Chapter 11

Campylobacter jejuni Capsule Polysaccharide Conjugate Vaccine

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Campylobacter jejuni is one of the most common causes of human diarrheal disease worldwide. Campylobacteriosis cases are primarily sporadic, but outbreaks are not uncommon. In developed countries Campylobacteriosis cases are about 50/100,000 per year, but in developing countries, particularly in Southeast Asia, the incidence is estimated to be at least 10 times higher. In these endemic regions, this high incidence represents a life threatening risk, especially towards the pediatric population. *C. jejuni* is considered a zoonotic disease in which the major source of contamination is through the consumption of poultry. In humans, the infectious dose can be as low as 500-1,000 bacteria with symptoms being variable and dependent on the bacterial strain and host factors. Some patients only present mild abdominal pain and mild to no diarrhea, while the most severe cases are associated with severe abdominal cramping, accompanied with fever, headaches, myalgia, and large volumes of mucous and bloody diarrhea that can last for several days. If left untreated, severe *C. jejuni* cases can be lethal. *C. jejuni* infections have also been associated with the development of Guillain-Barré syndrome (an autoimmune disease triggered by *C. jejuni* lipooligosaccharide sub-structures that mimic glanglioside forms) inflammatory bowel syndrome,

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reactive arthritis and stunting in children from developing countries. In this piece, we describe the development of a *C. jejuni* vaccine candidate in which the protective antigens are the cell-surface capsular polysaccharides (CPSs), with focus on (i) CPS discovery highlighting the two key structural features of *C. jejuni* CPSs, 6-deoxy-heptoses of unsual configurations and variably linked *O*-methyl-phosphoramidate moities; (ii) conjugation of *C. jejuni* CPSs to carrier proteins using new methodology for CPS activation (TEMPO-mediated oxidation); and (iii) protection studies in a non-human primate model. Collectively, the data obtained has allowed us to advance our prototype *C. jejuni* CPS conjugate vaccine through cGMP production and onto an ongoing phase I human clinical trial.

Campylobacter jejuni Overview

Campylobacter is one of the most common causes of human diarrheal disease worldwide. However, popular awareness has remained limited compared to other enteric pathogens like *Salmonella* spp., *Vibrio cholerae*, or *Shigella* spp. This may be due to its relative recent discovery: it was not until the early 1960's that the genus *Campylobacter* was identified and in the mid 1970's that its isolation methods and growing media were established (*1*, *2*). Since then, this genus has been given increasing attention and data has shown that *C. jejuni* is the most common *Campylobacter* sp. to be associated with human diarrheal diseases (*3*).

In developed countries Campylobacteriosis cases range from 14 to about 57 cases/100,000/year in US and Europe, respectively (*4*, *5*). Campylobacteriosis cases are primarly sporadic but outbreaks are not uncommon. In developing countries, in particular in Southeast Asia where *C. jejuni* is endemic, the incidence is estimated to be at least 10 times higher (*6*, *7*). This high incidence represents a concerning life threatening risk, especially towards the pediatric population in these endemic regions.

C. jeuni is considered a zoonotic disease. The major source of contamination is through the consumption of poultry, but other sources like unpasteurized milk or water have been referenced (*8*). *C. jejuni* is considered a chicken commensal. The bacteria asymptomatically colonize the mucus of the intestinal epithelium of the intestine and preferentially the ceca (*9*). The contamination of the chicken meat occurs during the slaughtering process (*10*).

The infectious dose can be as low as 500-1,000 bacteria. Following ingestion, 24h to 96h incubation are necessary before the appearance of Campylobacteriosis symptoms (*11*). Campylobacteriosis symptoms are quite variable and depend on the bacterial strain and host factors. Some patients will present mild abdominal pain and mild to no diarrhea, whilst the most severe cases are associated with severe abdominal cramping, accompanied with fever, headaches, myalgia, and large volumes of mucous and bloody diarrhea that can last for several days (*12*). Most cases are self-limiting but severe cases require hospitalization and are treated

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with re-hydratation fluids and antibiotics (*13*). If left untreated, severe cases can be lethal.

C. jejuni infections have also been linked autoimmune pathologies, such as the development of Guillain-Barré syndrome (GBS) and Miller-Fisher syndrome, an autoimmune disease triggered by specific lipooligosaccharide (LOS) structures present at the surface of the bacteria (*14*). It is believed that one out of a thousand patients, one will develop GBS (*14*). More recently, *C. jejuni* has been associated with inflammatory bowel syndrome (IBS) (*15*), reactive arthritis (*16*), a rare form of mucosa-associated lymphoid tissue (MALT) lymphoma called immunoproliferative small intestinal disease (IPSID) (*17*), and stunting in childs from developing countries (*18*).

Campylobacter jejuni Polysaccharide Capsule Discovery

The first evidence pointing towards the presence of polysaccharides (PS) akin to capsular polysaccharides (capsule, CPS) in *Campylobacter* was obtained in the early 1990s, when structural analysis revealed surface PSs in *C. lari* that were not attached to lipid A or LOS regions (*19*). Following this, the first publically available *C. jejuni* sequenced genome (clinical isolate NCTC 11168) was published in 2000 and also suggested the production of CPS by *Campylobacter* (*20*). However, the *C. jejuni* genome sequence did not clearly identify genetic determinants of pathogenicity. No common or known virulence factors were identified in the *C. jejuni* genome and, compared to other enteropathogens, no obvious acquired pathogenic genetic features (ex-pathogenicity islands) were found. Nevertheless, other whole genome sequencing revealed gene sequences presenting similarities with genes (*kps* family) involved in biosynthesis and transport of CPS to the cell surface. Bacterial CPS is considered a virulence factor due to its role in survival and evasion of the host immune system. The physical location and composition of CPS also prevent host recognition/access of antibody toward the bacterial cell surface proteins (*21*).

One PS moiety was later demonstrated to be attached to a phospholipid anchor (*22*, *23*). The genes involved in PS transport and attachment to the phospholipid anchor are genetically conserved between the different capsule types, while the locus responsible for the biosynthesis of the capsule (nestled between the *kpsC* and *kpsF* genes) is variable (*24–26*).

The high molecular weight CPS is the main serodeterminant of the Penner serotyping scheme. This serological typing method has been recognized as the gold standard *C. jejuni* isolate typing methodology for over 3 decades. This method requires antiserum generated by boiling a *C. jejuni* bacterial culture in water. Following centrifugation, the soluble, heat-stable (HS) fraction is used to vaccinate rabbits for serum production and establishing an HS antiserum reference bank. The HS antisera are used to type strains by monitoring passive agglutination on a glass microscope slide (*27*, *28*). The method recognizes a total of 47 *C. jejuni* serotypes. Due to the limitation of serum bank maintenance, the Penner serotyping method has been gradually replaced by more adapted typing methods such as multiplex PCR methods (*26*, *29*).

As suggested by the number of Penner serotypes identified, C. *jejuni* sp. present several capsule types. Based on sequenced capsule biosynthesis loci comparison and cross-reactions of Penner sera, the total of 47 capsule serotypes can be collapsed in 35 groups (*26*). The capsule loci compositions reflect and thus support this phenotypic complexity, whereknown C. *jejuni* CPS biosynthesis loci genes vary from 11 to more than 30 genes (*26*). Not surprisingly, this CPS serological and genetic diversity results in CPS structural diversity.

Structural Features of *Campylobacter jejuni* Capsules

C. jejuni produces structurally variable structures, reflecting the serological and genetic differences between these different CPS groups described in the previous section. These structural differences are further widened due to the presence of phase variation. Phase variation is a process where genes are turned on and off at high frequency, and this process occurs in *C. jejuni* due to the presence of homopolymeric tracts of 8-10 cytosine nucleotide residues in specific genes that cause slipped-strand mispairing (*30*). Slipped-strand mispairing is a high frequency, reversible, erroneous addition or subtraction of C residues during DNA replication at these poly(C) tracts, resulting in a frameshift mutation and ultimately conversion from a functional to non-functional protein product and vice-versa (*31*, *32*). Phase variation results in CPS biosynthetic enzymes being turned on and off, which ultimately affects expression of CPS structural modifications and even cell surface CPS expression itself (*15*, *33*). Phase variation allows structurally related CPS structures to be expressed by strains within a given serotype complex. For this reason, phase variation mechanisms most likely evolved to provide a mechanism of host immune system and predatory phage escape.

Even though the genes involved in *C. jejuni* CPS biosynthesis are variable in numbers and functions, some genetic and structural similarities are still observed between different CPS serotypes. A unique feature of many C. *jejuni* CPS serotypes is modification with O-methyl-phosphoramidate (MeOPN). The *cjj1415-1418* operon represents the core MeOPN biosynthesis genes, which is present in and highly conserved between *C. jejuni* serotypes expressing MeOPN (*34*). The functions of these four gene products are currently unclear, as is the mechanism of MeOPN biosynthesis. However, a recent study showed that *cjj1418* encodes an L-glutamine kinase enzyme that attaches a phosphate group from ATP onto the side chain amido nitrogen group, forming the P-N bond (*35*), although the enzymatic mechanism has not been experimentally defined. In contrast to the MeOPN biosynthesis genes, the MeOPN transferase genes are not as well conserved between strains, which would be expected given that the MeOPN group is attached to different monosaccharide hydroxyl groups or monosaccharide residues. Phosphoramidate (i.e.-MeOPN without O-methylation) is also sometimes detected on *C. jejuni* CPS (*34*, *36*), but at this point it is unclear whether phosphoramidate is a true CPS modification, an intermediate product in MeOPN biosynthesis, or an MeOPN extraction artifact. Indeed, the MeOPN linkage itself is highly acid- and base-labile and hence will hydrolyze

during methods of CPS extraction or monosaccharide analysis methods (*37*). An understanding of the MeOPN biosynthetic pathway may help verify whether phosphoramidate is of biological significance.

The MeOPN modification is not known to be naturally produced by any other living organisms. Therefore, the MeOPN modification is not only unique from a natural standpoint but also from the immune system's perspective, so a *C. jejuni* CPS glycoconjugate vaccine should include the MeOPN modifications to ensure that this rare and unique modification is available for recognition and memory by the immune system.

Other CPS modifications occur in specific CPS serotypes including methylation, acetylation, and dehydration. An interesting feature of all these modifications, including the MeOPN modification, is that they tend to occur non-stoichiometrically. One potential reason for this is phase variation of the genes involved in these modifications, which would cause the fraction of phase-on cells within a *C. jejuni* population to only express the CPS modification while the phase-off cells do not. However, other factors such as slow enzyme kinetics, low substrate concentrations, or possibly even artefactual loss of these modifications during CPS extraction may play a role.

Besides the unique MeOPN modification, the presence of heptose residues in unusual configuration is relatively unique to C. *jejuni* CPS. All strains of *C. jejuni* that contain heptoses in their CPS share four highly conserved enzymes that convert D-sedoheptulose-7-P to GDP-D-*glycero*-D-mannoheptose. With the exception of HS2, the strains that are capable of expressing a heptose in their CPS contain a gene *dmhA* that encodes a putative heptose-6-dehydratase enzyme to generate 6-deoxyheptose units. In addition, known *C. jejuni* CPS structures contain multiple unique isomeric forms of heptoses (*gluco*, *manno*, talo, *ido*, *gulo*, *altro*) as well as L configurate heptoses. CPS loci of HS8, HS10, HS42 and HS44 harbor two distinct copies of putative C4 reductases (MlghC/DdahC homologues) annotated as *fcl* and *fcl2* (*24*). Future sequencing and CPS structural analysis may reveal additional heptose isomeric forms and their responsible enzymes. Future studies of other *C. jejuni* CPS structures are hence expected to further uncover unique CPS structural components.

Known *Campylobacter jejuni* Capsular Polysaccharides

Serotype Complex HS1/HS44

The HS1/HS44 complex consists of Penner serotypes HS1 and HS44 as well as strains typed HS1 and HS44 (HS1/44), all of which contain genetically related bioshyntesis loci (*26*). The first structural studies on a HS1 CPS identified a linear teichoic-acid like CPS [→4)-α-D-Gal*p*-(1→2)-D-Gro-(1-P→]$_n$ (*38*, *39*). At this time, gangliosides in the core regions of C. jejuni were also detected (*40*). Further analysis confirmed the aforementioned teichoic-acid backbone sequence in HS1/44 seroclass CPS and in the HS1 type strain ATCC 43429, but also revealed the presence of fructofuranose branch [→4)-[β-D-Fru*f*-(2→2)]-α-D-Gal*p*-(1→2)-D-Gro-(1-P→]$_n$ (*41*). The D-glycerol-1-phosphate linkage is a phosphodiester rather than a glycosidic linkage, such connection has also been observed in the

HS53 CPS structure (described below). In some cases, the D-Gal*p* residue can be non-stoichiometrically modified at the 3-position with another β-Fru*f* residue. Additionally, both the stoichiometric and non-stoichiometric β-Fru*f* residues can be non-stoichiometrically modified at the 3-position with MeOPN. Exploratory studies on seroclass HS44 have revealed that it produces two distinct CPSs, a teichoic-acid CPS similar to that in HS1 and HS1/44, and a heptan CPS composed of 6-deoxy-*galacto*-heptose, 6-deoxy-*altro*-heptose and its methylated derivative 6-deoxy-3-*O*-methyl-*altro*-heptose (*42*). This HS44 heptan CPS is also decorated with MeOPN units (*42*).

Serotype Complex HS2

The structure of the HS2 type strain NCTC11168 is [→2)-β-D-Rib*f*-(1→5)-β-D-Gal*f*NAc-(1→4)-α-D-Glc*p*A6EtN-(1→]$_n$ (*43*). NCTC 11168, as described above, was both the first *C. jejuni* strain whose gene sequence and *kps* locus was uncovered. *C. jejuni* HS2 (NCTC 11168), along with strains of HS23/36 and HS19 (*44*, *45*), was one of the first strain discovered to harbor the MeOPN modification, which specifically occurs non-stoichiometrically on the 3-OH group of Gal*f*NAc.This CPS structure is further decorated with other non-stoichiometric modifications including a phase-variable CH_2OH modification at C7 of the α-D-GlcpA6EtN residue forming α-D-GlcpA6NGro, D-*glycero*-α-L-*gluco*-heptose at the 3-OH of the Glc*p*A residue (which was originally thought to be stoichiometric but later found to be non-stoichiometric), 3-O-methyation of the non-stoichiometric heptose, and 6-dehydration of the non-stoichiometric heptose.

Serotype Complex HS3

The CPS structure of the HS3 type strain ATCC 43431 (TGH9011) is [→3)-L-*glycero*-α-D-*ido*-Hep*p*-(1→4)-α-D-Gal*p*-(1→]$_n$ (*46*). The L-*glycero*-α-D-*ido*-Hep residue occurs non-stoichiometrically in the 6-deoxy form with MeOPN units non-stoichiometric present its position 2 (*47*). The Gal residue of HS3 CPS may also be substituted at position 3 with 3-hydroxypropanoyl. Genetic and biochemical evidence have shown that the genes *dmhA* and *dmhB* encode a heptose-C6-dehydratase and heptose-C4-reductase enzyme, respectively, required for 6-deoxyheptose formation. Putative *dmhA* and *dmhB* genes have been identified in the TGH9011 genome, and the *dmhA* gene has a poly(C) tract present that can cause slipped-strand mispairing and hence phase-variation in 6-deoxyheptose expression, which likely explains the non-stoichiometric 6-deoxyheptose modification in the HS3 CPS structure.

Serotype Complex HS4

The HS4 complex consists of multiple Penner serotypes including the HS4, HS13, HS16, HS43, HS50, HS62, HS64, and HS65 serotypes that contain genetically identical capsule biosynthetic loci (*25*). Some strains, such as the HS4/13/64 strain CG8486, cross-react with multiple

Penner antisera. The HS4/13/64 strain CG8486 strain has the CPS structure [→4)-β-D-Glc*p*NAc-(1→3)-L-*glycero*-β-D-*ido*-Hep*p*-(1→]$_n$ or [→4)-β-D-Glc*p*NAc-(1→3)-6-deoxy-β-D-*ido*-Hep*p*-(1→]$_n$ (*48*). A number of the *ido*-heptopyranose units were observed to carry O-methyl phosphoramidate moieties at the O-2 or O-7 position (*48*). An HS13 strain has been investigated in our laboratories, and found to contain a Glc unit (in the place of the GlcNAc residue seen in HS4 strains) [→4)-β-D-Glc*p*-(1→3)-6-deoxy-β-D-*ido*-Hep*p*-(1→]$_n$.

Serotype Complex HS5

The CPS structure of HS5 consists of the main structure [→6)-[α-3,6-dideoxy-*ribo*-Hep*p*-(1→2,6)]-α-D-*glycero*-D-*manno*-Hep*p*-(1→3)-[α-3,6-dideoxy-*ribo*-Hep*p*-(1→2)]-glucitol-(6→P]$_n$, with non-stoichiometric MeOPN modification at the 7-OH position of the α-3,6-dideoxy-*ribo*-Hep*p* residues (Figure 1). The HS5 CPS is the first *C. jejuni* serotype reported to contain either the α-3,6-dideoxy-*ribo*-Hep*p* or glucitol residue (*49*). In addition to the main structure, there are also three less abundant variations of the CPS structure that can be observed: [→6)-α-D-*glycero*-D-*manno*-Hep*p*-(1→2)-glucitol-(6→P]$_n$, [→6)-[α-3,6-dideoxy-*ribo*-Hep*p*-(1→2)]-α-D-*glycero*-D-*manno*-Hep*p*-(1→2)-glucitol-(6→P]$_n$, and [→6)-[α-3,6-dideoxy-*ribo*-Hep*p*-(1→2)]-α-D-*glycero*-D-*manno*-Hep*p*-(1→3)-[α-3,6-dideoxy-*ribo*-Hep*p*-(1→2)]-glucitol-(6→P]$_n$, all with non-stoichiometric MeOPN modification at the 7-OH position of the α-3,6-dideoxy-*ribo*-Hep*p* residues (*49*).

Serotype Complex HS6/7

In the HS6/7 complex, two carbohydrate structures were identified termed Structure A and Structure B in the HS6 type strain 81116 (RM1863) and occurring in a molar ratio of about 3:1, A:B, respectively. Structure A has the structure [→3)-β-D-Glc*p*-(1→2)-α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→3)-α-D-Glc*p*(1→]$_n$, with non-stoichiometric O-acetylation occurring at the 3-OH of the α-D-Glc*p*A residue and 6-OH position of the α-D-Glc*p* residue; Structure B was determined to be [→3)-β-D-Glc*p*NAc-(1→6)-α-D-Glc*p*-(1→4)-(β-D-Glc*p*NAc-(1→3))-α-D-Gal*p*-(1→]$_n$ (*50*). Structure B may be associated with Lipid A.

Serotype Complex HS10

The CPS of HS10 seroclass is composed of a GalNAc backbone with branches of 6-deoxy-L-*galacto*-heptofuranose: [→3)-[6d-α-L-*gal*-Hep*f*-(1→4)-α-D-GalNAc-(1→]$_n$ (*51*, *52*). Some of the 6-deoxy-L-*galacto*-heptofuranose units carry a MeOPN at position 3 (*52*).

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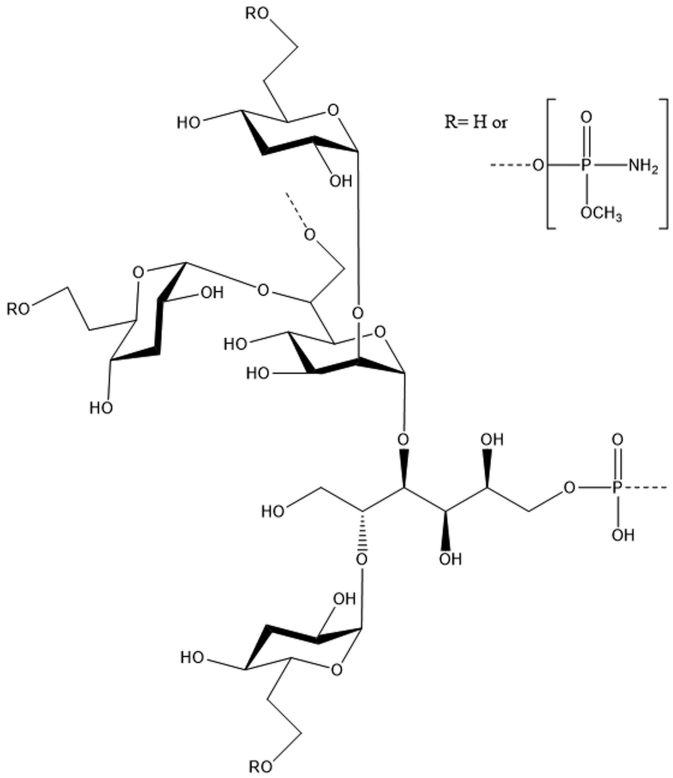

Figure 1. A structural variant of the oligosaccharide-phosphate repeating block of C. jejuni HS5 (49).

Serotype Complex HS15

The HS15 type strain ATCC 43442 core CPS structure is [→3)-6d-α-L-*gulo*-Hep*p*-(1→3)-α-L-Ara*f*-(1→]$_n$. This CPS structure has a non-stoichiometric α-L-Ara*f* residue non-reducing end (*53*), but the mechanism by which this α-L-Ara*f* is added to the HS15 core CPS unit remains unknown. No MeOPN was detected during structural analysis of the ATCC 43442 CPS, but the MeOPN biosynthesis and transferase genes are annotated to be present in the HS15 CPS bioshyntesis locus. Thus, the ATCC 43442 strain used for structural analysis likely contained a phase-off MeOPN transferase gene. For this reason, the location(s) of the MeOPN modification(s) on HS15 CPS are unknown at this time. The 6-deoxy group of the heptose appears was stoichiometric during structural studies. However, since the *dmhA* heptose dehydratase gene is phase-variable and hence non-stoichiometric in most C. *jejuni* strains containing *dmhA* and 6-dexoxyheptose, it may be possible that this 6-dehydration of *glycero*-β-D-altro-Hep*f* is also phase variable but is 100% phase-on in the ATCC 43442 strain used in these studies.

Serotype Complex HS19

The HS19 core CPS structure of strain ATCC 42446 (MK104) is [→4)-β-D-Glc*p*A6NGro-(1→3)-β-D-Glc*p*NAc-(1→]$_n$ (*54*). While the 6NGro group is stoichiometrically present on the β-D-Glc*p*A6NGro residue, a non-stoichiometric α-L-sorbofuranose (α-L-Sor*f*) ketohexose group occurs at the 2-OH of the β-D-Glc*p*A6NGro residue. Similarly to the β-Fru*f* ketohexose residues of HS1/44 CPS, the α-L-Sor*f* ketohexose residue is also highly acid-labile. Additionally, a non-stoichiometric MeOPN modification was identified, which was shown to occur on the 4-OH group of the β-D-Glc*p*NAc residue. Interestingly, structural analysis also revealed an appreciable amount of phosphoramidate in place of MeOPN at the 4-OH of GlcNAc. As described above, the biological significance of phosphoramidate on C. *jejuni* CPS remains to be determined.

Serotype Complex HS23/36

The main CPS structures of the HS23/36 complex are [→3)-α-D-Gal*p*-(1→2)-6d-3-*O*-Me-α-D-*altro*-Hep*p*-(1→3)-β-D-Glc*p*NAc-(1]$_n$ or [→3)-β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*-(1→2)-D-*glycero*-α-D-*altro*-Hep*p*-(1→]$_n$ (*44*, *55*). The HS23/36 seroclass consists of strains that are type HS23, HS36, and both HS23 and HS36 (HS23/36) by the Penner serotyping scheme, and sequencing of the CPS biosynthesis loci has revealed >97% nucleotide identity between the HS23 type strain, the HS36 type strain, and the HS23/36 strain 81-176. The trisaccharide repeat structure of HS23/36 also contains multiple non-stiochiometric modifications. The heptose residue can be methylated at the O-3 position and/or dehydrated at the 6-position. Moreover, three phase-variable MeOPN modifications can occur on the Gal residue at the 2-OH, 4-OH, or 6-OH group. The *cjj1420* MeOPN transferase was recently shown to transfer MeOPN to the 4-OH of Gal, while the *cjj1435* gene encodes a bifunctional MeOPN transferase that transfers MeOPN to both the 2-OH and 6-OH positions. The *cjj1435* MeOPN transferase preferentially adds the MeOPN to the 2-OH position vs. 6-OH position of Gal, but the mechanistic reason for this preference is currently unclear. Moreover, whether multiple MeOPN modifications can occur on a single Gal residue is unclear at this time. Interestingly, the *waaC* gene encoding a heptosyltransferase outside the CPS locus is required for 3-O-methylation of heptose (*44*) and may also play a role in the MeOPN modification of HS23/36 CPS. However, whether this enzyme acts as a methyltransferase enzyme or if this gene is indirectly required for heptose methylation and MeOPN biosynthesis has yet to be experimentally determined.

Serotype Complex HS41

The HS41 strain 176.83 (a non-type strain) core CPS structure is [→2)-β-L-Ara*f*-(1→2)-6d-β-D-*altro*-Hep*f*-(1→2)-α-D-Fuc*f*-(1→]$_n$ and of [→2)-β-L-Ara*f*-(1→2)-6d-β-D-*altro*-Hep*f*-(1→2)-β-6d-L-Alt*f*-(1→]$_n$ (*56*). The only difference between the two structures is that the α-D-Fuc*f* is substituted with 6d-β-L-Alt*f*. α-D-Fuc*f* and 6d-β-L-Alt*f* only vary from one another in that they are C5 epimers,

indicating that a non-stoichiometric C5 epimerase enzyme is responsible for these two different residues. To date, this C5 epimerase enzyme has yet to be identified and, until this enzyme is identified, it is unclear which monosaccharide residue represents the "core" monosaccharide residue of the HS41 CPS repeat unit. The HS41 CPS biosynthesis locus lacks genes involved in MeOPN biosynthesis, highly suggesting that HS41 CPS does not contain the MeOPN modification. The HS41 CPS structure is unique among known C. *jejuni* CPS in that the heptose is in the furanose rather than pyranose ring form. The 6-deoxy form of this heptofuranose was stoichiometric in these structural studies (*11*, *28*). Nvertheless, as described above for HS15 CPS structure, it cannot be ruled out that this modification is actually phase-variable and merely 100% phase-on in the 176.83 strain used in these studies.

Serotype Complex HS53

The HS53 type strain RM1221 has a relatively unique poly-heptose core CPS structure of [→3)-β-6d-D-*manno*-Hep*p*-(1→3)-α-6d-D-*manno*-Hep*p*-(1→3)-α-6d-D-*manno*-Hep*p*-(1-P→]$_n$ (*57*). The HS53 core CPS structure can be non-stoichiometrically modified with the ketohexose D-xylulose (D-*threo*-pent-2-ulose) in the furanose form (D-Xlu*f*) at the 2- and/or 4-OH positions of the β-6d-D-*manno*-Hep*p* residue, although the two D-Xlu*f* anomeric configurations are still unclear. Similarly to the non-stoichiometric ketohexose groups in HS1/44 and HS19 CPS, the α-D-Xlu*f* linkage in HS53 is highly acid-labile. Similarly to HS41 CPS biosynthesis locus, the HS53 locus lacks genes responsible for MeOPN biosynthesis. The HS53 CPS biosynthesis locus contains a putative *dmhA* gene, which correlate to the presence of the 6-deoxy form of D-*manno*-Hep*p* in the HS53 CPS structure. As described above for the HS15 and HS41 CPS structures, it cannot be ruled out that the 6-deoxy form of 6d-D-*manno*-Hep*p* is actually phase-variable and merely 100% phase-on in the RM1221 strain used in these studies.

Campylobacter jejuni Glycoconjugate Vaccine

C. jejuni CPS present a myriad of unique structural and conformational features representing unique targets for an anti-*C. jejuni* vaccine. Still, the range of unique CPS structures (albeit similar within serotype clusters), numerous phase-variable modifications, repeated *C. jejuni* infections observed in children in endemic areas, and repeated *C. jejuni* infection by the same serotype indicate that further study is required to fully understand the specificity of the immune response after *C. jejuni* exposure. (*18*, *58*). Nevertheless, conjugate vaccine development and manufacturing efforts are intricate and require optimization of multiple factors, from CPS discovery to CPS conjugation methodology.

Valency

Based on Penner serotyping, at least 35 different CPS structures exist within the C. *jejuni* sp. Due to cost, an efficient C. *jejuni* conjugate vaccine needs to target the most prevalent CPS types responsible for campylobacteriosis. A systematic review of the last 30 years of Penner serotyping analysis revealed that the HS4 complex, HS1/44, and HS2 CPS serotypes accounted for more than half of the sporadic Campylobacteriosis cases. Unfortunately, the majority of the typing was performed on cases from developed countries. Data is lacking from other regions of the world where the vaccine is needed the most. To address this deficiencyof serotyping data in endemic regions, a PCR based typing methods was developed that is currently able to characterize the 35 C. *jejuni* CPS groups. This typing system was employed to type over 2,100 *C. jejuni* human isolates collected from Africa (*59*), Middle East , Southeast Asia (*24*, *25*), and South America. Results presented in Figure 2 demonstrate a CPS distribution quite different than the one observed in developed countries. Nevertheless, the results suggest that the HS4 complex is the most prevalent serotype and that there are regional differences of CPS distribution. This preliminary study demonstrates that a CPS conjugate vaccine targeting about 10 types would cover ~70% of Campylobacteriosis cases within these endemic regions.

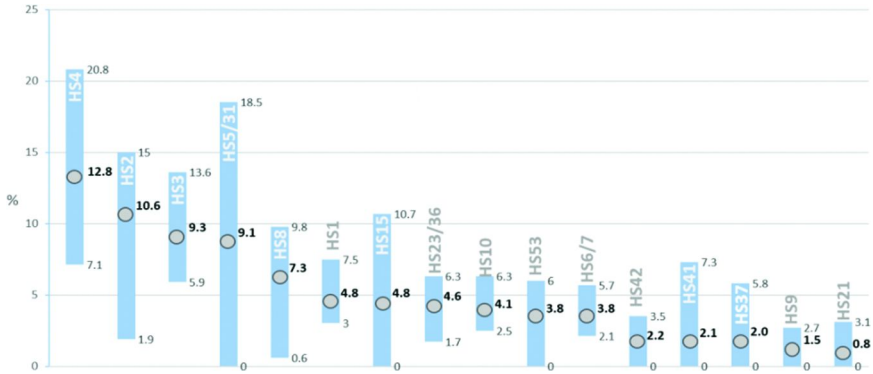

Figure 2. C. jejuni serotype distribution from the analysis of 2332 clinical isolates by CPS multiplex PCR (996 from Southeast Asia, 531 from Peru. 367 from Bangladesh, 120 from Israel and 318 from Egypt). Other serotypes constituted 15%.

Choice of Protein Carrier

The prototype *C. jejuni* glycoconjugate vaccine utilized a non-toxic diphtheria toxin protein, CRM_{197}, as the carrier (*60*). CRM_{197} has the advantage of being commercial available in large quantities and high purity as well as containing a large number of "privileged" lysine groups available for conjugation to activated *C. jejuni* CPS via reductive amination or carbodiimide chemistry (*60*, *61*). A shortcoming of CRM_{197} as the protein carrier is that humans are now routinely immunized against diphtheria toxin in diphtheria vaccines and

other glycoconjugate vaccines containing CRM_{197} as the protein carrier (e.g.-the pneumococcal Prevnar13® vaccine). Therefore, the immune system may not have sufficient time to generate a sufficiently strong *C. jejuni* CPS-specific immune response before pre-existing immunity against the diphtheria toxin carrier clears the glycoconjugate from the body. Also, CRM_{197} does not generate any additional immune coverage against *C. jejuni* or other important pathogens to which humans are not routinely immunized. Therefore, future studies on *C. jejuni* vaccines will evaluate cell surface C. *jejuni* proteins as potential protein carriers in the *C. jejuni* glycoconjugate vaccine, which may help broaden vaccine coverage against *C. jejuni* by targeting multiple cell surface antigens in addition to CPS. The protein carriers may also be vaccine candidates against other enteric pathogens besides *C. jejuni* to generate a multivalent diarrheal disease vaccine, such as enterotoxigenic *Escherichia coli* fimbriae proteins.

Campylobacter jejuni Capsule Conjugate Vaccine Syntheses and Immunogenicity

Conjugate vaccines have been widely used and have been safe and efficacious in preventing disease against a number of encapsulated bacteria including *Hemophilus influenzae* type B, *Streptococcus pneumoniae*, and *Neisseria meningiditis*. Furthermore, multivalent glycoconjugate vaccine platforms have been successfully developed and marketed to include a quadrivalent *N. meningiditis* vaccine, 7- and 13-valent *S. pneumoniae* vaccines, and a multipathogen combination vaccine including *H. influenzae* type B and *N. meningiditis* conjugates. Because there are 35 CPS types, an efficacious *C. jejuni* conjugate vaccine will be multivalent. The valency required to provide broad protection is yet uncertain, however, recent data discussed above suggest that a final vaccine formulation containing ~10 CPS types may provide adequate coverage. Immunization of mice with escalating doses of purified *C. jejuni* CPS delivered alone or adsorbed to aluminum hydroxide demonstrated that the *C. jejuni* polysaccharide alone is poorly immunogenic (*60*). These data are consistent with a T cell-independent immune response induced by unconjugated polysaccharides. However, polysaccharide conjugation to a carrier protein has been shown to induce a T cell-dependent immune response and enhance immunogenicity of many bacterial capsule polysaccharides. Conjugation strategies have been developed by us several key *C. jejuni* CPS types (HS1, HS2, HS3, HS4/13/64, HS5, HS10, HS15, HS23/36 and HS53) to the diphtheria toxin protein CRM_{197}, and the CPS-CRM_{197} conjugates have proven immunogenic (*53*, *60*). Stoichiometric oxidation of the CPSs, to generate aldehydes or carboxylic acid moieties for conjugation to amines in the protein, were carried out by the well known periodate oxidation method (*60*) or by oxidation with 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (*61*). Below, we describe key experiements carried out with some of the representative aforementioned conjugate vaccines.

The prototype CPSHS23/36-CRM_{197} vaccine has been the most extensively tested of the three conjugates (*60*). Monteiro and colleagues compared vaccination intervals and conjugate dose by immunizing mice subcutaneously at 0-2-4, 0-4-8

or 0-6-12 week intervals with 1, 5 or 25 μg of CPSHS23/36-CRM_{197} (dosed by total conjugate weight) delivered without an adjuvant. Immunization with CPSHS23/36-CRM_{197} primarily generated anti-CPSHS23/36 IgG responses and only the 25 μg dose at 4- and 6-week intervals induced significant levels of anti-CPSHS23/36 IgM compared to baseline. Anti-CPSHS23/36 IgG responses increased as a function of both antigen dose and increasing intervals between vaccinations (Figure 3A). At 2-week intervals, higher doses of CPSHS23/36-CRM_{197} in the 5 and 25 μg groups induced a significant increase in IgG responses compared to baseline titers. Regardless of the dose of CPSHS23/36-CRM_{197}, all mice immunized at 4- or 6-week intervals seroconverted by a 4-fold rise criteria and this IgG response persisted >26 weeks after vaccination (Figure 3A).

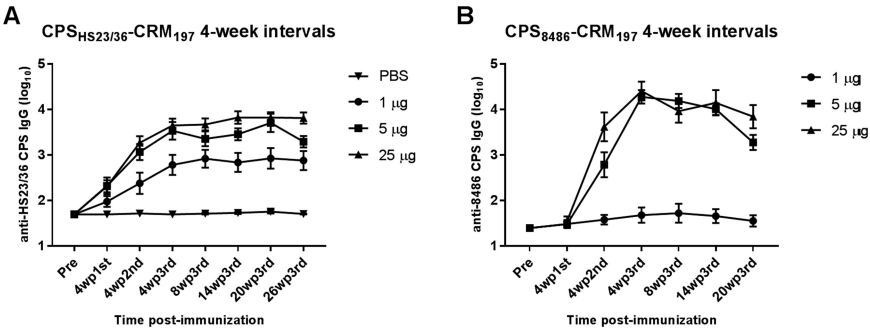

Figure 3. Immunogenicity of $CPS_{HS23/36}$-CRM_{197} (A) and CPS_{8486}-CRM_{197} (B) conjugates in mice. Mice were immunized subcutaneously three times with the respective conjugate or PBS at 4-week intervals with the indicated doses. Data represent mean log_{10} anti-CPS titer ± SEM for each group at the indicated time point post 1st, 2nd or 3rd vaccination. Pre, pre-vaccination; 4wp1st, 4-weeks post 1st dose; 4wp2nd, 4-weeks post 2nd dose; 4wp3rd, 4-weeks post 3rd. Data are adapted from Monteiro et al. (60).

The pattern of response is consistent with boosting of immunological memory that can be best elicited only after an interval of 4 or more weeks have elapsed between prime and boost vaccinations. Only a modest induction of CPS-specific serum IgA was observed in mice immunized with 5 or 25 μg at 4-week intervals. Importantly, mice immunized with 5 or 25 μg CPSHS23/36-CRM_{197} at either 2-, 4-, or 6-week intervals showed significant protection following intranasal challenge with *C. jejuni* strain 81-176. All CPSHS23/36-CRM197 vaccinated animals showed a significant reduction in illness index score after challenge compared to the sham immunized mice. These data suggest that anti-CPSHS23/36 IgG responses may be sufficient to mediate protection from *C. jejuni* infection in mice although the mechanism that affords this protection remains unknown.

There are currently no reliable small animal models of *C. jejuni* enteric infections and intestinal pathogenesis. While piglets, ferrets, rats, chickens and insects have been used as models, mouse models have required genetic manipulations or have failed to exhibit enteric disease. Indeed,

CPSHS23/36-CRM_{197} vaccine efficacy in the mouse intranasal challenge model described above may not translate into efficacy in a true enteric *C. jejuni* challenge. To examine conjugate vaccine efficacy in an enteric model, Monteiro and colleagues utilized a New World non-human primate *C. jejuni* enteric challenge model where infection of *Aotus nanycmaae* with high inoculum doses of 1010-1012 CFU of strain 81-176 induce diarrheal disease (*62*). The CPSHS23/36-CRM_{197} vaccine was administered to *A. nancymaae* subcutaneously at 1, 5 and 25 μg (total conjugate weight) with aluminum hydroxide in three doses at 6-week intervals (*60*). A dose dependent anti-CPS IgG response was observed where increasing doses of CPSHS23/36-CRM_{197} induced higher levels of anti-CPSHS23/36 IgG and only the 25 μg group had significantly higher IgG titers compared to the sham vaccinated animals (Table 1).

Animals were challenged with 1011 CFU of 81-176 nine weeks following the last immunization and a statistically significant trend towards protection from diarrhea was observed with increasing vaccine dose. Diarrhea attack rates were 40%, 20% and 0% in the 1, 5 and 25 μg groups, respectively, compared to a 60% attack rate in the sham vaccinated control animals (Table 1). A follow-on study was conducted with two new batches of CPSHS23/36-CRM_{197} to assess lot-to-lot reproducibility. Three doses of 25 μg of each conjugate plus aluminum hydroxide were administered at 6-week intervals. Immunized monkeys showed significantly higher anti-CPSHS23/36 IgG responses compared to control animals and all vaccinated animals were protected from 81-176 challenge. Interestingly, all monkeys were colonized with the challenge strain regardless of the vaccine dose administered demonstrating that anti-CPSHS23/36 IgG responses generated by the CPSHS23/36-CRM_{197} vaccine delivered with aluminum hydroxide are sufficient to protect against diarrheal disease, but not against *C. jejuni* colonization.

There were no significant differences in serum anti- CPSHS23/36 IgA responses between vaccinated and control animals and fecal IgA responses were not measured. The mechanism by which IgG affords protection from diarrhea but not colonization remains unclear, however, preliminary analysis indicates that vaccination with CPSHS23/36-CRM_{197} induces functional serum bactericidal IgG titers and we are currently exploring whether these responses contribute to protection in this model.

A second *C. jejuni* CPS conjugate vaccine that has been developed and evaluated is based on the CPS of strain CG8486, described above that types as HS4/13/64 (*60*), conjugated to CRM_{197}. The CPS8486-CRM_{197} vaccine was delivered to mice subcutaneously at 1, 5 and 25 μg (dosed by total conjugate weight) at 4-week intervals. While the low 1 μg dose failed to induce anti-CPS8486 IgG, 5 and 25 μg of CPS8486-CRM_{197} induced high levels of anti-CPS8486 IgG and 90% of the animals seroconverted (Figure 3B). In addition, animals in the 5 and 25 μg groups were protected from intranasal challenge with CG8486 which is similar to the efficacy observed with the CPSHS23/36-CRM_{197} vaccine in this model.

Table 1. Immunogenicity and protective efficacy of three lots of $CPS_{HS23/36}$-CRM_{197} against diarrheal disease in *Aotus nancymaae*. Data are adapted from Monteiro *et al.* (*60*).

Study	*Vaccine and dose*	*N*	*Mean anti-CPS IgG titer (log_{10})*	*Diarrhea attack rate (%) (no. of positive animals/total no.)*
1	PBS	5	2.54	60 (3/5)
	$CPSCPS_{HS23/36}$-CRM 1 μg	5	3.57	40 (2/5)
	$CPSCPS_{HS23/36}$-CRM 5 μg	5	5.38	20 (1/5)
	$CPSCPS_{HS23/36}$-CRM 25 μg	5	6.47	0 (0/5)
2	PBS	10	2.30	70 (7/10)
	$CPSCPS_{HS23/36}$-CRM Batch A 25 μg	9	5.09	0 (0/9)
	$CPSCPS_{HS23/36}$-CRM Batch B 25 μg	5	5.24	0 (0/5)

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ACS Symposium Series; American Chemical Society: Washington, DC, 2018.

Another *C. jejuni* conjugate vaccine synthesized and characterized is based on the HS15 strain ATCC 43442 conjugated to CRM_{197} (*53*). A dose of 25 μg of the $CPSHS15\text{-}CRM_{197}$ vaccine was administered to mice with aluminum hydroxide at 4-week intervals and anti-HS15 CPS antibodies were detected in the serum two-weeks after the third immunization. Although the antibody response to the $CPSHS15\text{-}CRM_{197}$ vaccine was not well defined in this study due to a difficulties using the HS15 CPS in standard immunologic assays.

Conjugation methods using periodate oxidation (Figure 4) or TEMPO-mediated oxidation (Figure 5) for the HS53 CPS to CRM_{197} and other proteins have also been developed (*49*) and immunogenicity of this vaccine has recently been tested in mice. Gel-electrophoresis (Figure 6) has become the tool of choice to confirm and analyze the products of conjugations.

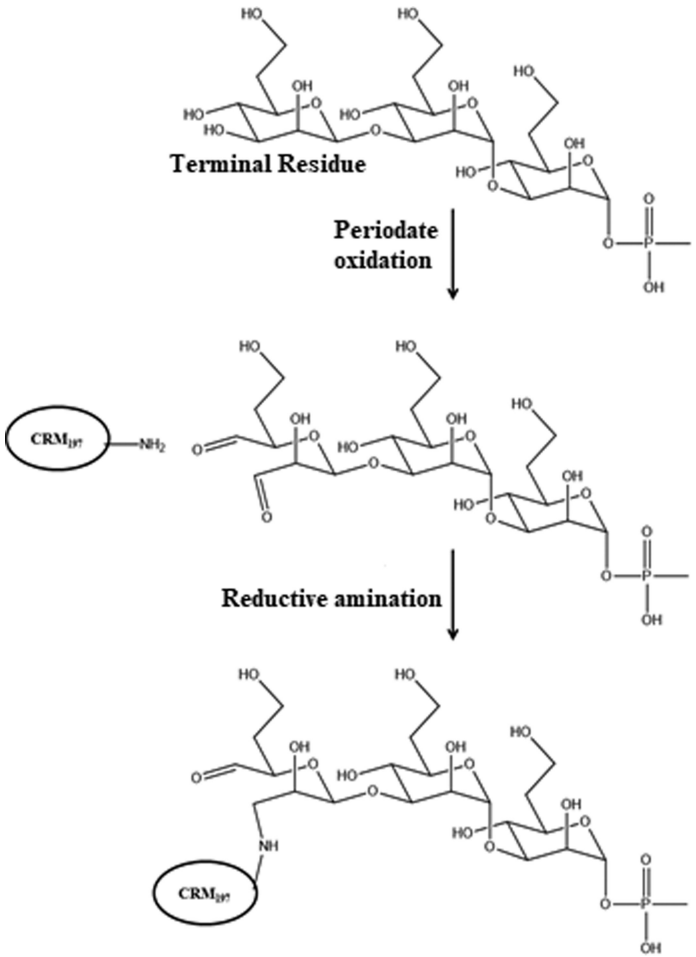

Figure 4. Conjugation of C. jejuni HS53 CPS to protein carrier CRM_{197} by periodate oxidation/reductive amination method. The monosaccharide at the non-reducing end was first oxidized with periodate and then conjugated coupled to a protein by reductive amination (49).

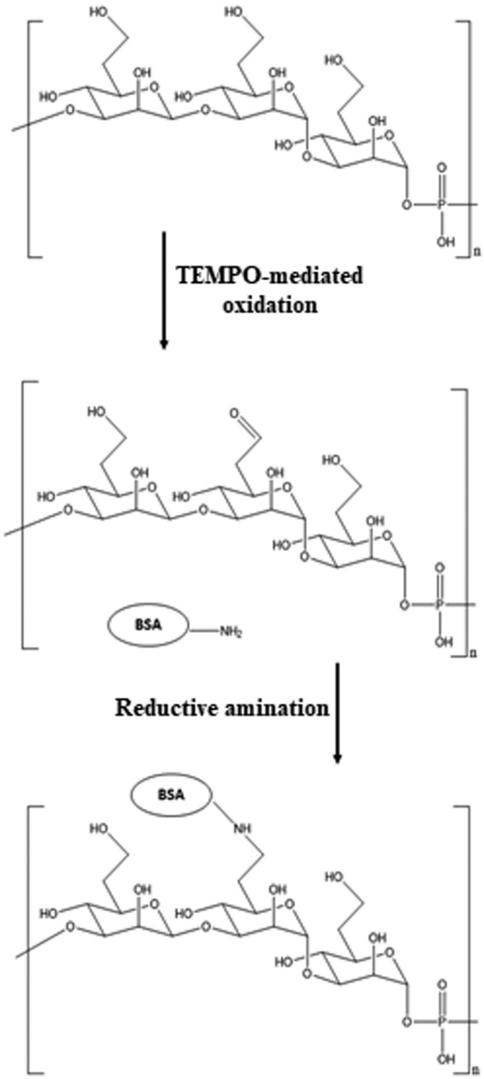

Figure 5. Activation of C. jejuni HS53 CPS by TEMPO-mediated oxidation. 10% of the primary hydroxyls (C-7 of heptoses) in the CPS were first stoichiometrically oxidized with TEMPO/bleach to aldehyde or carboxylic acid, and then coupled to a protein by reductive amination (in case of aldehyde) or carbodiimide chemistry (in case of carboxylic acid) (61).

Animals were dosed with increasing amounts of the CPSHS53-CRM_{197} conjugate at 0.5, 3.5 and 10 µg (dosed by total CPS weight, not total conjugate weight) at 4-week intervals without an adjuvant. Lower doses of 0.5 and 3.5 µg were not immunogenic after three doses, however, 10 µg induced anti-HS53 CPS IgG responses at levels comparable to titers observed with the CPSHS23/36-CRM_{197} vaccine (Figure 7). Conjugates containing the CPSs of

HS1, HS2, HS3, HS5 and HS10 have also been observed to raise antibodies against the corresponding CPSs (*47*, *49*, *52*, *63*). Upcoming experiments aim to optimize conjugation methods and analytical methods to evaluate these and other new CPS-CRM_{197} conjugate vaccines.

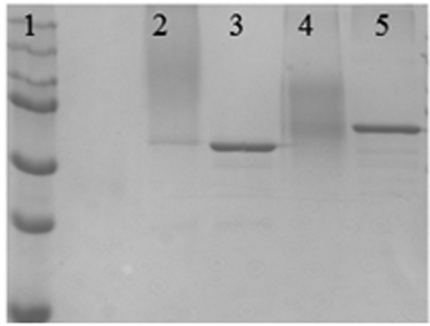

Figure 6. Analysis of CPSHS53 conjugates by gel-electrophoresi0s. Lane 1: molecular weight marker; Lane 2: CPSHS53-CRM197 conjugate obtained via periodate oxidation/reductive amination method; Lane 3: CRM197; Lane 4: CPSHS53-BSA conjugate generated by TEMPO-mediated oxidation/carbodiimide method; Lane 5: BSA (49).

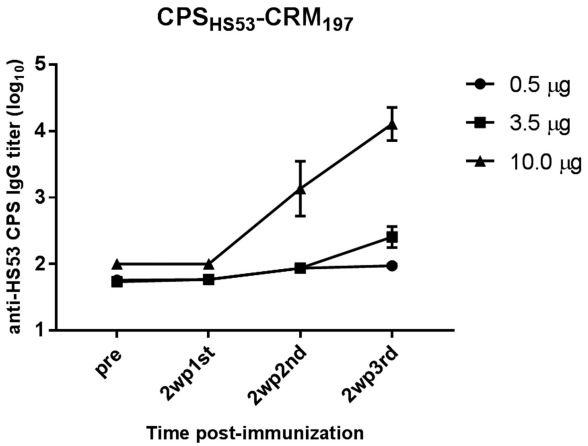

Figure 7. Immunogenicity of CPS_{HS53}-CRM_{197} conjugate vaccine in mice. Mice were immunized subcutaneously three times with the CPS_{HS53}-CRM_{197} conjugate at 4-week intervals with the indicated doses. Data represent mean log_{10} anti-CPS_{HS53} titer ± SEM for each group at the indicated time point post 1st, 2nd or 3rd vaccination. Pre, pre-vaccination; 2wp1st, 2-weeks post 1st dose; 2wp2nd, 2-weeks post 2nd dose; 2wp3rd, 2-weeks post 3rd.

The prototype HS23/36-CRM_{197} conjugate vaccine has been produced under cGMP and is now being evaluated in a phase I human clinical trial. Future studies must also target combination of monovalent *C. jejuni* conjugate vaccines into multivalent formulations. As conjugation methods for additional CPS types are established, monovalent and multivalent formulations will be examined. Preliminary studies have indicated that two monovalent CPS conjugates can be successfully combined into a bivalent formulation where immunogenicity to each CPS type is maintained. In addition, pathogen-relevant proteins are being explored as carriers for *C. jejuni* CPS conjugate vaccines. *C. jejuni* proteins and Enterotoxigenic *Escherichia coli* (ETEC) fimbriae proteins have been successfully conjugated to *C. jejuni* CPS in our laboratories and show promise of developing a conjugate vaccine that induce not just CPS-specific antibody responses, but also the potential for antibody and T cell-mediated responses against pathogen-relevant carrier proteins (*49*, *63*, *64*).

Conclusions

C. jejuni represents a threat to global health and a vaccine would be welcomed. *C. jejuni* exposes sero specific CPSs, whose main characteristic is the presence of 6-deoxy-heptoses and MeOPN moieties. Each serotype complex generates CPSs with specific 6-deoxy-heptoses (many of unusual configuration) and MeOPN decorations at selected linkage sites. Strains that fall within a serotype complex share structural characteristics, in that only a single CPS conjugate, containing only a CPS from a single strain, affords protection against the other strains of the same serotype family.

Immunogenic CPS-CRM_{197} conjugates have been made for all significant serotypes. So far, the CPS conjugate of serotype complex HS23/36 (strain 81-176) has been the most studied, and protection experiments in a non-human primate model have shown full efficacy in preventing diarrhea against HS23/36 strains. The prototype HS23/36 CPS conjugate vaccine was manufactured under cGMP and it is now in a phase I human clinical trial. Eventually, a *C. jejuni* vaccine will have to be multivalent. Recent trials have shown that a divalent vaccine, composed of HS23/36 and HS3 CPS conjugates, is indeed capable of equally raising antibodies against both serotypes. This encouraging result opens the door to a future multivalent *C. jejuni* vaccine that may be used globally.

Acknowledgments

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