Synthesis of *Campylobacter* and *Shigella* glycoconjugate Vaccines

by

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Abstract

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*Campylobacter jejuni* is considered one of the major causes of gastroenteritis worldwide. *C. jejuni* is the major cause of bovine abortion as well. The development of anti-*Campylobacter* vaccines based on the capsular polysaccharide of *C. jejuni* conjugated to carrier protein has proved its effectiveness and efficacy. The current research includes the characterization of the CPS of *C. jejuni* serotypes HS:1, HS:1/8, and HS:8. The structure was confirmed to have the same backbone as HS:1 previously characterized with the addition of an MeOPN at the Gal-6 position which has not been reported before. HS:8 CPS showed the presence of extra heptoses in different conformations and still needs more extensive investigation. The research introduces the development of prototype vaccines against *C. jejuni* strain HS:2, and HS1/8. In addition, the research introduces a multi-agent vaccine against *S. flexneri* serotype 2a which is conjugated to ETEC, and BSA by TEMPO-mediated oxidation.
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List of Abbreviations

1D  One Dimensional
2D  Two Dimensional
AA  Alditol Acetates
Ac  Acetate
AKA also known as
BSA Bovine serum albumin
C. jejuni Campylobacter jejuni
Cfa Colonization Factor Antigen of Enterotoxigenic Escherichia coli (ETEC)
COSY Correlation Spectroscopy
CPS Capsular Polysaccharide
CRM$_{197}$ Cross Reactive Material (Protein from Diphtheria toxin mutant)
D Dextro
DCM Dichloromethane
dH$_2$O deionized water
EDC 1-ethyl-(3-dimethylaminopropyl) carbodiimide
EI Electron Impact Ionization
ELISA Enzyme Linked Immunosorbant Assay
f Furanose
Gal Galactose
GalNAc N-Acetyl Galactosamine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>GBS</td>
<td>Guillain-Barre Syndrome</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glucoronic acid</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-Acetyl Glucosamine</td>
</tr>
<tr>
<td>Gro</td>
<td>Glycerol</td>
</tr>
<tr>
<td>Hep</td>
<td>Heptose</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Correlation</td>
</tr>
<tr>
<td>HS</td>
<td>Heat Stable (Penner serotyping)</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>L</td>
<td>Laevo</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipooligosaccharide</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass per charge</td>
</tr>
<tr>
<td>MeOPN</td>
<td>O-methyl phosphoramidate</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)-ethansulfonic acid buffer</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut Off</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>p</td>
<td>Pyranose</td>
</tr>
<tr>
<td>ppm</td>
<td>Part Per Million</td>
</tr>
</tbody>
</table>
Rha  Rhamnose
Rib  Ribose

*S. flexneri*  *Shigella flexneri*

SDS-PAGE  Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

TFA  Trifluoroacetic acid

TOCSY  Total Correlation Spectroscopy

α  alpha

β  beta

δ  Chemical Shift
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CHAPTER 1: Introduction

1.1. Bacterial cell wall

The bacterial cell wall gives the cell its rigidity and determines cell shape. The structure is used to divide bacteria into two broad classes that can be distinguished according to a technique called Gram staining. Despite the differences between the two groups of bacteria, they both have the same principal components in their cell walls: an inner cytoplasmic membrane, surrounded by a peptidoglycan layer consists of alternating N-acetylglucosamine (GlcNAc), and N-acetylneuramic acid [1].

Gram-positive bacteria, such as *Streptococcus*, *Staphylococcus*, and *Clostridium* species, have a cell wall that consists of a thick peptidoglycan layer, which makes up to 90% of the cell wall, and a single plasma membrane [2].

Gram-negative bacteria like *Salmonella*, *Shigella*, *Escherichia*, and *Campylobacter* species have a cell wall that is more structurally complex. The peptidoglycan layer comprises only 5-20% of the cell wall and is located between an inner and an outer membrane [1]. The outer membrane of Gram-negative bacteria is coated with a protruding lipopolysaccharide (LPS) structure that has a major role in host-microorganism interactions. The LPS comprises of three regions: the lipid A that is a glucosamine based phospholipid anchors the LPS to the outer membrane [3]. The middle region of the LPS is the non-repeating core oligosaccharide and has an inner core which is joined to lipid A and consists of sugars like 2-keto-3-deoxy-octanoate (Kdo), and L-glycero-D-manno-heptose. The outer core of the oligosaccharide joins the O-antigen, and it is usually comprises of hexoses and hexose amines [4]. Finally, the distal O-antigen
that is composed at least of 20 different sugar units that are found to be strain specific, and varies among species from the same strain [4]. The virulence among strains varies depending on the composition and length of the O-antigen. Typical Gram negative bacterial cell wall is illustrated in Figure 1.1 [5].

![Gram-negative bacterial cell wall diagram](image)

**Figure 1.1.** Gram-negative bacterial cell wall representing LOS, LPS, and CPS. Adapted from Raetz and Whitfield [3] with modifications

### 1.2. *Campylobacter jejuni*

*C. jejuni* infections are the leading cause of bacterial gastroenteritis worldwide. *Campylobacter* are small, spiral shaped, microaerophilic Gram-negative organisms. *Campylobacter* species are characterized by their rapid spinning motions [6, 7], provided by the presence of either a unipolar or bipolar flagella [8], which allows them to move in viscous environments at a speed of up to 75 µm/s [9]. *Campylobacter* was first observed
in 1886 by Theodor Escherich in stool samples from children with diarrhea. In 1913, a related *Vibrio* microorganism was identified by McFaydean and Stockman in the fetal tissue of aborted sheep. This was followed later by the description of the isolation of *Campylobacter* from blood samples isolated from children in 1957 by King [10]. The development of selective isolation media for *Campylobacter* from human feces in early 1970’s allowed Belgian microbiologists to isolate *Campylobacter* for the first time from stool samples of patients with diarrhea [10]. *Campylobacter* is currently considered the leading cause of bacterial enteritis.

The *Campylobacter* genus has 19 identified species. The serological classification of *Campylobacter* strains is split into two types of identification methods. The Lior serotype that differentiates *Campylobacter* species: *C. lari, C. jejuni, and C. coli*, according to heat-labile antigens. The Penner serotyping differentiates *C. jejuni* and *C. coli* according to heat-stable antigens [11].

1.2.1. *Campylobacteriosis*

*C. jejuni* is known to be one of the most causative microorganisms of bacterial gastroenteritis worldwide, especially among children [12]. It has been found that infection with *C. jejuni* is 2-7 times more likely to occur than enteritis caused by *Salmonella* species, *Shigella* species, or *Escherichia coli* [8]. Studies have shown that this microorganism causes 400 million cases of diarrhea annually [11]. The distribution of the infection is age and sex related. Studies showed that it affects children of less than one year, and adults between 15-44 years of age. In addition, infection is more often observed in males than in females [8]. While the infection is more likely to occur any
time during the year, it has been noted that the occurrence of the infection peaks in the summer and fall [13].

*C. jejuni* infection most often results from handling or consuming poultry, where animals are the reservoir carrying this pathogen. Drinking untreated water or unpasteurized milk is another potential source for outbreaks [14]. Recent studies found a relationship between the consumption of certain medications, like proton pump inhibitors (Lozec, Prevacid), and the possibility of having *Campylobacter* enteritis as well [15]. Responsible for approximately 80 million cases infecting people, is travel from developed countries to countries where campylobacteriosis is endemic [11].

*C. jejuni* colonizes the epithelium of the human intestine; only 500-800 colony forming units (CFU) are needed to produce infection in humans [11]. Clinical manifestations are diverse depending on host and pathogen factors, that can range from asymptomatic to severe gastroenteritis characterized by diarrhea ranging from mild watery to severe bloody diarrhea which can be accompanied by nausea, vomiting, and fever [16]. Extra intestinal manifestations like reactive arthritis or Guillain-Barré syndrome may also occur [17].

Although the infection is usually self-limiting, and patients need only replacement of fluids and electrolytes, drug intervention, in some severe cases might be needed. Fluroquinolones were highly active till the 1990’s after the license of their use in farm animals allowed the development of resistance. Currently, no flouroquinolones are allowed to be used in poultry in the US (FDA). Erythromycin is still the drug of choice for the treatment of severe or complicated enteritis [13]. The increase in the incidence of
the infection and the subsequent increase of resistance toward the regular antibiotics produce the urgency for the development of an anti-Campylobacteriosis vaccine.

1.2.2. Post infection sequelae

Guillain-Barré Syndrome (GBS) is an autoimmune disorder and the most common cause of neuromuscular paralysis in most developed countries [18]. It is defined as an acute motor symmetrical weakness of the extremities [18]. It is believed that GBS and Miller Fisher Syndrome (variant of GBS that includes ophthalmoplegia, ataxia, and tendon reflex loss) are developed 1-3 weeks after having an infection [19, 20]. In every 1000 Campylobacteriosis cases there is at least one case of GBS; approximately 20% of these cases are left with certain disability and 5% die [10]. Studies of C. jejuni strains associated neuropathy found out that sialylation of the LOS is required [21] due to the mimicry between the lipooligosaccharides of the microorganism and the carbohydrate structure of human gangliosides [22-24]. Yet, there are some other factors contributing to GBS occurrence since almost half of the strains isolated from persons not suffering from GBS expressed sialylated LOS [21].

1.2.3. C. jejuni infection in Poultry

C. jejuni can be carried in the intestine of healthy sheep without any signs of clinical symptoms [25], but it is known to be the major cause of ovine abortion worldwide, being responsible for at least 23% of the aborted cases [26]. The infection occurs in susceptible ewes by ingestion of infected material which will lead to bacteremia followed by infection in the placenta that will lead eventually to abortion in the third trimester of pregnancy [27]. Once a case of abortion occurs, the rest of the healthy pregnant ewes will
be exposed to *Campylobacter* organisms due to contact with the infected fetus, placenta and uterine discharge, which leads to fetal infection that might be asymptomatic however, the infected ewes will die as a result from uterine sepsis and septicemia if the dead fetus retained in utero [25]. The usual approach for the treatment of *C. jejuni* infection is by giving antibiotics like tetracycline, which is the only approved treatment in the United States. Tetracycline resistant strains have been observed lately, and one strain that has been proved to resist tetracycline and was responsible for the majority of the infections is IA3902 clone (HS1/8), that was first isolated from aborted fetus in an outbreak in 2006 [25]. Available vaccines against fetal abortion in the United States; like CampyVax4 (A Quadri-valent vaccine against three *C. fetus* serotypes and one *C. jejuni* strain) has proven to be effective with a limited cross-protection between different serotypes, and it was developed against the most predominant strains at the time of the vaccine production [25]. IA3902 clone (HS1/8) has not only been responsible for *Campylobacter* associated abortion in sheep, it has also been proved that this clone is transmitted to humans, and it is considered to be of a high risk to human health [28].

### 1.3. *C. jejuni* Surface Carbohydrates

*C. jejuni* unlike most other Gram-negative bacteria does not express a LPS (lipid A, core oligosaccharide, and O-chain), instead it expresses a low molecular weight lipooligosaccharide (LOS) (Core oligosaccharide attached to lipid A), and a high molecular weight capsular polysaccharide (CPS) with no attachment to lipid A and it is the outer most layer of the bacterial cell wall [23, 29].

#### 1.3.1. Lipooligosaccharide of *C. jejuni*
The complete structures of LOS produced by several C. jejuni strains have been characterized [23, 30-32]. The structure of the inner region of the LOS is highly conserved, consisting mainly of trisaccharides: one 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo), and two L-glycero-D-manno-Heptose (LD-manno-Hep). The LD-manno-Hep that is attached to Kdo is substituted by glucose at the C4 position, and can be substituted by phosphoethanolamine (PEA) at either C6, or C7 in all the LOS structures that has been characterized. In the LOS of serotypes HS:1, HS:2 (figure 1.2), the other LD-manno-Hep is substituted by a glucose at the C2 position. The outer core region is more variable, and might include hexoses, amino sugars, and N-acetyl-neuraminic acid (Neu5Ac, aka sialic acid). This sialic acid structurally mimics the human gangliosides, especially GM1, and GM2 (figure 1.2). The terminal region of the LOS in serotype HS:2 resembles the saccharide structure in human ganglioside GM1 [23], and the LOS terminal region of serotype HS:1 mimic the saccharide structure of GM2. This mimicry leads to the development of the previously mentioned autoimmune disease GBS. The disease results from the production of antibodies against the LOS structure which ends up also causing an attack on the closely related human gangliosides [23, 24]. It has been always a challenge to purify the CPS from the cell wall of the bacteria, in order to include it in a prototype vaccine. More studies and experiments are conducted on the C. jejuni strains to overcome this problem. One method is by producing a mutant that lacks the Neu5Ac that is believed to be the major substituent which mimics the human gangliosides. C. jejuni PG3588, is an HS:1 mutant that lacks the Neu5Ac on the LOS, and it is discussed in details in Chapter 4 of the current study.
Figure 1. Structure of common monosaccharides in the LOS of *C. jejuni*. Kdo: 2-keto-3-deoxy-D-manno-octulosonic acid, LD-manno-Hep: L-glycero-D-manno-Heptose, and Neu5Ac: N-acetyl-neuraminic acid
Figure 1.3. A. C. jejuni serostrain HS:2 LOS, B: C. jejuni strain HS:1 LOS, and the structural mimicry with human GM1 in C, and GM2 in D [23], (Glc: glucose, Gal: galactose, GalNAc: N-acetyl-galactosamine, Neu5Ac: 5-acetyl-neuraminic acid, Hep: heptose, PEA: phosphoethanolamine).

1.3.2. Capsular Polysaccharide of C. jejuni

Capsular polysaccharides usually serve as means for the cells interaction with the outer environment, cell adherence and invasion [23, 33]. C. jejuni is able to express different types of CPS that are structurally complex and highly variable, which is mainly due to the phase-variable modifications such as amino glycerol, O-methyl, ethanolamine, and the O-methyl phosphoramidate (MeOPN) group [33, 34]. The presence of MeOPN (figure 1.4) was first detected in nature in the repeating capsular polysaccharide unit of
C. jejuni strain NCTC 11168 (HS:2) [35]. MeOPN was then found to be expressed in around 70% of C. jejuni isolates [34]. The MeOPN modification was found to enhance the adsorption of the host cell surface and infection [36], impart resistance to serum killing [34], and modulate the immune system by a possible avoidance to immune response, and establishing colonization [34, 37]. Genes responsible for making MeOPN have been found to promote colonization and disease in humans [38]. It can be expressed on a hexose as it is the case in the repeating unit of HS:23/36 at the C-2 of the α-D-Gal, or it can be expressed on a NAc sugar (C-3 of the β-D-GalfNAc in strain HS:2). It can be expressed on the different heptoses in the CPS of C. jejuni as in strain HS:4 (C-2, or C-7 of the 6d-β-D-ido-Hep). There are four identified genes required for the biosynthesis of MeOPN cj1415-cj1418 in C. jejuni strain HS:2, whereas the genes that are responsible for adding the MeOPN on the different sugar moieties are phase variable genes. For example cj1421, and cj1422 are the genes responsible for the addition of MeOPN to C-3 of the β-D-GalfNAc, and to C-4 of the D-glycero-α-L-gluco-Hep of the C. jejuni HS:2 [34, 36], respectively. Overall the CPS of C. jejuni is highly variable, and many of C. jejuni CPS structures were characterized as shown in Table 1.1. The virulence of the unique heptoses that garnish the CPS of some strains of C. jejuni is still unknown [39]. The modified heptose derivatives and the unusual ring configuration of some CPS structures of C. jejuni strains (shown in Table 1.1) are believed to have a role in the host-bacterial interactions [39]. The unique structures of the CPS of C. jejuni strains can be considered as common motifs when developing a multivalent vaccine [40].
Figure 1.4. The chemical structure of the O-methylphosphoramidate. R is the attachment site at the CPS repeating unit.

Table 1.1. Capsular polysaccharide structures isolated from *C. jejuni* serotypes [22, 23, 32, 41-47]

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Structure</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS:1</td>
<td>→4)-β-D-Gal-(1→2)-(R)-Gro-(1-P→</td>
<td>With two Fruf branches at C2 and C3 of Gal. Fruf are substituted with MeOPN at C3</td>
</tr>
</tbody>
</table>
| NCTC 11168 (HS:2) | 6-O-Me-D-glycero-α-L-Glc-Hep  
<pre><code>     | ↓ 3                         | GlcA has 2-amino-2-deoxyglycerol at C6, MeOPN at C3 of Gal/NAc, and C4 of Hep |
</code></pre>
<p>|            | →2)-β-D-Ribf-(1→5)-β-D-Gal/NAc-(1→4)α-D-GlcA6(N-Gro)-(1→)                  |                                                                      |
| HS:3       | →3)-(3-hydroxypropanoyl)-L-α-D-ido-Hep-(1→4)α-D-Gal-(1→)                   |                                                                      |
| HS:4 CG8486| →3)-6d-β-D-ido-Hep-(1→4)-β-D-GlcNAc-(1→)                                  | MeOPN at C2 and/or C7 of 6d-β-ido-Hep                                |</p>
<table>
<thead>
<tr>
<th>Serotype</th>
<th>Structure</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS:15</td>
<td>$\rightarrow 3)-\alpha$-Ara-$\rightarrow 3$)-6-(\alpha)-gulo-Hep-$\rightarrow 3$)</td>
<td>C4 of (\beta)-D-GlcNAc is substituted with MeOPN or OH</td>
</tr>
<tr>
<td>HS:19</td>
<td>$\rightarrow 4)-\beta$-D-GlcA6(N-glycerol)-$\rightarrow 3$)-(\beta)-D-GlcNAc-$\rightarrow 3$)</td>
<td></td>
</tr>
<tr>
<td>HS:23/36</td>
<td>$\rightarrow 3$)-(\beta)-D-GlcNAc-$\rightarrow 3$)-(\alpha)-D-Gal-$\rightarrow 2$)-6d-$\rightarrow 3$)-OMe-$\rightarrow 3$)-(\alpha)-D-altro-Hep-$\rightarrow 3$)</td>
<td>C2 of (\alpha)-D-Gal is either MeOPN, or OH</td>
</tr>
<tr>
<td>HS:41</td>
<td>$\rightarrow 2$)-(\beta)$-L-Alaf-$\rightarrow 2$)-(\beta)$-D-altro-Hepf-$\rightarrow 2$)-(\beta)$-L-6d-Altf-$\rightarrow 3$) or (\rightarrow 2$)-(\beta)$-L-Alaf-$\rightarrow 2$)-(\beta)$-D-altro-Hepf-$\rightarrow 2$)-(\alpha)$-D-Fucf-$\rightarrow 3$)</td>
<td></td>
</tr>
<tr>
<td>HS:53</td>
<td>$\rightarrow 3$)-(\beta)$-D-6d-manno-Hep-$\rightarrow 3$)-(\alpha)$-6d-D-manno-Hep-$\rightarrow 3$)-(\alpha)$-6d-D-manno-Hep-$\rightarrow 3$)</td>
<td>At C2 and/or C4 a substitute (\alpha)-D-Xlu</td>
</tr>
<tr>
<td>NCTC 81116</td>
<td></td>
<td>Ratio 3:1</td>
</tr>
</tbody>
</table>

1.4. *Shigella flexneri*

*Shigella* is Gram-negative anaerobes, members of the family *Enterobacteriaceae*. The genus contains four species, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*, and *Shigella dysentriae* [48]. Based on the bacterial surface O-antigen (O-Ag) of *Shigella*; species are further divided in to serotypes. *Shigella flexneri* O-antigen with the exception
of serotypes 6, and 6a; has the similar backbone of a tetrasaccharide repeating unit of [rhamnose I-rhamnose II-rhamnose III- GlcNAc]. Further serogrouping depends on the addition of glucosyl, or acetyl groups, and gives rise to 13 serotypes [4, 48].

Shigellosis or bacillary dysentery is caused by *Shigella*. It is caused by the colonization of, and destruction of colonic mucosa, which leads to local inflammation and bleeding [49]. *Shigella*, unlike *Campylobacter* has no animal reservoir, and is usually transmitted through direct consumption of contaminated water or food, or through direct contact with human feces [50]. The main symptoms of shigellosis include diarrhea with frequent bloody stool, fever, and abdominal cramps that last for five to seven days. Treatment of shigellosis using antibiotics aimed usually to reduce the duration of infection, which reduces the duration of diarrheal attacks, and therefore, reduces the transmission of the disease to others. Shigellosis is responsible for more than 120 million cases of dysentery worldwide [50], and it is responsible for the death of more than one million people each ear, where 60% are children under the age of 5 years mainly in developing countries. In addition, shigellosis contributes to many infections among travelers each year. Of the most common predominant serotypes causing shigellosis in the developing countries is *S. flexneri* serotype 2a, while in the developed countries is *S. sonnei* [51]. Vaccine development against shigellosis has recently concentrated on using the O-Ag repeating unit conjugated to a carrier protein, which have proved to be effective and safe [52].

1.5. Introduction to vaccinology
Vaccine discovery is one invention that has had the most positive effect on the quality of public health over the past century. Vaccines against diphtheria, tetanus, polio, measles, mumps, rubella, pneumococcus, meningitis, and hepatitis B, have all contributed to the decline of mortality and morbidity during childhood and infancy by 97-99% [53]. Vaccines are no longer only used to prevent the diseases, they are also used in new approaches to treat serious diseases like cancer, and immune-mediated diseases [54], and they are being developed to aid in the improvement of life for impact groups, such as autistic patients [55].

Traditional approaches in bacterial vaccine development use the whole microorganism either killed or live attenuated. These preparations showed protective immunity after administration, but safety concerns regarding the use of unnecessary and unwanted molecules that may be of risk to humans are still to be considered [54]. Later approaches use part of the microorganism either purified proteins, purified polysaccharides, or detoxified extracellular toxoids instead of the whole organism [56].

The development of a carbohydrate vaccine focuses on the safety and efficacy of the preparation. These subunit antigens are usually located at the surface of the cell and can be easily recognized by the immune system. Purified bacterial polysaccharides are poorly immunogenic in infants, and they don’t activate memory cells and do not generate long term immunity [57]. The antibodies produced by B cells of infants produce immunoglobulins M (IgM), which is not enough to elicit long term protection against invasive pathogens. This problem of poor immunogenicity can be solved by covalently linking the polysaccharide to a carrier protein which can enhance the response of the
immune system and the production of immunoglobulin G (IgG) [58, 59]. Currently used carrier proteins that are known to be effective and safe include diphtheria toxoid, and the non-toxic diphtheria toxin cross reactive material CRM197. Generating a glycoconjugate vaccine requires the structural identification of the CPS and knowing the possible activation sites in which the carrier protein to be attached [58]. Glycoconjugation has been a successful approach in many commercialized vaccines (Haemophilus influenzae type B, Neisseria meningitides, Streptococcus pneumonia) [60], and it has been shown the presence of antibodies in vaccinated infants after 6 weeks of life [61]. A prototype vaccine against Campylobacteriosis has been developed by the Monteiro group [29], In 2014; the vaccine was approved by the United States Food and Drug Administration for Phase I human clinical trials, and is proved to be safe in humans.

Glycoconjugate vaccine against certain microorganisms remains a challenge due to the variation among serotypes of bacterial strains. In the case of Campylobacter jejuni, research has been conducted to verify the most abundant epitope of the CPS among strains; this requires the structural characterization of the CPS of the most predominant serotypes worldwide. In recent research it was determined that eight of the C. jejuni serotypes are responsible for 50.4% of all of the reported infections. These serotypes are: the HS:4 complex, HS:1/44, HS:2, HS:11, HS:5/31, HS:8/17, HS:6/7, and HS:3 [62]. In addition to C. jejuni strains; we are looking in the production of a multi-agent vaccine containing the antigenic epitopes of the most prevalent serotypes of the major bacterial species involved in gastroenteritis worldwide (C. jejuni, S. flexneri, and E. coli).
1.6. Introduction to the enteric pathogens used in this study for the development of glycoconjugate vaccine

1.6.1. *Campylobacter jejuni* serotype HS:1

The CPS of *C. jejuni* strain HS:1 was characterized fully in 2005 [41]. The CPS as denoted in figure 1.5 is a repeating unit of \( [\rightarrow 4]-\beta-D\text{-Gal}-(1\rightarrow 2)-(R)-\text{Gro}-(1\rightarrow \text{P}] \), that is further substituted with fructofuranose (Fruf) at C-2, and C-3 of the Gal. The Fruf moiety is further substituted with MeOPN at the C-3 position. In this study, three strains that are related to HS:1; HS1 mutant (PG 3588), HS:1/8 (PG 3352), and HS:8 (PG 2827) will be discussed. Strain HS:1/8 has a major importance in both zoonotic and human health perspectives. A monovalent vaccine containing the CPS of this strain conjugated to a carrier protein will be a glycoconjugate prototype vaccine to be used in sheep. The other HS:1 strain in this study is PG 3588, which is a mutant of HS:1, This strain of HS1 is a mutant at the orf 4, which will have a truncated LOS to ensure maximal purification of the CPS. Structural characterization of the CPS and preliminary results for the LOS structure will be presented.
Figure 1. Structure of the CPS of *C. jejuni* HS:1; Gal: Galactose, Fruf: fructofuranose, MeOPN: O-methyl-phosphoramidate.

1.6.2. *Campylobacter jejuni* strain HS:2

*C. jejuni* HS:2 LOS and CPS molecular structures were identified by Michael et al. 2002 [23]. The CPS showed interesting features (Figure 1.6), like the presence of N-acetylgalactosamine in a furanose ring form that was the first report of a NAc sugar in a furanose form in nature, and the L-gluco-heptopyranose which was the first demonstration of the L-gluco conformer in nature as well. There was an observation of glycerol and O-methyl modifications in the CPS repeats as well. In 2003, it was the first report of the novel modification of the CPS which was detected in *C. jejuni* strain HS:2, which is MeOPN, it was detected on the C3 of the β-D-Gal/NAc [35]. In 2007, a study showed that one serotype of HS:2 expresses another MeOPN on the C-4 of the 6-O-Me-
D-glycero-α-L-gluco-Hep [33]. In addition to that, different variants of the *C. jejuni* strain HS:2 expresses another O-Me group at C-3 of the 6-O-Me-D-glycero-α-L-gluco-heptopyranose, and the GlcA amidated at C-6 can be substituted with 2-amino-2-deoxyglycerol, or ethanolamine [33, 35]. All these features that characterize and specify the CPS of HS:2 makes it highly variable, and gives a wide range of possibilities [36]. This study will focus on the characterization of one variant of *C. jejuni* HS:2 CPS and make a prototype vaccine by conjugation to carrier proteins.

\[\alpha-D-GlcA\]

\[\beta-D-Gal/NAc\]

\[\beta-D-Ribf\]

\[D-glycero-\alpha-L-gluco-Hep\]

\[R_1 = H, \text{ or MeOPN}\]

\[R_2 = H, \text{ or CH}_3\]

\[R_3 = 2\text{-amino-2-deoxyglycerol, or ethanolamine}\]

1.6.3. Shigella flexneri 187 serotype 2a

The structure of the O-specific chain of Shigella flexneri type 2a has been previously characterized [4, 48]. It is known to have the same tetrasaccharide backbone that is further glycosylated at the position 4 of rhamnose III, and non-stoichiometrically O-acetylated at C3, and C6 of rhamnose I, and the GlcNAc, respectively as shown in Figure 1.7 [63, 64].

Figure 1. 7. Structure of the O-Ag of S. flexneri serotype 2a; Rha: rhamnose, Glc: glucose, and GlcNAc: N-acetylglucosamine
The O-Ag of *S. flexneri* will be included in a di-agent vaccine against two major bacterial pathogens involved in gastroenteritis (*S. flexneri*, and Enterotoxigenic *Escherichia coli*). ETEC is considered to be of the leading causes of gastroenteritis beside *Campylobacter* and *Shigella* species. The proposed research introduces the conjugation of the TEMPO-mediated oxidized *S. flexneri* O-Ag to the antigenically distinct colonization factor antigens (CFA) from ETEC, and BSA by EDC-mediated coupling.
Chapter 2: Aim of the research

Diarrheal disease is ranked fourth as a cause of death worldwide, and it is increasing to be a leading cause of mortality and morbidity [65]. Of the main microorganisms: Campylobacter, Shigella, and Escherichia species. C. jejuni being responsible for almost 400 million cases annually, and it has shown an increased resistance to antimicrobials that has been used, which makes the development of a multivalent vaccine based on the CPS of the most predominant strains of C. jejuni, that will be then conjugated to a carrier protein; an alternative way to defeat this bacteria.

The current research focuses on the characterization of the CPS of many C. jejuni serotypes; HS:1, HS:1/8, and HS:8 that are genetically related, and the development of a glycoconjugate vaccine against serotype HS:1/8 after. It also reveals the structure of a strain of C. jejuni HS:2, and the development of a glycoconjugate against that strain type.

The research looks into the efficiency and effectivity of conjugating S. flexneri serotype 2a to ETEC via TEMPO mediated oxidation, and EDC-mediated coupling.
Chapter 3: Experimental procedures typically used in the structural characterization of CPS

3.1. Bacterial Growth, Extraction and purification

3.1.1. Extraction of the bacteria from whole cells

3.1.1.1. Campylobacter jejuni serostrains HS:2, HS:1 PG 3588 and PG 3352

The bacterial cells were obtained from the US Naval Medical Department of enteric diseases US NAVY. The cell masses were grown in 10 L of porcine brain heart infusion broth (BHI) media and phenol inactivated. The cell pellet was received as a frozen sample and freeze dried before applying it to a hot water/phenol extraction to obtain the (CPS) [66]. This type of extraction has been proved to be effective and retains the CPS repeating unit structure of various strains of C. jejuni [29, 42, 43, 55]. The extraction was accomplished by dispersing the dried bacterial cells in 500 mL water/phenol mixture (3:2 ratio by volume), and heated at 75 degrees centigrade, with continuous stirring for 6 hours. This was followed by cooling the solution by placing in an ice bath overnight in order to get a complete separation into two layers where the aqueous layer containing the carbohydrates will be on top and the phenol layer on the bottom. The aqueous layer is then extracted and replaced with fresh deionized water and the extraction was repeated for another two days in order to recover as much as possible of the polysaccharides. Although the collected aqueous layer contains the carbohydrates, it is still carrying traces of phenol. The aqueous layer will be then dialyzed against running deionized water overnight using a dialysis bag of a molecular weight cut off
(MWCO) 1000 Dalton in order to get rid of any phenol traces. The resultant liquid is freezed and then freeze-dried for the following step of purification.

3.1.1.2. Shigella flexneri serotype 2a PG 187

The bacterial cells were obtained from US NAVY; they were grown in Luria Broth (LB) flasks. A total of 21.48 g of the wet cell mass was received. It was freeze dried for extraction. The cell mass was extracted by the hot water/phenol method described in 2.1.1.1.

3.1.2. Ultracentrifugation

The obtained polysaccharide for C. jejuni strain HS:2, strain HS:1/8, and strain HS8, were subjected to ultracentrifugation using a Beckman coulter Optima LE-80K ultracentrifuge at 40000 rpm and 4 °C for 24 hours, which allows the separation of the CPS which will be in the supernatant from the gel like pellet containing the LOS. C. jejuni strain HS:1, was ultra-centrifuged at 10000 rpm and 4 °C for 6 hours using Beckman coulter J2-MC. The supernatant containing CPS was collected and freeze dried for the next step of purification. Pellet containing the LOS was dried for further analysis.

3.1.3. Mild Acetic Acid treatment

3.1.3.1. Campylobacter jejuni strain HS:1 LOS

The obtained pellet from ultracentrifugation containing the LOS was subjected to a mild acetic acid treatment to remove the lipid A part that is attached to the LOS [23]. The pellet was dispersed in a 1.5% acetic acid solution and heated to 100 °C with continuous stirring for 2 hours until a precipitate was formed. The solution was then
transferred into a glass centrifuge tube and centrifuged at 4000 × g for 1 hour. The supernatant containing the LPS was then collected and freeze dried for further analysis.

3.1.3.2. *Shigella flexneri* PG187

The dried aqueous layer containing the lipopolysaccharide was subjected to a 1.5% acetic acid treatment at 100 °C for 3 hours to remove lipid A. The solution was then transferred into a centrifuge tube and centrifuged at 4000 × g for 1 hour [63]. The supernatant containing the core polysaccharide was then freeze dried.

3.1.4. Size exclusion chromatography

The obtained polysaccharide from either *C. jejuni* different strains, or *S. flexneri* were further purified through size exclusion chromatography column, using a Bio-Rad P2 gel (Polyacrylamide gel), or G50 gel column, using deionized water as eluent. Positive fractions containing the desired sugar, usually the first fractions after void volume contains the CPS, will then be freeze-dried. The structure identification was confirmed by nuclear magnetic resonance (NMR), and gas chromatography/mass spectrometry (GC/MS) of the alditol acetates derivatives by the monosaccharide composition analysis.

3.2. Chemical Analysis for the structural determination of the polysaccharide

3.2.1. Gas Chromatography/Mass Spectrometry

Sugar composition is determined using the alditol acetate method (Scheme 3.1) [67]. The alditol acetate analysis of polysaccharides begins with hydrolysis of the
polysaccharide to its monomeric sugars using trifluoroacetic acid. The resultant monomeric residues will be reduced by adding sodium borodeuteride (NaBD₄) in water, and the reaction will be left at room temperature overnight. Excess borodeuteride is removed using 5% acetic acid in 95% methanol evaporation three times. The monomeric units are then acetylated at the free hydroxyl sites using acetic anhydride. Alditol acetates are then extracted using dichloromethane (DCM) through a sodium sulfate column to remove any residual acetic anhydride from the acetylated sugars. The resulting alditol acetates are now volatile and can be analyzed by gas chromatography-mass spectrometry (GC-MS). A DB-17 column in a Thermo Finnigan Polaris-Q gas GC-MS was used to detect the relevant alditol acetate results for the C. jejuni HS:2. S. flexneri GC-MS results were conducted at the Advanced Analytical Center (AAC) at the University of Guelph using the Bruker GC-MS running with the scion triple quadrupole (TQ) detector. The MS is operated at 70 eV in the electron ionization (EI) mode. The retention times of the monosaccharides were recognized by comparing to standards developed in our lab.

In gas chromatography, the sample evaporates after injection; the components will be adsorbed on the stationary phase and elutes by the mobile phase (inert gas; helium) in different rates depending on the components physical and chemical properties. Mass spectroscopy will ensure the identification of these components. GC-MS is widely used in the separation and identification of carbohydrates. For carbohydrates to be detected by a GC, they should be volatile. The oligosaccharide will be hydrolyzed in to monosaccharide units, monosaccharides will present in different tautomer in solutions and will give rise to many peaks, therefore, those units will be further reduced to form
stable alditols to give rise to a single peak for each monosaccharide in the chromatogram, which will be further acetylated to be volatile enough for the GC [67].

Primary determination of the individual monosaccharides will be by comparing the retention times resulted from the GC, and the standards that has been known. Analyzing the fragmentation pattern of each individual monosaccharide will confirm the identity. Carbon-carbon bonds of the alditol acetates are cleaved to produce primary fragments, which are further cleaved to give secondary fragments resulting from the loss of acetic acid (60 mass units), or the loss of ketene (42 mass units).
Scheme 3.1. Monosaccharide composition analysis, the alditol acetate method.
3.2.2. Nuclear magnetic resonance experiments (NMR)

One dimensional (1D) $^1$H, and $^{31}$P NMR experiments were conducted using a Bruker AMX 400 MHz spectrometer equipped with a Bruker Cryoprobe™ prodigy probe at 297 K. Two dimensional (2D) experiments were conducted on Bruker AVANCE ultra-shield 600 MHz spectrometer equipped with cryoprobe at 297 K unless it is otherwise stated. Samples were prepared by exchanging with deuterium oxide (D$_2$O) three times before it was transferred to NMR tube using a 600 µl of D$_2$O. External standards that were used: 3-trimethylsilyl-tetradequeto sodium propionate (TSP) δ$_H$ 0 ppm for $^1$H NMR, and ortho-phosphoric acid was used as the standard for $^{31}$P NMR (δ$_p$ 0 ppm). 1D and 2D NMR experiments were used in order to identify and confirm sugar moieties in our polysaccharides. The HOD peak at room temperature may interfere with the anomeric resonances and running the experiments under high temperatures will displace it upfield which expose the anomeric resonances underneath it. A series of 2D experiments were conducted to assist in structural characterization. The following 2D experiments were performed: $^1$H-$^1$H correlation spectroscopy (COSY), $^1$H-$^{13}$C Heteronuclear single quantum correlation spectroscopy (HSQC), $^1$H-$^{13}$C Heteronuclear multiple bond correlation spectroscopy (HMBC), and $^1$H-$^{31}$P HMBC NMR.

1D $^1$H spectra NMR analysis (Figure 2.2) provides general information on the carbohydrate components. It reveals the anomeric configuration of the monosaccharide residues in the polysaccharide chain. The anomeric proton region is typically found at the region δ 4.4-5.5 ppm, while other ring proton resonances found in the region δ 3.0-4.2 ppm [68-70]. Normally, α-anomers have a downfield shift compared to a β-anomer. In addition, the vicinal coupling constants can aid in the determination of the configuration,
where the $\alpha$- anomeric protons shows a small coupling constant (1-4 Hz), and the $\beta$-anomeric protons has a large coupling constant (7-8 Hz) [68]. The information of some non-sugar moieties can be obtained like the NAc, OAc, and O-Me group presence in the repeating unit. $^{31}$P NMR aids in revealing the phosphorylated substituents; phosphodiester groups ($\delta_P$ -1.00 -1.50 ppm), and MeOPN groups at around ($\delta_P$ 13.50-14.80 ppm) ppm. $^{13}$C NMR also reveals characteristic chemical shifts; anomeric carbons at the range 90-110 ppm, hydroxymethyl carbons 60-65 ppm, the 6-deoxy methylene carbons 30-35 ppm, and the region 170-176 ppm reflects the presence of either a carbonyl of NAc, or a carboxylic group [70]. 1D Total Correlation Spectroscopy TOCSY is a technique that gives correlations between all protons of the same spin system, one peak is selected and usually is the anomeric proton and will be irradiated and it will detect all the protons in a stepwise process in the same spin system within the ring. Although 1D experiments provides a wide range of information, further structural characterization using a 2D NMR experiments is required.
Figure 3.1. Illustration of chemical shifts of carbohydrates in a 1D $^1$H NMR spectra.

2D NMR experiments for fully assigning each monosaccharide protons in a single ring system using $^1$H-$^1$H COSY, where it usually starts from assigning one known proton resonance (anomeric, or a methyl resonance in the 6-deoxy sugars) then correlating the ring protons in a step-wise manner. COSY for more than two monosaccharides, usually has a bulk in the ring region and prevents the assignments of all protons in the spin system. 2D $^1$H-$^{13}$C HSQC generates cross peaks for two different atoms separated by one bond. In this experiment we can detect the number of ring systems, and correlate each
proton previously assigned by a COSY to its carbon. 2D HMBC experiments identify proton nuclei with either carbon in the case of $^1$H-$_{13}$C HMBC, or phosphorous in the case of $^1$H-$_{31}$P HMBC; that are separated by more than one bond.

3.3. Synthesis of the Capsular Polysaccharide Glycoconjugate Vaccine

3.3.1. Periodate oxidation of the CPS of *C. jejuni* Strain HS:2 and reductive amination of the oxidized CPS to a carrier protein

Oxidation of *C. jejuni* HS:2 CPS was activated using a fresh buffer solution of 0.04 M sodium metaperiodate (NaIO$_4$) in 0.1 M sodium acetate (NaOAc) [29]. The pH is adjusted to 4.0 using glacial acetic acid and was left at 4 °C for 72 hours. The oxidation was then halted using ethylene glycol. The excess of the unoxidized material was cleared out by dialyzing the solution against running deionized water using a molecular weight cut off 1000 Da. The resultant oxidized CPS is then freeze-dried.

Reductive amination of the oxidized CPS was then conducted. Fresh borate buffer was prepared by dissolving boric acid and borax in deionized water, pH of the solution is adjusted to 9.00. Carrier protein CRM$_{197}$ was added in a ratio of 2:1 CPS$_{HS:2}$: CRM$_{197}$. The compounds were dissolved in buffer solution, and the reducing agent sodium cyanoborohydride was added. The mixture was stirred at room temperature for 24 hours, followed by 48 hours of stirring at 37 °C. The resulting conjugate was then dialyzed against running deionized water using a dialysis bag of a molecular weight cut off 25,000 Da for 2 days, and then freeze-dried. Periodate oxidation is based on the presence of vicinal diols in the repeating saccharide, where the oxidizing agent periodate will form two aldehyde groups by breaking the C-C bond as illustrated in scheme 3.2. The
reductive amination will involve the attack of the nitrogen atom to the carbonyl carbon of the aldehyde to form an intermediate carbinolamine that will lose H$_2$O to form an imine. This imine will be further reduced by a reducing agent sodium cyanoborohydride to form the secondary amine [71].

**Scheme 3. 2.** Schematic representation of the periodate oxidation of vicinal diols followed by reductive amination of the aldehyde to the primary amine (carrier protein) to form a secondary amine [72].

**3.3.2. TEMPO-mediated oxidation of CPS and EDC coupling of the oxidized CPS to a carrier protein**

Oxidation of polysaccharides in aqueous media on the basis of using stable nitroxy radicals like 2,2,6,6-Tetramethylpiperidine-1-oxyl radical (TEMPO), which acts as a secondary oxidant that targets primary alcohols and oxidizes them to carboxylates in
the presence of a primary oxidant like hypochlorite [73]. This method was developed and improved in the Monteiro research lab to oxidize CPS derived from different bacteria, scheme 3.3 [74, 75]. This technique was successfully improved to oxidize only 2-3 monosaccharide units in each saccharide chain to avoid the disruption of the repeating unit. TEMPO oxidation was applied to CPS from C. jejuni strain HS:2, HS1/8, and S. flexneri O-Ag. General procedure followed was: 0.1 mg of TEMPO suspended in a 2 ml 1 M sodium acetate (NaOAc) buffer, and 1.5 mg of sodium bromide to be added to the CPS, and stirred in an ice bath at 0 °C. 10 µL sodium hypochlorite was then added and left for 24 hours. The reaction will be stopped by the addition of ethanol, and the oxidized CPS will be dialyzed using dialysis bags (MWCO 1 KDa) under running deionized water for 24 hours, and it will be collected and freeze dried after. The coupling of the activated CPS was achieved by dissolving the oxidized CPS in 2 ml of 0.5 M 2-(N-morpholino)-ethanesulfonic acid (MES) buffer, and the pH of the solution was adjusted to around 5.5 using 0.5 M HCl, followed by the addition of 10 µL of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), which then will be followed by the addition of the carrier protein. The reaction vial stirred at room temperature for 24 hours, then stirred for another 48 hours at 37 °C. The conjugate then was dialyzed against running deionized water using dialysis bags (MWCO 25.000 Da) to remove unconjugated protein for 3 days. The conjugate was then collected, freeze dried, and sent to the NAVY to be further analyzed.
Scheme 3.3. Schematic diagram of TEMPO-mediated oxidation of a monosaccharide [73], where hydroxyl amine, nitroxy radical, and nitrosonium ion are redox TEMPO forms

3.4. Immunogenicity studies of the prototype Vaccines

3.4.1. Gel Electrophoresis (SDS-PAGE) analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. This was done by the US NAVY. This technique depends on the mass and charge of the molecule that controls the separation on a gel which will be stained with a staining reagent (Coomassie Brilliant Blue). The vaccine should be separated at a higher molecular weight than the CPS, or the carrier protein alone.

3.4.2. Immunoblot Studies of Conjugate Vaccines

The immunoblot analysis was performed in the US NAVY, using western blot analysis to transfer the glycoconjugate on a SDS-PAGE gel to nitrocellulose membrane.
The incubated membrane blotted with the glycoconjugate was then incubated with whole cell anti-sera from the bacterial strains used to serve as the primary antibodies (*C. jejuni* strains HS:2, HS1/8 whole cells), and *S. flexneri* used commercial anti-Shigella serum. The horseradish peroxidase-coupled goat anti-rabbit IgG was used as a secondary antibody. The membrane was then washed with a coloring stain TMB (SkyTeK). An image will then be obtained.
Chapter 4: Results and Discussion: The Capsular Polysaccharides of *C. jejuni*
serotypes HS:1, HS 1/8, and HS:8 and the conjugation and immunology of the CPS vaccine of HS:1/8

4.1. Structural characterization of the CPS of *C. jejuni* strains HS:1, and HS:1/8

4.1.1. Isolation and purification of the CPSs

The cell paste of all strains *C. jejuni* serotype HS:1 (PG 3588), HS:1/8 (PG 3522), and HS:8 (PG 2827) were obtained from the US NAVY. The cell masses were extracted with the regular hot water/phenol extraction [66]. The aqueous layer was then collected, and ultra-centrifuged to get the supernatant containing the CPS, and the gel like pellet containing the LOS and traces of CPS as well. The aqueous layer was further purified through a P2 size exclusion chromatography. The pellet was then acid treated with 1.5% acetic acid. The supernatant containing the sugar was then diluted with dH$_2$O and centrifuged at a 5000 g for 3 hours to be freeze dried. Further purification was then obtained using a G50 gel column. Positive fractions were collected for further analysis.

4.1.2. NMR Analysis for the CPS of *C. jejuni* strain HS:1 (PG 3588)

1D $^1$H NMR (Figure 4.1 A) was obtained on the CPS to confirm the identity of the repeating unit, which looked exactly as the previously characterized HS:1 CPS [41], and showed in Figure 1.5 showing two alpha anomic protons at $\delta$ 5.21 (residue A), and $\delta$ 5.39 (residue A'). The anomic peaks suggested as Gal with (residue A) and without (Residue A) substitution at the C-2, and C-3 positions with Fru$^f$ [41]. $^{31}$P NMR (Figure 4.1 B) showed signals as expected at $\delta_p$ 1.12 for the phosphates, and surprisingly it showed two distinct peaks at the region of MeOPN $\delta_p$ 14.14, and $\delta_p$ 14.50 ppm, which
differs from the reported MeOPN of the *C. jejuni* strain HS:1 CPS that has one MeOPN in its structure. Fruf residue of *C. jejuni* is acid labile substituent as has been reported previously [41], Fruf being an acid labile group and knowing that MeOPN is attached at the C-3 of it; a mild acid treatment was obtained on the CPS to get a defructosylated CPS, in which if the MeOPN is retained after that means that the other MeOPN is attached to the Gal residue. 1D $^1$H NMR of the defructosylated CPS (Figure 4.2 A) showed the anomeric resonance at $\delta$ 5.21 that corresponds to the $\alpha$-D-Gal residue without the Fruf substitution. 2D $^1$H-$^1$H COSY (Figure 4.2 B) was obtained to assign the ring protons (H2 $\delta$ 3.88, H3 $\delta$3.90, H4 $\delta$4.54). H5 $\delta$ 4.18 was assigned from the H6 $\delta$ 3.75 proton resonance. Gro resonances were found to be H1/1′ $\delta$ 4.05/4.12, H2 $\delta$ 3.98, and H3/3′ 3.78/3.82. 1D $^1$H TOCSY (Figure 4.3) were obtained to further confirm the assignments, where the anomeric proton when irradiated was able to observe H2, H3, and H4. Further irradiation of H4 observed H5, which was able to observe H6-6′ at $\delta$ 3.75. All carbon resonances were assigned using a 2D $^1$H-$^{13}$C HSQC (Figure 4.3). All proton and carbon resonances are summarized in table 4.1. The defructosylated CPS retained one peak at the region of MeOPN, which suggested that it is attached to the Gal residue. 2D $^1$H-$^{31}$P HMBC (Figure 4.4) showed a strong cross peak at ($\delta_H$ 4.54/ $\delta_P$ 1.14), and ($\delta_H$ 4.05, 4.11/ $\delta_P$ 1.14) confirmed the presence of the phosphodiester and its attachment to the Gro at the C4 of Gal through a phosphodiester, which is consistent with the previously characterized HS:1 CPS type strain of having a teichoic-acid polysaccharide. The cross peak at $\delta$ 14.04 which corresponds to MeOPN, showed a cross peak at ($\delta_H$ 3.75/$\delta_P$ 14.04) that suggested the attachment of MeOPN at the C6 of Gal, and this is the first time to report such a connection in a variant of HS:1.
Full assignment of the defructosylated CPS made it easier to fully assign the sugars of the intact CPS of strain PG 3588. 2D $^1$H-$^{31}$P HMBC was conducted to reveal the MeOPN connection (Figure 6.6). It shows a strong cross peak at ($\delta_H$ 4.84/$\delta_P$ 14.45), and ($\delta_H$ 3.74/$\delta_P$ 14.20) which suggests the attachment of MeOPN at C3 position of the Fru$_f$ and the C6 of the Gal residue, respectively.

![Figure 4.1](image)

**Figure 4.1.** A: 1D $^1$H NMR spectrum of the CPS of *C. jejuni* strain PG 3588, B: 1D $^{31}$P NMR spectrum of *C. jejuni* strain PG 3588 CPS; A: Gal, A’: Gal substituted with Fruf at C-2, and C-3. NMR experiments were conducted at 297 K on a 400 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.
Figure 4. 2. A: $^1$H NMR, B: 2D $^1$H-$^1$H COSY of *C. jejuni* strain PG 3588 defructosylated CPS (A: $\alpha$-D-Gal, B: Gro). The NMR experiments were obtained at 297 K on a 600 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.
Figure 4. 3. 1D TOCSY of C. jejuni strain PG 3588 defructosylated CPS. * represents the irradiated peak of interest (A: α-D-Gal), on a 600 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.
Figure 4. 4. A: $^1$H-$^{13}$C HSQC of C. jejuni strain PG 3588 defructosylated CPS, B: is the magnification for the area $\delta$ 3.70-$\delta$ 3.90 to show the methyl cross peaks of the MeOPN (A: $\alpha$-D-Gal, B: Gro). The NMR experiment was obtained at 297 K on a 600 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.

Figure 4. 5. $^1$H-$^{31}$P HMBC NMR of C. jejuni defructosylated CPS strain PG 3588 (A: $\alpha$-D-Gal, B: Gro) on a 400 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.

Table 4. 1. Compiled $^1$H, and $^{13}$C NMR chemical shifts of C. jejuni PG 3588 defructosylated CPS. All NMR data were obtained at 297 K.

<table>
<thead>
<tr>
<th>Sugar residue</th>
<th>H1/1′ C1</th>
<th>H2 C2</th>
<th>H3/3′ C3</th>
<th>H4 C4</th>
<th>H5 C5</th>
<th>H6/6′ C6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-D-Gal</td>
<td>5.21</td>
<td>3.88</td>
<td>3.90</td>
<td>4.54</td>
<td>4.18</td>
<td>3.75</td>
</tr>
<tr>
<td></td>
<td>100.84</td>
<td>71.05</td>
<td>71.10</td>
<td>77.31</td>
<td>73.45</td>
<td>63.42</td>
</tr>
<tr>
<td>Gro</td>
<td>4.05/4.12</td>
<td>3.98</td>
<td>3.78/3.82</td>
<td>63.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>67.23</td>
<td>79.81</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Following the assignment of the defructosylated CPS of *C. jejuni* HS:1 PG 3588, it was easier to assign the intact CPS. 2D $^1$H-$^{13}$C HSQC (Figure 4.6 A), and $^1$H-$^{31}$P HMBC (Figure 4.6 B) aided in the confirmation of the structure. *C. jejuni* HS:1 PG 3588 is a repeating unit of $[\rightarrow 4]-\beta-D-Gal-(1\rightarrow 2)-(R)-Gro-(1\rightarrow]$ that is further substituted with Fruf at C-3 and C-4 of the Gal, it has two MeOPN residues one is attached at the C-3 position of Fruf, and the other MeOPN is attached at the C-6 of the Gal which is the first report of such an attachment of MeOPN in HS:1. Table 4.2 has the proton and carbon chemical shifts for the intact CPS.
Figure 4.6. A $^1$H-$^{13}$C HSQC NMR (600 MHz), B $^1$H-$^{31}$P HMBC (400 MHz) spectrum of C. jejuni PG 3588, A is $\alpha$-D-Gal, A' is Gal with residue C Fru. NMR experiments were obtained at 297 K. The sample was dissolved in 600 µl D$_2$O.

Table 4.2. Compiled $^1$H, and $^{13}$C NMR chemical shifts of C. jejuni HS:1 PG 3588 (δ, ppm); All Chemical shifts were obtained at 297 K.

<table>
<thead>
<tr>
<th>Sugar residue</th>
<th>H1/H1'</th>
<th>H2</th>
<th>H3/H3'</th>
<th>H4</th>
<th>H5</th>
<th>H6/H6'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1</td>
<td>C2</td>
<td>C3</td>
<td>C4</td>
<td>C5</td>
<td>C6</td>
</tr>
<tr>
<td>$\alpha$-Gal with Fru'</td>
<td>5.39</td>
<td>4.27</td>
<td>4.34</td>
<td>4.69</td>
<td>4.18</td>
<td>3.74/3.75</td>
</tr>
<tr>
<td></td>
<td>100.59</td>
<td>70.25</td>
<td>71.16</td>
<td>79.03</td>
<td>73.58</td>
<td>63.58</td>
</tr>
<tr>
<td>$\alpha$-Gal</td>
<td>5.21</td>
<td>3.89</td>
<td>3.89</td>
<td>4.54</td>
<td>4.18</td>
<td>3.74/3.75</td>
</tr>
</tbody>
</table>
4.1.3. Preliminary NMR results for the LOS of *C. jejuni* strain HS:1 PG 3588

The 1D $^1$H NMR of the *C. jejuni* strain PG 3588 LOS revealed anomeric protons in the region δ 4.40 - δ 5.50 (Figure 4.7). It showed the ring proton resonances at the region δ 3.20 - δ 4.60, which was crowded as a result of overlapping. 2D $^1$H-$^{13}$C HSQC (Figure 4.9) helped in assigning the anomeric peaks. It showed three alpha resonances at δ 5.42, δ 5.10, and at δ 5.06 that correspond to two L-glycero-α-D-manno-heptose, and α-Gal, respectively. Another three beta resonances were noted at δ 4.88, δ 4.93, and δ 4.44 that corresponds to two β-Glc, and β-Gal compared to the previously characterized LOS [76,77]. 1D $^{31}$P NMR (Figure 4.8) was obtained to see if the LOS retained the phosphoenalolamine (PEA) residues. It showed interestingly 2 peaks that correspond to PEA at δ 1.30, and δ 2.26 ppm, and a phosphate group at δ -0.396 which is carried over from lipid A. Although 2D $^1$H-$^{13}$C HSQC (figure 4.9) confirmed the absence of the characteristic peaks corresponds to the Neu5Ac at the region δ 1.7, and δ 2.5 ppm. *C. jejuni* PG 3588 LOS was compared with other mutants of the HS:1 strain (Figure 4.10). It showed that the LOS of the wild type strain HS:1( Figure 4.10; Lane 1) has a higher molecular weight than the mutated strains, and the strain PG 3588 (Figure 4.10; Lane 3) has even a lower molecular weight than the strain that has been known to lack Neu5Ac.
Electron spray ionization mass spectroscopy (ESI-MS) was done on *C. jejuni* PG 3588 LOS by the AAC at the University of Guelph to detect the molecular weight. Figure 4.11 A that has the ESI-MS ran in a positive ion detection mode showed a peak at m/z 1416 that corresponds to a Kdo, 4 hexoses, 2 heptoses, and a PEA plus sodium ion. In addition, ESI-MS was ran in the negative ion detection to confirm the findings and it showed a peak at m/z 1392 that corresponds to the presence of a Kdo, 4 hexoses, 2 heptoses, and a PEA. All the previous results confirmed the site of mutation and the occurrence at the outer core region of the LOS, it also proved the absence of the sialic acid part in the LOS. The LOS of *C. jejuni* HS:1 PG 3588 structure is proposed in Figure 4.12.

![Figure 4.7. 1D $^1$H NMR of the LOS of *C. jejuni* strain PG 3588. The NMR experiment was obtained at 297 K on a 600 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.](image)
Figure 4. 8. 1D $^{31}$P NMR of the LOS of *C. jejuni* strain PG 3588. The NMR experiment was obtained at 297 K on a 400 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.

Figure 4. 9. 2D $^1$H-$^1$C HSQC of *C. jejuni* PG 3588 LOS. The NMR experiment was conducted at 297 K on a 600 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.
Figure 4. SDS-PAGE showing the estimated molecular weight of the; Lane 1: LOS of *C. jejuni* HS:1 wild type; Lane 2: LOS of a *C. jejuni* HS:1 mutant that lacks the Neu5Ac (Cst mutant), and Lane 3: LOS of *C. jejuni* PG 3588.
Figure 4. ESI-MS results of *C. jejuni* PG 3588 LOS, where A: is in the positive ion detection, and B: is in the negative ion detection.

### 4.1.4. NMR analysis of *C. jejuni* strain HS:1/8 (PG 3352)

*C. jejuni* strain HS:1/8 as it is denoted from the name, is related genetically to HS:1. This strain capsular locus looks exactly the same as HS:1, with minor differences in genes. 1D $^1$H NMR of the *C. jejuni* strain PG 3352 (Figure 4.7) showed two alpha anomeric protons at δ 5.21 (residue A), and δ 5.39 (residue A'). It showed a ring region between δ 3.22- δ 4.88 ppm, which is consistent with the published structure of HS:1 [41]. The anomeric peaks suggested as Gal with (residue A') and without (residue A) substitution at the C-2, and C-3 positions with Fruf [41]. In order to further confirm the structure, a 2D $^1$H-$^{13}$C HSQC was obtained (Figure 4.13). 1D $^{31}$P NMR (Figure 4.12 B) was obtained in order to confirm the whole integrity of the CPS. It showed signals as expected at δ_p 1.12 for the phosphates, and it showed two distinct peaks at the region of
MeOPN $\delta_p$ 14.14, and $\delta_p$ 14.50 ppm, which has the same chemical shifts of the characterized HS:1 PG 3588. In order to confirm the attachment sites of MeOPN, 2D $^1$H-$^{31}$P HMBC was obtained. The cross peaks at the MeOPN region were ($\delta_H$ 4.84/ $\delta_P$14.50) originated from the MeOPN attached to the C3 of the Fruf' being the most predominant peak, and the other cross peak was at ($\delta_H$ 3.75/ $\delta_P$ 14.14), which suggests the attachment of the MeOPN at the C-6 position of the Gal as has been proved previously for C. jejuni HS:1 PG 3588. In addition, the 2D $^1$H-$^{31}$P HMBC showed a strong cross peak at ($\delta_H$ 4.54/ $\delta_P$ 1.14), and ($\delta_H$ 4.05, 4.11/ $\delta_P$ 1.14) confirmed the presence of the phosphodiester and its attachment to the C1/C1’ of the glycerol, and to the C4 of the Gal.
Figure 4. 13C NMR of the C. jejuni HS1/8 PG 3352 (A), and 31P NMR (B). The NMR experiment was acquired at 297 K on a 400 MHz spectrometer. The sample was dissolved in 600 µl D₂O.

Figure 4. 14C HSQC NMR for C. jejuni PG 3352 CPS, where A, A': Gal without and with Fruf. B: Gro, and C, C': Fruf with out and with MeOPN substitution. The experiment was done at 297 K on a 600 MHz spectrometer. The sample was dissolved in 600 µl D₂O.
Figure 4. 15. 2D $^1$H-$^{31}$P HMBC NMR for the C. jejuni HS:1/8 PG 3352 CPS, A, A’ Gal with out and with Fruf, B: Gro, C': Fruf with MeOPN. The NMR experiment was acquired at 297 K on a 400 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.
4.1.5. Synthesis of the glycoconjugate vaccine of *C. jejuni* strain HS:1/8 PG 3352 CPS

The glycoconjugate vaccine was obtained by the method TEMPO-mediated oxidation followed by EDC-mediated coupling. The oxidation was accomplished using 10% TEMPO to the possible primary alcohols presented most probably at the primary hydroxyl groups presented at either C1, or C6 of the Fru\(\text{f}\). The integrity of the CPS was confirmed by NMR (Figure 4.15). 1D \(^1\text{H}\) NMR showed reduction in the anomeric peak of residue \(\text{A}'\) that suggested the occurrence of the oxidation at the Fru\(\text{f}\). The following step was to conjugate to a proper carrier protein CRM\(_{197}\) to form the vaccine, and this was obtained by EDC-mediated coupling.
Figure 4. 16. 1D $^1$H NMR of *C. jejuni* strain HS1/8 PG 3352 A: $^1$H NMR of the intact CPS, and B: TEMPO-mediated oxidized CPS. The experiment was acquired at 297 K on a 600 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.

SDS-PAGE analysis was carried out and showed that the *C. jejuni* CPS$_{HS1/8}$-CRM$_{197}$ was not visualized on the SDS-PAGE as shown in (figure 4.16 Lane 2). The absence of any bands through the PAGE not even a band corresponding to unconjugated CRM$_{197}$ was surprising, taking in to account the immunoblot analysis results of the antisera raised against the whole cells of *C. jejuni* type strain HS:1, which recognized the CPS$_{HS1/8}$-CRM$_{197}$ conjugate vaccine (Figure 4.17; lane 2). This finding suggested the presence of the vaccine. The immunoblot shows some reactivity against CRM$_{197}$ (Figure
4.17; lane3), which has been seen previously and can be due to previous exposure of the rabbits to CRM$_{197}$.

**Figure 4.17.** SDS-PAGE analysis of the *C. jejuni* TEMPO oxidized CPS$_{HS1/8}$-CRM$_{197}$ conjugate (Lane 1: Precision plus protein markers; Lane 2: CPS$_{HS1/8}$-CRM$_{197}$ conjugate; Lane 3: CRM$_{197}$).
Figure 4. 18. Immunoblot analysis of the C. jejuni TEMPO oxidized CPS\textsubscript{HS:1/8}-CRM\textsubscript{197} conjugate with antisera raised against C. jejuni HS:1 whole cells (Lane 1: Precision plus protein markers; Lane 2: CPS\textsubscript{HS:1/8}-CRM\textsubscript{197} conjugate; Lane 3: CRM\textsubscript{197}; Lane 4: C. jejuni type strain HS:1 whole cells)

4.2. Structural characterization of the CPS of C. jejuni strain HS:8 PG 2827

C. jejuni strain HS:8 is genetically related to C. jejuni strain HS:1/8 having some differences in the genetic locus. The genetic locus contains genes required to make heptoses and deoxy-heptoses, and it has no genes responsible for MeOPN transferase [77]. The purification of C. jejuni HS:8 was so complicated and needed multiple ultracentrifugation, and many applications of size exclusion chromatography, yet I was not able to separate the CPS from the N-linked glycans and parts of the LOS. N-linked glycans has been seen in many C. jejuni strains [22, 41, 42].
4.2.1. Monosaccharide composition analysis by GC-MS

Monosaccharide composition analysis was obtained by the alditol acetate method. The GC profile (Figure 4.18) showed that the CPS of *C. jejuni* strain HS:8 was composed mainly of galactose, 3-O-Me-6d-altro-Hep, 3-O-Me-6d-gulo-Hep, 6d-altro-Hep. It also showed traces of glucose and mannose originating from fructose that completely converts in to a mixture of mannitol and glucitol during the reduction step in alditol acetates. GC profile also showed ribose, GalNAc, and L-glycero-D-manno-Hep from LOS. The sugar ring configuration of 3-O-Me-6d-altro-Hep, 3-O-Me-6d-gulo-Hep, and 6d-altro-Hep were assigned by comparing the relative retention time with alditol acetate standards prepared in our laboratory.

GC-MS profile for each monosaccharide was investigated to confirm the identity of each monosaccharide. The fragmentation pattern of Glc originating from the reduction of fructose, and Gal (Figure 4.19 A, B) showed the characteristic primary fragments of a hexose from the cleavage of acetoxyl-carrying carbon atoms, including m/z 362, m/z 290, m/z 218, and m/z 146. Whereas secondary fragments were observed from loss of acetic acid, and ketene, such as m/z 188, m/z 128, m/z 86 from the primary fragment m/z 290, m/z 302, m/z 260, m/z 200, and m/z 140 from the m/z 362.
The fragmentation pattern in figure 4.20 A, and B showed the mass spectra of two 3-O-Me-6d-heptitol acetates including 3-O-Me-6d-altro-Hep, 3-O-Me-6d-gulo-Hep, respectively showed primary fragments resulting from the cleavage between carbon-carbon bonds carrying methoxyl group of the alditol acetates. Primary peaks m/z 190, m/z 275 were mainly detected from the side of the methoxyl bearing carbons. Secondary fragments were detected by loss of acetic acid (60 mass units), or the loss of ketene (42 mass units). Secondary fragment such as m/z 155, m/z 130 were from the primary fragments m/z 215, and m/z 190, and secondary fragment m/z 88 was from m/z 130, and the fragment m/z 113 from m/z 155 by the loss of ketene.

The fragmentation pattern at 41.32 minutes (Figure 4.21) of 6d-altro-Hep has the primary fragments m/z 375, m/z 303, m/z 231, m/z 290, m/z 218, m/z 159, m/z 146, and m/z 73, and the secondary fragments resulting from the loss of ketene, or acetic acid such as m/z 171, and m/z 129 from the primary fragment m/z 231, m/z 129, the fragments m/z 188, and m/z 128 were from the primary fragment m/z 290, and the fragments m/z 210, m/z 141 were from the primary fragment m/z 303.
Figure 4.20. Fragmentation pattern of the alditol acetates of A: glucose, and B: galactose
4.2.2. NMR results

1D $^1$H NMR spectrum of C. jejuni HS:8 CPS (Figure 4.22 A) showed broad overlapping peaks between δ 4.80 - δ 5.50 ppm, which represents the anomeric protons. These overlapping peaks suggested the presence of α and β anomeric sugars. Further 2D NMR experiments are required to confirm the identity of the anomeric protons. It showed also proton resonances at δ 1.94, and δ 2.07, which are characteristic of the 6-deoxy moiety originating from 3-O-Me-6d-altro-Hep, 3-O-Me-6d-gulo-Hep, and 6d-altro-Hep. Other proton resonances were observed at δ 3.47 - δ 3.52 originating from the methyl
group from 3-O-Me-6d-altro-Hep, and 3-O-Me-6d-gulo-Hep. The proton resonance between δ 3.50 and δ 4.90 represents the overlapping sugar ring proton resonances that needs further 2D NMR experiments. 1D $^{31}$P NMR was obtained in order to check for the presence of any phosphorus substituents (Figure 4.22 B). It showed a phosphorous resonance at $\delta_P$ 1.11 which suggests the presence of a phosphodiester bridge that has been seen in both *C. jejuni* strains HS:1, and HS:1/8. Although the genetic locus of strain HS:8 didn’t contain any genes responsible for the synthesis of MeOPN, $^{31}$P NMR revealed the presence of MeOPN groups that can be seen at the resonance $\delta_P$ 14.20, which has been seen in *C. jejuni* strains HS:1, and HS:1/8, where the MeOPN was attached to the C-6 of the Gal.

2D $^1$H-$^1$H HSQC revealed the presence of the anomeric resonances corresponds to the α-Gal with (residue A') and without (residue A) the Fruf substitution at $\delta$ 5.39, and $\delta$ 5.23, respectively. It also revealed the presence of another 6 anomeric resonances that originates from the three heptoses noted from the GC-MS results. This finding suggests that one monosaccharide or more expressed in the repeating unit might have different substitutions as it was seen with the Gal residue in *C. jejuni* HS:1 when represents two anomeric shifts corresponds to the Gal with and without the substitution with Fruf.
Figure 4. 1D NMR of the CPS of *C. jejuni* strain HS:8 A: $^1$H NMR, B: $^{31}$P NMR. NMR experiments were obtained at 297 K on a 400 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.
Figure 4. 2D $^1$H-$^{13}$C HSQC NMR for the CPS of C. jejuni strain HS:8 PG 2827; A’, and A are α-Gal with and without Fruf substitution. The NMR experiment was obtained at 297 K on a 600 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.
4.2.3. Summary of the proposed structure of *C. jejuni* strain HS:8 PG 2827 CPS

The CPS of *C. jejuni* strain HS:8 is a complicated structure that has a similar backbone structure as *C. jejuni* strain HS:1. It has the α-Gal with the addition of two fructofuranose residues at C-2, and C-3 of the Gal. The CPS has another three monosaccharides; 3-O-Me-6d-altro-Hep, 3-O-Me-6d-gulo-Hep, 6d-altro-Hep that has been seen in the GC-MS and further confirmed the presence of more anomeric resonances in the NMR spectra. It has at least one MeOPN group as has been depicted from $^{31}$P NMR spectra that is most probably attached to the C-3 of the Fru$f$ residues due to the fact that it has the same phosphorus chemical shift seen above in *C. jejuni* HS:1, and HS:1/8, and the presence of the cross peak ($\delta_H$ 4.88, $\delta_C$ 81.81) in the $^1$H-$^{13}$C HSQC that has the same chemical shift as the C-3 of the Fru$f$ with MeOPN. The structure of *C. jejuni* strain HS:8 is a complicated structure that needs extensive experiments includes more purification and followed by an extensive 2D NMR experiments, and linkage type analysis.

4.1.6. Future work

*C. jejuni* strain HS:1 PG 3588, and HS1/8 CPS structure has revealed a CPS structure that is identical to that of the previously characterized *C. jejuni* strain HS:1 [2]. It has the teichoic acid-like repeating unit which consists of 4-substituted α-galactose and a glycerol phosphate [\(\rightarrow4\)-α-Gal-(1→2)-Gro-(1-], with the addition of two fructofuranose residues at C-2, and C-3 of the Gal each of which is further substituted with MeOPN residue at the C-3 position. Both strains have an extra MeOPN at the C-6 of the Gal, which is the first report of such a connection in HS:1. *C. jejuni* strain HS1/8 has been a problematic strain in the last few years, it was responsible for most of abortion
cases in sheep, and it started to appear in human isolates. In order to make a prototype vaccine against this type strain, The CPS was oxidized and conjugated by the TEMPO-mediated oxidation, followed by EDC-mediated coupling to the carrier protein CRM\textsubscript{197}. The vaccine was not recognized by the SDS-PAGE although there was a mild immunogenic activity in the immunoblot with antisera raised against whole cells of \textit{C. jejuni} strain HS:1. This might be as a result of an inadequate oxidation sites at the repeating unit to attach with the protein and that would be solved by using higher concentrations of TEMPO in the oxidation. \textit{C. jejuni} strain HS:8 which is genetically related to strain HS:1 has revealed the presence of the \textit{α}-Gal backbone that is further substituted to Fruf at C-2, and C-3, and this CPS structure needs further purification and experimental analysis to characterize the full CPS structure.
Chapter 5: Results and discussions: Analysis of *C. jejuni* strain HS:2 CPS, conjugation and immunology of HS:2 vaccine

5.1. Structural Characterization of *C. jejuni* serostrain HS:2 CPS

5.1.1. Isolation and purification of the CPS of *C. jejuni* strain HS:2

The cell paste was obtained from the US NAVY. The cells were then extracted in a hot water/phenol method [66]. The aqueous layer obtained was then dialyzed against deionized water. Ultracentrifugation at a 40000 rpm for 24 hours at 4 °C followed. The supernatant was then further purified using a P-2 gel column. Positive fractions were then freeze dried and saved for further analysis.

5.1.2. Monosaccharide composition analysis by GC-MS

Monosaccharide composition was achieved by the alditol acetate analysis method [78]. The retention times of the purified CPS were compared to commercial available sugar standard derivatives on GC, and by interpreting the fragmentation pattern on GC/MS. The GC profile (Figure 5.1) revealed that the CPS extracted from the *C. jejuni* strain HS2 was composed of ribose (Rib) at 27.49 min, glucose (Glc) at 36.99 min, N-acetyl-galactosamine (GalNAc) at 52.44 min. In addition, the GC profile showed the presence of almost equal amounts of 3,6-di-O-methyl-D-glycero-L-gluco-heptopyranose at 44.09 min, and 3-O-methyl-D-glycero-L-gluco-heptopyranose at 47.77 min. The monosaccharide composition analysis of *C. jejuni* HS:2 revealed the same CPS composition as it was previously published [23, 33, 79].
GC-MS profile for each monosaccharide was investigated to confirm the identity of each monosaccharide. The fragmentation pattern of ribose (Figure 5.2 A) showed characteristic primary fragments from the cleavage of acetoxylic-carrying carbon atoms, including m/z 146, m/z 218, m/z 290, m/z 73, m/z 145, and m/z 217. In addition, secondary fragments were produced from the loss of acetic acid (60 mass units) and ketene (42 mass units), as in m/z 188, m/z 128 from the primary fragment m/z 290, m/z 116 from m/z 218, m/z 86 from m/z 146.
Figure 5.2 B revealed the mass spectrum of glucose from glucoronic acid. Primary fragments were observed, including m/z 362, m/z 290, m/z 218, and m/z 146. Whereas secondary fragments were observed from loss of acetic acid, and ketene, such as m/z 188, m/z 128, m/z 86 from the primary fragment m/z 290, m/z 302, m/z 260, m/z 200, and m/z 140 from the m/z 362.

The fragmentation pattern of the 3,6-di-O-Me-D-glycero-\(\alpha\)-L-gluco-heptopyranose as seen in (Figure 5.3 A). Primary fragments were detected from the cleavage of the carbon-carbon bonds carrying methoxyl group of the alditol acetate chain. The two characteristic primary peaks are m/z 190, and m/z 305. Secondary fragments will be as a result of a loss of an acetic acid, ketene, or methanol (32 mass units), such as m/z 143, and m/z 97 from the primary fragment m/z 305, and the m/z 130 from the primary m/z 190. The fragmentation pattern showed in (Figure 5.3 B) for the 6-O-Me-D-glycero-\(\alpha\)-L-gluco-heptopyranose showed a characteristic primary fragments m/z 406, m/z 117, and secondary fragments m/z 212, m/z 170 from the primary fragment m/z 406.

The fragmentation pattern of the GalNAc (Figure 5.4) shows the characteristic primary fragments m/z 360, m/z 289, m/z 217, and m/z 145. In addition, subsequent secondary fragments were produced from primary fragments by the loss of acetic acid or ketene, for example, m/z 318, m/z 300 (loss of acetic acid and ketene from m/z 360), and the m/z 139, m/z 199, m/z 259, and m/z 318 from the primary fragment m/z 360.
Figure 5. 2. GC-MS profile of the alditol acetates of: A. β-D-ribofuranose, and B. glucose from glucuronic acid of *C. jejuni* HS:2 CPS
Figure 5. 3. GC-MS profile of the alditol acetates of: A. 3,6-di-O-Me-D-glycero-α-L-glco-heptopyranose, and B. 6-O-Me-D-glycero-α-L-gluco-heptopyranose of C. jejuni HS:2 CPS
5.1.3. NMR results

\(^1\)H NMR of the CPS revealed the presence of four anomeric resonances. CPS has a \(\beta\)-D-Ribf (A) at \(\delta\) 5.37, \(\alpha\)-D-GlcA (B) at \(\delta\) 5.13, \(\beta\)-D-GalNAc (C) at \(\delta\) 5.25, and D-glycero-\(\alpha\)-L-gluco-Hep (D) at \(\delta\) 5.587 were reported in agreement with the CPS structure reported for the NCTC11168 strain (Figure 5.5 A) \[23, 33, 35\]. Two anomeric signals at \(\delta\) 5.02, and \(\delta\) 4.92 corresponds to \(\beta\)-D-GalNAc with and without MeOPN at the C3, respectively. The \(^1\)H NMR spectrum revealed a singlet at \(\delta\) 2.06 originating from the methyl group which is characteristic to the N-acetyl moiety from the GalNAc. Two peaks at \(\delta\) 3.50 and \(\delta\) 3.62 originated from the O-methyl groups attached to C3, and C6 of

Figure 5.4. GC-MS profile of the alditol acetates of Gal/NAc of \(C. jejuni\) HS:2 CPS.
the D-glycero-α-L-gluco-heptopyranose respectively. The $^1$H NMR spectra showed a broad range of overlapping sugar ring proton resonances between δ 3.50 and δ 4.90. The two peaks at the δ 3.77 and δ 3.79 corresponds to the methyl groups of the MeOPN residue attached to either the C-3, or C-4 of the β-D-GalNAc, and D-glycero-α-L-gluco-Hep, respectively. $^{31}$P NMR (Figure 5.5 B) shows two peaks at the resonances $\delta_p$ 13.18, and $\delta_p$ 13.62 corresponding to the MeOPN residues [33]. The GC-MS and NMR results confirmed the structure to agree with the CPS structure previously reported for serotype HS:2 [3, 5, 6]. Figure 5.5 shows the 1D $^1$H NMR spectra of CPS structure of the HS:2 serotype.
Figure 5. A) $^1$H NMR of the CPS of *C. jejuni* serostrain HS:2 where A,B,C,D are the anomeric proton resonances for $\beta$-D-Ribf, $\alpha$-D-GlcA, $\beta$-D-GalNAc, and D-glycero-L-gluco-Hep respectively. B) $^{31}$P NMR spectrum of the *C. jejuni* HS:2 revealed two (MeOPN) peaks at $\delta_P$ 13.18, and $\delta_P$ 13.62. The experiments were obtained at 297 K on a 400 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.
Figure 5.6. A: 2D $^1$H-$^{13}$C HSQC (600 MHz), B: 2D $^1$H-$^{31}$P HMBC (400 MHz) for *C. jejuni* serostrain HS:2. The NMR experiments were obtained at 297 K. The sample was dissolved in 600 µl D$_2$O.
**Figure 5.7.** CPS structure of *C. jejuni* serostrain HS:2 CPS, where A is β-D-Rib, B is α-D-GlcA which is amidated at C6 with 2-amino-2-deoxy glycerol, C is β-D-GalNAc, and D is D-glycero-L-guco-Hep.
Table 5.1. Compiled \(^1\)H and \(^{13}\)C NMR chemical shift data from \(C.\) jejuni serostrain HS:2 CPS collected from many 1D and 2D NMR experiments. All experiments were collected at 297 K. TSP was used as an external standard.

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<th>H4</th>
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5.2. Synthesis of the glycoconjugate vaccine of \(C.\) jejuni strain HS:2 CPS

5.2.1. Periodate Oxidation of the CPS and conjugation of the oxidized CPS by reductive amination

The potential site for oxidation will be on the D-glycero-L-gluco-Hep, due to the fact that the predominant methylation is on the C-6 of the D-glycero-L-gluco-Hep, and the 3-O-Me will be less frequent, and the C-4 position is not always occupied with MeOPN. They will provide the vicinal diols that serve as the oxidation site between C-2, and C-3, or C-3, and C-4 of the D-glycero-L-gluco-Hep. Following oxidation, the intact structure of the CPS was confirmed by NMR. The activated CPS containing the aldehyde
groups was then conjugated to a carrier protein via reductive amination. The structural integrity of the CPS was confirmed by NMR.

The SDS-PAGE analysis of the \( C. jejuni \) CPS\textsubscript{HS:2} –CRM\textsubscript{197} conjugate (Figure 5.8) revealed a band of the same molecular weight of CRM\textsubscript{197}, and showed a smear of a higher molecular weight, that confirms the presence of the conjugate with the CPS, and this suggested that cross-linked glycoconjugate (lattice model) was formed. It also shows the presence of some unconjugated CRM\textsubscript{197}, which needs further purification in order to get rid of the unconjugated CRM\textsubscript{197}.

The immunoblot analysis (Figure 5.9 Lane 4) showed that the antisera raised against the whole cells of \( C. jejuni \) HS:2 recognized the glycoconjugate \( C. jejuni \) CPS\textsubscript{HS:2} –CRM\textsubscript{197}. In (figure 5.9 lane 1) the immunoblot shows that the anti-CRM recognizes the conjugate, which suggests that the rabbit appears to have had pre-existing antibodies to CRM, which has been frequently observed.
Figure 5. SDS-PAGE analysis of periodate oxidation of *C. jejuni* CPS$_{HS:2}$–CRM$_{197}$ glycoconjugate vaccine. Lane 1: protein markers, Lane 2: CPS$_{HS:2}$–CRM$_{197}$ conjugate, Lane 3: unconjugated CRM$_{197}$. 
Figure 5.9. Immunoblot analysis of *C. jejuni* CPS<sub>HS:2</sub>–CRM<sub>197</sub> conjugate with antisera raised against CRM<sub>197</sub> (lane 1: *C. jejuni* CPS<sub>HS:2</sub>–CRM<sub>197</sub> conjugate, and lane 2: CRM<sub>197</sub>), and with antisera against *C. jejuni* HS:2 whole cells (lane 4: *C. jejuni* CPS<sub>HS:2</sub>–CRM<sub>197</sub> conjugate, and lane 5: CRM<sub>197</sub>), Lane 3: protein markers.

5.2.2. Oxidation of *C. jejuni* HS:2 by TEMPO mediated oxidation and the following conjugate by EDC mediated coupling

An alternative method was used in the activation of the CPS of *C. jejuni* HS:2 by using TEMPO-mediated oxidation. It is always a good idea to look into other ways that might produce better immunogenic activity. Having a complicated structure and the presence of primary hydroxyls through the structure of serostrain HS:2 made another possibility for the activation of the CPS, which is TEMPO mediated oxidation.

20 mg of the CPS was activated using 0.25 mg of TEMPO, which leads to the activation of the CPS which was then conjugated to a carrier protein CRM<sub>197</sub> by EDC-
mediated coupling. The integrity of the intact CPS was further confirmed by NMR as shown in (Figure 5.10). SDS-PAGE analysis (Figure 5.11 lane 2) showed that the C. jejuni CPS_{HS:2}–CRM_{197} conjugate was successful by the appearance of a small band with a higher molecular weight than the protein itself, however, there was a band with a smaller molecular weight, which suggested the presence of free unconjugated CPS that might be as a result of inadequate oxidation sites for the protein coupling. The immunoblot showed that antisera raised against the whole cells of C. jejuni HS:2 recognized the TEMPO-derived CPS_{HS:2}–CRM_{197} conjugate (Figure 5.12).

Following the assurance of the integrity of the conjugate, it was prepared for immunogenicity studies in mice (Figure 5.13). This study was performed at Spring Valley Laboratories, USA. The study used 3 groups of mice each has 5. The mice groups were immunized with 5, and 25 µg by weight with alum as adjuvant, and another group was immunized with 5 µg using poly (I:C). The groups were immunized three times. The blood IgG levels were measured two weeks after the third immunization. The IgG levels showed an increase after immunization with 25 µg comparing to the others. More experiments should be done in order to prove the effectiveness and safety of the vaccine. Challenging mice with a whole cell C. jejuni HS:2 will be the next step.
Scheme 5. 1. Schematic diagram for the TEMPO oxidation of the CPS of *C. jejuni* strain HS:2 followed by EDC mediated coupling.
Figure 5. 1D $^1$H NMR spectrum of *C. jejuni* HS:2 10% TEMPO oxidized CPS (A) compared to the inactivated CPS (B). The NMR experiment was obtained at 297 K on a 600 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.
Figure 5. SDS-PAGE analysis of *C. jejuni* CPS$_{HS:2}$ –CRM$_{197}$ glycoconjugate vaccine by TEMPO-mediated oxidation. Lane 1: protein markers, Lane 2: CPS$_{HS:2}$ –CRM$_{197}$ conjugate, Lane 3: unconjugated CRM$_{197}$.
Figure 5.12. Immunoblot analysis of *C. jejuni* CPS<sub>HS2</sub>–CRM<sub>197</sub> conjugate via 10% TEMPO mediated oxidation with antisera raised against *C. jejuni* HS:2 whole cells (lane 1: protein marker, lane 2: *C. jejuni* CPS<sub>HS2</sub>–CRM<sub>197</sub> conjugate, and lane 3: CRM<sub>197</sub>).
Figure 5. 13. \textit{C. jejuni} CPS_{HS:2}-CRM_{197} vaccine by TEMPO-mediated oxidation Immunogenicity study.

5.3. Future Work

\textit{C. jejuni} strain HS:2 CPS was conjugated using two types of conjugation, and the latest conjugation was promising in the means of immunogenicity studies. More investigations need to be done on the level of immunogenicity studies and compared it to the other \textit{C. jejuni} strain 81176 prototype glyconjugate vaccine. The oxidation of the CPS might need some modifications in order to get the more oxidation sites of the CPS to be connected to the carrier protein.
Chapter 6: Results and Discussion: A Dual Conjugate Vaccine against *Shigella flexneri* 187 and ETEC

6.1. Structural Characterization of the LPS of *S. flexneri* 187

6.1.1. Extraction and Purification of the LPS

The cell mass was obtained from the US NAVY. The LPS was extracted following the hot water-phenol extraction [66]. The aqueous layer was dialyzed under running deionized water overnight, and freeze dried. The recovered LPS was delipidated using 2% acetic acid at 100 °C for 3.5 hours till a precipitation occurred, which was removed by centrifugation (5000 × g, 3 h), the supernatant containing the LPS was then further purified using size exclusion chromatography on a Sephadex G-50 column, using deionized water as eluent [63]. Positive fractions containing the O-specific chain were dried for analysis.

6.1.2. GC-MS results

The purified LPS sugar composition analysis (figure 6.1) showed the presence of characteristic peaks at retention times 19.68, 30.87, and 37.17 min that correspond to rhamnose (Rha), Glc, and GlcNAc, respectively. These findings were in agreement with the structure of the O-Ag repeating unit previously characterized for *S. flexneri* 2a. GC/MS profile showed Gal peak, that has been known to be part of the core polysaccharide of *S. flexneri* [51].
Figure 6. 1. GC/MS of alditol acetate derivatives of the LPS of *S. flexneri* 2a; (Rha: rhamnose, Glc: glucose, Gal: galactose, and GlcNAc: N-acetylglucosamine).

The fragmentation pattern of the monosaccharides was used to further confirm the identity of individual sugars. The fragmentation pattern of Rha (Figure 6.2 A) showed the characteristic intensities for a 6-deoxy sugar. Primary fragments resulting from the cleavage of two acetoxyl carrying carbons were at m/z 87, m/z 159, m/z 231, m/z 117, and m/z 303. In addition, subsequent secondary fragments were produced from primary fragments by the loss of acetic acid (60 mass units), or ketene (42 mass units), such as m/z 141, m/z 201 from the primary fragment m/z 303, and m/z 171, secondary fragments m/z 129, m/z 69 from m/z 231, secondary fragments m/z 99 from m/z 159, and secondary fragments m/z 157, and m/z 115 from m/z 217. The fragmentation pattern of Glc (Figure 6.2 B) showed primary peaks at m/z 361, m/z 289, m/z 146, m/z 217, m/z 218, m/z 145, m/z 290, m/z and m/z 73. Secondary fragments were detected by loss of acetic acid or ketene, such as m/z 215, m/z 155, and m/z 130 from the primary fragment m/z 275, and the m/z 215, and m/z 190, m/z 88 from the primary fragment m/z 130, and the m/z 113,
and m/z 131 from the primary fragment m/z 275. The fragmentation pattern of the GlcNAc (Figure 6.2 C) showed the primary peaks m/z 217, and m/z 145. Subsequent loss of ketene, and acetic acid produced the secondary fragments m/z 85 from the primary fragment m/z 145, the secondary fragment m/z 318 from m/z 360, the secondary fragment m/z 259 from m/z 361. The secondary fragment m/z 199 from 259, and by subsequent loss of an acetic acid (60 mass units) the secondary fragment m/z 139 could be seen.
Figure 6. 2. Fragmentation pattern of the alditol acetates of A: rhamnose, B: galactose, and C: GlcNAc of *S. flexneri* 2a O-Ag
6.1.3. NMR Data

The 1D $^1$H NMR (Figure 6.3) confirmed the integrity of the O-Ag repeating unit. The presence of the anomeric peaks in the region between $\delta$ 4.83-$\delta$ 5.20 agrees with the previously characterized in literature [51, 63, 80]. The region of the spectrum depicting the anomeric protons was a bit crowded which made it difficult to determine the number of sugar residues and needed further analysis. The $^1$H NMR showed the characteristic methyl signal of the Rha moiety at $\delta$ 1.35 ppm, and it revealed the methyl signals in the region $\delta$ 2.08-2.22 that corresponds to the methyl groups of the O-Ac, and N-Ac of the LPS. 2D $^1$H-$^{13}$C HSQC was then obtained to assign the carbon shifts (Figure 6.4). It revealed the presence of seven alpha anomers in the region $\delta$ 4.84-$\delta$ 5.20 ppm that corresponds to the three Rha residues, and the glucose with and without the O-Ac at the Rha I C-3, and showed three beta anomers corresponds to the GlcNAc residue at $\delta$ 4.71, and GlcNAc with O-Ac at the C-3 of RhaI at $\delta$ 4.53, and the GlcNAc with O-Ac at the C-6 of the GlcNAc itself at $\delta$ 4.75 ppm. It is of a major importance here to mention that the whole repeating unit was affected with the O-Ac of the C-3 of the RhaI. All the sugars showed different anomeric shifts although the ring region was pretty much the same. On the other hand, the O-Ac at the C-6 of the GlcNAc affected only the GlcNAc residue and showed different anomeric shifts with slight differences in the ring region. These findings were in agreement with the previously characterized structure [63]. Another interesting carbon shift was noted in the HSQC was the shift at ($\delta_H$ 5.07, $\delta_C$ 75.14) which is due to a proton shift that is attached to a carbon bearing an O-acetyl group, and corresponds to the C-3 of the O-Ac RhaI, compared to the proton shift of the non-acetylated oxygen at $\delta$ 3.89. The non-acetylated O-6 of GlcNAc has a proton shift of H-6/H-6′ $\delta$ 3.80, $\delta$ 3.90,
whereas in the acetylated O6 of the GlcNAc H-6/H-6' will show a shift of δ 4.32, δ 4.41ppm which also matches previously assigned O-Ag structure [63]. All proton and carbon chemical shifts are compiled and summarized in Table 6.1.

Figure 6.3. 1D $^1$H NMR of S. flexneri serotype 2a LPS. The NMR experiment was conducted at 297 K on a 600 MHz spectrometer. The sample was dissolved in 600 µl D$_2$O.
Figure 6. 4. 2D \(^1\)H-\(^{13}\)C HSQC of the LPS of \textit{S. flexneri} serotype 2a. The NMR experiment was conducted at 297 K on a 600 MHz spectrometer. The sample was dissolved in 600 \(\mu\)l D\(_2\)O.
Table 6. 1. Compiled proton and carbon chemical shifts data for the *S. flexneri* serotype 2a LPS. All NMR experiments were obtained at 297 K.

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<tr>
<th>Sugar Residue</th>
<th>H1 C1</th>
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<th>H3 C3</th>
<th>H4 C4</th>
<th>H5 C5</th>
<th>H6/H6’ C6</th>
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<th>H4 C4</th>
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6.2. Synthesis of the glycoconjugate vaccine of the *S. flexneri* serotype 2a LPS

The presence of a primary alcohol at the Glc residue provides site for conjugation by TEMPO-mediated oxidation, which will be followed by EDC-mediated coupling to the proper carrier protein. 20 mg of the purified LPS was activated using 0.25 mg of TEMPO, which leads mainly to the activation at the primary alcohol of the Glc at C-6 resulting in the formation of carboxylic acid that will serve as the site of attachment to the carrier protein. After activation of the LPS, it was conjugated to Cfa-EB carrier protein, or BSA. The resultant activated LPS and conjugates were analyzed for the presence of the intact LPS by NMR.

SDS-PAGE analysis (Figure 6.4, lane 4) showed the success of the LPS$_{52a}$-BSA conjugate by the detection of a smear at a higher molecular weight than the native protein. The LPS$_{52a}$-Cfa conjugate (Figure 6.4, lane 2) on the other hand showed only a band at a lower molecular weight which suggested the presence of unconjugated LPS of *S. flexneri*. Cfa protein is a sensitive protein that should be kept under very low temperatures, which can attribute to the fact that the conjugate didn’t work as a result of handling and shipping as well. Yet the oxidation and conjugation procedure was proved to be successful by the LPS$_{52a}$-Cfa conjugate. The immunoblot against commercial anti-*Shigella* serum showed a strong reactivity of the LPS$_{52a}$-BSA conjugate (Figure 6.5 Lane 4), which supports the previous findings, although the LPS$_{52a}$-Cfa conjugate was not so promising from the SDS-PAGE, looking at the immunoblot some reactivity was observed (Figure 6.5 Lane 2).
Figure 6. 5. SDS-PAGE analysis of the *S. flexneri* serotype 2a; Lane 1: precision plus protein markers, lane 2: LPS\textsubscript{S2a}-Cfa conjugate, Lane 3: Cfa protein, Lane 4: LPS\textsubscript{S2a}-BSA conjugate, and Lane 5: BSA protein.
Figure 6. Immunoblot analysis of the *S. flexneri* serotype 2a of the TEMPO oxidized LPS$_{2a}$-Cfa conjugate with commercial anti-*Shigella* serum; Lane 1: Precision plus protein markers, Lane 2: LPS$_{2a}$-Cfa conjugate, Lane 3: LPS$_{2a}$-BSA conjugate, and Lane 4: *S. flexneri* whole cells.

6.3. Discussion and Future work

*S. flexneri* being one of the leading causes of gastroenteritis worldwide, due to the fact that most of the strains are sharing the same LPS structure, made a glyconjugate vaccine one of the main approaches for the development of anti-*Shigella* vaccine. The current study provided a prototype multi-agent vaccine against *S. flexneri*, and *E. coli* in a step toward the development of a multi-agent vaccine that includes *C. jejuni* CPS of the most dominant strains worldwide.
References


[56] Plotkin, S. A. *Clinic. and Vac. Imm.,*** **2009**, *16*, 1709-1719.


تم بحمد الله