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Saccharide/protein conjugate vaccines for *Bordetella* species: preparation of saccharide, development of new conjugation procedures, and physico-chemical and immunological characterization of the conjugates

Joanna Kubler-Kielb[1*], **Evgeny Vinogradov**[2], **Gil Ben-Menachem**[1], **Vince Pozsgay**[1], **John B. Robbins**[1], and **Rachel Schneerson**[1]

1*National Institute of Child Health and Human Development, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD, 20892, USA*

2*Institute for Biological Sciences, National Research Council, 100 Sussex Dr., Ottawa ON, K1A 0R6, Canada*

Abstract

Bordetellae are Gram-negative bacilli causing respiratory tract infections of mammals and birds. Clinically important are *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. *B. pertussis* vaccines have been successful in preventing pertussis in infants and children. Veterinary vaccines against *B. bronchiseptica* are available, but their efficacy and mode of action are not established. There is no vaccine against *B. parapertussis*. Based on the concept that immunity to non-capsulated Gram-negative bacteria may be conferred by serum IgG anti-LPS we studied chemical, serological and immunological properties of the O-specific polysaccharides (O-SP) of *B. bronchiseptica* and *B. parapertussis* obtained by different degradation procedures. One type of the *B. parapertussis* and two types of *B. bronchiseptica* O-SP were recognized based on the structure of their non-reducing end saccharide; no cross-reaction between the two *B. bronchiseptica* types was observed. Competitive inhibition assays showed the immunodominance of the non-reducing end of these O-SP. Conjugates of *B. bronchiseptica* and *B. parapertussis* O-SP were prepared by two methods: using the Kdo residue exposed by mild acid hydrolysis of the LPS or the core glucosamine residue exposed by deamination of the LPS, for binding to an aminooxylated protein. Both coupling methods were carried out at a neutral pH, room temperature, and in a short time. All conjugates, injected as saline solutions at a fraction of an estimated human dose, induced antibodies in mice to the homologous O-SP. These methodologies can be applied to prepare O-SP-based vaccines against other Gram-negative bacteria.

Keywords

bordetella; LPS; vaccine; conjugate

*Corresponding author: Tel: (301)-496-6141, Fax: (301)-480-3442, Email: kielbj@mail.nih.gov.

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1. Introduction

Vaccination has been proven effective for preventing infection of humans and animals by *Bordetella* spp. Killed whole cell and subunit vaccines have been used to immunize infants and children against *Bordetella pertussis*, the cause of pertussis, a highly contagious, severe respiratory infection especially of young children. No vaccine is available against *B. parapertussis* which causes a milder and less frequent form of pertussis in humans and a respiratory infection in sheep [1]. *B. pertussis* is confined to humans. Infection with *B. parapertussis* does not confer immunity to pertussis [2]. *B. bronchiseptica* causes serious respiratory infections in a variety of hosts: kennel cough in dogs, atrophic rhinitis in piglets, bronchopneumonia in rabbits and guinea pigs [3]. Rarely, *B. bronchiseptica* infects humans, mostly young children, animal handlers and immuno-compromised individuals [4]. Cellular and subcellular veterinary vaccines are available for this pathogen but they are of limited efficacy [5,6,7]. Among all bordetellae only *B. pertussis* expresses pertussis toxin [8,9].

Serum IgG anti-LPS has been shown to confer immunity to Gram-negative bacteria [10,11, 12]. Monoclonal antibodies to *B. pertussis* LPS were shown to have complement-dependent bactericidal activity [13]. The LPS of all three bordetellae is of low molecular weight, < 10 kDa, rendering their isolated saccharides non-immunogenic. *B. pertussis* LPS is comprised of a Lipid A domain and a branched dodecasaccharide, composed of unusual sugars with free amino and carboxylic groups [14]. By SDS-PAGE, *B. pertussis* LPS shows two bands, A and B. Band B contains lipid A and a branched nonasaccharide core, Band A contains further substituted Band B by a trisaccharide unit. An almost identical core structure to *B. pertussis* was reported for *B. bronchiseptica* LPS [15]. The core region of *B. parapertussis* has a shorter heptasaccharide structure and does not contain the Band A trisaccharide [14]. In contrast to *B. pertussis*, which produces no O-specific polysaccharide (O-SP), *B. bronchiseptica* and *B. parapertussis* synthesize short O-SP containing about 12– 18 sugars. Initially, it was reported that the O-SP of both these organisms is identical and composed of a linear polymer of 1,4-linked 2,3-diacetamido-2,3-dideoxy-α-galacturonic acid (GalNAc3NAcA) [16]. Later, serological differences between *B. bronchiseptica* strains were ascribed to the structural variations of the non-reducing end-groups of the O-SP [17,18]. Similar observations were made for the serotypes Ogawa and Inaba of *Vibrio cholerae* O1 that differ only by a methyl group at the non-reducing end of Ogawa [19] and for *Salmonella* O40 and O43 serotypes [18].

The objectives of this study were to define and correlate structural and immunological data of *B. bronchiseptica* and *B. parapertussis* O-SP to enable proving experimental vaccines of wide coverage. Different conjugation methods were devised and the serum antibody responses elicited by these investigational vaccines in young outbred mice were assayed.

2. Materials and methods

2.1. Bacteria and cultivation

B. bronchiseptica ATCC 10580, Rb50 (ATCC BAA-588), and *B. parapertussis* ATCC 15989 were obtained from ATCC (Manassas, VA). *B. bronchiseptica* 15374, 3145 and *B. parapertussis* 12822 were obtained from Dr. M. Perry (NRC, Ottawa, Canada). Bacteria were grown on Bordet-Gengou (BG) agar plates and transferred to Stainer-Scholte (S-S) media [20]. After 16 to 24 hours of cultivation at 37 °C with shaking in buffled flasks, bacteria were harvested by centrifugation, killed by boiling for 1 h and stored at −20 °C for LPS extraction. *Haemophilus ducreyi* LPS used as a control was a gift from Teresa Lagergård (Göteborg, Sweden).

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2.2. Oligosaccharides

LPS was isolated by hot phenol-water extraction and purified by enzyme treatment and ultracentrifugation as described [21]. Two methods were used for LPS degradation: (1) LPS (100 mg) was heated in 10 ml 1 % acetic acid for 60 min at 100 °C, ultracentrifuged at 35000 rpm for 5h at 4 °C and the carbohydrate-containing supernatant was passed through a 1 ×100 cm column of BioGel P-4 in pyridine/acetic acid/water buffer (4/8/988 ml) monitored with a Knauer differential refractometer. Twenty eight mg of O-SP were recovered from the void volume; (2) LPS (100 mg) was deaminated in 18 ml of a solution containing 30% acetic acid/ 5% sodium nitrite/water (1/1/1) for 6 h, at room temperature, on a magnetic stirrer, followed by ultracentrifugation [22]. The supernatant was freeze-dried and purified on the BioGel P-4 column as above. Twenty three mg of saccharide fraction, designated as O-SP_{deam}, were recovered from the void volume.

For isolation of oligosaccharides used in competitive inhibition assays, LPS was dissolved in anhydrous HF (100 mg LPS, 8 ml HF, 25°C, 24 h), then evaporated at room temperature, in a hood, on a plastic Petri dish. The residue was dissolved in pyridine/acetic acid/water buffer (4/8/988 ml), passed through a 2.5 × 80 cm column of Sephadex G-50 and further purified by HPLC on 250 × 9.5 mm Phenomenex Aqua column in 0.1 % TFA (for the first 10 min), then with a gradient of 0–50 % acetonitrile in 0.1 % TFA. The effluent was monitored by UV 220 nm absorption. Isolated oligosaccharides contained average 15 repeats of diacetamidouronic acid (average molecular mass of 4158 Da, as assayed by MALDI-TOF) and were chemically characterized previously [17].

2.3. Conjugation

2.3.1. O-SP conjugates—Bovine serum albumin (BSA, Sigma, St. Louis, MO) was derivatized to aminooxylated derivatives in a two step procedure: (1) BSA was treated with succinimidyl 3-(bromoacetamido)propionate (SBAP, Pierce, Pittsburgh, PA) to introduce thiol-reactive bromoacetamido moieties (BSA-Br); (2) BSA-Br was coupled with *O*-(3-thiopropyl)hydroxylamine, a heterobifunctional linker, to form the aminooxylated protein through stable thioether linkages (BSA-ONH_2) as described [23]. For conjugation, BSA-ONH_2 (5 mg) was reacted with 10 mg of O-SP of *B. bronchiseptica* 10580 (Bb10580), *B bronchiseptica* Rb50 (BbRb50) or *B. parapertussis* 10595 (Bpp15989) in 1.5 ml Buffer A (PBS, 0.1% glycerol, 5 mM EDTA), at pH 5.7, for 15 hours with stirring, at room temperature. The reaction mixture was passed through a 1 × 100 cm Sephadex G-100 column in 0.2 M NaCl and the void volume fraction characterized by protein concentration, immunodiffusion, SDS-PAGE and MALDI-TOF spectroscopy. The conjugates were designated BSA-ONH_2/Bb10580 (#1), BSAONH_2/ BbRb50 (#2) and BSA-ONH_2/Bpp15989 (#3).

2.3.2. O-SP_{deam} conjugates—BSA-ONH_2 (5 mg) were reacted with 10 mg of O-SP_{deam} of the listed strains using the described conditions. The products were designates BSA-ONH_2/$Bb10580_{deam}$ (#4), BSA-ONH_2/$BbRb50_{deam}$ (#5) and BSA-ONH_2/$Bpp15989_{deam}$ (#6).

2.4. Immunization

5–6 weeks-old female NIH Swiss Webster mice were injected s.c. 3 times at 2 weeks intervals with 2.5 μg saccharide as a conjugate in 0.1 ml PBS. Groups of 10 mice were exsanguinated 7 days after the second or third injections [24]. Controls received PBS. Hyperimmune sera against *B. bronchiseptica* strains 10580 and Rb50, and against *B. parapertussis* strain 15989 were prepared with heat-killed whole bacteria as described [25].

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2.5. Analytic

Protein concentration was measured by the method of Lowry [26]. SDS-PAGE used 14% gels according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Double immunodiffusion was performed in 1 % agarose gel in PBS. Endotoxic activity was measured by the limulus amebocyte lysate assay as described by the manufacturer (Cambrex, Walkersville, MD).

2.6. Spectroscopy

MALDI-TOF mass spectra of the derivatized carrier proteins and of the conjugates were obtained with an OmniFlex MALDI-TOF instrument (Bruker Daltonics, Billerica, MA) operated in the linear mode. Samples for analysis were desalted and 1 µl, mixed with 20 µl of sinnapinic acid matrix made in 30% CH_3CN and 0.1% trifluroacetic acid. Next, 1 µl of mixture was dried on the sample stage and placed in the mass spectrometer. NMR spectra were recorded at 30°C in D_2O on a Varian UNITY INOVA 600 instrument using acetone as reference for both proton (2.225 ppm) and carbon (31.5 ppm) spectra. Varian standard programs COSY, NOESY (mixing time of 400ms), TOCSY (spinlock time 120 ms), HSQC, and gHMBC (long-range transfer delay 70 ms) were used [17].

2.7. Antibodies

Serum IgG antibodies were measured by ELISA [27]. Nunc Maxisorb plates were coated with *B. bronchiseptica* 10580, Rb50 or *B. parapertussis* 15989 LPS at 5 µg/ml in PBS containing 1% trichloroacetic acid and assayed as described [28], with the modification of 1 % HSA in PBS instead of BSA was used in a blocking step. The optimal concentration of the coating antigen was determined by checkerboard titration. PA MRX Dynatech reader was used. Antibody levels were calculated relative to the hyperimmune standard serum diluted 1:20,000 for *B. bronchiseptica* 10580; 1:15,000 for *B. bronchiseptica* Rb50 and 1:10,000 for *B. parapertussis* 15989, each assigned a value of 1000 ELISA Units (EU). Results were computed with an ELISA data processing program provided by the Biostatistics and Information Management Branch, CDC [29].

Competitive inhibition ELISA was done by incubating hyperimmune mice sera, diluted to the concentration that gave an A_{405} absorption of about 1.0, with 10 or 50 µg of inhibitor per well, for 1 h at 37 °C and overnight at 4°C. The assay was then continued as above. Sera at the same dilution with and without inhibitor were compared. Percent inhibition was defined as (A_{405} adsorbed serum/ A_{405} non-adsorbed serum) × 100%.

3. Results

3.1. Chemical characterization of LPS

B. bronchiseptica 10580, 15374, 5137 and *B. parapertussis* 15989 and 12822 strains had an alanine substituent at the terminal non-reducing GalNAc3NAcA residue of the O-SP and thus was designated as an ‘ala-type’. *B. bronchiseptica* Rb50 had an O-methyl lactic acid substituent at the terminal non-reducing end of the O-SP, thus representing a ‘lac-type’ (Figure 1). The second GalNAc3NAcA residue from non-reducing-end (penultimate) was present as a free acid in *B. bronchiseptica* 10580, Rb50 and 5137 and was amidated in *B. bronchiseptica* 15374 and in *B. parapertussis* 15989 and 12822 strains.

3.2. Immunological characterization of LPS

Immunodiffusion studies showed the following: (1) no cross-reaction between ‘ala-type’ and ‘lac-type’ LPS; (2) the ‘ala-type’ LPS from *B. bronchiseptica* 10580 and *B. parapertussis* 15989 were cross-reactive but with a spur of *B. bronchiseptica* 10580 LPS over that of *B.*

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parapertussis 15989 when tested against *B. bronchiseptica* 10580 antiserum. Using *B. parapertussis* 15989 serum, a spur of *B. parapertussis* 15989 LPS over that of *B. bronchiseptica* 10580 was seen (Figure 2). These two strains differ in their penultimate residue, being a free acid in *B. bronchiseptica* 10580 LPS and amidated in *B. parapertussis*15989. The LPS of *B. bronchiseptica* 15374, which like *B. parapertussis* 15989 LPS was an 'ala-type' and had amidated penultimate sugar, showed a line of identity with *B. parapertussis* 15989 LPS tested against anti-*B. parapertussis* 15989. Competitive inhibition ELISA corroborated the immunodiffusion data. Oligosaccharides of 'ala-type' did not inhibit binding of antibodies to 'lac-type' LPS (Table 1) and conversely, oligosaccharides of 'lac-type' did not inhibit binding of antibodies to 'alatype' LPS. Serum raised against *B. parapertussis* 15989 of 'ala-type' LPS was inhibited only 50% by *B. bronchiseptica* 10580 oligosaccharides also of 'ala-type'. Conversely, serum raised against *B. bronchiseptica* 10580 LPS was inhibited only 50% by *B. parapertussis* 15989 'oligosaccharides. This partial inhibition may be ascribed to the amidation of penultimate sugar in *B. parapertusis* 15989 LPS and lack of it in *B. bronchiseptica* 10580 LPS.

3.3. Characterization of conjugates

Schemes for preparing conjugates by derivatization of BSA with *O*-(3-thiopropyl) hydroxylamine in a two step procedure followed by a reaction with a carbonyl group of either acetic acid hydrolyzed (Conjugate #1,2,3) or deaminated (Conjugate #4,5,6) O-SP to form a stable oxime linkage are shown in Figure 3. Both procedures yielded high molecular mass conjugates, assayed by MALDI mass spectrometry (Table. 2). The number of O-SP chains per protein was calculated based on the known molecular mass of the aminoxylated BSA, the O-SP chains and the conjugate (Figure 4). For example, the average molecular mass of the aminooxylated BSA was 74.1 kDa, the average molecular mass of the O-SP from *B. bronchiseptica* strain 10580 was 6588 Da and that of the conjugate of 135 kDa (135 kDa - 74.1 kDa / 6.588 kDa) leading to an estimate of 9 O-SP chains per one BSA molecule (Conjugate #1). The average molecular mass of deaminated O-SP (O-SP_{deam}) from the same strain was 5108 Da, and that of the conjugate was 130 kDa. Therefore we estimated 11 O-SP chains per one BSA molecule (Conjugate #4).

The amount of saccharide used for conjugation was in excess (twice the weight of protein) to ensure maximal binding. The conjugate yield was 65–75% for the protein and 30–35% for the sugar. All conjugates had < 5 EU/μg, endotoxin levels, compared to 15000 EU/μg of *B. bronchiseptica* 10580 LPS.

3.4. Antibodies to O-SP

The conjugates varied in their O-SP type ('ala-type' or 'lac-type'), the chain length (O-SP_{deam} is shorter by about 1700 Da than the O-SP and in the density of the O-SP/O-SP_{deam} per protein (9–14 chains per BSA molecule), Table 2. All conjugates reacted with the hyperimmune sera raised against the homologues strains and with the anti-protein serum by a line of identity. All conjugates were immunogenic in mice (Table 2) and the following observations were made: (1) antibodies to 'ala-type': Conjugate #4 of *B. bronchiseptica* 10580, made with O-SP_{deam} was more immunogenic than Conjugate #1 made with *B. bronchiseptica* 10580 O-SP (GM=55 *vs* 4.9; *P*=0.01); Conjugate #6 of *B. parapertussis* 15989 made with O-SP_{deam} also induced higher antibody levels than Conjugate #3, prepared with *B. parapertussis* 15989 O-SP but the difference was not statistically significant (GM=15.6 *vs* 1.2; *P* =NS); (2) antibodies to 'lac-type': conjugate #2 of *B. bronchisepti*ca Rb50 made with O-SP was more immunogenic than Conjugate #5 made with *B. bronchisepti*ca Rb50 O-SP_{deam} (GM = 132 vs 3.5; *P*< 0.001); (3) only one of six conjugates, #4 of *B. bronchiseptica* 10580_{deam} cross-reacted with *B. parapertussis* 15989 LPS (both 'ala-type').

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4. Discussion

Immunization with whole cell and subunit vaccines has been used to protect humans against *B. pertussis* and animals against *B. bronchiseptica*. The licensed pertussis vaccines confer incomplete efficacy on an individual basis, probably because pertussis toxin antibodies do not kill the organism directly, however herd immunity contributes to the almost complete protection with the wide vaccine usage [46,1]. An additional component inducing bactericidal antibodies, such as anti-LPS, could improve pertussis vaccine individual effectiveness. Veterinary vaccines against *B. bronchiseptica* are available but their efficacy is limited and challenge studies were performed with the homologues strains only [47,5]. Several formulations are licensed, for instance Bronchi-Shield (Fort Dodge, Overland Park, KS), a Canine Adenovirus Type 2-Parainfluenza-Bordetella Bronchiseptica Vaccine, composed of modified live viruses and avirulent live bacteria, used for vaccination of healthy dogs and puppies eight weeks of age or older; Bronchicine (Pfeizer, NY), prepared from *B. bronchiseptica* extracts; or Protex-Bb (Intervet, Millsboro, DE), an intranasal vaccine containing avirulent live *B. bronchiseptica*, recommended for vaccination of healthy kittens and cats. Our studies are predicated on the assumption that LPS-based *Bordetella* vaccines could be of benefit.

O-SP-based vaccines were immunogenic in adults and in children [11,27]. *Shigella sonnei* O-SP conjugates were effective in adults [10]. Monoclonal antibodies to the core oligosaccharide of *B. pertussis* LOS exhibited bactericidal activity and reduced colonization with these bacteria in the lungs and tracheas of mice [13]. We hypothesize that the O-SP from *B. parapertussis* and *B. bronchiseptica* conjugated to an immunogenic carrier protein will stimulate production of protective levels of LPS antibodies. Vaccine of *B. pertussis* LPS prepared according to our scheme could add to protection afforded by current pertussis vaccine.

In this work we provided immunological verification to previously described structural variations at the terminal non-reducing end of *B. bronchiseptica* O-SP [18] and expanded the studies. A similar serospecifity attributed to the differences in methylation of the non-reducing end sugar of the O-SP was observed in *V. cholerae* O1 Ogawa and Inaba [19]. The terminal residues of *B. bronchiseptica* O-SP were shown to be immunodominant as no cross reaction between the two types: the ‘ala-type’, and the ‘lac-type’, was observed. *B. parapertussis* O-SP was described previously as structurally identical to that of *B. bronchiseptica*, a homopolymer of 1,4-linked 2,3-diacetamido-2,3-dideoxy-α-galacturonic acid (GalNAc3NAcA) [16]. *B. parapertussis* strains analyzed here expressed this homopolymer chain and belonged exclusively to the ‘ala-type’. *B. parapertussis* O-SP differed however from some *B. bronchiseptica* O-SP in the amidation of the penultimate non-reducing end residue. Immunodiffusion and competitive inhibition ELISA showed that this difference influences serospecificity. Immunogenicity of several types of O-SP-protein conjugate corroborated this assertion. High levels of antibodies were induced only to the homologues strains. Conjugates of ‘lac-type’ O-SP did not induce antibodies to ‘ala-type’ O-SP of the same species and conjugates prepared with *B. parapertussis* O-SP did not induce antibodies to *B. bronchiseptica* O-SP, though both belonged to the same ‘ala-type’ but differed in the amidation of the penultimate non-reducing end sugar of the O-SP. Survey of disease isolates will determine the number of O-SP types to be included in a comprehensive *B. bronchiseptica* vaccine.

We report new methods for binding O-SP to a carrier protein by one-point attachment at their reducing end. Many protocols have been proposed for synthesizing this type of conjugates with reductive amidation being the most popular [30,31,32,33,34]. The choice of conjugation methods is restricted by the pH and temperature sensitivities of both vaccine components. The types of chemical linkages between the O-SP and the protein as well as the saccharide chain

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length and the chain density on the protein were shown to influence the serum antibody responses to both vaccine components [35,36,37,38,39,40]. Here we studied two conjugation methods of O-SP either by their Kdo or their glucosamine residues. Conjugation was performed between the carbonyl group on the terminal reducing end of the O-SP and an amiooxy group of a bifunctional linker bound to the protein [23,41,42,43]. The carbonyl group was introduced into the O-SP either by deamidation of the GlcN, a first non-reducing end sugar of Band B or by the Kdo released by acetic acid hydrolysis, proposed to undergo degradation to carbonyl containing anhydro-Kdo [44,45,46]. This is an efficient reaction carried out under mild conditions and in a short time. Conjugates thus prepared preserved the external non-reducing end of the O-SP and induced antibodies to both conjugate components. This method can be used for preparing O-SP-based clinical vaccines.

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Abbreviations

GM, geometric mean; SBAP, *N*-succinimidyl 3-(bromoacetamido) propionate; GalNAc3NAcA, 2,3-diacetamido-2,3-dideoxy-L-galacturonic acid; Buffer A, PBS, 0.1% glycerol, 5 mM EDTA, pH 7.4; s.c., subcutaneously.

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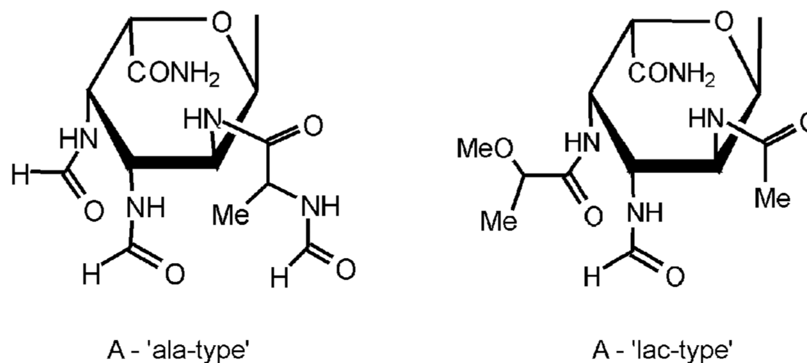
O-SP NON-REDUCING END SACCHARIDES



Bordetella bronchiseptica strain 10580 ('ala-type') O-SP

2-N-(N-formyl-L-alanyl)-3,4-di-N-formyl-2,3,4-triamino-2,3,4-trideoxy-α-L-GalA(NH_2)-[(1→4)-α-L-GalNAc3NAcA-]$_n$→→CORE

Bordetella bronchiseptica strain Rb50 ('lac-type') O-SP

2-N-acetyl-3-N-formyl-4-N-(2-methoxypropanoyl)-2,3,4-triamino-2,3,4-trideoxy-α-L-GalA(NH_2)-[(1→4)-α-L-GalNAc3NAcA-]$_n$→→CORE

Fig. 1.

Non-reducing end-group types of *B. bronchiseptica* O-SP.

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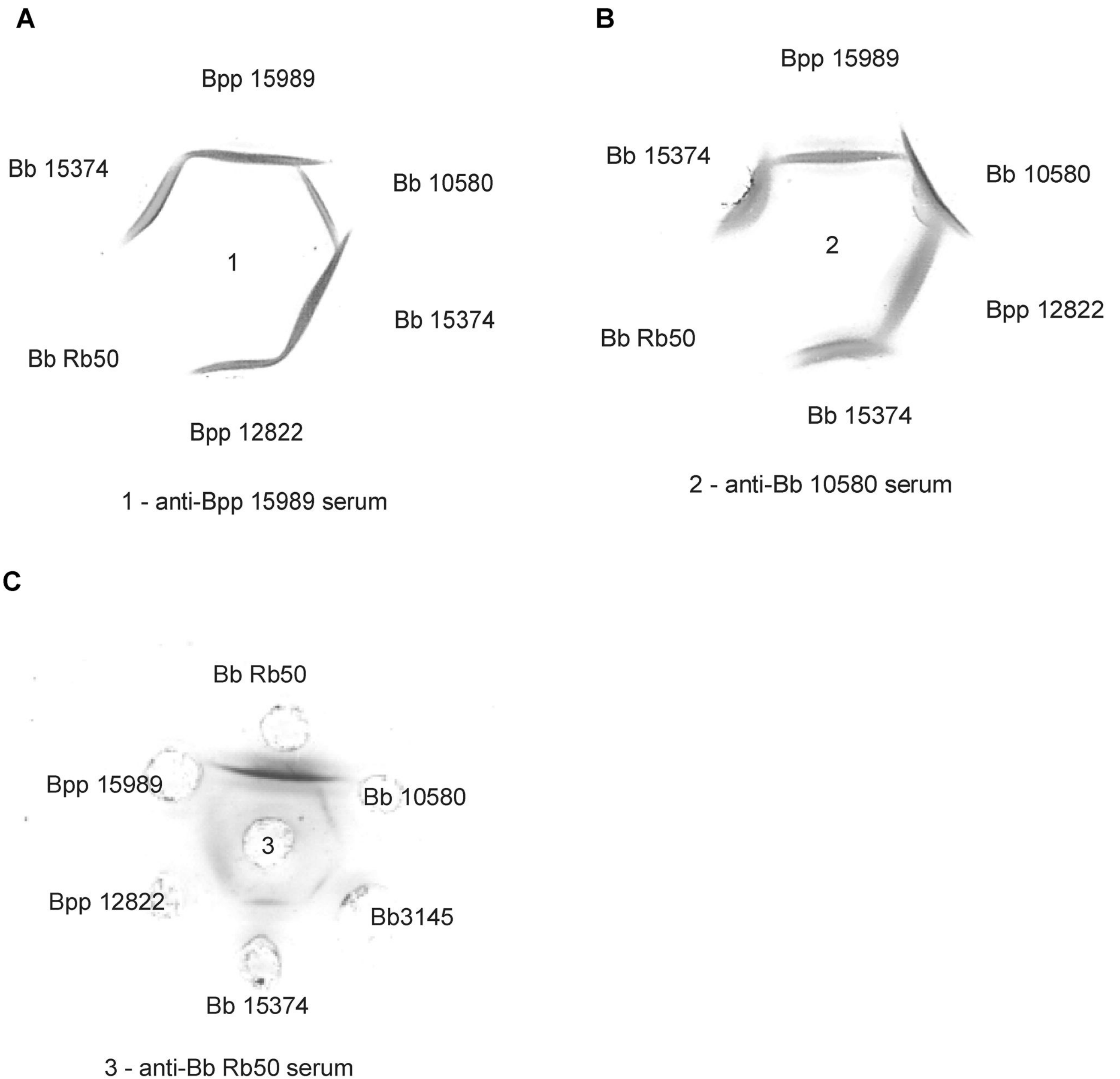

Fig. 2.

Immunodifusion assays of anti-*B parapertussis* (Bpp) 15989 (A) and anti-*B. bronchiseptica* (Bb) 10580 (B) with *Bordetella* LPS. The LPS from Bpp15989 forms a spur over Bb10580 LPS, both ‘ala-type’, when tested against anti-Bpp15989 serum; Bb10580 LPS forms a spur over Bpp15989 using anti-Bb10580 serum. No reaction was seen between with BbRb50 LPS (‘lac’ type) and the two antisera.

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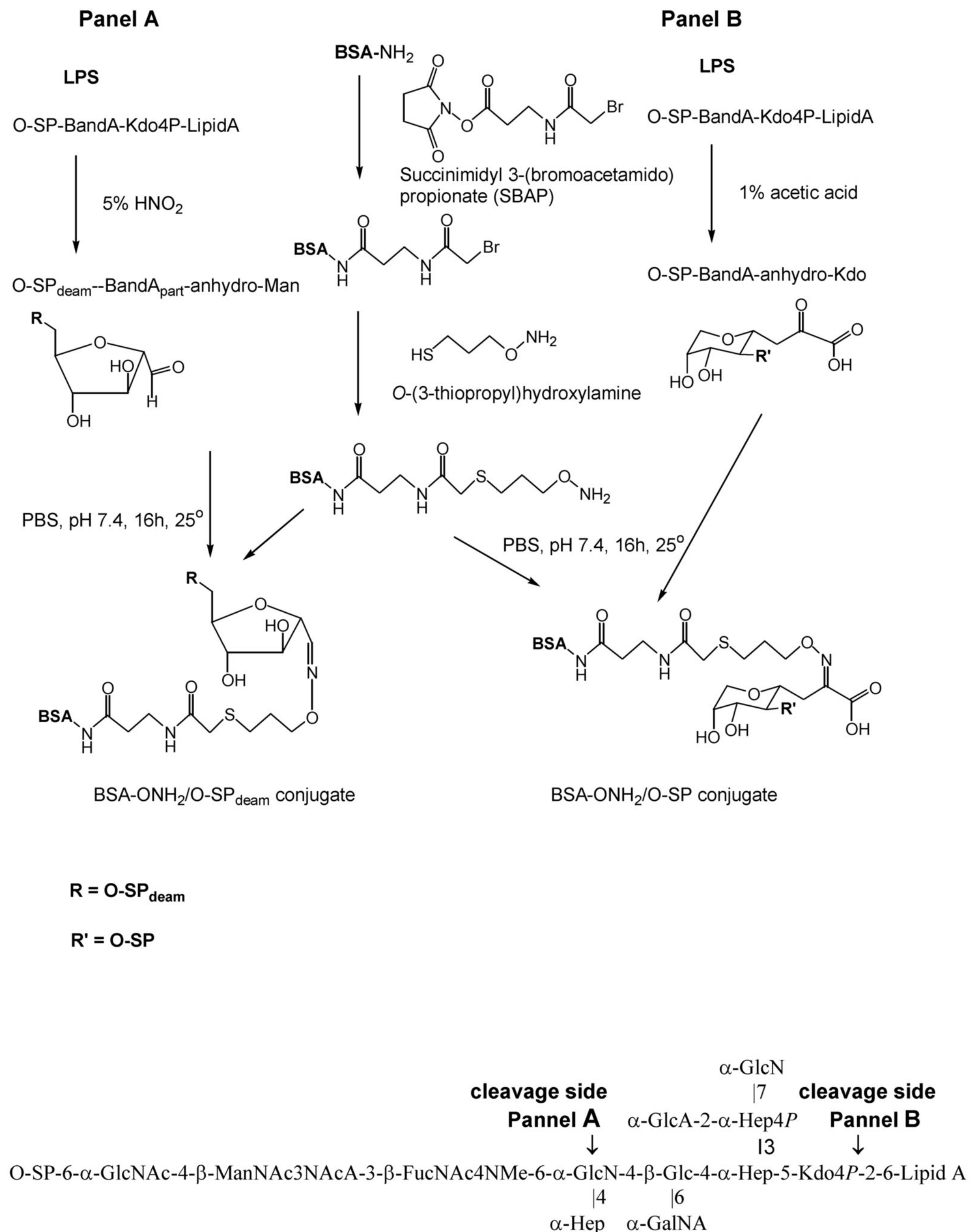

Fig. 3.

Schemes of conjugation of *B. bronchiseptica* and *B. parapertussis* O-SP to protein Protein was first amiooxylated in a two step procedure and reacted with the carbonyl group on the terminal reducing end introduced into the O-SP either by deamidation of the GlcN, a first non-reducing end sugar of Band B (Panel A) or by acetic acid hydrolysis of the Kdo converting it to anhydro-Kdo (Panel B). Conjugates preserved the external nonreducing end of the O-SP. Below, the structure and the cleavage sides of *B. bronchiseptica* LPS.

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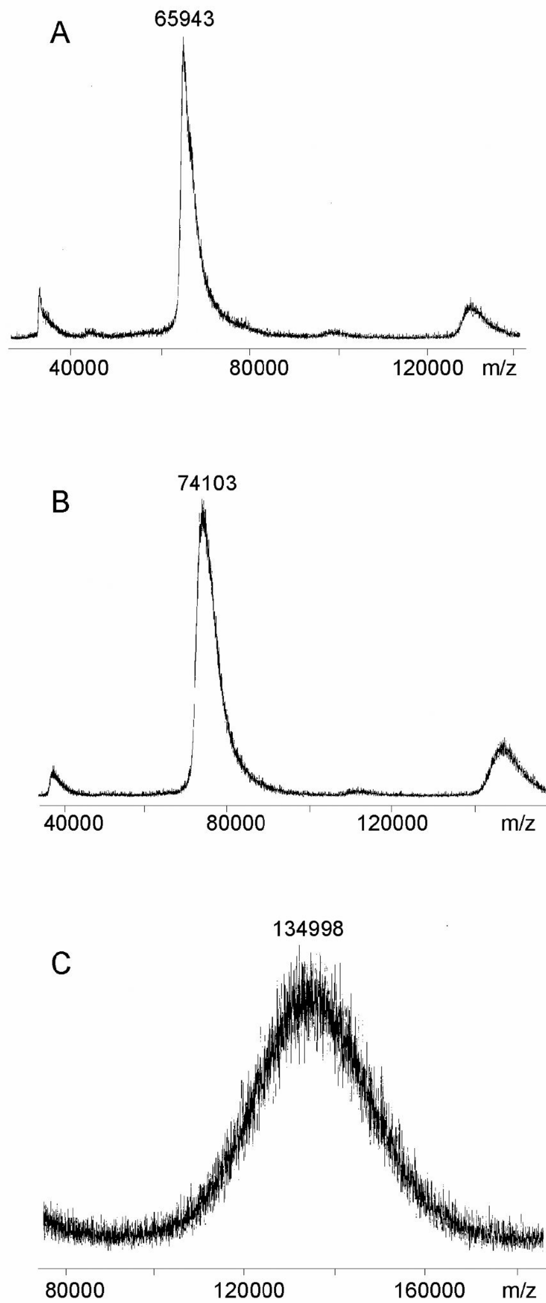

Fig. 4.
MALDI-TOF of conjugates. (A) BSA, (B) aminooxylated BSA (BSA-ONH_2), (C) BSA-ONH_2/Bb10580 Conjugate #1 (C).

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Table 1

Competitive inhibition ELISA. Plates were coated with *B. bronchiseptica* 10580, Rb50 or *B. parapertussis* 15989 LPS at 5 ug/ml and reacted with anti-*B. bronchiseptica* 10580, anti-Rb50 or anti-*B. parapertussis* 15989 hyperimmune mice sera. Inhibitors were used at 50 ug/well.

Inhibitor	O-SP end-group	Amidation of 2nd O-SP sugar	% of inhibition of hyperimmune whole cell sera		
			anti-Bb10580	anti-BbRb50	anti-Bpp15989
Bb 10580 O-SP	Ala	−	92	2	50
Bb 110H O-SP	Ala	−	96	3	45
Bb Rb50 O-SP	Lac	−	0	93	1
Bb 512 O-SP	Lac	−	1	95	3
Bpp 15989 O-SP	Ala	+	42	5	97
Bpp 15311 O-SP	Ala	+	50	3	98
H. ducreyi O-SP	na[1]	na	0	0	0

[1] na - not applicable

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Table 2

Composition and GM of mouse IgG of anti-*B. bronchiseptica* 10580, Rb50 and *B. parapertussis* 15989 LPS induced by conjugates of O-SP and O-SP_{deam} bound to bovine serum albumin (BSA). Mice (10 per group) were injected with 2.5 μg of saccharide as a conjugate per mouse, s.c., 3 times, 2 weeks apart and bled one week after last injection..

#	Conjugate	Mol. mass [kDa][1]	Ratio protein:sugar (wt:wt)	Mol O-SP/ Mol Protein	ELISA Units after 3rd injection		
					Coating antigen		
					10580 LPS	Rb50 LPS	15989 LPS
1	BSA-ONH_2/Bb10580	135	1 : 0.9	9	4.9	0.3	0.4
2	BSA-ONH_2/BbRb50	137	1 : 0.9	9	2.4	132.0	0.4
3	BSA-ONH_2/Bpp15989	165	1 : 1.4	14	0.3	0.3	1.2
4	BSA-ONH_2/ $Bb10580_{deam}$	130	1 : 1.0	11	55.0	0.6	4.8
5	BSA-ONH_2/ $BbRb50_{deam}$	105	1 : 0.5	8	0.1	3.5	0.3
6	BSA-ONH_2/ $Bpp15989_{deam}$	116	1 : 0.7	10	0.5	0.1	15.6

[1] Molar mass assayed by Maldi , Mol mass of BSA-ONH_2 was 74.1 kDa