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(54) **METHODS AND COMPOSITIONS FOR IMMUNE PROTECTION AGAINST EXTRA-INTESTINAL PATHOGENIC E. COLI**

VERFAHREN UND ZUSAMMENSETZUNGEN ZUM IMMUNSCHUTZ GEGEN EXTRAINTESTINALE PATHOGENE E. COLI

MÉTHODES ET COMPOSITIONS POUR UNE PROTECTION IMMUNITAIRE CONTRE E. COLI PATHOGÈNE EXTRA-INTESTINALE

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(73) Proprietors:
- **GlaxoSmithKline Biologicals S.A.**
  1330 Rixensart (BE)
- **Janssen Pharmaceuticals, Inc.**
  Titusville, NJ 08560 (US)

(72) Inventors:
- **POOLMAN, Jan, Theunis**
  2011 CT Haarlem (NL)
- **JACQUEMYN, Bert**
  2800 Mechelen (BE)
- **ABBANAT, Darren, Robert**
  Cornwall, NY 12518 (US)
- **YON, Patricia, Ibarra**
  4112 Solothum (CH)
- **HERMANS, Peter, Wilhelmus, Maria**
  6852 PG Huissen (NL)
- **KOWARIK, Michael, Thomas**
  8055 Zurich (CH)
- **WETTER, Michael, Lukas**
  8047 Zurich (CH)
- **KEMMLER, Stefan, Jochen**
  8048 Zurich (CH)
- **HAUPTLE, Micha, Andres**
  8952 Zurich-Schlieren (CH)
- **GAMBILLARA, Veronica**
  8706 Meilen (CH)

Processed by Luminess, 75001 PARIS (FR)

(Cont. next page)

- **MALLY, Manuela**
  8105 Watt (CH)

(74) Representative: **J A Kemp LLP**
80 Turnmill Street
London EC1M 5QU (GB)

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Remarks:

•The complete document including Reference Table(s) and the Sequence Listing(s) can be downloaded from the EPO website

•The file contains technical information submitted after the application was filed and not included in this specification

Description

FIELD OF THE INVENTION

[0001] This invention relates to compositions and the compositions for use in methods for inducing an immune response against extra-intestinal pathogenic *Escherichia coli* (ExPEC) to thereby provide immune protection against diseases associated with ExPEC. In particular, embodiments of this invention relate to multivalent vaccines containing conjugates of *E. coli* polysaccharide antigens O25B, O1A, O2, and O6A each covalently bound to a detoxified exotoxin A of *Pseudomonas aeruginosa* carrier protein and uses of the vaccines to provide effective immune protection against ExPEC infection.

BACKGROUND OF THE INVENTION

[0002] Extra-intestinal pathogenic *E. coli* (ExPEC) are normally harmless inhabitants of human gut. However, ExPEC strains can possess virulence factors for the colonization and infection of sites outside of the gastrointestinal tract to cause diverse and serious invasive diseases, resulting in significant morbidity, mortality, and costs annually (see, e.g., Johnson et al., J Lab Clin Med. 2002;139(3):155-162; Kohler et al., Int J MedMicrobiol. 2011;301(8):642-647; Foxman, Am J Med. 2002;113 Suppl 1A:5S-13S; and Russo et al., Microbes Infect. 2003;5(5):449-456). ExPEC strains are the most common cause of urinary tract infection (UTI). They are also a contributor to surgical site infections and neonatal meningitis (Johnson et al., 2002; and Russo et al., 2003), associated with abdominal and pelvic infections and nosocomial pneumonia, and are occasionally involved in other extra-intestinal infections such as osteomyelitis, cellulitis, and wound infections. All these primary sites of infection can result in ExPEC bacteremia (Russo et al., 2003).

[0003] Bacterial resistance to antibiotics is a major concern in the fight against bacterial infection, and multi-drug resistant (MDR) E. coli strains are becoming more and more prevalent (see, e.g., Schito et al., 2009, Int. J. Antimicrob. Agents 34(5):407-413; and Pitout et al., 2012, Expert Rev. Anti. Infect. Ther. 10(10): 1165-1176). The emergence and rapid global dissemination of ExPEC sequence type 131 is considered the main driver of increased drug resistance, including multi-drug resistance (Johnson et al., Antimicrob Agents Chemother. 2010; 54(1):546-550; Rogers et al., J Antimicrob Chemother. 2011; 66(1):1-14). This clone is found in 12.5% to 30% of all ExPEC clinical isolates, mostly exhibits serotype O25B:H4, and shows high levels of fluoroquinolone resistance, which is often accompanied by trimethoprim/sulfamethoxazole resistance (Rogers et al, 2011, and Banerjee et al., Antimicrob Agents Chemother. 2014; 58(9):4997-5004).

[0004] The O-antigen serotype is based on the chemical structure of the O polysaccharide antigen, the outer membrane portion of the lipopolysaccharide (LPS) in a Gram-negative bacterium. More than 180 E. coli O-antigens have been reported (Stenutz et al., FEMS Microbial Rev. 2006; 30: 382-403). ExPEC infection can be caused by any serotype. Although there is an overrepresentation of certain serotypes in ExPEC infection, surface polysaccharides from ExPEC isolates nonetheless exhibit considerable antigenic diversity, which makes the development of an ExPEC vaccine based on surface polysaccharides extremely challenging (Russo et al., Vaccine. 2007; 25: 3859-3870). Also, certain O-antigens may be poorly immunogenic. Furthermore, based on studies from Pneumococcal conjugate vaccines, when a number of serotypes can cause a disease, the vaccine composition, such as the choice of serotypes for inclusion in a vaccine and the dosage levels of the included serotypes, can be critical, since use of a vaccine against certain serotypes may potentially increase carriage of and disease from serotypes not included in the vaccine, or even a serotype that is included in the vaccine but only weakly effective in immunizing against the serotype (Lipsitch, Emerging Infectious Diseases; 1999, 5:336-345). Ideally, a vaccine should maximize its beneficial effects in the prevention of disease caused by serotypes included in the vaccine, while minimizing the risk of added disease from increased carriage of non-vaccine serotypes.

[0005] Accordingly, there is a need in the art for vaccines against ExPEC. In particular, there exists a need for an ExPEC vaccine based on surface polysaccharides that can be used to provide effective immune protection against ExPEC O25B serotype and other serotypes prevalent among ExPEC.

[0006] The following further documents are also referred to:

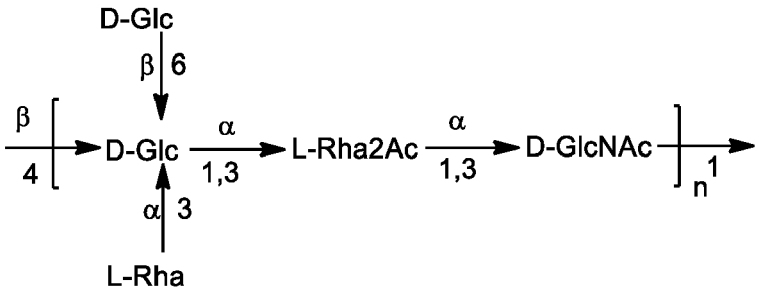
- Cross et al(1994) Journal of Infectious Diseases 170(4): 834-840 which is concerned with the safety and immunogenicity of a polyvalent *Escherichia coli* vaccine in human volunteers;
- Cryz et al (1995) Vaccine 13(5): 449-453 which is concerned with synthesis and characterization of a polyvalent *Escherichia coli* O-polysaccharide-toxin A conjugate vaccine;
- Feldman et al (2005) PNAS USA 102(8): 3016-3021 which is concerned with engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli;* and
- WO 2014/037585 which is concerned with bioconjugates comprising modified antigens and uses thereof.

BRIEF SUMMARY OF THE INVENTION

[0007] It has been surprisingly discovered that an *E. coli* O25B antigen appears to be somewhat less immunogenic than other *E. coli* O-antigens (e.g., O1A, O2, and O6A) when tested as conjugates of the O-antigens each covalently bound to a detoxified exotoxin A of *P. aeruginosa* (EPA) carrier protein, and that vaccination with a composition containing EPA conjugates of *E. coli* O25B antigen and EPA conjugates of one or more additional *E. coli O*-antigens at an appropriate dose and ratio provides an improved immune response against the ExPEC O25B serotype and the one or more additional ExPEC O-serotypes. Accordingly, the present invention provides a composition comprising a first concentration of an *E. coli* O25B antigen polysaccharide, and a second concentration of each of an *E. coli* O1A antigen polysaccharide, an *E. coli* O2 antigen polysaccharide and an *E. coli* O6A antigen polysaccharide, wherein the ratio of the first concentration to the second concentration is 2:1, each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA) carrier protein, and the first concentration is 10 to 36 μg/ml.

[0008] The present invention further provides a multivalent immune composition comprising an *E. coli* O25B antigen polysaccharide at a first dose of 5 to 18 μg, and an *E. coli* O1A antigen polysaccharide, an *E. coli* O2 antigen polysaccharide and an *E. coli* O6A antigen polysaccharide each at a dose that is independently 50% of the first dose, wherein each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA) carrier protein

[0009] In one embodiment, the present invention provides multivalent immune composition comprising an *E. coli* O25B antigen polysaccharide having the structure of Formula O25B':



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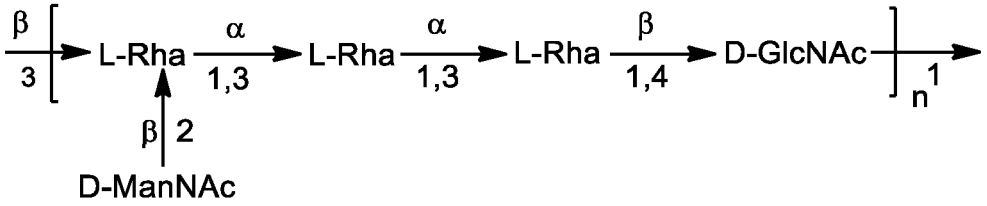
an *E. coli* O1A antigen polysaccharide having the structure of Formula O1A':



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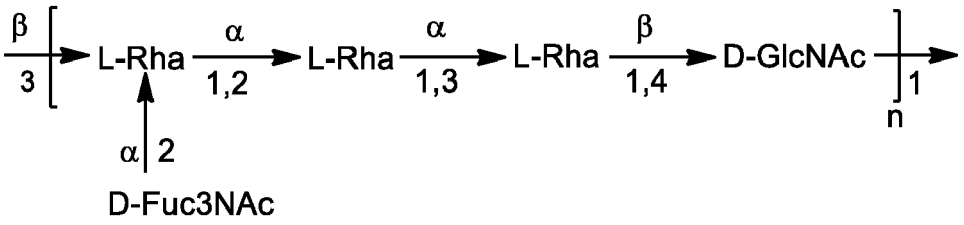
an *E. coli* O2 antigen polysaccharide having the structure of Formula O2':



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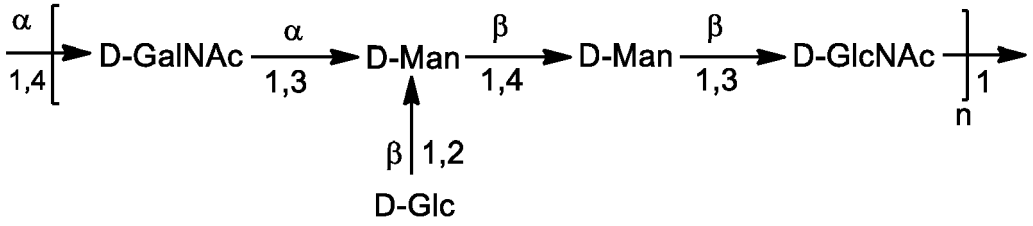
and an *E. coli* O6A antigen polysaccharide having the structure of Formula O6A':



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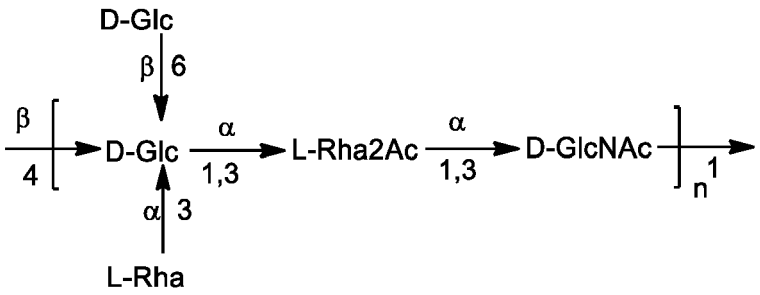
wherein n is independently an integer of 5 to 25, and
each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a carrier protein having the amino acid sequence of SEQ ID NO:1; and the concentrations of the *E. coli* O25B, O1A, O2, O6A antigen polysaccharides in the compositions are respectively 16:8:8:8 μg/ml, or 32:16:16:16 μg/ml.

[0010] In one preferred embodiment, the present invention provides a composition of the present invention for use in a method of inducing an immune response to extra-intestinal pathogenic *E. coli* (ExPEC) in a subject in need thereof, comprising administering to the subject a composition of the present invention.

[0011] The present invention further provides an *E. coli* O25B antigen polysaccharide for use in a method of inducing an immune response to extra-intestinal pathogenic *E.coli* (ExPEC) in a subject in need thereof, comprising administering to the subject a first effective amount of an *E. coli* O25B antigen polysaccharide, and a second effective amount of each of an *E. coli* O1A antigen polysaccharide, an *E. coli* O2 antigen polysaccharide and an *E. coli* O6A antigen polysaccharide, wherein the ratio of the first effective amount to the second effective amount is 2:1, each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA) carrier protein, and the first effective amount is 5 to 18 μg per administration. In one embodiment, the invention provides an *E.coli* O25B antigen polysaccharide for use in a method of inducing an immune response to extra-intestinal pathogenic *E.coli* (ExPEC) in a subject in need thereof, comprising administering to the subject an *E. coli* O25B antigen polysaccharide having the structure of Formula O25B':



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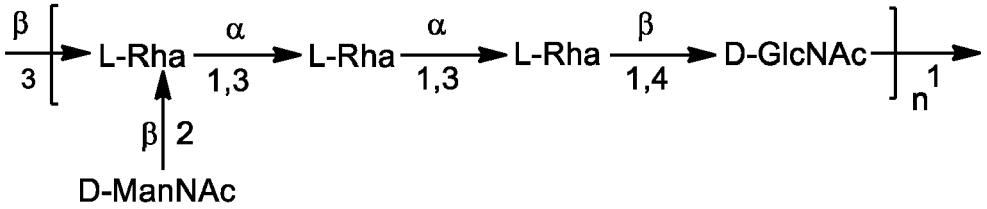
an *E. coli* O1A antigen polysaccharide having the structure of Formula O1A':



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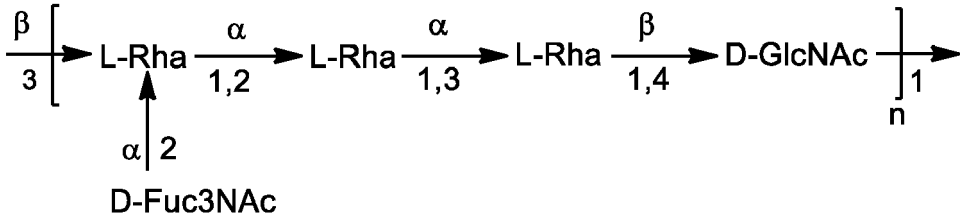
an *E. coli* O2 antigen polysaccharide having the structure of Formula O2':



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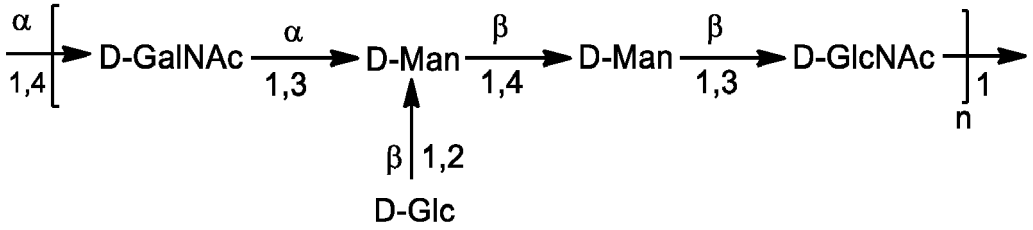
and an *E. coli* O6A antigen polysaccharide having the structure of Formula O6A':



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wherein n is independently an integer of 5 to 25,
each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a carrier protein having the amino acid sequence of SEQ ID NO: 1, and the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are administered at 8:4:4:4 μg, or 16:8:8:8 μg per administration.

[0012] According to a preferred embodiment, the immune response induced by a composition of the present invention prevents an invasive ExPEC disease caused by ExPEC serotypes O1A, O2, O6A and O25B in a human subject in need thereof. Diseases associated with ExPEC or ExPEC diseases include, but are not limited to, urinary tract infection, surgical-site infection, bacteremia, abdominal or pelvic infection, pneumonia, nosocomial pneumonia, osteomyelitis, cellulitis, pyelonephritis, wound infection, meningitis, neonatal meningitis, peritonitis, cholangitis, soft-tissue infections, pyomyositis, septic arthritis, and sepsis. Preferably, the human subject is an at-risk human subject, who has or is at risk of having an invasive ExPEC disease, such as an elderly human, a hospitalized patient, a human child, an immunocompromised human, a pregnant woman, people with diabetes or wound injuries, people who recently had or are scheduled to have a surgery, etc.

[0013] According to an embodiment of the invention, each of the O-antigen is covalently bound to the EPA carrier protein at the Asn residue of a glycosylation sequence comprising Asp (Glu)-X-Asn-Z-Ser (Thr) (SEQ ID NO: 3), wherein X and Z are independently selected from any natural amino acid except Pro. In a preferred embodiment, the EPA carrier protein comprises the amino acid sequence of SEQ ID NO: 1. In another embodiment, the O-antigen is covalently bound to the EPA carrier protein at a polysaccharide-to-protein weight ratio of 1:7 to 1:2, preferably, 1:5 to 1:2. For example, in an O-antigen/EPA conjugate according to an embodiment of the invention, the weight of the O-antigen can be 15% to 50%, or 20% to 40%, of the weight of the EPA.

[0014] Another aspect of the invention relates to a process of making a composition according to an embodiment of the invention, the process comprising combining the *E. coli* O25B antigen polysaccharide, the *E. coli* O1A antigen polysaccharide, the *E. coli* O2 antigen polysaccharide and the *E. coli* O6A antigen polysaccharide to thereby obtain the composition. The process comprises combining the *E. coli* O25B, O1A, O2 and O6A antigens, each independently covalently bound to the EPA carrier protein, in one composition.

[0015] Also disclosed is the use of a composition as disclosed herein for the manufacture of a vaccine or medicament for inducing an immune response to ExPEC or for preventing or treating a disease associated with ExPEC in a subject in need thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings. It should be understood that the invention is not limited to the precise embodiments shown in the drawings.

[0017] In the drawings:

Figure 1 is an exemplary representation of the glycoconjugate vaccine production platform: the cytoplasm (marked in grey) of the host cell contains all the DNA constructs necessary for the recombinant production of the O-antigen/EPA

conjugate in the periplasm (marked in white) of the host cell;
Figure 2 is a detailed schematic representation of the protein glycosylation process;
Figures 3A-3C depict the opsonization indices (OIs) obtained with sera derived from rats pre-immunization (empty circles) compared to 42 days post-immunization (filled squares) with one priming dose and two booster doses of indicated doses of monovalent vaccine; Figure 3A: O2-EPA immunization; Figure 3B: O6A-EPA immunization; and Figure 3C: O25B-EPA immunization;
Figure 4 shows the ELISA titers obtained with sera from human subjects vaccinated with a placebo or a tetravalent vaccine comprising *E. coli* antigens O1A, O2, O6A and O25B at 4 μg polysaccharide per serotype; a significant increase in the ELISA titers between post- (30 days after injection) and pre-injection (day 1) was observed only in the vaccinated groups (* represents statistical significance, wherein multiple * represent increased degree of significance; ns, no significant difference); and
Figures 5A-5D depict the OIs obtained with sera derived from human subjects vaccinated with a tetravalent vaccine comprising *E. coli* antigens O1A, O2, O6A and O25B at 4 μg polysaccharide per serotype; immune response as indicated by OI against placebo and components of the tetravalent vaccine; Figure 5A: O1A-EPA; Figure 5B: O2-EPA; Figure 5C: O6A-EPA; and Figure 5D: O25B-EPA; pre-injection (Day 1) and post-injection (30 days after injection), wherein a significant increase in the OI between post- and pre-injection (indicated by *, multiple * represents increased degree of significance) was observed only in the vaccinated groups; ns, no significant difference.

DETAILED DESCRIPTION OF THE INVENTION

[0018] Discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is for the purpose of providing context for the invention. Such discussion is not an admission that any or all of these matters form part of the prior art with respect to any inventions disclosed or claimed.

[0019] Unless defined otherwise, all technical and scientific terms used herein have the same meaning commonly understood to one of ordinary skill in the art to which this invention pertains. Otherwise, certain terms cited herein have the meanings as set in the specification. It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

[0020] As used herein, the terms "O polysaccharide," "O-antigen", "O antigen", "O-antigen polysaccharide," "O-polysaccharide antigen" and the abbreviation "OPS", all refer to the O antigen of Gram-negative bacteria, which is a component of the lipopolysaccharide (LPS) and is specific for each serotype or sero(sub)type of the Gram-negative bacteria. The O antigen usually contains repeating units (RUs) of two to seven sugar residues. As used herein, the RU is set equal to the biological repeat unit (BRU). The BRU describes the RU of an O-antigen as it is synthesized *in vivo.*

[0021] As used herein, the terms "conjugate" and "glycoconjugate" all refer to a conjugation product containing an *E. coli* O antigen covalently bound to a carrier protein. The conjugate can be a bioconjugate, which is a conjugation product prepared in a host cell, wherein the host cell machinery produces the O antigen and the carrier protein and links the O antigen to the carrier protein, e.g., via N-links. The conjugate can also be prepared by other means, for example, by chemical linkage of the protein and sugar antigen.

[0022] As used herein, the term "effective amount" in the context of administering an O antigen to a subject refers to the amount of the O antigen that is sufficient to induce a desired immune effect or immune response in the subject. In certain embodiments, an "effective amount" refers to the amount of an O antigen which is sufficient to produce immunity in a subject to achieve one or more of the following effects in the subject: (i) prevent the development or onset of an ExPEC infection, preferably an invasive ExPEC disease, or symptom associated therewith; (ii) prevent the recurrence of an ExPEC infection, preferably an invasive ExPEC disease, or symptom associated therewith; (iii) prevent, reduce or ameliorate the severity of an ExPEC infection, preferably an invasive ExPEC disease, or symptom associated therewith; (iv) reduce the duration of an ExPEC infection, preferably an invasive ExPEC disease, or symptom associated therewith; (v) prevent the progression of an ExPEC infection, preferably an invasive ExPEC disease, or symptom associated therewith; (vi) cause regression of an ExPEC infection or symptom associated therewith; (vii) prevent or reduce organ failure associated with an ExPEC infection; (viii) reduce the chance or frequency of hospitalization of a subject having an ExPEC infection; (ix) reduce hospitalization length of a subject having an ExPEC infection; (x) increase the survival of a subject with an ExPEC infection, preferably an invasive ExPEC disease; (xi) eliminate an ExPEC infection, preferably an invasive ExPEC disease; (xii) inhibit or reduce ExPEC replication; and/or (xiii) enhance or improve the prophylactic or therapeutic effect(s) of another therapy.

[0023] An "effective amount" can vary depending upon a variety of factors, such as the physical condition of the subject, age, weight, health, etc.; route of administration, such as oral or parenteral; the composition administered, such as the target O antigen, the other co-administered O antigens, adjuvant, etc.; and the particular disease for which immunity is desired. When the O antigen is covalently bound to a protein carrier, the effective amount for the O antigen is calculated based on only the O antigen polysaccharide moiety in the conjugate.

[0024] As used herein, the term "in combination," in the context of the administration of two or more O antigens or

compositions to a subject, does not restrict the order in which O antigens or compositions are administered to a subject. For example, a first composition can be administered prior to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 16 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 16 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second composition to a subject.

[0025] As used herein, "subject" means any animal, preferably a mammal, most preferably a human, to who will be or has been vaccinated by a composition according to an embodiment of the invention. The term "mammal" as used herein, encompasses any mammal. Examples of mammals include, but are not limited to, cows, horses, sheep, pigs, cats, dogs, mice, rats, rabbits, guinea pigs, monkeys, humans, etc., most preferably a human. In some embodiments, a subject is a human infant. In another embodiment, a subject is a human child. In another embodiment, a subject is a human adult. In a specific embodiment, a subject is an at-risk human adult. In another embodiment, a subject is an elderly human. In another embodiment, a subject is a human infant, including a premature human infant and a human infant born at term. In another embodiment, a subject is a human toddler. The terms "subject" and "patient" may be used herein interchangeably.

[0026] As used herein, the term "premature human infant" refers to a human infant born at less than 37 weeks of gestational age.

[0027] As used herein, the term "human infant" refers to a newborn to 1 year old human.

[0028] As used herein, the term "human toddler" refers to a human that is 1 year to 3 years old.

[0029] As used herein, the term "human child" refers to a human that is 1 year to 18 years old.

[0030] As used herein, the term "human adult" refers to a human that is 18 years or older.

[0031] As used herein, the term "at-risk human adult" refers to a human that is 18 years or older who is more prone to ExPEC infection than the average human adult population. Examples of "at-risk human adult" include, but not limited to, elderly humans, immunocompromised humans, pregnant women, people with diabetes or wound injuries, people who recently had or are scheduled to have a surgery, etc.

[0032] As used herein, the term "elderly human" refers to a human that is 55, preferably 60, more preferably 65, years or older.

[0033] As used herein, an "invasive ExPEC disease" is defined as isolation and identification of ExPEC from normally sterile body sites in a subject presenting with an acute illness consistent with bacterial infection.

[0034] As used herein, an "immunological response" or "immune response" to an antigen or composition refers to the development in a subject of a humoral and/or a cellular immune response to the antigen or an antigen present in the composition.

[0035] It has been surprisingly discovered in the invention that *E. coli* O25B antigen conjugated to an EPA carrier protein appears to be less immunogenic than the other *E. coli O*-antigens (e.g., O1A, O2, and O6A) conjugated to the EPA carrier protein. This discovery leads to further investigation into the dosage of *E. coli* O25B antigen and the dosage ratios of various *E. coli* O antigens within a multivalent vaccine, thus the development of multivalent vaccines and immunization methods based on *E. coli* O antigens for improved immune responses against the O25B serotype and other serotypes of ExPEC.

Epidemiology

[0036] Studies on the serotype distribution of *E. coli* causing ExPEC disease indicate that 10 predominant O serotypes could cover an estimated 60-80% of ExPEC infections, assuming coverage of subportions of the non-typeable strains. See, e.g., Tables 1A-1C below. In both UTI and bacteremia target populations, serotypes O1, O2, O6, and O25 were identified as the four most prevalent *E. coli* serotypes, among which, serotype O25 was the most prevalent *E. coli* serotype in the bacteremia. It was also found that, for an O antigen serotype that is composed of distinct, yet structurally and antigenically related subtypes, one subtype may be more prevalent among the clinical isolates than the others. For example, O1A, O6A and O25B antigens were determined to be the more frequent subtypes among the analyzed more recent clinical strains or isolates for 01, 06 and O25 serotypes, respectively. See related disclosure on epidemiology studies in International Patent application No. PCT/EP2015/053739, published as WO 2015/124769).

Table 1A: Distribution of the most common UTI-associated *E. coli* serotypes from a collection of 1841 urine samples collected in Switzerland in 2012. Shown is the serotype distribution of samples from a relevant subpopulation of 671 subjects, and the distribution from all** samples.

Most prevalent *E. coli* serotypes associated with UTI			
O-serotype	Community acquired UTI in 18-70 years old* (n=67 1)	O-serotype	Community and hospital acquired UTI in all ages ** (n=1871)
6	10.75%	2	8.75%
2	9.55%	6	8.47%
25	6.87%	25	8.37%
1	5.52%	75	4.56%
4	5.37%	1	4.29%
75	4.78%	8	3.86%
8	3.43%	18	3.53%
18	3.28%	4	3.26%
15	3.28%	15	2.39%
73	2.24%	73	2.17%
16	2.24%	16	1.85%
7	1.94%	7	1.68%

Table 1B: Prevalence of most common UTI-associated serotypes from selected literature ranging from 1987- 2011 and from retrospectively analyzed US data from 2000-2011 (ECRC).

INDICATION	**TOTAL UTI**	**CYSTITIS**	**PYELONEPHRITIS**	US 2000-2010
	available data from 1860 isolates	available data from 1089 isolates	available data from 373 isolates	315 (all UTI specimen except fecal, all ages, F+M)*
Serotype				
O1	4.8%	4.1%	5.4%	7.0%
O2	7.1%	4.9%	15.3%	14.0%
O4	7.8%	6.0%	3.2%	3.2%
O6	16.9%	16.3%	7.8%	18.7%
O7	3.3%	2.4%	2.4%	1.9%
O8	1.7%	3.2%	0.8%	3.5%
O15	0.6%	1.5%	0.8%	1.3%
O16	4.3%	3.2%	7.2%	1.9%
O18	7.0%	7.1%	6.7%	7.0%
O21	Na	Na	Na	1.3%
O22	0.6%	0.6%	0.5%	0.0%
O25	3.0%	4.8%	0.5%	8.6%
O75	7.5%	6.0%	8.6%	3.8%
O83	1.9%	0.7%	0.5%	1.3%
O20				1.6%

(continued)

INDICATION	TOTAL UTI	CYSTITIS	PYELONEPHRITIS	US 2000-2010
	available data from 1860 isolates	available data from 1089 isolates	available data from 373 isolates	315 (all UTI specimen except fecal, all ages, F+M)*
O77				2.2%
O82				1.9%
others and non typeable/ not available	33.3%	39.2%	40.2%	
other O-types (NT not available)				21.0%
*Number of non-typeable was not available				

Table 1C: Distribution of the most common bacteremia-associated *E. coli O*-serotypes from a collection of 860 blood isolates collected in the US and EU in the period 2011-2013. Indicated is the relative O-serotype distribution of the samples.

O-serotype	Bacteremia in ≥ 60 years old US/EU 2011-2013 (n=860)
25	19.2
2	8.8
6	8.3
1	7.8
75	3.3
4	2.8
16	2.7
18	2.7
15	2.3
8	2.0
153	1.6
73	1.6

[0037] A novel O25 agglutinating clone has recently emerged in *E. coli* isolated from hospital settings, and this is named O25B. For O-serotype O25, it was found using subtyping analysis by PCR that the vast majority is actually of the O25B subtype (in a study of 24 tested clinical isolates with an O25 agglutination positive phenotype, 20 were assigned to the O25B subtype while the remaining 4 were assigned to the O25A subtype, and in the bacteremia population that was studied then, 56 of 57 studied O25 serotype isolates were typeable as O25B). It has been confirmed that autologous antisera recognize the autologous antigen better than the non-autologous antigen, and therefore inclusion of the O25B antigen into a vaccine can provide better protection against the predominant O25B clinical strains of the O25 group than inclusion of the O25A antigen would do (see, e.g., International Patent application No. PCT/EP2015/053739, published as WO 2015/124769). Results showed that an O25B vaccine can give rise to sera that cross-react with an O25A antigen, thus also providing immune response against O25A serotype (*Id.*). Compositions according to embodiments of the invention comprise an *E. coli* O25B antigen conjugated to an EPA carrier protein and other *E. coli* O-antigens conjugated to the EPA carrier protein.

Compositions Comprising *E. coli* O Antigen Conjugates

[0038] In one general aspect, the invention relates to a multivalent vaccine containing O-antigen serotypes found predominantly among *E. coli* clinical isolates, which can be used to provide active immunization for the prevention of disease caused by ExPEC having the O-antigen serotypes contained in the vaccine. In particular, the present invention provides a multivalent immune composition comprising an *E. coli* O25B antigen polysaccharide at a first dose of 5 to 18 μg, and an *E. coli* O1A antigen polysaccharide, an *E. coli* O2 antigen polysaccharide and an *E. coli* O6A antigen polysaccharide each at a dose that is independently 50% of the first dose, wherein each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA) carrier protein.

[0039] Preferably, the at least one additional *E. coli* O antigen used in compositions of the disclosure is prevalent among the *E. coli* clinical isolates. Examples of such additional O antigens include, but are not limited to, *E. coli* O1, O2, O4, O6, O7, O8, 015, 016, 018, 021, O73, O75 and 0153 antigens. Depending on the need, the composition can include more than one additional *E. coli* O antigens, such as two, three, four, five, six, seven, eight or nine additional *E. coli* O antigens, to provide immune protection against multiple *E. coli* serotypes in addition to *E. coli* O25B serotype. In a preferred instance, the additional *E. coli* O-antigen is selected from the group consisting of *E. coli* O1, O2 and O6 antigens. More preferably, the additional *E. coli* O-antigen is selected from the group consisting of *E. coli* O1A, O2 and O6A antigens.

[0040] In one embodiment, a composition of the invention comprises a first concentration of an *E. coli* O25B antigen polysaccharide, and a second concentration of each of an *E. coli* O1A antigen polysaccharide, an *E. coli* O2 antigen polysaccharide and an *E. coli* O6A antigen polysaccharide, wherein the ratio of the first concentration to the second concentration is 2:1, each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA) carrier protein, and the first concentration is 10 to 36 μg/ml. In a preferred embodiment, each of the *E. coli* O1A, O2 and O6A antigens is independently present at a concentration of at least 5 μg/ml, more preferably, at a concentration of least 8 μg/ml.

[0041] A composition according to an instance t of the disclosure contains *E. coli* O25B antigen at a concentration that is same or higher than the concentration of any of the additional O antigens in the composition. For example, the composition can have *E. coli* O25B antigen at a first concentration of, e.g., 10, 16, 24, 32 or 36 μg/ml, and one or more additional *E. coli O*-antigens each at a concentration that is, *e.g.* 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the first concentration. When the composition contains more than one additional O antigen, all of the additional O antigens can have the same concentration that is 10 to 100% of the *E. coli* O25B antigen concentration in the composition. Alternatively, the additional O antigens can also have different concentrations, each of which is independently 10-100% of the *E. coli* O25B antigen concentration. Preferably, the composition comprises 32 μg/ml *E. coli* O25B antigen and independently 16 to 32 μg/ml each of the one or more additional O antigens, wherein each of the *E. coli* O25B antigen and the additional *E. coli* O-antigens is independently covalently bound to an EPA carrier protein. In another preferred embodiment, the composition comprises 16 μg/ml *E. coli* O25B antigen, and independently 8 μg/ml to 16 μg/ml of each of the one or more additional O antigens, wherein each of the *E. coli* O25B antigen and the additional *E. coli* O-antigens is independently covalently bound to an EPA carrier protein.

[0042] In one preferred embodiment, the invention relates to a composition comprising an *E. coli* O25B antigen at a first concentration of 10 to 36 μg/ml, and a second concentration of each of an *E. coli* O1A antigen, an *E. coli* O2 antigen and an *E. coli* O6A antigen, wherein each of the *E. coli* O25B, O1A, O2 and O6A antigens are independently covalently bound to an EPA carrier protein, and the ratio of the first concentration to the second concentration is 2:1.

[0043] Preferably, the composition comprises the *E. coli* O25B, O1A, O2 and O6A antigens at a weight ratio of 2:1:1:1, and the composition comprises 32 μg/ml of the *E. coli* O25B antigen, wherein each of the O-antigen is covalently bound to an EPA carrier protein. In another preferred embodiment, the composition comprises the *E. coli* O25B, O1A, O2 and O6A antigens at a weight ratio of 2:1:1:1, and the composition comprises 16 μg/ml of the *E. coli* O25B antigen, wherein each of the O-antigen is covalently bound to an EPA carrier protein. More preferably, each of the *E. coli* O25B, O1A, O2 and O6A antigens are independently covalently bound to an EPA carrier protein having the amino acid sequence of SEQ ID NO: 1, and each of the O-antigen and EPA carrier protein conjugate is made in a cell, i.e., being a bioconjugate. Most preferably, the *E. coli* O25B, O1A, O2 and O6A antigens comprise, respectively, the structures of formula O25B', formula O1A', formula O2' and formula O6A' described *infra.*

[0044] The compositions described herein are useful in the treatment and prevention of infection of subjects (e.g., human subjects) with ExPEC. In certain embodiments, in addition to comprising *E. coli* O-antigens covalently bound to an EPA carrier protein, the compositions described herein comprise a pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of a Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier," as used herein in the context of a pharmaceutically acceptable carrier, refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition is administered. Saline solutions and aqueous

dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

[0045] In a specific embodiment, provided herein is a composition comprising an *E. coli* O25B antigen covalently bound to an EPA carrier protein, and the additional *E. coli* O antigens each covalently bound to the EPA carrier protein.

[0046] Also disclosed herein is a composition comprising (i) a bioconjugate comprising an *E. coli* O25B antigen covalently bound to an EPA carrier protein, and (ii) a bioconjugate comprising an *E. coli* O1A antigen covalently bound to an EPA carrier protein.

[0047] Also disclosed herein is a composition comprising (i) a bioconjugate comprising an *E. coli* O25B antigen covalently bound to an EPA carrier protein, and (ii) a bioconjugate comprising an *E. coli* O2 antigen covalently bound to an EPA carrier protein.

[0048] Also disclosed herein is a composition comprising (i) a bioconjugate comprising an *E. coli* O25B antigen covalently bound to an EPA carrier protein, and (ii) a bioconjugate comprising an *E. coli* O6A antigen covalently bound to an EPA carrier protein.

[0049] Also disclosed herein is a composition comprising an *E. coli* O25B bioconjugate comprising an *E. coli* O25B antigen covalently bound to an EPA carrier protein, and two or more bioconjugates selected from the group consisting of: (i) an *E. coli* O1A bioconjugate comprising an *E. coli* O1A antigen covalently bound to an EPA carrier protein; (ii) an *E. coli* O2 bioconjugate comprising an *E. coli* O2 antigen covalently bound to an EPA carrier protein; and (iii) an *E. coli* O6A bioconjugate comprising an *E. coli* O6A antigen covalently bound to an EPA carrier protein.

[0050] Also disclosed herein is a composition that comprises: (i) an *E. coli* O25B bioconjugate comprising an *E. coli* O25B antigen covalently bound to an EPA carrier protein; (ii) an *E. coli* O1A bioconjugate comprising an *E. coli* O1A antigen covalently bound to an EPA carrier protein; (iii) an *E. coli* O2 bioconjugate comprising an *E. coli* O2 antigen covalently bound to an EPA carrier protein; and (iv) an *E. coli* O6A bioconjugate comprising an *E. coli* O6A antigen covalently bound to an EPA carrier protein, wherein (i), (ii), (iii), and (iv) are formulated in a single formulation.

[0051] Also disclosed herein is a composition that comprises: (i) an *E. coli* O25B bioconjugate comprising an *E. coli* O25B antigen covalently bound to an EPA carrier protein; (ii) an *E. coli* O1A bioconjugate comprising an *E. coli* O1A antigen covalently bound to an EPA carrier protein; (iii) an *E. coli* O2 bioconjugate comprising an *E. coli* O2 antigen covalently bound to an EPA carrier protein; and (iv) an *E. coli* O6A bioconjugate comprising an *E. coli* O6A antigen covalently bound to an EPA carrier protein, wherein (i), (ii), (iii), and (iv) are formulated in individual compositions that are administered in combination.

[0052] In certain embodiments, the foregoing compositions optionally comprise an EPA carrier protein covalently linked to an *E. coli* O-antigen other than *E. coli* O1A, O2, O6A, and O25B. Other *E. coli* O antigens include, but are not limited to, the additional O antigens listed in Tables 1A-1C above.

[0053] The compositions provided herein can be used for eliciting an immune response in a host to whom the composition is administered, i.e., are immunogenic. Thus, the compositions described herein can be used as vaccines against ExPEC infection, and can comprise any additional components suitable for use in a vaccine. The compositions described herein are multivalent formulations, e.g., at least tetravalent formulations comprising bioconjugates of *E. coli* O-antigens of the O25B, O1A, O6A, and O2 serotypes/subserotypes.

[0054] In certain embodiments, the compositions described herein additionally comprise a preservative, such as the mercury derivative thimerosal. In a specific embodiment, the pharmaceutical compositions described herein comprise 0.001% to 0.01% thimerosal. In other embodiments, the pharmaceutical compositions described herein do not comprise a preservative.

[0055] In certain embodiments, the compositions described herein (e.g., the immunogenic compositions) comprise, or are administered in combination with, an adjuvant. The adjuvant for administration in combination with a composition described herein may be administered before, concomitantly with, or after administration of said composition. In some embodiments, the term "adjuvant" refers to a compound that when administered in conjunction with or as part of a composition described herein augments, enhances and/or boosts the immune response to a bioconjugate, but when the adjuvant compound is administered alone does not generate an immune response to the bioconjugate. In some embodiments, the adjuvant generates an immune response to the poly bioconjugate peptide and does not produce an allergy or other adverse reaction. Adjuvants can enhance an immune response by several mechanisms including, e.g., lymphocyte recruitment, stimulation of B and/or T cells, and stimulation of macrophages. In certain preferred embodiments, the compositions described herein do not comprise an adjuvant besides the bioconjugates, and/or are not administered in combination with an adjuvant besides the bioconjugates (in case the bioconjugates would comprise some intrinsic adjuvant properties, these would be disregarded and no extrinsic adjuvant would be added in these embodiments).

[0056] Specific examples of adjuvants include, but are not limited to, aluminum salts (alum) (such as aluminum hydroxide, aluminum phosphate, and aluminum sulfate), 3 De-O-acylated monophosphoryl lipid A (MPL) (see United

Kingdom Patent GB2220211), MF59 (Novartis), AS03 (GlaxoSmithKline), AS04 (GlaxoSmithKline), polysorbate 80 (Tween 80; ICL Americas, Inc.), imidazopyridine compounds (see International Application No. PCT/US2007/064857, published as International Publication No. WO2007/109812), imidazoquinoxaline compounds (see International Application No. PCT/US2007/064858, published as International Publication No. WO2007/109813) and saponins, such as QS21 (see Kensil et al., in Vaccine Design: The Subunit and Adjuvant Approach (eds. Powell & Newman, Plenum Press, NY, 1995); U.S. Pat. No. 5,057,540). In some embodiments, the adjuvant is Freund's adjuvant (complete or incomplete). Other adjuvants are oil in water emulsions (such as squalene or peanut oil), optionally in combination with immune stimulants, such as monophosphoryl lipid A (see Stoute et al., 1997, N. Engl. J. Med. 336, 86-91). Another adjuvant is CpG (Bioworld Today, Nov. 15, 1998).

[0057] In certain embodiments, the compositions described herein are formulated to be suitable for the intended route of administration to a subject. For example, the compositions described herein may be formulated to be suitable for subcutaneous, parenteral, oral, intradermal, transdermal, colorectal, intraperitoneal, and rectal administration. In a specific embodiment, the pharmaceutical composition may be formulated for intravenous, oral, intraperitoneal, intranasal, intratracheal, subcutaneous, intramuscular, topical, intradermal, transdermal or pulmonary administration. In certain embodiments, the compositions described herein are administered by intramuscular injection.

[0058] In certain embodiments, the compositions described herein additionally comprise one or more buffers, e.g., Tris-buffered saline, phosphate buffer, and sucrose phosphate glutamate buffer.

[0059] In certain embodiments, the compositions described herein additionally comprise one or more salts, e.g., Tris-hydrochloride, sodium chloride, calcium chloride, potassium chloride, sodium phosphate, monosodium glutamate, and aluminum salts (e.g., aluminum hydroxide, aluminum phosphate, alum (potassium aluminum sulfate), or a mixture of such aluminum salts). In one embodiment, a composition of the invention comprises the bioconjugates described herein in a Tris-buffered saline (TBS) pH 7.4 (e.g. containing Tris, NaCl and KCl, e.g. at 25 mM, 137 mM and 2.7 mM, respectively).

[0060] The compositions described herein can be included in a container, pack, or dispenser together with instructions for administration.

[0061] The compositions described herein can be stored before use, e.g., the compositions can be stored frozen (e.g., at about -20°C or at about -70°C); stored in refrigerated conditions (e.g., at about 4°C); or stored at room temperature.

Methods/Uses

[0062] In another general aspect, the invention provides an *E. coli* O25B antigen polysaccharide for use in a method of inducing an immune response to extra-intestinal pathogenic *E. coli* (ExPEC) in a subject in need thereof, comprising administering to the subject a first effective amount of an *E. coli* O25B antigen polysaccharide, and a second effective amount of each of an *E. coli* O1A antigen polysaccharide, an *E. coli* O2 antigen polysaccharide and an *E. coli* O6A antigen polysaccharide, wherein the ratio of the first effective amount to the second effective amount is 2: 1, each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA) carrier protein, and the first effective amount is 5 to 18 μg per administration.. Preferably, the immune response is effective to prevent or treat a disease associated with ExPEC in the subject in need thereof.

[0063] Preferably, the at least one additional *E. coli* O antigen used in the methods and uses disclosed herein is prevalent among the *E. coli* clinical isolates. Examples of such additional O antigens include, but are not limited to, *E. coli* O1, O2, O4, O6, O7, O8, 015, 016, 018, 021, O73, O75 and 0153 antigens. Depending on the need, more than one additional *E. coli* O antigens, such as two, three, four, five, six, seven, eight or nine additional *E. coli* O antigens, can be administered to provide immune protection against multiple *E. coli* serotypes in addition to *E. coli* O25B serotype.

[0064] Preferably, a composition of the present invention is for use in a method that induces an immune response in a subject in need thereof against ExPEC serotypes O25B, and one or more of the additional *E. coli* O-antigens selected from the group consisting of *E. coli* O1A, O2 and O6A antigens. The composition of the present invention may be for use in a method that comprises administering to a subject in need thereof an *E. coli* O25B antigen at a first effective amount of 5 to 18 μg per administration, and *E. coli* O1A, O2 and O6A antigens each at an effective amount that is 50% of the first effective amount, wherein each of the *E. coli* O25B antigen and the additional *E. coli* O-antigens is independently covalently bound to an EPA carrier protein. In a preferred embodiment, each of the *E. coli* O1A, O2 and O6A antigens is independently administered at an effective amount of at least 3 μg per administration, more preferably, at an effective amount of at least 4 μg per administration.

a method according to the disclosure, the administered effective amount of *E. coli* O25B antigen is same or higher than the administered effective amount of any of the additional O antigens. For example, the *E. coli* O25B antigen can be administered at a first effective amount of 4 to 24 μg per administration, and the at least one additional *E. coli* O-antigen can be administered at a second effective amount that is, e.g., 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the first effective amount. When more than one additional O antigens are administered in combination, all of the additional O antigens can be administered at the same effective amount that is 10-100% of the first effective amount

for the *E. coli* O25B antigen. The additional O antigens can also be administered at different effective amounts each of which is independently 10-100% of the first effective amount for the *E. coli* O25B antigen. Preferably, the *E. coli* O25B antigen is administered at the first effective amount of 5 μg to 18 μg, and the at least one additional O antigen is administered at a second effective amount that is independently 50% to 100% of the first effective amount.

[0065] In one embodiment a composition according to the invention is provided for use in a method of inducing an immune response to ExPEC in a subject in need thereof that comprises administering to the subject an *E. coli* O25B antigen at a first effective amount of, e.g., 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 μg per administration, and *E. coli* O1A, O2 and O6A antigens each at an effective amount that is independently, 50% of the first effective amount, wherein each of the *E. coli* O25B antigen and the additional *E. coli* O-antigens is independently covalently bound to an EPA carrier protein.

[0066] In one preferred embodiment, a composition of the invention is provided for use in a method of inducing an immune response to ExPEC in a subject in need thereof, comprising administering to the subject an *E. coli* O25B antigen at a first effective amount of 5 to 18 μg per administration, and a second effective amount of each of an *E. coli* O1A antigen, an *E. coli* O2 antigen and an *E. coli* O6A antigen, wherein each of the *E. coli* O25B, O1A, O2 and O6A antigens are independently covalently bound to an EPA carrier protein, and the ratio of the first effective amount to the second effective amount is 2:1. Preferably, the *E. coli* O25B, O1A, O2 and O6A antigens are administered to the subject at a dosage ratio of 2:1:1:1, and the *E. coli* O25B antigen is administered at 5 μg, 8 μg or 16 μg per administration. Also preferably, the *E. coli* O25B, O1A, O2 and O6A antigens are administered to the subject in one composition.

[0067] Disclosed herein are methods and uses of compositions for inducing an immune response to ExPEC in a subject in need thereof, comprising administering to the subject an *E. coli* O25B antigen covalently bound to an EPA carrier protein, and at least one additional *E. coli* O antigen covalently bound to the EPA carrier protein. In a specific instance, the compositions described herein are used to vaccinate a human subject to induce a protective immunity against ExPEC infection of the human subject.

[0068] Further disclosed herein are methods of inducing the production of opsonophagocytic antibodies against ExPEC in a subject in need thereof, comprising administering to the subject an *E. coli* O25B antigen covalently bound to an EPA carrier protein, and at least one additional *E. coli* O antigen covalently bound to the EPA carrier protein.

[0069] In one embodiment, said subject has an ExPEC infection at the time of administration. In another embodiment, said subject does not have an ExPEC infection at the time of administration. Examples of infections caused by ExPEC include, but are not limited to, urinary tract infection, surgical-site infection, bacteremia, abdominal or pelvic infection, pneumonia, nosocomial pneumonia, osteomyelitis, cellulitis, wound infection, meningitis, neonatal meningitis, peritonitis, cholangitis, soft-tissue infections, pyomyositis, septic arthritis, and sepsis. Preferably, the infection caused by ExPEC is an invasive ExPEC disease caused by ExPEC serotypes of which antigens are included in the compositions according to embodiments of the invention.

[0070] The compositions of the invention may be for use in methods of inducing an immune response in a subject described herein that result in vaccination of the subject against infection by the ExPEC strains whose O-antigens are present in the composition(s). When an O-antigen subtype is used, the invention can also induce immune response to another O-antigen subtype having similar antigenicity.

[0071] In a specific embodiment, the immune response induced by a method or composition described herein is effective to prevent and/or treat an infection caused by *E. coli* of the O25 serotype. In a specific embodiment, said O25 serotype is O25B. In another specific embodiment, said O25 serotype is O25A.

[0072] In a specific embodiment, the immune response induced by a method or composition described herein is effective to prevent and/or treat an infection caused by *E. coli* of the O25 serotype, e.g. O25B serotype, and O1 serotype, e.g. O1A serotype.

[0073] In a specific embodiment, the immune response induced by a method or composition described herein is effective to prevent and/or treat an infection caused by *E. coli* of the O25 serotype, e.g. O25B serotype, and O2 serotype.

[0074] In a specific embodiment, the immune response induced by a method or composition described herein is effective to prevent and/or treat an infection caused by *E. coli* of the O25 serotype, e.g. O25B serotype, and O6 serotype, e.g. O6A serotype.

[0075] In a specific embodiment, the immune response induced by a method or composition described herein is effective to prevent and/or treat an infection caused by *E. coli* of the O25 serotype (e.g. O25B and/or O25A), and two or more of the following *E. coli* serotypes: O1 (e.g., O1A, O1B, and/or O1C), O2, and/or O6 (e.g., O6A and/or O6GlcNAc).

[0076] In a specific embodiment, the immune response induced by a method or composition described herein is effective to prevent and/or treat an infection caused by each of the following *E. coli* serotypes: O25 (e.g., O25B and/or O25A), O1 (e.g., O1A, O1B, and/or O1C), O2, and O6 (e.g., O6A and/or O6GlcNAc).

[0077] In a specific embodiment, the immune response induced is effective to prevent and/or treat an infection caused by *E. coli* of the O25B serotype, and an *E. coli* serotype other than O1, O2, O6, or O25, including, but not limited to, the additional O serotypes listed in Tables 1A-1C.

[0078] In order to immunize a subject against an ExPEC infection, the subject can be administered a single composition

described herein, wherein said composition comprises *E. coli* O25B antigen, and one, two, three, four, or more additional *E. coli* O antigens described herein, each covalently bound to an EPA carrier protein. Alternatively, in order to treat a subject having an ExPEC infection or immunize a subject against an ExPEC infection, the subject can be administered multiple compositions described herein in combination. For example, a subject can be administered a composition comprising *E. coli* O25B antigen conjugated to an EPA carrier protein, in combination with the administration of two, three, four, or more compositions comprising additional O antigen conjugates according to embodiments of the invention.

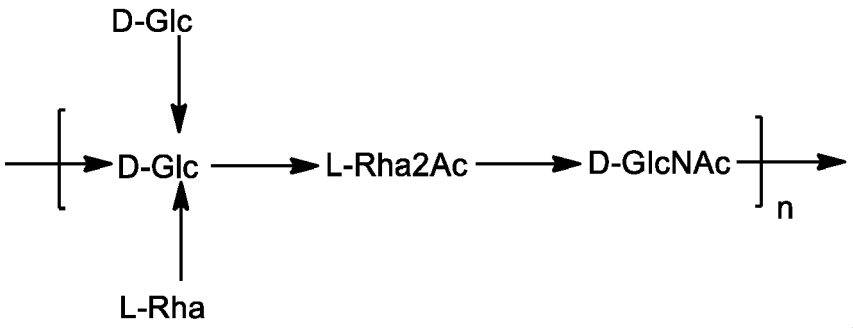
[0079] In certain embodiments, the immune response induced in a subject following administration of a composition described herein is effective to prevent or reduce a symptom resulting from an ExPEC infection, preferably in at least 30%, more preferably at least 40%, such as at least 50%, of the subjects administered with the composition. Symptoms of ExPEC infection may vary depending on the nature of the infection and may include, but are not limited to: dysuria, increased urinary frequency or urgency, pyuria, hematuria, back pain, pelvic pain, pain while urinating, fever, chills, and/or nausea (e.g., in subjects having a urinary tract infection caused by ExPEC); high fever, headache, stiff neck, nausea, vomiting, seizures, sleepiness, and/or light sensitivity (e.g., in subjects having meningitis caused by ExPEC); fever, increased heart rate, increased respiratory rate, decreased urine output, decreased platelet count, abdominal pain, difficulty breathing, and/or abnormal heart function (e.g., in subjects having sepsis caused by ExPEC).

[0080] In certain embodiments, the immune response induced in a subject following administration of a composition described herein is effective to reduce the likelihood of hospitalization of a subject suffering from an ExPEC infection. In some embodiments, the immune response induced in a subject following administration of a composition described herein is effective to reduce the duration of hospitalization of a subject suffering from an ExPEC infection.

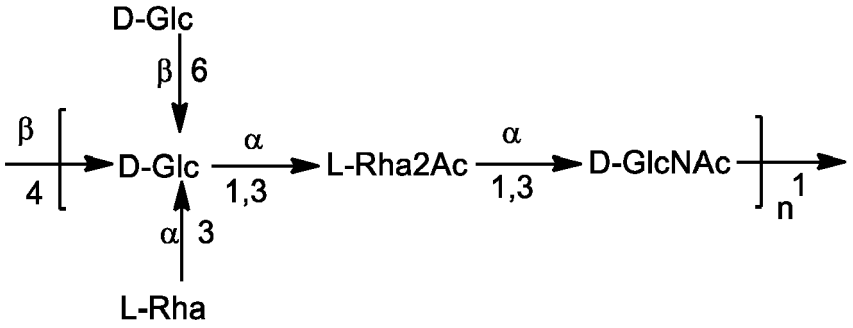
***E. coli* O-antigens**

[0081] Embodiments of the invention relate to compositions and the compositions for use in methods relating to *E. coli* O25B antigen and one or more additional *E. coli* O antigens. Preferably, the additional O antigen is prevalent among the clinical isolates of *E. coli.* Examples of *E. coli* antigens that can be used in the invention include, but are not limited to, the *E. coli* O25B, O1A, O2, and O6A antigens.

[0082] As used herein an "*E. coli* O25B antigen" refers to an O antigen specific to the *E. coli* O25B serotype. In one embodiment, an *E. coli* O25B antigen comprises the structure of Formula O25B:



preferably, the *E. coli* O25B antigen comprises the structure of Formula O25B':

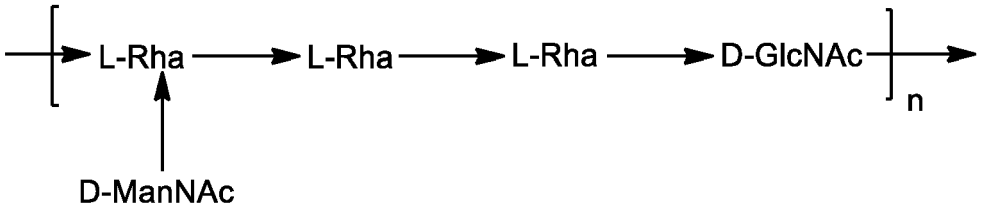


wherein the n in Formula O25B or Formula O25B' is an integer of 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5, 10 to 30, 15 to 30, 20 to 30, 25 to 30, 5 to 25, 10 to 25, 15 to 25, 20 to 25, 10 to 20, or 15 to 20. In one embodiment of the invention, the n in Formula O25B or Formula O25B' is an integer of 10-20.

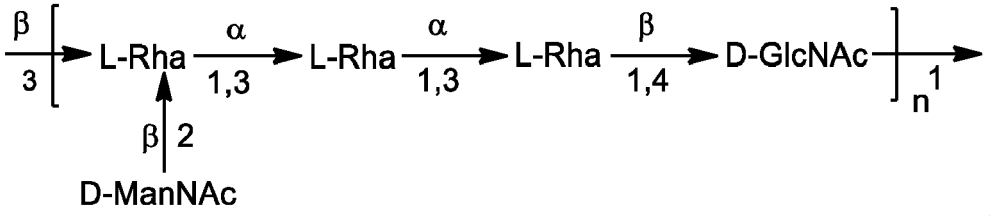
[0083] Preferably, a population of *E. coli* O25B antigens having the structure of Formula O25B, more preferably Formula O25B', is used in compositions and methods according to embodiments of the invention, wherein the n of at least 80% of the *E. coli* O25B antigens in the population is an integer of 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5, 10 to 30, 15 to 30, 20 to 30, 25 to 30, 5 to 25, 10 to 25, 15 to 25, 20 to 25, 10 to 20, or 15 to 20. In one embodiment of the invention, the n of at least 80% of the *E. coli* O25B antigens in the population is an integer of 10-20.

[0084] As used herein, an "*E. coli* O1 antigen" refers to an O antigen specific to the *E. coli* O1 serotype. In one embodiment, an *E. coli* O1 antigen is an *E. coli* O1A antigen.

[0085] As used herein, an "*E. coli* O1A antigen" refers to an O antigen specific to the *E. coli* O1A serotype. In one embodiment, an *E. coli* O1A antigen comprises the structure of Formula O1A:



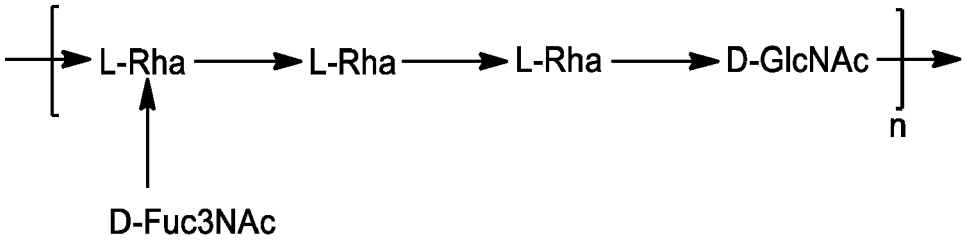
preferably, the *E. coli* O1A antigen comprises the structure of Formula O1A':



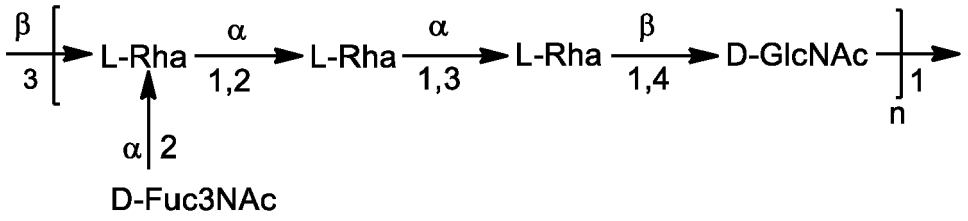
wherein the n in Formula O1A or Formula O1A' is an integer of 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5, 10 to 30, 15 to 30, 20 to 30, 25 to 30, 5 to 25, 10 to 25, 15 to 25, 20 to 25, 10 to 20, or 15 to 20. In one embodiment, the n in Formula O1A or Formula O1A' is an integer of 7-15.

[0086] Preferably, a population of *E. coli* O1A antigens having the structure of Formula O1A, more preferably Formula O1A', is used in compositions and methods according to embodiments of the invention, wherein the n of at least 80% of the *E. coli* O1A antigens in the population is of 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5, 10 to 30, 15 to 30, 20 to 30, 25 to 30, 5 to 25, 10 to 25, 15 to 25, 20 to 25, 10 to 20, or 15 to 20. In one embodiment, the n of at least 80% of the *E. coli* O1A antigens in the population is an integer of 5-15.

[0087] As used herein, an "*E. coli* O2 antigen" refers to an O antigen specific to the *E. coli* O2 serotype. In one embodiment, an *E. coli* O2 antigen comprises the structure of Formula O2:



preferably, the *E. coli* O2 antigen comprises the structure of Formula O2':



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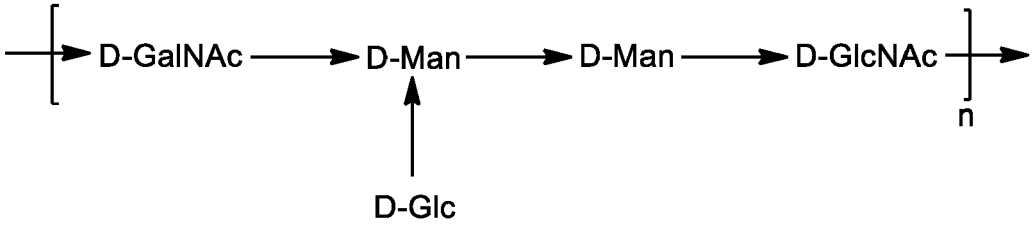
wherein the n in Formula O2 or Formula O2' is an integer of 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5, 10 to 30, 15 to 30, 20 to 30, 25 to 30, 5 to 25, 10 to 25, 15 to 25, 20 to 25, 10 to 20, or 15 to 20. In one embodiment, the n in Formula O2 or Formula O2' is an integer of 8-16.

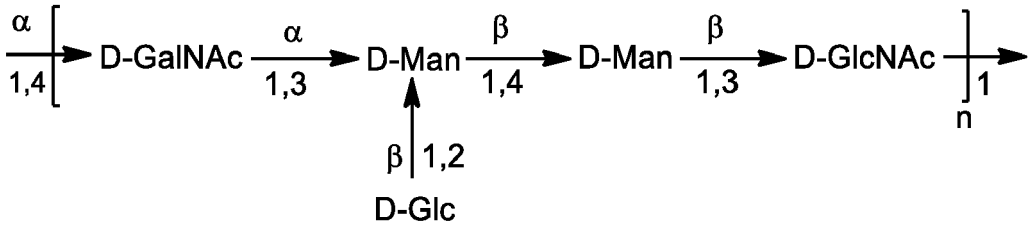
[0088] Preferably, a population of *E. coli* O2 antigens having the structure of Formula O2, more preferably Formula O2', is used in compositions and methods according to embodiments of the invention, wherein the n of at least 80% of the *E. coli* O2 antigens in the population is of 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5, 10 to 30, 15 to 30, 20 to 30, 25 to 30, 5 to 25, 10 to 25, 15 to 25, 20 to 25, 10 to 20, or 15 to 20. In one embodiment, the n of at least 80% of the *E. coli* O2 antigens in the population is an integer of 5-20.

[0089] As used herein, an "E. *coli* O6 antigen" refers to an O antigen specific to the *E. coli* O6 serotype. In one embodiment, an *E. coli* O6 antigen is an *E. coli* O6A.

[0090] As used herein, an "*E. coli* O6A antigen," also referred to as "*E. coli* O6K2 antigen" or "*E. coli* O6Glc antigen," refers to an O antigen specific to the *E. coli* O6A serotype. In one embodiment, an *E. coli* O6A antigen comprises the structure of Formula O6A:



preferably, the *E. coli* O6A antigen comprises the structure of Formula O6A':



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wherein the β 1, 2 linkage is also named β2 linkage, the n in Formula O6A or Formula O6A' is an integer of 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5, 10 to 30, 15 to 30, 20 to 30, 25 to 30, 5 to 25, 10 to 25, 15 to 25, 20 to 25, 10 to 20, or 15 to 20. In one embodiment, the n in Formula O6A or Formula O6A' is an integer of 8-18.

[0091] Preferably, a population of *E. coli* O6A antigens having the structure of Formula O6A, more preferably Formula O6A', is used in compositions and methods according to embodiments of the invention, wherein the n of at least 80% of the *E. coli* O6A antigens in the population is of 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5, 10 to 30, 15 to 30, 20 to 30, 25 to 30, 5 to 25, 10 to 25, 15 to 25, 20 to 25, 10 to 20, or 15 to 20. In one embodiment, the n of at least 80% of the *E. coli* O6A antigens in the population is an integer of 5-20.

[0092] In a preferred embodiment, a composition of the invention comprises *E. coli* O25B antigens having the structure of formula O25B', wherein the n of at least 80% of the *E. coli* O25B antigens in the population is an integer of 10-20; *E. coli* O1A antigens having the structure of formula O1A', wherein the n of at least 80% of the *E. coli* O1A antigens in the population is an integer of 5-15; *E. coli* O2 antigens having the structure of formula O2', wherein the n of at least 80% of the *E. coli* O2 antigens in the population is an integer of 5-20; and *E. coli* O6A antigens having the structure of formula O6A', wherein the n of at least 80% of the *E. coli* O6A antigens in the population is an integer of 5-20, wherein each of the O-antigens is covalently bound to an EPA carrier protein having the amino acid sequence of SEQ ID NO:1.

[0093] An *E. coli* O antigen useful in the invention can be produced by methods known in the art in view of the present disclosure. For example, they can be produced from a cell, preferably a recombinant cell that is optimized for the biosynthesis of the O antigen. See, e.g., relevant disclosure on the nucleic acids, proteins, host cells, production methods, etc., for *E. coli* O antigen biosynthesis in WO 2006/119987, WO 2009/104074, International Patent Application No. PCT/EP2015/053739 (published as WO 2015/124769), Ihssen et al., 2010, Microbial Cell Factories 9, 61.

EPA Carrier Protein

[0094] According to embodiments of the invention, each *E. coli* O antigen is covalently bound to an EPA carrier protein (see, e.g., Ihssen et al., 2010, Microbial Cell Factories 9, 61). Various detoxified EPA variants have been described in literature and can be used as EPA carrier proteins in the conjugates described herein.

[0095] In certain embodiments, the EPA carrier proteins used in the conjugates described herein are EPAs modified in such a way that the protein is less toxic and/or more susceptible to glycosylation. For example, detoxification can be achieved by mutating and deleting the catalytically essential residues, such as L552V and ΔE553, according to Lukac et al., Infect Immun, 56: 3095-3098, 1988 and Ho et al., Hum Vaccin, 2:89-98, 2006. In a specific embodiment, the carrier proteins used in the generation of the conjugates described herein are EPAs modified such that the number of glycosylation sites in the carrier proteins is optimized in a manner that allows for lower concentrations of the protein to be administered, e.g., in an immunogenic composition, in its bioconjugate form.

[0096] In certain embodiments, the EPA carrier proteins are EPAs modified to include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more glycosylation sites than would normally be associated with the carrier protein (e.g., relative to the number of glycosylation sites associated with the carrier protein in its native/natural, e.g., "wild-type" state). In specific embodiments, introduction of glycosylation sites is accomplished by insertion of glycosylation consensus sequences (e.g., Asn-X-Ser(Thr), wherein X can be any amino acid except Pro (SEQ ID NO: 2); or preferably Asp(Glu)-X-Asn-Z-Ser(Thr), wherein X and Z are independently selected from any natural amino acid except Pro (SEQ ID NO:3) (see WO 2006/119987)) anywhere in the primary structure of the EPA protein. In one particular embodiment, the EPA carrier protein comprises 4 consensus glycosylation sequences Asp/Glu-X-Asn-Z-Ser/Thr (SEQ ID NO: 3), and has an amino acid sequence as provided in SEQ ID NO: 1.

[0097] In certain embodiments, the EPA carrier protein can be produced together with a signal sequence (such as a signal peptide for *E. coli* DsbA, *E. coli* outer membrane porin A (OmpA), *E. coli* maltose binding protein (MalE), etc.) that targets the carrier protein to the periplasmic space of the host cell that expresses the carrier protein. The EPA carrier protein can also be modified to a "tag," i.e., a sequence of amino acids that allows for the isolation and/or identification of the carrier protein.

[0098] An EPA carrier protein useful in the invention can be produced by methods known in the art in view of the present disclosure. See, e.g., relevant disclosure in e.g., Ihssen et al., 2010, Microbial Cell Factories 9, 61, and in WO 2006/119987, WO 2009/104074, and International Patent application No. PCT/ EP2015/053739 (published as WO 2015/124769) .

Conjugates

[0099] In certain embodiments, a host cell can produce an *E. coli* O antigen and an EPA carrier protein, and covalently bind the O antigen to the EPA carrier protein to form a bioconjugate useful in the invention. See, e.g., relevant disclosure in e.g., Ihssen et al., 2010, Microbial Cell Factories 9, 61, and in WO 2006/119987, WO 2009/104074, and International Patent application No. PCT/ EP2015/053739 (published as WO 2015/124769).

[0100] Alternatively, the glycoconjugates can be prepared by chemical synthesis, i.e., prepared outside of host cells (*in vitro*). For example, the *E. coli* O-antigens described herein, e.g., O25B antigen, can be conjugated to carrier proteins using methods known to those of skill in the art, including by means of using activation reactive groups in the polysaccharide/ oligosaccharide as well as the protein carrier. See, e.g., Pawlowski et al., 2000, Vaccine 18:1873-1885; and Robbins et al., 2009, Proc Natl Acad Sci USA 106:7974-7978. Such approaches comprise extraction of antigenic polysaccharides/ oligosaccharides from host cells, purifying the polysaccharides/oligosaccharides, chemically activating the polysaccharides/oligosaccharides, and conjugating the polysaccharides/ oligosaccharides to a carrier protein.

[0101] Bioconjugates have advantageous properties over glycoconjugates made *in vitro,* e.g., bioconjugates require less chemicals in manufacture and are more consistent and homogenous in terms of the final product generated. Thus, bioconjugates are preferred over chemically produced glycoconjugates.

[0102] In a specific embodiment, the EPA carrier protein is N-linked to an *E. coli* O-antigen useful in the invention. For example, the *E. coli* O antigen is linked to the Asn residue in a glycosylation sequence of a carrier protein, such as Asn-X-Ser(Thr), wherein X can be any amino acid except Pro (SEQ ID NO: 2), preferably Asp(Glu)-X-Asn-Z-Ser(Thr), wherein X and Z are independently selected from any natural amino acid except Pro (SEQ ID NO: 3).

[0103] The conjugates described herein can be purified by any method known in the art for purification of a protein,

for example, by chromatography (e.g., ion exchange, anionic exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. See, e.g., Saraswat et al., 2013, Biomed. Res. Int. ID#312709 (p. 1-18); see also the methods described in WO 2009/104074. The actual conditions used to purify a particular conjugate will depend, in part, on the synthesis strategy (e.g., synthetic production vs. recombinant production) and on factors such as net charge, hydrophobicity, and/or hydrophilicity of the bioconjugate, and will be apparent to those having skill in the art.

Combination Therapies

[0104] In certain embodiments, a composition described herein is administered to a subject in combination with one or more other therapies (e.g., antibacterial or immunomodulatory therapies). The one or more other therapies can be beneficial in the treatment or prevention of an ExPEC infection or can ameliorate a symptom or condition associated with an ExPEC infection. In some embodiments, the one or more other therapies are pain relievers or anti-fever medications. In certain embodiments, the therapies are administered less than 5 minutes apart to less than 1 week apart. Any anti-bacterial agents known to one of skill in the art (e.g. antibiotics) may be used in combination with a composition described herein.

Patient Populations

[0105] In certain embodiments, a composition described herein is for use in a method where it is administered (or applied) to a naive subject, *i.e.,* a subject that does not have an ExPEC infection or has not previously had an ExPEC infection. In one embodiment, a composition described herein is for use in a method where it is administered (or applied) to a naive subject that is at risk of acquiring an ExPEC infection.

[0106] In certain embodiments, a composition described herein is for use in a method where it is administered (or applied) to a subject who has been or was previously diagnosed with an ExPEC infection. In some embodiments, a composition described herein is for use in a method where it is administered (or applied) to a subject infected with ExPEC before symptoms manifest or symptoms become severe (*e.g.*, before the patient requires hospitalization).

[0107] In certain embodiments, a composition described herein is for use in a method where it is administered (or applied) to a subject who has been diagnosed with an uropathogenic *E. coli* (UPEC) infection. In some embodiments, a composition described herein is for use in a method where it is administered (or applied) to a subject suffering from reoccurring urinary tract infections. In some embodiments, a composition (or method) described herein is administered (or applied) to a subject suffering from reoccurring urinary tract infections, but is healthy at the moment of treatment. In some embodiments, a composition described herein is for use in a method where it is administered (or applied) to a subject having or at risk of acquiring bacteremia or sepsis.

[0108] In some embodiments, a subject to be administered (or applied) a composition described herein is an animal. In certain embodiments, the animal is a canine. In certain embodiments, the animal is a feline. In certain embodiments, the animal is a horse. In certain embodiments, the animal is a cow. In certain embodiments, the animal is a mammal, e.g., a horse, swine, rabbit, mouse, or primate. In a preferred embodiment, the subject is a human.

[0109] In certain embodiments, a subject to be administered (or applied) a described herein is a human subject, preferably, a human subject at risk of having an invasive ExPEC disease. In certain embodiments, a subject to be administered (or applied) a composition described herein is a human adult more than 50 years old. In certain embodiments, a subject to be administered (or applied) a composition described herein is a human adult more than 55, more than 60 or more than 65 years old.

[0110] In certain embodiments, a subject to be administered (or applied) a composition described herein is a human child. In certain embodiments, a subject to be administered (or applied) a composition described herein is a human child. In certain embodiments, a subject to be administered (or applied) a composition described herein is a human infant, including a premature human infant. In some embodiments, a subject to be administered (or applied) a composition described herein is a human toddler. In certain embodiments, a subject to be administered (or applied) a composition described herein is not an infant of less than 6 months old.

[0111] In certain embodiments, a subject to be administered (or applied) a composition described herein is an individual who is pregnant. In certain embodiments, a subject to be administered (or applied) a composition described herein is a woman who has given birth 1, 2, 3, 4, 5, 6, 7, or 8 weeks earlier.

[0112] In certain embodiments, a subject to be administered (or applied) a composition (described herein is an individual at increased risk of ExPEC, *e.g.*, an immunocompromised or immunodeficient individual, an individual scheduled for surgery or recently undergone a surgery, an individual having a wound injury, an intensive care unit (ICU) or critical care unit (CCU) patient, etc. In certain embodiments, a subject to be administered (or applied) a composition (or method) described herein is an individual in close contact with an individual having or at increased risk of ExPEC infection.

[0113] In certain embodiments, a subject to be administered (or applied) a composition described herein is a health

care worker. In certain embodiments, a subject to be administered (or applied) a composition described herein is immunocompromised (e.g., suffers from HIV infection) or immunosuppressed.

[0114] In certain embodiments, a subject to be administered (or applied) a composition described herein has diabetes. In certain embodiments, a subject to be administered (or applied) a composition described herein has multiple sclerosis.

[0115] In certain embodiments, a subject to be administered (or applied) a composition described herein has a condition that requires them to use a catheter, such as a urinary catheter. In certain embodiments, a subject to be administered (or applied) a composition described herein has a spinal cord injury.

[0116] In certain embodiments, the subject is a male who will undergo or has recently undergone a prostate biopsy.

[0117] In a preferred embodiment, the subject to be administered (or applied) a composition described herein is an at-risk human adult in need of immunization for the prevention of invasive ExPEC disease caused by ExPEC serotypes O1A, O2, O6A and O25B. Examples of at-risk human include, but are not limited to, those described herein *supra.* Other examples of at-risk human include, e.g., individuals having transrectal ultrasonography with prostate needle biopsy (TRUS-PNB) or recurrent urosepsis, residents of a long term care facility (LTCF), long term care (LTAC)-assisted living, pre-surgery patients (including but not limited to, patients scheduled for genito-urinary/abdominal surgery); pre-dialysis patients, pre-dialysis, etc.

Dosage and Frequency of Administration

[0118] Administration of the conjugates of O-antigens and an EPA carrier protein and/or composition thereof can be done via various routes known to the clinician, for instance subcutaneous, parenteral, intravenous, intramuscular, topical, oral, intradermal, transdermal, intranasal, etc. In one embodiment, administration is via intramuscular injection.

[0119] According to embodiments of the invention, the dosage level of *E. coli* O25B antigen covalently should be no less, preferably more, than the dosage levels of the other *E. coli* O antigens used in a composition or a method the composition of the invention is for use in, wherein each of the *E. coli* O antigens is covalently bound to an EPA carrier protein. The precise dosage to be employed in the formulation and method will depend on the route of administration, and the seriousness of the infection, and should be decided according to the judgment of the practitioner and each subject's circumstances.

[0120] In certain embodiments of the invention, exemplary dosages for *E. coli* O25B antigen range from 5 to 18 μg of O25B antigen per administration, and the exemplary dosages for each of the additional *E. coli* O antigens to be used in combination with the *E. coli* O25B antigen range from 10% to 100% of the dosage of *E. coli* O25B antigen, wherein each of the *E. coli* O antigens is covalently bound to an EPA carrier protein. In certain embodiments, an exemplary dosage for an *E. coli* O25B glycoconjugate is, e.g., 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 μg of O25B antigen per administration, and an exemplary dosage for another *E. coli* O glycoconjugate to be used in combination with the *E. coli* O25B glycoconjugate is, e.g., 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the dosage for the *E. coli* O25B glycoconjugate, wherein the dosage is calculated by the amount of the O antigen in the O glycoconjugates per administration.

[0121] In certain embodiments of the invention, a composition of the present invention is for use in a method where a subject in need thereof is administered with 0.5 ml of a composition according to the invention.

[0122] In certain embodiments, an exemplary dosage for per administration to a human subject corresponds to 0.5 ml of a composition containing a first concentration of about 8-48 μg/mL, e.g., about 8, 12, 16, 20, 24, 28, 32, 36, 40, 44 or 48 μg/mL, of *E. coli* O25B antigen covalently bound to an EPA carrier protein, and a concentration of 10% to 100% of the first concentration of one or more additional *E. coli* O antigens covalently bound to the EPA carrier protein.

[0123] In certain embodiments, an exemplary dosage for per administration to a human subject corresponds to 0.5 ml of a composition containing a concentration of about 16 μg/mL of *E. coli* O25B antigen covalently bound to an EPA carrier protein, about 8 μg/mL of *E. coli* O1A antigen covalently bound to an EPA carrier protein, about 8 μg/mL of *E. coli* O2 antigen covalently bound to an EPA carrier protein, and about 8 μg/mL of *E. coli* O6A antigen covalently bound to an EPA carrier protein.

[0124] In certain embodiments, an exemplary dosage for per administration to a human subject corresponds to 0.5 ml of a composition containing a concentration of about 32 μg/mL of *E. coli* O25B antigen covalently bound to an EPA carrier protein, about 16 μg/mL of *E. coli* O1A antigen covalently bound to an EPA carrier protein, about 16 μg/mL of *E. coli* O2 antigen covalently bound to an EPA carrier protein, and about 16 μg/mL of *E. coli* O6A antigen covalently bound to an EPA carrier protein.

[0125] In certain embodiments, *E. coli* O-antigen conjugates, preferably bioconjugates, described herein or a composition described herein is for use in a method where it is administered to a subject once as a single dose. In certain embodiments, *E. coli* O-antigen conjugates, preferably bioconjugates, described herein or a composition described herein is for use in a method where it is administered to a subject as a single dose followed by a second dose 3 to 6 weeks later. In accordance with these embodiments, booster inoculations can be administered to the subject at 6 to 24 month intervals following the second inoculation. In certain embodiments, the booster inoculations can utilize a different

E. coli O-antigen, bioconjugate, or composition. In some embodiments, the administration of the same *E. coli O*-antigen conjugate, or composition can be repeated and the administrations can be separated by at least 1 day, 2 days, 3 days, 5 days, 7 days, 10 days, 15 days, 30 days, 45 days, 2 months, 75 days, 3 months, or at least 6 months. In certain embodiments, an *E. coli* O-antigen conjugate described herein or a composition described herein is for use in a method where it is administered to a subject as a single dose once per year. In certain embodiments, an *E. coli* O-antigen conjugate described herein or a composition described herein is for use in a method where it is administered to a subject as a single dose once per n years, n being for instance about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 years.

[0126] In certain embodiments, an *E. coli* O-antigen conjugate described herein or a composition described herein is for use in a method where it is administered to a subject as 2, 3, 4, 5 or more doses 2 weeks, 3 weeks, 4 weeks, 5 weeks or 6 weeks apart. In some embodiments, 2, 3, 4, 5 or more doses of an *E. coli* O-antigen conjugate described herein or a composition described herein are administered to a subject 2, 3, 4, 5 or 6 weeks apart. In certain embodiments, the *E. coli* O-antigen conjugates, or composition administered is the same each time. In certain embodiments, the *E. coli* O-antigen conjugate, or composition administered is different each time.

Assays

[0127] The ability of the conjugates/compositions described herein to generate an immune response in a subject can be assessed using any approach known to those of skill in the art in view of the present disclosure.

Assay for Assessing Ability of Bioconjugates to Induce an Immune Response

[0128] In some embodiments, the ability of a bioconjugate to generate an immune response in a subject can be assessed by immunizing a subject (e.g., a mouse) or set of subjects with the bioconjugate and immunizing an additional subject (e.g., a mouse) or set of subjects with a control (e.g., a placebo). The subjects or set of subjects can subsequently be challenged with ExPEC and the ability of the ExPEC to cause disease (e.g., UTI) in the subjects or set of subjects can be determined. Those skilled in the art will recognize that if the subject or set of subjects immunized with the control suffer(s) from disease subsequent to challenge with the ExPEC but the subject or set of subjects immunized with a bioconjugate(s) or composition thereof described herein suffer less from or do not suffer from disease, then the bioconjugate is able to generate an immune response in a subject. The ability of a bioconjugate(s) or composition thereof described herein to induce antiserum that cross-reacts with an O-antigen from ExPEC can be tested by, e.g., an immunoassay, such as an ELISA.

In Vitro Bactericidal Assays

[0129] The ability of the conjugates/compositions described herein to generate an immune response in a subject can be assessed using a serum bactericidal assay (SBA) or opsonophagocytotic killing assay (OPK), which represents an established and accepted method that has been used to obtain approval of glycoconjugate-based vaccines. Such assays are well-known in the art and, briefly, comprise the steps of generating and isolating antibodies against a target of interest (e.g., an O-antigen, e.g., O25B, of *E. coli*) by administering to a subject (e.g., a mouse) a compound that elicits such antibodies. Subsequently, the bactericidal capacity of the antibodies can be assessed by, e.g., culturing the bacteria in question (e.g., *E. coli* of the relevant serotype) in the presence of said antibodies and complement and - depending on the assay - neutrophilic cells and assaying the ability of the antibodies to kill and/or neutralize the bacteria, e.g., using standard microbiological approaches.

HITS

[0130] Also disclosed herein is a pack or kit comprising one or more containers filled with one or more of the ingredients of the compositions described herein, such as one or more *E. coli* O antigens and/or conjugates of the *E. coli* O antigens covalently bound to an EPA carrier protein. Optionally associated with such container(s) can be a notice or instructions in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. The kits can be used in the above methods of treatment and immunization of subjects.

[0131] The following examples of the invention are to further illustrate the nature of the invention. It should be understood that the following examples do not limit the invention and that the scope of the invention is to be determined by the appended claims.

EXAMPLES

O-Antigen Bioconjugates

[0132] O1A-EPA, O2-EPA, O6A-EPA and O25B-EPA bioconjugates containing, respectively, *E. coli* O1A, O2, O6A and O25B covalently linked to the glycosylation sites of an EPA protein carrier can be produced, purified, and characterized as described in, e.g., Ihssen et al., 2010, Microbial Cell Factories 9, 61, and in WO 2006/119987, WO 2009/104074, and International Patent application No. PCT/ EP2015/053739 (published as WO 2015/124769). The bioconjugates are synthesized using recombinant *E. coli* cells, which express the polysaccharide-synthesizing enzymes of the different O-serotypes in the presence of oligosaccharyltransferase PglB, and a protein carrier (EPA). In this approach, the glycoconjugate vaccine can be expressed in the periplasm of *E. coli,* extracted and purified through a biochemical process illustrated in Figure 1 and Figure 2. Table 2 indicates host strains used for the production of conjugates according to an embodiment of the invention.

Table 2: Host strains for production of preclinical, toxicology study and clinical batches

Product	Strain	EPA expression plasmid	PglB expression plasmid
EPA-O1A	W3110 Δ*rfb::rfb(upec032)* Δ*waaL*	pGVXN1076	pGVXN970
EPA-O2	W3110 Δ*rfb::rfb(upec116)* Δ*waaL*	pGVXN1076	pGVXN971
EPA-O6A	W3110 Δ*rfb::rfb(CCUG11309)* Δ*waaL*	pGVXN659	pGVXN114
EPA-O25B	W3110 Δ*rfb::rfb(upec138)* Δ*waaL* Δ*gtrABS*	pGVXN1076	pGVXN970

[0133] For example, for O25B-EPA production, a strain with a genomically integrated O25B cluster was constructed: W3110 ΔwaaL ΔgtrABS Δ*rfb*O16::*rfb*(upec138), which was transformed with plasmids pGVXN1076 (which expresses the EPA having the amino acid sequence of SEQ ID NO:1) and pGVXN970 (which expressed the oligosaccharyl transferase PelB) (WO/2009/104074). This strain was constructed starting from strain W3110 by the methods of Datsenko and Wanner (2000, Proc Natl Acad Sci USA 97: 6640-6645) and a homologous recombination technique for site directed integration of large inserts into bacterial chromosomes (see WO 2014/057109). The *rfb* cluster related to the O25B antigen was cloned from *E. coli* strain upec138, which is positive for O25B. The recombinant host cells produced O25B/EPA bioconjugates in the periplasm. The resulting O25B bioconjugates were characterized using standard release and characterization assays. Bioconjugates were purified using two consecutive anionic exchange and size exclusion chromatography steps, yielding 98.1 % pure O25B bioconjugate preparations.

[0134] Similarly, host strains for recombinant production of O1A-EPA, O2-EPA, and O6A-EPA were constructed (Table 2). These strains include the *rfb* clusters related to O1A, O2 and O6A cloned from *E. coli* strain upec032, upec116 and CCUG11309, respectively. Bioconjugates of O1A-EPA, O2-EPA, and O6A-EPA were produced from these recombinant host cells, and purified using methods known in the art in view of the present disclosure.

[0135] SDS PAGE quantification was used for purity analysis. Sugar to protein ratios were calculated based on sugar quantification by the anthrone assay (see Laurentin and Edwards, 2003, Anal Biochem 315, 143-145) and the BCA assay for protein concentration. Analytical size exclusion chromatography showed a monomeric state of the particles in agreement with the expected hydrodynamic radius of EPA with attached glycan chains.

[0136] The bioconjugates and an un-glycosylated EPA reference standard were analyzed by size-exclusion chromatography with multi-angle light scattering (SEC-MALS), in order to quantify the degree of mono- and di-glycosylation of the individual bioconjugates, and to determine the molecular mass (MW) of the protein carrier and of the O-PS attached to it. The samples were separated on a TSKgel-G3000 SWxl column in phosphate buffer (pH 7.0; 50 mM NaCl, 150 mM sodium phosphate) and monitored by UV (214 and 280 nm), refractive index (RI) and multi angle light scattering (MALS).

Vaccine Compositions

[0137] This Example illustrates the vaccine compositions useful for the invention.

Table 3-1: Vaccine Compositions

Ingredient	**Amount (μg/mL)**	
Active substance	ExPEC4V	Composition 3
O-antigen polysaccharide		

(continued)

Ingredient	Amount (μg/mL)	
Active substance	ExPEC4V	Composition 3
E. coli O1A	8	8
E. coli O2	8	8
E. coli O6A	8	8
E. coli O25B	8	16
Carrier Protein		
EPA	109	Expected: 126
Excipients		
TBS buffer, containing	pH 7.4	
Tris	25 mM	
NaCl	137 mM	
KCl	2.7 mM	

Table 3-2: Vaccine Compositions

Ingredient	Amount (μg/mL)	
Active substance	Composition 1	Composition 2
O-antigen polysaccharide		
E. coli O1A	32	16
E. coli O2	32	16
E. coli O6A	32	16
E. coli O25B	32	32
Carrier Protein		
EPA	Expected: 436	Expected: 251
Excipients		
TBS buffer, containing	pH 7.4	
Tris	25 mM	
NaCl	137 mM	
KCl	2.7 mM	

[0138] Each of the above illustrated liquid vaccine compositions is packaged in a vial ready for injection. The vaccine products should be stored at +2°C to +8°C.

[0139] The active substances in the vaccine composition are glycosylated proteins (bioconjugates composed of the EPA protein carrier covalently linked to an *E. coli* O antigen polysaccharide) and the dose is calculated based on the content of the polysaccharide moiety (O antigen) only.

[0140] The dose of the EPA carrier protein depends on the polysaccharide-to-protein ratio. The estimated polysaccharide-to-protein ratio was between 15% and 50% depending on the O-antigen serotypes, i.e., the weight of polysaccharide in a conjugate is about 15% to 50% of the weight of the EPA protein carrier in the conjugate. For each serotype in ExPEC4V, the polysaccharide-to-protein ratio was quantified, e.g., the amount of polysaccharide (O-antigen) was measured by the anthrone assay (see Laurentin and Edwards, 2003, Anal Biochem 315, 143-145) and the bicinchoninic acid (BCA) assay was used to measure the protein concentration. For each serotype in Products 1-3, the value of EPA provided in Tables 3-1 and 3-2 is the expected value based on the analytical results from ExPEC4V.

[0141] Stability of the tetravalent vaccine compositions (O25B, O1A, O2 and O6 bioconjugates) was tested during over a 3 month period. The studies included accelerated and stress storage conditions to identify degradation pathways. These studies demonstrate that the active substances and the tetravalent vaccine compositions are stable for at least three months, and thus are suitable vaccine compositions with respect to stability.

Repeated Dose Toxicity Study in Rats

[0142] A good laboratory practice (GLP) toxicity study with a 14-day recovery period was conducted in Sprague-Dawley rats to assess the toxicity and local tolerance of a vaccine composition following 2 intramuscular (i.m.) injections (in quadriceps femoris muscle) on Days 1 and 14. Reversibility, persistence, and delayed occurrence of any changes were assessed after a 14-day recovery period (i.e., on Day 28). The design of the Phase 1-enabling repeated dose toxicity study in the rat is outlined in Table 4.

Table 4: Design of Repeated Dose Toxicity Study in the Rat

Group	Dose level	Concentration (μg/mL)	Dose volume (mL)[b]	Main Animals (n)[c]	Recovery Animals (n)[d]
Vehicle[a]	-	-	0.5	10 M + 10 F	5M+5F
ExPEC4V	4 μg polysaccharide per O-antigen = total of 16 μg polysaccharide per dose + 48 μg per protein carrier EPA dose	polysaccharide per O-antigen: 8 μg/mL + protein carrier EPA: 96 μg/mL	0.5	10M+ 10F	5M+5F

EPA = ExoProtein *A*/*Pseudomonas aeruginosa* exotoxin A, detoxified form used as protein carrier;
F = female,
M = male
Note: Day 1 = start of treatment
[a] The vehicle group was administered the formulation buffer (vehicle control)
[b] Animals received 2 injections of 0.25 mL per dosing occasion (left and right hind leg). Animals were dosed on 2 occasions: Day 1 and Day 14
[c] Main animals were euthanized on Day 17
[d] Recovery animals were euthanized on Day 28, ie 14 days after the second treatment

[0143] A dose of 4 μg per O-antigen polysaccharide (PS) of ExPEC4V (total PS dose of 16 μg) was tested in this study. This dose is equivalent to the maximum dose that was evaluated in the Phase 1 clinical study described below. Hence the full human dose as used in Phase 1 was administered in the rat GLP toxicology study. The study was performed with a nonclinical batch which was representative for the batch used in the Phase 1 clinical study.

[0144] No mortalities were observed during the study, nor any treatment related clinical signs (including body temperature) or ophthalmological observations. Furthermore there were no toxicologically relevant, adverse effects on body weight, body weight gain, food consumption, or hematology, clinical chemistry, coagulation, and urinalysis parameters. There were no test article-related macroscopic findings or differences in organ weights at the end of the treatment (Day 14) and the recovery period (Day 28).

[0145] No adverse test article-related microscopic findings were observed. Non-adverse minimal to mild microscopic findings (interstitial inflammation, degeneration/necrosis of myofiber and mixed inflammatory cell infiltrates) were noted at the injection sites in the quadriceps femoris muscle at the end of the treatment period in both the vehicle and treated group. These findings were therefore considered to be not related to administration of the test article, but a result of the dosing procedure (ie, i.m. injection). At the end of the recovery period, Day 1-injection site muscles had recovered, while at the Day 14-injection site, residual minimal mixed cellular inflammation/infiltrates were seen in the muscle, suggesting ongoing recovery in both vehicle and treated animals. Overall, the vaccine was well tolerated and no adverse treatment-related effects were noted.

[0146] Immunogenicity of the vaccine has been confirmed, inducing higher serum immunoglobulin G (IgG) titers towards the 4 O-antigens in the vaccinated group compared with vehicles that received only formulation buffer.

Repeated Dose Toxicity Study in Rabbits

[0147] A GLP repeated dose toxicity study with a 3-week recovery period was conducted in NZW rabbits (TOX11163, draft report) to assess the toxicity and local tolerance of ExPEC4V following 3 i.m. injections (Days 0, 14, and 28) given 2 weeks apart. Reversibility, persistence, and delayed occurrence of any changes were assessed on Day 49, after a 3-week treatment-free period following the 3rd injection on Day 28. The design of the Phase 2-enabling repeated dose toxicity study in the rabbit can be found in Table 5.

Table 5: Design of Repeated Dose Toxicity Study in the Rabbit

Group	Dose level[a] (μg/dose)	Concentration (μg/mL)	Volume injected per dosing	ADM[d]	Dosing days	Number of Animals	
						Terminal[e]	Recovery[e]
1	-	0	2x1 mL	ADM 1, 2 ADM 3, 4 ADM 5, 6	Day 0, Day 14, Day 28	5M + 5F	5M+5F
2	32[b]	32	1 mL	ADM 1 ADM 3 ADM 5	Day 0, Day 14, Day 28	5M + 5F	5M + 5F
3	64[c]	32	2x1 mL	ADM 1, 2 ADM 3, 4 ADM 5, 6	Day 0, Day 14, Day 28	5M + 5F	5 M+5 F

ADM: administration site; F = female; M = male
Note: Day 0 = start of treatment; Group 1 received saline (control group)
[a] Total O-antigen polysaccharide (1:1:1:1 ratio for O1A, O2, O6A and O25B serotypes, respectively)
[b] 8 μg polysaccharide per serotype + 109 μg total EPA carrier protein
[c] 16 μg polysaccharide per serotype + 218 μg total EPA carrier protein
[d] ADM1: left - lower part m. biceps femoris; ADM2: right - lower part m. biceps femoris; ADM3: left - upper part m. biceps femoris; ADM4: right - upper part m. biceps femoris; ADM5: left - m. quadriceps femoris; ADM6: right - m. quadriceps femoris
[e] Terminal animals were euthanized on Day 30 and recovery animals on Day 49

[0148] A maximum dose of 16 μg per O-antigen PS of ExPEC4V (total PS dose of 64 μg, together with 218 μg EPA carrier protein) was tested in this study. This dose is 4 times higher than the maximum dose that was tested previously in the Phase 1-enabling GLP toxicity study in the rat and is equivalent to the maximum PS (and EPA) dose that is evaluated in the Phase 2 clinical study described below. Hence the full (maximum) human dose as to be used in Phase 2 was administered in this rabbit GLP toxicology study.

[0149] The study was performed with the ExPEC4V (containing 32 μg/mL total PS) that was used in the Phase 1 clinical study described below. This batch is considered representative for the vaccine composition that is used in the Phase 2 clinical study described below (containing 128 μg/mL total PS), as the same drug substances are used for both vaccine compositions.

[0150] No mortalities were observed during the study. There were no effects on body temperature, body weight, body weight gain, food consumption, ophthalmology, skin evaluation (Draize scoring), clinical chemistry and C-reactive protein.

[0151] Females receiving 64 μg ExPEC4V exhibited non-adverse, minimally decreased hemoglobin levels at the end of the treatment and recovery period, with minimal decreases in total RBC count and hematocrit at the end of the recovery period. Fibrinogen was minimally increased 1 week after the 1st injection and at the end of the treatment period in females, but no changes were observed anymore at the end of the treatment-free (recovery) phase.

[0152] Shortly after dosing, dark discoloration of the subcutis was noted in some animals of the ExPEC4V-dosed groups, which correlated with the foci/areas of discoloration that were seen at the sites injected on Day 28 in all groups (including control group) at necropsy (Day 30). No abnormalities were noted at the end of the recovery period. Histopathologically multiple foci of mixed inflammatory cell infiltrates (minimal to slight) were seen mainly at the Day 28 injection sites in ExPEC4V-dosed animals. At the end of the recovery period (Day 49) only 1 female in the high dose group exhibited mixed inflammatory cell infiltrates at the injection sites of Day 28, indicating (ongoing) recovery.

[0153] Within the draining medial iliac lymph nodes, production (in germinal centers) and sequestration of lymphoblastic cells was seen within the paracortex and/or medullary cords of ExPEC4V -dosed rabbits at the end of the treatment period, resulting in increased overall cellularity. Furthermore lymph nodes were larger in both sexes which correlated with an increased weight in females. These findings were not seen at the end of the recovery period. An increased

number of germinal centers was noted in the spleen of treated males and females at the end of the treatment and recovery period, and was accompanied by an increase in spleen weight in both sexes at the end of the treatment period.

[0154] These findings are considered non-adverse and related to the immune response to the vaccine administration.

[0155] Immunogenicity of the vaccine was confirmed as serum IgG levels against all 4 O-antigen serotypes as well as EPA were elevated in males and females.

[0156] Overall, vaccination of rabbits (3 i.m. injections, 2 weeks apart) with ExPEC4V doses containing up to 64 μg total PS was safe and well tolerated. All treatment-related effects observed are considered to reflect a normal, non-adverse response induced by the vaccine administration.

Functionality of Antibody Responses Induced by Vaccines in Rats

[0157] To assess the functional activity of vaccine-induced antibody responses of O25B, O1A, O2 and O6A bioconjugates, sera from rats vaccinated with monovalent or tetravalent vaccines containing O25B, O1A, O2 and O6A EPA bioconjugates, each alone or in combination, were analyzed using opsonophagocytic killing (OPK) assays, which measure in vitro complement- and antibody-dependent phagocytosis and killing of bacteria, e.g., *E. coli.* The OPK assay measures the ability of serum to facilitate opsonophagocytosis and killing of different *E. coli* serotypes. In 96-well plates, defined dilutions of the sample sera were incubated, in each well, with bacteria from one of the four vaccine-specific *E. coli* serotypes, a defined amount of HL60 cells, and baby rabbit complement. After incubation, a proportion of the mixture was spotted onto tryptic soy agar (TSA) and the number of bacterial colonies was counted. The ability of the antibodies to bind the bacterial cells and activate deposition of the complement and mediate uptake and killing of the bacteria by HL60 cells was expressed as opsonic titer. The opsonic titer or opsonization index (OI) corresponds to the dilution of the sera killing 50% of the bacterial cells. Opsonic indices for pre- and post-immune sera are provided. At least a 4-fold increase of OI from pre- to post-immune is considered significant.

[0158] *E. coli* was pre-opsonized with dilutions of serum from vaccinated rats, incubated with complement and phagocytes (differentiated HL60 cells), and the colony forming units (CFUs) were determined. Subsequently, the maximum % killing and Opsonization Indices (OI: serum dilution killing of 50% of *E. coli*) were calculated. *E. coli* selected for OPK testing were OC 24453 (serotype O2), OC 24781 (serotype O6A) and OC 24176 (serotype O25B).

[0159] As shown by the results depicted in Figures 3A-3C, monovalent vaccines containing O2-EPA, O6A-EPA and O25B-EPA induced robust antibody responses in rats, and such antibody responses are functional in killing *E. coli* from these serotypes.

[0160] Table 6 shows the total OI titers for the O-antigens O2, O6A and O25B from rats immunized with the tetravalent vaccine with either 0.4 or 4 μg per O-antigen. The titers were determined in two separate experiments. The 0.4 μg dose induced significant OIs in all animals for the O2 and O6A serotypes. For O25B, 3/8 animals showed a significant increase in OI following immunization with the 0.4 μg dose. Compared to the 0.4 μg dose, the 4 μg dose induced lower OI increases for O2 in all animals. 3/8 animals showed OI increases when the sera from the 4 μg dose group were tested on O25B *E. coli.*

[0161] The data confirm that a tetravalent vaccine is able to elicit O-antigen-specific opsonic antibodies against O2, O6A and O25B in animals, demonstrating that the vaccine compositions described herein induce antibody responses against *E. coli* serotypes from which O-antigens are included in the vaccine, and that such antibody responses are functional in killing *E. coli* from these serotypes.

Table 6: OIs against *E. coli* O2, O6A and O25B. OIs for individual pre-vaccination and post 3 vaccination sera from two separate experiments are shown for all animals.

	Tetravalent-EPA Rat Serum Opsonization Indices (OI)											
	O2 *E. coli*				O6 *E. coli*				O25 *E. coli*			
	0.4 ug Dose		4 ug Dose		0.4 ug Dose		4 ug Dose		0.4 ug Dose		4 ug Dose	
Animal No.	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
1: Pre-vacc	6	7	5	0	17	6	6	16	2'404	2'082	0	0
Post vacc	**>16384**	**1'476**	**293**	**32**	**202**	**226**	**2'045**	**2'821**	**1'847**	**1'578**	**9**	**0**
2: Pre-vacc	21	11	11	20	11	90	0	0	0	0	0	0
Post vacc	**11'148**	**>16384**	**150**	**120**	**436**	**475**	**10'262**	**11'460**	**0**	**0**	**4**	**0**
3: Pre-vacc	6	6	0	0	0	5	0	0	0	0	0	0
Post vacc	**11'073**	**>16384**	**46**	**19**	**98**	**37**	**7'959**	**8'597**	**6**	**0**	**355**	**197**
4: Pre-vacc	5	5	5	6	23	17	0	0	0	0	0	0
Post vacc	**>16384**	**63**	**57**	**45**	**108**	**116**	**2'189**	**4'488**	**0**	**0**	**70**	**26**
5: Pre-vacc	7	0	0	4	30	8	8	7	0	0	0	0
Post vacc	**10'413**	**7'050**	**105**	**108**	**>16,384**	**12'672**	**3'107**	**7'564**	**0**	**0**	**105**	**69**
6: Pre-vacc	8	0	8	7	299	164	5	0	269	154	0	0
Post vacc	**89**	**34**	**24**	**17**	**1'725**	**1'475**	**540**	**896**	**0**	**0**	**0**	**0**
7: Pre-vacc	9	9	6	6	18	21	22	5	0	0	0	0
Post vacc	**>16384**	**>16384**	**109**	**92**	**1'249**	**1'863**	**160**	**143**	**1'130**	**630**	**9**	**8**
8: Pre-vacc	4	6	6	5	26	22	0	0	0	0	0	0
Post vacc	**5'058**	**4'201**	**39**	**25**	**6'590**	**3'826**	**288**	**656**	**3'336**	**1'986**	**0**	**0**
Pre-vacc Av	8	5	5	6	53	42	5	3	334	280	0	0
Post-vacc Av	**10'867**	**7'747**	**103**	**57**	**3'349**	**2'586**	**3'319**	**4'578**	**790**	**524**	**69**	**37**

Effects in Human - Phase I Study

[0162] ExPEC4V has been tested in a first-in-human Phase 1 study, which enrolled a total of 194 subjects. This Phase 1 study is a randomized, placebo-controlled, multicenter study. The study was conducted in a single-blind manner as the investigator and study staff knew the randomization group of the subjects while the subjects were required to be blinded to their randomization group at all times.

[0163] The objective of this first-in-human study was to evaluate the safety, immunogenicity, and efficacy of the candidate vaccine in healthy women aged ≥18 to ≤70 years with a history of recurrent urinary tract infection (rUTI). The primary objective of the study was the comparison of solicited and unsolicited adverse events (AEs) and serious adverse events (SAEs) between subjects who received ExPEC4V and subjects who received placebo. The main secondary objectives included immunogenicity parameters, the number of symptomatic UTI episodes caused by *E. coli* vaccine-serotypes, and the rate of occurrence and clinical symptoms of vaccine-serotype-specific *E. coli* UTI.

[0164] Eligible subjects were required to have at least 3 independent UTI episodes in the last 12 months or at least 2 independent UTI episodes in the previous 6 months; at least 1 of the independent UTI episodes had to be due to a culture-confirmed *E. coli* infection. A total of 194 subjects were enrolled and randomized to a single i.m. dose of 0.5 mL of ExPEC4V or placebo (see Table 7). The enrollment was done in a staggered approach to assess Day 14 safety data before proceeding to the next phase:

1. The first 8 subjects were randomized (3:1) to a dose of 1 μg of each PS, or placebo
2. The following 8 subjects were randomized (3: 1) to a dose of 4 μg of each PS, or placebo
3. The remaining 178 subjects were randomized (1:1) to a dose of 4 μg of each PS, or placebo.

Table 7: the demographic and baseline characteristics of the subjects enrolled in the Phase 1 study.

	Placebo N = 95	ExPEC4V		Total N = 194
		1 μg polysaccharide per serotype[a] N = 6	4 μg polysaccharide per serotype[a] N = 93	
Age (years)				
N	95	6	93	194
Mean (SD)	42.1 (15.9)	28.1 (9.6)	42.0 (17.6)	41.6 (16.7)
Median (range)	42.6 (18, 71)	23.1 (20, 43)	38.5 (19, 72)	39.6 (18, 72)
Race				
N	95	6	93	194
Caucasian	91 (96%)	6 (100%)	87 (94%)	184 (95%)
Other	4 (4%)	0	6 (6%)	10 (5%)
Weight (kg)				
N	95	6	93	194
Mean (SD)	63.1 (11.0)	59.7 (10.3)	63.8 (11.0)	63.3 (10.9)
Median (range)	61 (46.2, 95.0)	56.5 (50, 79)	63 (44, 105)	61 (44, 105)
Body Mass Index (kg/m^2)				
N	95	6	93	194
Mean (SD)	23.2 (4.1)	21.1 (4.3)	23.3 (3.8)	23.2 (3.9)
Median (range)	22 (17.8, 33.7)	19.9 (17.5, 29.4)	22.7 (17.6, 34.3)	22.2 (17.5, 34.3)
Menopausal Status				
N	95	6	93	194
Premenopausal	64 (67%)	6 (100%)	57 (61%)	127 (65%)
Child Bearing Potential				
N	64	6	57	127
Yes	55 (86%)	6 (100%)	54 (95%)	115 (91%)

[a]The ExPEC4V doses contain O-antigen polysaccharides of the 4 ExPEC serotypes O1A, O2, O6A, and O25B

[0165] At first visit, eligible subjects that have provided informed consent were screened and compliance for inclusion/exclusion criteria was confirmed. Blood was drawn and urine was collected. At visit 2 (day 1), each subject received one intramuscular injection of 0.5 ml of solution (ExPEC4V or placebo) in the deltoid muscle. The reduced dose of the candidate vaccine contained 1 μg of each polysaccharide (total 4 μg polysaccharide). The target dose of the candidate vaccine contained 4 μg of each polysaccharide (total 16 μg polysaccharide).

[0166] Safety was evaluated based on solicited local (pain, erythema, and swelling at the injection site) and systemic (fever, i.e., body temperature ≥38°C) AEs collected in a diary from Day 1 postvaccination until Day 7 and on AEs and SAEs collected until Day 270 (end of study visit). Immunogenicity was evaluated by qualified enzyme-linked immunosorbent assays (ELISA) and opsonophagocytic killing (OPK) assays using serum from blood samples taken prevaccination on Day 1 and postvaccination on Days 30 and 270.

[0167] Descriptive statistics (n, mean, standard deviation, median and ranges for continuous variables, frequencies and percentages for categorical variables) are provided by treatment group and/or visit, where applicable. All data are listed by subject, treatment group and, where applicable, visit. All subjects from Group B receiving placebo are combined to form the placebo treatment group.

Safety

[0168] To date, none of the following has been identified from the Phase 1 study (which is still on-going): adverse drug reactions, significant clinical laboratory abnormality, cardiovascular, pulmonary, central nerve system, renal, or other significant adverse effects, overdose. Occurrence of adverse events and severe adverse events were comparable between the placebo and vaccinated groups.

Immunogenicity - Total Antibody Titer

[0169] To assess the immunogenicity of the vaccine components, sera from women participating in the clinical study were obtained and analyzed by ELISA to quantify IgG against the four different O-antigens included in the tetravalent vaccine (*E. coli* O1, *E. coli* O2, *E. coli* O6, and *E. coli* O25B). Total Day 1 (prevaccination) and Day 30 serum IgG antibody titers were assessed by a qualified ELISA optimized for each serotype isolate using purified serotype-specific O-antigen as primary assay antigen. Total IgG antibody titers per serotype were calculated using a 4-parameter logistic curve fit to determine per sample half maximal effective concentration (EC_{50}) values.

1 ug polysaccharide per serotype in ExPEC4V (N = 6)

[0170] Six subjects received an ExPEC4V dose of 1 μg PS per serotype (4 μg total PS). Analysis of serotype-specific immune responses of these subjects by Day 30 showed the proportion of subjects with a ≥2-fold increase in total antibodies per serotype was 50% (serotype O1A), 83% (serotype O2), 50% (serotype O6A), and 67% (serotype O25B). The proportion of subjects with a ≥4-fold increase in antibody titers was lower, i.e., 17% (serotype O6A), 33% (serotype 1A), 33% (serotype 2) and 50% (serotype O25B).

[0171] Analysis of responses of the 1 μg PS per serotype dose group yielded similar magnitude increases across the 4 serotypes. For serotypes O1A, O2, O6A, and O25B comparing Day 30 to Day 1, median fold increases in antibody titers were 2.5, 3.7, 2.2, and 4.1, respectively. Individual subject fold increases ranged from 1 to 6 for serotypes O1A and O2, from 1 to 7 for serotype O6A, and from 1 to 11 for serotype O25B. Geometric mean titer (GMT) values on Day 30 were 4,053, 13,768, 1,236, and 227 for the respective 4 serotypes (Table 8), representing approximately a 2.2 - 3.5 fold increase over Day 1 GMT values.

Table 8: GMT and 95% Confidence Intervals in ELISA-Determined Total Antibody Titers from Day 1 (Prevaccination) to Day 30 - Phase 1 Study

		Placebo N = 95		ExPEC4V			
				1 μg polysaccharide per serotype[a] N = 6		4 μg polysaccharide per serotype[a] N = 93	
Antibody		**Day 1**	**Day 30**	**Day 1**	**Day 30**	**Day 1**	**Day 30**
O1A	GMT	1,895	1,887	1,720	4,053	1,807	9,460
	95% CI	1,515 - 2,369	1,517 - 2,347	633 - 4,672	2,160 - 7,605	1,489 - 2,191	7,511 - 11,916
O2	GMT	3,529	3,502	4,266	13,768	2,855	27,973
	95% CI	2,926 - 4,257	2,910 - 4,214	1,731 - 10,511	6,516 - 29,091	2,279 - 3,576	22,026 - 35,526
O6A	GMT	943	953	558	1,236	920	4,475

(continued)

		Placebo N = 95		ExPEC4V			
				1 μg polysaccharide per serotype[a] N = 6		4 μg polysaccharide per serotype[a] N = 93	
Antibody		**Day 1**	**Day 30**	**Day 1**	**Day 30**	**Day 1**	**Day 30**
	95% CI	777 - 1,145	789 - 1,151	292 - 1,067	799 - 1,911	743 - 1,138	3,608 - 5,549
O25B	GMT	285	282	64	227	261	2,164
	95% CI	211 - 384	209 - 381	16-254	53-976	188 - 363	1,676 - 2,794

4 μg polysaccharide per serotype ExPEC4V (N = 93)

[0172] In comparison to the dose containing 1 μg PS per serotype (4 μg total PS), administration of an ExPEC4V dose of 4 μg PS per serotype (16 μg total PS) yielded a more robust immune response. Analysis of these subjects by Day 30 showed the proportion of subjects with a ≥2-fold increase in total antibodies per serotype was 81% (serotype O1A), 92% (serotype O2), 80% (serotype O6A), and 82% (serotype O25B). The proportion of subjects with a ≥4-fold increase in antibody titers ranged from 57% (serotypes O1A and O6A) to 80% (serotype O2), which is lower than the proportion with a 2-fold increase, but notably higher than observed with the dose containing 1 μg PS per serotype. See also Figure 4, a robust immune response to each of O1A, O2, O6A, and O25B was observed, and that a significant increase in the ELISA titers between post (30 days after injection) and pre-injection (day 1) was observed only in the vaccinated groups (V_Day 30 v.s. V_Day 1), but not in the placebo groups (P_Day 30 v.s. P_Day 1).

[0173] Analysis of responses of the 4 μg PS per serotype group yielded median fold increases that were larger than those of the 1 μg PS per serotype dose group. For serotypes O1A, O2, O6A, and O25B comparing Day 30 to Day 1, EC50 median fold increases were 4.6, 9.4, 4.9, and 5.9, respectively. The magnitude and variability in the fold increases were greater with this dose group, ranging from 1 to 96 for serotype O1A, from 1 to 165 for serotype O2, from 0 to 61 for serotype O6A, and from 1 to 579 for serotype O25B. GMT values on Day 30 were 9,460, 27,973, 4,475, and 2,164 for the respective 4 serotypes (Table 8), representing approximately a 4.9 to 9.8 fold increase over Day 1 GMT values.

Conclusion

[0174] These interim results show a vaccine-specific immune response in healthy subjects administered the 1 μg PS per serotype ExPEC4V dose, and a comparatively greater increase in the immune response with the higher 4 μg PS per serotype ExPEC4V dose, over a 30-day observational period. The lack of relevant change in antibody titers of the 95 subjects in the placebo group over this period suggests the antibody response in ExPEC4V recipients is vaccine-mediated, and is not due to environmental exposure to ExPEC bacteria.

[0175] These data indicate an overall increase in the antibody titers associated with the higher 4 μg PS per serotype dose compared to the 1 μg PS per serotype dose. Although these results suggest greater variability associated with the titers of the higher dose group compared to the lower dose group, the small number of subjects in the 1 μg per subject dose limit the interpretation of any observed differences. Differences were also observed within each dose group in the relative IgG titers per serotype, with the titer for antibodies to the O25B antigen being the lowest (Table 8).

Functional Antibody Response

[0176] OPK assays were used to assess the functional antibody response of women participating in the clinical study. Sera were collected from study participants. *E. coli* was pre-opsonized with dilutions of serum from the vaccinated women, incubated with complement and phagocytes (differentiated HL60 cells), and the remaining colony forming units (CFUs) was determined. Subsequently, the maximum percent killing and Opsonization Indices (OI: serum dilution killing of 50% of *E. coli*) were calculated. *E. coli* selected for OPK testing were OC 24452 (serotype O1A), OC 24453 (serotype O2), OC 24454 (serotype O6A), and OC 24176 (serotype O25B).

[0177] Day 1 and Day 30 sera were assessed for functional antibodies (measured as the opsonization index [OI], or serum concentration yielding a 50% decrease in *E. coli* colony forming units) by an OPK assay, optimized using selected serotype O1A, O2, O6A, or O25B ExPEC strains, with human complement and HL60 phagocytic cells. Functional antibody titers for serotype O25B are included in the interim analysis based on preliminary assessments of titer accuracy and reproducibility. For all serotypes, functional antibody titers are determined from measurements of *E. coli* op-

sonophagocytic-mediated killing using the NICE program, developed by the U.S. National Institute of Standards and Technology, and the Opsititer3 program, developed and licensed from the University of Alabama. For the interim analysis, OPK titers were determined for the 194 subjects, including 95 subjects receiving placebo, 6 subjects receiving the ExPEC4V 1 μg PS (per serotype) vaccine and 93 subjects receiving the ExPEC4V 4 μg PS (per serotype) vaccine.

Placebo recipients (N = 95)

[0178] As observed with ELISA testing, placebo recipients (95 subjects) showed similar OPK responses for Day 1 and Day 30 sera vs ExPEC4V serotypes, with little or no observed change to most respective per-subject OPK titer values. These results indicate a stable functional antibody titer for most or all placebo subjects over this time period.

1 μg polysaccharide per serotype ExPEC4V (N = 6)

[0179] Six subjects received an ExPEC4V dose of 1 μg PS per serotype (4 μg total PS). Analysis of serotype-specific immune responses of these subjects by Day 30 showed the proportion of subjects with a ≥2-fold increase in total antibodies per serotype was 33% (serotype O1A), 67% (serotype O2), and 0% (serotypes O6A and O25B). For serotypes O1A and O2, the proportion of subjects with a ≥4-fold increase in antibody titers decreased to 17% and 50%, respectively.

[0180] For serotypes O1A, O2, O6A, and O25B comparing Day 30 to Day 1, median fold increases in antibody titers were 1.0, 4.8, 0.9, and 1.0, respectively. Individual subject fold increases ranged from 0.6 to 8.1 for serotype O1, from 0.3 to 9.5 for O2, from 0.8 to 1.3 for serotype O6A, and from 0.5 to 1.5 for serotype O25B. Geometric mean titer (GMT) values on Day 30 were 429, 1834, 1,136, and 51 for the respective 4 serotypes, representing approximately a 1.0 - 2.8 fold increase over Day 1 GMT values.

4 μg polysaccharide per serotype ExPEC4V (N = 93)

[0181] Administration of an ExPEC4V dose of 4 μg PS per serotype (16 μg total PS) yielded a functional immune response for all ExPEC 4V serotypes. Analysis of these subjects by Day 30 showed the proportion of subjects with a ≥2-fold increase in OI values per serotype was 63% (serotype O1A), 90% (serotype O2), 33% (serotype O6A), and 55% (serotype O25B). The proportion of subjects with a ≥4-fold increase in OI values ranged from 20% (serotype O6A) to 82% (serotype O2); as expected, these proportions were consistently lower than those observed for the ≥2-fold increase.

[0182] For serotypes O1A, O2, O6A, and O25B comparing Day 30 to Day 1, OI median fold OPK titer increases were 3.5, 14.7, 1.4, and 2.5, respectively. The magnitude of the per subject fold increases with this dose group ranged from 0.5 to >292 for serotypes O1A and O2, from 0.3 to 26.4 for serotype O6A, and from 0.1 to 272.8 for serotype O25B. As depicted in Figures 5A-5D, a robust functional immune response to each of O1A, O2, O6A, and O25B was observed, and a significant increase in the OI between post- and pre-injection was observed only in the vaccinated groups, not the placebo groups.

[0183] GMT values on Day 30 were 950.5, 4,132, 1,542, and 414.7 for the respective 4 serotypes (Table 9), representing approximately a 2- to 14-fold increase over Day 1 GMT values. These data indicate an overall increase in the Day 30 functional antibody titers associated with the 4 μg PS per serotype dose, across all ExPEC 4V serotypes.

Table 9: GMT and 95% Confidence Intervals in OPK-Determined Functional Antibody Titers from Day 1 (Prevaccination) to Day 30 - Phase 1 Study

		Opsonization Index[a]					
		Placebo N = 95		ExPEC4V			
				1 μg polysaccharide per serotype[b] N = 6		4 μg polysaccharide per serotype[b] N = 93	
Antibody		**Day 1**	**Day 30**	**Day 1**	**Day 30**	**Day 1**	**Day 30**
O1A	**GMT 95% CI**	156 126.4 - 192.5	161 131 - 197.8	288 94 - 881	429 203 - 910	192.5 158.9 - 233.3	950.5 692.8 - 1,304.1
O2	**GMT 95% CI**	341.9 275.2 - 424.8	341.6 273.8 - 426.3	652 214 - 1980	1834 425 - 7913	301.3 237.6 - 382.2	4,132 3,034.9 - 5,625.

(continued)

		Opsonization Index[a]					
		Placebo N = 95		ExPEC4V			
				1 μg polysaccharide per serotype[b] N = 6		4 μg polysaccharide per serotype[b] N = 93	
Antibody		Day 1	Day 30	Day 1	Day 30	Day 1	Day 30
O6A	GMT 95% CI	692.6 589.4 - 813.8	715.1 614.5 - 832.2	1147 794 - 1657	1136 749 - 1721	790.6 676.7 - 923.7	1,542 1,263.7 - 1,881.
O25B	GMT 95% CI	86.6 58.4 - 128.5	97.3 64.3 - 147.2	52 27 - 103	51 28 - 92	114 74.6 - 174.5	414.7 273.5 - 629

Conclusion

[0184] These interim results show a vaccine-specific functional immune response in healthy subjects administered the 4 μg PS per serotype ExPEC4V dose, over a 30-day observational period. The lack of significant change in antibody titers of the 95 subjects in the placebo group over this period (Figures 5A-5D and Table 9) is consistent with ELISA results and supports the conclusion that an antibody response to vaccination has been demonstrated across the 4 ExPEC serotypes.

[0185] Interim immunogenicity results from the Phase 1 study show for both total (ELISA) and functional (OPK) antibody titers a vaccine-specific immune response in healthy subjects administered the 1 μg PS per serotype ExPEC4V dose, and a comparatively greater increase in the immune response with the higher 4 μg PS per serotype ExPEC4V dose, over a 30-day observational period. Comparison with serotype-specific fold increases observed with ELISA testing, the OPK assay showed similar response levels for serotypes O1A and O2 for both assays, but somewhat lower levels of OPK % subject responses with serotypes O6A and O25B. The selective decrease in OPK response of some subjects for serotypes O6A and O25B is under investigation.

[0186] Notably, the GMT value in OPK-determined functional antibody titer for O25B antigen is lower than those for the other antigens (O1A, O2 and O6A). The OPK assay has been accepted as a better surrogate assay for immune protection induced by the PS conjugate vaccine against *Streptococcus pneumoniae* (Prevenar®), since the ELISA may not differentiate nonprotective low-avidity antibodies from protective high-avidity antibodies (Kim et al., Clin Diagn Lab Immunol. 2003 10(4):616-21)

Effects in Human - Phase II Study

[0187] Based on the interim results from the Phase I study, a Phase II study will be conducted. This randomized, double-blind, placebo-controlled multicenter study is planned to evaluate safety, tolerability, and immunogenicity of 5 different doses in men and women in stable health, stratified by age: ≥18 to <50 years old (N=275) and ≥50 years old (N=560).

[0188] Two vaccine compositions, i.e., Products 1 &2 provided in Table 3-2, having two different polysaccharide concentrations using the same active substances as those used in ExPEC4V will be used in the Phase II study. More specifically, the Composition 1 formulation contains 32, 32, 32, 32 μg/ml per O-antigen polysaccharide (PS) of the *E. coli* serotypes O1A, O2, O6A and O25B, respectively, without adjuvant. The Composition 2 formulation contains 16, 16, 16, 32 μg/ml per O-antigen PS of the *E. coli* serotypes O1A, O2, O6A and O25B, respectively, without adjuvant.

[0189] Different dosages and ratios of the active substances will be tested in the phase II study. More specifically, the enrolled subjects will be randomized and divided into six arms: (i-v) five different doses of candidate vaccine and (vi) placebo. Each subject will receive a single i.m. dose of Composition 1, Composition 2 or placebo. The target dose for the *E. coli O*-antigens O1A: O2: O6A: O25B per injection of Composition 1 or 2 is: 4:4:4:4 μg (*i.e.,* the same as the highest dose used in the phase I study above), 4:4:4:8, 8:8:8:8, 8:8:8:16 and 16:16:16:16 μg. The objective of the study is to assess the safety, immunogenicity, and efficacy of the 5 different doses of the tetravalent *E. coli* bioconjugate vaccine.

Sequences

[0190]

Description	SEQUENCE	SEQ ID NO.
Detoxified EPA protein comprising 4 optimized N-glycosylation sequences	GSGGGDQNATGSGGGKLAEEAFDLWNECAKA CVLDLKDGVRSSRMSVDPAIADTNGQGVLHYS MVLEGGNDALKLAIDNALSITSDGLTIRLEGGV EPNKPVRYSYTRQARGSWSLNWLVPIGHEKPS NIKVFIHELNAGNQLSHMSPIYTIEMGDELLAK LARDATFFVRAHESNEMQPTLAISHAGVSVVM AQAQPRREKRWSEWASGKVLCLLDPLDGVYN YLAQQRCNLDDTWEGKIYRVLAGNPAKHDLDI KDNNNSTPTVISHRLHFPEGGSLAALTAHQACH LPLEAFTRHRQPRGWEQLEQCGYPVQRLVALY LAARLSWNQVDQVIRNALASPGSGGDLGEAIR EQPEQARLALTLAAAESERFVRQGTGNDEAGA ASADVVSLTCPVAKDQNRTKGECAGPADSGD ALLERNYPTGAEFLGDGGDVSFSTRGTQNWTV ERLLQAHRQLEERGYVFVGYHGTFLEAAQSIV FGGVRARSQDLDAIWRGFYIAGDPALAYGYAQ DQEPDARGRIRNGALLRVYVPRWSLPGFYRTG LTLAAPEAAGEVERLIGHPLPLRLDAITGPEEEG GRVTILGWPLAERTVVIPSAIPTDPRNVGGDLD PSSIPDKEQAISALPDYASQPGKPPREDLKLGSG GGDQNAT	1
N-glycosylation consensus sequence	Asn-X-Ser(Thr), wherein X can be any amino acid except Pro	2
N-glycosylation consensus sequence	Asp(Glu)-X-Asn-Z-Ser(Thr), wherein X and Z are independently selected from any natural amino acid except Pro	3

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[0191]

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Claims

1. A composition comprising a first concentration of an *E. coli* O25B antigen polysaccharide, and a second concentration of each of an *E. coli* O1A antigen polysaccharide, an *E. coli* O2 antigen polysaccharide and an *E. coli* O6A antigen polysaccharide, wherein the ratio of the first concentration to the second concentration is 2: 1, each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA) carrier protein, and the first concentration is 10 to 36 μg/ml.

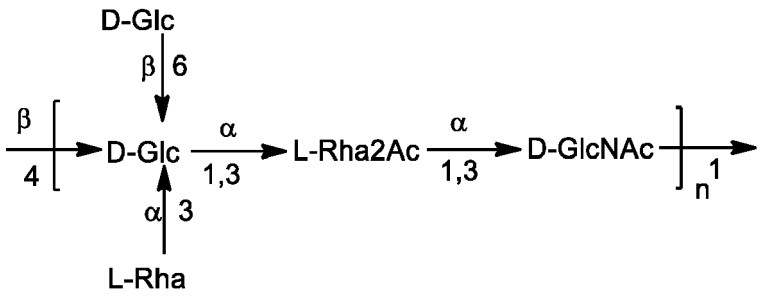
2. The composition of claim 1, comprising the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides at a weight ratio of 2:1:1:1, wherein the first concentration is 10, 16, 24, 32 or 36 μg/ml.

3. The composition of any one of claims 1 or 2, comprising 16 μg/ml of the O25B antigen polysaccharide.

4. The composition of any one of claims 1 or 2, comprising 32 μg/ml of the O25B antigen polysaccharide.

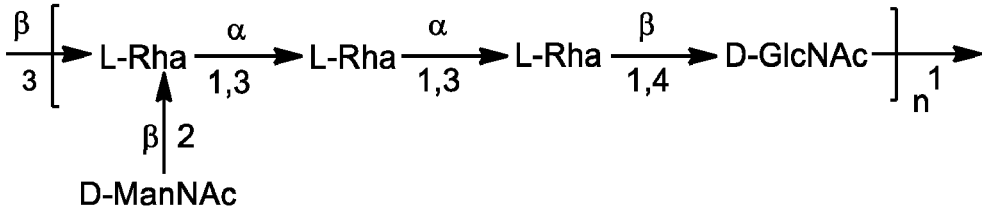
5. A multivalent immune composition comprising an *E. coli* O25B antigen polysaccharide at a first dose of 5 to 18 μg, and an *E. coli* O1A antigen polysaccharide, an *E. coli* O2 antigen polysaccharide and an *E. coli* O6A antigen polysaccharide each at a dose that is independently 50% of the first dose, wherein each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA) carrier protein.

6. A multivalent immune composition comprising an *E. coli* O25B antigen polysaccharide having the structure of Formula O25B':



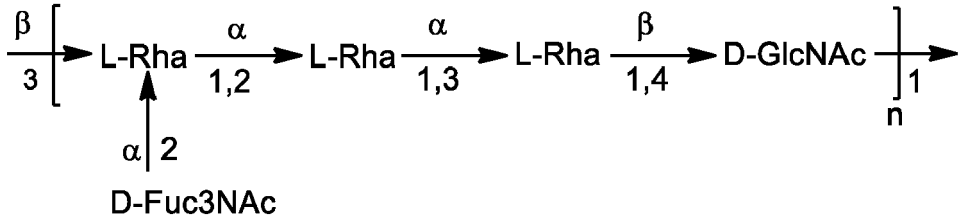
,

an *E. coli* O1A antigen polysaccharide having the structure of Formula O1A':



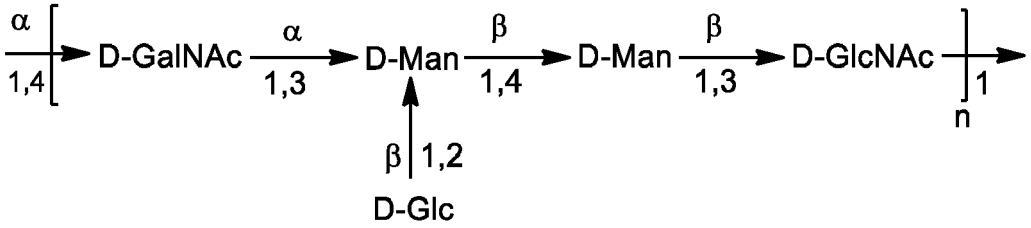
,

an *E. coli* O2 antigen polysaccharide having the structure of Formula O2':



,

and
an *E. coli* O6A antigen polysaccharide having the structure of Formula O6A':



,

wherein n is independently an integer of 5 to 25, and
each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a carrier protein having the amino acid sequence of SEQ ID NO:1; and the concentrations of the *E. coli* O25B, O1A, O2, O6A antigen polysaccharides in the compositions are respectively 16:8:8:8 μg/ml, or 32:16:16:16 μg/ml.

7. A composition of any one of claims 1 to 6 for use in a method of inducing an immune response to extra-intestinal pathogenic *E.coli* (ExPEC) in a subject in need thereof, the method comprising administering to the subject said composition.

8. An *E. coli* O25B antigen polysaccharide for use in a method of inducing an immune response to extra-intestinal pathogenic *E. coli* (ExPEC) in a subject in need thereof, comprising administering to the subject a first effective amount of an *E. coli* O25B antigen polysaccharide, and a second effective amount of each of an *E. coli* O1A antigen polysaccharide, an *E. coli O2* antigen polysaccharide and an *E. coli* O6A antigen polysaccharide, wherein the ratio of the first effective amount to the second effective amount is 2: 1, each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a detoxified exotoxin A of *Pseudomonas aeruginosa* (EPA) carrier protein, and the first effective amount is 5 to 18 μg per administration.

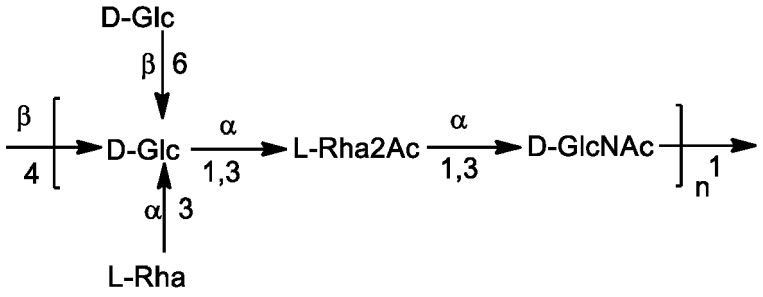
9. An *E. coli* O25B antigen polysaccharide for use in the method of claim 8, wherein the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are administered at a dosage ratio of 2:1:1:1, wherein the first effective amount is 6, 8, 10, 12, 14, 16, or 18 μg per administration.

10. An *E. coli* O25B antigen polysaccharide for use in the method of any one of claims 7to 9, wherein 8 μg of the *E. coli*_O25B antigen polysaccharide is administered per administration.

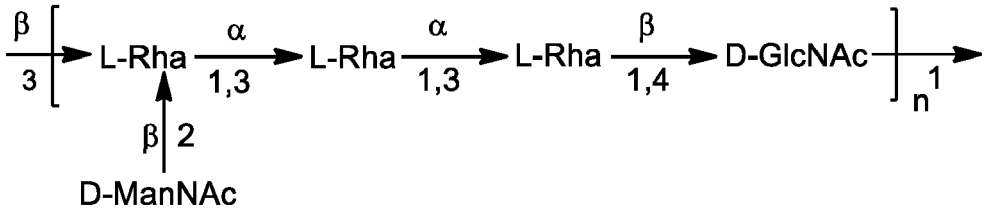
11. An *E. coli* O25B antigen polysaccharide for use in the method of any one of claims 7 to 9, wherein 16 μg of the *E. coli* O25B antigen polysaccharide is administered per administration.

12. An *E. coli* O25B antigen polysaccharide for use in the method of any one of claims 7 to 11, wherein the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are administered together in one composition.

13. An *E. coli* O25B antigen polysaccharide for use in a method of inducing an immune response to extra-intestinal pathogenic *E.coli* (ExPEC) in a subject in need thereof, the method comprising administering to the subject an *E. coli* O25B antigen polysaccharide having the structure of Formula O25B':

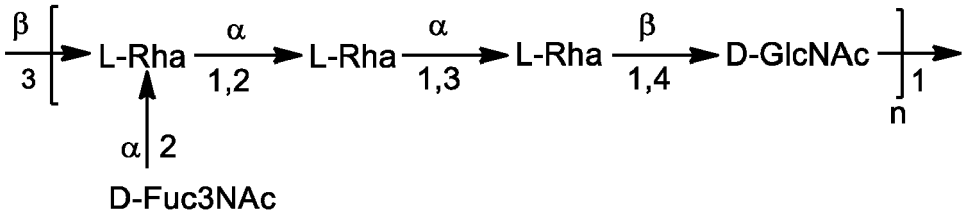


an *E. coli* O1A antigen polysaccharide having the structure of Formula O1A':



,

an *E. coli* O2 antigen polysaccharide having the structure of Formula O2':



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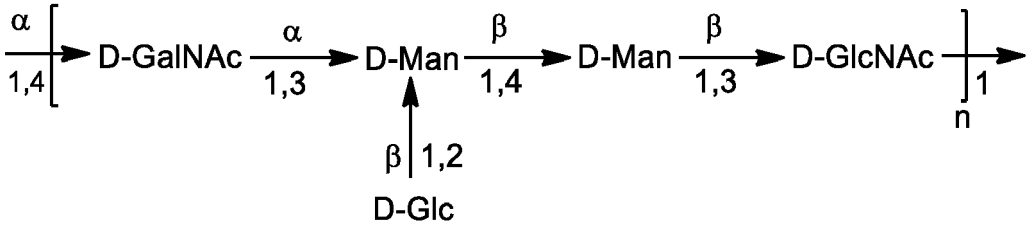
and an *E. coli* O6A antigen polysaccharide having the structure of Formula O6A':



,

wherein n is independently an integer of 5 to 25,
each of the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are independently covalently bound to a carrier protein having the amino acid sequence of SEQ ID NO: 1, and the *E. coli* O25B, O1A, O2 and O6A antigen polysaccharides are administered at 8:4:4:4 μg, or 16:8:8:8 μg per administration.

14. An *E. coli* O25B antigen polysaccharide for use in the method of any one of claims 7 to 13, wherein the immune response limits the severity of or prevents an invasive ExPEC disease caused by ExPEC serotypes O1A, O2 and

O6A and O25B in an at-risk human subject, for example wherein the adult human subject has or is at risk of having an invasive ExPEC disease selected from the group consisting of urinary tract infection, a surgical-site infection, an abdominal or pelvic infection, pneumonia, nosocomial pneumonia, osteomyelitis, cellulitis, sepsis, bacteremia, a wound infection, pyelonephritis, meningitis, neonatal meningitis, peritonitis, cholangitis, soft-tissue infections, pyomyositis and septic arthritis.

15. A process of making a composition of any one of claims 1 to 6, comprising combining the *E. coli* O25B antigen polysaccharide, the *E. coli* O1A antigen polysaccharide, the *E. coli O2* antigen polysaccharide and the *E. coli* O6A antigen polysaccharide to thereby obtain the composition.

Patentansprüche

1. Zusammensetzung, die Folgendes umfasst: eine erste Konzentration eines *E. coli* 025B-Antigen-Polysaccharids und eine zweite Konzentration von jeweils einem *E. coli* 01A-Antigen-Polysaccharid, einem *E. coli* 02-Antigen-Polysaccharid und einem *E. coli* 06A-Antigen-Polysaccharid, wobei das Verhältnis der ersten Konzentration zur zweiten Konzentration 2:1 beträgt, jedes der *E. coli* O25B-, O1A-, O2- und O6A-AntigenPolysaccharide unabhängig voneinander kovalent an ein entgiftetes Exotoxin A von *Pseudomonas aeruginosa* (EPA) Trägerprotein gebunden ist und die erste Konzentration 10 bis 36 μg/ml beträgt.

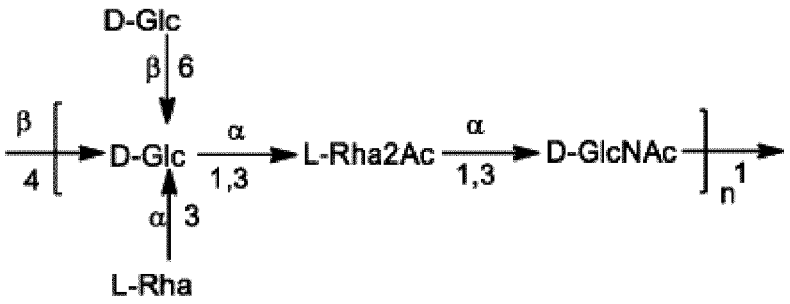
2. Zusammensetzung nach Anspruch 1, die die *E. coli*-Antigenpolysaccharide O25B, O1A, O2 und O6A in einem Gewichtsverhältnis von 2:1:1:1 umfasst, wobei die erste Konzentration 10, 16, 24, 32 oder 36 μg/ml beträgt.

3. Zusammensetzung nach einem der Ansprüche 1 oder 2, die 16 μg/ml des O25B-Antigenpolysaccharids umfasst.

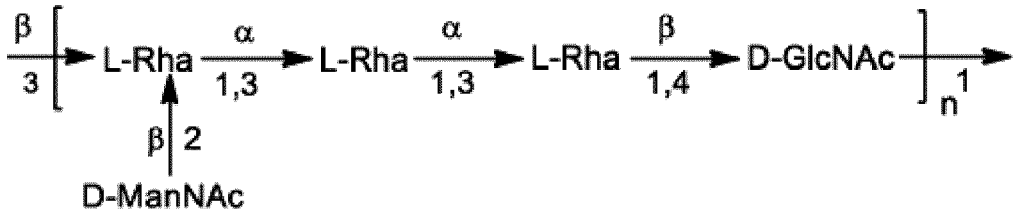
4. Zusammensetzung nach einem der Ansprüche 1 oder 2, die 32 μg/ml des O25B-Antigenpolysaccharids umfasst.

5. Multivalente Immunzusammensetzung, die Folgendes umfasst: ein *E. coli* O25B-Antigen-Polysaccharid in einer ersten Dosis von 5 bis 18 μg und ein *E. coli* O1A-Antigen-Polysaccharid, ein *E. coli* 02-Antigen-Polysaccharid und ein *E. coli* O6A-Antigen-Polysaccharid jeweils in einer Dosis, die unabhängig voneinander 50% der ersten Dosis beträgt, wobei jedes der *E. coli* O25B-, O1A-, O2- und O6A-Antigenpolysaccharide unabhängig voneinander kovalent an ein entgiftetes Exotoxin A von *Pseudomonas aeruginosa* (EPA) Trägerprotein gebunden ist.

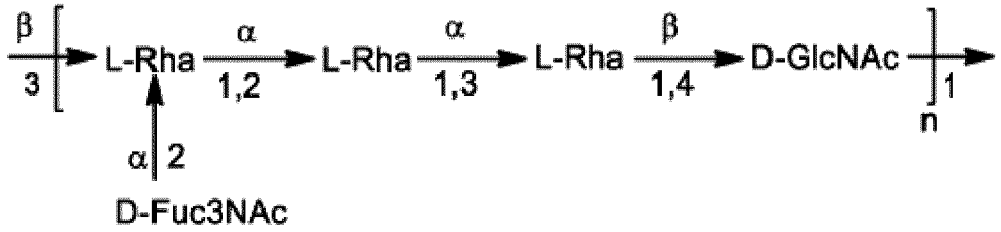
6. Multivalente Immunzusammensetzung, die Folgendes umfasst: ein *E. coli* O25B-Antigenpolysaccharid mit der Struktur der Formel O25B':



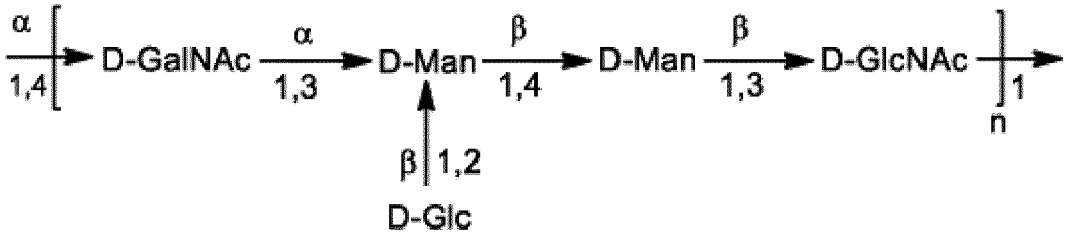
ein *E. coli* O1A-Antigen-Polysaccharid mit der Struktur der Formel O1A':



ein *E. coli* O2-Antigenpolysaccharid mit der Struktur der Formel O2':



und ein *E. coli* O6A-Antigenpolysaccharid mit der Struktur der Formel O6A':



,

wobei n unabhängig eine ganze Zahl von 5 bis 25 ist, und
jedes der *E. coli* O25B-, O1A-, O2- und O6A-Antigenpolysaccharide unabhängig voneinander kovalent an ein Trägerprotein mit der Aminosäuresequenz von SEQ ID NO: 1 gebunden ist; und die Konzentrationen der *E. coli* O25B-, O1A-, O2- und O6A-Antigenpolysaccharide in den Zusammensetzungen jeweils 16:8:8:8 μg/ml oder 32:16:16:16 μg/ml betragen.

7. Zusammensetzung nach einem der Ansprüche 1 bis 6 zur Verwendung in einem Verfahren zur Induktion einer Immunantwort auf extraintestinale pathogene *E.coli* (ExPEC) in einem Subjekt, das diese benötigt, wobei das Verfahren die Verabreichung der Zusammensetzung an das Subjekt umfasst.

8. *E. coli* 025B-Antigen-Polysaccharid zur Verwendung in einem Verfahren zur Induzierung einer Immunreaktion auf extraintestinale pathogene *E.coli* (ExPEC) bei einem bedürftigen Subjekt, das die Verabreichung von Folgendem an das Subjekt umfasst: einer ersten wirksamen Menge eines *E. coli* O25B-Antigen-Polysaccharids und einer zweiten wirksamen Menge von jeweils einem *E. coli* O1A-Antigen-Polysaccharid, einem *E. coli* 02-Antigen-Polysaccharid und einem *E. coli* 06A-Antigen-Polysaccharid, wobei das Verhältnis der ersten wirksamen Menge zur zweiten wirksamen Menge 2:1 beträgt, jedes der *E. coli* O25B-, O1A-, O2- und 06A-Antigenpolysaccharide unabhängig voneinander kovalent an ein entgiftetes Exotoxin A von *Pseudomonas aeruginosa* (EPA) Trägerprotein gebunden ist und die erste wirksame Menge 5 bis 18 μg pro Verabreichung beträgt.

9. *E. coli* 025B-Antigen-Polysaccharid zur Verwendung in dem Verfahren nach Anspruch 8, wobei die *E. coli* O25B-, O1A-, O2- und O6A-Antigen-Polysaccharide in einem Dosierungsverhältnis von 2:1:1:1 verabreicht werden, wobei die erste wirksame Menge 6, 8, 10, 12, 14, 16 oder 18 μg pro Verabreichung beträgt.

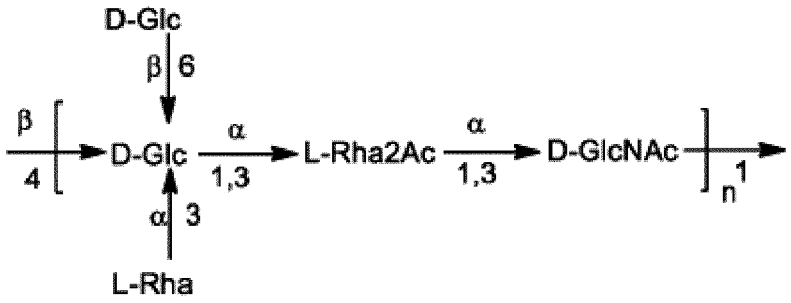
10. *E. coli* 025B-Antigen-Polysaccharid zur Verwendung in dem Verfahren nach einem der Ansprüche 7 bis 9, wobei 8 μg des *E. coli* O25B-Antigen-Polysaccharids pro Verabreichung verabreicht werden.

11. *E. coli* 025B-Antigen-Polysaccharid zur Verwendung in dem Verfahren nach einem der Ansprüche 7 bis 9, wobei 16 μg des *E. coli* O25B-Antigen-Polysaccharids pro Verabreichung verabreicht werden.

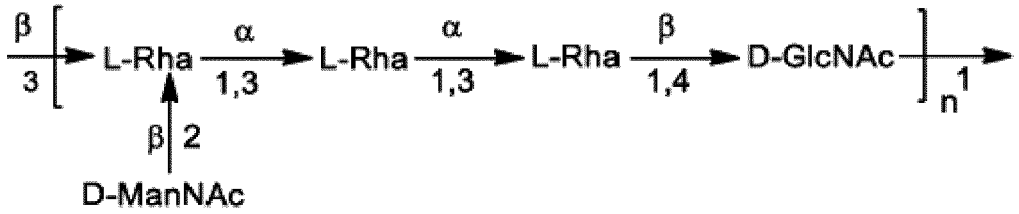
12. *E. coli* 025B-Antigenpolysaccharid zur Verwendung in dem Verfahren nach einem der Ansprüche 7 bis 11, wobei die *E. coli* O25B-, O1A-, O2- und O6A-Antigenpolysaccharide zusammen in einer Zusammensetzung verabreicht werden.

13. *E. coli* 025B-Antigen-Polysaccharid zur Verwendung in einem Verfahren zur Induktion einer Immunantwort auf extraintestinale pathogene *E. coli* (ExPEC) in einem Subjekt, das dessen bedarf, wobei das Verfahren die Verab-

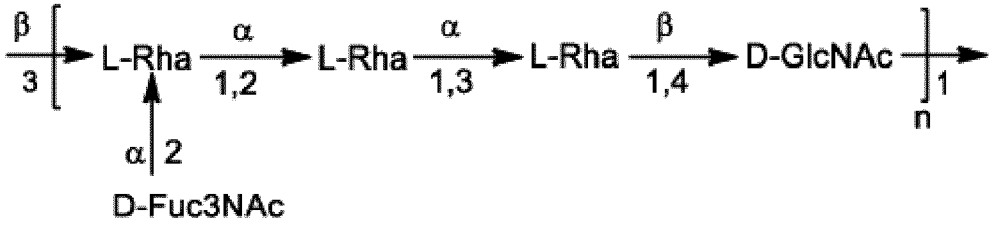
reichung von Folgendem an das Subjekt umfasst: eines *E. coli* 025B-Antigen-Polysaccharids mit der Struktur der Formel 025B':



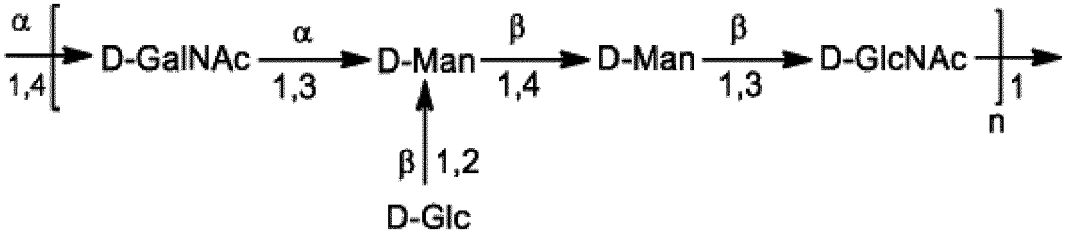
eines *E. coli* O1A-Antigen-Polysaccharids mit der Struktur der Formel O1A':



eines *E. coli* O2-Antigenpolysaccharids mit der Struktur der Formel O2':



und eines *E. coli* O6A-Antigenpolysaccharids mit der Struktur der Formel O6A':



wobei n unabhängig eine ganze Zahl von 5 bis 25 ist,
jedes der *E. coli* O25B-, O1A-, O2- und O6A-Antigenpolysaccharide unabhängig voneinander kovalent an ein Trägerprotein mit der Aminosäuresequenz von SEQ ID NO: 1 gebunden ist, und die *E. coli* O25B-, O1A-, O2- und O6A-Antigenpolysaccharide in einer Menge von 8:4:4:4 μg oder 16:8:8:8 μg pro Verabreichung verabreicht werden.

14. *E. coli* 025B-Antigenpolysaccharid zur Verwendung in dem Verfahren nach einem der Ansprüche 7 bis 13, wobei die Immunantwort den Schweregrad einer invasiven ExPEC-Erkrankung, die durch die ExPEC-Serotypen O1A, O2 und O6A und O25B verursacht wird, bei einem gefährdeten menschlichen Subjekt begrenzt oder verhindert, zum Beispiel, wobei das erwachsene menschliche Subjekt eine invasive ExPEC-Erkrankung hat oder gefährdet ist, eine invasive ExPEC-Erkrankung zu haben, ausgewählt aus der Gruppe bestehend aus Harnwegsinfektion, eine Infektion

an der Operationsstelle, eine Bauch- oder Beckeninfektion, Lungenentzündung, nosokomiale Lungenentzündung, Osteomyelitis, Zellulitis, Sepsis, Bakteriämie, eine Wundinfektion, Pyelonephritis, Meningitis, neonatale Meningitis, Peritonitis, Cholangitis, Weichteilinfektionen, Pyomyositis und septische Arthritis.

15. Verfahren zur Herstellung einer Zusammensetzung nach einem der Ansprüche 1 bis 6, das das Kombinieren des *E. coli* 025B-Antigen-Polysaccharids, des *E. coli* 01A-Antigen-Polysaccharids, des *E. coli* 02-Antigen-Polysaccharids und des *E. coli* 06A-Antigen-Polysaccharids umfasst, um dadurch die Zusammensetzung zu erhalten.

Revendications

1. Composition comprenant une première concentration d'un polysaccharide d'antigène O25B de *E. coli,* et une deuxième concentration de chacun d'un polysaccharide d'antigène 01A de *E. coli,* d'un polysaccharide d'antigène O2 de *E. coli* et d'un polysaccharide d'antigène O6A de *E. coli,* dans laquelle le rapport entre la première concentration et la deuxième concentration est de 2:1, chacun des polysaccharides d'antigènes O25B, O1A, O2 et O6A de *E. coli* est indépendamment lié de manière covalente à une protéine porteuse d'exotoxine A détoxifiée de *Pseudomonas aeruginosa* (EPA), et la première concentration est de 10 à 36 μg/ml.

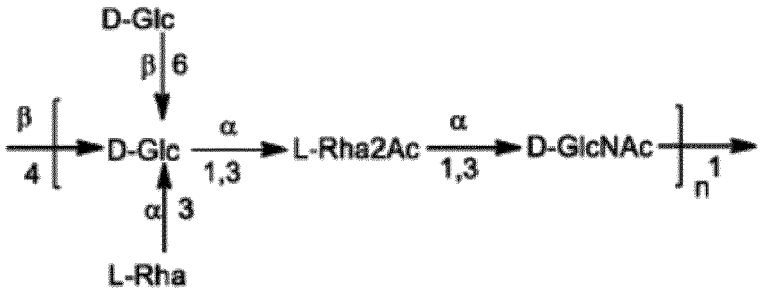
2. Composition selon la revendication 1, comprenant les polysaccharides d'antigènes O25B, O1A, O2 et O6A de *E. coli* à un rapport en poids de 2:1:1:1, dans laquelle la première concentration est de 10, 16, 24, 32 ou 36 μg/ml.

3. Composition selon l'une quelconque des revendications 1 ou 2, comprenant 16 μg/ml du polysaccharide d'antigène O25B.

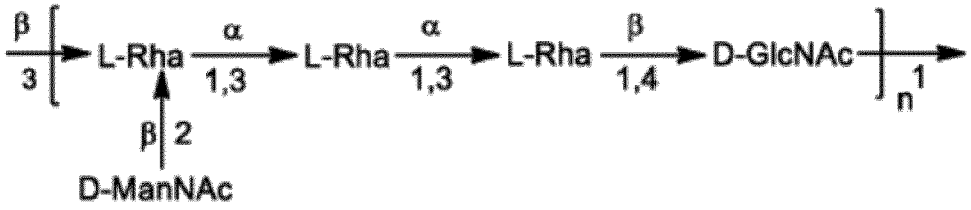
4. Composition selon l'une quelconque des revendications 1 ou 2, comprenant 32 μg/ml du polysaccharide d'antigène O25B.

5. Composition immunitaire multivalente comprenant un polysaccharide d'antigène O25B de *E. coli* à une première dose de 5 à 18 μg, et un polysaccharide d'antigène O1A de *E. coli,* un polysaccharide d'antigène O2 de *E. coli* et un polysaccharide d'antigène O6A de *E. coli* chacun à une dose qui est indépendamment de 50 % de la première dose, dans laquelle chacun des polysaccharides d'antigènes O25B, O1A, O2 et O6A de *E. coli* est indépendamment lié de manière covalente à une protéine porteuse d'exotoxine A détoxifiée de *Pseudomonas aeruginosa* (EPA).

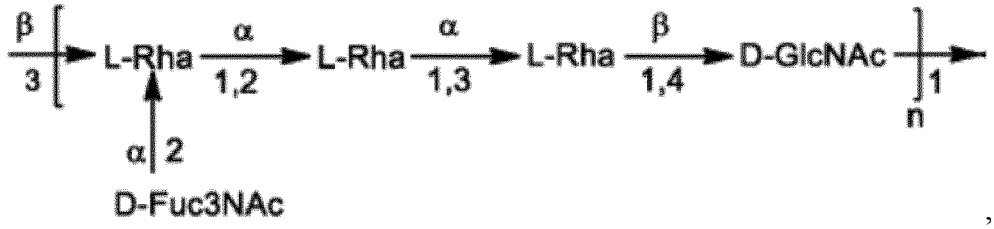
6. Composition immunitaire multivalente comprenant un polysaccharide d'antigène O25B de *E. coli* ayant la structure de formule O25B' :



un polysaccharide d'antigène O1A de *E. coli* ayant la structure de formule O1A' :



un polysaccharide d'antigène O2 de *E. coli* ayant la structure de formule O2' :



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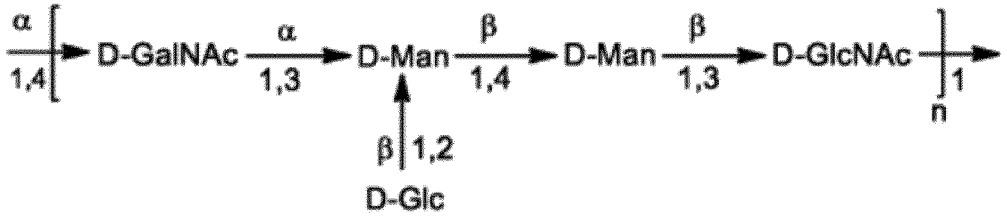
et un polysaccharide d'antigène O6A de *E. coli* ayant la structure de formule O6A' :



,

où n est indépendamment un entier de 5 à 25, et

chacun des polysaccharides d'antigènes O25B, O1A, O2 et O6A de *E. coli* est indépendamment lié de manière covalente à une protéine porteuse ayant la séquence d'acides aminés de SEQ ID N° : 1 ; et
les concentrations des polysaccharides d'antigènes O25B, O1A, O2, O6A de *E. coli* dans les compositions sont respectivement de 16:8:8:8 μg/ml, ou de 32:16:16:16 μg/ml.

7. Composition selon l'une quelconque des revendications 1 à 6 pour une utilisation dans un procédé d'induction d'une réponse immunitaire au pathogène *E. coli* extra-intestinal (ExPEC) chez un sujet qui en éprouve le besoin, le procédé comprenant l'administration au sujet de ladite composition.

8. Polysaccharide d'antigène O25B de *E. coli* pour une utilisation dans un procédé d'induction d'une réponse immunitaire au pathogène *E. coli* extra-intestinal (ExPEC) chez un sujet qui en éprouve le besoin, comprenant l'administration au sujet d'une première quantité efficace d'un polysaccharide d'antigène O25B de *E. coli,* et d'une deuxième quantité efficace de chacun d'un polysaccharide d'antigène O1A *de E. coli,* d'un polysaccharide d'antigène O2 de *E. coli* et d'un polysaccharide d'antigène O6A de *E. coli,* dans lequel le rapport entre la première quantité efficace et la deuxième quantité efficace est de 2:1, chacun des polysaccharides d'antigènes O25B, O1A, O2 et O6A de *E. coli* est indépendamment lié de manière covalente à une protéine porteuse d'exotoxine A détoxifiée de *Pseudomonas aeruginosa* (EPA), et la première quantité efficace est de 5 à 18 μg par administration.

9. Polysaccharide d'antigène O25B de *E. coli* pour une utilisation dans le procédé de la revendication 8, dans lequel les polysaccharides d'antigènes O25B, O1A, O2 et O6A de *E. coli* sont administrés à un rapport posologique de 2:1:1:1, dans lequel la première quantité efficace est de 6, 8, 10, 12, 14, 16, ou 18 μg par administration.

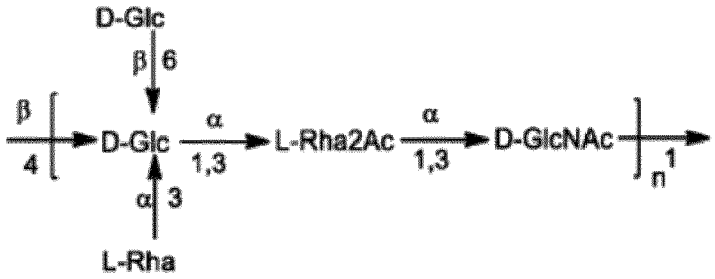
10. Polysaccharide d'antigène O25B de *E. coli* pour une utilisation dans le procédé de l'une quelconque des revendications 7 à 9, dans lequel il est administré 8 μg du polysaccharide d'antigène O25B de *E. coli* par administration.

11. Polysaccharide d'antigène O25B de *E. coli* pour une utilisation dans le procédé de l'une quelconque des revendications 7 à 9, dans lequel il est administré 16 μg du polysaccharide d'antigène O25B de *E. coli* par administration.

12. Polysaccharide d'antigène O25B de *E. coli* pour une utilisation dans le procédé de l'une quelconque des revendications 7 à 11, dans lequel les polysaccharides d'antigènes O25B, O1A, O2 et O6A de *E. coli* sont administrés ensemble dans une composition.

13. Polysaccharide d'antigène O25B de *E. coli* pour une utilisation dans un procédé d'induction d'une réponse immunitaire au pathogène *E. coli* extra-intestinal (ExPEC) chez un sujet qui en éprouve le besoin, le procédé comprenant

l'administration au sujet d'un polysaccharide d'antigène O25B de *E. coli* ayant la structure de formule O25B' :



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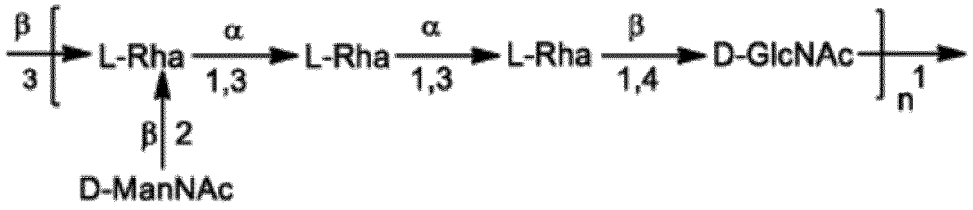
d'un polysaccharide d'antigène O1A de *E. coli* ayant la structure de formule O1A' :



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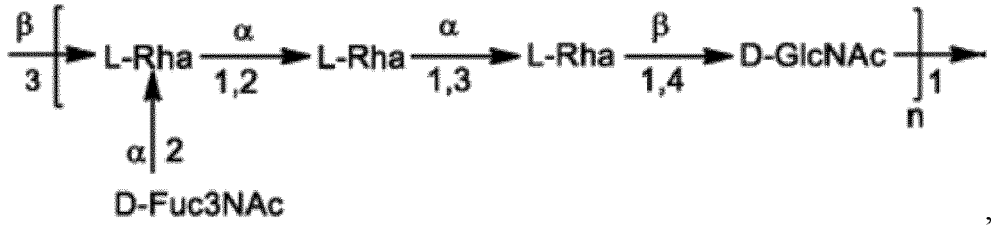
d'un polysaccharide d'antigène O2 de *E. coli* ayant la structure de formule O2' :



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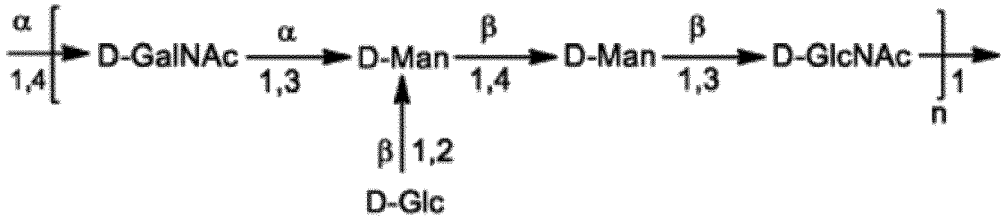
et d'un polysaccharide d'antigène O6A de *E. coli* ayant la structure de formule O6A' :



où n est indépendamment un entier de 5 à 25, et

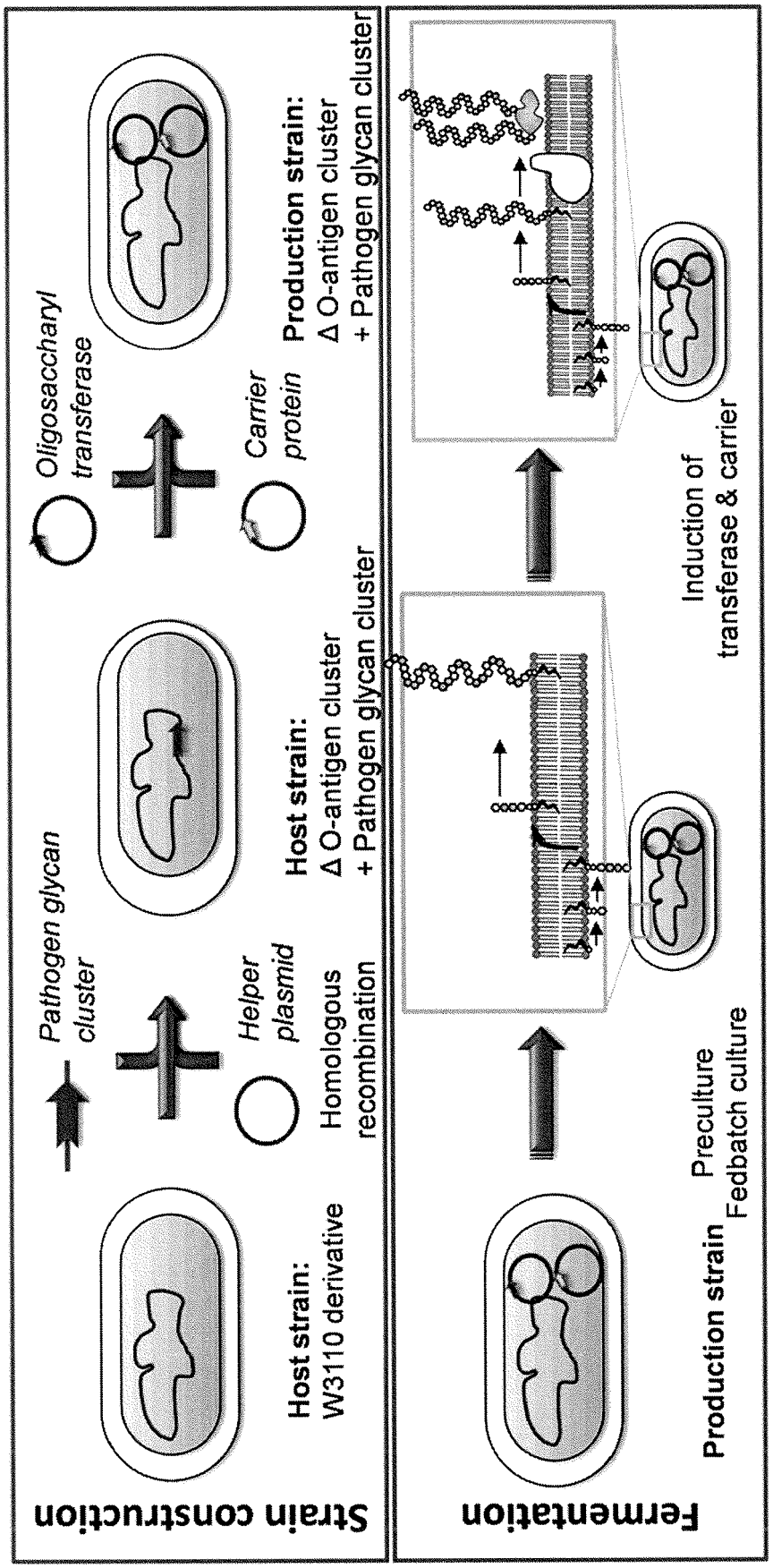
chacun des polysaccharides d'antigènes O25B, O1A, O2 et O6A de *E. coli* est indépendamment lié de manière covalente à une protéine porteuse ayant la séquence d'acides aminés de SEQ ID N° : 1, et
les polysaccharides d'antigènes O25B, O1A, O2, O6A de *E. coli* sont administrés à 8:4:4:4 μg, ou à 16:8:8:8 μg par administration.

14. Polysaccharide d'antigène O25B de *E. coli* pour une utilisation dans le procédé de l'une quelconque des revendications 7 à 13, dans lequel la réponse immunitaire limite la gravité d'une maladie ExPEC invasive causée par les sérotypes ExPEC O1A, O2 et O6A et O25B ou la prévient chez un sujet humain à risque, par exemple dans lequel le sujet humain adulte est atteint ou risque d'être atteint d'une maladie ExPEC invasive choisie dans le groupe constitué par une infection des voies urinaires, une infection de site chirurgical, une infection abdominale ou pel-

vienne, la pneumonie, la pneumonie nosocomiale, l'ostéomyélite, la cellulite, la septicémie, la bactériémie, une infection de plaie, la pyélonéphrite, la méningite, la méningite néonatale, la péritonite, la cholangite, une infection des tissus mous, la pyomyosite et l'arthrite septique.

15. Processus de production d'une composition de l'une quelconque des revendications 1 à 6, comprenant la combinaison du polysaccharide d' antigène O25B *de E. coli,* du polysaccharide d'antigène O1A de *E. coli,* du polysaccharide d'antigène O2 de *E. coli* et du polysaccharide d'antigène O6A de *E. coli* pour ainsi obtenir la composition.

Figure 1


Strain construction
Host strain:
W3110 derivative
Pathogen glycan cluster
Helper plasmid
Homologous recombination
Host strain:
Δ O-antigen cluster
+ Pathogen glycan cluster
Oligosaccharyl transferase
Carrier protein
Production strain:
Δ O-antigen cluster
+ Pathogen glycan cluster
Fermentation
Production strain
Preculture
Fedbatch culture
Induction of transferase & carrier

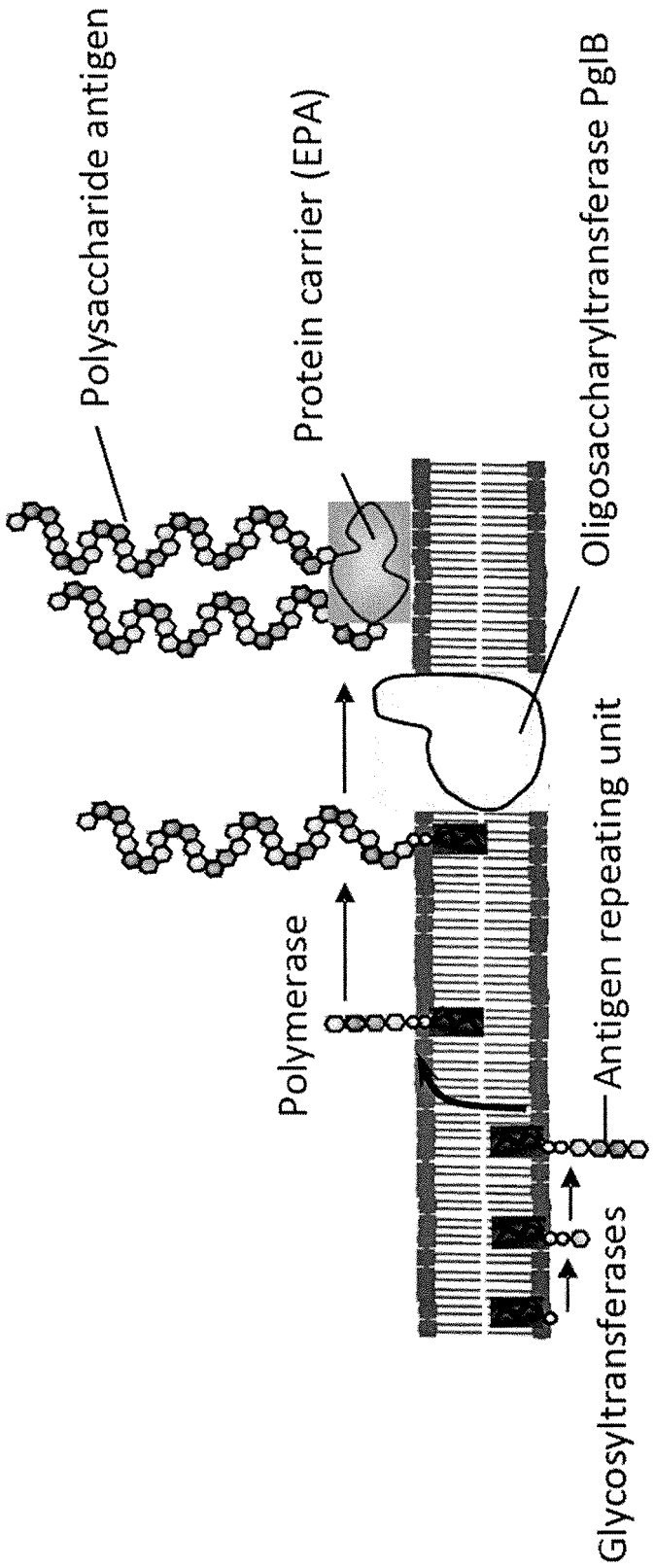

Polysaccharide antigen
Protein carrier (EPA)
Oligosaccharyltransferase PglB
Antigen repeating unit
Polymerase
Glycosyltransferases

Figure 2

Figure 3A

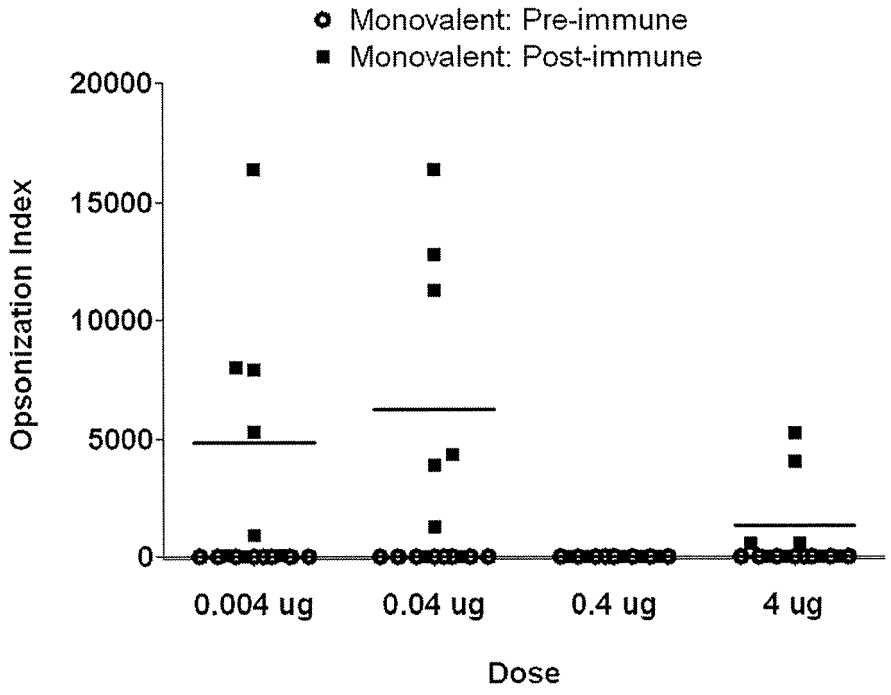


Figure 3B

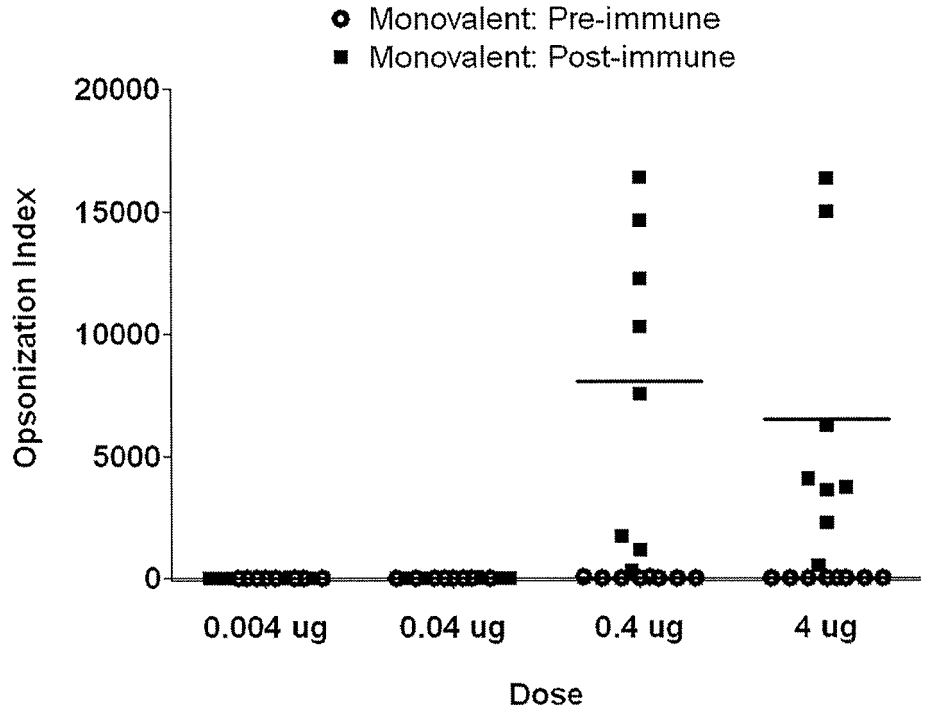


Figure 3C

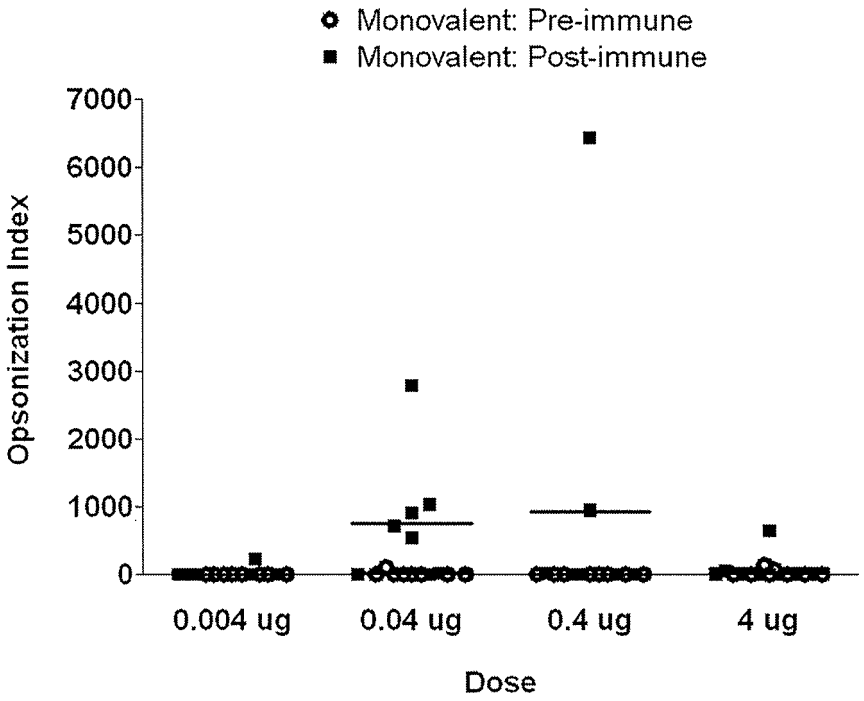
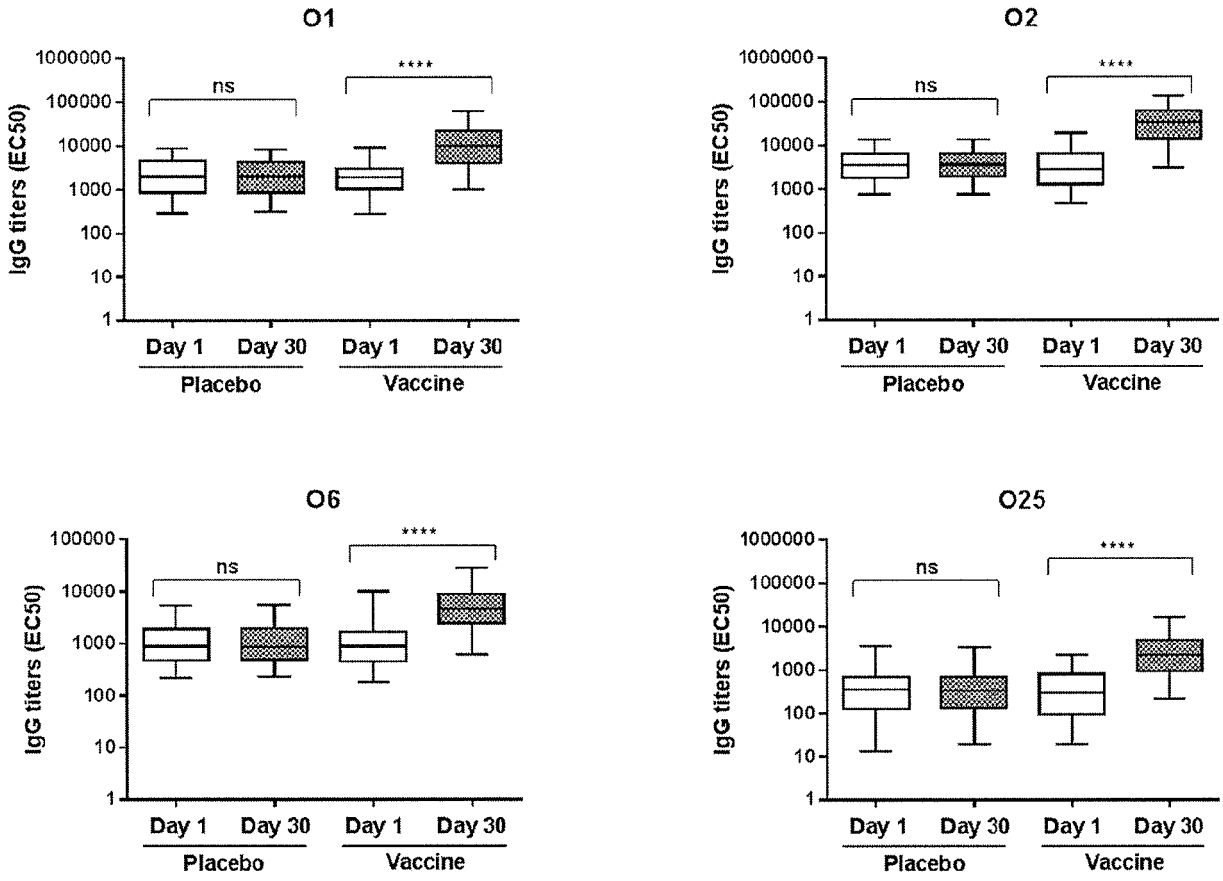
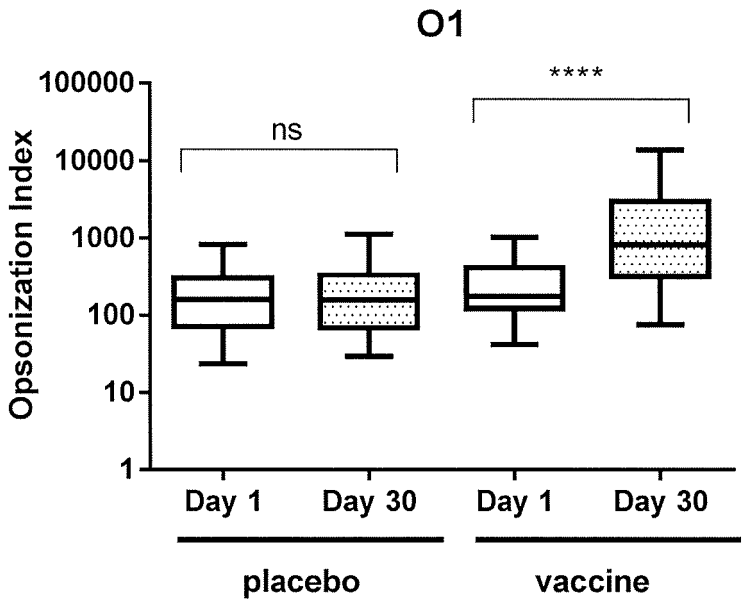

Monovalent: Pre-immune
Monovalent: Post-immune
Opsonization Index
7000
6000
5000
4000
3000
2000
1000
0
0.004 ug
0.04 ug
0.4 ug
4 ug
Dose

Figure 4



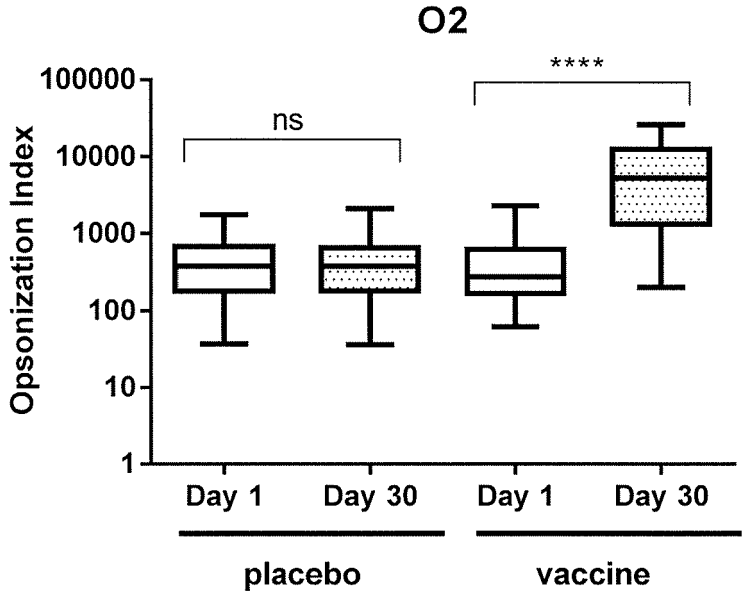
Paired t test; ns: not significant; **** P < 0.0001; Box plot median, 5% and 95% percentile

Figure 5A



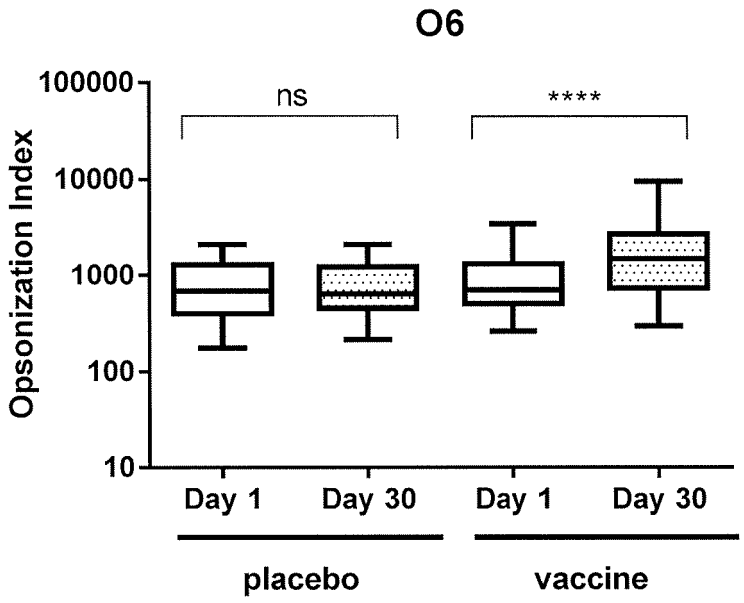
Paired t test of log-transformed values: ns, not significant; ****P<0.0001;
Box plot median, 5% and 95% percentile

Figure 5B



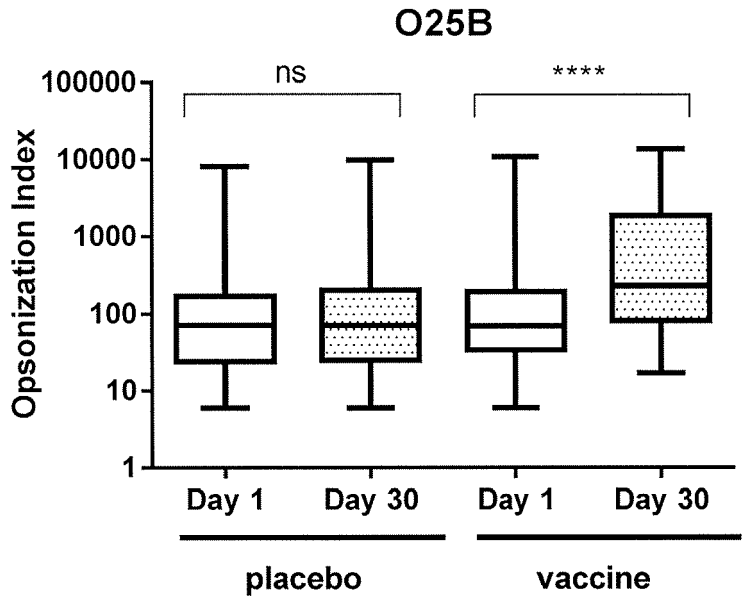
Paired t test of log-transformed values: ns, not significant; ****P<0.0001;
Box plot median, 5% and 95% percentile

Figure 5C



Paired t test of log-transformed values: ns, not significant; ****P<0.0001;
Box plot median, 5% and 95% percentile

Figure 5D



Paired t test of log-transformed values: ns, not significant; ****P<0.0001;
Box plot median, 5% and 95% percentile

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