

# A capsule conjugate vaccine approach to prevent diarrheal disease caused by *Campylobacter jejuni*

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**Abbreviations:** GBS, Guillain-Barré syndrome; LOS, lipooligosaccharide; LPS, lipopolysaccharide

*Campylobacter jejuni* is a major cause of diarrheal disease and results in high levels of morbidity and economic loss in both industrialized and developing regions of the world. To date, prior vaccine approaches have failed to confer protection against this enteric pathogen. Key challenges to the development of a practical *Campylobacter* vaccine for human use include a lack of understanding of *Campylobacter* pathogenesis and well-defined immune correlates of protection. With the discovery that *C. jejuni* expresses a capsule polysaccharide associated with virulence, a conjugate vaccine approach is currently being evaluated. Conjugate vaccines have been successfully developed and implemented against other invasive mucosal pathogens including *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Hemophilus influenzae*. Furthermore, *Shigella*-based conjugate vaccines based on lipopolysaccharide have shown promising results in field trials. A prototype *C. jejuni* conjugate vaccine is currently entering human testing.

## Introduction

*Campylobacter jejuni* is among the most common causes of diarrheal disease worldwide.<sup>1–4</sup> *C. jejuni* is a zoonotic pathogen and humans are most often infected by consumption of contaminated poultry, water, or raw milk.<sup>5–12</sup> The incidence of disease varies widely, ranging from hyperendemic levels (40 000/100 000 children less than 5 years old) in developing regions of the world, especially Southeast Asia,<sup>4</sup> to endemic levels (20–100/100 000 population) occurring as sporadic disease in young adults and infants in developed countries.<sup>13</sup> *C. jejuni* represent a significant cause of traveler's diarrhea in persons from industrialized nations visiting hyperendemic regions.<sup>14</sup> Although infection is not usually associated with mortality in developed countries, *Campylobacter* infections result in a high level of morbidity and loss of productivity. In the United States alone, it is estimated that greater than one million cases of diarrhea can be attributed to *Campylobacter* infection each year.<sup>15</sup> In addition to acute diarrhea, *C. jejuni* is associated with a number of post infectious serious sequelae, including Guillain-Barré syndrome (GBS),<sup>16</sup> reactive arthritis<sup>17</sup>,

and irritable bowel syndrome (IBS).<sup>3,18</sup> Moreover, an association of repeated *C. jejuni* infections with malnutrition has been reported in the developing world.<sup>19,20</sup> The molecular basis for the association of *C. jejuni* with GBS is recognized to be due to the mimicry between lipooligosaccharide (LOS) chains of most *C. jejuni* strains, such that antibodies directed toward LOS cross react with human peripheral nerves.<sup>21</sup>

As a result of acute diseases and post-infectious sequelae, it has been estimated that in the US it costs over 2000 USD for the treatment of the average *Campylobacter* case and over 1.7 billion USD annually for all cases, and the quality-adjusted life years (QALYs) associated with *Campylobacter* disease totals 13 300 annually.<sup>22</sup> Despite its significant impact on human health, relatively little is known regarding what constitutes protective immunity against this pathogen, and there is currently no licensed vaccine to prevent disease caused by *C. jejuni*.

## Campylobacter Virulence/Pathogenesis

*C. jejuni* is considered an invasive enteric pathogen, but the molecular details of its pathogenesis remain enigmatic, due largely to the lack of reliable, non-primate animal models of disease. Flagella are critical to virulence for the organism to swim through the mucus lining of the intestine and to serve as a secretory organelle for a variety of proteins, some of which appear to play roles in invasion.<sup>23–27</sup> *C. jejuni* invasion is similar to that of *Shigella spp.*, in that both usually invade the intestinal epithelium and are not generally found systemically, in contrast to *Salmonella spp.* The process of invasion of epithelial cells results in an influx of immune cells and cytokine release that is manifest as inflammatory diarrhea.<sup>28</sup>

Genome sequencing of numerous *C. jejuni* strains has failed to identify many virulence factors that are found in other, better-characterized pathogens. This suggests that *C. jejuni* utilizes unique pathogenic mechanisms. Indeed, one of the biggest surprises from the first genome sequence was the presence of an ABC capsular transport system, analogous to those found in a sub-set of other encapsulated pathogens. Studies from several labs quickly confirmed that the saccharide previously thought to be a lipopolysaccharide (LPS) was, in fact, a capsule.<sup>29,30</sup> Mutation in the *C. jejuni* capsule transport system demonstrated that the capsule was the major serodeterminant of the Penner typing scheme, a passive slide hemagglutination assay that recognizes 47 different serotypes.<sup>30,31</sup> Capsule characterization was quickly followed by studies linking

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this structure to virulence.<sup>32</sup> Isogenic non-encapsulated mutants were demonstrated to be attenuated in an infant ferret model of diarrheal disease,<sup>32</sup> exhibited reduced invasion of a human epithelial cell line in vitro,<sup>32</sup> reduced colonization of chicken ceca<sup>33</sup>, and were more sensitive to complement mediated killing.<sup>34,35</sup> More recent studies have also indicated that the polysaccharide capsule can be immunomodulatory.<sup>34,36</sup>

### Special Challenges to *C. jejuni* Vaccine Development

The association of *C. jejuni* with GBS poses special problems to vaccine development. The use of any whole cell vaccine approach, although originally evaluated (see below), is precluded due to safety concerns. Similarly, the standard phase 2B challenge that is generally done to evaluate the efficacy of vaccines cannot be performed with *C. jejuni* strains expressing ganglioside mimics, although some early volunteer studies were done with such strains prior to our current understanding of GBS pathogenesis. However, despite the fact that the vast majority of *C. jejuni* strains express one or more ganglioside mimics in their outer LOS core, there have been some strains described that lack such mimicry. More recently, a human challenge model has been developed using such a strain, as outlined below.

### Lessons Learned from Experimental *Campylobacter* Infections

Through the use of experimental *Campylobacter* infections in humans, much has been learned regarding pathogenesis of disease as well as immune responses to *C. jejuni*. In the first human challenge, conducted by Black et al.,<sup>37</sup> healthy adults were experimentally infected with either *C. jejuni* strain 81–176 or strain A3249. For challenge studies using strain 81–176, doses ranged from 10<sup>6</sup>–10<sup>9</sup> colony forming units (CFU), and the percentage of volunteers who developed diarrhea was 46%. Despite no apparent dose effect, this work established that experimental infection produces signs and symptoms that are comparable to those observed from natural infections in developed countries. In these studies, volunteers who fell ill following oral challenge with 10<sup>8</sup>–10<sup>9</sup> CFU strain 81–176 exhibited significant increases in *Campylobacter*-specific IgA and IgM. Interestingly, volunteers who were infected and did not become ill did not show any increase in *C. jejuni*-specific antibodies over the observation period. Volunteers who had exhibited illness during primary infection with strain 81–176 (n = 7) were subjected to a homologous re-challenge one month after recovery. Although 5/7 veterans displayed stool colonization, no diarrheal disease was observed in these previously infected volunteers. In contrast, 6/12 control volunteers developed illness following oral challenge.

Tribble et al. also examined the duration of protection in humans experimentally infected with escalating doses of *C. jejuni* strain 81–176.<sup>38</sup> Robust immune responses (systemic and mucosal) were observed in all naïve individuals following challenge,

irrespective of dose. Volunteers initially infected with 10<sup>9</sup> CFU were selected for homologous re-challenge studies in which some individuals would be challenged 28–49 days (short-term volunteers (STV) or 1 year [long-term volunteers (LTV)] following primary infection. STV (8/8) were completely protected against illness following re-challenge whereas 4 out of 7 LTV displayed outcomes ranging from mild to severe illness. Moreover, LTV exhibited an attenuated illness compared with naïve control volunteers.

These early human challenge studies using *C. jejuni* strain 81–176, which expresses GM2 and GM3 ganglioside mimics,<sup>39</sup> were conducted prior to our understanding that such structures are closely associated with GBS. This risk led to the development of a new refined human challenge model using *C. jejuni* strain CG8421 that naturally lacks ganglioside mimicry in its core.<sup>40</sup> Dose finding studies determined that a reduced inoculum (10<sup>5</sup> CFU) of strain CG8421 led to an improved diarrhea attack rate (93%) compared with what was achieved using strain 81–176 in which 10<sup>9</sup> CFU was needed to achieve an attack rate of >75%.<sup>38,41</sup> Furthermore, *Campylobacter*-specific humoral and cell-mediated immune responses were comparable to what had been previously observed during experimental infection with strain 81–176. Surprisingly, following a homologous re-challenge 3 mo after initial recovery, individuals that had recovered from a primary infection with strain CG8421 (n = 8) were not protected against illness following secondary infection, with 8/8 volunteers experiencing campylobacteriosis and no reduction in symptoms.<sup>42</sup>

The studies utilizing *C. jejuni* strain 81–176 are important in demonstrating that immunity can be acquired following recovery from experimental *Campylobacter* infection, and can be long lasting.<sup>37,38</sup> However, these human challenge studies lack adequate descriptions of protective antigens and precise mechanisms of immunity. Although *Campylobacter*-specific immune responses were measured in these studies, measurements were made against relatively crude mixtures of extracted cell surface proteins from *C. jejuni* with no true measure of the specificity of the immune response (e.g., anti-capsular IgG).<sup>37,38,41,42</sup> Another critical point to remember is that there are key differences between the two challenge models that likely influence the acquisition of immunity against homologous re-challenge. These differences include inocula size, duration of infection, and time to re-infection, all of which can affect the generation of long-lasting memory responses.<sup>43,44</sup> Thus, although homologous protection was not observed in humans re-challenged with CG8421, this should not diminish the value nor preclude the use of this challenge model in assessing the efficacy of candidate vaccines against disease caused by *C. jejuni*.

### Previous *C. jejuni* Vaccine Approaches

There have been limited numbers of potential *C. jejuni* vaccines that have advanced to human testing and most have been reviewed recently.<sup>45</sup> NMRC developed a killed, whole cell vaccine based on strain 81–176 in the 1990s which was immunogenic and safe during Phase 1 clinical evaluation, but was unsuccessful

in a Phase 2b challenge with the same strain.<sup>45</sup> A subunit protein vaccine based on the flagellin of *C. jejuni* also underwent phase 1 testing but was only moderately immunogenic.<sup>45</sup> More recently, ACE Biosciences developed another protein subunit vaccine, ACE 393, that was not efficacious in phase 2B trials with CG8421 (<http://www.tdvaccines.com/index.php?module=front:home&action=content&cid=7&menuid=11>; Clinical trials.gov; NCT00859716).

## Capsule Conjugate Vaccines

Capsule conjugate vaccines have an excellent track record with regards to safety and efficacy. Conjugate vaccines have been successfully developed and implemented against other invasive mucosal pathogens, notably *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Hemophilus influenzae*.<sup>46,47</sup> These vaccines, which are administered parenterally, elicit IgG antibodies that function to prevent invasion of these pathogens (see below). There are no licensed conjugate vaccines against enteric pathogens, however. In fact, most enteric pathogens do not express polysaccharide capsules, *C. jejuni* being the main exception to this rule. Moreover, enteric vaccines are generally designed to elicit secretory IgA antibodies in the intestine rather than IgG.<sup>48</sup> However, conjugate vaccines based on the LPS of *Shigella* have shown good efficacy in field trials.<sup>49,50</sup> Based on these results, a conjugate vaccine approach is being evaluated for its ability to prevent diarrheal disease caused by *C. jejuni*. It should also be mentioned that, unlike LOS, there has been no ganglioside mimicry associated with any *C. jejuni* capsular polysaccharide described to date, so there should be no association of this glycan with GBS.

Capsular polysaccharides are thymus-independent (TI-2) antigens that generally do not elicit potent immune responses.<sup>51-53</sup> Although adults can generate antibody responses to purified capsules administered as a vaccine,<sup>54</sup> children less than 2 years of age do not develop strong responses to TI-2 antigens.<sup>55</sup> Furthermore, it has been determined that to elicit B cell responses against TI-2 antigens, the polysaccharide must have a mass in excess of 100 kDa with a minimum of 20 epitopes per polymer.<sup>56</sup> Based on limited data, the capsule polysaccharide of *C. jejuni* is < 10 kDa, and it seems unlikely that free *C. jejuni* CPS would be capable of inducing effective antibody responses.<sup>57</sup> B cell responses to TI-2 antigens are characterized by the production of short-lived, low-affinity IgM.<sup>51,52</sup> The generation of long-lived, high affinity IgG (i.e., class switched) is dependent upon B cells receiving cognate T cell help.<sup>58</sup> In a conjugate vaccine, TI-2 antigens are covalently linked to a carrier protein to optimize immune responses. The protein component allows responding B cells to receive costimulatory signals from T cells that allow IgG antibody class switching, and the generation of memory cells possessing antibody with a higher avidity for CPS.<sup>58,59</sup>

To evaluate a capsule conjugate approach against *C. jejuni*, CPS was purified from *C. jejuni* strain 81-176 (HS23/36) and conjugated to a mutant diphtheria toxin subunit, CRM<sub>197</sub>, using reductive amination.<sup>57</sup> To determine if this research grade vaccine was immunogenic, BALB/c mice were immunized subcutaneously

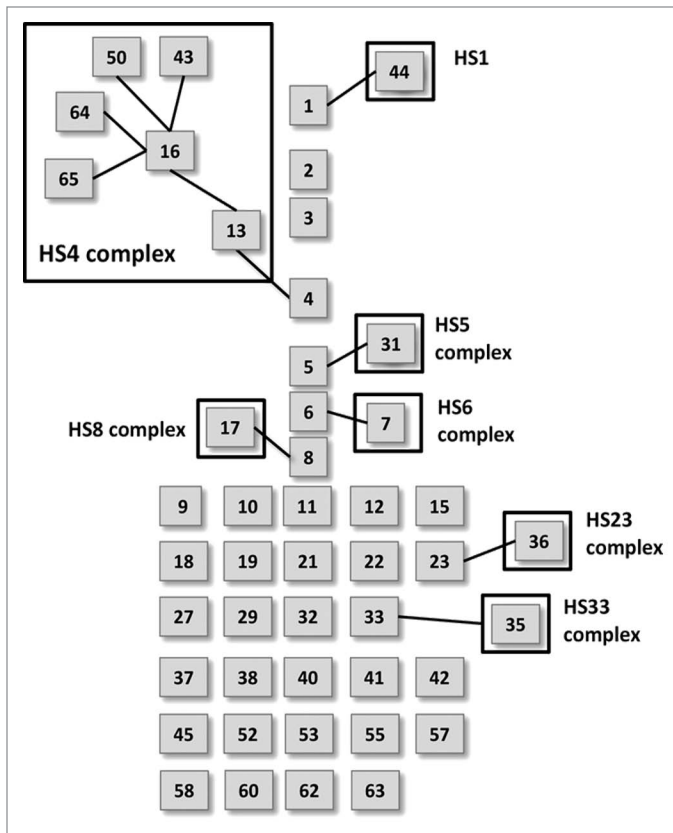
with escalating amounts (1, 5, or 25 µg vaccine by weight) of CPS-CRM<sub>197</sub> co-administered with Alhydrogel (alum). These doses correspond to approximately 0.1, 0.5, and 2.5 µg of conjugated polysaccharide respectively. Three doses were administered at 2-, 4-, or 6-wk intervals. Regardless of regimen, vaccination with all dose levels induced significant anti-CPS IgG responses in immunized mice compared with baseline levels with maximal titers appearing after the third dose. Additionally, immunized mice displayed elevated levels of serum CPS-specific IgG for up to 26 wk following the final immunization. This was the first evidence that a *C. jejuni* CPS-CRM<sub>197</sub> conjugate vaccine was capable of inducing robust, long-lived antibody responses.

Although a *C. jejuni* conjugate vaccine was demonstrated to be immunogenic in mice and protective in an intranasal mouse infection model, it remained unclear if immunization could prevent diarrhea caused by *C. jejuni*. Previously, a model of *Campylobacter*-induced diarrheal disease that mimics aspects of human illness was developed in *Aotus nancymaae* monkeys.<sup>60</sup> First, before determining if CPS-CRM<sub>197</sub> could confer protection against illness, *A. nancymaae* monkeys were immunized with escalating doses of research grade CPS-CRM<sub>197</sub> to assess the immunogenicity of the conjugate.<sup>57</sup> Subcutaneous immunization of *A. nancymaae* with escalating amounts (1, 5, and 25 µg by weight corresponding to approximately 0.1, 0.5, and 2.5 µg of polysaccharide, respectively) of a research-grade CPS-CRM<sub>197</sub> vaccine demonstrated a clear dose-dependent effect on CPS-specific IgG production. Vaccinates receiving either 5 or 25 µg of conjugate vaccine per dose demonstrated significantly increased levels of serum anti-capsular IgG compared with baseline titers following a three dose regimen. Interestingly, no increases in specific IgA production was observed in serum samples of any group.

To assess the protective efficacy of a research-grade CPS-CRM<sub>197</sub> conjugate vaccine, *A. nancymaae* non-human primates were immunized with three doses (25 µg/dose by weight, corresponding to 2.5 µg/dose conjugated CPS) at 6-wk intervals.<sup>57</sup> A group of control animals (n = 10) received saline as a sham treatment. A total of 9 wk following the immunization series, monkeys were orally challenged with ~10<sup>11</sup> CFU of *C. jejuni* strain 81-176. Immunized *A. nancymaae* demonstrated statistically significant protection against diarrhea caused by *C. jejuni* challenge, with 100% (14/14) exhibiting no diarrhea compared with 70% (7/10) of the animals in the control group that developed diarrhea. Therefore, these initial experiments in mice and non-human primates established that a conjugate vaccine comprised of capsule polysaccharide from *C. jejuni* covalently linked to CRM<sub>197</sub> was immunogenic and conferred protection against diarrheal disease.

## Valency Questions

Any effective conjugate vaccine against *C. jejuni* will have to be multivalent, but the number of serotypes that would be included remains an open question. As mentioned above, capsule is the sero-determinant of the Penner serotyping scheme, of which there are 47 *C. jejuni* serotypes.<sup>31</sup> Some of these serotypes



**Figure 1.** Illustration of the 47 described *C. jejuni* Penner serotypes and its 35 complexes.

fall into cross-reacting complexes (Fig. 1). Moreover, recent sequence analyses of the capsule loci of Penner type strains indicate that variation in the capsule loci associates with serotype differences, but the 47 described serotypes can be collapsed into 35 complexes based on DNA content (Poly, unpublished). While this number appears large, it is important to remember that *S. pneumoniae* has 97 serotypes. The first pneumococcal vaccine licensed by Wyeth in 2000 was based on the 7 most common *S. pneumoniae* serotypes identified in North America and Europe and showed a high efficacy. This vaccine was recently optimized to a total of 13 serotypes including the emerging 19A serotype. Like the licensed vaccine for pneumococcal disease, an effective *C. jejuni* CPS conjugate vaccine needs to be formulated to protect against the most common/virulent *C. jejuni* strains identified in developing countries where the disease is hyperendemic.

A systematic review of Penner serotyping published in the past 30 y was recently performed. A total of 59 studies, compiling almost 22000 sporadic human cases from 5 continents were reviewed,<sup>61</sup> and three serotypes, HS1, HS2, and HS4, were the most common in all sites comprising as high as 50% of the strains. However, the majority of the strains examined were from Europe (87%) and very few from hyper endemic areas where the burden of disease is higher, like South America where no data were reported. Due to its complexity and cost, Penner serotyping has been gradually replaced in the last decade by more efficient molecular typing methods including Pulse field

gel electrophoresis (PFGE) and multi locus sequence typing (MLST).<sup>62</sup> To gather additional information on CPS distribution in developing countries, Poly et al. developed a multiplex PCR method to rapidly determine capsule types. The initial description covered 14 major capsule types,<sup>62</sup> but the method has now been expanded to cover all 47 Penner types (Poly, manuscript in preparation) and has been deployed to numerous labs worldwide. Preliminary results indicate that in addition to HS1, HS2, and HS4, an additional 4 to 6 CPS types would cover >70% of *C. jejuni* CPS types worldwide.

## Correlates of Protection

Currently there are no well-defined correlates of protection against diarrheal disease caused by *Campylobacter*. Tribble et al. demonstrated that protection against homologous re-challenge with *C. jejuni* strain 81–176 correlated with in vitro IFN- $\gamma$  production by peripheral blood mononuclear cells (PBMCs), and that protective responses were observed above a threshold value of 400 pg/ml.<sup>38</sup> In addition, a protective role for IgA has been suggested given the biology of *Campylobacter*.<sup>38</sup> Currently, there are no studies detailing the human immune response to the capsule polysaccharide of *C. jejuni* following either experimental or natural infection. However, multiple studies examining natural infection have provided evidence that age-related increases in serum antibodies against *Campylobacter* are associated with resistance to infection and illness,<sup>63–66</sup> and in initial studies performed in *A. nancymae* where monkeys were immunized with a *C. jejuni* capsule conjugate vaccine, significant levels of CPS-specific serum IgG were observed with no concomitant increase in IgA levels. As mentioned previously, following challenge these immunized monkeys were completely protected against diarrheal disease, demonstrating that protection in this model correlates with anti-capsular antibody responses.<sup>57</sup> Although these studies demonstrate this link clearly, the precise mechanism of immunity remains to be determined.

Correlates of protection are established for pathogens in which licensed conjugate vaccines have been developed. Specific assays have been developed to measure vaccine-induced responses against pneumococcal and meningococcal disease. What these assays have in common is that they are measures of functional antibody activity. Protection against meningitis correlates with the serum bactericidal titer that is achieved following immunization,<sup>67–69</sup> whereas the correlate for protection against pneumococcal disease is the opsonophagocytic activity of immune serum.<sup>70,71</sup> Bactericidal activity is conferred by the ability of an antibody, generally IgM or IgG, to activate the classical complement pathway leading to formation of the membrane attack complex, which can directly kill bacteria. In contrast antibodies with opsonophagocytic functions bind pathogens and allow for uptake into phagocytes via Fc receptors. Common between the aforementioned pathogens and *Campylobacter* is that all are invasive, mucosal pathogens, and their polysaccharide capsules are critical for serum resistance. Thus, the evaluation of functional antibodies elicited by immunization with a *C. jejuni* conjugate vaccine is merited.

## Future Work

Recently, a prototype cGMP-produced *C. jejuni* CPS-CRM<sub>197</sub> monovalent conjugate vaccine based on the HS23/36 capsule type (CJCV1) was produced under contract with a contract research organization. This vaccine was immunogenic in mice and is currently being evaluated in a toxicology study. Following pre-clinical testing, future plans are to evaluate CJCV1 in a first-in-human Phase 1 clinical trial for safety and immunogenicity. As our candidate vaccine moves into clinical evaluations, the human challenge model utilizing *C. jejuni* strain CG8421 (HS23/36) will be of great value in assessing the efficacy of this prototype cGMP-produced conjugate vaccine in a Phase 2b clinical trial. This will establish a proof-of-principle that a conjugate vaccine can protect against diarrhea caused by *C. jejuni* in humans. However, additional work is needed to advance the goal of creating a multivalent *C. jejuni* conjugate vaccine for human use. Key to the development of a practical vaccine is identifying the valency needed to provide adequate vaccine coverage and identifying correlates of protection against diarrheal disease.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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