terminal heart puncture on day 70. Two rabbits also received control immunisations, which consisted of the *O*-deacylated carbohydrate (50 μg per rabbit (RCDC4-5)) admixed with the same amount of protein (HSA) as in the glycoconjugate and appropriate adjuvant, with the same boosting and sera recovery schedule.

Five Balb/C mice (6–8 weeks old) were also immunised intra-peritoneally with the HSA-BMPH-SH-de-*O*-LTA conjugate (MCDV1-5): two with 10 μg and three with 5 μg of conjugated carbohydrate per immunisation with Sigma adjuvant for the prime immunisation and boosts. The mice were boosted on days 21 and 42; sera were recovered following trial bleed on day 35 and terminal heart puncture on day 56. Additionally, eight mice received control immunisations, which comprised two mice (MCDC6–7) receiving *O*-deacylated carbohydrate (10 μg per mouse) admixed with the same amount of protein (HSA) as in the glycoconjugate, two mice (MCDC10–11) receiving purified LTA (10 μg per mouse) admixed with the same amount of protein (HSA) as in the glycoconjugate, two mice (MCDC8–9) receiving *O*-deacylated carbohydrate (10 μg per mouse) alone and two mice (MCDC12–13) receiving native LTA (10 μg per mouse) alone, all with the same boosting and sera recovery schedule.

Conjugate 2: HSA-BrAc-SH-LTA and HSA-BrAc-SH-de-O-LTA

Six New Zealand white rabbits (1.5–2 kg) were immunised subcutaneously with the HSA-BrAc-SH style glycoconjugates. Three rabbits received 50 μg of HSA-BrAc-SH-LTA conjugate (RCLV1-3) and three rabbits received 50 μg of HSA-BrAc-SH-de-*O*-LTA conjugate (RCOV1-3) as 2×0.5 mL per immunisation with incomplete Freund's adjuvant for the prime immunisation and boosts. The rabbits were boosted on days 28 and 56; sera were recovered following trial bleed on day 42 and terminal heart puncture on day 70. Four rabbits also received control immunisations, which consisted of the LTA (2 rabbits RCLC4-5) or the *O*-deacylated LTA (2 rabbits RCOC4-5) (50 μg per rabbit) admixed with the same amount of protein

(HSA) as in the glycoconjugate and appropriate adjuvant, with the same boosting and sera recovery schedule.

Ten Balb/C mice (6–8 weeks old) were also immunised intraperitoneally with the HSA-BrAc-SH style glycoconjugates. Five mice received the HSA-BrAc-SH-LTA conjugate (MCLV1–5) with 5 μg of conjugated carbohydrate per immunisation and five mice received the HSA-BrAc-SH-de-*O*-LTA conjugate (MCOV 1–5) with 5 μg of conjugated carbohydrate per immunisation with Sigma adjuvant for the prime immunisation and boosts. The mice were boosted on days 21 and 42; sera were recovered following trial bleed on day 35 and terminal heart puncture on day 56. Additionally, six mice received control immunisations, which comprised three mice receiving *O*-deacylated LTA (5 μg per mouse (MCOV6-8)) admixed with the same amount of protein (HSA) as in the glycoconjugate and three mice receiving the native LTA (5 μg per mouse (MCLV6-8)) admixed with the same amount of protein (HSA) as in the glycoconjugate, all with the same boosting and sera recovery schedule.

Conjugate 3: ExoA-BMPH-SH-de-O-LTA

Three New Zealand white rabbits (1.5–2 kg) were immunised subcutaneously with the ExoA-BMPH-SH-de-*O*-LTA conjugate receiving 25 μg of conjugate (RCXV1–3) as 2×0.5 ml per immunisation with incomplete Freund's adjuvant for the prime immunisation and boosts. The rabbits were boosted on days 28 and 56; sera were recovered following trial bleed on day 42 and terminal heart puncture on day 70. Two rabbits also received control immunisations, which consisted of the *O*-deacylated LTA (50 μg per rabbit (RCXC4–5)) admixed with the same amount of protein (ExoA) as in the glycoconjugate and appropriate adjuvant, with the same boosting and sera recovery schedule.

Five Balb/C mice (6–8 weeks old) were also immunised intra-peritoneally with the ExoA-BMPH-SH-de-O-LTA conjugate (MCXV1–5) receiving the conjugate with 5 μg of conjugated carbohydrate per immunisation with Sigma adjuvant or the prime immunisation and boosts. The mice were boosted on days 21 and 42; sera were recovered following trial bleed on day 35 and terminal heart puncture on day 56. Additionally, three mice received control immunisations, which comprised of receiving *O*-deacylated LTA (5 μg per mouse (MCXC6–8)) admixed with the same amount of protein (ExoA) as in the glycoconjugate, all with the same boosting and sera recovery schedule.

Whole cell ELISA

Whole cell ELISA was performed to determine whether sera recognized whole cells from various strains of *C. difficile*. Briefly, wells of Nunc Maxisorp EIA plates were coated with 100 μL of formalin-killed bacteria (optical density at 620 nm [OD620] of 0.08) in PBS for 18 h in a 37 °C drying oven and then brought to 22–24 °C before use. Plates were blocked with 1 % bovine serum albumin (BSA)-PBS for 1 h at 22–24 °C, wells were washed with PBS–0.05 % Tween 20 (PBS-T), and incubated with sera for 3 h at 22–24 °C. Following washing with PBS-T, alkaline phosphatase-labeled goat anti-mouse IgG (or goat anti-rabbit Ig) (Cedarlane Laboratories) diluted 1:1,000 (mice) 1:3,000 (rabbits) in 1 % BSA-PBS was added for 1 h at 22–24 °C. The plates were then washed and developed with Phosphatase Substrate System (Kirkegaard and Perry Laboratories). After 60 min OD was measured at A_{405nm} using a microtiter plate reader.

Immunofluorescence

In order to determine if antibodies in immune serum could access LTA epitopes on the bacterial cell surface, immunofluorescence on live *C. difficile* vegetative cells was performed. *C. difficile* was cultured to mid-log phase in BHIS broth without shaking in a MiniMacs anaerobic chamber at 37 °C. The cells were centrifuged to remove the broth, re-suspended in PBS and then 10 μL was air dried onto glass coverslips. The bacteria were heat fixed to the coverslip by passing through a Bunsen flame 5–6 times, and then were blocked with 5 % Bacto skim milk (Difco, Sparks, USA)-PBS for 30 min at room temperature. The cells were incubated for 45 min at room temperature in 50 μL of either the pre- or post-immune (D70) anti-LTA serum (RCDV2, RCLV2, RCXV2) at a dilution of 1:100 in PBS. The coverslips were washed with PBS-0.1 % Tween 20 (PBS-T), then incubated for 45 min at room temperature with 50 μL goat anti-rabbit IgG Alexafluor 488 antibody (Invitrogen, Eugene, Oregon, USA) at a 1:1000 dilution. The coverslips were washed with PBS, mounted with Vectashield-DAPI (Vector Laboratories, Burlington, Canada) then examined with a Zeiss microscope (Axiovert 200 M).

In order to determine if antibodies in immune serum would react with *C. difficile* spore surface, immunofluorescence on *C. difficile* spores was performed.

C. difficile was cultured on BHIS agar plates in a MiniMacs anaerobic chamber at 37 °C for 7 days to allow spore formation. Spores were purified by heat inactivating any vegetative cells for 20 min at 60 °C then by multiple washes in ice cold H_2O. Once purified, 10 μL of spores was air dried onto glass coverslips. The bacterial spore preparation was heat fixed to the coverslip by passing through a Bunsen flame 5–6 times, then were blocked with 5 % Bacto milk (Difco)-PBS for 30 min at room temperature. The spores were incubated for 45 min at room temperature in 50 μl of either the pre- or post-immune (D70) anti-LTA serum (RCDV2, RCXV2) at a dilution of 1:100 in PBS-T. The coverslips were washed with PBS then incubated for 45 min at room temperature with 50 μL goat anti-rabbit IgG Alexafluor 488 antibody (Invitrogen, Eugene, Oregon,

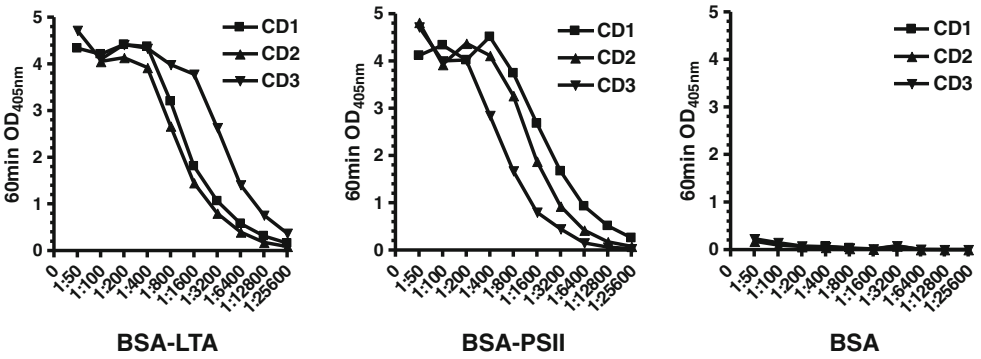


Fig. 1 Titration curves for the polyclonal sera derived from rabbit immunisations with killed whole cells of *C. difficile* strains 630 (CD1) and R20291 (CD2 & CD3) against purified BSA conjugates of the LTA and PS-II antigens

USA) at a 1:1000 dilution. The coverslips were washed with PBS, mounted with Vectashield-DAPI (Vector Laboratories, Burlington, Canada) then examined with a Zeiss microscope (Axiovert 200 M). For all images representative cells are shown. Experiments were performed in duplicate on at least three independent occasions.

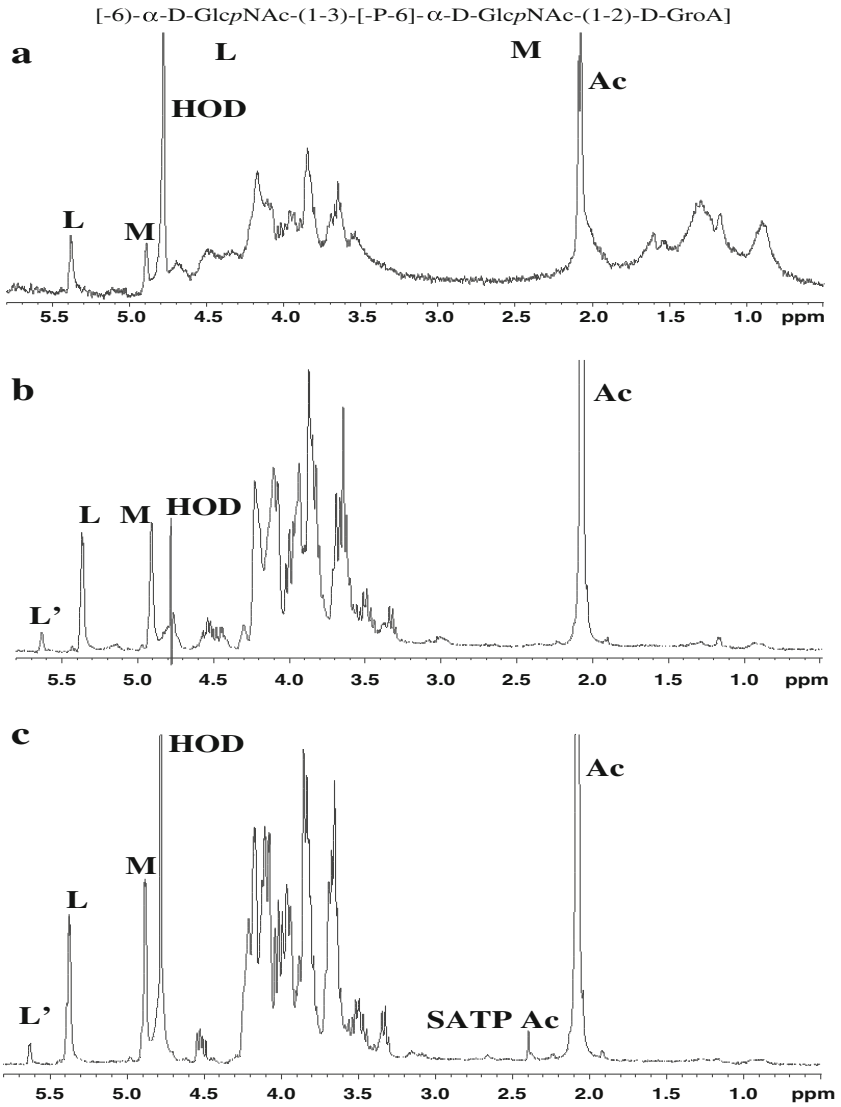


Fig. 2 NMR spectra of the LTA from *C. difficile* strain 630 **a** before; **b** after *O*-deacylation and **c** after O-deacylation and linker incorporation. The repeat unit structure is inset and labeled according to the original structural paper [22]. Anomeric protons, HOD signal and acetyl groups are also indicated

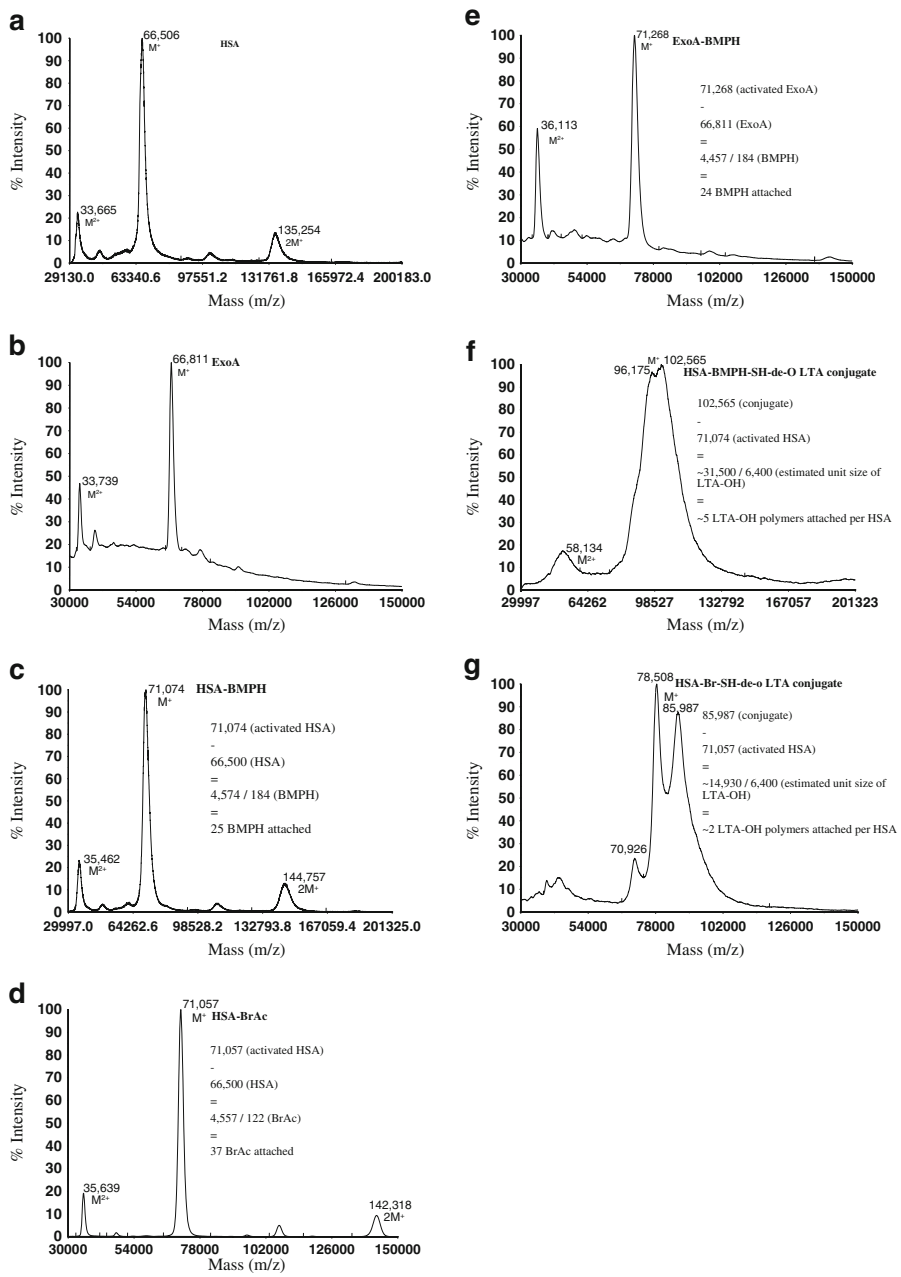


Fig. 3 MALDI-MS analyse s of; **a** HSA, **b** ExoA, **c** HSA-BMPH, **d** HSA-BrAc, **e** ExoA-BMPH, **f** HSA-BMPH-SH-de-O-LTA conjugate and **g** HSA-BrAc-SH-de-O-LTA conjugate

Results

LTA is recognised following whole cell immunisations

Figure 1 illustrates the ELISA determination of recognition of LTA and PS-II purified from *C. difficile* 630 (LTA_{630} and $PSII_{630}$) with titration of post-immune rabbit sera following immunization with bacterial cells. Plates were coated with 1 μg of conjugated LTA_{630} or 1 μg of conjugated PS-II_{630} per well. The polyclonal antisera raised to either *C. difficile* 630 or *C. difficile* R20291 (O27 ribotype) revealed a good titer towards both LTA_{630} and towards PS-II_{630}.

Conjugates were prepared as detailed in the Materials and Methods and efficiency of each step of the strategy was examined by MS and or NMR as appropriate.

Table 1 Comparison of conjugation linker strategies, carbohydrate loading and carrier protein to generate titers to LTA

Conjugate	Carbohydrate : Protein molar ratio	Rabbit sera OD_{405nm} @1:400[1]	Mice sera OD_{405nm} @1:160[2]
ExoA-BMPH-SATP-de-O-LTA	3.5:1	1.8	1.7
HSA-BMPH-SATP-de-O-LTA	5:1	1.6	1.15
HSA-BrAc-SATP-de-O-LTA	1.5:1	0.4	0.34
HSA-BrAc-SATP-LTA	nd[3]	1.33	0.1

[1] Rabbit sera OD is an average of the 3 immunised rabbits measured after 60 min incubation with substrate

[2] Mice sera OD is an average of the 5 immunised mice measured after 60 min incubation with substrate

[3] Not determined

Characterisation of *O*-deacylation of LTA

The extent and specificity of *O*-deacylation achieved by treatment with 14 % NH_4OH was effectively monitored by NMR as evidenced by the loss of the signals for the CH_2 residues at 0.5 to 1.5 ppm (Fig. 2a, b)

Attachment of linker

A thiol containing linker was attached to the LTA as detailed in the Material and Methods and characterised by NMR, which revealed the acquisition of a singlet at 2.4 ppm corresponding to the methyl protons of the acetate protecting group consistent with attachment of the linker molecule (Fig. 2c).

Purification of *Pseudomonas aeruginosa* ExoA

ExoA was expressed as a recombinant protein in *E. coli* BL21-CodonPlus (DE3)-RIL. ExoA was purified *via* an ion exchange column and gel filtration. Protein yield after the gel filtration step was approximately 25 mg L^{-1} liquid media. The calculated molecular weight of ExoA is 66 kDa. An SDS-PAGE of the expected product demonstrated that a band of 66 kDa was isolated to a high degree of purity (data not shown).

Activation of protein carrier

Carboxyl groups of the carrier proteins, HSA and ExoA were activated with a maleimide containing linker (BMPH) as described in the Material and Methods and characterised by MALDI-MS which revealed that ~25 carboxyl residues had been activated with BMPH as evidenced by a mass increase of ~4.6 kDa (Fig. 3c, e) over inactivated HSA/ExoA (Fig. 3a, b). Similarly, amino groups of HSA were activated with a bromoacetyl linker (BrAc) as described in the Materials and Methods and characterized by MALDI-MS which revealed that ~37 amino

Table 2 ELISA determination of recognition of whole cells from *C. difficile* strains and other Clostridial species (as indicated) with post-immune rabbit sera (D70) following immunisations with the ExoA-BMPH-SH-de-O-LTA glycoconjugate. ELISA values after 60 min. at OD_{405nm} are detailed. Cells were killed with formalin, washed with water and resuspended at the same OD prior to plating; Dilutions are shown in parentheses. nd = not determined

C. difficile Strain	Conjugate Rabbit Sera (1:200)				
	RCXV1	RCXV2	RCXV3	RCXC4	RCXC5
Cd630	0.997	1.008	0.877	0.106	0.229
QCD	1.265	1.194	1.009	0.075	0.152
R20291	1.353	1.319	1.109	0.061	0.132
M120	1.041	1.034	0.878	0.056	0.127
CM26	1.435	1.234	1.015	0.069	0.145
106-01	1.350	1.303	1.148	0.063	0.152
Cd196	1.694	1.507	1.469	0.159	0.819
001-01	1.540	1.456	1.454	0.122	0.661
Cd20	1.803	1.659	1.463	0.192	0.754
B1-14	1.508	1.432	1.208	0.115	0.151
B1-11	1.668	1.552	1.330	0.140	0.209
B1-9	1.586	1.369	1.261	0.132	0.162
B1-6	1.813	1.682	1.646	0.130	0.177
CM121	1.138	1.158	0.980	0.040	0.122
CM56	1.569	1.449	1.322	0.125	0.240
O6CD130	1.442	1.392	1.365	0.083	0.291
29975	1.687	1.526	1.494	0.156	0.308
M13876	1.423	1.339	1.275	0.121	0.119
M16256	1.417	1.375	1.330	0.120	0.120
B1-1	1.742	1.628	1.563	0.118	0.158
052694	1.582	1.593	1.462	0.140	0.187
B1-7	1.726	1.646	1.614	0.122	0.578
M26195	1.542	1.460	1.361	0.223	0.128
M23257	1.592	1.618	1.693	0.176	0.702
M46846	1.779	1.591	1.437	0.163	0.601
LIV022	1.495	1.468	1.298	0.178	0.122
TL178	1.372	1.232	1.116	0.055	0.050
LIV024	1.760	1.431	1.393	0.101	0.161
TL176	1.551	1.342	1.218	0.117	0.079
CD305	0.679	0.711	0.626	0.025	0.040
CF5	1.620	1.481	1.326	0.084	0.124
M6510	1.327	1.084	1.024	0.072	0.110
TL174	1.330	1.193	1.000	0.040	0.039
M68	1.044	1.045	0.870	0.057	0.144
M6317	1.283	1.290	1.023	0.062	0.070
M7465	1.456	1.200	1.125	0.088	0.086
M9349	1.352	1.245	1.148	0.074	0.068
M13340	1.443	1.359	1.160	0.098	0.169
VPI 10463	2.043	1.734	1.358	0.116	0.134
D0724491	0.917	0.949	0.813	0.153	0.153
D0835450	1.375	1.232	1.081	0.135	0.104
955289	1.214	1.215	1.064	0.178	0.097
C. perfringens	0.216	0.957	0.307	0.157	0.171
C. sporogenes	0.137	0.153	0.202	0.128	0.149
C. barati	0.230	0.272	0.411	0.178	0.176
C. butyricum	1.818	1.672	1.549	0.104	0.129
C. subterminale	1.884	1.697	1.458	0.131	0.134

Table 2 (continued)

C. difficile Strain	Conjugate Rabbit Sera (1:200)				
	RCXV1	RCXV2	RCXV3	RCXC4	RCXC5
C. bifermentans	1.674	1.466	1.309	0.129	0.140
C. botulinum A6	0.138	0.191	0.290	0.111	0.152
C. botulinum E Russ	0.053	0.064	0.096	0.045	0.047

groups had been activated with BrAc as evidenced by a mass increase of ~4.5 kDa (Fig. 3d) over inactivated HSA (Fig. 3a).

Characterisation of conjugation products

Following de-protection of the SATP-activated carbohydrate to expose the thiol moiety, activated proteins were conjugated to the carbohydrate as described above. Conjugation products were purified as described and monitored by MALDI-MS suggesting that ~5 carbohydrate molecules had been attached per maleimide activated HSA protein by virtue of a mass increase of ~31.5 kDa overall that corresponds to 5 units of ~6.5 kDa for each carbohydrate unit attached, suggesting that the de-*O*-LTA polymer attached to HSA was approximately 15 repeat units in length (Fig. 3f). Similarly MALDI-MS revealed that only 2 carbohydrate molecules had been attached per bromo-acetyl activated HSA protein by virtue of a mass increase of ~14.9 kDa overall that corresponds to ~2 units of ~6.5 kDa for each carbohydrate unit attached, suggesting that the de-*O*-LTA polymer attached to HSA was approximately 15 repeat units in length (Fig. 3g). MALDI spectra were not able to be obtained for the intact LTA molecule containing conjugates, nor the ExoA conjugate. In the case of the ExoA conjugate, we performed sugar and protein assays which revealed a ratio of 3–4 carbohydrate molecules per protein. Carbohydrate loading information is summarised in Table 1.

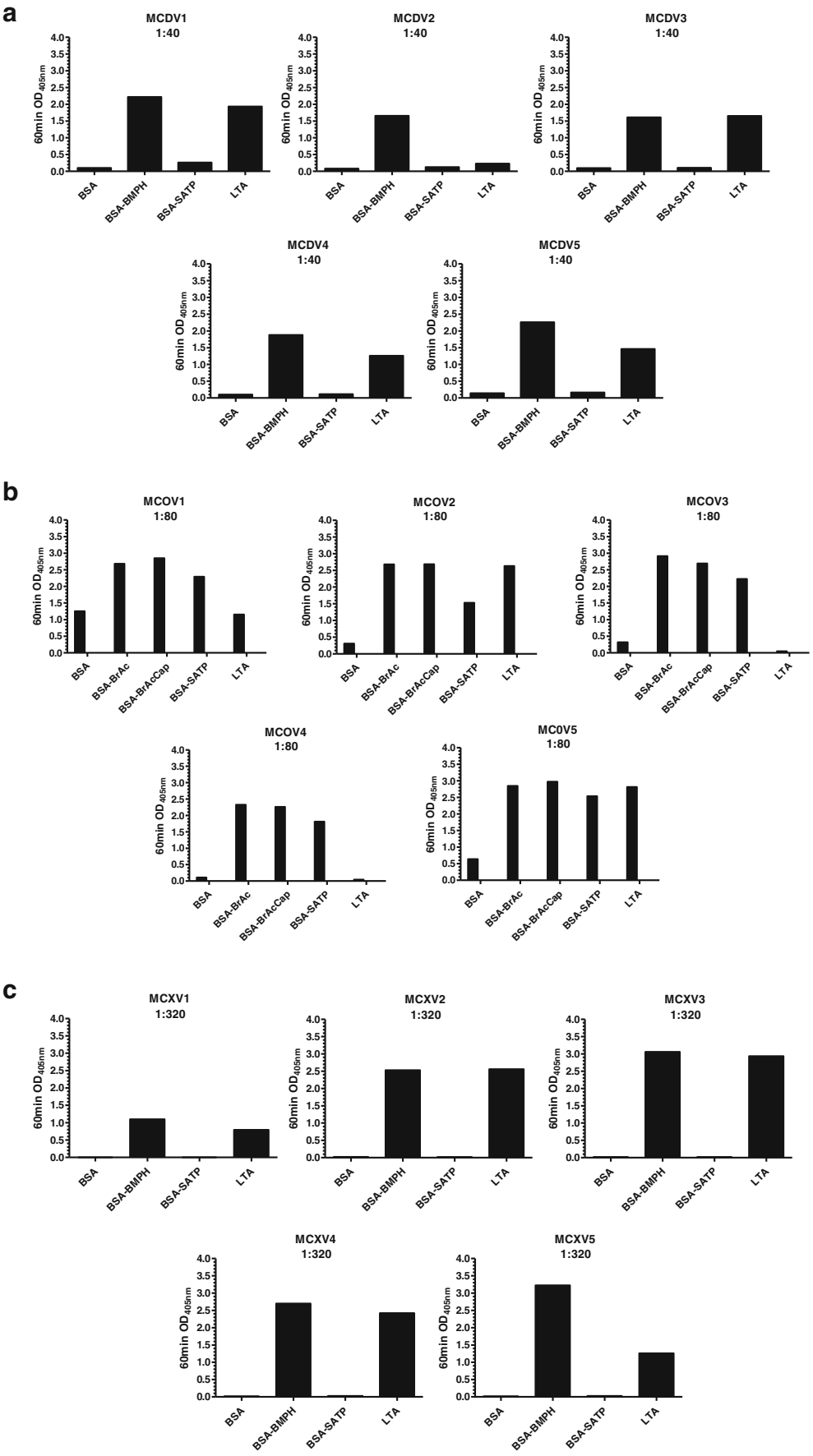
Immunogenicity of glycoconjugates

Animals were immunised, based on conjugated carbohydrate with a prime and two boosts strategy. The low numbers of animals used in this intial study precluded any statistical analyses but the following observations were made. Derived sera were initially titrated against the purified LTA antigen (Table 1), revealing that the BMPH linker strategy was superior to the bromo-acetyl linker strategy in terms of generating higher titers and the ExoA protein appeared to be a better carrier protein than HSA in this same regard. For the bromo-acetyl style conjugates we were able to compare the de-*O*-acylated and native LTA antigen, which suggested that the native antigen provoked a stronger immune response in rabbits but this was not corroborated in mice. The derived conjugate sera from each conjugation strategy were subsequently shown to be broadly cross reactive against all strains of *C. difficile* that we tested as shown for ExoA conjugate mice and rabbit sera (Tables 2 and 3, other serum Tables S1, S2, S3 and S4). We also examined whether the LTA conjugate derived sera could recognise other clostridial species in order to evaluate the specificity of the sera. Clearly not all clostridial species are recognised, but. *C. butyricum, C. subterminale* and *C. bifermentans* were recognised by the LTA specific sera (Table 2 and Tables S1, S3 and S4).

There was also a significant and somewhat immunodominant response to the linkers as revealed by testing mice derived sera against an irrelevant protein, bovine serum albumin (BSA) alone or BSA with the different linkers utilised in the conjugation strategies attached (Fig. 4). For both conjugates 1 & 3 that utilised a maleimide (BMPH) and thiol (SATP) linker strategy, it was clear that the maleimide linker (BMPH) was recognised by the immune sera, whereas the thiol linker (SATP) did not provoke an immune response (Fig. 4a, c). Interestingly, for conjugate 2 that employed the bromo-acetyl linker (BrAc) and thiol (SATP) linker strategy, the thiol linker (SATP) in this presentation was now immunogenic (Fig. 4b). Similar results were obtained

Table 3 ELISA determination of recognition of whole cells from *C. difficile* strains (as indicated) with post-immune mice sera (D56) following immunisations with the ExoA-BMPH-SH-de-O-LTA glycoconjugate. ELISA values after 60 min. at OD_{405nm} are detailed. Cells were killed with formalin, washed with water and resuspended at the same OD prior to plating; Dilutions are shown in parentheses

C. difficile Strain	Conjugate Mouse Sera (IgG 1:80)							
	MCXV1	MCXV2	MCXV3	MCXV4	MCXV5	MCXC6	MCXC7	MCXC8
Cd630	0.643	1.625	1.377	0.987	0.932	0.036	0.043	0.140
QCD	0.395	1.480	1.187	0.714	0.637	0.040	0.056	0.151
R20291	0.275	1.190	0.827	0.470	0.368	0.032	0.037	0.106
M68	0.182	1.033	0.622	0.345	0.292	0.022	0.027	0.077
M120	0.182	0.638	0.703	0.356	0.283	0.025	0.040	0.064
CM26	0.418	1.296	1.013	0.665	0.638	0.026	0.070	0.122


a
MCDV1
1:40
MCDV2
1:40
MCDV3
1:40
MCDV4
1:40
MCDV5
1:40
60min OD405nm
BSA
BSA-BMPH
BSA-SATP
LTA
b
MCOV1
1:80
MCOV2
1:80
MCOV3
1:80
MCOV4
1:80
MC0V5
1:80
BSA
BSA-BrAc
BSA-BrAcCap
BSA-SATP
LTA
c
MCXV1
1:320
MCXV2
1:320
MCXV3
1:320
MCXV4
1:320
MCXV5
1:320

◀ **Fig. 4** ELISA data for IgG titers of polyclonal sera raised in mice against conjugates **a** HSA-BMPH-SH-de-O-LTA conjugate, **b** HSA-BrAc-SH-de-O-LTA conjugate and **c** ExoA-BMPH-SH-de-O-LTA conjugate against the antigens as indicated. BSA-BrAcCap refers to the BrAc linker capped with cysteine. Titers utilised for each sera are as indicated

with the rabbit sera (data not shown). Clearly a response to LTA is observed in all presentations but it would be preferable to not provoke a competing linker response.

Immunofluorescence at the bacterial cell surface

In order to determine if antibodies in immune serum could access LTA epitopes on bacterial cell surface, immunofluorescence on live *C. difficile* vegetative cells was performed.

The accessibility to the cell surface and cross reactivity to live vegetative cells of *C. difficile* strains 630 and R20291 was demonstrated by the binding activity of the post immune serum in immunofluorescence experiments comparing pre- and post-(D70) immune serum from rabbits RCXV2 (Fig. 5a) and RCDV2 and RCLV2 (supplemental Fig. 1). No fluorescence was observed with any of the strains when pre-immune serum was used. This illustrates that the derived sera is specifically recognising a conserved accessible epitope on the surface of live *C. difficile* vegetative cells.

Immunofluorescence of *C. difficile* spores

In order to determine if antibodies in immune serum would react with *C. difficile* spore surface, immunofluorescence on *C. difficile* 630 and R20291 spores was performed.

RCXV2 and RCDV2 immune sera are shown to bind to spores (Fig. 5b and supplemental data respectively). No binding was observed when pre-immune serum was used.

Discussion

In our previous study we had identified a novel lipoteichoic acid like polymer on the surface of a range of *C. difficile* cells [22]. In this study we have demonstrated that the LTA antigen is immunogenic in the context of the bacterial cell and this molecule is visible and accessible on the cell surface, in a similar fashion to the previously characterized PS-II antigen [19]. We have developed a novel glycoconjugation strategy, in which we chose to exploit the free amino functionality present in approximately 33 % of the repeating units, to conjugate the carbohydrate polymer to the carrier protein. In this way we have prepared conjugates with either intact or *O*-deacylated LTA molecules linked to either HSA or ExoA. Following immunisation in both mice and rabbits we have established a consistent immune response to the LTA carbohydrate. Our

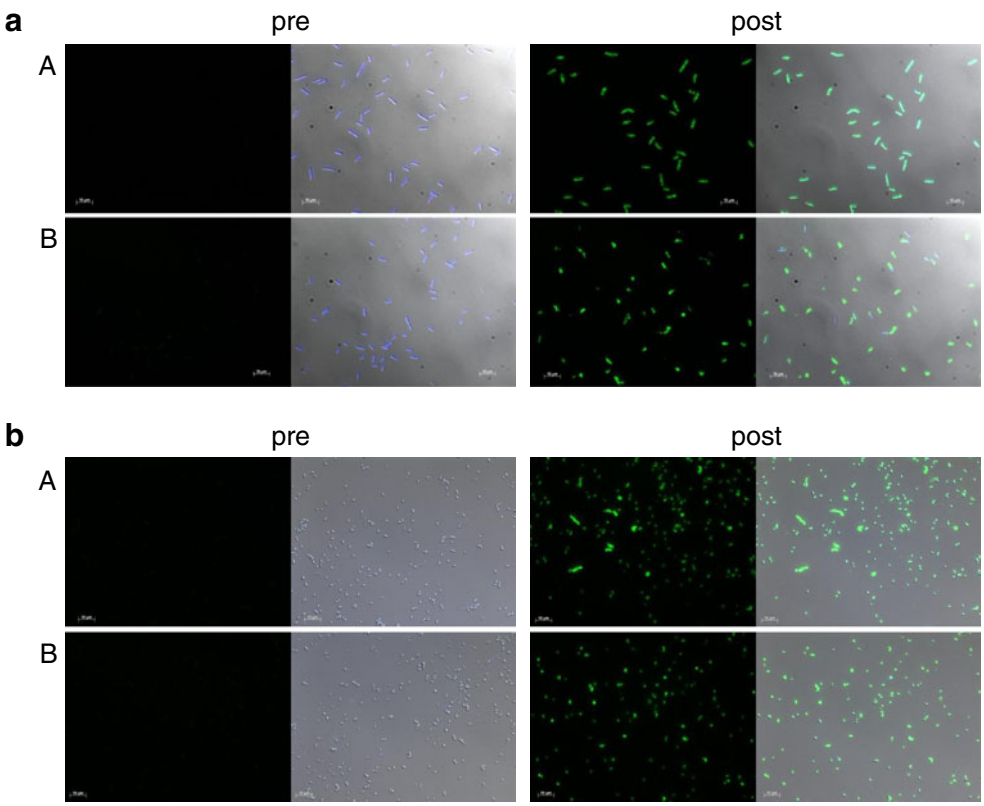


Fig. 5 Immunofluorescence of pre- and post-immune D70 rabbit RCXV2 sera *vs.* **a** vegetative cells and **b** spores of *C. difficile*; A, 630 and B, R20291. Anti-LTA binding was visualised with anti-rabbit IgG Alexafluor 488. The first column is the 488 channel only; the second column merges 488, DAPI stain visualising nucleic acids and transmitted light channels together. Representative cells are shown

results suggested that as expected the ExoA protein provoked a more immunogenic response to the LTA antigen than the HSA protein. In our future studies we will examine alternative protein carriers with a view to utilise proteins from *C. difficile*, with the aim of improving the immune response by increasing the density of antibody deposition on the bacterial cell surface and thus the potential to eradicate this bacterium with a vaccination strategy. We also observed that the maleimide based linker (BMPH) was better than the bromo-acetyl linker in terms of generating higher titers. However a significant immune response to the linkers was observed, which may be diluting the immune response to the target antigen. We had identified previously that the utilization of linkers to conjugate carbohydrates to the carrier protein resulted in an immune response to the linkers themselves, though we rationalized that this might not be the case here as we were using polymeric rather than oligomeric structures [27–29]. We did not attempt to cap unused maleimido functionalities as we were wary that we could prove the completeness of this capping procedure given the constraints of the MALDI spectra resolution. Furthermore, we had identified in our *Moraxella catarrhalis* study [28], that even when the maleimide functionality was not terminally exposed it was still immunogenic, thus casting further doubts as to the benefit of a capping reaction. Our subsequent studies will evaluate alternate conjugation strategies whereby the linker response is avoided.

In previous work by Adamo *et al.* it was observed that antibodies raised to the PSII conjugate did not uniformly label all bacterial cells in a population [19], whereas in this study antibodies to the LTA conjugate did label all vegetative cells in the population (see Fig. 5a). This suggests that the LTA antigen may be less susceptible to heterogeneous expression on the cell surface and as such be a preferential conjugate vaccine candidate. In addition LTA-conjugate serum reactivity towards *C. difficile* spores was also demonstrated providing preliminary evidence that either LTA or a component which cross reacts with LTA is present on the spore surface. We did observe some variability in spore reactivity with LTA antiserum presumably due to differences in stages of maturation of individual spores within the preparations.

The post-immune sera was also utilised to establish that the LTA polymer is highly conserved on the surface of a wide range of *C. difficile* species encompassing a variety of geographical locations and animal species, including strains causing serious hospital outbreaks. We also observed some cross-reactivity to other clostridial species including *C. butyricum, C. subterminale* and *C. bifermentans*, but not to *C. perfringens, C. sporogenes, C. barati* and *C. botulinum*, illustrating that although the LTA antigen is conserved amongst some clostridial species it is not common to all. Overall the conservation in structure amongst *C. difficile* strains as well as ubiquitous cell surface expression and accessibility makes the LTA polymer an attractive vaccine candidate.

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