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(54) Title: COMBINED ENTEROTOXIGENIC ESCHERICHIA COLI AND CAMPYLOBACTER JEJUNI RECOMBINANT CONSTRUCT
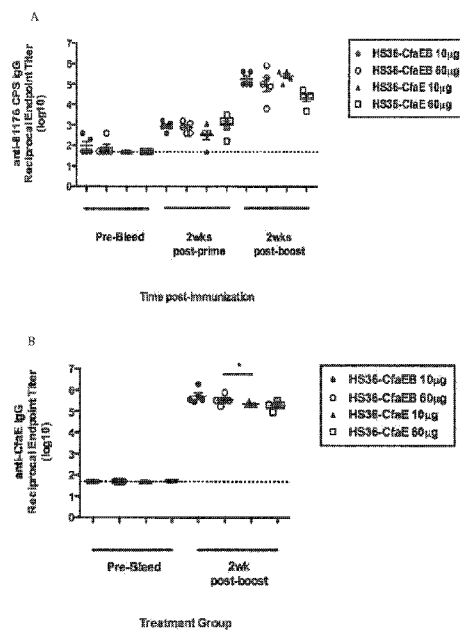
FIG. 4

(57) **Abstract**: The inventive subject matter relates to a construct comprising antigens derived from multiple enterobacteria including *Campylobacter jejuni* capsule polysaccharide polymer, enterotoxigenic *Escherichia coli* recombinant polypeptide construct and lipopolysaecharide from *Shigella spp.*. The subject invention also relates to a method of inducing an immune response utilizing the inventive composition.

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COMBINED ENTEROTOXIGENIC *ESCHERICHIA COLI* AND *CAMPYLOBACTER JEJUNI* RECOMBINANT CONSTRUCT

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application is a Continuation-in-Part to U.S. Nonprovisional application 11/340,003, filed January 10, 2006, which claims priority to U.S. Provisional application 60/642,771 filed January 11, 2005, and a Continuation-in-Part to U.S. Nonprovisional application 11/524,057 filed September 20, 2006, which claims priority to U.S. Provisional application 60/722,086, filed September 21, 2005, and a Continuation-in-Part to U.S. Nonprovisional Application 14/048,264, filed October 8, 2013, which claims priority to U.S. Provisional application 61/727,943, filed November 19, 2012, the contents of which are herein incorporated by reference. This application also claims priority to U.S. Provisional application 62/054,454, filed 24 September 2014, U.S. Provisional application 62/127,927, filed March 4, 2015, U.S. Provisional application 62/165,301, filed May 22, 2015, U.S. Provisional application 62/127,935, filed March 4, 2015, and U.S. Provisional application 62/075,399, filed November 5, 2014, the contents of which are herein incorporated by reference.

BACKGROUND OF INVENTION

Field of the Invention

[0002] The inventive subject matter relates to a recombinant construct against enterotoxigenic *Escherichia coli* and *Campylobacter jejuni* comprising a combined anti-ETEC recombinant polypeptide construct and *C. jejuni* campsule polysaccharide.

Description of Related Art

[0003] Enterotoxigenic *Escherichia coli* (ETEC), *Shigella*, spp. and *Campylobacter*

jejuni (CJ) are major causes of bacterial diarrhea worldwide. Both pathogens are a serious

health threat to western travelers and young children in resource-limited countries,

making them apt target populations for a single or dual pathogen vaccine against ETEC

and CJ. No FDA-licensed vaccines are available for either pathogen.

[0004] ETEC causes an estimated 210 million cases of diarrhea and 380,000 deaths

annually among infants and young children. Moreover, ETEC is the most common cause

of travelers' diarrhea. ETEC causes diarrhea ranging in severity from mild illness to

severe cholera-like purging. There are two major virulence factors, adhesive fimbriae,

dubbed colonization factors (CFs), and enterotoxins. Surface-expressed CFs, consisting

of complex protein heteropolymers, mediate adherence to the small intestinal epithelium

to initiate colonization within this privileged host niche. ETEC produce one or both of

two different enterotoxins, a heat-labile (LT) and a heat-stable enterotoxin (STI). LT and

STI intoxicate epithelial cells, resulting in fluid and electrolyte secretion and clinical

diarrhea. LT is highly immunogenic and a potent adjuvant, while STI is a small, poorly

immunogenic peptide.

[0005] Prevalent CFs and a non-toxic form of the LT (or its congener cholera toxin (CT))

have been the focus for several strategies to develop an ETEC vaccine. Such antigens

have been used individually or bundled as components of a whole-cell killed vaccine, live

vaccines vectored by attenuated ETEC or other enterobacterial species (e.g., *Shigella* and

Vibrio cholerae 01), and purified protein vaccines. None has yet been shown to confer

sufficiently high and broad levels of protection. The weight of evidence from clinical

trials indicates that anti-LT immunity confers short-term protection against LT-producing

ETEC. There is also evidence to show that certain CFs function as protective antigens.

There are, however, significant challenges for ETEC vaccine development. For one,

about half of all ETEC express only STI, for which anti-LT immunity is not thought to be

effective, thus necessitating anti-CF or anti-bacterial immunity. Also, the diversity of

ETEC CFs poses issues for achievement of sufficiently broad coverage with inclusion of

a realistic number of CFs.

SUMMARY OF THE INVENTION

[0006] The invention relates to an immunogenic construct comprising a polypeptide

construct expressing enterotoxigenic *Escherichia coli* (ETEC) fimbrial subunits

combined with a *Campylobacter jejuni* capsule polysaccharide or *Shigella* spp

lipopolysaccharide (LPS).

[0007] In a preferred embodiment, one or more *Camplobacter jejuni* capsule

polysaccharides are conjugated to one or more Escherichia coli enterotoxigenic

recombinant polypeptide constructs. In another embodiment, *Shigella* LPS is conjugated

to the ETEC polypeptide construct.

[0008] *Campylobacger jejuni* is associated with induction of Guillain-Barré Syndrome

(GBS), a post-infectious polyneuropathy that can result in paralysis. The association is

due to molecular mimicry between the sialic acid containining-outer core of the

lipooligosaccharide (LOS) and human gangliosides (5, 6, 89, 91). Thus, antibodies

generated against LOS cores result in an autoimmune response to human neural tissue.

Use of capsule polysaccharide from *C. jejuni* can induce an immune response without the possible induction of Guillain-Barré Syndrome.

[0009] In a preferred embodiment, the composition comprises an ETEC recombinant polypeptide construct design wherein major or minor subunits, derived from the same ETEC fimbrial type, are connected, via polypeptide linkers, and stabilized by donor strand complementation. The C-terminal most ETEC major subunit is connected, via a linker, to a donor strand region from an ETEC major subunit, which can be either homologous or heterologous to the C-terminal major subunit. The immunogenic composition can comprise a whole or an immunogenic fragment, containing a donor β strand region, of the ETEC fimbrial major or minor subunits. In some construct examples, in order to avoid inadvertent association of subunits, especially in CS6 subunits to each other, major ETEC fimbrial subunits can contain an N-terminal deletion of 14 to 18 amino acids.

[0010] In another embodiment one or more of the above constructs are connected, via a polypeptide linker, to form a multipartite fusion construct, wherein the subunits derived from multiple fimbrial types are expressed. In this embodiment, the fimbrial subunits can be derived from any ETEC fimbrial type, including, but not limited to: ETEC class 5 fimbriae type, including class 5a, 5b or 5c; ETEC CS3; and ETEC CS6.

[0011] The embodied multipartite construct can contain a deletion of the N-terminal region of one or more fimbrial subunits to avoid undesirable associations with other monomers or multimers and to remove reduce amino acid sequence length between polypeptides to reduce the protease cleavage.

[0012] DNA encoding the ETEC recombinant polypeptide construct can be used to express a polypeptide for attachment to *C. jejuni* or *Shigella* LPS. As such, an object of the invention also includes a use of a construct for immunizing mammals, including humans, by a composition comprising antigens from multiple bacterial species, including ETEC, *C. jejuni* and *Shigella* strains. The embodied use comprises one or more priming administrations of the combination construct. The priming dose can be subsequently followed by one or more boosting doses.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Illustration of inventive construct design wherein major or minor subunits, derived from the same ETEC fimbrial type are connected, via polypeptide linkers and stabilized by donor strand complementation. The construct can contain a deletion of the N-terminal region of the N-terminal subunit. This feature prevents undesirable association with other monomers or multimers. The C-terminal subunit is stabilized by a donor β strand, connected to the subunit via a polypeptide linker, wherein the donor β strand is either derived from a homolgous subunit, which is defined as a subunit that is the same as the subunit the donor strand is stabilizing or from a heterologous subunit, defined as derived from a subunit that is different still from the same fimbrial type.

FIG. 2 illustrates a multipartite construct wherein multiple compositions, illustrated in FIG. 1, are connected via a polypeptide linker. The first subunit, is a major or minor (e.g. ETEC class 5 adhesin) ETEC fimbrial subunit. One or more major ETEC fimbrial subunits are then connected to the first subunit and to each other via a linker, wherein the subunits are stabilized by donor strand complementation. The C-terminal most ETEC

major subunit is connected, via a linker, to a donor strand region from an ETEC major

subunit, which can be either homologous or heterologous to the terminal major subunit.

In some construct examples, in order to avoid inadvertent association of subunits,

especially in CS6 subunits to each other, major ETEC fimbrial subunits can contain an N-

terminal deletion of 14 to 18 amino acids.

FIG. 3. SDS-PAGE and immunoblots of conjugate vaccines. A. Analyses of CfaE-

HS36 conjugate. Lane 1-3 are stained with Gel Code Blue. Lane 1, Precision Plus

Protein standards (BioRad); lane 2, CfaE; lane 3, CfaE-HS36 conjugate. Lanes 4-5 are

immunodetected with anti-CfaE antibodies. Lane 4, CfaE; lane 5, CfaE-HS36 conjugate.

Lanes 6-7 are immunodetected with antibodies to whole cells of 81-176 (HS36). Lane 6,

CfaE-HS36 conjugate; lane 7, proteinase K digested whole cells of 81-176. B. Analyses

of CfaEB-HS36 conjugate. Lane 1-3 are stained with Gel Code Blue. Lane 1, Precision

Plus Protein standards (BioRad); lane 2, CfaEB lane 3, CfaEB-HS36 conjugate. Lanes 4-

5 are immunodetected with anti-CfaE antibodies. Lane 4, CfaEB; lane 5, CfaEB-HS36

conjugate. Lanes 6-7 are immunodetected with antibodies to whole cells of 81-176

(HS36). Lane 6, CfaEB-HS36 conjugate; lane 7, proteinase K digested whole cells of 81-

176. The molecular weights of the protein markers are shown on the left.

FIG. 4. *C. jejuni* anti-CPS (A) or ETEC anti-CfaE (B) induced by HS36 conjugated to

CfaE or CfaEB in mice.

FIG. 5. Functional antibodies, evidenced by HAI titer, induced in mice immunized with

HS36 conjugate vaccines.

FIG. 6. Summary of synthesis of polysaccharide construct and conjugation to CRM$_{197}$.

FIG. 7. Synthesis of aminopentanyl OMe-phosphoramidate galactoside. Reagent and

conditions: (a) TrCl, pyridine, 95%; (b) AllBr, NaH, DMF, 0 °C, 89%; (c) CAN, CH$_3$CN,

H$_2$O, 0 °C; then CCl$_3$CN, K$_2$CO$_3$, CH$_2$Cl$_2$, 57% over 2 steps; (d) HO(CH$_2$)$_5$NPhth,

TMSOTf, CH$_2$Cl$_2$, 65%; (e) 80% AcOH, 80 °C, 78%; (f) PCl$_2$O$_2$Me$_2$, Et$_3$N, CH$_2$Cl$_2$, then

NH$_3$(g), 27%; (g) PdCl$_2$, MeOH, 75%, (h) H$_2$NNH$_2$, EtOH, 82%.

FIG. 8. Capsule cross-reactivity to 6-MeOPN-Gal with antibodies to multiple conjugate

immunogenic compositions.

FIG. 9. Serology of A. nancymaae immunized with CfaEB-HS23-36 construct. Day 0

verses day 140.

FIG. 10. HAI titers of A. nanacymaae against ETEC strain H10407 expressing Cfa 1.

FIG. 11. Immune response of mice against HS3 capsule (top panel) and against CS6

(bottom panel) following immunization with an CssBA-HS3 conjugate vaccine. The

vaccine was administered at two doses, either 5 µg or 25 µg by weight.

FIG. 12. Immune response of mice to HS4 capsule (top panel) and to LTB (bottom

panel) following immunization with an LTB-HS4 conjugate vaccine. The vaccine was

administered at two doses, 5 ug or 25 ug by weight.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0013] The term enterobacteria , as used herein, refers to enterotoxigenic *Escherichia*

coli (ETEC), *Campylobacter jejuni* or *Shigella* spp., which include: *Shigella dysenteriae*,

Shigella flexneri, Shigella Boydii or *Shigella sonnei*. As used herein, an enterobacteria

polysaccharide polymer is a polysaccharide polymer derived from enterobacteria. The

term "polysaccharide antigen" as used herein refers to a capsule polysacchride derived

from *Campylobacter jejuni* (*C. jejuni* or *Campylobacter jejuni* capsule) or a

lipopolysaccharide derived from *Shigella* spp.. As used herein, "polysaccharide" refers

to two or more monosaccharide units composing a carbohydrate polymer molecule. A

"polysaccharide polymer" refers to two or more polysaccharide molecules connected

together.

[0014] The terms "polypeptide," "peptide," and "protein" as used herein can be

interchangeably used, and refer to a polymer formed of two or more amino acid residues,

wherein one or more amino acid residues are naturally occurring amino acids. The term

"amino acid sequence" refers to the order of the amino acids within a polypeptide. As

used, herein, "oligomer" are polypeptides sequences comprising relatively few amino

acids.

[0015] The term "recombinant polypeptide", "recombinant polypeptide construct", or

"recombinant protein", as used herein, refers to polypeptides or proteins produced by

recombinant DNA techniques, i.e., produced from cells transformed by an exogenous

DNA construct encoding the desired polypeptide or the desired protein. The term

"recombinant construct" refers to the DNA encoding the recombinant polypeptide,

recombinant polypeptide construct or recombinant protein.

[0016] The term "donor strand" or "donor β strand" refers to the N-terminal region of an

ETEC fimbrial subunit that associates with another ETEC fimbrial subunit in donor

strand complementation.

[0017] The term "immunogenic composition" refers to a formulation containing proteins

or polypeptides or polysaccharides or polysaccharide polymers that induce a humoral

and/or cellular immune response. The term "immunogenic coverage" or "spectrum of coverage" refers to the induction of humoral and/or cellular immune response against specific strains of bacteria under the "coverage." The term "immunogenic fragment" refers to a polypeptide containing one or more B- or T-cell epitopes and is of sufficient length to induce an immune response or to be recognized by T- or B-cells. The term "derivative" refers to a polypeptide or nucleic acid sequence with at least 80% identity with sequence of the identified gene. In this context, "identity" refers to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues that are the same, when aligned for maximum correspondence. Where some sequences differ in conservative substitutions, i.e., substitution of residues with identical properties, the the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Percent similarity refers to proportion of identical and similar (conserved change) residues.

[0018] "Fimbriae" are defined as projections or filaments on ETEC bacteria and are composed of major subunits, as in the case of CS3 and CS6 fimbriae or major and minor subunits, as in the case of class 5a, 5b and 5c ETEC. "Fibrillae" are narrow projections from a bacteria. CS3 and CS6 fimbriae can also be termed fibrillae due to their narrow characteristic. The term "fimbrial subunit" refers to the proteins that comprise ETEC fimbriae and is used interchangeably with "pilin." "Pilin", therefore, can refer to a "major" or "minor" "fimbrial subunit" that comprise ETEC fimbriae. A "minor fimbrial subunit" refers to the adhesin protein at the tip of class 5 ETEC fimbriae and is expressed in stoichiometrically low amounts compared to "major" subunits. The "minor fimbrial subunits"include, but are not limited to, CfaE, CsfD, CsuD, CooD, CosD, CsdD, CsbD

and CotD. "Major fimbrial subunits" refers to the ETEC fimbrial proteins represented in

stoichiometrially larger amounts in ETEC fimbriae, compared to "minor fimbrial

subunits." "Major fimbrial subunits" include the ETEC class 5 proteins: CfaB, CsfA,

CsuA2, CsuA1, CooA, CosA, CsdA, CsbA, CotA; the ETEC CS3 proteins: CstH, CstG;

and the ETEC CS6 proteins: CssA, and CssB.

[0019] The pathogenesis of *Campylobacter jejuni* remains poorly understood in

comparison with ETEC and the organism shares few virulence factors with better-

characterized pathogens. *C. jejuni* is unusual, however, among enteric pathogens in that

it expresses a polysaccharide capsule (CPS) that is one of its few confirmed virulence

factors.

[0020] Because of the importance of ETEC and *C. jejuni* as pathogenic agents, a

combined ETEC-CJ composition was constructed in order to afford protection against

both agents. In one embodiment, a recombinant polypeptide construct, comprising

fimbrial subunits from Class 5 ETEC strains is fused to a capsule polysaccharided from

the *C. jejuni* strain 18-176.

[0021] In a preferred embodiment, one or more recombinant polypeptide ETEC

constructs, comprising the ETEC fimbrial adhesion, are conjugated to isolated *C. jejuni*

capsule polysaccharide (CPS). One or more of a number of ETEC recombinant

constructs can be conjugated to one or more of a number of C. jejuni capsule

polysaccharide structures. In the inventive construct, the ETEC recombinant construct

operates both as an immunogen against ETEC and as a protein carrier molecule,

presenting the *C. jejuni* polysaccharide. Examples of ETEC recombinant polypeptides

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and *C. jejuni* capsule polysaccharides that can be incorporated into a combined structure

are given in the following examples.

[0022] In a preferred embodiment, the ETEC polypeptide construct can not only serve as

antigen against ETEC but also serve as a protein carrier for polysaccharide antigens, such

as *C. jejuni* capsule polysaccharide.

Example 1: Conjugation of ETEC polypeptides to C. jejuni capsule polysaccharide

(CPS)

[0023] In a preferred embodiment, ETEC recombinant polypeptides or polypeptide

constructs are conjugated to *C. jejuni* CPS. The CPS can be derived from a number of *C.*

jejuni strains. In the embodiment, any CPS of any *C. jejuni* strain is envisioned to be

conjugated to ETEC recombinant polypeptide constructs. Alternatively, *Shigella* LPS

can be conjugated to ETEC recombinant polypeptide constructs.

[0024] The overall method of conjugating includes oxidizing *C. jejuni* CPS, for example,

with $NaIO_4$ in sodium acetate (pH 4.0). Oxidized CPSs were desalted with a 5 kDa

cutoff membrane by stirred ultrafiltration, which is subsequently lypholized. ETEC

proteins are then added. The stoichiometery protein to CPS can vary, however, a typical

ratio is 1:2 protein to CPS by mass. The concentration of components can be by any

method. However, for example, polysaccharide concentration was determined by

antrhone assay and protein concentration was determined. $NaCNBH_3$ is then added. The

conjugates are subsequently desalted by ultrafiltration and lyophilized. CPS (or *Shigella*

LPS), ETEC proteins and conjugates were analyzed, for example by SEC-HPLC.

Conjugates were also analyzed by SDS polyacrylaminde gel electrophoresis (PAGE) and

Gel Code ® Blue (Pierce, Biotechnology, Inc, Lombard, IL) staining. Conjugates were

detected by antibody-based assay using anti-CPS and anti-ETEC protein.

[0025] As an example of ETEC recombinant polypeptide and *C. jejuni* conjugation, the

CPS from the *C. jejuni* strain 81-176 was conjugated to ETEC recombinant polypeptide

construct CfaE (class 5 ETEC adhesin) or to the recombinant polypeptide construct CfaE

linked, via a polypeptide linker, to the major subunit CfaB.

[0026] CPS *C. jejuni* capsule was purified from *Campylobacter jejuni* strain 81-176

(PG3208). This mutant, in which the *galT* gene was insertionally inactivated by

chloraphenicol cassette, lacks all ganglioside mimicry in its lipoligosaccharide (LOS)

core.

[0027] The cells were grown in porcine Brain-Heart Infusion (BHI) broth and sonicated

to inactivate the cells. The CPS was extracted by hot water/phenol method previously

employed for the same organism (Chen, et al., Carbohyd. Res. 243: 1034 (2008)). Cells

were immersed in a water/phenol mixture (3:2 ratio by volume), which was heated to

67°C with stirring for 4 hours. The suspension was cooled and separation of the mixture

into two separate layers (the aqueous layer and the phenol layer) and extraction of the

aqueous layer was performed. The aqueous layer was removed and the phenol/water

extraction was repeated on residue, to maximize the yield. Aqueous layers from two

extractions were pooled and boiled fro 1.5 hours with the aditon of acetic acid to a pH of

3.5. The aqueous layer was dialyzed gainst running water for 2 days and concentrated

using a Millipore concentrator cell with a 5 kDa cutoff membrane. Trace amounts of

residual RNA were removed by digestion with benzonase enzyme at 90 u/ml in 50 mM

Tris-CCl/1 mM MgCl₂, pH 8 overnight at 37°C. Benzonase was removed from CPS, then

desalted and concentrated using stirred ultrafiltration with 30 and 5 kDa MWCO disc

membranes, respectively.

[0028] The isolated CPS was oxidized with adding 40 mg of CPS to 40 mM $NaIO_4$ in

sodium acetate pH 4 in the dark at 4°C for 2 days. Oxidized CPS was desalted with

5kDa cutoff membrane by stirred ultrafiltration and was subsequently lyophilized.

[0029] Prior to conjugation the ETEC proteins, for example dscCfaE and dscCfaEB,

were transferred to 0.1M borate buffer at pH 9.0. Oxidized CPS was added to each

ETEC protein at a ratio of 1:2, protein to CPS by mass, and then $NaCNBH_3$ was added at

2 times mass equivalent to CPS. The reaction was incubated 1 day at room temperature

and 6 days at 37°C in the dark with continuous stirring. The conjugates were desalted by

stirred ultirafiltration with 30 kDa membrane and lyophilized. Conjugates of the CPS to

dscCfaE and dscCfaEB was conducted by SEC-HPLC. and polyacrylamide gel (PAGE)

(12.5%) electrophoresis.

[0030] In PAGE analysis, immunodetected with rabbit polyclonal antibodies to whole

cells of 81-176 was used to detect CPS and to CfaE. The results of this study are shown

in FIG. 3. Immunoblotting of both conjugates with anti-CfaE antisera confirmed that the

proteins ran as high molecular weight conjugates with conjugates with apparent masses

ranging from just higher than the mass of each respective protein to >250 kDa.

Immunoblotting with antisera to formalin fixed whole cells of C. jejuni 81-176 confirms

that capsular polysaccharide was conjugated to the proteins. As illustrated in FIG. 3, no

unconjugated protein remained in either conjugation.

[0031] The results of FIG. 3 were confirmed in SEC-HPLC. In the SEC-HPLC,

unoxidized and oxidized CPSs, ETEC proteins and conjugates were analyzed using SEC-

HPLC with a TSKgel-G2000SW$_{xl}$ column (30 cm x 7.8 mm ID) and TSKgel SW guard

column run on an ICS-5000 Dionex system with 0.1 M phosphate at pH 6.8, 0.1 M

sodium sulfate and 5% acetonitrile at 0.6 ml/min flow rate. Samples were monitored at

214 nm with Ultimate 3000 variable wavelength detector and RI detector, both from

Dionex.

[0032] The results of the SEC-HPLC are shown in FIG. 4 for dscCfaEB and in FIG. 5 for

dscCfaE. Analysis by matrix-assisted laser desorption/ionization (MALDI) is shown in

FIG. 6, for dscCfaEB and FIG. 7 for CfaE.

[0033] Detection of the conjugates by refractive index (RI) on SEC-HPLC revealed that

45% and 50% of the polysaccharide remained unconjugated with the CfaE and CfaEB

conjugates respectively. This is summarized in Table 1, which also illustrates that the

conjugated molar ratio of CPS to CfaE was 4.8:1 and that of CPS to CfaEB was 4.4:1.

Table 1

	CfaE conjugate		CfaEB conjugate	
	CPS	CfaE	CPS	CfaEB
Final product (includes unconjugated CPS) % yield	49%		63%	
Sugar : protein mass ratio in final product	2	1	1.85	1
% unconjugated with respect to final product by SEC-HPLC RI detection	45%		50%	
Conjugated mass ratio	1	1.5	1	2.3
Molecular weight	5.5 kDa	41 kDa	5.5 kDa	57 kDa
Conjugated molar ratio	**4.8**	**1**	**4.4**	**1**

Example 2: Anti-Class 5 ETEC, CS3 or CS6 constructs

[0034] Anti-ETEC constructs that are contemplated to be conjugated to *C. jejuni* polysaccharide comprise the structures as illustrated in FIG. 1 and FIG. 2. FIG. 1 illustrates the basic recombinant construct design. As diagrammed in FIG. 1 the construct design comprises one, or more ETEC major or minor fimbrial subunits or fragments of major fimbrial subunits, containing the donor strand, derived from the same ETEC fimbrial type, which are connected, via polypeptide linkers and stabilized by donor strand complementation. The construct can contain a deletion of the N-terminal region of the N-terminal subunit. This feature prevents undesirable associations with other monomers or multimers. The C-terminal subunit is connected to and stabilized by a donor β strand, connected to the subunit via a polypeptide linker, wherein the donor β strand is either derived from the adjacent subunit (i.e., homologous) or from a different subunit of the same fimbrial type (i.e., heterologous).

[0035] FIG. 2 illustrates the basic multipartite construct, wherein multiple constructs as in FIG. 1, are connected forming a recombinant construct comprising two or more fimbrial types. As illustrated in FIG. 1, major or minor subunits from the same fimbrial type are connected via a polypeptide linker sequence. In the multipartite construct, two or more constructs, as in FIG. 1, are connected, via a linker polypeptide.

[0036] In the multipartitie construct design, as in the basic design (compare FIG. 1 with FIG. 2), the first subunit (N-terminal) is a major or minor ETEC fimbrial subunit. Each additional subunit is connected to adjacent subunits via a polypeptide linker that enables rotary freedom of the molecular components. The subunits are associated with and stabilized via a donor strand complementation from a C-terminally adjacent subunit via a donor β strand, connected via a linker polypeptide, to the C-terminus of the stabilized

subunit. In some embodiments, subunits can contain a deletion of 14 to 18 amino acids from its N-terminal end. Additionally, specific constructs can be constructed with or without signal peptides of 18 to 22 amino acids and with or without histidine tags at the C-terminus.

[0037] In the multipartite construct, subunits from the same fimbrial type are directly connected. Groupings of subunits from the same fimbrial type are then connected to other groupings of subunits from other fimbrial types. Fimbrial types include, but are not limited to ETEC class 5a, 5b, 5c, CS3 and CS6. For example a single construct can include subunits derived from any two or more of class 5a, 5b, 5c, CS3 and CS6 fimbrial types.

[0038] Multiple linker sequences can be utilized in connecting the individual subunits. Examples of specific linkers include the tetrapeptide of SEQ ID No. 5. Another example is a tri-glycine linker (i.e., G-G-G). In the inventive construct, *in cis* donor strand complementation is used to stabilize adhesins and adhesin-pilin fusions for representative Class 5a, 5b, and 5c adhesins.

[0039] The contemplated composition is designed to enable as wide a range of coverage of ETEC strains as possible. As such, in one embodiment, the contemplated composition and use is aimed at inducing immunogenic response against class 5a, 5b, 5c ETEC, as well as ETEC strains expressing CS3 or CS6 fimbrial components.

[0040] In a preferred embodiment, recombinant polypeptide ETEC constructs are conjugated to *C. jejuni* capsule polysaccharide (CPS). One or more of a number of ETEC recombinant constructs can be conjugated to one or more of a number of C. jejuni capsule polysaccharide structures. Examples of Class 5 ETEC recombinant polypeptides

are listed in Table 2. In Table 2, minor subunits are stabilized by connection, via a

polypeptide linker, to associated major subunits. Alternatively, a 12-16 amino acid donor

strand, derived from the associated major subunit is connected to the minor subunit via a

polypeptide linker. These polypeptides can also be linked as per FIG. 1 and FIG. 2 to

lead to the example constructs listed in Table 3. These examples can then be conjugated

to isolated *C. jejuni* capsule polysaccharide, as in Example 1.

Table 2

Immune coverage (fimbrial types)	Subunit		SEQ ID No. Full length sequences including spd[1] (DNA/polypeptide))	SEQ ID No. Mature sequences (DNA/polypeptide)[2]
Class 5a	CfaE		56/57	115/58
	CfaB		59/60	116/61
	CsfD		64/65	117/88
	CsfA		62/63	118/89
	CsuD		70/71	119/90
	CsuA2		68/69	120/91
	CsuA1		66/67	121/92
Class 5b	CooD		74/75	122/93
	CooA		72/73	123/94
	CsdD		78/79	124/95
	CsdA		76/77	125/96
	Cos D		82/83	133/97
	CosA		80/81	126/98
	CsbD		44/45	127/46
	CsbA		47/48	128/49
Class 5c	CotD		50/51	129/52
	CotA		53/54	130/55
CS3	CstH		84/85	131/99
	CstG		86/87	132/101
CS6	CssA		134/135	1/2
	CssB		136/137	3/4

[1] "spd" refers to signal peptide. The mature polypeptide sequence, therefore, would be
the full length minus the signal peptide.
[2] DNA sequence encodes mature protein.

Table 3

Fimbriae class represented	Construct (adhesin-pilin) example[1]	SEQ ID No. (Protein/DNA)[3]
Class 5a	dsc_{14CsfA}CfaE-CfaB-CsuA2-CsfA	103/104
Class 5b	dsc_{14CsbA}CsbD-CsbA-ntd$_{15}$dsc$_{14CooA}$CooA[2]	105/106
Class 5b	dsc_{15CooA}CsbD-CsbA-CooA[2]	107/108
Class 5c	dsc_{14CotA}CotD-CotA	109/110

[1]dsc refers to donor strand complementation. The number and subunit refers to the N-terminal amino acids of length represented by the number from the subunit indicated that is connected at the C-terminus of the construct and is serving to stabilize the C-terminal construct. For example, "dsc_{14CsfA}" refers to the N-terminal 14 amino acids of CsfA connect to the C-terminus of the construct.

[2]Linkers polypeptides are GGG rather than DNKQ.

[3]Sequence in example contains a Leu-Glu-His$_6$ at the C-terminus.

[0041] An important feature of the anti-ETEC construct is the enhanced immune recognition of the fimbrial adhesion. The minor subunits (i.e., ETEC adhesin) of ETEC Class 5 fimbriae are stoichiometrically represented in very low numbers relative to the major subunit. Therefore, an important feature of the recombinant constructs is the vastly improved stoichiometric representation of the minor subunit in order to enhance immune recognition of the minor subunit. Additionally, since fimbrial subunits, such as CfaE, are relatively susceptible to proteolytic degradation outside of the fimbrial structure, stabilization of the adhesin is also important. Therefore, constructs are designed to express ETEC subunits stabilized from misfolding and degradation by donor strand complementation.

[0042] The donor β strand is provided by the major fimbrial subunit. For example, in the case of CfaE, stabilization is provided by the N-terminal region of CfaB. Engineering of dscCfaE by incorporation of a donor peptide strand from the N-terminus of the CFA/I major subunit CfaB at its C-terminus transformed an insoluble, unwieldy native, recombinant protein into a stable immunogenic composition (Savarino, U.S. Patent application publication no. 20060153878 (13 July 2006)), which is incorporated by reference, herein.

[0043] Based on its atomic structure, dscCfaE is folded into a native, β-sandwich conformation, consisting of two half-barrels, comprising the N-terminal adhesin domain (CfaEad) a short α-helical connector, and the C-terminal pilin domain (CfaEpd). The molecule is functional in that it directly mediates MRHA of bovine and human erythrocytes, and generates neutralizing antibodies that act to inhibit MRHA and decorate the tips of CFA/I fimbriae on immunoelectron microscopy.

[0044] A fusion protein was engineered by genetic insertion of the coding sequence for mature major structural subunit of ETEC adhesin, such as CfaB, to the 3'-end of the minor subunit, such as CfaE. This concept was disclosed in Savarino, U.S. Patent application (11/340,003, filed January 10, 2006), which is incorporated, herein. This molecule contains all three domains of the CFA/I fimbriae (i.e., ad, pd, and major subunit) in a ratio of 1:1:1, rather than that found in native fimbriae (ca. 1:1:1000).

[0045] A number of observations indicate the suitability of dscCfaE (cloned from ETEC strain E7473) as a vaccine antigen. First, sequencing of 31 different wild type alleles of *cfaE* from ETEC isolates of varying geographic origin and serotypes, show that the gene and predicted polypeptide sequence are nearly invariant, with three different

nonsynonymous nucleotide changes at one site each in only five of these 31 alleles

(Chattopadhyay, et al., J. Biol. Chem., 287(9): 6150-6158 (2012)). Hence, the target

protein shows uniformity in natural ETEC bacterial populations.

[0046] Additionally, CfaE, a Class 5a fimbrial adhesin, is 80-81% identical with the

other Class 5a minor subunits proteins adhesins CsuD of CS14 fimbriae and CsfD of CS4

fimbriae. CsuD and CsfD share 94% identity. This is considerably higher than the

average identity with other Class 5b and 5c fimbrial adhesins (mean 50% identity).

[0047] Moreover, rabbit anti-dscCfaE serum cross-neutralizes CS4- and CS14-ETEC in

the hemagglutination assay (HAI). A number of vaccination studies have been performed

in small (rabbit and mice) and large (monkeys and cows) animals with various routes of

administration and adjuvant combinations showing that dscCfaE is a potent immunogen

that can elicit systemic and mucosal antibodies which recognize dscCfaE and CFA/I and

are neutralizing (as measured by HAI assay).

[0048] An embodiment includes anti-class 5 ETEC constructs based on the construct

design illustrated in FIG. 1, whereby the N-terminal subunit is an ETEC class 5 minor

(i.e., adhesin) subunit, listed in Table 2, including CfaE, CsfD, CsuD, CooD, CsdD,

CosD, CsbD and CotD, connected, via a polypeptide linker, to one or more ETEC major

subunits, from the same ETEC class 5 type, listed in Table 2. The polypeptide linker can

be any of a number of polypeptide sizes. In a preferred embodiment, the linker is a

tetrapeptide with the polypeptide sequence of SEQ ID No. 5. The C-terminal class 5

subunit is connected to a donor β strand, derived from a homologous subunit and is

typically 12-19 amino acids. In alternative embodiments, one or more major subunit can

include a deletion of 12 to 16 amino acids from the N-terminal region of the subunit.

[0049] The design in FIG. 1, utilizes the concepts disclosed in Savarino, U.S. Patent

application (11/340,003, filed January 10, 2006)), including donor strand

complementation to provide stabilized class 5 ETEC adhesin. Due to the homology of

ETEC class 5 minor subunits and major subunits, FIG. 1 further contemplates multiple

constructs incorporating the fimbrial subunits of Table 2, or derivatives of these

polypeptides or DNA sequences.

[0050] The construct design, illustrated in FIG. 1, incorporates the donor strand

complementation stabilization features of Savarino (U.S. Patent application (11/340,003,

filed January 10, 2006)), and furthers it by incorporating multiple major subunits, from a

specific ETEC type, into a single adhesin-pilin construct. For example, multiple class 5b

major subunits can be connected to a class 5b adhesin (i.e, minor subunit). Embodiments

include adhesin-pilin constructs containing Csb D (ETEC Class 5b fimbrial adhesin) and

Cot D (ETEC Class 5c fimbrial adhesin). Examples, for illustration, of embodiments of

adhesin-pilin ETEC class 5 adhesin-pilin constructs, representing Class 5a, 5b and 5c are

shown in Table 3.

CS6 and CS3

[0051] Rabbit model (RITARD) studies suggest the colonization factor CS6 and CS3 has

immune-protective potential (Svennerholm, et al., Infect. Immun. 56: 523-528 (1988);

Svennerholm, et al., Infect. Immun. 58: 341-346 (1990)). As such, an important

technical goal is to reproduce a stabilized CS6 expressing recombinant structure

expressing CS6 antigens that maximally elicits antibody responses inhibitory to CS6-

directed adhesion.

[0052] Unlike class 5 ETEC fimbriae, the fimbrial structures may function as polyadhesins rather than monadhesins (Zavialov, et al., FEMS Microbiol. Rev. 31: 478-514 (2007)). Extrapolation from related fimbriae, assembly of ETEC CS6 and CS3 may be mediated by a donor strand complementation mediated process through association of a CS6 or CS3 subunit with the N-terminal donor strand region of an adjacent subunit. Additionally, protection against misfolding and proteolytic degradation may also be afforded through donor strand complementation.

[0053] Association of monomers of CS3 and CS6 was evaluated by visualization of the subunit proteins under denaturing and non-denaturing conditions in polyacrylamide gel electrophoresis (PAGE). For both CS3 and CS6 monomers, under denaturing conditions the proteins migrating at the expected sizes. Under non-denaturing conditions multiple size (i.e., ladders) are seen formed by multimeric association of the subunits.

CS6 fimbriae

[0054] CS6 fimbriae comprise CssA and CssB. Whereas the two CS3 major subunits show little to no variation in polypeptide sequences, modest variation in CS6 proteins is observed. For example, greater than 90% identity is found in CS6 protein CssA and greater than 95% identity is found in CssB allotypes. Both CS6 structural proteins exhibit a relatively low level of variation (i.e., greather than 90% amino acid conservation), with greater variation in CssA and the mutations randomly distributed along the CssA polypeptide.

[0055] In order to design an effective immunogenic composition that would be suitable for inclusion in a vaccine formulation a number of criteria were devised for determination

of suitable constructs. These included the ability to maintain a structure without unwanted self-association or assembly; thermostability; and ability to generate anti-CS6 IgG and IgA antibody levels similar to those elicited by immunization with CS6.

[0056] Monomeric CS6 subunit assembly appears to be mediated by donor strands from adjacent CS6 subunits, as discussed above. It is hypothesized that interaction to form these stable structures is mediated by inter-subunit interaction through donor strand complementation. Donor strand complementation also affords protection against misfolding and proteolytic degradation. Therefore, in a preferred embodiment, multimeric CS6 constructs were developed to take advantage of these attributes of donor strand complementation. Additionally, multimeric expression provides more efficient manufacture over production of monomers.

[0057] In one embodiment, a construct conjugated to *C. jejuni* comprises a multimeric CS6 with one or more of the CS6 subunits, CssA and CssB, or allelic variation or derivatives, with the construct design configuration illustrated in FIG. 1. In a preferred embodiment, the construct comprises a dimer of CssB and CssA with CssB N-terminal to CssA (i.e., CssB-CssA).

[0058] As illustrated in FIG. 1, or FIG. 2, CS6 subunit association is stabilized by *in cis* donor β strand complementation. Donor strand complementation is afforded by linking a CS6 subunit at its C-terminus, to the donor β strand region of another CS6 subunit, via a tetrapeptide linker. The linker can be any of a number of polypeptide regions. However, in a preferred embodiment, the linker is either as in SEQ ID No. 5 or a triglyicine linker. In the case of a terminal CS6 subunit, stabilization is provided by donor β strand, connected at its C-terminus, from a homologous or heterologous CS6 subunit.

Homologous subunit is defined as two subunits of the same form (e.g., CssA OR CssB).

Heteterologous subunits are of different forms (e.g., one is derived from CssA the other

from CssB. The CS6 donor β strand is typically the N-terminal 14-16 amino acid region

of CS6 subunit. The recombinant protein can be constructed with or without

hexahistidine affinity tags, which are typically on the C-terminus.

[0059] Additionally, to prevent recombinant polypeptide constructs forming molecular

associations resulting in un-desirable non-covalent oligomer formation, in a preferred

embodiment, the N-terminal 14-16 amino acids of the N-terminal CS6 subunit is deleted.

As an illustration, "dsc$_{B14}$CssBA" would contain a heterologous donor strand (i.e., "dsc"),

from CS6 CssB, inserted at the C-terminus of the construct. In this case, the donor strand

is 14 amino acids in length, as indicated by the "14." Similarly, a constructed designated

"ntd$_{15}$dsc$_{A}$CssBA" would contain a homologous donor strand at the C-terminus of the

construct and also comprises a deletion of the N-terminal amino acid region (termed

"ntd").

[0060] Examples of constructs comprise one or more CS6 subunits with amino acid

sequences sequences selected from the group consisting of SEQ ID No. 2 (CssA) or SEQ

ID No. 4 (CssB), or derivatives of these polypeptides. The DNA sequence for CssA is

SEQ ID No. 1 and for CssB, SEQ ID No. 3. The subunits are connected by a polypeptide

linker sequences. In a preferred embodiment, the linker is a tetrapeptide with the amino

acid sequence of SEQ ID No. 5.

CS3 fimbriae

[0061] Savarino (U.S. Patent application No. 11/340,003 (2006)) discloses donor strand complementation stabilized ETEC constructs. Embodiments of this application incorporate the donor strand stabilization of CstH and adds the second CS3 subunit, CstG. Embodiments herein add additional features found to be important for stabilization of the CS3 subunits and immunogenicity against CS3. CS3 comprises CstH and CstG. The CS3 structural protein CstH is invariant. CstG is also highly conserved, showing 99-100% identity in polypeptide sequence for 39 wildtype CS3 genes sequenced. Similiarly, although some variation CstG is observed, it is also relatively invariant, with 99-100% amino acid conservation.

[0062] CS3 contains both CstG and CstH, in near equal amounts. Therefore, dimeric constructs were devised incorporating CstG and CstH, according to the template construct design of FIG. 1.

[0063] In one embodiment a polypeptide construct conjugated to C. jejuni capsule polysaccharide comprises a CS3 s construct designed according to FIG. 1. In FIG. 1, CS3 constructs comprise one or more CS3 fimbrial subunits connected via a polypeptide linker. The C-terminal fimbrial subunit is connected, via a polypeptide linker, to a donor β strand region of a CS3 fimbrial subunit. The C-terminal donor β strand can be derived from the same CS3 subunit to which it is connect (i.e., homologous) or derived from a different subunit (i.e., heterologous). The polypeptide linker can be any number of polypeptide regions, however, in a preferred embodiment, the linker is a tetrapeptide of the sequence of SEQ ID No. 5, or a triglycine (i.e., G-G-G). The donor β strand region is the N-terminal 14 - 16 amino acids of the mature CstH or CstG protein. In alternatives

of this embodiment, the first 14 - 18 amino acids of the N-terminal region of the N-terminal most subunit is deleted to avoid undesirable associations.

[0064] In a preferred embodiment, the CS3 construct is a dimer. Although other examples are contemplated using the design of FIG. 1, as an illustrative example, the recombinant polypeptide construct can be configured as "dsc$_{16CstH}$CstG-(linker)-CstH". In this example, the mature CstG polypeptide (SEQ ID No. 101) or full length polypeptide sequence (SEQ ID No. 87) is connected at its C-terminus to CstH polypeptide (SEQ ID No. 99), via a polypeptide linker. In this example, the CstH polypeptide, is connected, at its C-terminus, to a donor β strand region of 16 amino acids derived from CstH via a polypeptide linker.

[0065] Other examples can include constructs, according to FIG. 1. In other examples, the C-terminal donor β strand can be either homologous (derived from the same subunit) or heterologous (derived from a different subunit) to the C-terminal most CS3 fimbrial subunit.

Construction of multipartite fusion constructs

[0066] Immunity to multiple strains of ETEC is important to obtain the greatest extent of anti-ETEC immunity. Toward this goal, recombinant polypeptide constructs were developed comprising two or more subunits derived from different ETEC fimbrial types according to the design illustrated in FIG. 2 to form multipartite fusion constructs. As used, herein, multipartite fusion or multipartite fusion constructs are recombinant polypeptide constructs according to FIG. 2. In this design, different ETEC fimbrial types are defined as fimbrial proteins derived from fimbriae of different strain ETEC types, as listed in Table 4 or 5, or deriviates of these polypeptides or DNA sequences. For

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example, the fimbrial type "CS3" comprises CstH and CstG. The fimbrial type "CS6"

comprises CssA and CssB. The fimbrial types of Class 5 ETEC include the fimbrial

types Class 5a, Class 5b and Class 5c.

[0067] In a preferred embodiment, major and/or minor subunits, derived from the same

ETEC fimbrial type are connected, via polypeptide linkers, and stabilized by donor β

strand complementation, as illustrated in FIG. 1. A multipartite fusion comprises one or

more fimbrial subunits of the same fimbrial type, as in FIG. 1, connected to one or more

fimbrial subunits derived from a different fimbrial type as illustrated in FIG. 2.

[0068] In one embodiment, the multipartite fusion construct can include a deletion of the

N-terminal region of one or more fimbrial subunits, but is preferably on the N-terminal

most fimbrial subunit for a given ETEC fimbrial type, as illustrated in FIG. 2. This

feature prevents undesirable associations with other monomers or multimers. The size of

the deletion of the N-terminal region is 14 to 18 amino acids. In other embodiments,

multipartite fusion constructs comprising Class 5 adhesins do not contain a deletion of

the N-terminal region.

[0069] As illustrated in FIG. 2, the C-terminal subunit, for an ETEC fimbrial type, is

connected to and stabilized by a donor β strand, connected to the subunit via a

polypeptide linker, wherein the donor β strand is either that derived from the adjacent

subunit (i.e., homologous) or from a different subunit of the same fimbrial type (i.e.,

heterologous). The size of the N-terminal donor strand depends on the fimbrial type and

subunit stabilized. In preferred embodiments, for class 5 fimbrial subunits, the donor β

strand, derived from the N-terminal region of the class 5 subunit stabilized, is 12 to 16

amino acids. For CS3 and CS6 subunits, the donor β strand is 14 to 16 amino acids. As

mentioned above, the construct can contain a deletion of the N-terminal region of the N-terminal subunit. This feature prevents undesirable associations with other monomers or multimers. The size of the deletion of the N-terminal region is 14 to 18 amino acids.

[0070] As illustrated in FIG. 2 multiple constructs as in FIG. 1 are connected forming a recombinant polypeptide construct comprising two or more ETEC fimbrial types. In this way, one or more major or minor subunits, derived from the same ETEC fimbrial type, are connected via polypeptide linkers and stabilized by donor strand complementation. In another embodiment, one or more glycine residues separates different ETEC fimbrial types, acting as a "swivel" means between the ETEC types. The glycine residue, due to its small, unbranched molecular characteristics, enables rotary freedom of the molecular components. Subunits derived from the same fimbrial type (as in FIG. 1) are connected by a polypeptide linker, with the subunits stabilized by donor strand complementation. As shown in FIG. 2, the C-terminal subunit of each ETEC fimbrial type is stabilized by a donor β strand that is homologous or heterologous to the C-terminal subunit of that fimbrial type.

[0071] In other embodiments, the construct can contain an N-terminal deletion at the N-terminus of the entire construct as well as an additional deletion, of 14 to 18 amino acids, at the N-terminus of the first "internal" subunit that is of a different fimbrial type. This is illustrated in FIG. 2. In the case of the deletion on the N-terminus of the "internal" subunit, the deletion serves to shorten the length between subunits, thus reducing the likelihood of misfolding and proteolytic cleavage. In another embodiment, a donor β strand, derived from a homologous or heterologous subunit, is inserted at the C-terminus of the C-terminal CS6 or CS3 subunit. For class 5 fimbrial subunits, the donor β strand,

derived from the N-terminal region of the class 5 subunit that is stabilized, is 12 to 16

amino acids. For example, in preferred embodiments, CfaB is stabilized by a 14 amino

acid donor β strand; CsfA by a 14 amino acid donor β strand; CsbA by a 15 amino acid

donor β strand, CooA by a 14 amino acid donor β strand and CotA by a 14 amino acid

donor β strand. For CS3 and CS6 subunits, the donor β strand is 14 to 16 amino acids,

with preferred embodiments of CS3 fimbrial subunits (i.e., CstH or CstG) stabilized by a

16 amino acid donor β strand derived from CstH or CstG; and CS6 fimbrial subunits (i.e.,

CssA or CssB) stabilized with a 16 amino acid donor β strand derived from CssA or

CssB. However, other donor β strand lengths are envisioned.

[0072] The inventive compositions can utilize different linker sequences. In a preferred

embodiment, the linker contains the amino acid sequence of SEQ ID No. 5. In another

embodiment, the linker is a tri-glycine linker. In other embodiments, the C-terminal end

of the construct contains a histidine tag for purification of the construct.

[0073] In the inventive construct, *in cis* donor strand complementation is used to stabilize

adhesins and adhesin-pilin fusions for representative Class 5a, 5b, and 5c adhesins. For

each adhesin target group, in a preferred embodiment, the compositions are constructed

with the intent of eliciting anti-adhesive immune responses. Further towards this goal,

Class 5 multipartite fusions comprising Class 5 adhesin minor subunits are typically

construct such that the adhesin (i.e., minor fimbrial subunit) is located at the N-terminus

of the constructed with the minor fimbrial subunit linked at its C-terminus to one or more

major subunits, followed at the terminal end of the construct with the donor β-strand of

the last major subunit.

[0074] Other embodiments include constructs comprising Class 5a adhesin CfaE

tandemly linked at its C-terminus to one or more of CfaB (CFA/1 major subunit), CsuA2

(CS14 major subunit) and CsfA (CS4 major subunit); Class 5b adhesin CsbD tandemly

linked at its C-terminus to one or more of CsbA (CS17 major subunit), which shares high

identity to the CS19 pilin subunit CsdA, and CooA (CS1 major subunit), which shares

high identity to the PCFO71 pilin subunit CosA; and Class 5c adhesin CotD tandemly

linked at its C-terminus to CotA (CS2 major subunit).

[0075] Embodiments of ETEC multipartite fusion constructs are illustrated in Table 4

and 5. In this embodiment, constructs comprise any major or minor ETEC fimbrial

subunit from Table 2 in multiple combinations, connected by linker polypeptides and

stabilized from proteolytic degradation by donor strand complementation utilizing the

design illustrated in FIG. 2. Table 2 lists the ETEC fimbrial subunits (major and minor

subunits) than can be used and incorporated into the multipartite fusion construct design

of FIG. 2, which can then be conjugated to *C. jejuni* capsule polysaccharide or *Shigella*

LPS. Any subunit, therefore, is combined with one or more other ETEC major subunits

from any ETEC fimbrial phenotypic type, including Class 5a, 5b, 5c, CS3 and CS6.

[0076] The recombinant polypeptide construct motif comprises a whole or immunogenic

fragment of a minor or major ETEC fimbrial subunit connected at its C-terminal end to a

linker. The linker is connected at its C-terminus to a whole major ETEC fimbrial subunit

or a polypeptide donor strand of an ETEC major structural subunit, derived from the

same fimbrial type. The whole ETEC major subunit or donor strand polypeptide is then

connected, via a linker at its C-terminal end, to one or more additional major structural

fimbrial subunits, derived from the same fimbrial type, from Table 2.

[0077] The strategy for selecting and developing specific genetic fusion constructs is guided, in part, by the phylogenetic and antigenic relatedness of subunits. For example, constructs containing Class 5a, 5b and 5c pilin subunits are selected based on the relatedness of minor and major subunits within a particular ETEC fimbrial class (i.e., class 5a, 5b or 5c). As such, adhesin (i.e., minor fimbrial subunit) from a specific fimbrial type (e.g., Class 5a) are linked to Class 5a major subunits. Further selection of subunits is guided and based on epidemiological study analysis in order to achieve optimum immunogenic coverage of ETEC strains. What C. jejuni capsule polysaccharide to conjugate is predicated primarily on epidemiological data suggesting pathogenicity of the strain providing the capsule polysaccharide. Although many C. *jejuni* strains exist, most are not pathogenic.

[0078] In the multipartite constructs listed in Table 4 and 5 the linker polypeptide, depending on the example construct, can comprise a four (4) amino acid sequence (tetrapeptide) or a tri-glycine. Also, as illustrated in FIG. 2, the subunits are interconnected and stabilized by donor strand complementation, which is denoted by "dsc". In this nomenclature, the fimbrial subunit derivation is also indicated. For example, in the construct "dsc_{16CstH} CstG-CstH-(G)-$ntd_{15}dsc_{16CssA}$CssA-CssB", the N-terminal CS3 subunit "CstG" is connected, via a linker, to the CS3 subunit "CstH", which is connected, via a linker, to a donor strand of 16 amino acids derived from "CstH." Similarly, the N-terminal CS6 subunit "CssB" is connected, via a linker, as illustrated in FIG. 2, to a 16 amino acid donor strand derived from "CssA." In this example, donor strand complementation of the "CssB" subunit is via a heterologous donor strand (i.e., derived from "CssA)."

[0079] In Table 4 and 5 the examples contain a "G" (i.e., glycine) to provide a "swivel." Also, in some examples, the N-terminal region of N-terminal CS6 subunit is deleted

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(delineated by "ntd") to avoid undesirable association with other CS6 subunits, as described above. It should be noted that, in addition to the examples illustrated in Table 4 or 5 (or Table 3), other combinations of major and minor subunits are contemplated utilizing the construct design illustrated in FIG. 2 and the fimbrial subunits of Table 2. In some sequences listed, a six (6) histidine (i.e., His₆) tag is inserted. The constructs can be designed to include the histidine (i.e., His₆) tag or designed without this tag region. Additionally, some sequences contain the signal peptide (designated "spd" in Table 2 and 3) region. Constructs can be constructed with or without this region, as well, which may be added to improve manufacturing efficiency of the multipartite fusion construct.

Table 4

Fimbrial type (SEQ ID No. DNA/Protein)	Examples of CS3 containing constructs[1,2,4,5]
Class 5a/CS3 (6/7)	dsc_{14CfaB}-CfaE-CfaB-(G^3)-$ntd_{18}dsc_{16CstH}$CstG-CstH
Class 5a/CS3 (8/9)	dsc_{14CsfA}CfaE-CfaB-CsuA2-CsfA-(G)-$ntd_{18}dsc_{16CstH}$CstG-CstH
Class 5b/CS3 (10/11)	dsc_{14CsbA}CsbD-CsbA-$ntd_{15}dsc_{14CooA}$CooA-(G)-$ntd_{18}dsc_{16CstH}$ CstG-CstH
Class 5c/CS3 (12/13)	dsc_{14CotA}CotD-CotA-(G)-ntd_{18} dsc_{16CstH}CstG-CstH
CS3/toxin fusion (36/37)	dsc_{16CstH}CstG-CstH-sCTA2
LTB multimeric composition (38/39)	LTB_5
CS3/CS6 (14/15)	dsc_{16CstH}CstG-CstH-(G)-$ntd_{15}dsc_{16CssA}$CssA-CssB
CS6/CS3 (34/35)	$ntd_{14}dsc_{16CssB}$CssB-CssA-(G)-$ntd_{18}dsc_{16CstH}$CstG-CstH

[1] All combinations can include a histidine (i.e., His₆) at the C-terminal end.

[2] Subunits can be linked via either DNKQ or tri-glycine linker.

[3] (G) refers to glycine residue introduced to provide a "swivel."

[4] "ntd" refers to N-terminal deletion (excised from mature protein) with extent of deletion (i.e., amino acids) indicated.

[5] "dsc" refers to span of N-terminal residues from donor β-strand, its amino acid length and its source.

Table 5

Fimbrial type (SEQ ID No. DNA/Protein)	Examples of CS6 containing constructs
CS6/CS3 (34/35)	ntd$_{14}$dsc$_{16CssB}$CssB-CssA-(G)-ntd$_{18}$dsc$_{16CstH}$CstG-CstH
CS3/CS6 (32/33)	dsc$_{16}$CstG-CstH-(G)-ntd$_{14}$dsc$_{16CssB}$CssB-CssA
Class 5b/CS6 (28/29)	spd$_{19}$ dsc$_{14CotA}$CotD-CotA-(G)-ntd$_{14}$dsc$_{16CssB}$-CssB-CssA
Class 5b/CS6 (30/31)	dsc$_{14CotA}$CotD-CotA-(G)-ntd$_{14}$dsc$_{16CssB}$-CssB-CssA
Class5b/CS6 (24/25)	spd$_{19}$dsc$_{15CsbA}$CsbD-(GGG)-CsbA-(GGG)-ntd$_{14}$dsc$_{14CooA}$CooA-(G)-(GGG)-ntd$_{14}$dsc$_{16CssB}$CssB-CssA
Class 5b/CS6 (26/27)	dsc$_{15CsbA}$CsbD-(GGG)-CsbA-(GGG)-ntd$_{14}$dsc$_{14CooA}$CooA-(G)-(GGG)-ntd$_{14}$dsc$_{16CssB}$CssB-CssA
Class 5a/CS6 (16/17)	dsc$_{14CfaB}$CfaE-CfaB-(G)-ntd$_{16}$dsc$_{16CssA}$CssB-CssA
Class 5a/CS6 (113/114)	dsc$_{14CfaB}$CfaE-CfaB-(G)-ntd$_{16}$dsc$_{16CssB}$CssB-CssA
Class 5a/CS6 (18/19)	dsc$_{14CfaB}$CfaE-CfaB-(G)-ntd$_{16}$dsc$_{16CssB}$CssA-CssB
Class 5a/CS6 (111/112)	dsc$_{14CfaB}$CfaE-CfaB-(G)-ntd$_{16}$dsc$_{16CssA}$CssA-CssB
CS3/CS6 (101/102)	dsc$_{16CssA}$CssA-CssB-(G)-ntd$_{18}$dsc$_{16CstH}$CstG-CstH
Class 5a/CS6 (22/23)	dsc$_{14CsfA}$CfaE-CfaB-CsuA2-CsfA-(G)-ntd$_{14}$dscCssB-CssA
Class 5a/CS6 (20/21)	spd$_{22}$ dsc$_{14CsfA}$CfaE-CfaB-CsuA2-CsfA-(G)-ntd$_{14}$dscCssB-CssA
CS6-chimera (40/41)	ntd$_{14}$dsc$_{16CssB}$CssB-CssA-sCTA2
CS6-chimera (42/43)	ntd$_{15}$dsc$_{16CssA}$CssA-CssB-sCTA2

[1] All combinations can include a histidine (i.e., His$_6$) at the C-terminal end.

[2] Subunits can be linked via either DNKQ or tri-glycine (GGG) linker. In preferred embodiments, DNKQ is used, except where indicated with (GGG).

[3] (G) refers to glycine residue introduced to provide a "swivel."

[4] "spd" refers signal peptide. Number indicates number of amino acids.

[5] "ntd" refers to N-terminal deletion (excised from mature protein) with extent of deletion (i.e., amino acids) indicated.

[6] "dsc" refers to span of N-terminal residues from donor β-strand, its amino acid length and its source.

[0080] In another embodiment, recombinant polypeptide constructs can contain a C-terminal toxin A subunit, such as cholera toxin A2 (CTA) to form a chimeric molecule. In this embodiment, a full-length or truncated CTA2 is connected to CS6 or CS3 multimeric recombinant polypeptide construct, such as a CS6 or CS3 dimer.

[0081] Examples of these toxin constructs are illustrated in Table 4 and 5. In these constructs, the LTB gene and the CS3 or CS6 – toxin chimera are separately expressed. LTB, once expessed, would self assemble to form a pentameric structure. The ensuing LTB multimeric composition (i.e., LTB$_5$) and CS3 or CS6 –toxin chimera then non-covalently associate to form a holotoxin-like heterohexamer.

[0082] Although other examples are contemplated, the sequences of examples of illustrative chimeric constructs, containing a C-terminal toxin component, are illustrated in Table 4 (for CS3) and Table 5 (for CS6).

[0083] For CS3-chimeric molecules, one or more CS3 fimbrial subunits are connected, as in FIG. 1, via a polypeptide linker, preferably a tetrapeptide or triglycine. The C-terminal most CS3 fimbrial subunit is then connected to a donor β strand, via a polypeptide linker. The donor strand can be homologous or heterologous to the C-terminal fimbrial subunit. The donor strand is then connected to a toxin fragment, such as CTA2. The CS3-chimera example shown in Table 4, comprise the polypeptide sequence of SEQ ID No. 37, which is encoded by the DNA sequence of SEQ ID No. 36. In this example, the N-terminal fimbrial subunit is CstG with a pelB leader (22 amino acids) connected at its N-terminal end (see FIG. 13). However, different ordering of CS3 fimbrial subunit units is contemplated. Also, in this example, the CstH is connected, via a polypeptide linker, to a 16 amino acid donor strand derived from the N-terminal 16 amino acids of CstH, which

is connected to an A2 toxin fragment (i.e., CTA2). In a preferred embodiment, LTB is

also expressed. LTB comprises the amino acid sequence of SEQ ID No. 39 and is

encoded by the nucleotide sequence of SEQ ID No. 38. Once expressed, the LTB

sequence would self assemble into a pentamer and associate, non-covalently, with the

CS3-chimera to form a hetero-hexameric holotoxin-like structure.

[0084] CS6 toxin chimera examples are also illustrated in Table 5. For CS6 chimeras, as

in CS3, one or more CS6 fimbrial subunits are connected via a polypeptide linker,

preferably a tetrapeptide or triglycine. The C-terminal most CS6 fimbrial subunit is then

connected to a donor β strand, via a polypeptide linker. The donor strand can be

homologous or heterologous to the C-terminal fimbrial subunit. The donor strand is then

connected to a toxin component (e.g., CTA2). In a preferred embodiment, like for CS3,

the chimera is co-expressed, with LTB, which self assembles into a pentamer to form a

non-covalent association with the chimeric adhesion-toxoid fusion molecule.

[0085] Although many additional combinations are possible, in the examples shown in

Table 5, the constructs are dimers of CS6 subunits, connected via a tetrapeptide linker,

with the C-terminal fimbrial subunit connected, via a tetrapeptide linker to a donor β

strand. The donor β strand can be homologous or heterologous to the C-terminal most

fimbrial subunit. However, in the examples in Table 5 the donor strands are heterologous

to the C-terminal fimbrial subunit. The donor strand is then connected to a cholera toxin

A2 (CTA2) subunit. The polypeptide sequences of one of the examples is as in SEQ ID

No. 43, which is encoded by the nucleotide sequence of SEQ ID Nos. 42. In this

example, the N-terminal subunit is CssA, with the N-terminal 15 amino acids of the

mature CssA sequence deleted. In this example, a pelB leader sequence (22 amino acids) was also added, which is illustrated in FIG. 14.

Example 3: C. jejuni capsule polysaccharides

[0086] Recent development of a molecular CPS typing system re-enforced the strong correlation between CPS and Penner types (Poly, et al., J. Clin. Microbiol. 49: 1750 (2011)). Both Penner serotyping and molecular CPS typing have revealed the predominance of a handful of CPS types worldwide. Also, despite over 60 Penner serotypes having been identified, most Campylobacter diarrheal disease is caused by *C. jejuni* expressing only a limited number of serotypes. Therefore, only selected strains of C. jejuni, predicated on epidemiological studies, provides suitable candidate strains for development of vaccine compositions. However, despite the importance of this organism to human disease, there are no licensed vaccines against *C. jejuni.*

[0087] *C. jejuni* capsule polysaccharide (CPS) was extracted from C. jejuni strains selected based on their association with diarrheal disease. CPS from bacteria was extracted by hot water–phenol extraction for 2 h at 70 °C. The aqueous layer was dialyzed (1000 Da) against water followed by ultracentrifugation to separate the CPS from the LOS. The supernatant material containing the CPS was subjected to size-exclusion chromatography (Sephadex G50) for further purification to yield the intact CPSs. Monosaccharide composition was performed using a procedure amenable to the alditol acetate method (Chen, et al., Carbohydr. Res. 343: 1034 (2008)) with the alditol acetates being analyzed in a ThermoFinnigan POLARIS™-Q (Thermo Fisher Scientific, Inc, Waltham, MA) gas chromatograph/mass spectrometer (GC/MS) using a DB-17

capillary column. The sugar linkage types were characterized by characterization of the

permethylated alditol acetates by GC/MS as previously described (Chen, et al.,

Carbohydr. Res. 343: 1034 (2008)). The NMR experiments were performed on a Bruker

400 MHz spectrometer (Bruker Corporation, Billeria, MA) equipped with a Bruker cryo

platform at 295 K with deuterated trimethylsilyl propanoic acid and orthophosphoric acid

as external standards. The structures of important pathogenic *C. jejuni* capsule

polysaccharides are shown in Table 6.

Table 6

Capsule type	Polysaccharide structure
HS1	→4)-a-D-Galp-(1→2)-Gro-(1→P→ 3　2 ↑　↑ 1　1 [MeOPN]→3)-Fruf　Fruf-(3←[MeOPN]
HS44	→4)-a-D-Galp-(1→2)-Gro-(1→P→
HS3	→4)-[P→3]-alpha-D-Gal-(1→3)-[P→2/7]-6-d-alpha-D-ido-Hep-(1→; or →4)-[P→3]-alpha-D-Gal-(1→3)-[P→2]-L-glycero-alpha-D-ido-Hep-(1→ (where P represents O-methyl-phosphoramidate)
HS4/13/64	→3)-6-deoxy-beta-D-ido-Heptose-(1→4)-beta-D-GlcNAc-(1→.
HS23/36	[→3)-α-D-Gal-(1→2)-6d-α-D-altro-Me-Hep-(1→3)-β-D-GlcNAc-(1→]$_n$
HS15	[→3)-α-Araf-(1→3)-6-d-α-gulo-Hepp-(1→]$_n$
HS10	[→3-β-GalpNAc-(1→]$_n$; 4 ↑ 6-d-α-gal-Hep 3 ↑ MeOPN
HS13	MeOPN ↓ 7 [→4)-β-Glcp-(1→3)-6-d-α-ido-Hepp]$_n$
HS13	MeOPN ↓ 7 [→4)-β-Glcp-(1→3)-LD-ido-Hepp]$_n$
HS2	[MeOPN] ↓ 4 (3,6,-O-Me)-D-glycero-α-L-glc-Hepp 1 ↓ [→2)-β-D-Ribf-(1→5)-β-D-GalfNAc-(1→4-α-D-GlcpA6-(1→]$_n$ 3　　　　　　　5 ↑　　　　　　↑ [MeOPN]　　　[MeOPN]

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[0088] Additionally, the capsule polysaccharide from the HS5 strain of C. jejuni can be attached. HS 5 contains a complex of variations of polysaccharides. These include the following structures:
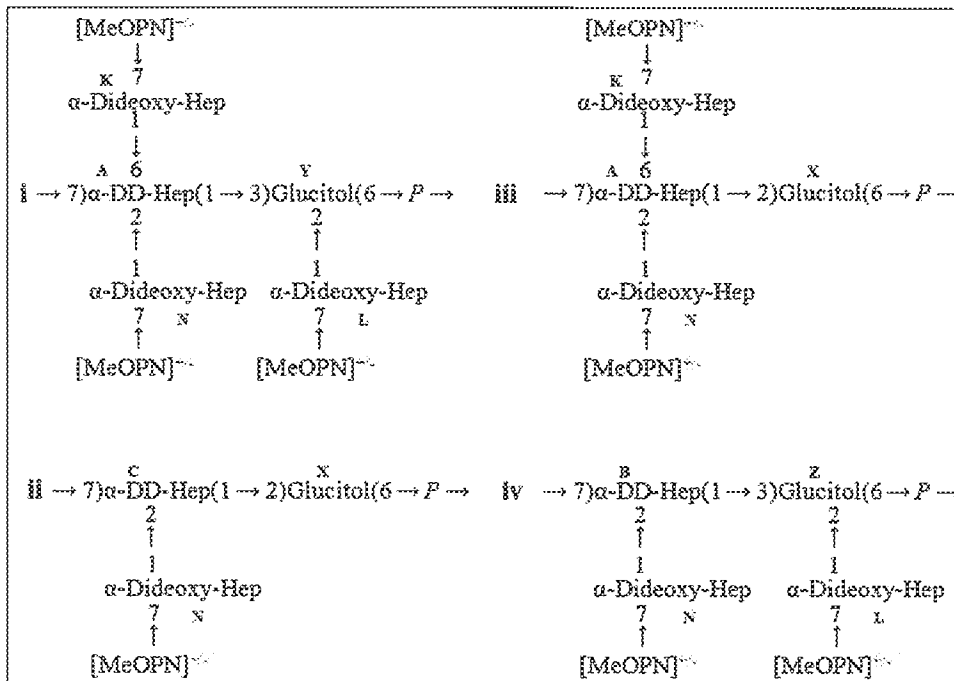
Example 4: Induction of immune response by ETEC-Campylobacter capsule conjugates.

[0089] Induction of an immune response by the conjugates was evaluated. In these studies, BALB/c mice were immunized with escalating amounts of vaccines administered with alhydrogel[®] (Sergent Adjuvants, Clifton, NJ). Mice received a total of two immunization at a 4-week interval.

[0090] The results of these studies is illustrated in FIG. 4. As shown in FIG. 4, two weeks following the first immunization, mice immunized with HS36-CfaEB (10 μg, 60

μg) and HS36-CfaE (60 μg) exhibited significant levels of serum IgG antibodies specific

against HS36 CPS (p< 0.05) (see FIG. 4 (A)), comparared to pre-immune sera.

Following two immunizations all groups of immunized animals had antibody levels that

were significant increased (p<0.05) compared to levels observed after only one

immunization. This effect was not dose dependent at the vaccine doses tested.

[0091] Furthermore, as illustrated in FIG. 4 (B), antibodies against CfaE was also

determined by ELISA. As shown in FIG. 4(B), all mice immunized with the conjugate

vaccine possessed significant levels of anti-CfaE IgG (p<0.05) for CPS – CfaE or CPS

CfaEB. No dose dependent effects were observed and all groups displayed similar levels

of CfaE-specific IgG.

[0092] The data shown in FIG. 4 illustrates that the conjugate vaccine comprising an

ETEC adhesin-based carrier protein conjugated to a *C. jejuni* CPS is capable of inducing

an immune response against both bacterial components, i.e., *C. jejuni* CPS and ETEC

CfaE.

[0093] To determine the levels of functional anti-adhesive antibody generated by HS36

conjugate vaccines, serum samples were tested by hemagglutination inhibition assay

(HAI) assay in order to measure functional anti-adhesive antibodies present in the

immune mouse serum.

[0094] The HAI assays were conducted by evaluating samples from each animal. The

samples were initially diluted 1:8, then diluted two-fold over a wide range of dilutions.

Each serum dilution was incubated with an equal volume of CFA/I$^+$ ETEC bacteria

(strain H10407), which further diluted the serum 1:2. The final lowest dilution tested

dwas 1:16, which was the limit of detection (LOD). The pre-incubated mixture was

subsequently mixed with bovine erythrocytes in the presence of 0.5% mannose in U-

bottom 96-well plates. In the absence of anti-adhesive antibodies, the erythrocytes

formed visible agglutinated "buttons" of cells. In the presence of anti-adheisve

antibodies, agglutination was inhibited. The HAI titer was the highest serial dilution that

completely inhibited agglutination. If there was not detectable inhibition at the lowest

serum dilution of 1:16, the samples were assigned a value of one-half of the detection

limit (i.e., 8) for computational purposes.

[0095] The results of the HAI analysis are illustrated in FIG. 5. Prior to immunization,

pooled serum contained HAI titers below the the assay's level of detection. Following

immunization, all groups of mice displayed significantly (p<0.05) higher levels of anti-

adhesive antibodies in their sera compared to pre-immune titers. Genrally, mice

immunized with HS36-CfaEB conjugate vaccine exhibited higher HAI titers. However,

the only significance difference (P<0.05) observed was between mice immunized with an

HS36-CfaEB (60 µg) and HS36-CfaE (10 µg).

Example 5: Immune response against multiple MeOPN-6-Gal

Synthesis of polysaccharide construct

[0096] A polysaccharide constructed was synthesized as shown in FIG. 6. Starting from

a previously reported compound 4-methoxyphenyl-α-D-galactopyranoside (see also FIG.

7, structure 1) (Comfort, et al., Biochem. 46: 3319-3330 (2007)), trityl group was

selectively introduced to C-6. Originally, benzoylation was performed on compound

(FIG. 7, structure 2), however extensive migration observed during the introduction of

MeOPN lead us to look for a more suitable protecting group. Therefore, allyl groups were

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selected to protect the C-2, C-3 and C-4 positions which were resistant to migration. Allyl

groups were later deprotected with catalytic hydrogenolysis which proved to be

compatible with the MeOPN modification.

[0097] As shown in FIG. 6 and FIG. 7, after allyl groups were installed, an amino-

pentanyl linker was introduced to the anomeric position as a site for conjugation. Starting

from galactoside (FIG. 7, structure 3), 4-methoxyphenyl group (OMP) was first removed

with cerium ammonium nitrate (CAN). The corresponding hemiacetal was then

converted into trichloroacetimidate donor . 5-Amino-*N*-phthalimido-pentanyl linker was

then introduced with TMSOTf as activator at 0 °C. Compound 5 (FIG. 7) was collected

with 65% as the β anomer and 29% as the α anomer. The removal of trityl group gave a

free 6-hydroxyl group for modification.

[0098] The strategy for the introduction of MeOPN group was inspired by a similar

reaction initially proposed by C. Mara et al, Bioorg. Med. Chem. Lett. 6180-6183 (2011).

Compound 6 (FIG. 6 and FIG. 7) was treated with commercially available methyl

dichlorophosphate in the presence of triethyl amine, followed by ammonolysis. Due to

the chirality nature of the newly introduced MeOPN (R and S), product 7 (FIG. 7,

structure 7) was collected as a mixture of two diastereoisomers. ^{1}H NMR was able to

confirm that product 7 (FIG. 7) was indeed a 1:1 mixture of two diastereoisomers,

revealing two sets of signals throughout the spectrum, such can be seen for anomeric and

O-Me signals. The reaction yielded a mixture of side products, the most abundant being

the O-Me group being replaced by a second NH_2, accounting for the poor yield of this

reaction.

[0099] Allyl and phthlimido protecting groups were removed with palladium (II) chloride

and hydrazine respectively, generating product 9 (FIG. 7, structure 9). Similar to

compound 7 (FIG. 7), a mixture of diastereoisomers is apparent in NMR. Although not

optically pure, the ^{31}P NMR result agrees with native MeOPN-containing

polysaccharides, having a phosphorous signals around 14 ppm.[3] ^{1}H-^{31}P HMBC NMR

experiment was able to confirm that the MeOPN was introduced to the O-6 position,

showing correlation signal with O-Me as well as the H-6 signals.

Induction of immunity against MeOPN-6-Gal

[00100] In one embodiment, galactose modified at the 6 carbon with O-methyl

phosphoramidate (MeOPN-6-Gal) is used to induce immunity against multiple C. jejuni

strains, even those strains not expressing MeOPN-6-Gal. As illustrated in FIG. 8, the

monosaccharide construct MeOPN-6-Gal was recognized by antibody against capsule

polysaccharide isolated from HS23/36, conjugated to CRM$_{197}$. Unexpectedly, antibody

against polysaccharide from HS4, conjugated to CRM$_{197}$, also elicited an equivalent

response, as anti-HS23/36 CRM$_{197}$ conjugate, against MeOPN-6-Gal. Also, anti-HS1-

CRM$_{197}$, also reacted to MeOPN-6-Gal, although to a somewhat less extent.

[00101] The strong cross-reactivity with MeOPON-6-Gal exhibited against HS23/36

and HS4 antibody may be explained by the the fact that MeOPN-6-Gal share epitopic

structures with HS23/36 and HS4 capsule polysaccharides. One explanation may be that

the MeOPN group in both HS23/36 and HS4 is to a primary hydroxyl. The cross reaction

of MeOPN-6-Gal (HS23/36) with HS4, which contains MeOPN-7-6d-β-D-*ido*-Heptose,

was unexpected, but may be due to the linkage of MeOPN to primary hydroxyl positions on both sugars. This feature is illustrated in FIG 8 by the arrow.

Example 6: Immunogenic composition against C. jejuni and enterotoxigenic Escherichia coli (ETEC) using a combined C. jejuni capsule/ETEC construct

[00102]   A synthetic conjugate vaccine strategy can be developed to protect against multiple enteric pathogens.   Most efforts at development of vaccines against bacterial enteric pathogens are limited to a specific pathogen.   The ability to combine vaccines against multiple, antigenically variable pathogens in a single, multi-valent, injectable vaccine would greatly simplify approaches to prevent acquisition and transmission of these pathogens worldwide. Globally, ETEC and *C. jejuni* are among the leading causes of bacterial diarrheal disease. In addition CJ has been causally linked to several serious sequelae including Guillain Barré Syndrome, irritable bowel syndrome, and reactive arthritis. Moreover, recent studies have indicated an association between CJ infections and malnutrition and growth stunting in young children in resource-limited settings.

[00103]   Using conventional methods, we have developed conjugate vaccines containing CJ polysaccharide capsules that have proven to be immunogenic in multiple animal species and to confer protection against *C. jejuni* diarrhea in NHP. The newer synthetic approach is based on recent data that the immunodominant epitope on CJ polysaccharide capsule conjugate vaccines is the MeOPN modification found on different sugars in different capsule types.

[00104]   Therefore, an immunogenic platform against both *C. jejuni* and ETEC can be created by linking synthetic MeOPN-sugars to different ETEC protein antigens. The approach could also be extended to include *Shigella* lipopolysaccharides (synthetic or detoxified) conjugated to ETEC proteins. Thus, this platform could form the basis of a multivalent vaccine against three major bacterial diarrheal pathogens. Conjugation can also serve as a protein carrier to enhance immunogenicity of the Campylobacter construct.

[00105]   It is envisioned to conjugate the construct of Examples 3 - 5 to an ETEC construct. The overall method of conjugating includes oxidizing *C. jejuni* CPS, for example, with $NaIO_4$ in sodium acetate (pH 4.0). Oxidized CPSs were desalted with a 5 kDa cutoff membrane by stirred ultrafiltration, which is subsequently lypholized. ETEC proteins are then added. The stoichiometery protein to CPS can vary, however, a typical ratio is 1:2 protein to CPS by mass. The concentration of components can be by any method. However, for example, polysaccharide concentration was determined by antrhone assay and protein concentration was determined by Pierce 660 protein assay or the BCA assay. $NaCNBH_3$ is then added. The conjugates can then be subsequently desalted by ultrafiltration and lyophilized. CPS, ETEC proteins and conjugates were analyzed, for example by SEC-HPLC or by SDS polyacrylaminde gel electrophoresis (PAGE), or other methods.

Example 7: Non-human primate response

[00106]   The immunogenicity of CfaE-HS23/36 and CfaEB-HS23/36 conjugates was

observed in mice, as well as induction of hemagglutination inhibition (HAI) titers against

Cfal in mice. The amino acid sequence of the dscCfaE construct used is SEQ ID No.

138 (nucleotide sequence is SEQ ID No. 139). The dsc$_{19}$CfaE amino acid sequence is

SEQ ID No. 143 (nucleotide sequence is SEQ ID No. 142). The amino acid sequence for

dsc$_{19}$CfaEB is SEQ ID No. 141 (nucleotide sequence is SEQ ID No. 140).

[00107] The CfaEB-HS23/36 conjugate was down-selected in order to proceed to

studies in *Aotus nancymaae*. This non-human primate (NHP) model was selected

because it has been used as a diarrheal disease model for both ETEC and *C. jejuni*. We

synthesized a lot of the CfaEB-HS23/36 vaccine that was sufficient in size for three NHP

studies by reductive amination. The first such study, which is the only one that has been

completed, was a dose finding study followed by a C. jejuni challenge.

[00108] The design of this NHP study is shown in Table 7. Animals (6 per group) were

immunized three times at days 0, 42, and 84. The CfaEB-HS23/36 vaccine was given

subcutaneously at either 0.5 ug or 3.5 ug polysaccharide (PS) adjuvanted with aluminum

hydroxide. The ratio of PS to protein in the vaccine was roughly 1:1 so this was

equivalent to 0.5 or 3.5 ug of CfaEB per dose. The 3.5 ug dose was also given

intradermally (ID) with poly-IC as adjuvant. This was done to bridge to previous work

done using ID immunizations with CfaEB alone. Similarly, another group was given

HS23/36-CRM197 subcutaneously to bridge to previous work with the same capsule

conjugated to another protein. Finally, the control group was immunized with PBS. On

day 148 the animals were all challenged with 4 x 10^{11} CFU of CG8421, an HS23/36

strain.

Table 7: Design of NHP study

Group	Route	CfaEB-CPS	CPS-CRM197	Alum (ug)	Poly IC (ug)	PBS
1	SC	0.5	-	300	-	-
2	SC	3.5	-	300	-	-
3	ID	3.5	-	-	100	-
4	SC	-	3.5	300	-	-
5	SC	-	-	-	-	+

[00109] Animals were observed for diarrheal disease daily for 10 days following challenge. Diarrhea was defined as two or more days of consecutive of stools that were $\geq$ grade 3. The results are summarized in Table 8. Only 3/5 animals in the PBS group developed diarrhea for an attack rate of 60%. Note that one animal was eliminated because it developed diarrhea prior to challenge. The mean time to onset of disease in this negative control group was 2.3 days and the mean duration of illness was 5.3 days. The attack rate in the animals immunized with the HS23/36-CRM197 vaccine was 33% (2/6), with a mean onset of disease of 2 days and a mean duration of illness of 4 days (45% efficacy). Animals that were immunized with CfaEB-HS23/36 intradermally with poly IC also showed an attack rate of 33% with a mean onset of 1.5 days and a duration of two days (45% efficacy). The animals immunized subcutaneously with CfaEB-CPS showed between 67-100% efficacy against diarrheal disease. The attack rate in the group immunized with 0.5 ug of the vaccine was 0 (0/5, with one animal that vomited after challenge being eliminated) and the attack rate in the group immunized with 3.5 ug of the vaccine was 20% (1/5, with one animal being eliminated due to diarrhea prior to challenge). The single animal in this group that did develop diarrhea had a later onset of disease (day 9). There were no significant differences among the control group and any of the immunized animals due to the small numbers of animals per group.

Table 8. Results of challenge with C. jejuni CG8421.

Group	Vaccine	#ill/total	Attack rate (%)	Mean days to onset of diarrhea (range)	Mean days of illness (range)	Protective efficacy
1	CfaEB-CPS (0.5 ug) + alum	0/5*	0	0	0	100
2	CfaEB-CPS (3.5 ug) + alum	1/5**	20	9	2	67
3	CfaEB-CPS (3.5 ug) + poly IC	2/6	33	1.5 (1-4)	2 (2-4)	45
4	CRM-CPS + alum	2/6	33	2 (1-3)	4 (2-6)	45
5	PBS	3/5**	60	2.3 (1-6)	5.3 (2-10)	--

* One animal vomited after challenge and was excluded
** Animals were excluded from analyses due to diarrheal onset prior to challenge

[00110]   Serology results are shown in Fig. 9.   Immune responses to CPS and to CfaE were measured by ELISA. Animals in groups 1, 2 and 3 displayed IgG responses to both antigens and and IgA response to CfaE. Hemagglutination inhibition (HAI) titers against ETEC strain H10407 expressing CfaI fimbriae were determined and are shown in Fig. 10. The results indicate that HAI titers were detected in animals in groups 1, 2 and 3, with group 2 showing the highest titers.

Example 8: Synthesis and immunogenicity of additional combinations of ETEC-Campylobacter capsule conjugates

[00111]   CssBA-HS3 vaccine. CssBA is a recombinant form of the two subunits of CS6 that are fused together. This protein was conjugated to capsule from an HS3 strain by TEMPO oxidation.   The conjugates were analyzed by SDS-PAGE and immunoblotting. Purified CssBA has a predicted Mr of 31.8 kDa. The conjugate of CssBA-HS3 CPS runs

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as two bands, one slightly smaller than CssBA and one that runs at approximately 60

kDa. The bands in the conjugate react with both anti-CssBA antiserum and antibodies to

whole cells of HS3, indicating that polysaccharide has been conjugated to the protein.

[00112] Mice were immunized subcutaneously with three doses of the vaccine given at

4 week intervals. Doses were 5 ug by weight or 25 ug by weight. Animals were bled at

day 0 and two weeks after each immunization and the response to CssBA and to CPS

were determined by ELISA. The results, shown in Fig. 11, indicate that there was a

robust response to both the protein and the polysaccharide at both doses.

[00113]   LTB-HS4 vaccine. LTB is the binding component of the heat labile

enterotoxin of ETEC. Recombinant LTB, which is not toxic, was conjugated to the

polysaccharide capsule of an HS4 strain by reductive amination. The conjugate was

analyzed by immunoblotting as shown in Fig. 12. Immunodetection with anti-LTB

antiserum revealed a single band for LTB at approximately 10 kDa. The conjugate

contained 4 major bands ranging from ~20kDa->75kDa that were reactive with both anti-

LTB and anti-HS4 antiserum, indicating successful conjugation.

[00114]   Mice were immunized with three doses of either 5 or 25 ug (by weight) of the

LTB-HS4 conjugate subcutaneously at 4 week intervals and the serum immune response

was determined. The results, shown in Fig. 12, indicate that there was a robust immune

response to both the HS4 capsule and to LTB at both doses.

Example 9: Conjugation to Shigella lipopolysaccharide (LPS)

[00115]   There are four species of Shigella, a human pathogen cause diseases such as

diarrhea and bacilliary dysentaery: *Shigella dysenteriae, Shigella flexneri, Shigella*

boydii and *Shigella sonnei* are important enteropathogens Strains of *Shigella* spp. Express

long-chain lipopolysaccharides. The chemical structures for many strains has been

determined (see Liu, et al., FEMS Microbiol. Rev. 32: 627-653 (2008)).

[00116] An object of this invention, is a *Shigella* LPS-ETEC construct. The construct

comprises an ETEC construct, as the above examples, conjugated, to a *Shigella* LPS, as

an alternative or in addition to *C. jejuni* capsule polysaccharide. It is envisioned that any

of the *Shigella* spp. can be conjugated to the ETEC construct. As an example, the

Shigella flexneri 2a LPS is illustrated, as a potential LPS structure that can be conjugated

to an ETEC construct, as follows:

α-D-Glc(1-4)

|

–2)-α-L-Rha*p*-(1-2)-α-L-Rha*p*-(1-3)-α-L-Rha*p*-(1-3)-β-D-GlcNAc*p*(1-.
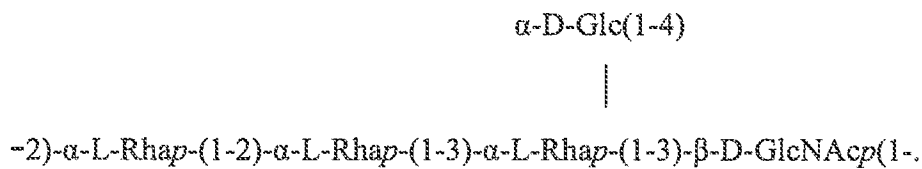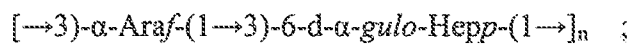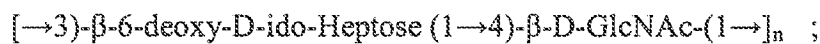
What is claimed is:

1.   A multi-agent immunogenic construct, comprising a *Campylobacter jejuni* capsule polysaccharide conjugated to a protein carrier, wherein said protein carrier comprises an *Escherichia coli* enterotoxigenic recombinant polypeptide construct.

2.   The multi-agent immunogenic construct of claim 1, wherein said *Escherichia coli* recombinant polypeptide construct comprises a minor or major subunit connected to one or more major fimbrial subunits or immunogenic fragments, thereof, of the same fimbrial type, via a polypeptide linker, and wherein each of the one or more major fimbrial subunits contain a donor β strand and are also connected to each other via a polypeptide linker wherein the C-terminal Escherichia coli fimbrial major subunit is connected, via a linker, to a C-terminal donor β strand derived from a major Escherichia coli fimbrial subunit that is homologous or heterologous to the immediately N-terminal major subunit, or wherein said recombinant polypeptide construct is connected to one or more additional recombinant polypeptide constructs, wherein each of the constructs contain fimbrial subunits derived from a different fimbrial type than any of the other constructs and wherein the the recombinant polypeptide construct can contain a C-terminal histidine tag at the C-terminus.

3.   The multi-agent immunogenic construct of claim 1, wherein the multi-agent immunogenic construct comprises *Shigella* lipopolysaccharide in place of or in addition to the *Campylobacter jejuni* capsule polysaccharide.

4.   The multi-agent immunogenic construct of claim 1, wherein said molar ratio of Campylobacter jejuni or *Shigella* spp. lipopolysaccharide to *Escherichia coli* recombinant protein carrier is 1:1 to 5:1.
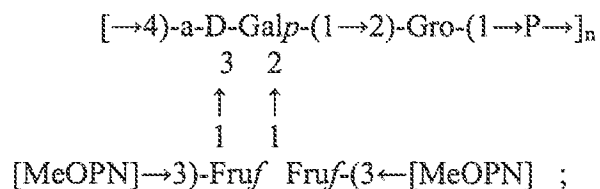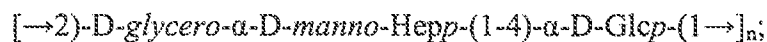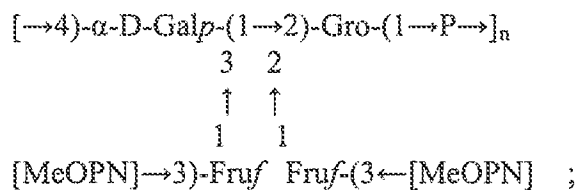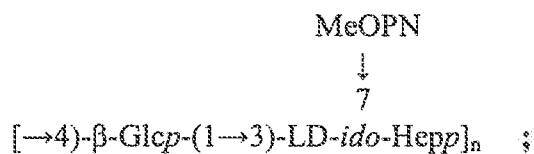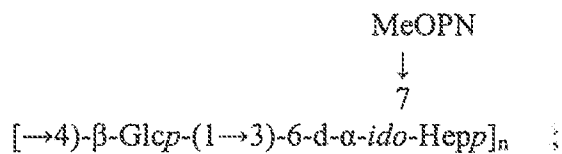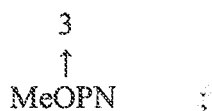
5.   The multi-agent immunogenic construct of claim 2, wherein said minor or major *Escherichia coli* fimbrial subunits are derived from ETEC strains selected from the group consisting of Class 5, CS3 and CS6.

6.   The multi-agent immunogenic construct of claim 2, wherein the *Escherichia coli* recombinant polypeptide construct comprises an amino acid sequence selected from the group consisting of SEQ ID Nos. 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 41, 43, 102, 103, 105, 107, 109, 112, 114, 138, 141 and 143.

7.   The multi-agent immunogenic construct of claim 2, wherein the *Escherichia coli* recombinant polypeptide construct is encoded by the nucleotide sequence of SEQ ID Nos. 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 40, 42, 101, 104, 106, 108, 110, 111, 113, 139, 140, and 142.

8.   The multi-agent immunogenic construct of claim 2, wherein said *Campylobacter jejuni* polysaccharide is a repeating trisaccharide structure having the formula selected from the group consist of:
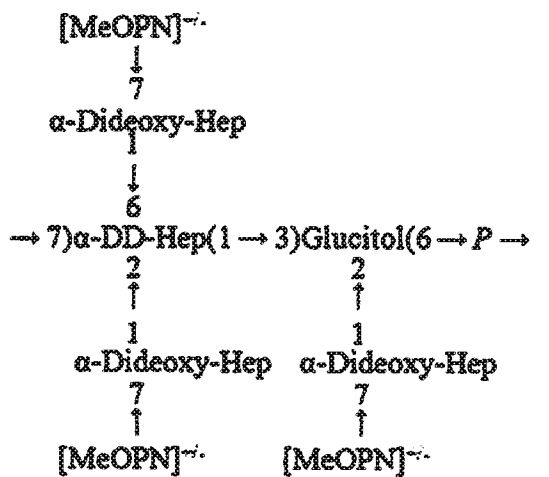
$[\rightarrow 3)\text{-}\alpha\text{-D-Gal-}(1\rightarrow 2)\text{-6d-}\alpha\text{-D-}altro\text{-Me-Hep-}(1\rightarrow 3)\text{-}\beta\text{-D-GlcNAc-}(1\rightarrow]_n$;

$[\rightarrow 3)\text{-}\beta\text{-6-deoxy-D-ido-Heptose }(1\rightarrow 4)\text{-}\beta\text{-D-GlcNAc-}(1\rightarrow]_n$;

$[\rightarrow 3)\text{-}\alpha\text{-Ara}f\text{-}(1\rightarrow 3)\text{-6-d-}\alpha\text{-}gulo\text{-Hep}p\text{-}(1\rightarrow]_n$;

$[\rightarrow 3\text{-}\beta\text{-Gal}p\text{NAc-}(1\rightarrow]_n;$
           4
           ↑
   6-d-α-*gal*-Hep

$$\begin{array}{c} 3 \\ \uparrow \\ \text{MeOPN} \end{array} \quad ;$$

$$\begin{array}{c} \text{MeOPN} \\ \downarrow \\ 7 \end{array}$$
$$[\rightarrow 4)\text{-}\beta\text{-Glc}p\text{-}(1\rightarrow 3)\text{-}6\text{-d-}\alpha\text{-}ido\text{-Hep}p]_n \quad ;$$

$$\begin{array}{c} \text{MeOPN} \\ \downarrow \\ 7 \end{array}$$
$$[\rightarrow 4)\text{-}\beta\text{-Glc}p\text{-}(1\rightarrow 3)\text{-LD-}ido\text{-Hep}p]_n \quad ;$$

$$[\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 2)\text{-Gro-}(1\rightarrow P\rightarrow)]_n$$
$$\begin{array}{cc} 3 & 2 \\ \uparrow & \uparrow \\ 1 & 1 \end{array}$$
$$[\text{MeOPN}]\rightarrow 3)\text{-Fru}f \quad \text{Fru}f\text{-}(3\leftarrow[\text{MeOPN}] \quad ;$$

$$[\rightarrow 2)\text{-D-}glycero\text{-}\alpha\text{-D-}manno\text{-Hep}p\text{-}(1\text{-}4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow]_n;$$

$$[\rightarrow 4)\text{-a-D-Gal}p\text{-}(1\rightarrow 2)\text{-Gro-}(1\rightarrow P\rightarrow)]_n$$
$$\begin{array}{cc} 3 & 2 \\ \uparrow & \uparrow \\ 1 & 1 \end{array}$$
$$[\text{MeOPN}]\rightarrow 3)\text{-Fru}f \quad \text{Fru}f\text{-}(3\leftarrow[\text{MeOPN}] \quad ;$$

$[\rightarrow 3)\text{-L-beta-D-ido-Hep-}(1\text{->}4)\text{-beta-D-Glc-}(1\rightarrow]_n$, with non-stoichiometric substitution

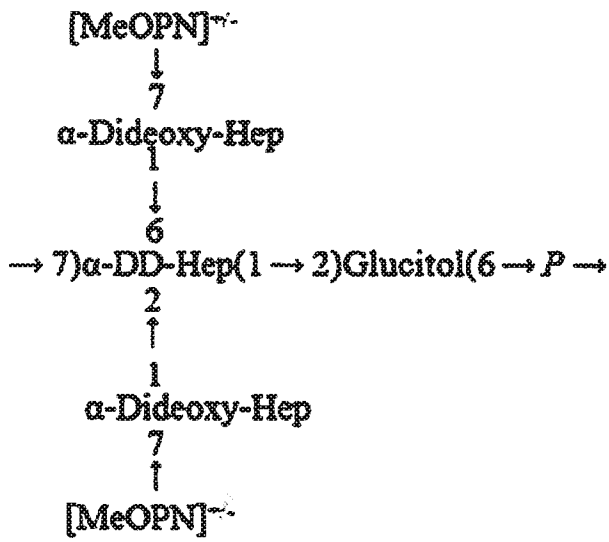of O-methyl-phosphoramidate at position 2 of L-glycero-beta-D-ido-heptose;

[→3)-6d-beta-D-ido-Hep-(1->4)-beta-D-Glc-(1→]$_n$, derived from HS13, with non-

stoichiometric substitution of O-methyl-phosphoramidate at position 2 or/and 7 of 6-

deoxy-beta –D-ido-heptose;

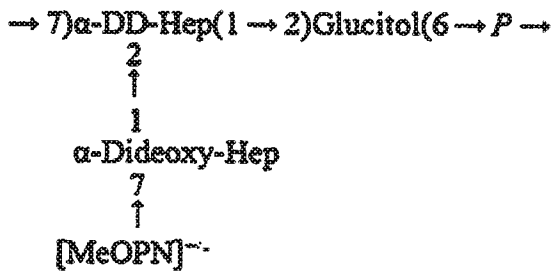[→3)-L-beta-D-ido-Hep-(1->4)-beta-D-Glc-(1→]$_n$;

[→3)-L-alpha-D-ido-Hep-(1->4)-alpha-Gal-(1→]$_n$, with non-stoichiomoetric

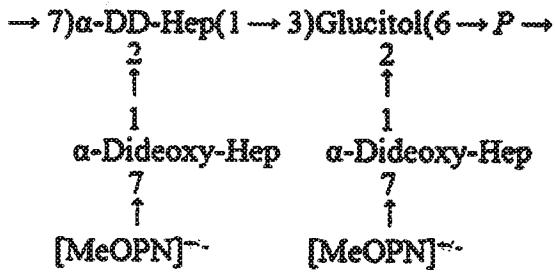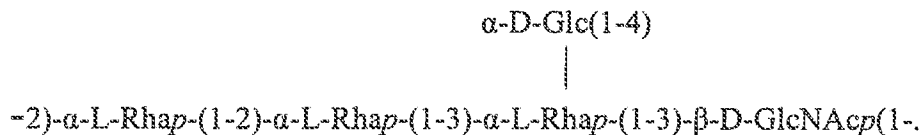substitution O-methyl-phosphoramidate at position 2 of 6-deoxy-alpha-D-ido-heptose;

```
        [MeOPN]⁻·
            ↓
            7
       α-Dideoxy-Hep
            ↓
            6
 → 7)α-DD-Hep(1 → 3)Glucitol(6 → P →
        2                 2
        ↑                 ↑
        1                 1
  α-Dideoxy-Hep   α-Dideoxy-Hep
        7                 7
        ↑                 ↑
   [MeOPN]⁻·       [MeOPN]⁻·
                          ;
```

```
          [MeOPN]⁻·
             ↓
             7
      α-Dideoxy-Hep
             1
             ↓
             6
→ 7)α-DD-Hep(1 → 2)Glucitol(6 → P →
             2
             ↑
             1
        α-Dideoxy-Hep
             7
             ↑
          [MeOPN]⁻·
                                                ;
```

```
→ 7)α-DD-Hep(1 → 2)Glucitol(6 → P →
             2
             ↑
             1
        α-Dideoxy-Hep
             7
             ↑
          [MeOPN]⁻·
                                                ;
```

```
→ 7)α-DD-Hep(1 → 3)Glucitol(6 → P →
             2                    2
             ↑                    ↑
             1                    1
      α-Dideoxy-Hep      α-Dideoxy-Hep
             7                    7
             ↑                    ↑
          [MeOPN]⁻·          [MeOPN]⁻·
                                              ; and
```

[→3)-6d-alpha-D-ido-Hep-(1->4)-alpha-Gal-(1→]ₙ, derived from HS3, HS13 and HS50

with non-stoichiometric substititution of O-methyl-phosphoramidate at position 2 of L-

glycero-alpha-D-ido-heptose, wherein "n" is 1 to 100.

9.   The multi-agent immunogenic construct of claim 3, wherein said Shigella

lipopolysaccharide has the structure:

$$\alpha\text{-D-Glc}(1\text{-}4)$$
$$|$$
$$-2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\text{-}2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\text{-}3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\text{-}3)\text{-}\beta\text{-D-GlcNAc}p(1\text{-.}$$

10.   The multi-agent immunogenic construct of claim 2, wherein said donor β strand

contains 12 to 16 amino acids.

11.   The multi-agent immunogenic construct of claim 2, wherein said N-terminus of

said minor or major subunit contains an 18-22 amino acid signal peptide.

12.   The multi-agent immunogenic construct of claim of claim 2, wherein the amino

acid sequence of said polypeptide linker is the amino acid sequence of SEQ ID No. 5 or a

tri-glyicine.

13.   The multi-agent immunogenic construct of claim of claim 2, wherein one or more

major subunits contain a deletion of the 14 to 18 N-terminal amino acids.

14.   The multi-agent immunogenic construct of claim 5, wherein said *Escherichia coli*

fimbrial minor subunit is selected from the group consisting of CfaE, CsfD, CsuD, CooD,

CsbD, CosD, CsdD, CotD and wherein said major subunit is selected from the group

consisting of CfaB, CsfA, CsuA2, CooA, CsdA, CosA, CsbA, CotA, CstG, CstH, CssA,

and CssB, or immunogenic fragments or derivatives, thereof.

15.   The multi-agent immunogenic construct of claim 14, wherein said amino acid

sequence of said *Escherichia coli* fimbrial minor subunit is selected from the group

consisting of SEQ ID Nos. 45, 46, 51, 52, 57, 58, 65, 71, 75, 79, 83, 88, 90, 93, 95, and

97, or derivatives thereof, and wherein said amino acid sequence of said *Escherichia coli*

fimbrial major subunit is selected from the group consisting of SEQ ID Nos. 2, 4, 48, 49,

54, 55, 60, 61, 63, 67, 69, 73, 77, 81, 85, 87, 89, 91, 92, 94, 96, 98, 99, 101, 135, and

137, or derivatives thereof.

16.   A method of inducing an immune response against *C. jejuni* strains, wherein said

method induces an immune response against one or more enterobacteria selected from the

group consisting of *C. jejuni* strains, *Escherichia coli*, and *Shigella*, comprising the steps:

        a. administering the multi-agent immunogenic composition of claim

            2 at a dose range of 0.1 µg to 10 mg per dose;

        b. administering a boosting dose of said capsule polysaccharide

            composition at a dose range of 0.1 µg to 10 mg per dose.

17.   The method of claim 16, wherein said *Campylobacter jejuni* capsule

polysaccharide comprises the polysaccharide structures of claim 8.

18.   The method of claim 16, wherein said multi-agent immunogenic composition

comprises the construct of claim 3.

19.   The method of claim 16, wherein said *Escherichia coli* enterotoxigenic

recombinant polypeptide construct wherein said *Escherichia coli* fimbrial minor subunit

is selected from the group consisting of SEQ ID Nos. 45, 46, 51, 52, 57, 58, 65, 71, 75,

79, 83, 88, 90, 93, 95, and 97, or derivatives thereof, and wherein said amino acid

sequence of said *Escherichia coli* fimbrial major subunit is selected from the group

consisting of SEQ ID Nos. 2, 4, 48, 49, 54, 55, 60, 61, 63, 67, 69, 73, 77, 81, 85, 87, 89,

91, 92, 94, 96, 98, 99, 101, 135, and 137, or derivatives thereof.

20.   The method of claim 16, wherein the *Escherichia coli* recombinant polypeptide

construct comprises an amino acid sequence selected from the group consisting of SEQ

ID Nos. 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 41, 43, 102, 103, 105,

107, 109, 112, 114, 138, 141, and 143.

21.   The method of claim 16, wherein the *Escherichia coli* recombinant polypeptide

construct is encoded by the nucleotide sequence of SEQ ID Nos. 6, 8, 10, 12, 14, 16, 18,

20, 22, 24, 26, 28, 30, 32, 34, 36, 40, 42, 101, 104, 106, 108, 110, 111, 113, 139, 140, and

142.

22.   The method of claim 16, wherein said donor β strand contains 12 to 16 amino

acids.

23.   The multi-agent immunogenic construct of claim 16, wherein said N-terminus of

said minor or major subunit contains an 18-22 amino acid signal peptide.

24.   The multi-agent immunogenic construct of claim 16, wherein the amino acid

sequence of said polypeptide linker is the amino acid sequence of SEQ ID No. 5 or a tri-

glyicine.

25.   The multi-agent immunogenic construct of claim16, wherein one or more major

subunits contain a deletion of the 14 to 18 N-terminal amino acids.

One or more subunits (with or without signal peptide) derived from same fimbrial type (e.g., class 5, CS3 or CS6)

Donor β-strand from adjacent (N-terminal) subunit (homologous) or heterologous subunit

with or without N-terminal deletion (ntd)

Polypeptide linker

= Donor β-strand region (from major subunit)

FIG. 1

Subunits derived from   Subunits derived from second (or
first fimbrial type   more) fimbrial type

First minor or major subunit (with   +/- Glycine
or without signal peptide (spd))   spacer

N   C

with or without
N-terminal
deletion (ntd)
                    One or more   One or more   Donor β-strand from
                    major subunits   major subunits   adjacent (N-terminal)
                                                                             subunit or heterologous

                                         with or without N-
                                         terminal deletion
                                         (ntd)
        Donor β-strand from
        adjacent (N-terminal)   = Linker
        subunit or heterologous
        subunit
                                                               =   Donor β-strand
                                                                   region (from
                                                                   major subunit)

FIG. 2

FIG. 3

SUBSTITUTE SHEET (RULE 26)

A

B

FIG. 4

FIG. 5

SUBSTITUTE SHEET (RULE 26)

FIG. 6

FIG. 7

Structure of MeOPN-modified monosaccharide in each CPS	Spot of MeOPN-6-Gal	Detecting antiserum
MeOPN-6-Gal		Anti-HS23/36 CRM conjugate
MeOPN-7-6d-β-D-		Anti-HS4-CRM conjugate
MeOPN-2-β-D-fru		Anti-HS1-CRM conjugate
MeOPN-2-L-glycero-α-D-		Anti-HS3-CRM conjugate
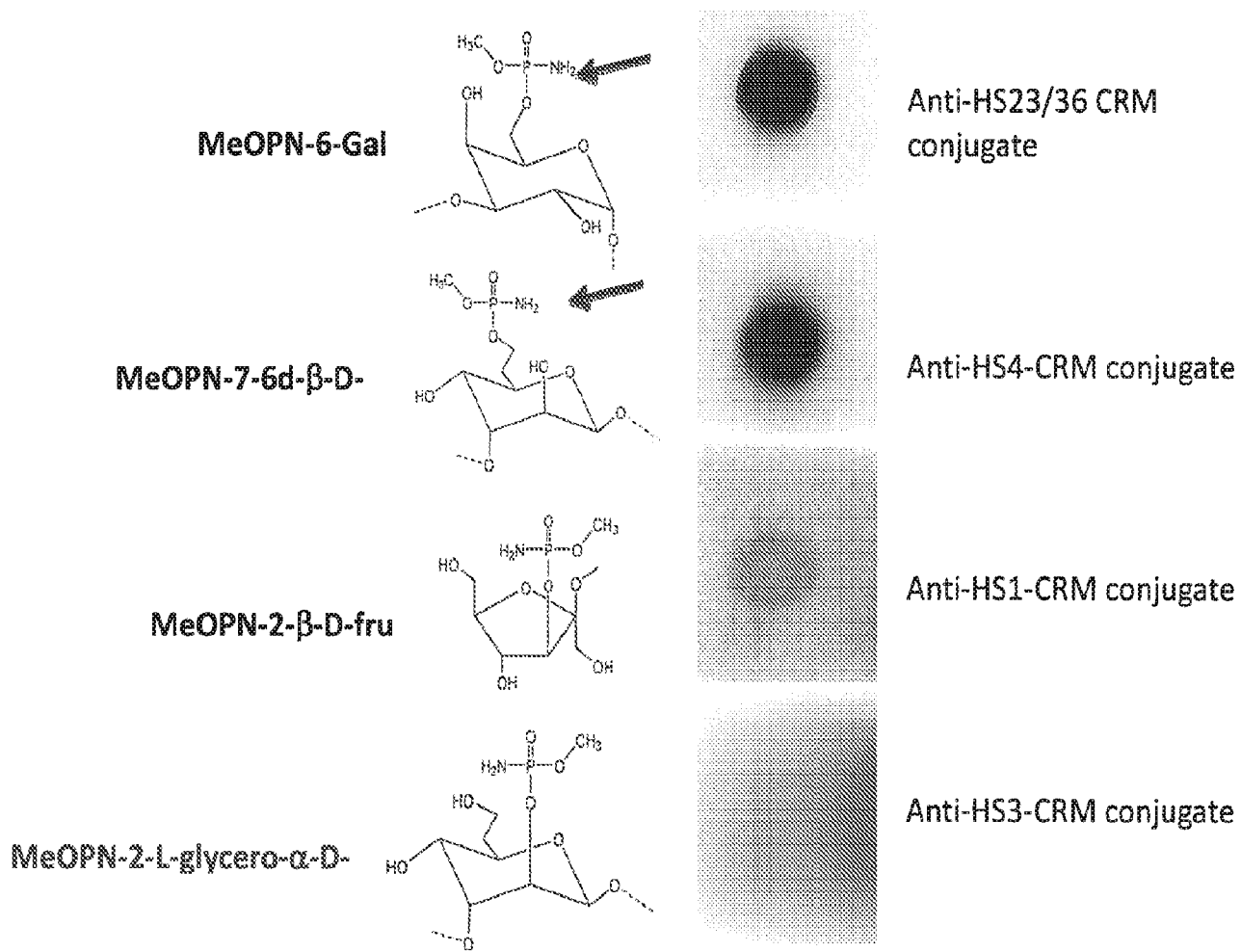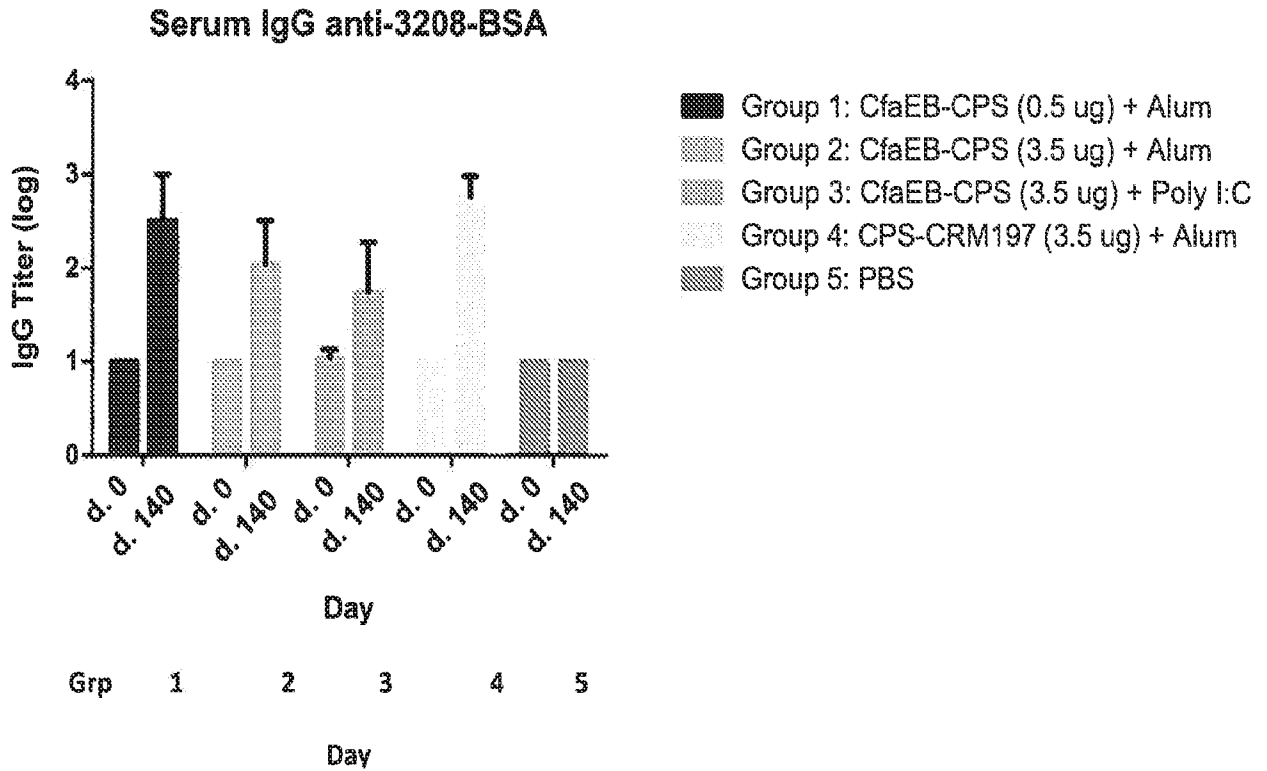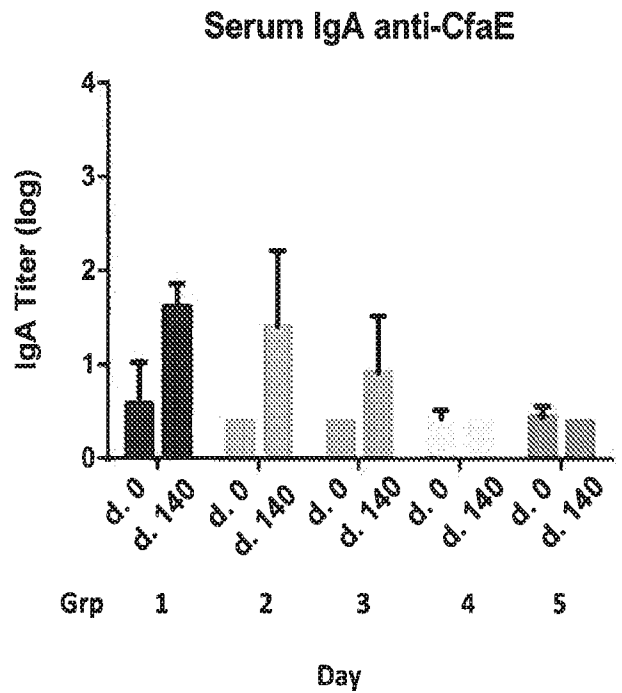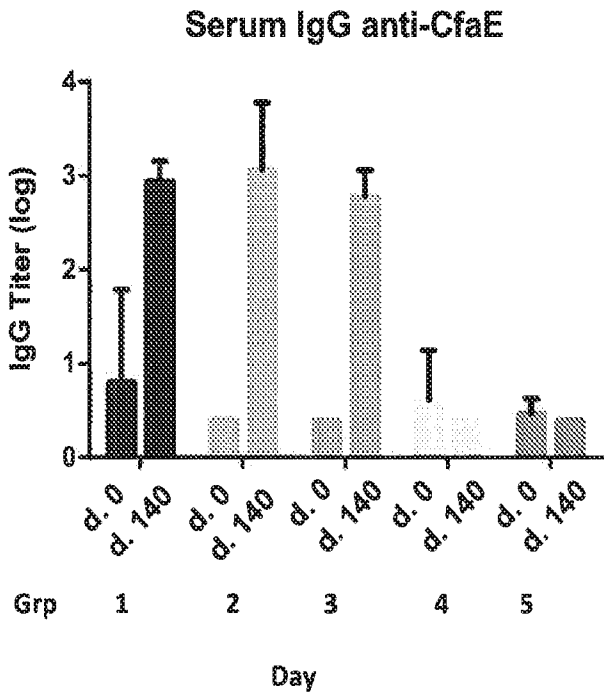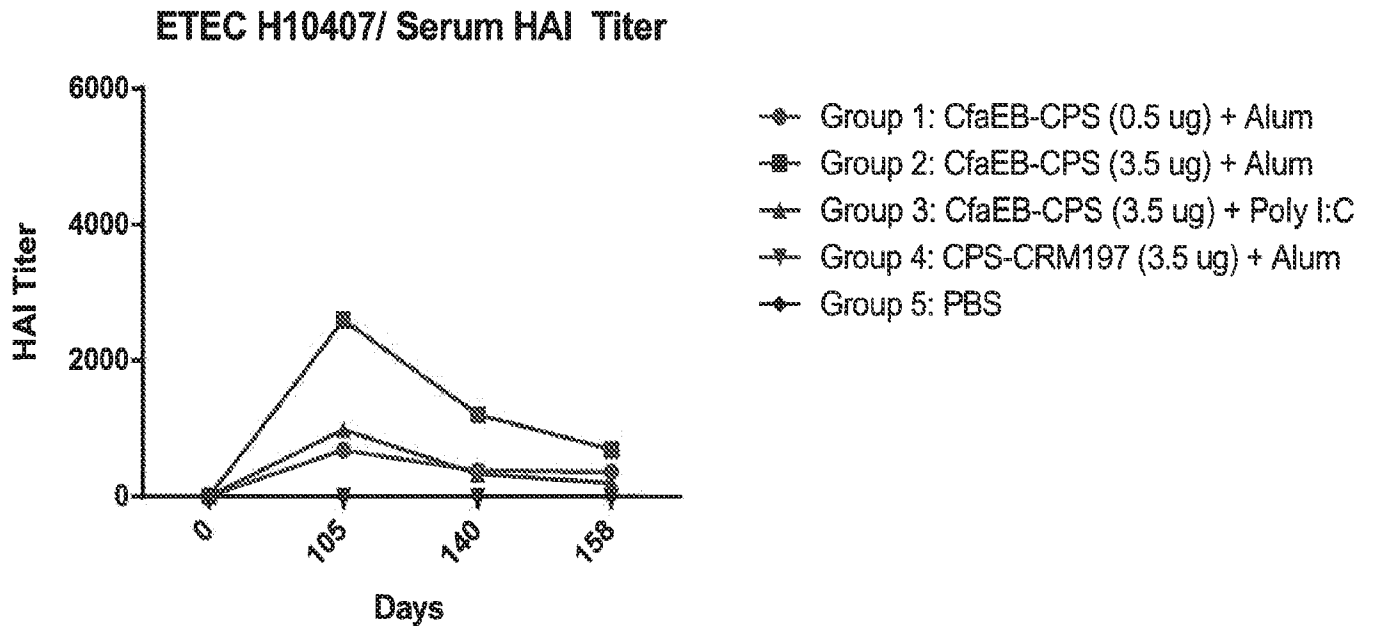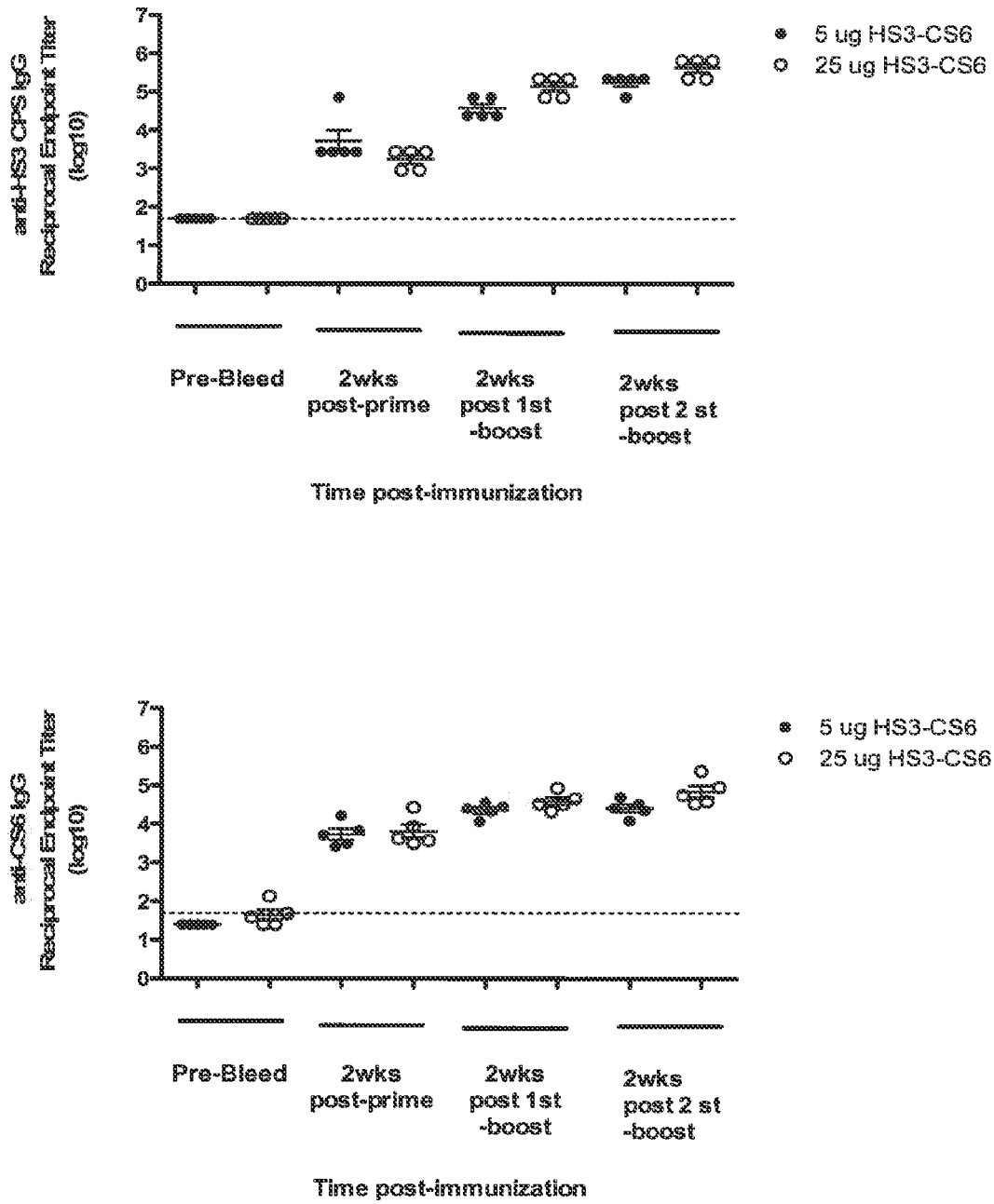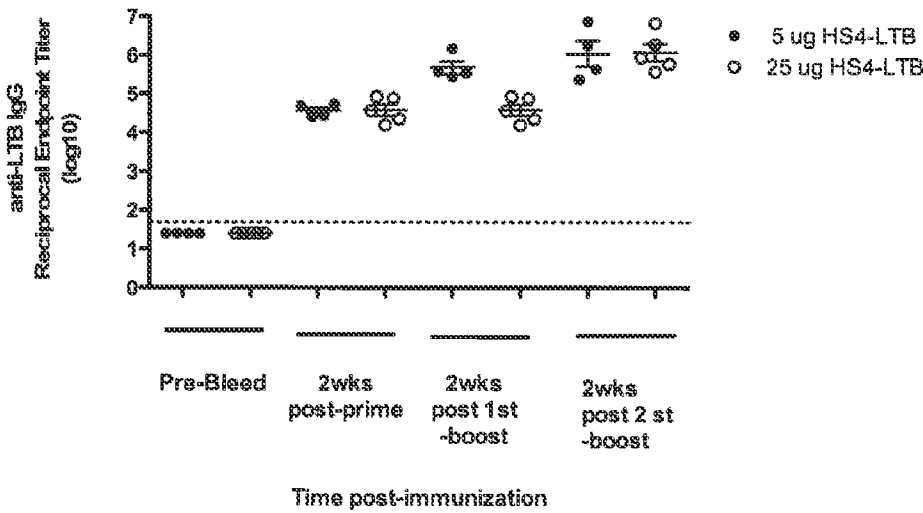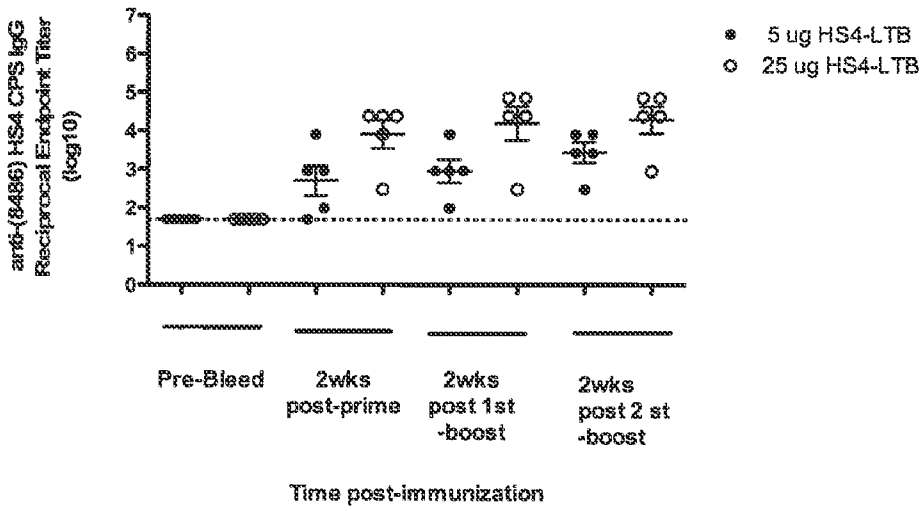
FIG. 8

A.

Serum IgG anti-3208-BSA

- ■ Group 1: CfaEB-CPS (0.5 ug) + Alum
- Group 2: CfaEB-CPS (3.5 ug) + Alum
- Group 3: CfaEB-CPS (3.5 ug) + Poly I:C
- Group 4: CPS-CRM197 (3.5 ug) + Alum
- Group 5: PBS

B.

Serum IgG anti-CfaE

Serum IgA anti-CfaE

FIG. 9

ETEC H10407/ Serum HAI Titer

Group 1: CfaEB-CPS (0.5 ug) + Alum
Group 2: CfaEB-CPS (3.5 ug) + Alum
Group 3: CfaEB-CPS (3.5 ug) + Poly I:C
Group 4: CPS-CRM197 (3.5 ug) + Alum
Group 5: PBS

FIG. 10

SUBSTITUTE SHEET (RULE 26)

FIG. 11

FIG. 12

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/106 (2016.01)
CPC - A61K 39/105 (2015.12)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 39/00, 39/106, 39/108, 39/385 (2016.01)
CPC - A61K 39/00, 39/105, 39/0258, 39/0283, 2039/60, 2039/6031, 2039/6037; C07K 14/245 (2015.12)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/194.1, 197.11, 234.1; 536/123.1
CPC - A61K 39/00, 39/105, 39/0258, 39/0283, 2039/60, 2039/6031, 2039/6037; C07K 14/245 (2015.12) (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patent, PubMed
Search terms used: immunogenic CAMPYLOBACTER JEJUNI polysaccharide ESCHERICHIA COLI enterotoxigenic FIMBRIAL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0300173 A1 (GUERRY et al) 08 December 2011 (08.12.2011) entire document	1, 2, 4-8, 12, 16, 20, 21, 24
Y	WO 2014/077977 A1 (THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY OF THE NAVY) 22 May 2014 (22.05.2014) entire document	1, 2, 4-8, 12, 16, 20, 21, 24
A	US 2012/0321658 A1 (BIEMANS et al) 20 December 2012 (20.12.2012) entire document	1, 2, 4-8, 12, 16, 20, 21, 24
A	US 2011/0097357 A1 (FERNANDEZ et al) 28 April 2011 (28.04.2011) entire document	1, 2, 4-8, 12, 16, 20, 21, 24
P,X	US 2015/0258201 A1 (GUERRY et al) 17 September 2015 (17.09.2015) entire document	1, 2, 4-8, 12, 16, 20, 21, 24

☐ Further documents are listed in the continuation of Box C.		☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 January 2016	**1 0 FEB 2016**

Name and mailing address of the ISA/	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450	Blaine R. Copenheaver
Facsimile No. 571-273-8300	PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

| Box No. I | Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet) |

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

    a. ☒ forming part of the international application as filed:

        ☒ in the form of an Annex C/ST.25 text file.

        ☐ on paper or in the form of an image file.

    b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

    c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

        ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

        ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 6 and 7 were searched.

Box No. II   Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 17, 18
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III   Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
see Extra Sheet(s).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

   1, 2, 4-8, 12, 16, 20, 21, and 24 to the extent that they read on the Campylobacter jejuni capsule polysaccharide [->3)-n-D-Gal (1 >2) 6d α D-altru-Me-I lep-(I->3)-β-D-GlcNAc-(1->] and an Escherichia coli enterotoxigenic recombinant polypeptide of SEQ ID NO:7.

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)

Continued from Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-16 and 19-25 are drawn to a multi-agent immunogenic construct, and a method comprising the same.

The first invention of Group I+ is restricted to a multi-agent immunogenic construct, and a method comprising the same, the multi-agent immunogenic construct comprising a Campylobacter jejuni capsule polysaccharide, wherein the Campylobacter jejuni capsule polysaccharide is selected to be [->3)-α-D-Gal-(1->2)-6d-α-D-altro-Me-Hep-(1->3)-β-D-GlcNAc-(1->]n, where n is 1; and an Escherichia coli enterotoxigenic recombinant polypeptide, wherein the Escherichia coli enterotoxigenic recombinant polypeptide construct is selected to be SEQ ID NO: 7, which is encoded by the polynucleotide of SEQ ID NO: 6. It is believed that claims 1, 2, 4-8, 12, 16, 20, 21, and 24 read on this first named invention and thus these claims will be searched without fee to the extent that they read on the Campylobacter jejuni capsule polysaccharide [->3)-α-D-Gal-(1->2)-6d-α-D-altro-Me-Hep-(1->3)-β-D-GlcNAc-(1->] and an Escherichia coli enterotoxigenic recombinant polypeptide of SEQ ID NO:7.

Applicant is invited to elect additional C. jejuni capsule polysaccharides with specified structure and/or E. coli enterotoxigenic recombinant polypeptides with specified SEQ ID NO to be searched in a specific combination by paying additional fee for each set of election. An exemplary election would be a multi-agent immunogenic construct, and a method comprising the same, the multi-agent immunogenic construct comprising a Campylobacter jejuni capsule polysaccharide, wherein the Campylobacter jejuni capsule polysaccharide is selected to be [->3)-β-6-deoxy-D-ido-Heptose(1->4)-β-D-GlcNAc-(1->]n, where n is 1; and an Escherichia coli enterotoxigenic recombinant polypeptide, wherein the Escherichia coli enterotoxigenic recombinant polypeptide construct is selected to be SEQ ID NO: 9, which is encoded by the polynucleotide of SEQ ID NO: 8. Additional C. jejuni capsule polysaccharides with specified structure and/or E. coli enterotoxigenic recombinant polypeptides with specified SEQ ID NO will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element, requiring the selection of alternatives for the C. jejuni capsule polysaccharides and the E. coli enterotoxigenic polypeptides, where the C. jejuni capsule polysaccharides are selected from the group of polysaccharides shown in claim 8 of the instant application; and where "the Escherichia coli recombinant polypeptide construct comprises an amino acid sequence selected from the group consisting of SEQ ID Nos. 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 41, 43, 102, 103, 105, 107, 109, 112, 114, 138, 141 and 143".

The Groups I+ share the technical features of a multi-agent immunogenic construct, comprising a Campylobacter jejuni capsule polysaccharide conjugated to a protein carrier, wherein said protein carrier comprises an Escherichia coli enterotoxigenic recombinant polypeptide construct. However, these shared technical features do not represent a contribution over the prior art.

Specifically, US 2011/0300173 A1 to Guerry et al. discloses a multi-agent immunogenic construct (immunogenic compositions, and method of using the compositions to elicit an immune response; against Campylobacter jejuni, Abstract; another object of the invention is an isolated C. jejuni capsule carbohydrate polymer that is conjugated to a carrier protein, Para. [0014]), comprising a Campylobacter jejuni capsule polysaccharide conjugated to a protein carrier (an isolated C. jejuni capsule carbohydrate polymer that is conjugated to a carrier protein, Para. [0014]), wherein said protein carrier comprises toxigenic polypeptide construct (although a number of carriers are contemplated, examples of carriers include CRM197 and tetanus toxoid. However, any carrier that is capable of enhancing the induction of an immune response is contemplated, Para. [0079]).

Further, WO 2014/077977 A1 to The United States of America Represented by the Secretary of the Navy et al. teaches wherein said protein carrier comprises an Escherichia coli enterotoxigenic recombinant polypeptide construct (The invention relates to a recombinant polypeptide construct expressing enterotoxigenic Escherichia coli (ETEC) fimbrial subunits. The composition is useful as an immunogenic composition against ETEC strains, Para. [0012]). It would have been obvious to one of ordinary skill in the art at the time of invention to modify the protein carrier of Guerry et al. to include an Escherichia coli enterotoxigenic recombinant polypeptide as taught by The United States of America Represented by the Secretary of the Navy et al. as a E. coli enterotoxigenic recombinant polypeptide would enhance the immunogenincity of immunogenic compositions comprising C. jejuni capsule polysaccharide (Guerry et al., Para. [0043]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.