Chapter 21

The Design of a *Clostridium difficile* Carbohydrate-Based Vaccine

Mario A. Monteiro

Abstract

Clostridium difficile vaccines composed of surface polysaccharides (PSs) have the potential to simultaneously control infection and colonization levels in humans. Hot water–phenol treatment of *C. difficile* biomass can extricate water-soluble PS-I and PS-II; and water- and phenol-soluble PS-III. *C. difficile* vaccines based on PS-II have attracted the most attention due its facile purification and ubiquitous expression by *C. difficile* ribotypes. Anti PS-II antibodies recognize both *C. difficile* vegetative cell and sporulating preparations and confer protection against *C. difficile* infection in a mouse model. The design of such an efficacious *C. difficile* PS-II conjugate vaccine is described here.

Key words *Clostridium difficile*, Vaccine, Polysaccharide, *C. difficile* PS-II, Conjugate, TEMPO-mediated conjugation

1 Introduction

Clostridium difficile surface polysaccharides (Table 1) are attractive vaccine candidates to control *C. difficile* disease and reduce colonization levels in the population. In previous reports [1, 2], we described the fine structures of two water-soluble *C. difficile* polysaccharides obtained from hot water–phenol extraction; polysaccharide I (PS-I), composed of a pentasaccharide phosphate repeat of rhamnose (Rha), glucose (Glc), and phosphate (P): [→4)-α-L-Rha*p*-(1-3)-β-D-Glc*p*-(1-4)-[α-L-Rha*p*-(1-3]-α-D-Glc*p*-(1-2)-α-D-Glc*p*-(1-P→]; and polysaccharide II (PS-II), made up of a hexasaccharide phosphate repeat with Glc, mannose (Man), *N*-acetyl-galactosamine (GalNAc), and P: [→6)-β-D-Glc*p*-(1-3)-β-D-Gal*p*NAc-(1-4)-α-D-Glc*p*-(1-4)-[β-D-Glc*p*-(1-3)]-β-D-Gal*p*NAc-(1-3)-α-D-Man*p*-(1-P→]. Water-soluble PS-I and PS-II attracted immediate attention as potential *C. difficile* vaccines and the corresponding oligosaccharide repeating blocks were promptly synthesized by several groups [3–7].

Sunil Thomas (ed.), *Vaccine Design: Methods and Protocols: Volume 1: Vaccines for Human Diseases*, Methods in Molecular Biology, vol. 1403, DOI 10.1007/978-1-4939-3387-7_21,

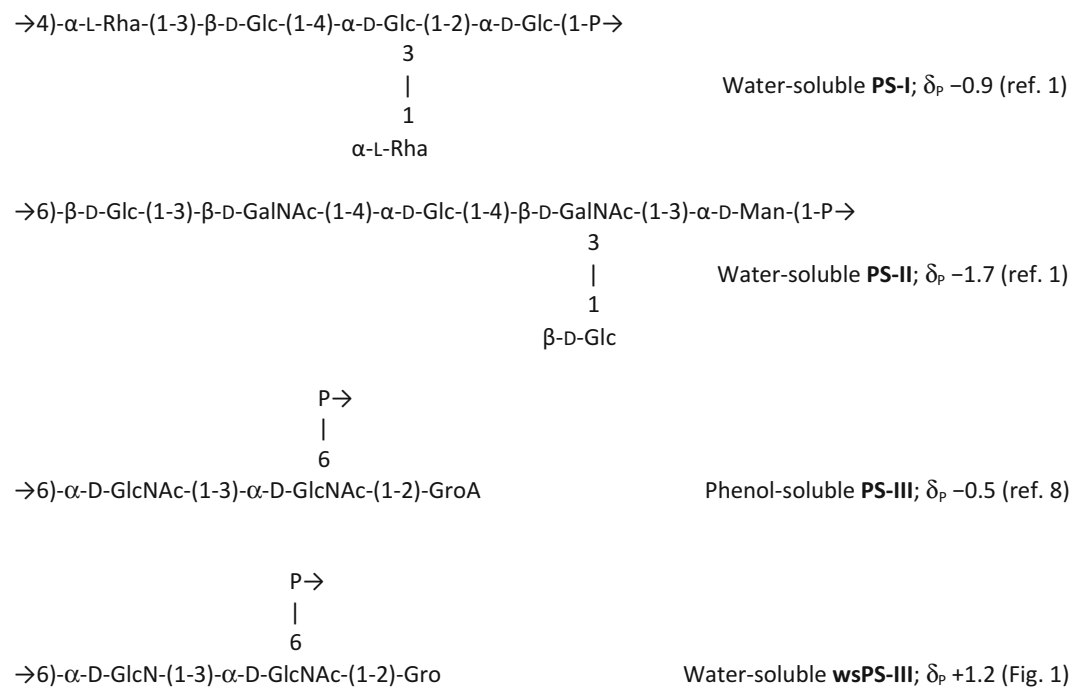

Table 1 The oligosaccharide phosphate repeating blocks of *C. difficile* polysaccharides and their characteristic ^{31}P NMR spectroscopy resonances

Along with the description of water-soluble PS-I and PS-II polysaccharides, early evidence was also obtained for another phosphorylated *C. difficile* glycan, a phenol-soluble polysaccharide, named PS-III, composed of P, Glc, *N*-acetyl-glucosamine (GlcNAc), and glycerol (Gro) [2]. This phenol-soluble PS has now also been fully analyzed and its repeating oligosaccharide synthesized [8–10]. The majority of the repeating units of the phenol-soluble PS-III were found to be composed of P, GlcNAc, and glyceric acid (GroA): →6)-α-GlcNAc-(1-3)-α-GlcNAc-[-6-P→]-(1-2)-GroA, with minor repeats of Glc and Gro moieties at the reducing-end regions. Akin to lipoteichoic-acids, fatty-acids capped the reducing-terminus of this phenol-soluble polysaccharide. A structural variant of the phenol-soluble PS-III has also been isolated from *C. difficile*, but in the aqueous phase, and with an alternate major repeating structure having glycerol (in place of glyceric acid) and 6-substituted glucosamine (in place of 6-substituted *N*-acetyl-glucosamine): →6)-α-D-GlcN-(1-3)-α-D-GlcNAc-[-6-P→]-(1-2)-Gro (submitted for publication). In the water-soluble PS-III, the combination of the negatively charged phosphate entity (PO_3^-) with the high content of positively charged glucosamine ($GlcNH_3^+$) affords this water-soluble PS-III a zwitterionic character, which may contribute in part for its solubilization in water.

A (1→4)-glucan has also been observed in *C. difficile* [11] and in two other Clostridia species, *C. butyricum* [12] and *C. botulinum* [13] and although in minor quantities, some sporulating preparations have been observed to contain Man units in the form of end-groups [α-Man-(1→], 2-linked linear units [→2)-α-Man-(1→] and 2,6-linked branch-points [→2,6)-α-Man-(1→] (Monteiro, unpublished results).

Fine structural validation of *C. difficile* polysaccharides is beyond the reach of non-carbohydrate vaccine scientists. Therefore, a rapid detection protocol for the characterization of *C. difficile* polysaccharides based on one-dimensional phosphorous (^{31}P) Nuclear Magnetic Resonance (NMR) spectroscopy is revealed here. This NMR-based evaluation of *C. difficile* PSs can be performed by any NMR center. Each *C. difficile* polysaccharide listed in Table 1 furnishes a distinctive ^{31}P NMR resonance, which can be used after size-exclusion chromatography for polysaccharide scrutiny. *C. difficile* water-soluble PS-I yields a ^{31}P NMR resonance at δ_P −0.9 [1], water-soluble PS-II at δ_P −1.7 [1], phenol-soluble PS-III at δ_P −0.5 [8], and water-soluble PS-III (called wsPS-III) at δ_P +1.2 (submitted for publication). Shown below in the Methods section, is an easy to follow collection of ^{31}P NMR spectra identifying water-soluble PS-II and water-soluble PS-III materials obtained from size-exclusion chromatography.

Several studies have shown that *C. difficile* polysaccharides and their synthetic oligosaccharide repeating blocks are antigenic and immunogenic [2, 4, 5, 7, 9, 11, 14]. However, in terms of an attractive vaccine candidate, only the native water-soluble PS-II absorbed instant interest, mainly due to the fact that PS-II is a conserved antigen ubiquitously expressed by *C. difficile* ribotypes [1, 2, 8, 15]. Other advantages of PS-II include its constant good yield and solubility in water, which facilitates purification protocols, and its low endotoxicity level of 3.77 EU/μg, well below that required for cGMP production (100 EU/μg). On the other hand, water-soluble PS-I and phenol-soluble PS-III carry some limitations; PS-I is not regularly produced by *C. difficile* and thus only the synthetic route can be reliably used in vaccine development; and phenol-soluble PS-III requires many manipulations to attain a cGMP acceptable product, because of the need for extensive phenol removal and high endotoxicity levels due to its fatty-acid content. The water-soluble PS-III overcame the purification obstacles of phenol-soluble PS-III, but no significant immunogenic studies have yet been carried out with this PS. For these reasons, the most advanced *C. difficile* carbohydrate-based vaccine data has been afforded by PS-II, and these pages describe such progress.

The most encouraging set of results have been yielded by a recent preclinical protection study, which showed that a parenteral vaccine, composed of PS-II adjoined to the immunostimulatory protein keyhole limpet hemocyanin (KLH), protected 90 % of

mice when challenged with an LD_{50} dose of *C. difficile* spores [15]. In this piece, the detailed instructions are given for: (1) hot water–phenol extraction of PS-II; (2) purification of PS-II by size-exclusion chromatography; (3) quick characterization of PS-II by ^{31}P NMR; (4) selective and stoichiometric activation of primary hydroxyls of PS-II by (2,2,6,6-Tetramethylpiperidin-1-yl)oxy (TEMPO); and (5) carbodiimide-dependent conjugation of PS-II to a protein carrier. The design of a *C. difficile* challenge study in an established mouse model to test the aforementioned PS-II conjugate vaccine is also explained.

2 Materials

2.1 Extraction of PS-II from C. difficile Biomass

1. High vacuum lyophilizer (220 V).
2. 99 % liquefied phenol (if crystallized, heat to liquefied form in microwave).
3. Dialyzed water.
4. Heating plate with magnetic stirring capability.
5. Water bath (or heating blanket).
6. Thermometer.
7. Round-bottom flask with stopper.
8. Magnetic stirrer.
9. Dialysis bag 1000 MWCO.

2.2 Purification and Rapid Characterization of C. difficile PS-II

1. Sephadex G-50.
2. Glass column (1 m × 1 cm).
3. Fraction collector.
4. Lyophilizer.
5. Deuterium oxide (D_2O).
6. NMR instrument with a ^{31}P NMR probe.
7. NMR spectroscopy tubes.
8. Ortho-phosphoric acid.

2.3 Stoichiometric Activation of C. difficile PS-II by TEMPO-Mediated Oxidation

1. Ingredients for oxidation of PS-II: TEMPO (0.6 mg), 1 M NaOAc, NaBr (9.0 mg), NaClO (4 %, 0.375 mL), deionized water (15 mL).
2. 4 dram vial.
3. *C. difficile* PS-II (60.0 mg).
4. Stir bar.
5. Ice bath.
6. Ethanol (0.5 mL).
7. 1000 MWCO dialysis bag.

2.4 Conjugation of the Partially Oxidized *C. difficile* PS-II to an Immunostimulatory Protein Carrier

1. Components for conjugation: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (200 μL), 10 ml of 0.5 M of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5); 5.0 M HCl (220 μL).
2. TEMPO oxidized PS-II (30.0 mg).
3. Protein carrier (15.0 mg).
4. Reaction vial.
5. Stir plate and stirrer.
6. 25,000 MWCO dialysis bag.

2.5 Evaluation of *C. difficile* PS-II Conjugate in a *C. difficile* Infection Mouse Model

1. C57BL6 mice.
2. *C. difficile* PS-II conjugate vaccine.
3. Adjuvant (Alum or KLH).
4. Antibiotic cocktail (Vancomycin and Clindamycin).
5. *C. difficile* spores.

3 Methods

3.1 Extraction of PS-II from *C. difficile* Biomass

1. In a 1 L round-bottom flask, *C. difficile* wet cell paste (2.5 g) is dissolved in 250 ml of deionized water and stirred at 70 °C for 30 min (a water bath or heating blanket may be used).
2. Following this, 200 ml of 99 % liquefied phenol is added and the hot water–phenol solution is stirred for 3 h. If water bath is used, use thermometer to control temperature.
3. Then, the round-bottom flask with the hot preparation is placed in an ice-bath and allowed to stay overnight.
4. On the following morning, the top distinguishable aqueous layer is collected and dialyzed against water for 2 days in a dialysis bag (1000 MWCO) mainly for phenol removal.
5. The dialyzed solution is then lyophilized and purified by size-exclusion chromatography (Subheading 3.2).

The water layer of all *C. difficile* ribotypes will afford PS-II and wsPS-III, but seldom will PS-I be detected (*see* **Note 1**). Extended dialysis of the phenol layer will yield phenol-soluble PS-III and protein- and ribonucleotide-rich material, and thus extended treatment with proteinase and ribonuclease is necessary obtain a purified phenol-soluble PS-III preparation.

3.2 Purification and Rapid Characterization of *C. difficile* PS-II

1. The lyophilized water layer (50 mg from 2.5 g wet paste) is dissolved in 5 ml of distilled water and placed at the top of a Sephadex G-50 column (3 cm × 1 m) with water as eluent.
2. A fraction collector, without a physical pump, accumulates 1 ml fractions.

3. The first ten tubes (fractions) consist of the void volume with the PS material eluting after fraction 10.
4. The fractions containing material may be detected by a UV (210 nm) or refractive index detector, or simply by freeze-drying the independent fractions.
5. The fractions containing PS-II can be easily identified by ^{31}P NMR spectroscopy (Fig. 1) due to the fact that PS-II and wsPS-III exhibit distinct ^{31}P NMR resonances, PS-II at δ_P −1.7 and wsPS-III at δ_P +1.2. Ortho-phosphoric acid is used as the external reference (δ_P 0.0). Figure 1 shows the typical elution profile of PS-II, which is usually accompanied by wsPS-III.
6. The material is dissolved once in 99 % D_2O and lyophilized and then it is dissolved again in 0.5 ml 99 % and placed in a 5 mm NMR tube.
7. At least 256 scans per each NMR experiment should be acquired. Here (Fig. 1), the first two fractions contained PS-II, but also small amounts of wsPS-III. Fractions 13–17 afforded pure PS-II, and pure wsPS-III was collected during the later fractions, fraction 22 and 23. Physicochemical analyses have shown that the average length of PS-II is seven repeating blocks. Fractions containing pure PS-II may now be pooled to yield on average 25–30 mg of bulk PS-II.

3.3 Stoichiometric Activation of *C. difficile* PS-II by TEMPO-Mediated Oxidation

1. TEMPO (0.6 mg) and NaBr (9.0 mg) are dissolved in deionized water (15 mL) by vigorous vortexing in a 4 dram vial. Then, NaOAc (1.23 g) is added (*see* **Note 2**).
2. PS-II (30.0 mg) and a stir bar are added to the buffer solution described above.
3. The reaction vial is kept in an ice bath and stirred for 10 min.
4. NaClO (4 %, 0.375 mL) is added drop wise at 0 °C.
5. The solution was kept at 0 °C for 24 h (stirred for 8 h and then kept in a fridge in an ice bath for 16 h).
6. For work up, ethanol (0.5 mL) is added and the reaction mixture dialyzed in a 1000 MWCO dialysis bag against water overnight.
7. The retentate is lyophilized to yield the partially (10 %) oxidized PS-II.

Monosaccharide analysis of the partially TEMPO-oxidized PS-II has revealed that the 3-substituted GalNAc is the unit that more prominently undergoes oxidation at its C-6 position (Fig. 2).

3.4 Conjugation of the Partially Oxidized *C. difficile* PS-II to an Immunostimulatory Protein Carrier

1. EDC (200 μL) is added to 10 mL of 0.5 M MES buffer (pH 5.5) and then neutralized with 5.0 M HCl (220 μL).
2. TEMPO oxidized PS-II (20.0 mg) is added and the mixture stirred for 15 min at room temperature.

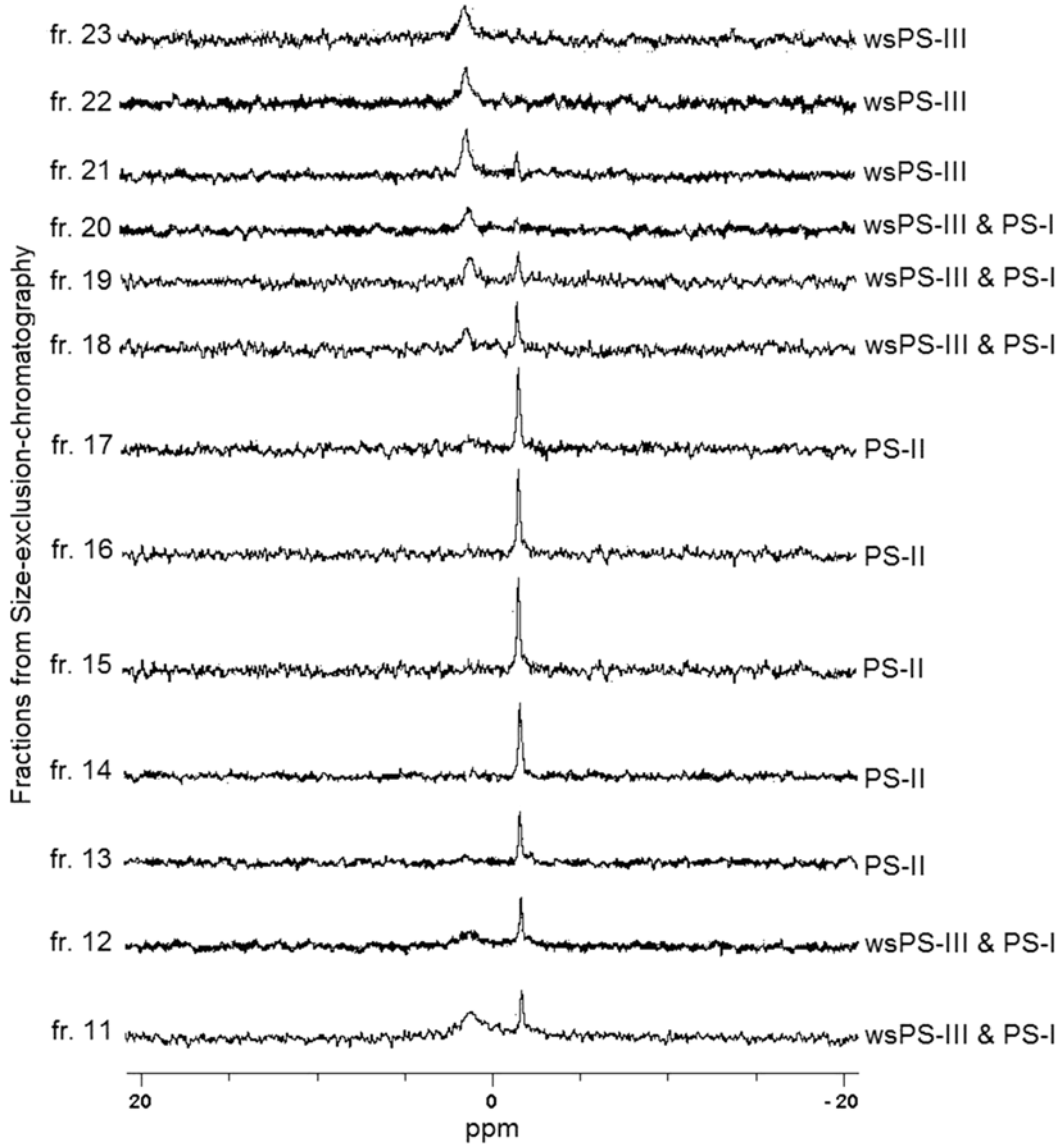

Fig. 1 ^{31}P NMR spectra of the fractions obtained by fractionation of the water layer through a Sephadex G-50 column. ^{31}P NMR can be used to identify the location of ***C. difficile*** PS-II through the detection of its distinctive resonance at –1.7 ppm

3. Following this, a carrier protein such as BSA, LTB, CRM_{197}, toxin [11] or KLH (10.0 mg) is transferred into the reaction vial with 10 mL of 0.5 M MES buffer (pH 5.5).
4. The reaction is stirred at room temperature for 1 day and then stirred at 37 °C for 2 days.
5. The reaction mixture is subsequently dialyzed in a 25,000 MWCO bag against water for 3 days (*see* **Note 3**).

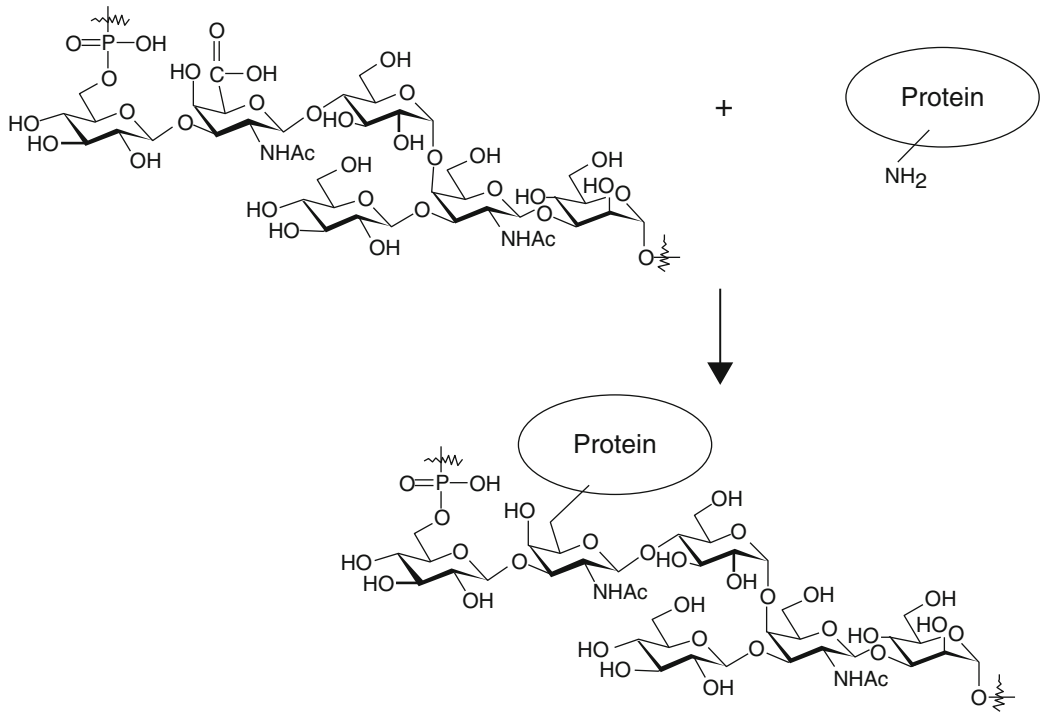

Fig. 2 The primary position of the three-substituted GalNAc is the site of preferential oxidation when ***C. difficile*** PS-II is subjected to stoichiometric TEMPO-mediated oxidation

6. The retentate is lyophilized to yield the PS-II conjugate.

Since the primary position of the 3-substituted GalNAc is the PS-II site most readily oxidized under the aforementioned conditions, the majority of protein ligation occurs at this point (Fig. 2).

3.5 Guidance for the Analysis of PS-II Conjugates

The resulting PS-II conjugates may be analyzed by matrix assisted laser desorption/ionization (MALDI) mass-spectrometry or by standard gel electrophoresis. However, due to the unreliable behavior of large molecular weight conjugates in MALDI mass-spectrometry, the widely used gel electrophoresis (Coomassie stain procedure that detect proteins) and Western blot analysis have became our methods of choice for rapid confirmation of conjugation between activated PS-II and protein carrier.

Gel electrophoresis [16] confirms that conjugation has occurred through the observation of a shift towards a higher molecular weight (Fig. 3a, b), and the reactivity of the PS-II conjugate with PS-II antisera in a Western Blot [17] confirms that the PS-II structure in the conjugate format retains and exposes the important immunogenic epitopes (Fig. 3c). The Western blot analysis of the PS-II conjugate must be performed with antisera raised previously by native PS-II. Also, if accessible, immunofluorescence [5] studies can also confirm that the antibodies raised by the PS-II conjugate recognize the native PS-II on the surface of *C. difficile*.

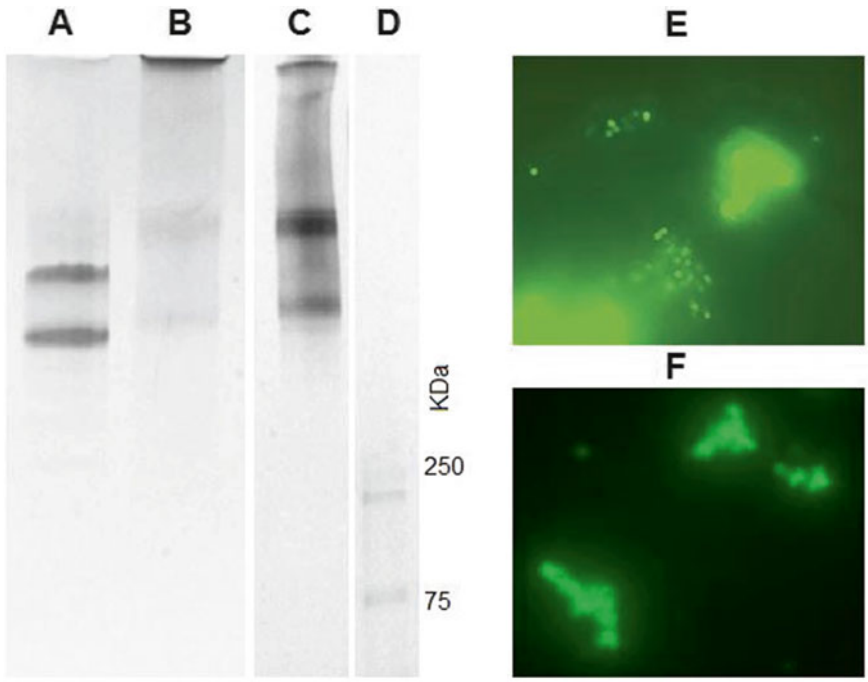

Fig. 3 (**a**) Gel electrophoresis (Coomassie stain) of KLH and (**b**) PS-II/KLH conjugate. The shift towards high molecular weight observed in (**b**) attests to the fact that conjugation between PS-II and KLH took place; (**c**) A Western Blot showing the recognition of PS-II in a conjugate format (PS-II/KLH) with PS-II antisera; (**d**) Standard molecular weigh marker; and immunofluorescence images illustrating that ***C. difficile*** vegetative cells (**e**) and sporulating preparations (**f**) are recognized by PS-II antibodies (performed by Dr. Martin Sagermann). These data were presented at the 7th Vaccine and ISV Annual Global Congress and at the International Conference on the Molecular Biology and Pathogenesis of the Clostridia [15]

The aforementioned procedures have been previously described in detail elsewhere [5, 16, 17], but as an illustration, Fig. 3 displays examples of these verifications of PS-II conjugates: (A) and (B) are gel electrophoreses of the carrier protein, KLH, and (B) is a gel electrophoresis of the PS-II/KLH conjugate; (C) is a Western Blot showing that anti PS-II antibodies (raised in rabbits against native PS-II beforehand) can detect PS-II in a PS-II/KLH conjugate produced by TEMPO-mediated activation; and (E) and (F) are immunofluorescence analyses showing that antibodies raised by a PS-II conjugate recognize the PS-II on *C. difficile* surface.

3.6 Guidance for the Evaluation of a C. difficile PS-II Conjugate in a C. difficile Infection Mouse Model

Animal vaccine models of *C. difficile* infection are still limited in scope and in the number of institutions that can perform such trials. In most cases, academic scientists have to work in partnership with industrial contract research organization to have their vaccines evaluated. For such purposes, a mouse model now exists, the C57BL6 *C. difficile* model [18], which can be used as a primary

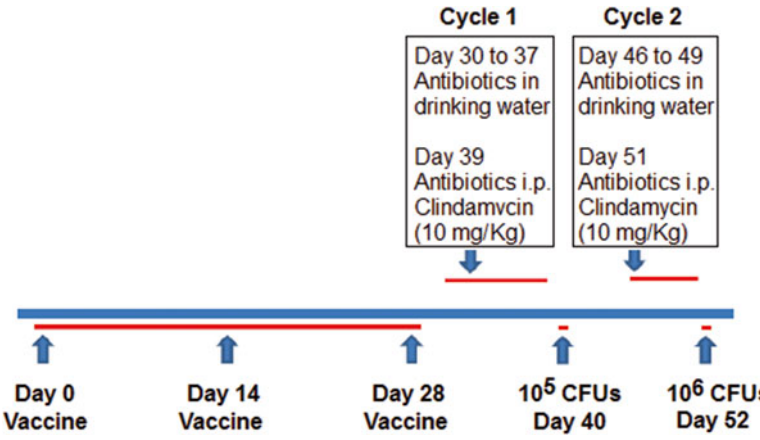

Fig. 4 Schematic representation of a C. difficile infection mouse model (C57BL6) challenge study that may be used to evaluate PS-II conjugate vaccines

test for a *C. difficile* vaccine candidate. Here, the approach taken to evaluate the efficacy of the *C. difficile* PS-II/KLH conjugate vaccine is described (Fig. 4) [15].

C57BL6 mice are vaccinated with three intramuscular and subcutaneous injections of 100 μg PS-II conjugate (in this case a PS-II/KLH vaccine produced via TEMPO-mediated activation) (*see* **Note 4**) admixed with an adjuvant (100 μg of KLH here) on Day 1, 14, and 28. Sera samples may be collected on Day 35 to analyze titers of anti PS-II IgG. Following vaccination, the mice were rendered susceptible to *C. difficile* with multiple antibiotics and were then challenged with an LD_{50} dose of *C. difficile* spores orally at Day 40 (Fig. 4). The animals that survive the *C. difficile* spore challenge are allowed to rest for 7 days. Then, the mice are exposed to another 3 day course of antibiotic pretreatment and challenged with a 10× LD_{50} dose of *C. difficile* spores to mimic a recurrent *C. difficile* infection in humans. In this case [15] 90 % (38 of 42) of the vaccinated mice survived the first *C. difficile* challenge (40 % of the unvaccinated mice survived), and all vaccinated animals survived the second challenge.

4 Notes

1. The water layer has been found not to contain oligonucleotide- or protein-related contents (these are found in the phenol layer). However, it is advisable that the fresh experimentalist have the aqueous content analyzed for RNA and protein contamination.
2. TEMPO does not dissolve well in 1 M NaOAc buffer; therefore do not reverse the two aforementioned steps [19].

3. Depending on the carrier protein being used, conjugation reactions between activated PS-II and protein carrier may yield very high molecular weight lattice-type conjugates. In these cases, one may observe particulates in the solution after dialysis. Even though these preparations may have low solubility, they may be also be used in vaccine studies.
4. Prior to challenge studies in animal models, the PS-II conjugate vaccine to be employed must be analyzed by Western Blot to confirm that it is recognized by anti PS-II antibodies (raised by previously performed immunogenicity studies).

Acknowledgements

This work was supported in part by a Discovery grant from the Natural Sciences and Engineering Research Council (Canada) to M.A.M. The KLH conjugation immunogenic analysis and mouse model studies were funded and carried out by Stellar Biotechnologies, Inc. (Herbert Chow, Martin Sagermann).

References

1. Ganeshapillai J, Vinogradov E, Rousseau J, Weese JS, Monteiro MA (2008) *Clostridium difficile* cell-surface polysaccharides composed of pentaglycosyl and hexaglycosyl phosphate repeating units. Carbohydr Res 343:703–710
2. Monteiro MA, Ganeshapillai J (2013) Polysaccharide immunogens from *Clostridium difficile*. US Patent 8597663 B2, 3 Dec 2013
3. Danieli E et al (2011) First synthesis of *C. difficile* PS-II cell wall polysaccharide repeating unit. Org Lett 13:378–381
4. Oberli MA et al (2011) A possible oligosaccharide-conjugate vaccine candidate for *Clostridium difficile* is antigenic and immunogenic. Chem Biol 18:580–588
5. Adamo R et al (2012) Phosphorylation of the synthetic hexasaccharide repeating unit is essential for the induction of antibodies to *Clostridium difficile* PSII cell wall polysaccharide. ACS Chem Biol 7:1420–1428
6. Martin CE, Weishaupt MW, Seeberger PH (2011) Progress toward developing a carbohydrate-conjugate vaccine against *Clostridium difficile* ribotype 027: synthesis of the cell-surface polysaccharide PS-I repeating unit. Chem Commun (Camb) 47:10260–10262
7. Jiao Y et al (2013) *Clostridium difficile* PSI polysaccharide: synthesis of pentasaccharide repeating block, conjugation to exotoxin B subunit, and detection of natural anti-PSI IgG antibodies in horse serum. Carbohydr Res 378:15–25
8. Reid CW et al (2012) Structural characterization of surface glycans from *Clostridium difficile*. Carbohydr Res 354:65–73
9. Martin CE et al (2013) Glycan arrays containing synthetic *Clostridium difficile* lipoteichoic acid oligomers as tools toward a carbohydrate vaccine. Chem Commun (Camb) 49: 7159–7161
10. Wouter FJ et al (2014) Total synthesis of five lipoteichoic acids of *Clostridium difficile*. Chemistry 20:13511–13516
11. Bertolo L et al (2012) *Clostridium difficile* carbohydrates: glucan in spores, PSII common antigen in cells, immunogenicity of PSII in swine and synthesis of a dual *C. difficile*-ETEC conjugate vaccine. Carbohydr Res 354:79–86
12. Hobson PN, Nasr H (1951) An amylopectin-type polysaccharide synthesised from sucrose by *Cl. butyricum*. J Chem Soc 1855–1857
13. Whyte JNC, Strasdine GA (1972) An intracellular alpha-D-glucan from *Clostridium botulinum*, type E. Carbohydr Res 25:435–441
14. Monteiro MA et al (2013) Carbohydrate-based *Clostridium difficile* vaccines. Expert Rev Vaccines 12:421–431

15. Chow H, Sagermann M, Ma Z, Vandentam G, Monteiro MA (2013) Vaccination with *Clostridium difficile* PSII polysaccharide antigen adjuvanted with KLH induced broad-based enhancement of adaptive immune responses and protection in mice. Paper presented at the 7th Vaccine and ISV Congress Sitges, Spain, 27–29 Oct 2013; and Pequegnat B, Sagermann M, Arroyo L, Chow H, Monteiro MA (2013) An anti-*C. difficile* PSII polysaccharide-KLH conjugate vaccine is efficacious in mice. Paper presented at the international conference on the molecular biology and pathogenesis of the Clostridia (ClostPath 8), Queensland, Australia, 22–26 Oct 2013
16. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
17. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76:4350–4354
18. Chen X, Katchar K, Goldsmith JD et al (2008) A mouse model of *Clostridium difficile*-associated disease. Gastroenterology 135:1984–1992
19. Ma Z, Bertolo L, Arar A, Monteiro MA (2011) TEMPO-mediated glycoconjugation: a scheme for the controlled synthesis of polysaccharide conjugates. Carbohydr Res 346:343–347