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(54) Title: MULTIVALENT PNEUMOCOCCAL POLYSACCHARIDE-PROTEIN CONJUGATE COMPOSITION

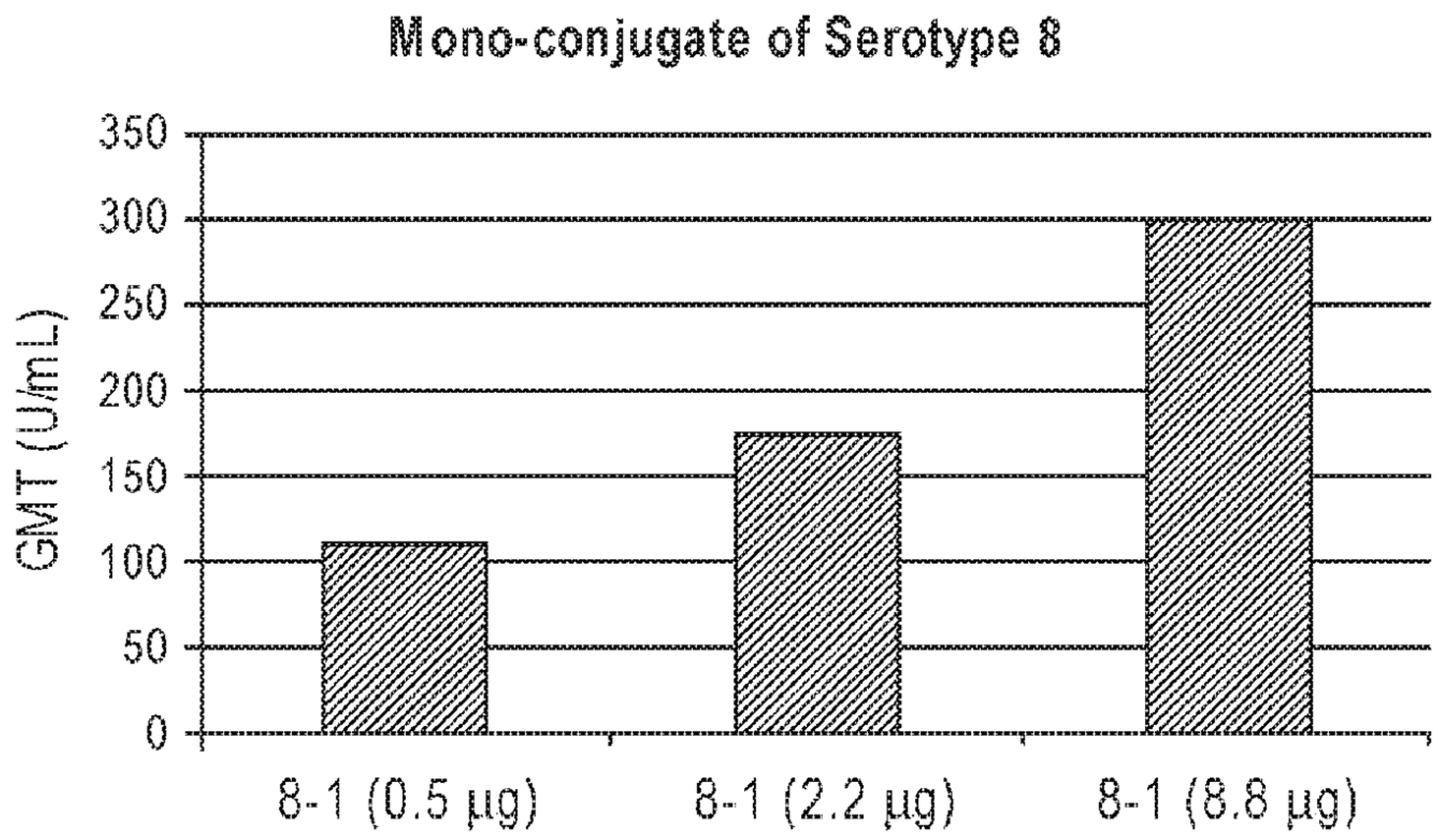

FIG. 1A

(57) Abrégé/Abstract:
Provided are mixed carrier, multivalent pneumococcal conjugate compositions comprising 20 different pneumococcal capsular polysaccharide-protein conjugates, wherein each of the conjugates includes a capsular polysaccharide from a different serotype of Streptococcus pneumoniae conjugated to either tetanus toxoid or CRM_{197}, wherein the Streptococcus pneumoniae serotypes are selected from 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F, wherein two of the capsular polysaccharides are conjugated to tetanus toxoid and the remaining capsular polysaccharides are conjugated to CRMig7, and wherein the two capsular polysaccharides that are conjugated to tetanus toxoid are selected from the group consisting of serotypes 1, 3, and 5. Also provided are methods of producing the mixed carrier, multivalent pneumococcal conjugate compositions and methods of using the same for prophylaxis against Streptococcus pneumoniae infection or disease in a subject.

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(54) Title: MULTIVALENT PNEUMOCOCCAL POLYSACCHARIDE-PROTEIN CONJUGATE COMPOSITION

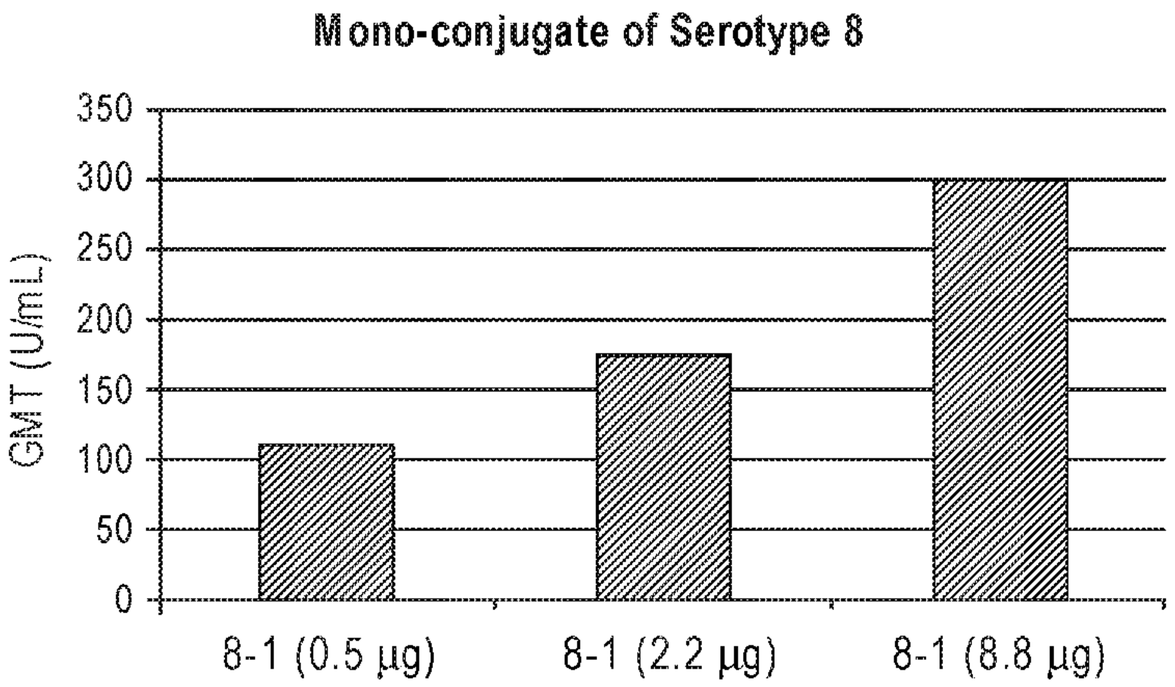

FIG. 1A

(57) Abstract: Provided are mixed carrier, multivalent pneumococcal conjugate compositions comprising 20 different pneumococcal capsular polysaccharide-protein conjugates, wherein each of the conjugates includes a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae* conjugated to either tetanus toxoid or CRM_{197}, wherein the *Streptococcus pneumoniae* serotypes are selected from 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F, wherein two of the capsular polysaccharides are conjugated to tetanus toxoid and the remaining capsular polysaccharides are conjugated to CRMig7, and wherein the two capsular polysaccharides that are conjugated to tetanus toxoid are selected from the group consisting of serotypes 1, 3, and 5. Also provided are methods of producing the mixed carrier, multivalent pneumococcal conjugate compositions and methods of using the same for prophylaxis against Streptococcus pneumoniae infection or disease in a subject.

[Continued on next page]

WO 2018/027126 A1

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MULTIVALENT PNEUMOCOCCAL POLYSACCHARIDE-PROTEIN CONJUGATE COMPOSITION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of, and relies on the filing date of, U.S. provisional patent application number 62/371,553, filed 5 August 2016 and U.S. provisional patent application number 62/525,945, filed 28 June 2017, the entire disclosures of which are herein incorporated by reference.

TECHNICAL FIELD

[0002] This application relates generally to mixed carrier, multivalent pneumococcal conjugate compositions, vaccines comprising the same and methods of using these compositions and vaccines for prophylaxis of *Streptococcus pneumoniae* infection or disease in a subject.

BACKGROUND

[0003] Pneumococcus (*Streptococcus pneumoniae*) is a Gram-positive, lancet-shaped, facultative anaerobic bacteria with over 90 known serotypes. Most *S. pneumoniae* serotypes have been shown to cause disease, with the 23 most common serotypes accounting for approximately 90% of invasive disease worldwide. Serotypes are classified based on the serological response of the capsular polysaccharides, the most important virulence factor for pneumococcus. Capsular polysaccharides are T-cell independent antigens that induce antibody production in the absence of T helper cells. T-cell independent antigens generally induce antibodies with low affinity and short-lived immune responses with little to no immunological memory.

[0004] Initial pneumococcal vaccines included combinations of capsular polysaccharides from different serotypes. These vaccines can confer immunity against *S. pneumoniae* in patients with developed or healthy immune systems, however, they were not effective in infants, who lack a developed immune system, and elderly subjects, who often have impaired immune function. To improve the immune response to pneumococcal vaccines, particularly in infants and elderly subjects, who are at higher risk to develop *S. pneumoniae* infection, capsular polysaccharides were conjugated to suitable carrier proteins to create pneumococcal

conjugate vaccines. Conjugation to a suitable carrier protein changes the capsular polysaccharide from a T-cell independent antigen to a T-cell dependent antigen. As such, the immune response against the conjugated capsular polysaccharide involves T helper cells, which help induce a more potent and rapid immune response upon re-exposure to the capsular polysaccharide.

[0005] There are at least two approaches to developing pneumococcal conjugate vaccines: the single carrier approach and the mixed carrier approach. The immunogenicity of different capsular polysaccharide conjugates may vary depending on the pneumococcal serotype and carrier protein used. In the single carrier approach, the capsular polysaccharides from different serotypes are conjugated to a single protein carrier. Pfizer's PREVNAR series of vaccines is an example of a single carrier approach where the different capsular polysaccharides are conjugated to the CRM_{197} protein carrier, a non-toxic variant of the diphtheria toxoid having a single amino acid substitution of glutamic acid for glycine. The 7-valent PREVNAR vaccine (PREVNAR) was first approved in 2000 and contains the capsular polysaccharides from the seven most prevalent serotypes: 4, 6B, 9V, 14, 18C, 19F and 23F. The 13-valent vaccine, PREVNAR 13, added the serotypes 1, 5, 7F, 3, 6A, and 19A to the CRM_{197} protein carrier. The protein carrier, CRM_{197}, used in the single carrier, PREVNAR vaccines has never been used as part of a mixed carrier system in a pneumococcal conjugate vaccine.

[0006] The second pneumococcal vaccine approach is the mixed carrier approach. In the mixed carrier approach, instead of using a single protein carrier, two or more protein carriers are used, with capsular polysaccharides from specific serotypes conjugated to a first protein carrier and capsular polysaccharides from different serotypes conjugated to at least a second, different protein carrier. For example, GlaxoSmithKline has developed SYNFLORIX, a 10-valent (serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F), mixed carrier, pneumococcal conjugate vaccine that uses *H influenzae* protein D, tetanus toxoid, and diphtheria toxoid as the protein carriers. In SYNFLORIX, serotypes 1, 4, 5, 6B, 7F, 9V, 14, and 23F are conjugated to protein D; serotype 18C is conjugated to tetanus toxoid; and serotype 19F is conjugated to diphtheria toxoid [7]. Serotype 3 was removed from the 11-valent precursor to SYNFLORIX because it did not show serotype-specific efficacy in an acute otitis media trial [1]. Another group, Aventis Pasteur, developed an 11-valent (serotypes 1, 3, 4,

5, 6B, 7F, 9V, 14, 18C, 19F, and 23F), mixed carrier, pneumococcal conjugate vaccine using diphtheria toxoid and tetanus toxoid as protein carriers [2, 3]. Capsular polysaccharides from serotypes 3, 9V, 14, and 18C can evoke a better response when conjugated to diphtheria toxoid than they do when conjugated to tetanus toxoid [2, 6]. Thus, serotypes 3, 6B, 14, and 18C were conjugated to diphtheria toxin and serotypes 1, 4, 5, 7F, 9V, 19F, and 23F were conjugated to tetanus toxoid. The development of this mixed carrier, pneumococcal vaccine was terminated due, in part, to technical reasons and the potential of a reduced response when combined with acellular pertussis vaccines [3]. Recently, serotype 5 as well as 1 was reported as having one of the lowest observed OPA titres from all PREVNAR 13 serotypes, in which there was an associated correlation between IgG titer and OPA activity [4]. Also it was suggested that for serotype 3, a much higher serum IgG concentration would be needed for protection [5].

SUMMARY

[0007] This application provides new and improved mixed carrier, multivalent pneumococcal conjugate compositions and vaccines comprising the same. In one aspect, this application provides a mixed carrier, multivalent pneumococcal conjugate composition, comprising 20 different pneumococcal capsular polysaccharide-protein conjugates, wherein each pneumococcal capsular polysaccharide-protein conjugate comprises a protein carrier conjugated to a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae*, wherein the *Streptococcus pneumoniae* serotypes are selected from 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F, wherein the protein carrier is CRM_{197} or tetanus toxoid, wherein two of the capsular polysaccharides are conjugated to tetanus toxoid and the remaining capsular polysaccharides are conjugated to CRM_{197}, and wherein the two capsular polysaccharides that are conjugated to tetanus toxoid are selected from the group consisting of serotypes 1, 3, and 5.

[0008] In one aspect, the mixed carrier, multivalent pneumococcal conjugate composition, comprises 20 different pneumococcal capsular polysaccharide-protein conjugates, wherein each pneumococcal capsular polysaccharide-protein conjugate comprises a protein carrier conjugated to a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae*, wherein the *Streptococcus pneumoniae* serotypes are selected from 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C,

19A, 19F, 22F, 23F, and 33F, wherein the protein carrier is CRM_{197} or tetanus toxoid, wherein two of the capsular polysaccharides are conjugated to tetanus toxoid and the remaining capsular polysaccharides are conjugated to CRM_{197}, and wherein the two capsular polysaccharides that are conjugated to tetanus toxoid are selected from the group consisting of serotypes 1, 3, and 5.

[0009] In some embodiments of the mixed carrier, 20-valent pneumococcal conjugate composition, the capsular polysaccharides from serotypes 1 and 5 are conjugated to tetanus toxoid, and the capsular polysaccharides from serotypes 3, 4, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}.

[0010] In another embodiment of the mixed carrier, 20-valent pneumococcal conjugate composition, the capsular polysaccharides from serotypes 1 and 3 are conjugated to tetanus toxoid, and the capsular polysaccharides from serotypes 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}.

[0011] In yet another embodiment of the mixed carrier, 20-valent pneumococcal conjugate composition, the capsular polysaccharides from serotypes 3 and 5 are conjugated to tetanus toxoid, and the capsular polysaccharides from serotypes 1, 4, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}.

[0012] In some embodiments, the mixed carrier, multivalent pneumococcal conjugate composition further comprises an adjuvant, such as an aluminum-based adjuvant, including, but not limited to aluminum phosphate, aluminum sulfate, and aluminum hydroxide.

[0013] Another aspect is directed to the use of the mixed carrier, 20-valent pneumococcal conjugate composition as a vaccine.

[0014] Yet another aspect is directed to a vaccine comprising the mixed carrier, 20-valent pneumococcal conjugate composition and a pharmaceutically acceptable excipient.

[0015] Yet another aspect is directed to a method for prophylaxis of *Streptococcus pneumoniae* infection or disease in a subject, such as a human, the method comprising administering a prophylactically effective amount of the mixed carrier, 20-valent pneumococcal conjugate compositions or a vaccine comprising the same to the subject.

[0016] In certain embodiments, the subject is a human who is at least 50 years old and the disease is pneumonia or invasive pneumococcal disease (IPD).

[0017] In other embodiments, the subject is a human who is at least 6 weeks old and the disease is pneumonia, invasive pneumococcal disease (IPD), or acute otitis media (AOM). In some embodiments, the human subject is 6 weeks to 5 years of age. In other embodiments, the human subject is 2 to 15 months of age or 6 to 17 years of age.

[0018] In certain embodiments, the mixed carrier, 20-valent pneumococcal conjugate composition or vaccine is administered by intramuscular injection. In certain embodiments, the mixed carrier, 20-valent pneumococcal conjugate composition or vaccine is administered as part of an immunization series.

[0019] The foregoing and other objects, features, and advantages of the mixed carrier, 20-valent pneumococcal conjugate compositions will become more apparent from the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] The Drawings included herein, which is comprised of the following Figures, is for illustration purposes only not for limitation.

[0021] Figures 1A-D show the *in vivo* dose-dependent antibody response of mono-conjugates of serotype 8 (Figure A), serotype 10A (Figure B), serotype 11A (Figure C), and serotype 15B (Figure D), as measured by the geometric mean titer (GMT).

DEFINITIONS

[0022] In order for the present disclosure to be more readily understood, certain terms are first defined below. Additional definitions for the following terms and other terms may be set forth through the specification.

[0023] As used in this specification and the appended claims, the singular forms “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise. Thus for example, a reference to “a method” includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0024] *Adjuvant:* As used herein, an "adjuvant" refers to a substance or vehicle that non-specifically enhances the immune response to an antigen.

[0025] *Administer:* As used herein, "administering" a composition to a subject means to give, apply or bring the composition into contact with the subject. Administration can be accomplished by any of a number of routes, such as, for example, topical, oral, subcutaneous, intramuscular, intraperitoneal, intravenous, intrathecal and intradermal.

[0026] *Approximately:* As used herein, the term "approximately" or "about," as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term "approximately" or "about" refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[0027] *Conjugate:* As used herein, and understood from the proper context, the terms "conjugate(s)" or "glycoconjugate(s)" refer to a *Streptococcus pneumoniae* polysaccharide conjugated to a carrier protein using any covalent or non-covalent bioconjugation strategy.

[0028] *Excipient*: As used herein, the term "excipient" refers to a non-therapeutic agent that may be included in a composition, for example to provide or contribute to a desired consistency or stabilizing effect.

[0029] *Mixed carrier*: As used herein, a mixed carrier, pneumococcal conjugate composition refers to a pneumococcal conjugate composition having more than one type of protein carrier.

[0030] *Multivalent:* As used herein, the term "multivalent" refers to a pneumococcal conjugate composition having pneumococcal capsular polysaccharides from more than one *Streptococcus pneumoniae* serotype.

[0031] *Mixed carrier, 16-valent pneumococcal conjugate composition*: As used herein, the term "mixed carrier, 16-valent pneumococcal conjugate composition(s)" refers to a composition comprising 16 different pneumococcal capsular polysaccharide-protein conjugates, wherein each pneumococcal capsular polysaccharide-protein conjugate comprises a protein carrier conjugated to a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae*, wherein the *Streptococcus pneumoniae* serotypes are 1, 3, 4, 5, 6A, 6B, 7F, 9V, 12F, 14, 18C, 19A, 19F, 22F, 23F, and 33F, wherein the protein carrier is CRM_{197} or tetanus toxoid, wherein two of the capsular

polysaccharides are conjugated to tetanus toxoid and the remaining capsular polysaccharides are conjugated to CRM_{197}, and wherein the two capsular polysaccharides that are conjugated to tetanus toxoid are selected from the group consisting of serotypes 1, 3, and 5. In some embodiments, the capsular polysaccharides from serotypes 1 and 5 are conjugated to tetanus toxoid, and the capsular polysaccharides from the remaining serotypes are conjugated to CRM_{197} (also referred to herein as PCV16-15TT). In another embodiment, the capsular polysaccharides from serotypes 1 and 3 are conjugated to tetanus toxoid, and the remaining capsular polysaccharides are conjugated to CRM_{197} (also referred to herein as PCV16-13TT). In yet another embodiment, the capsular polysaccharides from serotypes 3 and 5 are conjugated to tetanus toxoid, and the remaining capsular polysaccharides are conjugated to CRM_{197} (also referred to herein as PCV16-35TT).

[0032] *Mixed carrier, 20-valent pneumococcal conjugate composition*: As used herein, the term "mixed carrier, 20-valent pneumococcal conjugate composition(s)" refers to a composition comprising 20 different pneumococcal capsular polysaccharide-protein conjugates, wherein each pneumococcal capsular polysaccharide-protein conjugate comprises a protein carrier conjugated to a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae*, wherein the *Streptococcus pneumoniae* serotypes are 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F, wherein the protein carrier is CRM_{197} or tetanus toxoid, wherein two of the capsular polysaccharides are conjugated to tetanus toxoid and the remaining capsular polysaccharides are conjugated to CRM_{197}, and wherein the two capsular polysaccharides that are conjugated to tetanus toxoid are selected from the group consisting of serotypes 1, 3, and 5. In some embodiments, the capsular polysaccharides from serotypes 1 and 5 are conjugated to tetanus toxoid, and the capsular polysaccharides from the remaining serotypes are conjugated to CRM_{197}. In another embodiment, the capsular polysaccharides from serotypes 1 and 3 are conjugated to tetanus toxoid, and the remaining capsular polysaccharides are conjugated to CRM_{197}. In yet another embodiment, the capsular polysaccharides from serotypes 3 and 5 are conjugated to tetanus toxoid, and the remaining capsular polysaccharides are conjugated to CRM_{197}.

[0033] *Pharmaceutically acceptable excipient*: The pharmaceutically acceptable excipients useful in this disclosure are conventional. Remington's Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975),

describes compositions and formulations suitable for pharmaceutical delivery of one or more therapeutic compositions, including vaccines, and additional pharmaceutical agents. Suitable pharmaceutical excipients include, for example, 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. In general, the nature of the excipient will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, buffers, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid excipients can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, a surface active agent, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

[0034] *Prophylactically effective amount:* As defined herein, the term "a prophylactically effective amount" or "a prophylactically effective dose" refers to the amount or dose required to induce an immune response sufficient to delay onset and/or reduce in frequency and/or severity one or more symptoms caused by an infection with *Streptococcus pneumoniae*.

[0035] *Prophylaxis:* The term "prophylaxis," as used herein, refers to avoidance of disease manifestation, a delay of onset, and/or reduction in frequency and/or severity of one or more symptoms of a particular disease, disorder or condition (e.g., infection with *Streptococcus pneumoniae*). In some embodiments, prophylaxis is assessed on a population basis such that an agent is considered to provide prophylaxis against a particular disease, disorder or condition if a statistically significant decrease in the development, frequency, and/or intensity of one or more symptoms of the disease, disorder or condition is observed in a population susceptible to the disease, disorder, or condition.

[0036] *Subject*: As used herein, the term "subject" means any mammal, including mice, rabbits, and humans. In certain embodiments the subject is an adult, an adolescent

or an infant. In some embodiments, terms “individual” or “patient” are used and are intended to be interchangeable with “subject.”

DETAILED DESCRIPTION

[0037] The following description of the disclosed embodiment(s) and Examples is merely exemplary in nature and is in no way intended to limit the invention, its application, or uses.

[0038] This application provides new and improved mixed carrier, multivalent pneumococcal conjugate compositions and vaccines comprising the same. While the protein carrier, CRM_{197}, has previously been used in single carrier, pneumococcal conjugate vaccines, this application describes for the first time the use of CRM_{197} in a mixed carrier, pneumococcal conjugate vaccine.

[0039] As discussed above, the immunogenicity of different capsular polysaccharide conjugates may vary depending on the pneumococcal serotype and carrier protein used. This application describes the successful conjugation of serotype 3 to tetanus toxoid as part of a mixed carrier vaccine, notwithstanding previous teachings that serotype 3 was more immunogenic when conjugated to diphtheria toxoid rather than tetanus toxoid [2, 6]. It also discloses the unexpected finding that the antibody response to serotype 3 conjugated to tetanus toxoid in a mixed carrier, multivalent pneumococcal conjugate composition was about 4-fold and about 7-fold higher in PCV16 and PCV20 compositions, respectively, than when serotype 3 was conjugated to CRM_{197} in a single carrier, 13-valent pneumococcal conjugate composition (PREVNAR 13).

[0040] Further, the unexpected finding was not limited to serotype 3 but was also observed for the other serotypes conjugated to tetanus toxoid in a mixed carrier, multivalent pneumococcal conjugate composition. As shown in the Examples, conjugation of serotypes 1 and 3, 1 and 5, or 3 and 5 to tetanus toxoid in a mixed carrier, 16-valent or 20-valent pneumococcal conjugate composition with the remaining serotypes conjugated to CRM_{197} (e.g., PCV16-13TT, PCV16-15TT, and PCV16-35TT or PCV20-35TT, PCV20-13TT, and PCV20-15TT) consistently induced significantly enhanced antibody responses to the serotypes conjugated to tetanus toxoid as compared to the antibody responses (IgG response or MOPA titers) against the same serotypes conjugated to CRM_{197} in a single carrier, pneumococcal conjugate

composition (PREVNAR 13), validating this specific mixed carrier approach (tetanus toxoid and CRM_{197}) approach. The antibody response of the mixed carrier, multivalent pneumococcal conjugate compositions is summarized in the table below.

[0041] Table 1: Fold Increase in Antibody Response to Serotypes Conjugated to Tetanus Toxoid in Mixed Carrier Vaccine Compared to PREVNAR 13

	Fold Increase in Antibody Response Compared to PREVNAR 13					
Serotype	PCV16-13TT	PCV16-15TT	PCV16-35TT	PCV20-13TT	PCV20-15TT	PCV20-35TT
1 (IgG)	3X	3.9X	N/A	4.9X	1.7X	N/A
1 (MOPA)	6.3X	6.3X	N/A	19X	8.9X	N/A
3 (IgG)	5.5X	N/A	2.8X	1X	N/A	7.5X
3 (MOPA)	4X	N/A	4X	7.9X	N/A	4.4X
5 (IgG)	N/A	8.7X	3.2X	N/A	2.5X	1X
5 (MOPA)	N/A	5X	4X	N/A	3.6X	2X

[0042] The mixed carrier, 20-valent pneumococcal conjugate compositions described in this application also include pneumococcal serotypes not covered by the three pneumococcal conjugate vaccines currently available on the global market: PREVNAR (called Prevenar in some countries), SYNFLORIX and PREVNAR 13. Disease caused by pneumococcal serotypes not currently covered is on the rise, due, in part, to the development of antibacterial resistance, the increased number of immunocompromised patients, and lack of immune pressure. For example, none of the currently available pneumococcal conjugate vaccines includes serotype 12F. In addition, none of the currently available pneumococcal conjugate vaccines includes serotypes 8, 10A, 11A, 15B, 22F and 33F. The present disclosure demonstrates the successful implementation of serotypes 8, 10A, 11A, 12F, 15B, 22F, and 33F into a mixed carrier (tetanus toxoid and CRM_{197}), pneumococcal conjugate vaccine.

Mixed Carrier, Multivalent Pneumococcal Conjugate Compositions and Methods of Making the Same

[0043] In one aspect, this disclosure provides a mixed carrier, multivalent pneumococcal conjugate composition comprising 20 different pneumococcal capsular

polysaccharide-protein conjugates, wherein each pneumococcal capsular polysaccharide-protein conjugate comprises a protein carrier conjugated to a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae*, wherein the *Streptococcus pneumoniae* serotypes are 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F, wherein the protein carrier is CRM_{197} or tetanus toxoid, wherein two of the capsular polysaccharides are conjugated to tetanus toxoid and the remaining capsular polysaccharides are conjugated to CRM_{197}, and wherein the two capsular polysaccharides that are conjugated to tetanus toxoid are selected from the group consisting of serotypes 1, 3, and 5.

[0044] In some embodiments, the capsular polysaccharides from serotypes 1 and 5 are conjugated to tetanus toxoid, and the capsular polysaccharides from the remaining serotypes are conjugated to CRM_{197}. In another embodiment, the capsular polysaccharides from serotypes 1 and 3 are conjugated to tetanus toxoid, and the remaining capsular polysaccharides are conjugated to CRM_{197}. In yet another embodiment, the capsular polysaccharides from serotypes 3 and 5 are conjugated to tetanus toxoid, and the remaining capsular polysaccharides are conjugated to CRM_{197}.

[0045] In a polysaccharide-protein conjugate vaccine, a carrier protein is conjugated to a polysaccharide antigen primarily to help enhance the immune response (e.g. antibody response) to the polysaccharide antigen. Carrier proteins are preferably proteins that are non-toxic having little or no immunogenicity. Carrier proteins should be amenable to conjugation with a pneumococcal polysaccharide using standard conjugation procedures, as discussed in further detail below. The carrier proteins used in the mixed carrier, 20-valent pneumococcal conjugate compositions are tetanus toxoid (TT) and CRM_{197}, each of which has been used in the design of pneumococcal conjugate vaccines but never in the same, mixed carrier vaccine.

[0046] CRM_{197} is a non-toxic variant (i.e., toxoid) of diphtheria toxin that retains the immunologic properties of the wild type diphtheria toxin. CRM_{197} differs from the wild type diphtheria toxin at a single base in the structural gene, which gives rise to a single amino acid substitution from glutamic acid to glycine. CRM_{197} is typically isolated from cultures of *Corynebacterium diphtheria* strain C7 (β197) grown on casamino acids and yeast extract-based medium. CRM_{197} may be purified through ultra-filtration, ammonium sulfate precipitation, and ion-exchange chromatography. Alternatively, CRM_{197} can be prepared recombinantly in accordance with U.S. Patent No. 5,614,382, which is hereby incorporated by reference in its entirety. CRM_{197} has been used in the

design of pneumococcal conjugate vaccines but never as part of a mixed carrier vaccine.

[0047] Tetanus toxoid is prepared and used worldwide for large-scale immunization against tetanus (or lockjaw) caused by *Clostridium tetani*. Tetanus toxoid is also used both singly and in combination with diphtheria and/or pertussis vaccines. The parent protein, tetanus toxin, is generally obtained in cultures of *Clostridium tetani*. Tetanus toxin is a protein of about 150 kDa and consists of two subunits (about 100 kDa and about 50 kDa) linked by a sulfide bond. The toxin is typically detoxified with formaldehyde and can be purified from culture filtrates using known methods, such as ammonium sulfate precipitation (see, e.g., [7], [8]) or chromatography techniques, as disclosed, for example, in WO 1996/025425. Tetanus toxin may also be inactivated by recombinant genetic means.

[0048] Tetanus toxoid has also been used as a carrier protein in other vaccines, including pneumococcal conjugate vaccines. But it has never been used in a mixed carrier, pneumococcal conjugate vaccine in combination with CRM_{197}. The art also teaches away from conjugating serotype 3 to tetanus toxoid in a mixed carrier, pneumococcal conjugate vaccine because serotype 3 was shown to be more immunogenic when conjugated to diphtheria toxoid as compared to tetanus toxoid [2, 6].

[0049] The pneumococcal capsular polysaccharides used in the compositions and vaccines described herein, including the capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F, may be prepared from *Streptococcus pneumoniae* using any available technique, including standard techniques known to one of ordinary skill in the art, including, for example, those disclosed in WO 2006/110381, WO 2008/118752, WO 2006/110352, and U.S. Patent App. Pub. Nos. 2006/0228380, 2006/0228381, 2007/0184071, 2007/0184072, 2007/0231340, 2008/0102498 and 2008/0286838, all of which are incorporated by reference in their entireties. For example, each pneumococcal capsular polysaccharide serotype may be grown in culture medium (e.g., a soy-based medium). The cells are lysed, and individual polysaccharides may be purified from the lysate through centrifugation, precipitation, ultra-filtration, and/or column chromatography. In addition, the pneumococcal capsular polysaccharide can be produced using synthetic protocols.

[0050] Capsular polysaccharides of *Streptococcus pneumoniae* comprise repeating oligosaccharide units, which may contain up to 8 sugar residues. A capsular saccharide antigen may be a full length polysaccharide, or it may be reduced in size (e.g., a single oligosaccharide unit, or a shorter than native length saccharide chain of repeating oligosaccharide units). The size of capsular polysaccharides may be reduced by various methods known in the art, such as acid hydrolysis treatment, hydrogen peroxide treatment, sizing by a high pressure homogenizer, optionally followed by a hydrogen peroxide treatment to generate oligosaccharide fragments, or microfluidization.

[0051] The pneumococcal conjugate of each of the serotypes may be prepared by conjugating a capsular polysaccharide of each serotype to a carrier protein. The different pneumococcal conjugates may be formulated into a composition, including a single dosage formulation.

[0052] To prepare a polysaccharide-protein conjugate, the capsular polysaccharides prepared from each pneumococcal serotype may be chemically activated so that the capsular polysaccharides may react with a carrier protein. Once activated, each capsular polysaccharide may be separately conjugated to a carrier protein to form a glycoconjugate. The chemical activation of the polysaccharides and subsequent conjugation to the carrier protein may be achieved by conventional methods. For example, vicinal hydroxyl groups at the end of the capsular polysaccharides can be oxidized to aldehyde groups by oxidizing agents such as periodates (including sodium periodate, potassium periodate, or periodic acid), as disclosed, for example, in U.S. Pat. Nos. 4,365,170, 4,673,574 and 4,902,506, which are hereby incorporated by reference in their entireties. Polysaccharides may also be activated with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide is then coupled directly or via a spacer or linker group to an amino group on the carrier protein.

[0053] For example, the spacer could be cystamine or cysteamine to give a thiolated polysaccharide which could be coupled to the carrier via a thioether linkage obtained after reaction with a maleimide-activated carrier protein (for example using N-[y-maleimidobutyrloxy]succinimide ester (GMBS)) or a haloacetylated carrier protein (for example using iodoacetimide, N-succinimidyl bromoacetate (SBA; SIB), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-SIAB), N-succinimidyl iodoacetate (SIA) or

succinimidyl 3-[bromoacetamido]proprionate (SBAP)). Preferably, the cyanate ester (optionally made by COAP chemistry) is coupled with hexane diamine or adipic acid dihydrazide (AOH) and the amino-derivatized saccharide is conjugated to the carrier protein using carbodiimide (e.g., EOAC or EOC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described for example in WO 93/15760, WO 95/08348 and WO 96/129094.

[0054] The conjugation of the activated capsular polysaccharides and the carrier proteins may be achieved, for example, by reductive amination, as described, for example, in U.S. Patent Appl. Pub. Nos. 2006/0228380, 2007/0231340, 2007/0184071 and 2007/0184072, WO 2006/110381, WO 2008/079653, and WO 2008/143709, all of which are incorporated by reference in their entireties. For example, the activated capsular polysaccharides and the carrier protein may be reacted with a reducing agent to form a conjugate. Reducing agents which are suitable include borohydrides, such as sodium cyanoborohydride, borane-pyridine, sodium triacetoxyborohydride, sodium borohydride, or borohydride exchange resin. At the end of the reduction reaction, there may be unreacted aldehyde groups remaining in the conjugates. The unreacted aldehyde groups may be capped using a suitable capping agent, such as sodium borohydride (NaBH4). In an embodiment, the reduction reaction is carried out in aqueous solvent. In another embodiment the reaction is carried out in aprotic solvent. In an embodiment, the reduction reaction is carried out in DMSO (dimethylsulfoxide) or in DMF (dimethylformamide) solvent.

[0055] The activated capsular polysaccharides may be conjugated directly to the carrier protein or indirectly through the use of a spacer or linker, such as a bifunctional linker. The linker is optionally heterobifunctional or homobifunctional, having for example a reactive amino group and a reactive carboxylic acid group, 2 reactive amino groups or two reactive carboxylic acid groups.

[0056] Other suitable techniques for conjugation use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S--NHS, EOC, TSTU, as described, for example, in International Patent Application Publication No. WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with 1,1'-carbonyldiimidazole (CDI) (see Bethell et al. (1979) J. Biol. Chem. 254:2572-2574; Hearn et al. (1981) J. Chromatogr. 218:509-518) followed by reaction with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional

protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

[0057] The ratio of polysaccharide to carrier protein for pneumococcal conjugate vaccines is typically in the range 0.3–3.0 (w/w) but can vary with the serotype. The ratio can be determined either by independent measurement of the amounts of protein and polysaccharide present, or by methods that give a direct measure of the ratio known in the art. Methods include ^{1}H NMR spectroscopy or the use of SEC-HPLC-UV/RI with dual monitoring (e.g. refractive index and UV, for total material and protein content respectively) can profile the saccharide/protein ratio over the size distribution of conjugates as well as by SEC-HPLC-MALLS or MALDI-TOF-MS.

[0058] The polysaccharide-protein conjugates thus obtained may be purified and enriched by a variety of methods. These methods include concentration/diafiltration, column chromatography, and depth filtration. The purified polysaccharide-protein conjugates are combined to formulate a mixed carrier, 16-valent pneumococcal conjugate composition, which can be used as a vaccine.

[0059] Formulation of a vaccine composition can be accomplished using art-recognized methods. A vaccine composition is formulated to be compatible with its intended route of administration. The individual pneumococcal capsular polysaccharide-protein conjugates can be formulated together with a physiologically acceptable vehicle to prepare the composition. Examples of such vehicles include, but are not limited to, water, buffered saline, polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions.

[0060] In some embodiments, the mixed carrier, 20-valent pneumococcal conjugate composition further comprises an adjuvant. Adjuvants can include a suspension of minerals (alum, aluminum salts, such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, aluminum hydroxyphosphate sulfate, etc.) on which antigen is adsorbed; or water -in-oil emulsion in which antigen solution is emulsified in mineral oil (for example, Freund's incomplete adjuvant), sometimes with the inclusion of killed mycobacteria (Freund's complete adjuvant) to further enhance antigenicity. Immunostimulatory oligonucleotides (such as those including a CpG motif) can also be used as adjuvants (for example, see U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; 6,339,068; 6,406,705; and 6,429,199). Adjuvants also include biological molecules, such as lipids and costimulatory molecules.

Exemplary biological adjuvants include AS04 [9], IL-2, RANTES, GM-CSF, TNF-α, IFN-γ, G-CSF, LFA-3, CD72, B7-1, B7-2, OX-40L and 41 BBL.

[0061] In some embodiments, the adjuvant is an aluminum-based adjuvant. Typically, a single 0.5 ml vaccine dose is formulated to contain about 0.1 mg to 2.5 mg of the aluminum-based adjuvant. In other embodiments, a single 0.5 ml vaccine dose is formulated to contain between 0.1 mg to 2 mg, 0.1 mg to 1 mg, 0.1 mg to 0.5 mg, 0.1 mg to 0.2 mg, 0.125 mg to 2.5 mg, 0.125 mg to 0.5 mg, 0.125 mg to 0.2 mg or 0.125 to 0.25 mg of the aluminum-based adjuvant. In certain embodiments, a single 0.5 ml vaccine dose is formulated to contain about 0.125 mg of the aluminum-based adjuvant.

[0062] In particular embodiments, the adjuvant is selected from the group consisting of aluminum phosphate, aluminum sulfate, and aluminum hydroxide.

[0063] In particular embodiments, the adjuvant is aluminum phosphate.

[0064] In some embodiments, the composition is for use as a vaccine against an infection of *Streptococcus pneumoniae*.

Prophylactic Methods and Uses

[0065] In one aspect, this disclosure provides a vaccine comprising a mixed carrier, 20-valent pneumococcal conjugate composition and a pharmaceutically acceptable excipient. In some embodiments, the pharmaceutically acceptable excipient comprises at least a buffer, such as a succinate buffer, a salt, such as sodium chloride, and/or a surface active agent, such as a polyoxyethylene sorbitan ester (e.g., polysorbate 80). In some embodiments, the capsular polysaccharides from serotypes 1 and 5 are conjugated to the tetanus toxoid, and the capsular polysaccharides from serotypes 3, 4, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}.

[0066] In another embodiment, the capsular polysaccharides from serotypes 1 and 3 are conjugated to the tetanus toxoid, and the capsular polysaccharides from serotypes 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}.

[0067] In yet another embodiment, the capsular polysaccharides from serotypes 3 and 5 are conjugated to the tetanus toxoid, and the capsular polysaccharides from serotypes 1, 4, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}.

[0068] In some embodiments, the vaccine elicits a protective immune response in a human subject against disease caused by *Streptococcus pneumoniae* infection.

[0069] According to a further aspect, this disclosure provides a method for prophylaxis of *Streptococcus pneumoniae* infection or disease, the method comprising administering to a human subject a prophylactically effective amount of a mixed carrier, 20-valent pneumococcal conjugate composition or a vaccine comprising the same. The mixed carrier, 20-valent pneumococcal conjugate composition or vaccine comprising the same may be administered by any route, including, for example, by a systemic or mucosal route, as described below in further detail.

[0070] In certain embodiments, the human subject is an elderly subject and the disease is pneumonia or invasive pneumococcal disease (IPD). In certain embodiments, the elderly subject is at least 50 years old. In other embodiments, the elderly subject is at least 55 years old. In yet other embodiments, the elderly subject is at least 60 years old.

[0071] In other embodiments, the human subject is an infant and the disease is pneumonia, invasive pneumococcal disease (IPD), or acute otitis media (AOM). In certain embodiments, the infant is 0-2 years. In other embodiments, the infant is 2 to 15 months.

[0072] In yet another embodiment, the human subject is 6 weeks to 17 years of age and the disease is pneumonia, invasive pneumococcal disease (IPD) or acute otitis media (AOM). In certain embodiments, the human subject is 6 weeks to 5 years of age. In other embodiments, the human subject is 5 to 17 years of age.

[0073] The amount of conjugate in each vaccine dose or the prophylactically effective amount of the mixed carrier, multivalent pneumococcal conjugate composition may be selected as an amount that induces prophylaxis without significant, adverse effects. Such an amount can vary depending upon the pneumococcal serotype. Generally, each dose may include about 0.1 μg to about 100 μg of polysaccharide, specifically, about 0.1 to 10 μg, and, more specifically, about 1 μg to about 5 μg. Optimal amounts of components for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. For example, the amount for vaccination of a human subject can be determined by extrapolating an animal test result. In addition, the dose can be determined empirically.

[0074] In some embodiments, the vaccine or the mixed carrier, 20-valent pneumococcal conjugate composition may be a single 0.5 ml dose formulated to

contain about 1 µg to about 5 µg of each capsular polysaccharide; about 1 µg to about 25 µg of TT; about 20 µg to about 75 µg of CRM_{197}; and optionally about 0.1 mg to about 2.5 mg of elemental aluminum adjuvant. In some embodiments, the vaccine or the mixed carrier, 20-valent pneumococcal conjugate composition may be a single 0.5 ml dose formulated to contain about 2 µg to about 4 µg of each capsular polysaccharide except serotype 6B, which is present in an amount of about 4 µg to about 8 µg and optionally about 4 µg to about 5 µg. In some embodiments, the vaccine or the mixed carrier, 20-valent pneumococcal conjugate composition may be a single 0.5 ml dose formulated to contain about 2.0 µg of each capsular polysaccharide except serotype 6B, which is present in an amount of about 4.0 µg. In other embodiments, the vaccine or the mixed carrier, 20-valent pneumococcal conjugate composition may be a single 0.5 ml dose formulated to contain about 2.2 µg of each capsular polysaccharide except serotype 6B, which is present in an amount of about 4.4 µg. In some embodiments, the vaccine or the mixed carrier, 20-valent pneumococcal conjugate composition may be a single 0.5 ml dose formulated to contain about 2 µg to about 2.5 µg of the capsular polysaccharides of serotypes 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 22F, 23F, and 33F and about 4 µg to about 5 µg of the capsular polysaccharides of serotypes 1, 3, 6B, 12F, 19A, and 19F. In some embodiments, the vaccine or the mixed carrier, 20-valent pneumococcal conjugate composition may be a single 0.5 ml dose formulated to contain about 2.0 µg of the capsular polysaccharides of serotypes 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 22F, 23F, and 33F and about 4.0 µg of the capsular polysaccharides of serotypes 1, 3, 6B, 12F, 19A, and 19F. In other embodiments, the vaccine or the mixed carrier, 20-valent pneumococcal conjugate composition may be a single 0.5 ml dose formulated to contain about 2.2 µg of the capsular polysaccharides of serotypes 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 22F, 23F, and 33F and about 4.4 µg of the capsular polysaccharides of serotypes 1, 3, 6B, 12F, 19A, and 19F.

[0075] In some embodiments, the vaccine or the mixed carrier, 20-valent pneumococcal conjugate composition may be a single 0.5 ml dose formulated to contain about 2 µg to about 2.5 µg of the capsular polysaccharides of serotypes 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 22F, 23F, and 33F; about 4 µg to about 5 µg of the capsular polysaccharides of serotypes 1, 3, 6B, 12F, 19A, and 19F; about 5 µg to about 15 µg of TT; about 50 µg to about 60 µg of CRM_{197}; and optionally about 0.1 mg to about 0.2 mg of elemental aluminum adjuvant. In certain embodiments, the

mixed carrier, 20-valent pneumococcal conjugate composition or vaccine comprising the same further comprises sodium chloride and sodium succinate buffer as excipients.

[0076] In some embodiments, the mixed carrier, 20-valent pneumococcal conjugate composition may be formulated into a liquid formulation in which each of the pneumococcal capsular polysaccharides of serotypes 1 and 3 is conjugated to TT and the capsular polysaccharides from serotypes 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}. In one embodiment, each 0.5 mL dose may be formulated into a liquid containing: about 2.2 µg of each polysaccharide, except for 6B at about 4.4 µg; about 10 µg to about 15 µg of TT carrier protein (only for the serotypes 1 and 3) and about 50 µg to about 60 µg of CRM_{197} carrier protein; 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) as an adjuvant; and sodium chloride and sodium succinate buffer as excipients. In another embodiment, each 0.5 mL dose may be formulated into a liquid containing: about 2.0 µg of each polysaccharide, except for 6B at about 4.0 µg; about 10 µg to about 15 µg of TT carrier protein (only for the serotypes 1 and 3) and about 50 µg to about 60 µg of CRM_{197} carrier protein; 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) as an adjuvant; and sodium chloride and sodium succinate buffer as excipients.

[0077] In some embodiments, the mixed carrier, 20-valent pneumococcal conjugate composition may be formulated into a liquid formulation in which each of the pneumococcal capsular polysaccharides of serotypes 1 and 3 is conjugated to TT and the capsular polysaccharides from serotypes 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}. In one embodiment, each 0.5 mL dose may be formulated into a liquid containing: about 2.2 µg of the capsular polysaccharides of serotypes 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 22F, 23F, and 33F; about 4.4 µg of the capsular polysaccharides of serotypes 1, 3, 6B, 12F, 19A, and 19F; about 10 µg to about 15 µg of TT carrier protein (only for the serotypes 1 and 3) and about 50 µg to about 60 µg of CRM_{197} carrier protein; 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) as an adjuvant; and sodium chloride and sodium succinate buffer as excipients. In another embodiment, each 0.5 mL dose may be formulated into a liquid containing: about 2.0 µg of the capsular polysaccharides of serotypes 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 22F, 23F, and 33F; about 4.0 µg of the capsular polysaccharides of serotypes 1, 3, 6B, 12F, 19A, and 19F; about 10 µg to about 15 µg of TT carrier protein (only for the serotypes 1 and

3) and about 50 µg to about 60 µg of CRM_{197} carrier protein; 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) as an adjuvant; and sodium chloride and sodium succinate buffer as excipients.

[0078] In some embodiments, the mixed carrier, 20-valent pneumococcal conjugate composition may be formulated into a liquid formulation in which each of the pneumococcal capsular polysaccharides of serotypes 1 and 5 is conjugated to TT and the capsular polysaccharides from serotypes 3, 4, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}. In one embodiment, each 0.5 mL dose may be formulated into a liquid containing: about 2.2 µg of each saccharide, except for 6B at about 4.4 µg; about 5 µg to about 10 µg of TT carrier protein (only for the serotypes 1 and 5) and about 50 µg to about 60 µg of CRM_{197} carrier protein; about 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; and sodium chloride and sodium succinate buffer as excipients. In another embodiment, each 0.5 mL dose may be formulated into a liquid containing: about 2.0 µg of each saccharide, except for 6B at about 4.0 µg; about 5 µg to about 10 µg of TT carrier protein (only for the serotypes 1 and 5) and about 50 µg to about 60 µg of CRM_{197} carrier protein; about 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; and sodium chloride and sodium succinate buffer as excipients.

[0079] In some embodiments, the mixed carrier, 20-valent pneumococcal conjugate composition may be formulated into a liquid formulation in which each of the pneumococcal capsular polysaccharides of serotypes 1 and 5 is conjugated to TT and the capsular polysaccharides from serotypes 3, 4, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}. In one embodiment, each 0.5 mL dose may be formulated into a liquid containing: about 2.2 µg of the capsular polysaccharides of serotypes 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 22F, 23F, and 33F; about 4.4 µg of the capsular polysaccharides of serotypes 1, 3, 6B, 12F, 19A, and 19F; about 5 µg to about 10 µg of TT carrier protein (only for the serotypes 1 and 5) and about 50 µg to about 60 µg of CRM_{197} carrier protein; about 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; and sodium chloride and sodium succinate buffer as excipients. In another embodiment, each 0.5 mL dose may be formulated into a liquid containing: about 2.0 µg of the capsular polysaccharides of serotypes 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 22F, 23F, and 33F; about 4.0 µg of the capsular polysaccharides of serotypes 1, 3, 6B, 12F, 19A,

and 19F; about 5 µg to about 10 µg of TT carrier protein (only for the serotypes 1 and 5) and about 50 µg to about 60 µg of CRM_{197} carrier protein; about 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; and sodium chloride and sodium succinate buffer as excipients.

[0080] In some embodiments, the mixed carrier, 20-valent pneumococcal conjugate composition may be formulated into a liquid formulation in which each of the pneumococcal capsular polysaccharides of serotypes 3 and 5 is conjugated to TT and the capsular polysaccharides from serotypes 1, 4, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}. In one embodiment, each 0.5 mL dose may be formulated into a liquid containing: about 2.2 µg of each saccharide, except for 6B at about 4.4 µg; about 10 µg to about 15 µg µg of TT carrier protein (only for the serotypes 3 and 5) and about 50 µg to about 60 µg of CRM_{197} carrier protein; about 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; and sodium chloride and sodium succinate buffer as excipients. In another embodiment, each 0.5 mL dose may be formulated into a liquid containing: about 2.0 µg of each saccharide, except for 6B at about 4.0 µg; about 10 µg to about 15 µg µg of TT carrier protein (only for the serotypes 3 and 5) and about 50 µg to about 60 µg of CRM_{197} carrier protein; about 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; and sodium chloride and sodium succinate buffer as excipients.

[0081] In some embodiments, the mixed carrier, 20-valent pneumococcal conjugate composition may be formulated into a liquid formulation in which each of the pneumococcal capsular polysaccharides of serotypes 3 and 5 is conjugated to TT and the capsular polysaccharides from serotypes 1, 4, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}. In one embodiment, each 0.5 mL dose may be formulated into a liquid containing: about 2.2 µg of the capsular polysaccharides of serotypes 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 22F, 23F, and 33F; about 4.4 µg of the capsular polysaccharides of serotypes 1, 3, 6B, 12F, 19A, and 19F; about 10 µg to about 15 µg µg of TT carrier protein (only for the serotypes 3 and 5) and about 50 µg to about 60 µg of CRM_{197} carrier protein; about 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; and sodium chloride and sodium succinate buffer as excipients. In another embodiment, each 0.5 mL dose may be formulated into a liquid containing: about 2.0 µg of the capsular polysaccharides of serotypes 4, 5, 6A, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 22F, 23F,

and 33F; about 4.0 µg of the capsular polysaccharides of serotypes 1, 3, 6B, 12F, 19A, and 19F; about 10 µg to about 15 µg µg of TT carrier protein (only for the serotypes 3 and 5) and about 50 µg to about 60 µg of CRM_{197} carrier protein; about 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; and sodium chloride and sodium succinate buffer as excipients.

[0082] In some embodiments, the liquid formulation may be filled into a single dose syringe without a preservative. After shaking, the liquid formulation becomes a vaccine that is a homogeneous, white suspension ready for intramuscular administration.

[0083] The mixed carrier, 20-valent pneumococcal conjugate composition can be administered in a single injection or as part of an immunization series. For example, the mixed carrier, 20-valent pneumococcal conjugate composition can be administered 2, 3, 4, or more times at appropriately spaced intervals, such as, a 1, 2, 3, 4, 5, or 6 month interval or a combination thereof. In some embodiments, the mixed carrier, 20-valent pneumococcal conjugate composition is administered to an infant 4 times within the first 15 months of birth, including, for example, at about 2, 3, 4, and 12-15 months of age; at about 3, 4, 5, and 12-15 months of age; or at about 2, 4, 6, and 12 to 15 months of age. This first dose can be administered as early as 6 weeks of age. In another embodiment, the mixed carrier, 20-valent pneumococcal conjugate composition is administered to an infant 3 times within the first 15 months of birth, including, for example, at about 2, 4, and 11-12 months.

[0084] The mixed carrier, multivalent pneumococcal conjugate composition may also include one or more proteins from *Streptococcus pneumoniae*. Examples of *Streptococcus pneumoniae* proteins suitable for inclusion include those identified in International Patent Application WO02/083855, as well as those described in International Patent Application WO02/053761.

[0085] The mixed carrier, 20-valent pneumococcal conjugate composition can be administered to a subject via one or more administration routes known to one of ordinary skill in the art such as a parenteral, transdermal, or transmucosal, intranasal, intramuscular, intraperitoneal, intracutaneous, intravenous, or subcutaneous route and be formulated accordingly. The mixed carrier, 20-valent pneumococcal conjugate composition can be formulated to be compatible with its intended route of administration.

[0086] In some embodiments, the mixed carrier, 20-valent pneumococcal conjugate composition can be administered as a liquid formulation by intramuscular,

intraperitoneal, subcutaneous, intravenous, intraarterial, or transdermal injection or respiratory mucosal injection. The mixed carrier, 20-valent pneumococcal conjugate compositions can be formulated in liquid form or in a lyophilized form. In some embodiments, injectable compositions are prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. In some embodiments, injection solutions and suspensions are prepared from sterile powders or granules. General considerations in the formulation and manufacture of pharmaceutical agents for administration by these routes may be found, for example, in *Remington's Pharmaceutical Sciences*, 19th ed., Mack Publishing Co., Easton, PA, 1995; incorporated herein by reference. At present the oral or nasal spray or aerosol route (*e.g.*, by inhalation) are most commonly used to deliver therapeutic agents directly to the lungs and respiratory system. In some embodiments, a mixed carrier, 20-valent pneumococcal conjugate composition is administered using a device that delivers a metered dosage of composition. Suitable devices for use in delivering intradermal pharmaceutical compositions described herein include short needle devices such as those described in U.S. Patent No. 4,886,499, U.S. Patent No. 5,190,521, U.S. Patent No. 5,328,483, U.S. Patent No. 5,527,288, U.S. Patent No. 4,270,537, U.S. Patent No. 5,015,235, U.S. Patent No. 5,141,496, U.S. Patent No. 5,417,662 (all of which are incorporated herein by reference). Intradermal compositions may also be administered by devices which limit the effective penetration length of a needle into the skin, such as those described in WO1999/34850, incorporated herein by reference, and functional equivalents thereof. Also suitable are jet injection devices which deliver liquid vaccines to the dermis via a liquid jet injector or via a needle which pierces the stratum corneum and produces a jet which reaches the dermis. Jet injection devices are described for example in U.S. Patent No. 5,480,381, U.S. Patent No. 5,599,302, U.S. Patent No. 5,334,144, U.S. Patent No. 5,993,412, U.S. Patent No. 5,649,912, U.S. Patent No. 5,569,189, U.S. Patent No. 5,704,911, U.S. Patent No. 5,383,851, U.S. Patent No. 5,893,397, U.S. Patent No. 5,466,220, U.S. Patent No. 5,339,163, U.S. Pat. No. 5,312,335, U.S. Pat. No. 5,503,627, U.S. Pat. No. 5,064,413, U.S. Patent No. 5,520,639, U.S. Patent No. 4,596,556, U.S. Patent No. 4,790,824, U.S. Patent No. 4,941,880, U.S. Patent No. 4,940,460, WO1997/37705, and WO1997/13537 (all of which are incorporated herein by reference). Also suitable are ballistic powder/particle delivery devices which use compressed gas to accelerate vaccine in powder form through the outer layers of the

skin to the dermis. Additionally, conventional syringes may be used in the classical mantoux method of intradermal administration.

[0087] Preparations for parenteral administration include sterile aqueous or nonaqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, oils such as olive oil, and injectable organic esters such as ethyl oleate. Examples of oil include vegetable or animal oil, peanut oil, soybean oil, olive oil, sunflower oil, liver oil, synthetic oil such as marine oil, and lipids obtained from milk or eggs. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti- oxidants, chelating agents, and inert gases and the like.

[0088] The mixed carrier, 20-valent pneumococcal conjugate composition can be formulated in the form of a unit dose vial, multiple dose vial, or pre-filled syringe. A pharmaceutically acceptable carrier for a liquid formulation includes aqueous or nonaqueous solvent, suspension, emulsion, or oil. The composition may be isotonic, hypertonic, or hypotonic. However, it is desirable that the composition for infusion or injection is basically isotonic. Thus, isotonicity or hypertonicity may be advantageous for storage of the composition. When the composition is hypertonic, the composition can be diluted to isotonicity before administration. A tonicity agent may be ionic tonicity agent such as salt or non-ionic tonicity agent such as carbohydrate. The ionic tonicity agent includes, but is not limited to, sodium chloride, calcium chloride, potassium chloride, and magnesium chloride. The nonionic tonicity agent includes, but is not limited to, sorbitol and glycerol. Preferably, at least one pharmaceutically acceptable buffer is included. For example, when the composition is an infusion or injection, it is preferable to be formulated in a buffer with a buffering capacity at pH 4 to pH 10, such as pH 5 to pH 9, or, pH 6 to pH 8. The buffer may be selected from those suitable for United States Pharmacopeia (USP). For example, the buffer can be selected from the group consisting of a monobasic acid, such as acetic acid, benzoic acid, gluconic acid, glyceric acid, and lactic acid; a dibasic acid, such as aconitic acid, adipic acid, ascorbic acid, carbonic acid, glutamic acid, malic acid, succinic acid, and tartaric acid; a

polybasic acid such as citric acid and phosphoric acid; and a base such as ammonia, diethanolamine, glycine, triethanolamine, and TRIS.

[0089] The mixed carrier, 20-valent pneumococcal conjugate composition may comprise a surface active agent. Examples of the surface active agent include, but are not limited to, polyoxyethylene sorbitan ester (generally referred to as Tweens), in particular, polysorbate 20 and polysorbate 80; copolymers (such as DOWFAX) of ethylene oxide (EO), propylene oxide (PO), butylenes oxide (BO); octoxynols with different repeats of ethoxy(oxy-1,2-ethanediyl) group, in particular, octoxynol-9 (Triton-100); ethylphenoxypolyethoxyethanol (IGEPAL CA-630/NP-40); phospholipid such as lecithin; nonylphenol ethoxylate such as TERGITOL NP series; lauryl, cetyl, stearyl, oleyl alcohol-derived polyoxyethylene fatty ether (Brij surfactant), in particular, triethyleneglycol monolauryl ether (Brij 30); sorbitan ether known as SPAN, in particular, sorbitan trioleate (Span 85) and sorbitan monolaurate.

[0090] Mixtures of surface active agents such as Tween 80/Span 85 can be used. A combination of polyoxyethylene sorbitan ester such as Tween 80 and octoxynol such as Triton X-100 is also suitable. A combination of Laureth 9 and Tween and/or octoxynol is also advantageous. Preferably, the amount of polyoxyethylene sorbitan ester (such as Tween 80) included may be 0.01% to 1% (w/v), 0.01% to 0.1% (w/v), 0.01% to 0.05% (w/v), or about 0.02%; the amount of octylphenoxy polyoxyethanol or nonylphenoxy polyoxyethanol (such as Triton X-100) included may be 0.001% to 0.1% (w/v), in particular 0.005% to 0.02%; and the amount of polyoxyethylene ether (such as Laureth 9) included may be 0.1% to 20% (w/v), possibly 0.1% to 10%, in particular 0.1% to 1% or about 0.5%.

[0091] In some embodiments, the mixed carrier, 20-valent pneumococcal conjugate composition may be delivered via a release control system. For example, intravenous infusion, transdermal patch, liposome, or other routes can be used for administration. In one aspect, macromolecules such as microsphere or implant can be used.

[0092] The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples. These examples are described solely for the purpose of illustration and are not intended to limit the scope of the invention.

EXAMPLES

[0093] Example 1. Preparation of S. pneumoniae Capsular Polysaccharides

[0094] Cultivation of *S. pneumoniae* and purification of capsular polysaccharides were conducted as known to one of skill in the art. *S. pneumoniae* serotypes were obtained from the American Type Culture Collection (ATCC) (serotype 1: ATCC No. 6301; serotype 3: ATCC No. 6303; serotype 4: ATCC No. 6304; serotype 5: ATCC No. 6305; serotype 6A: ATCC No. 6306; serotype 6B: ATCC No. 6326; serotype 7F: ATCC No. 10351; serotype 9V: ATCC No. 10368; serotype 12F: ATCC No. 6312; serotype 14: ATCC No. 6314; serotype 18C: ATCC No. 10356; serotype 19A: ATCC No. 10357; serotype 19F: ATCC No. 6319; serotype 22F: ATCC No. 6322; serotype 23F: ATCC No. 6323; serotype 33F: ATCC No. 10370). Internal strains for serotypes 8, 10A, 11A, and 15B were used. *S. pneumoniae* were characterized by capsules and immobility, Gram-positive, lancet-shaped diplococcus, and alpha hemolysis in a blood agar medium. Serotypes were identified by Quelling test using specific anti-sera (US Patent No. 5,847,112).

[0095] Preparation of Cell Banks

[0096] Several generations of seed stocks were generated in order to expand the strains and remove components of animal origin (generations F1, F2, and F3). Two additional generations of seed stocks were produced. The first additional generation was cultured from an F3 vial, and the subsequent generation was cultured from a vial of the first additional generation. Seed vials were stored frozen (below -70℃) with synthetic glycerol as a cryopreservative. For cell bank preparation, all cultures were grown in a soy-based medium. Prior to freezing, cells were concentrated by centrifugation, spent medium was removed, and cell pellets were re-suspended in a fresh medium containing a cryopreservative (such as synthetic glycerol).

[0097] Culturing and Harvesting

[0098] Cultures from the working cell bank were inoculated into seed bottles containing a soy-based medium and cultured. After the target optical density (absorbance) was reached, the seed bottle was used to inoculate a fermentor containing the soy-based medium. The culturing was terminated when an optical density value started to be maintained constant. After terminating the culturing, sodium deoxycholate was added to the culture to lyse the cells. The resulting fermentor contents were cooled, and

protein precipitation was induced. Then, the mixture was centrifuged to remove precipitated proteins and cell debris.

[0099] Purification

[00100] The solution obtained from the centrifugation was filtered through a depth filter to remove the proteins and cell debris that had not precipitated in the centrifugation. The filtrate was concentrated on a 100 kDa MW membrane and the concentrate was diafiltered with 10 volumes of a 25 mM sodium phosphate buffer (pH 7.2) to obtain a sample. The sample was filtered to collect a supernatant from which polysaccharides were precipitated and filtered. The filtrate was concentrated on a 30 kDa membrane, and the concentrate was diafiltered using about 10 volumes of triple distilled water. After performing the diafiltration, the remaining solution was filtered through a 0.2 μm filter. An in-process control test was performed on the filtrate (appearance, remaining proteins, remaining nucleic acids, endotoxins, molecular weights, and the total amount of polysaccharides). The concentrate was sterile filtered and stored at -20℃.

[00101] Example 2. Preparation of Conjugate of *S. pneumoniae* Capsular Polysaccharide and Carrier Protein

[00102] Polysaccharides of different serotypes were activated following different pathways and then conjugated to a carrier protein, CRM_{197} or TT. Specifically, conjugates were prepared by conjugating each of the capsular polysaccharides of all serotypes to CRM_{197} and by conjugating each of the capsular polysaccharides of the serotypes 1, 3, and 5 to TT. The activation process includes reduction of the size of each capsular polysaccharide to the target molecular weight, chemical activation, and buffer exchange via ultrafiltration. The conjugates were purified using ultrafiltration and finally filtered through 0.2 μm filter. The process parameters such as pH, temperature, concentration, and time were as follows.

[00103] (1) Activation Process

[00104] Step 1: Hydrolysis

[00105] Reductive amination is a known method for conjugating polymers in which an amide bond is formed between a primary amine ($-NH_2$) group of a protein and an aldehyde of a saccharide. However, since *S. pneumoniae* polysaccharides do not have any aldehyde groups, an aldehyde group is added to the pneumococcal capsular polysaccharide. A diol structure of a monosaccharide can be oxidized by

sodium periodate ($NaIO_4$) to form an aldehyde group. The capsular polysaccharides from serotypes 1, 3, 4, 6A, 8, 11A, 12F, 14, 15B, 18C, 22F, and 33F were pre-treated as follows.

[00106] In the case of the serotype 1, sodium hydroxide (at a final base concentration of 0.05 M) was added to a solution of the capsular polysaccharide, and the solution was incubated at 50±2℃. The solution was then cooled to a temperature in a range of about 21℃ to about 25℃, and hydrochloric acid was added thereto to a final pH of 6.0±0.1, thereby stopping hydrolysis.

[00107] In the case of the serotype 3, 8, 11A, and 15B, hydrochloric acid (at a final acid concentration of 0.01 M) was added to a solution of the capsular polysaccharide, and the solution was incubated at 60±2℃. The solution was then cooled to a temperature in a range of about 21℃ to about 25℃, and 0.1M sodium phosphate was added thereto to a final pH of 6.0±0.1, thereby stopping hydrolysis.

[00108] In the case of the serotype 4, hydrochloric acid (at a final acid concentration of 0.1 M) was added to a solution of the capsular polysaccharide, and the solution was incubated at 45±2℃. The solution was then cooled to a temperature in a range of about 21℃ to about 25℃, and 1M sodium phosphate was added thereto to a final pH of 6.0±0.1, thereby stopping hydrolysis.

[00109] In the case of the serotype 6A, glacial acetic acid (at a final acid concentration of 0.1 M) was added to a solution of the capsular polysaccharide, and the solution was incubated at 60±2℃. The solution was then cooled to a temperature in a range of about 21℃ to about 25℃, and 1M sodium hydroxide was added thereto to a final pH of 6.0±0.1.

[00110] In the case of the serotype 12F, hydrochloric acid (at a final acid concentration of 0.01 M) was added to a solution of the capsular polysaccharide, and the solution was incubated at 70±2℃. The solution was then cooled to a temperature in a range of about 21℃ to about 25℃, and 0.1M sodium phosphate was added thereto to a final pH of the solution of 6.0±0.1, thereby stopping hydrolysis.

[00111] In the case of the serotypes 14 and 18C, glacial acetic acid (at a final acid concentration of 0.2 M) was added to a solution of the capsular polysaccharide, and the solution was incubated at about 91-96℃. The solution was then cooled to a

temperature in a range of about 21℃ to about 25℃, and 1M sodium phosphate was added thereto so that a final pH of the solution was 6.0±0.1.

[00112] In the case of the serotypes 22F and 33F, hydrochloric acid (at a final acid concentration of 0.01 M) was added to a solution of the capsular polysaccharide, and the solution was incubated at 60±2℃. The solution was then cooled to a temperature in a range of about 21℃ to about 25℃, and 0.1M sodium phosphate was added thereto to a final pH of 6.0±0.1, thereby stopping hydrolysis.

Each of the obtained capsular polysaccharides was diluted in water for injection (WFI), sodium acetate, and sodium phosphate to a final concentration between about 1.0 mg/mL and about 2.0 mg/mL.

[00113] Step 2: Periodate reaction

[00114] The sodium periodate molar equivalent for each pneumococcal saccharide activation was determined using total saccharide content. With thorough mixing, the oxidation reaction was allowed to proceed for 16 to 20 hours at 21℃ to 25℃ for all serotypes except for 1, 7F, and 19F, for which the temperature was 10℃ or less. During the conjugation process, maintaining an aldehyde concentration at an appropriate level provides for consistent and stable production of conjugates. A degree of production of the aldehyde is determined by a ratio between a concentration of the produced aldehyde and a concentration of saccharide, and this degree is related to a degree of oxidation (Do) which is set for each serotype as shown in Table 2 and Table 3.

Table 2. Range of Do for all serotypes to be conjugated to CRM_{197}

Serotype	**Range of Do**	**Serotype**	**Range of Do**
Serotype 1	4 to 10	Serotype 11A	1 to 15
Serotype 3	2 to 8	Serotype 12F	1 to 9
Serotype 4	1 to 5	Serotype 14	6 to 13
Serotype 5	2 to 6	Serotype 15B	1 to 17
Serotype 6A	5 to 15	Serotype 18C	6 to 14
Serotype 6B	7 to 13	Serotype 19A	7 to 13

Serotype	Range of Do	Serotype	Range of Do
Serotype 7F	2 to 8	Serotype 19F	6 to 12
Serotype 8	1 to 17	Serotype 22F	1 to 16
Serotype 9V	4 to 9	Serotype 23F	6 to 14
Serotype 10A	1 to 12	Serotype 33F	1 to 15

Table 3. Range of Do for serotypes 1, 3, and 5 to be conjugated to TT

Serotype	Range of Do
Serotype 1	1 to 15
Serotype 3	2 to 14
Serotype 5	1 to 15

[00115] Step 3: Ultrafiltration

[00116] The oxidized saccharide was concentrated and diafiltered with WFI on a 100 kDa MWCO ultrafilter (30kDa ultrafilter for serotype 1 and 5 kDa ultrafilter for serotype 18C). Diafiltration was conducted using 0.9% sodium chloride solution for serotype 1, 0.01 M sodium acetate buffer (pH 4.5) for serotypes 7F and 23F, and 0.01 M sodium phosphate buffer (pH 6.0) for serotype 19F. The permeate was discarded, and the retentate was filtered through a 0.2 μm filter.

[00117] Step 4: Lyophilization

[00118] For capsular polysaccharides of serotypes 3, 4, 5, 8, 9V, 10A, 14, 15B, 22F, and 33F that are to be conjugated to a carrier protein by using an aqueous solvent, mixed solution of polysaccharides and carrier protein was prepared without addition of sucrose, lyophilized, and then stored at -25℃ ± 5℃.

[00119] For capsular polysaccharides of serotypes 1 and 18C that are to be conjugated to a carrier protein by using an aqueous solvent, polysaccharides and carrier protein were independently prepared, without addition of sucrose, lyophilized, and then stored at -25℃ ± 5℃.

[00120] For capsular polysaccharides of serotypes 6A, 6B, 7F, 19A, 19F, and 23F that are to be conjugated to a carrier protein by using a DMSO solvent, a

predetermined amount of sucrose to reach a final sucrose concentration of 5%±3% was added to the activated saccharides, and the samples were independently prepared, lyophilized, and then stored at -25℃ ± 5℃.

[00121] For capsular polysaccharide of serotype 11A, a predetermined amount of sucrose to reach a final sucrose concentration of 20%±5% was added to the activated saccharide, and the polysaccharides and carrier protein were independently prepared, lyophilized, and then stored at -25℃ ± 5℃.

[00122] For capsular polysaccharide of serotype 12F, a predetermined amount of sucrose to reach a final sucrose concentration of 10%±5% was added to the activated saccharide, and the polysaccharides and carrier protein were independently prepared, lyophilized, and then stored at -25℃ ± 5℃.

[00123] (2) Conjugation process

[00124] Aqueous conjugation was conducted for serotypes 1, 3, 4, 5, 8, 9V, 10A, 14, 15B, 18C, 22F, and 33F, and DMSO conjugation was conducted for serotypes 6A, 6B, 7F, 11A, 12F, 19A, 19F, and 23F. Each of the capsular polysaccharides was conjugated to a carrier protein at a ratio of 0.2 to 2:1.

[00125] Step 1: Dissolution

[00126] **Aqueous Conjugation**

[00127] For serotypes 1, 3, 4, 5, 8, 9V, 10A, 14, 15B, 18C, 22F, and 33F, the lyophilized sample was thawed and equilibrated at room temperature. The lyophilized sample was reconstituted to a reaction concentration by using a sodium phosphate buffer solution at 23±2℃ at a ratio set for each serotype.

[00128] **Dimethyl sulfoxide (DMSO) Conjugation**

[00129] For serotypes 6A, 6B, 7F, 11A, 12F, 19A, 19F, and 23F, the lyophilized sample was thawed, equilibrated at room temperature, and reconstituted in DMSO.

[00130] Step 2: Conjugation Reaction

[00131] **Aqueous Conjugation**

[00132] For serotypes 1, 3, 4, 5, 8, 9V, 10A, 14, 15B, 18C, 22F, and 33F, the conjugation reaction was initiated by adding the sodium cyanoborohydride solution (100 mg/mL) to 1.0 to 1.4 moles sodium cyanoborohydride per mole of saccharide. However, for serotypes 1 and 3, the reaction was initiated by adding the sodium cyanoborohydride solution to 0.5 moles sodium cyanoborohydride per mole of saccharide.

[00133] For serotypes 1, 3, and 5 to be conjugated to TT, the conjugation reaction was initiated by adding the sodium cyanoborohydride solution (100 mg/mL) to 1.0 to 1.4 moles sodium cyanoborohydride per mole of saccharide, except for serotype 1 to 0.5 moles sodium cyanoborohydride per mole of saccharide.

[00134] The reaction mixture was incubated at 23℃ to 37℃ for 44 to 106 hours. The reaction temperature and time were adjusted by serotype. The temperature was then reduced to 23±2℃ and sodium chloride 0.9% was added to the reactor. Sodium borohydride solution (100 mg/mL) was added to achieve 1.8 to 2.2 molar equivalents of sodium borohydride per mole of saccharide. The mixture was incubated at 23±2℃ for 3 to 6 hours. This procedure reduced any unreacted aldehydes present on the saccharides. Then, the mixture was diluted with sodium chloride 0.9% and the diluted conjugation mixture was filtered using a 0.8 or 0.45 µm pre-filter.

[00135] **DMSO conjugation**

[00136] For capsular polysaccharides of serotypes 6A, 6B, 7F, 11A, 12F, 19A, 19F, and 23F, the conjugation reaction was initiated by adding the sodium cyanoborohydride solution (100 mg/mL) to a ratio of 0.8 to 1.2 molar equivalents of sodium cyanoborohydride per one mole of activated saccharide. WFI was added to the reaction mixture to a target concentration of 1 % (v/v), and the mixture was incubated for 12 to 26 hours at 23±2°C. 100 mg/mL of a sodium borohydride solution (typical 1.8 to 2.2 molar equivalents sodium borohydride per mole activated saccharide) and WFI (target 5% v/v) were added to the reaction and the mixture was incubated for 3 to 6 hours at 23±2℃. This procedure reduced any unreacted aldehydes present on the saccharides. Then, the reaction mixture was diluted with sodium chloride 0.9%, and the diluted conjugation mixture was filtered using a 0.8 or 0.45 µm pre-filter.

[00137] Step 3: Ultrafiltration

[00138] The diluted conjugate mixture was concentrated and diafiltered on a 100 kDa MWCO ultrafiltration filter or a 300 kDa MWCO ultrafiltration filter with a minimum of 15 volumes of 0.9% sodium chloride or buffer. Also, a type and pH of the buffer used in the process varied depending on each of the serotypes.

[00139] Step 4: Sterile Filtration

[00140] The retentate after the ultrafiltration was sterile filtered (0.2 µm), and in-process controls (appearance, free protein, free saccharide, molecular size distribution, sterility, saccharide content, protein content, pH, endotoxin, residual

cyanide, residual DMSO, saccharide identity, TT identity, and CRM_{197} identity) were performed on the filtered conjugates. The final concentrate was refrigerated and stored at 2℃ to 8℃.

[00141] Example 3. Formulation of Multivalent Pneumococcal Conjugate Vaccine

[00142] The desired volumes of final bulk concentrates obtained from Example 2 were calculated based on the batch volume and the bulk saccharide concentrations. After the 0.85% sodium chloride (physiological saline), polysorbate 80, and succinate buffer were added to the pre-labeled formulation vessel, bulk concentrates were added. The preparation was then thoroughly mixed and sterile filtered through a 0.2 µm membrane. The formulated bulk was mixed gently during and following the addition of bulk aluminum phosphate. The pH was checked and adjusted if necessary. The formulated bulk product was stored at 2℃ to 8℃.

[00143] The following three types of multivalent pneumococcal conjugate vaccine formulations were prepared and named PCV20-13TT, PCV20-15TT, and PCV20-35TT, respectively.

[00144] PCV20-13TT included polysaccharide-conjugates prepared by conjugating each polysaccharide of the serotypes 1 and 3 to TT and each polysaccharide of the serotypes 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F to CRM_{197}.

[00145] PCV20-15TT included polysaccharide-conjugates prepared by conjugating each polysaccharide of the serotypes 1 and 5 to TT and each polysaccharide of the serotypes 3, 4, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F to CRM_{197}.

[00146] PCV20-35TT included polysaccharide-conjugates prepared by conjugating each polysaccharide of the serotypes 3 and 5 to TT and each polysaccharide of the serotypes 1, 4, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F to CRM_{197}.

[00147] The PCV20-13TT and PCV20-35TT vaccine composition obtained included 2.2 µg of each saccharide, except for serotype 6B at 4.4 µg; 10 µg to 15 µg of TT (for serotypes 1, 3, and 5) and 50 µg to 60 µg of CRM_{197}; 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; 4.25 mg of sodium chloride; about 295 µg of a succinate buffer solution; and about 100 µg of polysorbate 80 in the total

of 0.5 ml dose. The PCV20-15TT composition in a total dose of 0.5 ml included 5 µg to 10 µg of TT (for serotypes 1 and 5) and 50 µg to 60 µg CRM_{197}, respectively, with the other components and contents thereof identical to those of PCV20-13TT.

[00148] Example 4: Immunogenicty of Mono-Conjugates (Serotypes 8, 10A, 11A, and 15)

[00149] As noted above, the mixed carrier, 20-valent pneumococcal conjugate compositions described herein include pneumococcal serotypes not covered by the three pneumococcal conjugate vaccines currently available on the global market. These pneumococcal serotypes that are not part of the currently available vaccines include serotypes 8, 10A, 11A, and 15B. Mono-conjugates of serotypes 8, 10A, 11A, and 15B were prepared as described in Example 2. The immunogenicity of these mono-conjugates was tested *in vivo*. More specifically, rabbits (New Zealand white, female, 2-3kg) were immunized with the mono-conjugates at 0 and 2 weeks. Antibody titers were measured at 4 weeks (28 days) and shown in Figures 1A-D. As shown in the Figures 1A-D, each mono-conjugate of serotypes 8, 10, 11A, and 15B showed a dose-dependent antibody response *in vivo*, as measured by the geometric mean titer (GMT).

[00150] Example 5. Immunogenicity of 16-valent Pneumococcal Conjugate Vaccine

[00151] Mixed carrier, 16-valent pneumococcal conjugate compositions, including PCV16-13TT, PCV16-15TT, and PCV16-35TT, were prepared using the general method outlined in Example 3. A control mixed carrier, 16-valent pneumococcal composition was also prepared and comprised CRM_{197} conjugated to capsular polysaccharides from *Streptococcus pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 12F, 14, 18C, 19A, 19F, 22F, 23F, and 33F ("PCV16-CRM197"). These 16-valent pneumococcal conjugate compositions were tested for the ability to induce an immunogenic response in rabbits. These immunogenic effects were characterized by antigen-specific ELISA for serum IgG concentrations and by opsonophagocytic assay (OPA) for antibody function. New Zealand White rabbits were immunized intramuscularly at week 0 and week 3 with a dose of 5% higher than the planned human clinical dose of each polysaccharide (2.31 µg of each polysaccharide, except for 6B at 4.62 µg) in the formulation or the human dose (2.2 ug of each polysaccharide, except for 6B at 4.4 ug). Sera were sampled every 3 weeks post immunization. Both

concentrations showed the same results.

[00152] A serotype-specific immune reaction with respect to PCV16-CRM197, PCV16-13TT, PCV16-15TT, and PCV16-35TT compositions was evaluated by IgG ELISA and a complement-mediated MOPA that measures a functional antibody.

[00153] *4-1. PCV16-CRM197*

[00154] **Serotype specific IgG concentration measurement**

[00155] Capsular polysaccharides (PnPs) for each serotype were coated on a 96-well plate at 0.5 μg/well to 1 μg/well. An equivalent amount of serum was sampled from each subject and was pooled by group. The serum pool was serially diluted by 2.5 times with an antibody dilution buffer comprising Tween 20 and CWPS 5 μg/mL and then reacted at room temperature for 30 minutes. The plate was washed 5 times with a washing buffer and then pre-adsorbed and diluted serum 50 μl was added to the coated well plate, followed by incubation at room temperature for 2 hours to 18 hours. The well plate was washed in the same way and then goat anti-Rabbit IgG-alkaline phosphatase conjugates were added to each well, followed by incubation at room temperature for 2 hours. Plates were washed as described above and 1 mg/mL p-nitrophenylamine buffer as substrate was added to each well and then reacted at room temperature for 2 hours. The reaction was quenched by adding 50 μl of 3 M NaOH and absorbances at 405 nm and 690 nm were measured. As a comparative example, the commercially available, 13-valent vaccine (PREVNAR13) was subjected to the same procedure. The results are shown in Table 4.

[00156] Table 4. IgG concentration (U/mL) for 16 serotypes at 3 weeks after secondary immunization

Serotype	PREVNAR13	PCV16-CRM197	Serotype	PREVNAR13	PCV16-CRM197
1	320.99	379.99	12F	-	120.44
3	436.85	653.84	14	482.05	502.6
4	1820.49	1948.29	18C	1731.07	2915.55
5	466.09	380.18	19A	993.68	672.2
6A	1064.69	1643.6	19F	863.32	1054.3
6B	326.94	552.58	22F	1.33	678.45

Serotype	PREVNAR13	PCV16-CRM197	Serotype	PREVNAR13	PCV16-CRM197
7F	1010.79	833.11	23F	329.11	185.97
9V	715.40	433.33	33F	4.58	499.3

[00157] PCV16-CRM197 was found to lead to good levels of serotype specific IgG concentrations for all 16 serotypes. For PCV16-CRM197, the serotypes common to the PCV16-CRM197 and PREVNAR13 showed serotype-specific IgG concentrations equivalent to or higher than that of PREVNAR13 and each of the newly added serotypes 12F, 22F, and 33F also showed a good level of serotype specific IgG concentration.

[00158] **Functional Immunogenicity Test (MOPA)**

[00159] Antibody functions were evaluated by testing serum in a MOPA assay. *S. pneumoniae* MOPA strain stored at -70℃ or lower was diluted to the corresponding final dilution fold so that a concentration of each strain was about 50,000 CFU/mL. An equivalent amount of serum was sampled from each subject, pooled by group and 2-fold serially diluted so that 20 μl of serum remained in a U-bottom plate. After diluting the sample, 10 μl of the strain prepared for each serotype was mixed with the diluted sample, and the mixture was allowed to react at room temperature for 30 minutes so that *S. pneumoniae* and the antibody were well mixed. A mixture of pre-differentiated HL-60 cells and complement was added and reacted in a CO_2 incubator (37℃) for 45 minutes. The temperature was reduced to stop phagocytosis and 10 μl of the reaction solution was spotted onto an agar plate pre-dried for 30 to 60 minutes, and then allowed to be absorbed onto the plate for 20 minutes until drying. A 25 mg/mL TTC stock solution was added to a prepared overlay agar, and an antibody appropriate for the corresponding strain was added thereto. The mixture was thoroughly mixed, and then about 25 mL of the mixture was added onto the plate and hardened for about 30 minutes. The completely hardened plate was incubated in a CO_2 incubator (37℃) for 12 to 18 hours and then colonies were counted. MOPA titer was expressed as a dilution rate at which 50% killings were observed. As a comparative example, the commercially-available, 13-valent vaccine (PREVNAR13) was subjected to the same procedure. The results are shown in Table 5.

[00160] Table 5. MOPA titers for 16 serotypes at 3 weeks after secondary immunization

Serotype	PREVNAR13	PCV16-CRM197	Serotype	PREVNAR13	PCV16-CRM197
1	128	128	12F	4	1024
3	512	1024	14	2048	1024
4	2048	2048	18C	1024	2048
5	512	256	19A	4096	2048
6A	4096	4096	19F	2048	2048
6B	4096	4096	22F	16	4096
7F	2048	1024	23F	2048	1024
9V	1024	512	33F	32	1024

[00161] All serotypes showed an excellent level of functional immunogenicity in PCV16-CRM197. For PCV16-CRM197, the serotypes common to PCV16-CRM197 and PREVNAR13 showed functional immunogenicity equivalent to or better than that of PREVNAR13 and each of the newly added serotypes 12F, 22F, and 33F also showed a high level of functional immunogenicity.

[00162] *4-2. PCV16-13TT*

[00163] The serotype specific IgG concentration and functional immunogenicity titer were measured in the same manner as in 4-1, except that PCV16-13TT was used instead of PCV16-CRM_{197}, and the results are shown as follows.

[00164] **Serotype specific IgG concentration measurement**

[00165] Table 6. IgG concentration (U/mL) for 16 serotypes at 3 weeks after secondary immunization

Serotype	PREVNAR13	PCV16-13TT	Serotype	PREVNAR13	PCV16-13TT
1	276.92	844.48	12F	0.37	354.00
3	539.40	2980.73	14	254.59	582.61

Serotype	PREVNAR13	PCV16-13TT	Serotype	PREVNAR13	PCV16-13TT
4	1000.76	1698.00	18C	3266.87	5553.58
5	303.20	184.49	19A	681.62	1702.05
6A	533.35	532.02	19F	528.77	1998.83
6B	172.75	451.18	22F	0.74	1583.58
7F	726.27	3449.73	23F	576.63	367.71
9V	647.71	725.14	33F	0.25	977.02

[00166] When the capsular polysaccharides of serotypes 1 and 3 were conjugated to TT, they showed significantly increased levels of serotype specific IgG compared to that obtained when the serotypes were conjugated to CRM_{197}. Also, each of the capsular polysaccharides of serotypes 12F, 22F, and 33F conjugated to CRM_{197} showed a decent level of serotype specific IgG concentration and serotypes 4, 6B, 7F, 14, 18C, 19A, and 19F showed higher levels of serotype specific IgG concentrations than in PREVNAR13.

[00167] **Functional immunogenicity test (MOPA)**

[00168] Table 7. MOPA titers for 16 serotypes at 3 weeks after secondary immunization

Serotype	PREVNAR13	PCV16-13TT	Serotype	PREVNAR13	PCV16-13TT
1	102	645	12F	4	1625
3	813	3251	14	2048	4096
4	2580	3251	18C	4096	4096
5	813	406	19A	2580	5161
6A	4096	4096	19F	2048	5161
6B	4096	6502	22F	8	5161
7F	2580	6502	23F	4096	3251
9V	2048	3251	33F	4	3251

When the capsular polysaccharides of serotypes 1 and 3 were conjugated to TT, the functional immunogenicity improved compared to that obtained when they were conjugated to CRM_{197}. Also, each of the capsular polysaccharides of serotypes 12F, 22F, and 33F conjugated to CRM_{197} showed excellent functional immunogenicity and each of the capsular polysaccharides of serotypes 4, 6B, 7F, 9V, 14, 19A, and 19F showed better functional immunogenicity than in PREVNAR13.

[00169] *4-3. PCV16-15TT*

[00170] The serotype specific IgG concentration and functional immunogenicity titer were measured in the same manner as in 4-1, except that PCV16-15TT was used instead of PCV16-CRM_{197}, and the results are shown as follows.

[00171] **Serotype specific IgG concentration measurement**

[00172] Table 8. IgG concentration (U/mL) for 16 serotypes at 3 weeks after secondary immunization

Serotype	PREVNAR13	PCV16-15TT	Serotype	PREVNAR13	PCV16-15TT
1	276.92	1083.23	12F	0.37	303.99
3	539.40	901.37	14	254.59	493.06
4	1000.76	2655.28	18C	3266.87	4075.62
5	303.20	2645.56	19A	681.62	937.41
6A	533.35	1460.65	19F	528.77	1355.08
6B	172.75	603.87	22F	0.74	1874.55
7F	726.27	2285.92	23F	576.63	607.40
9V	647.71	663.37	33F	0.25	880.54

[00173] When the capsular polysaccharides of serotypes 1 and 5 were conjugated to TT, the serotype specific IgG concentration significantly increased compared to that obtained when they were conjugated to CRM_{197}. Also, each of the capsular polysaccharides of serotypes 12F, 22F, and 33F conjugated to CRM_{197} showed a high level of serotype specific IgG concentration and each of the capsular polysaccharides of serotypes 3, 4, 6A, 6B, 7F, 14, 18C, 19A, and 19F showed a higher level of serotype specific IgG concentration than in PREVNAR13.

[00174] **Functional immunogenicity test (MOPA)**

[00175] Table 9. MOPA titers for 16 serotypes at 3 weeks after secondary immunization

Serotype	PREVNAR13	PCV16-15TT	Serotype	PREVNAR13	PCV16-15TT
1	102	645	12F	4	1290
3	813	1290	14	2048	2580
4	2580	4096	18C	4096	3251
5	813	4096	19A	2580	2580
6A	4096	6502	19F	2048	4096
6B	4096	6502	22F	8	6502
7F	2580	4096	23F	4096	3251
9V	2048	1290	33F	4	2580

[00176] When the capsular polysaccharides of serotypes 1 and 5 were conjugated to TT, the functional immunogenicity improved compared to that obtained when they were conjugated to CRM_{197}. Also, each of the capsular polysaccharides of serotypes 12F, 22F, and 33F conjugated to CRM_{197} showed excellent functional immunogenicity and each of the capsular polysaccharides of serotypes 3, 4, 6A, 6B, 7F, and 19F showed a higher level of functional immunogenicity than in PREVNAR13.

[00177] *4-4. PCV16-35TT*

[00178] The serotype specific IgG concentration and functional immunogenicity titer were measured in the same manner as in 4-1, except that PCV16-35TT was used instead of PCV16-CRM_{197}, and the results are shown as follows.

[00179] **Serotype specific IgG concentration measurement**

[00180] Table 10. IgG concentration (U/mL) for 16 serotypes at 3 weeks after secondary immunization

Serotype	PREVNAR13	PCV16-35TT	Serotype	PREVNAR13	PCV16-35TT
1	242.33	367.13	12F	0.25	334.45

Serotype	PREVNAR13	PCV16-35TT	Serotype	PREVNAR13	PCV16-35TT
3	656.91	1837.36	14	320.12	1055.422
4	1305.61	1786.84	18C	2920.75	3665.59
5	408.78	1316.12	19A	652.67	409.17
6A	737.55	957.85	19F	411.07	534.19
6B	167.41	322.61	22F	1.15	1176.6
7F	808.75	1357.46	23F	742.55	408.88
9V	775.28	966.22	33F	0.25	855.55

[00181] When the capsular polysaccharides of serotypes 3 and 5 were conjugated to TT, the serotype specific IgG concentration significantly increased compared to that obtained when they were conjugated to CRM_{197}. Also, each of the capsular polysaccharides of the serotypes 12F, 22F, and 33F conjugated to CRM_{197} had good serotype IgG concentration and each of the capsular polysaccharides of serotypes 1, 4, 6A, 6B, 7F, 9V, 14, 18C, and 19F showed a higher level of serum specific IgG concentration than in PREVNAR13.

[00182] **Functional immunogenicity test (MOPA)**

[00183] Table 11. MOPA titers for 16 serotypes at 3 weeks after secondary immunization

Serotype	PREVNAR13	PCV16-35TT	Serotype	PREVNAR13	PCV16-35TT
1	128	256	12F	4	512
3	512	2048	14	2048	4096
4	2048	4096	18C	1024	4096
5	512	2048	19A	4096	4096
6A	4096	4096	19F	2048	4096
6B	4096	4096	22F	16	4096
7F	2048	2048	23F	2048	2048

Serotype	PREVNAR13	PCV16-35TT	Serotype	PREVNAR13	PCV16-35TT
9V	1024	2048	33F	32	2048

[00184] When the serotypes 3 and 5 were conjugated to TT, the functional immunogenicity improved compared to that obtained when they were conjugated to CRM_{197}. Also, each of the capsular polysaccharides of serotypes 12F, 22F, and 33F conjugated to CRM_{197} showed excellent functional immunogenicity and each of the capsular polysaccharides of serotypes 1, 4, 9V, 12F, 14, 18C, and 19F showed a higher level of functional immunogenicity than in PREVNAR13.

[00185] These results show that mixed carrier, multivalent pneumococcal capsular polysaccharide conjugate compositions induce immunogenicity equivalent to or better than the single carrier, pneumococcal capsular polysaccharide conjugate vaccine, PREVNAR13. They also unexpectedly show that the antibody response to serotypes 1, 3, and/or 5 conjugated to tetanus toxoid in the mixed carrier compositions were significantly enhanced as compared to the antibody responses against the same serotypes conjugated to CRM_{197} in the single carrier PREVNAR 13 vaccine. In addition, they show that a mixed carrier, multivalent pneumococcal capsular polysaccharide conjugate compositions successfully induce antibody responses against the added serotypes, 12F, 22F, and 33F, providing broader serotype protection than the pneumococcal capsular polysaccharide conjugate vaccines currently on the market.

[00186] Example 6. Immunogenicity of 20-valent Pneumococcal Conjugate Vaccine

[00187] Mixed carrier, 20-valent pneumococcal conjugate compositions, including PCV20-13TT, PCV20-15TT, and PCV20-35TT, were prepared using the general method outlined in Example 3. These 20-valent pneumococcal conjugate compositions were tested for the ability to induce an immunogenic response in rabbits. These immunogenic effects were characterized by antigen-specific ELISA for serum IgG concentrations and by opsonophagocytic assay (OPA) for antibody function. New Zealand White rabbits were immunized intramuscularly at week 0 and week 3 with a dose of 5% higher than the planned human clinical dose of each polysaccharide (2.31 µg of each polysaccharide, except for 6B at 4.62 µg) in the formulation or the human

dose (2.2 ug of each polysaccharide, except for 6B at 4.4 ug). Sera were sampled every 3 weeks post immunization. Both concentrations showed the same results.

[00188] A serotype-specific immune reaction with respect to PCV20-13TT, PCV20-15TT, and PCV20-35TT compositions was evaluated by IgG ELISA and a complement-mediated MOPA that measures a functional antibody.

[00189] *6-1. PCV20-35TT*

[00190] **Serotype specific IgG concentration measurement**

[00191] Capsular polysaccharides (PnPs) for each serotype were coated on a 96-well plate at 0.5 μg/well to 1 μg/well. An equivalent amount of serum was sampled from each subject and was pooled by group. The serum pool was serially diluted by 2.5 times with an antibody dilution buffer comprising Tween 20 and CWPS 5 μg/mL and then reacted at room temperature for 30 minutes. The plate was washed 5 times with a washing buffer and then pre-adsorbed and diluted serum 50 μl was added to the coated well plate, followed by incubation at room temperature for 2 hours to 18 hours. The well plate was washed in the same way and then goat anti-Rabbit IgG-alkaline phosphatase conjugates were added to each well, followed by incubation at room temperature for 2 hours. Plates were washed as described above and 1 mg/mL p-nitrophenylamine buffer as substrate was added to each well and then reacted at room temperature for 2 hours. The reaction was quenched by adding 50 μl of 3 M NaOH and absorbances at 405 nm and 690 nm were measured. As a comparative example, the commercially available, 13-valent vaccine (PREVNAR13) was subjected to the same procedure. The results are shown in Table 12.

[00192] Table 12. IgG concentration (U/mL) for 20 serotypes at 4 weeks after secondary immunization

Serotype	PREVNAR13	PCV20-35TT	Serotype	PREVNAR13	PCV20-35TT
1	8856.5	53170.2	11A	547.7	30690.8
3	5310.1	39965.2	12F	343.9	14071.5
4	8831.8	93626.2	14	15202.0	39933.6
5	3890.0	4241.1	15B	905.6	26347.9
6A	24412.1	13284.2	18C	104985.9	106523.3
6B	7528.5	4120.1	19A	13799.6	8035.9
7F	61054.8	46334.1	19F	19124.3	39824.7
8	591.4	47418.7	22F	201.2	57170.0
9V	20912.3	50598.3	23F	8109.6	9615.9
10A	477.9	39935.5	33F	191.0	35957.0

[00193] The serotypes in PCV20-35TT that are common to PCV20-35TT and PREVNAR13 showed serotype-specific IgG concentrations equivalent to or higher than that of PREVNAR13 and each of the newly added serotypes 8, 10A, 11A, 12F, 15B, 22F, and 33F also showed a good level of serotype specific IgG concentration.

[00194] **Functional Immunogenicity Test (MOPA)**

[00195] Antibody functions were evaluated by testing serum in a MOPA assay. *S. pneumoniae* MOPA strain stored at -70°C or lower was diluted to the corresponding final dilution fold so that a concentration of each strain was about 50,000 CFU/mL. An equivalent amount of serum was sampled from each subject, pooled by group and 2-fold serially diluted so that 20 μl of serum remained in a U-bottom plate. After diluting the sample, 10 μl of the strain prepared for each serotype was mixed with the diluted sample, and the mixture was allowed to react at room temperature for 30 minutes so that *S. pneumoniae* and the antibody were well mixed. A mixture of pre-differentiated HL-60 cells and complement was added and reacted in a CO_2 incubator (37℃) for 45 minutes. The temperature was reduced to stop phagocytosis and 10 μl of the reaction solution was spotted onto an agar plate pre-dried for 30 to 60 minutes, and then allowed to be absorbed onto the plate for 20 minutes until drying. A 25 mg/mL TTC stock solution was added to a prepared overlay agar, and an antibody appropriate for the corresponding strain was added thereto. The mixture was thoroughly mixed, and then about 25 mL of the mixture was added onto the plate and hardened for about 30 minutes. The completely hardened plate was incubated in a CO_2 incubator (37℃) for 12 to 18 hours and then colonies were counted. MOPA titer was expressed as a dilution rate at which 50% killings were observed. As a comparative example, the commercially-available, 13-valent vaccine (PREVNAR13) was subjected to the same procedure. The results are shown in Table 13.

[00196] Table 13. MOPA titers for 20 serotypes at 4 weeks after secondary immunization

Serotype	PREVNAR13	PCV20-35TT	Serotype	PREVNAR13	PCV20-35TT
1	78	236	11A	-	2055
3	758	3360	12F	-	598
4	2389	2725	14	1855	1398
5	372	757	15B	-	956

Serotype	PREVNAR13	PCV20-35TT	Serotype	PREVNAR13	PCV20-35TT
6A	6375	3099	18C	6549	3904
6B	6798	5000	19A	5131	664
7F	2872	2434	19F	5197	2848
8	-	1705	22F	55	11337
9V	2026	928	23F	2064	1568
10A	-	1472	33F	-	1531

[00197] All serotypes showed an excellent level of functional immunogenicity. The serotypes in PCV20-35TT that are common to PCV20-35TT and PREVNAR13 showed functional immunogenicity equivalent to or better than that of PREVNAR13 and each of the newly added serotypes 8, 10A, 11A, 12F, 15B, 22F, and 33F also showed a high level of functional immunogenicity.

[00198] *6-2. PCV20-13TT*

[00199] The serotype specific IgG concentration and functional immunogenicity titer were measured in the same manner as in 6-1, except that PCV20-13TT was used instead of PCV20-35TT, and the results are shown as follows.

[00200] **Serotype specific IgG concentration measurement**

[00201] Table 14. IgG concentration (U/mL) for 20 serotypes at 4 weeks after secondary immunization

Serotype	PREVNAR13	PCV20-13TT	Serotype	PREVNAR13	PCV20-13TT
1	8856.5	43835.7	11A	547.7	26830.0
3	5310.1	5251.4	12F	343.9	8849.8
4	8831.8	56988.7	14	15202.0	25402.0
5	3890.0	12600.1	15B	905.6	12057.3
6A	24412.1	19211.1	18C	104985.9	70935.6
6B	7528.5	11142.8	19A	13799.6	12017.3
7F	61054.8	37449.3	19F	19124.3	39708.2
8	591.4	39845.1	22F	201.2	35974.6
9V	20912.3	23632.9	23F	8109.6	9397.4
10A	477.9	21153.0	33F	191.0	33665.6

[00202] The serotypes in PCV20-13TT that are common to PCV20-13TT and PREVNAR13 showed serotype-specific IgG concentrations equivalent to or higher than that of PREVNAR13 and each of the newly added serotypes 8, 10A, 11A, 12F, 15B, 22F, and 33F also showed a good level of serotype specific IgG concentration.

[00203] **Functional immunogenicity test (MOPA)**

[00204] Table 15. MOPA titers for 20 serotypes at 4 weeks after secondary immunization

Serotype	PREVNAR13	PCV20-13TT	Serotype	PREVNAR13	PCV20-13TT
1	78	1519	11A	-	1882
3	758	5989	12F	-	845
4	2389	7520	14	1855	3819
5	372	672	15B	-	879
6A	6375	6154	18C	6549	8139
6B	6798	6112	19A	5131	2072
7F	2872	2358	19F	5197	6146
8	-	2785	22F	55	13123
9V	2026	5282	23F	2064	2131
10A	-	2173	33F	-	1254

[00205] All serotypes showed an excellent level of functional immunogenicity. The serotypes in PCV20-13TT common to PCV20-13TT and PREVNAR13 showed functional immunogenicity equivalent to or better than that of PREVNAR13 and each of the newly added serotypes 8, 10A, 11A, 12F, 15B, 22F, and 33F also showed a high level of functional immunogenicity.

[00206] *6-3. PCV20-15TT*

[00207] The serotype specific IgG concentration and functional immunogenicity titer were measured in the same manner as in 6-1, except that PCV20-15TT was used instead of PCV20-35TT, and the results are shown as follows.

[00208] **Serotype specific IgG concentration measurement**

[00209] Table 16. IgG concentration (U/mL) for 20 serotypes at 4 weeks after secondary immunization

Serotype	PREVNAR13	PCV20-15TT	Serotype	PREVNAR13	PCV20-15TT
1	8856.5	15443.5	11A	547.7	23066.5
3	5310.1	26383.7	12F	343.9	9830.6
4	8831.8	15534.5	14	15202.0	11218.9
5	3890.0	9591.5	15B	905.6	6268.9
6A	24412.1	35326.2	18C	104985.9	56224.9
6B	7528.5	10561.9	19A	13799.6	4660.7
7F	61054.8	54145.8	19F	19124.3	25815.4
8	591.4	38313.5	22F	201.2	31025.9
9V	20912.3	34801.5	23F	8109.6	11888.4
10A	477.9	47071.9	33F	191.0	24332.6

[00210] The serotypes in PCV20-15TT that are common to PCV20-15TT and PREVNAR13 showed serotype-specific IgG concentrations equivalent to or higher than that of PREVNAR13 and each of the newly added serotypes 8, 10A, 11A, 12F, 15B, 22F, and 33F also showed a good level of serotype specific IgG concentration.

[00211] **Functional immunogenicity test (MOPA)**

[00212] Table 17. MOPA titers for 20 serotypes at 4 weeks after secondary immunization.

Serotype	PREVNAR13	PCV20-15TT	Serotype	PREVNAR13	PCV20-15TT
1	78	700	11A	-	1854
3	758	1677	12F	-	889
4	2389	6170	14	1855	1983
5	372	1371	15B	-	948
6A	6375	2750	18C	6549	4810
6B	6798	7229	19A	5131	1879
7F	2872	2508	19F	5197	5089
8	-	2016	22F	55	6676
9V	2026	2081	23F	2064	1347
10A	-	1049	33F	-	2606

[00213] All serotypes showed an excellent level of functional immunogenicity. The serotypes in PCV20-15TT common to PCV20-15TT and PREVNAR13 showed functional immunogenicity equivalent to or better than that of PREVNAR13 and each of the newly added serotypes 8, 10A, 11A, 12F, 15B, 22F, and 33F also showed a high level of functional immunogenicity.

[00214] While one or more exemplary embodiments have been described in the specification, it will be understood by those of ordinary skill in the art that various changes in form and details may be made therein without departing from the spirit and scope of the inventive concept as defined by the following claims.

REFERENCES

[00215] The following references are cited in the application and provide general information regarding the technical field and provide assays and other details discussed in the application. The following references are incorporated herein by reference in their entirety.

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WHAT IS CLAIMED IS:

1. A mixed carrier, multivalent pneumococcal conjugate composition, comprising 20 different pneumococcal capsular polysaccharide-protein conjugates, wherein each pneumococcal capsular polysaccharide-protein conjugate comprises a protein carrier conjugated to a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae*, wherein the *Streptococcus pneumoniae* serotypes are selected from 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F, wherein the protein carrier is CRM_{197} or tetanus toxoid, wherein two of the capsular polysaccharides are conjugated to tetanus toxoid and the remaining capsular polysaccharides are conjugated to CRM_{197}, and wherein the two capsular polysaccharides that are conjugated to tetanus toxoid are selected from the group consisting of serotypes 1, 3, and 5.

2. The mixed carrier, multivalent pneumococcal conjugate composition of claim 1, wherein the capsular polysaccharides from serotypes 1 and 5 are conjugated to tetanus toxoid, and the capsular polysaccharides from serotypes 3, 4, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}.

3. The mixed carrier, multivalent pneumococcal conjugate composition of claim 1, wherein the capsular polysaccharides from serotypes 1 and 3 are conjugated to tetanus toxoid, and the capsular polysaccharides from serotypes 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}.

4. The mixed carrier, multivalent pneumococcal conjugate composition of claim 1, wherein the capsular polysaccharides from serotypes 3 and 5 are conjugated to tetanus toxoid, and the capsular polysaccharides from serotypes 1, 4, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F are conjugated to CRM_{197}.

5. The mixed carrier, multivalent pneumococcal conjugate composition of any one of the preceding claims, further comprising an adjuvant.

6. The mixed carrier, multivalent pneumococcal conjugate composition of claim 5, wherein the adjuvant is an aluminum-based adjuvant.

7. The mixed carrier, multivalent pneumococcal conjugate composition of claim 6, wherein the adjuvant is selected from the group consisting of aluminum phosphate, aluminum sulfate, and aluminum hydroxide.

8. The mixed carrier, multivalent pneumococcal conjugate composition of claim 7, wherein the adjuvant is aluminum phosphate.

9. The use of the mixed carrier, multivalent pneumococcal conjugate composition of any of the preceding claims for prophylaxis against *Streptococcus pneumoniae* infection or disease in a subject.

10. A vaccine comprising the mixed carrier, multivalent pneumococcal conjugate composition of any one of claims 1-8 and a pharmaceutically acceptable excipient.

11. A method for prophylaxis of *Streptococcus pneumoniae* infection or disease in a subject, the method comprising administering a prophylactically effective amount of the mixed carrier, multivalent pneumococcal conjugate composition of any one of claims 1-8 or the vaccine of claim 10 to the subject.

12. The method of claim 11, wherein the subject is a human who is at least 50 years old and the disease is pneumonia or invasive pneumococcal disease (IPD).

13. The method of claim 11, wherein the subject is a human who is at least 6 weeks old and the disease is pneumonia, invasive pneumococcal disease (IPD), or acute otitis media (AOM).

14. The method of claim 13, wherein the subject is 6 weeks to 5 years of age, 2 to 15 months of age, or 6 to 17 years of age.

15. The use of claim 9 or the method of claim 11, wherein the subject is a human.

16. The method of any one of claims 11-15, wherein the mixed carrier, multivalent pneumococcal conjugate composition or the vaccine is administered by intramuscular injection.

17. The method of any one of claims 11-16, wherein the mixed carrier, multivalent pneumococcal conjugate composition or the vaccine is administered as part of an immunization series.

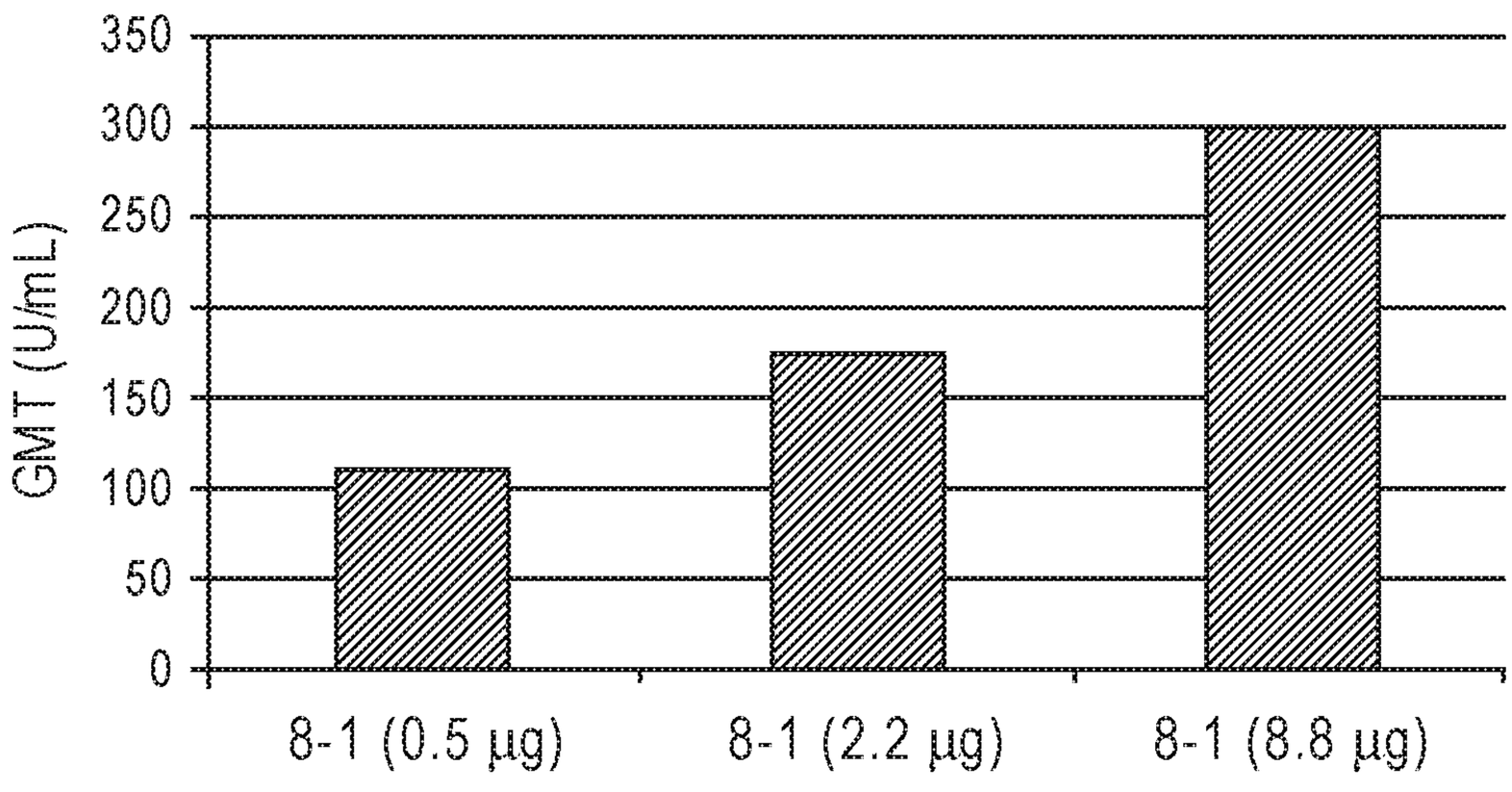
Mono-conjugate of Serotype 8
GMT (U/mL)
350
300
250
200
150
100
50
0
8-1 (0.5 µg)
8-1 (2.2 µg)
8-1 (8.8 µg)

FIG. 1A

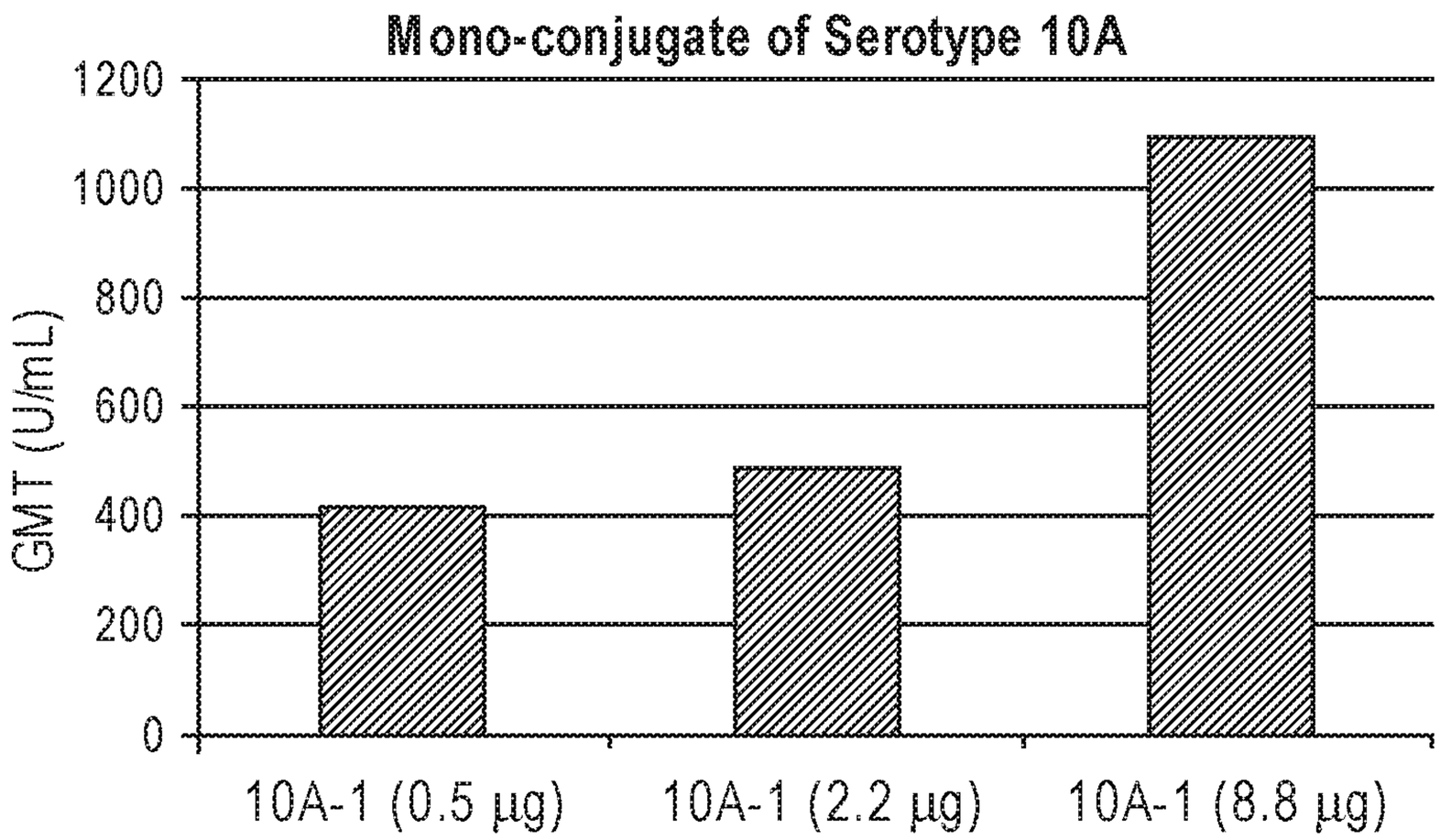
Mono-conjugate of Serotype 10A
GMT (U/mL)
1200
1000
800
600
400
200
0
10A-1 (0.5 µg)
10A-1 (2.2 µg)
10A-1 (8.8 µg)

FIG. 1B

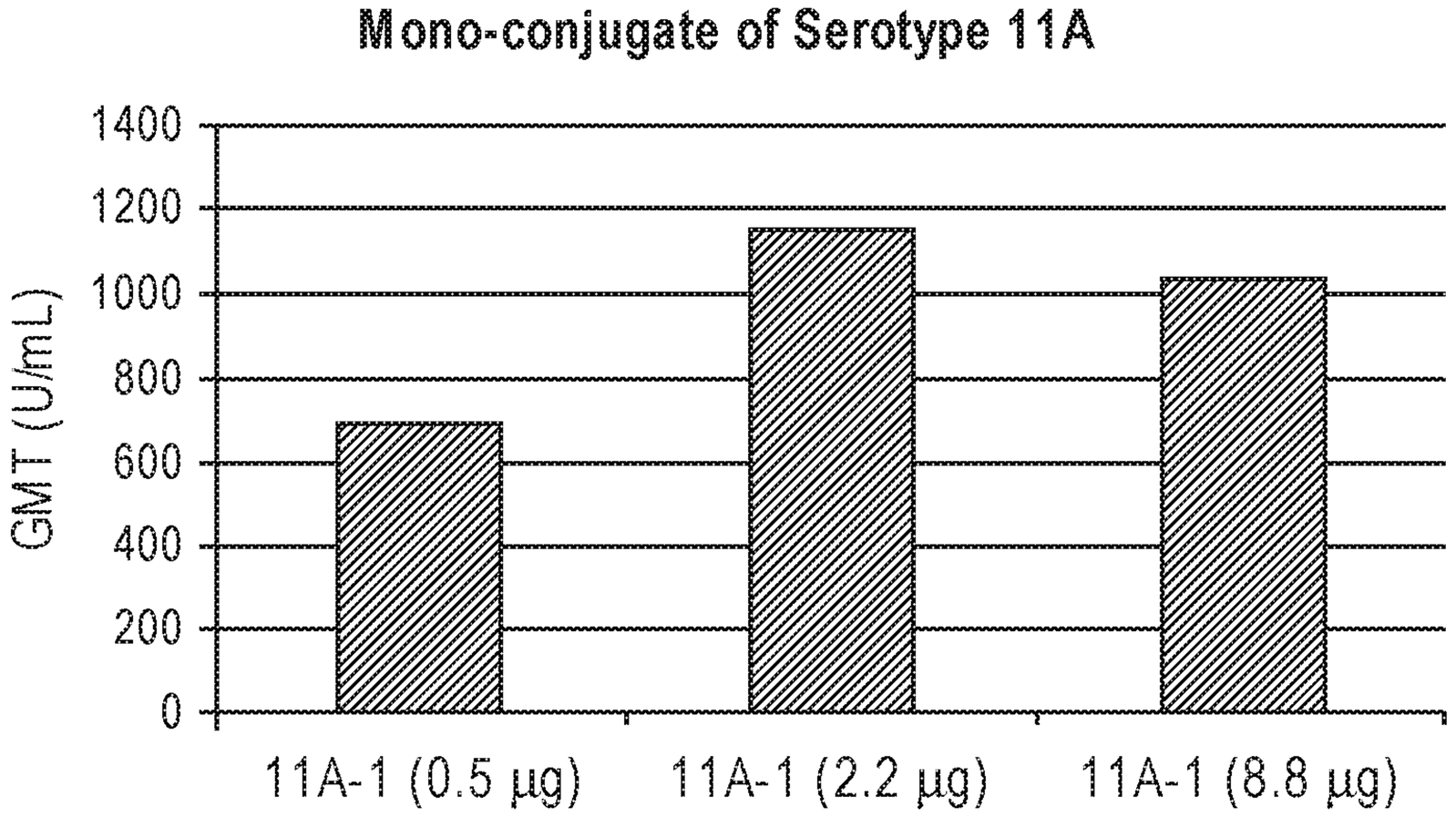
Mono-conjugate of Serotype 11A
GMT (U/mL)
1400
1200
1000
800
600
400
200
0
11A-1 (0.5 µg)
11A-1 (2.2 µg)
11A-1 (8.8 µg)

FIG. 1C

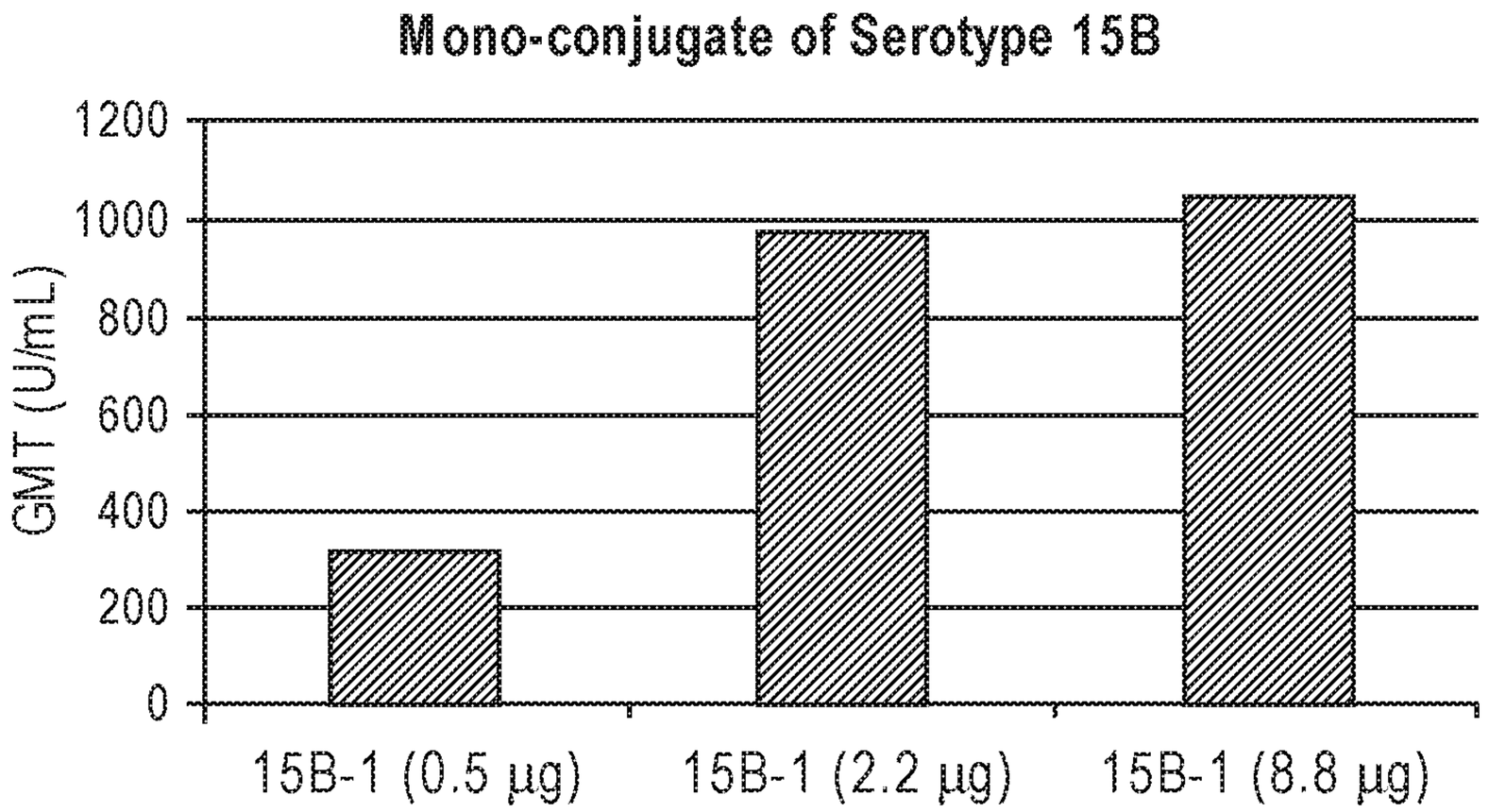
Mono-conjugate of Serotype 15B
GMT (U/mL)
1200
1000
800
600
400
200
0
15B-1 (0.5 µg)
15B-1 (2.2 µg)
15B-1 (8.8 µg)

FIG. 1D

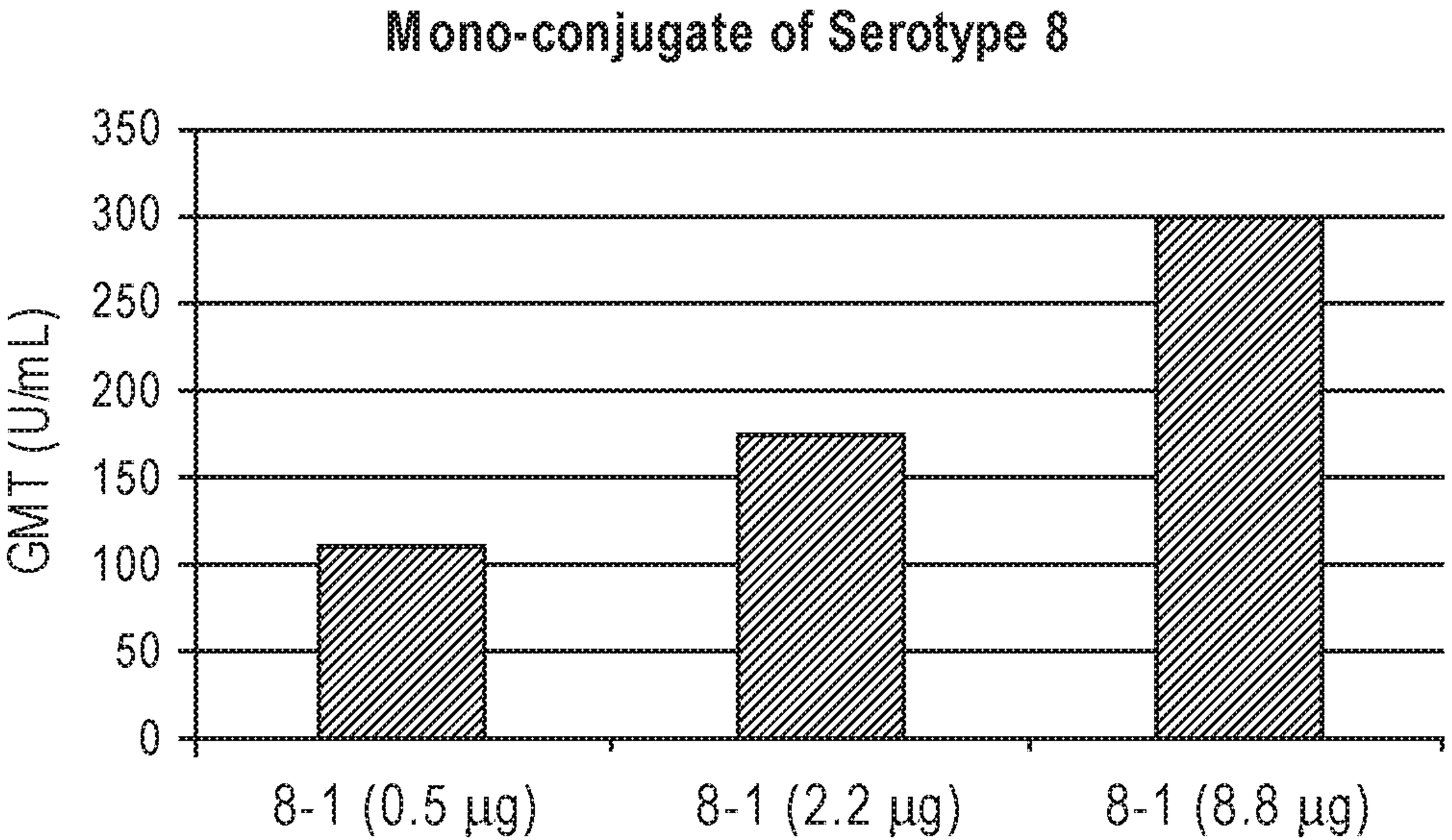
Mono-conjugate of Serotype 8
GMT (U/mL)
350
300
250
200
150
100
50
0
8-1 (0.5 µg)
8-1 (2.2 µg)
8-1 (8.8 µg)

FIG. 1A