Systems vaccinology : molecular signatures of immunity to Bordetella pertussis

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CHAPTER 4

Immunoproteomic profiling of *Bordetella pertussis* outer membrane vesicle vaccine reveals broad and balanced humoral immunogenicity

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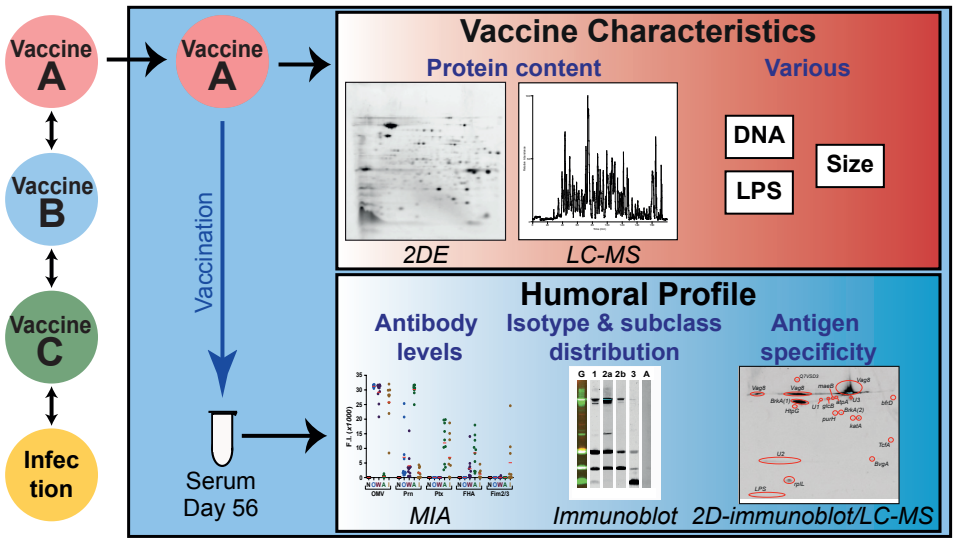
Abstract

The current resurgence of whooping cough is alarming and improved pertussis vaccines are thought to offer a solution. Outer membrane vesicle vaccines (omvPV) are potential vaccine candidates, but omvPV-induced humoral responses have not yet been characterized in detail. The purpose of this study was to determine the antigen composition of omvPV and to elucidate the immunogenicity of the individual antigens. Quantitative proteome analysis revealed the complex composition of omvPV. The omvPV immunogenicity profile in mice was compared to those of classic whole-cell vaccine (wPV), acellular vaccine (aPV) and pertussis infection. Pertussis-specific antibody levels, antibody isotypes, IgG subclasses, and antigen specificity were determined after vaccination or infection by using a combination of multiplex immunoassays, 2-dimensional immunoblotting and mass spectrometry. The vaccines and infection raised strong antibody responses, but large quantitative and qualitative differences were measured. The highest antibody levels were obtained by omvPV. All IgG subclasses (IgG1/IgG2a/IgG2b/IgG3) were elicited by omvPV and in a lower magnitude by wPV, but not by aPV (IgG1) or infection (IgG2a/b). The majority of omvPV-induced antibodies were directed against Vag8, BrkA and LPS. The broad and balanced humoral response makes omvPV a promising pertussis vaccine candidate.

Study design



Introduction

The resurgence of whooping cough in the vaccinated population [1-4] demands improved pertussis vaccines. Two types of pertussis vaccines are marketed: whole-cell (wPV) and acellular vaccines (aPV). Other types are under development, such as live attenuated and outer membrane vesicle pertussis vaccines (omvPV) [5, 6]. These vaccines differ largely in their composition. The first marketed vaccines were wPVs, which are inactivated bacteria that are naturally adjuvated with bacterial components, such as lipopolysaccharide (LPS) [6-8]. Despite an acceptable vaccine efficacy, wPVs were found to be reactogenic due to the presence of endotoxins. This lead to the development of alum-adjuvated aPVs containing one to five purified pertussis antigens: pertussis toxoid (Ptd), filamentous hemagglutinin (FHA), pertactin (Prn), and fimbriae types 2 and 3 (Fim2 and Fim3). Despite low reactogenicity of aPVs, waning immunity [9, 10] and persistent transmission of *Bordetella pertussis* [11] call for reevaluation of aPVs. To retain high vaccine compliance, the target product profile of an improved pertussis vaccine should include better efficacy compared to aPV while maintaining a low reactogenicity.

Currently, antibodies are used as correlates of vaccine-induced immunity for most commercial vaccines [12], including pertussis vaccines [13, 14]. The protective role of pertussis antibodies was supported by passive immunization studies in naive mice [15, 16], though cellular responses may also contribute to protection, at least during infection [17, 18]. Antibody responses can be characterized by several parameters, including (i) antigen specificity, (ii) titer, (iii) isotype and subclasses, and (iv) avidity. The IgG antibody subclass profile reflects the T-helper (Th) cell environment [19, 20]. For instance, vaccination with aPV results in the formation of IgG1 antibodies and Th2 cells [21, 22]. Contrarily, vaccination with wPV generates a Th1/Th17 response [23] which is thought to be important for protection against *B. pertussis* [17, 18, 23]. The functionality of the antibodies is determined by antigen specificity, avidity and antibody subclass. This determines the ability of antiserum to kill bacteria directly or by opsonophagocytic killing through activation of Fc-receptors (FcRs) on phagocytes, with an important role for FcγRs in the clearance of *B. pertussis* [24, 25]. For aPV vaccines, antibodies against pertussis toxin (Ptx) and Prn are considered to be protective against a *B. pertussis* infection [13, 26, 27]. For wPV, many immunogenic proteins have been identified by 2D-electrophoresis and western blotting [8, 28, 29]. The humoral immune response generated by omvPV is not well defined, although some major antigens were identified [30] and long-lasting immunity observed [31].

In this study, the systemic humoral response after immunization of mice with an experimental omvPV was compared with the response elicited by classic vaccines, aPV and wPV. Multiplex immunoassays, gel electrophoresis, immunoblotting and mass spectrometry were used to

identify the immunogenic antigens and the subclass profiles they induced. Because mice develop sterilizing immunity after a pertussis infection [18], antisera from *B. pertussis*-infected mice were included for comparison.

Materials and Methods

Vaccines

B. pertussis B1917 was heat-inactivated (30 min, 56°C) in PBS to produce wPV. Outer membrane vesicles from *B. pertussis* B1917 (omvPV) were produced as previously described [32] with minor changes. Both wPV and omvPV were diluted in PBS to a final concentration of 4 µg total protein per vaccine dose (300 µl). One human dose of a tetanus-diphtheria-3-component (25 µg pertussis toxoid, 25 µg filamentous hemagglutinin, and 8 µg pertactin) acellular pertussis vaccine (TDaP, Infanrix, GSK) was diluted in PBS (aPV) to a final concentration of 0.25 µg Ptd, 0.25 µg FHA, and 0.08 µg Prn per vaccine dose (300 µl).

Dynamic Light Scattering (DLS)

Particle size was determined by analyzing 0.5 ml of vaccine stock solutions diluted in PBS (1:100) at 25°C using a Zetasizer Nano-ZS (Malvern Instruments). For each sample, three records of 12 scans were obtained. The Z-average particle size and polydispersity index (PdI) were calculated using DTS Nano software (Malvern Instruments).

Double stranded (ds) DNA quantitation

Samples were diluted in Tris EDTA (TE) buffer (Invitrogen). Salmon sperm ds-DNA (Invitrogen) was used as standard (0 – 2500 ng/ml). In a V-bottom (black) 96-wells plate, 50 µl of each sample or standard was incubated with 50 µl of PicoGreen reagent solution (Invitrogen). Fluorescence intensity (excitation 480 nm, emission 520 nm) was measured by using a SynergyMx (BioTek Instruments, USA). Concentration of dsDNA was calculated based on the standard curve.

LPS analysis

LPS concentration was determined by analyzing fatty acid composition with a modified gas chromatography method [33]. The peak height of C14:0-3OH was used to quantify LPS, while C12:0-2OH was used as the internal standard. The molecular weight (4057 g/mol [34]) of *B. pertussis* LPS was used to calculate the LPS-concentrations.

Challenge culture

A stock suspension of *B. pertussis* strain B1917 was diluted with Verweij medium (BBio, Bilthoven, The Netherlands) to a final concentration of $5x10^6$ colony-forming units (cfu)/ml. The cfu concentration was confirmed by plating 100 µL of the bacterial suspension (after 2000-fold dilution in Verweij medium) on Bordet-Gengou agar plates (Cat. no. 254400, BD, The Netherlands). Plates were incubated for 4 days at 35°C and the number of colonies was counted by using a ProtoCOL colony counter (Synbiosis, Cambridge, United Kingdom).

Animal experiment

An independent ethical committee for animal experimentations of the Institute for Translational Vaccinology (Intravacc) approved the animal experiment. Female BALB/c mice (Harlan, The Netherlands), 8-week-old, were divided in five groups of ten animals and housed in cages (macrolon III including filter top). Group 1 remained naive. Groups 2, 3 and 4 were vaccinated twice with 300 µl omvPV (4 µg total protein), 300 µl wPV (4 µg total protein) and 300 µl aPV (1:100 HD in PBS), respectively, on day 0 and day 28. Vaccinations with omvPV, wPV, and aPV were administered subcutaneously in the left groin for primary vaccination, followed by booster vaccination in the right groin. Group 5 was intranasally infected under anesthesia (isoflurane/oxygen), with $2x10^5$ CFU *B. pertussis* B1917 in 40 µl Verweij medium on day 0. Mice in all groups were bled, under anesthesia (isoflurane/oxygen), by orbital bleeding and euthanized by cervical dislocation on day 56. Whole blood was collected in blood collection tubes (MiniCollect 0.8ml Z Serum Sep GOLD, Greiner Bio-One, Austria). After coagulation (10 min. at room temperature (RT)), sera were collected by centrifugation (10 min., 3000 g) and stored at -80°C for further use.

Multiplex immunoassay

Serum total IgA, IgG, and IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) levels against pertactin (Prn), filamentous hemagglutinin (FHA), pertussis toxin (Ptx), combined fimbriae type 2 and 3 antigens (Fim2/3), and outer membrane vesicles B1917 (OMV B1917) were determined using a multiplex immunoassay (MIA). Conjugation of OMVs and purified antigens to beads was performed as described previously [35]. Serum was diluted 100-fold or 1250-fold in PBS containing 0.1% Tween 20 and 3% bovine serum albumin and mixed 1:1 (v/v) with 25 µl conjugated beads (4,000 beads/region/well). After incubation with *R*-Phycoerythrin (RPE)-conjugated anti-mouse IgA (1:100), IgG (1:200), IgG1 (1:200), IgG2a (1:40), IgG2b (1:200) and IgG3 (1:200) (Southern Biotech), samples were analyzed by using a Bio-Plex system (Bio-Plex 200, BioRad). Results for antibody levels were illustrated with GraphPad Prism 6.04 (GraphPad Software Inc., USA), and presented in fluorescent intensity (F.I.).

Protein digestion by proteinase K treatment

For protein digestion, 5 µg *B. pertussis* B1917 was incubated (Overnight (ON), 56°C) with 5 µl (20 mg/ml) proteinase K (Proteinase K, recombinant, PCR Grade, Roche). Incubation was terminated by heating the sample for 5 minutes at 100°C.

Sample preparation

Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Pierce). For SDS-PAGE, *B. pertussis* B1917 (100 µg) was denatured in a total volume of 100 µl 8 M urea (GE Healthcare) in 500 mM bicarbonate (Merck) solution (pH 8.5, 1 h, RT). For 2DE, *B. pertussis* B1917 (100 µg) was centrifuged (1 min., 16200 g) and the pellet was dissolved and denatured in 100 µl DeStreak Rehydration Solution (GE Healthcare) (1h, RT).

Infrared labeling

IR680 label (15 µg) (Pierce) and IR800 label (100 µg) (Licor) were reconstituted in 100 µl and 25 µl water, respectively. For conjugation of IR800 label to secondary antibodies, 100 µl goat-anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3 or IgA (Southern Biotech) was mixed with 1 µl (4 µg) IR800 label. For IR680 labeling of *B. pertussis* proteins, 100 µg denatured proteins were mixed with 10 µl IR680 label with optimized concentrations. For 1DE gels and corresponding western blots, a 1:10 and 1:100 dilution of IR-680 stock (0.15 µg/ml) were used, respectively. For 2DE, an undiluted and 1:20 dilution of IR-680 stock (0.15 µg/ml) were used for gels and western blot, respectively. All samples were incubated for 1 hour (RT, in the dark). Subsequently, unbound IR label was removed from samples by using a ZEBA spin desalting column (Pierce) (2 min, 1500 g).

Isoelectric focusing (IEF)

After sample preparation and IR-labeling, samples for 2DE (25 µg *B. pertussis* proteins) were incubated (60 min., RT) in a total volume of 115 µl DeStreak Rehydration Solution plus 10 µl 250 mM DTT and 0.62 µl (0.5%) IPG Buffer pH 3-10NL (GE Healthcare). Immobiline DryStrip pH 3-10NL, 7 cm (GE Healthcare) were rehydrated (ON, RT) with protein sample in an IPGbox (GE Healthcare). Isoelectric focusing (IEF) was performed in an Ettan IPGphor 3 IEF system (GE Healthcare) according to the following conditions: 0.5 h at 300 V, 0.5 h at 1000 V, 1 h at 2000 V, 1 h at 3000 V, 1 h at 4000 V, and 1 h at 5000 V. After IEF, strips were equilibrated in 3 ml equilibration buffer (75 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and bromophenol blue) with DTT (65 mM) for 15 min. at RT, followed by 3 ml equilibration buffer with iodoacetamide (54 mM) for 15 min. at RT.

Gel electrophoresis

For SDS-PAGE, 10 µg *B. pertussis* (B1917) lysate, 1 µg of Prn P.69 (in house prepared [36]), Ptx (Kaketsuken, Japan), FHA (Kaketsuken, Japan), or Fim2/3 (Sanofi) were incubated (10 min., 100°C) with 3.3 µl reducing sample buffer (250 mM Tris, 8% SDS, 400 mM DTT, 40% Glycerol, 0.04% bromophenol blue) and loaded on a 10% NuPAGE bis tris 1.0-mm precast gel (Invitrogen). For 2DE, the equilibrated strip was placed on a 4-12% NuPAGE Novex bis-tris ZOOM gel (Invitrogen) and sealed with agarose sealing buffer (Bio-rad). Proteins were separated (SDS-PAGE, 45 min., 200 V) (2DE, 50 min., 200 V) with MES running buffer (Invitrogen) in a Xcell surelock minicell electrophoresis system (Invitrogen). Gels were washed in water and either stained with Coomassie (Imperial protein stain, Thermo Scientific), used for western blot or scanned using an Odyssey infrared imager (Westburg).

Western blotting

Nitro-cellulose membrane (Biorad), filters (Biorad) and gel were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol in 1 liter water). Subsequently, proteins were transferred (60 min., 60 mA) (TE77 semidry pwr transfer unit, Amersham Biosciences) from

the acrylamide gel to the nitro-cellulose membrane. Blot was blocked in block buffer (0.5% protifar in TBS-T, 20 mM Tris, 150 mM NaCl, 0.5% Tween-20 in 1 liter water) (ON, 4°C). Blot was incubated (2 h, RT) with pooled murine sera (100-fold diluted in block buffer) or a monoclonal anti-LPS antibody (Mab 88F3) (1:200) followed by frequent washing with TBS-T. Subsequently, secondary antibody incubation (1 h, RT) was performed using goat-anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3 or IgA (Southern Biotech) labeled with IR800 (Licor) (1:5000 diluted in block buffer), which was followed by frequent washing with TBS-T. Blots were scanned using an Odyssey infrared imager.

Delta2D analysis

Image warping and spot detection on 2DE gels and western blots were done by using Delta2D software (Version 4.5) (Decodon, Germany). Images of 2DEWB using five distinct sera (omvPV, wPV, aPV, infection and control) were merged by using the Delta2D group warping strategy for IgA, IgG and IgG subclasses individually. Subsequently, spot detection was performed and the intensities were measured in grey values.

In-gel protein digestion for LC-MS/MS analysis

Spots were manually excised from the acrylamide gel. In-gel digestion was performed according to a protocol based on Yan *et. al* [37]. Briefly, individual gel spots were cut in pieces of about 1 mm3 and washed three times with water, followed by washings with 50% acetonitrile and 100% acetonitrile for dehydration. Trypsin (Promega) digestion (pH 8.5, ON, 37°C) was performed in 50 mM triethyl ammonium bicarbonate. Peptides were extracted with three sequential steps of 5% formic acid solution and extracts were dried in a vacuum concentrator. Peptides were dissolved in formic acid/dimethyl sulfoxide (DMSO)/water (0.1/5/94.9% v/v) for LC-MS/MS analysis.

Protein digestion for LC-MS/MS analysis

Samples (omvPV or wPV) were diluted in a denaturation buffer containing 1 M guanidine hydrochloride (Gnd-HCl) and 50 mM triethyl ammonium bicarbonate, pH 8.5, to a final concentration of 0.5 mg/ml protein. For digestion, 100 µg of protein was digested with 2 µl of 0.25 µg/µl endoproteinase Lys-C (Roche) followed by incubation (4h, 37°C). Subsequently, digests were incubated (ON, 37°C) with 2 µl of 0.5 µg/µl trypsin (Promega). The samples were stored at –20 °C before dimethyl labeling. A common reference was prepared by mixing equal volumes of both omvPV and wPV digests. The samples and common reference were dimethylated by treatment with formaldehyde (CH_2O) and d2-formaldehyde (CD_2O) and sodium cyanoborohydride, respectively, and subsequently pooled in a 1:1 w/w ratio [38]. Solid-phase extraction was performed to remove excess reagents using C18 Sep-pack cartridges according to the manufacturer's protocol. Peptides were dried in a vacuum concentrator. Peptides were dissolved in formic acid/DMSO/water (0.1/5/94.9% v/v) for LC-MS/MS analysis.

Peptide identification by LC-MS/MS analysis

Samples were analyzed by nano-scale reversed-phase liquid chromatography electrospray mass spectrometry, according to the method by Meiring *et al.* [39]. The analysis was performed on LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Germany). Analytes were loaded on a trapping column (Reprosil-Pur C18-AQ 5 µm (Dr. Maish, Germany); 23 mm long x 100 µm inner diameter) with solvent A (0.1% (v/v) formic acid in water) in 10 min. at 5 µl/min. The analytes were separated by reversed-phase chromatography on an analytical column (Reprosil-Pur C18-AQ 3 µm (Dr. Maish, Germany); 36.2 cm long x 50 µm inner diameter) at a flow rate of 100-150 nl/min. A gradient was started with solvent B (0.1% (v/v) formic acid in acetonitrile): 6% to 28% in 130 min, 28% to 38% in 10 min and 90% for 10 min. After the gradient, the columns were equilibrated in 100% solvent A for 20 min at 100-150 nl/min. The peptides were measured by data-dependent scanning; comprising a MS-scan (m/z 300 – 1500) in the orbitrap with a resolution of 60,000 (FWHM), followed by collision-induced dissociation (LTQ) of the ten most abundant ions of the MS spectrum. The threshold value for these precursor ions was set at 1000 counts. The normalized collision energy was set at 35% and isolation width at 2.0 Da, activation Q to 0.250 and activation time to 30 ms. The maximum ion time (dwell time) for MS scans was set to 250 ms and for MS/MS scans to 1000 ms. Precursor ions with unknown and +1 charge states were excluded for MS/MS analysis. Dynamic exclusion was enabled (exclusion list with 500 entries) with repeat set to 1 and an exclusion duration of 15s. The background ion at 391.28428 was used as lock mass for internal calibration.

Data handling for total vaccine protein digests

Proteome Discoverer software (version 1.4.1.14, Thermo) was used for peak area determination, identification and relative quantification of the LC-MS/MS raw data. Identification of peptides was performed by searching MS/MS spectra against the protein database of *B. pertussis* Tohama (NCBI 257313) (3261 entries) and *B. pertussis* B1917 (3513 entries) [40] using the SEQUEST HT mode. Asparagine deamidation and methionine oxidation were set as variable modifications and lysine dimethylation (light) as a fixed modification. The data were searched with full trypsin cleavage specificity, allowing 2 miscleavages. Precursor ion and MS/MS tolerances were set to 5 ppm and 0.6 Da, respectively. Peptides were filtered to 1% FDR using Perculator (Proteome Discoverer, Thermo). Relative protein concentrations were calculated using the quantification module of Proteome Discoverer using default settings (light: 28.013 Da, heavy 32.055 Da). The molar concentration of proteins was estimated according to Silva *et. al.* [41], except that the peptides were dimethyl-labeled in our method. The molar concentrations were converted to mass concentrations by multiplying with the molecular masses of the proteins. The percentage of protein abundance for each individual protein relative to the total sum of all identified proteins was used to determine an estimated protein amount in one vaccine dose (4 µg total protein) of wPV and omvPV.

Data handling for in-gel protein digests

Proteome Discoverer software was used for identification of the LC-MS/MS raw data for in-gel digested proteins almost in a similar way as described in data handling for total vaccine protein digests. Asparagine deamidation and methionine oxidation were set as variable modification. The data were searched with full trypsin cleavage specificity, allowing 2 miscleavages. Precursor ion and MS/MS tolerances were set to 5 ppm and 0.6 Da, respectively. Peptides were filtered to 1% FDR using Perculator.

Bioinformatics

Functional enrichment with an over-representation analysis (ORA) of the detected *B. pertussis* proteins was carried out by using DAVID [42] based on Gene Ontology Biological Processes (GO-BP), Molecular Function (GO-MF) and Cellular Component (GO-CC).

Results

Characteristics and composition of pertussis vaccines

The outer membrane vesicle vaccine (omvPV), heat-inactivated whole-cell vaccine (wPV), and acellular vaccine (aPV) were characterized with respect to particle size, DNA content, LPS concentration and protein composition (Table 1). OMVs were about ten times smaller in diameter (114 nm) than the inactivated bacteria in the wPV (1156 nm). Further, the omvPV contained a small amount of DNA per vaccine dose (0.2 µg) when compared to wPV (1.2 µg), whereas aPV contained undetectable levels of DNA. In addition, the LPS concentration was slightly lower in omvPV compared to wPV.

Table 1. Characteristics of the vaccines studied.

Characteristic	omvPV	wPV	aPV
Dose	4 µg B. *pertussis* proteins	4 µg *B. pertussis* proteins	0.25 µg FHA, 0.25 µg Ptx, 0.08 µg Prn
Z-average particle size (d.nm ± SD)	114 ± 1	1156 ± 37	N/M[1]
Polydispersity Index (PdI)	0.22	0.19	N/M
DNA[2] (µg ± SD)	0.18 ± 0.02	1.19 ± 0.15	0
LPS[2,3] (µg)	1.0	1.5	N/M
Number of proteins	268	332	3
Additional Adjuvant	-	-	$Al(OH)_3$

[1] N/M = Not measured.
[2] Based on the vaccine dose of 4 µg total protein for omvPV and wPV.
[3] Analysis n = 1 (All other analysis n = 3)

LC-MS analysis was performed to characterize total vaccine protein composition of omvPV and wPV. In total, 268 and 332 proteins were identified and quantified in omvPV and wPV, respectively (Table S1). Obviously, these numbers do not cover the identification of all proteins present in both vaccines, as *B. pertussis* has more than 3500 genes. However, this LC-MS method identified and quantified the most abundant proteins present in the vaccines. The quantity of different proteins differed remarkably between omvPV and wPV. For both vaccine formulations, the top 25 of most abundant proteins is shown in Figure 1A. The 25 proteins that are most abundant in omvPV cover 86% of the total protein content in omvPV. Autotransporters Vag8 (34%) and BrkA (24%) are the major antigens. The other proteins of the top 25 contributed each less than 5% of the total protein amount. For wPV, the top 25 of proteins of this vaccine covered 67% of the total protein content. GroEL (17%) and FHA (11%) were the most abundant proteins in wPV.

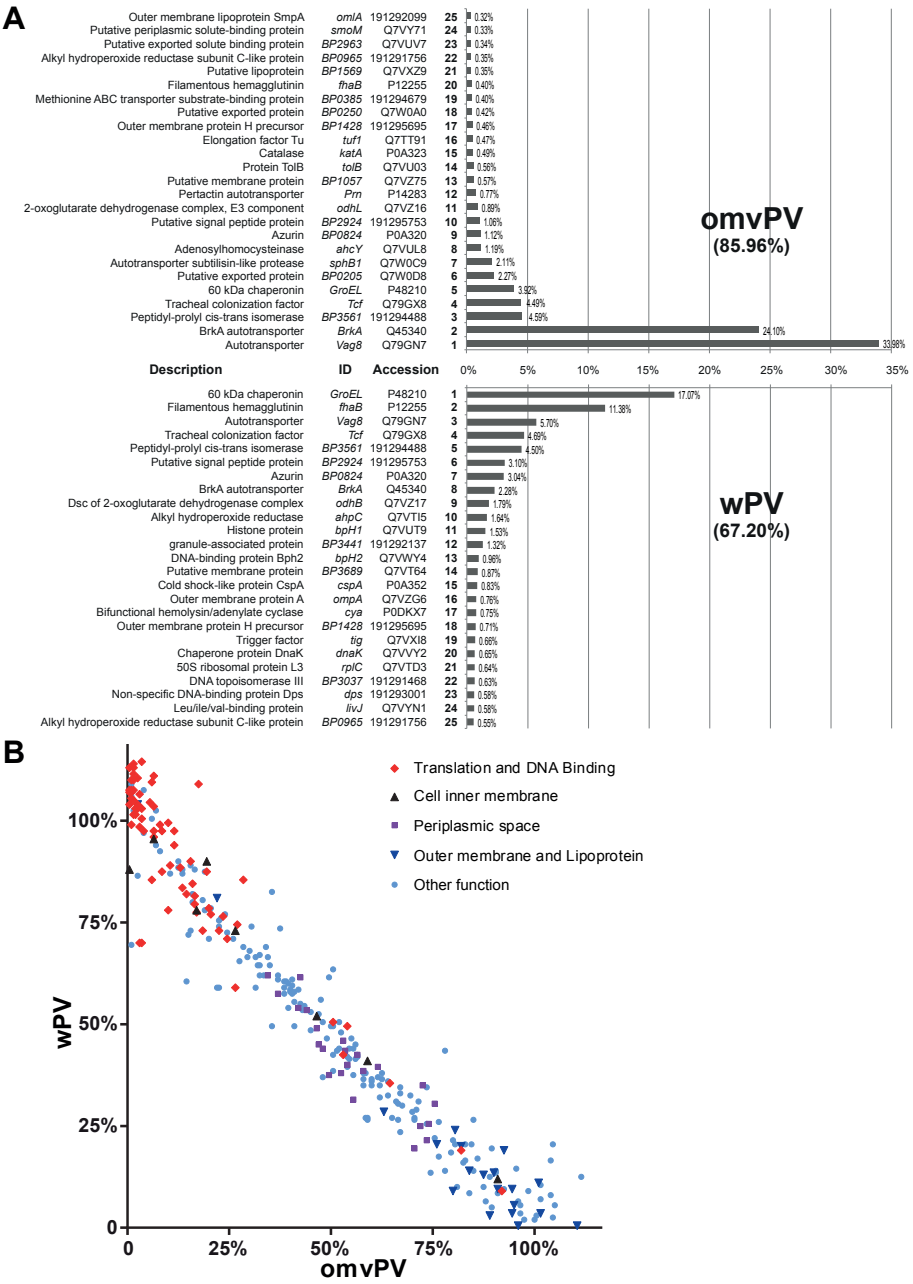


Figure 1 - Protein composition wPV and omvPV. **(A)** Proteins in omvPV and wPV were determined by quantitative LC-MS-analysis. Protein abundance is expressed as percentage of total detected protein content. The top 25 proteins cover 86.0% of the total protein content in the omvPV, compared to 67.2% in wPV. **(B)** For each individual protein, the amount in wPV was compared to omvPV and expressed as percentage as shown in the scatter plot. For instance, 50% - 50% indicate equal presence of a protein in both vaccines. Sum of percentages in omvPV and wPV is in principle 100%. Functional analysis indicates enrichment of proteins involved in translation, DNA binding and the cell inner membrane in wPV, while the outer membrane proteins and lipoproteins are enriched in the omvPV. Three technical replicates of both vaccines were characterized.

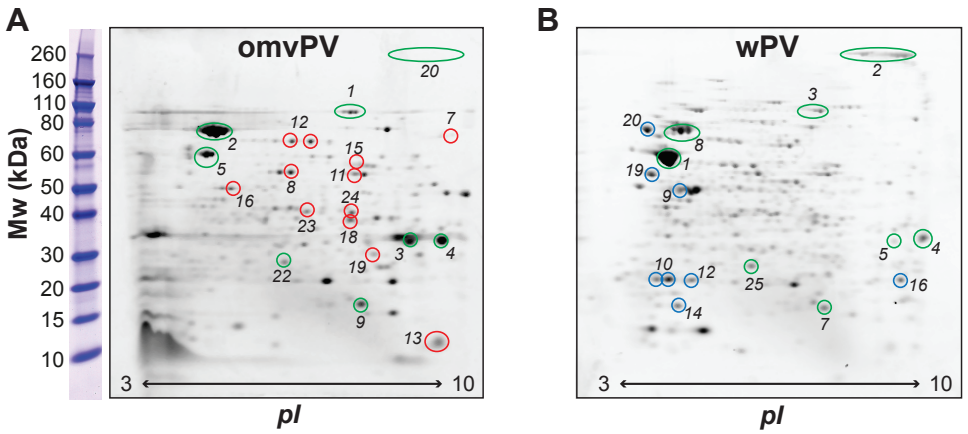


Figure 2 - Protein fingerprint of wPV and omvPV by 2-dimensional gel electrophoresis. Total protein lysates (25 µg total protein) of omvPV **(A)** and wPV **(B)** were separated by 2-dimensional gel electrophoresis. Gels were Coomassie stained and scanned on an Odyssey infrared imager (680 nm, Int. 5.0). Numbers in the gels correspond with the 25 most abundant proteins identified by LC-MS/MS analysis in either omvPV or wPV. Different colors correspond with proteins that are present in both vaccines (green), only present in omvPV (red), and only present in wPV (blue), respectively. For each vaccine, three technical replicates were characterized and one representative gel is presented.

Next, the relative amounts of each individual protein present in omvPV and wPV were compared between both vaccines (**Figure 1B**). In addition, functional analysis using DAVID [42] revealed that nuclear, cytoplasmic and inner membrane proteins were more abundant in wPV, whereas omvPV was enriched for outer membrane proteins and lipoproteins. Periplasmic proteins were present in similar amounts in both vaccines. The protein composition of aPV was not investigated, since it is specified by the manufacturer (one human dose contains 25 µg FHA, 25 µg Ptx and 8 µg Prn).

By using 2-dimensional electrophoresis (2DE), 182 spots were visualized in omvPV and 270 in wPV (**Figure 2A and 2B**, respectively). The most intense spots were isolated and the proteins present identified by LC-MS after in-gel protein digestion. The 25 most abundant proteins in omvPV as determined by total vaccine protein LC-MS analysis could also be recovered from the 2DE gels. For wPV, five proteins (bpH1, bpH2, cspA, rplC, BP3037) (see **Figure 1**) were not found by 2DE, presumably because their isoelectric point (pI) was outside the range (3-10) of the IPG-strip.

Serum levels and subclass distribution of antibodies against antigens in OMVs and aPV.

Serum IgG levels against purified antigens (Prn, Ptx, FHA and Fim2/3) as well as *B. pertussis* OMVs were determined in mice vaccinated subcutaneously with omvPV, wPV or aPV at day 0 and day 28. In addition, a group of mice was intranasally infected with *B. pertussis* at day 0. Vaccination with omvPV induced the highest concentration of anti-OMV IgG, followed by wPV and intranasal infection, whereas anti-OMV IgG was absent after aPV vaccination (**Figure 3A**

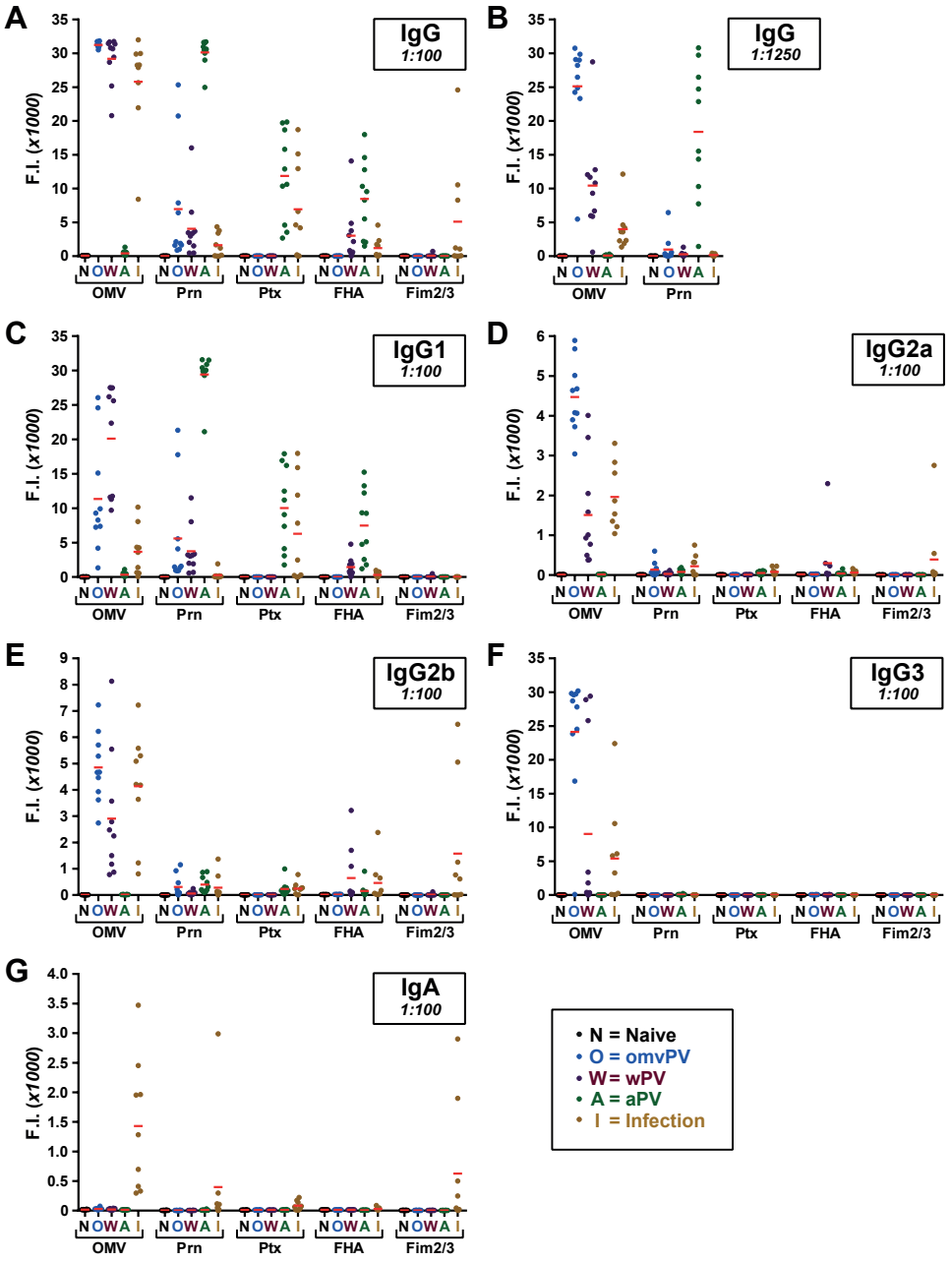
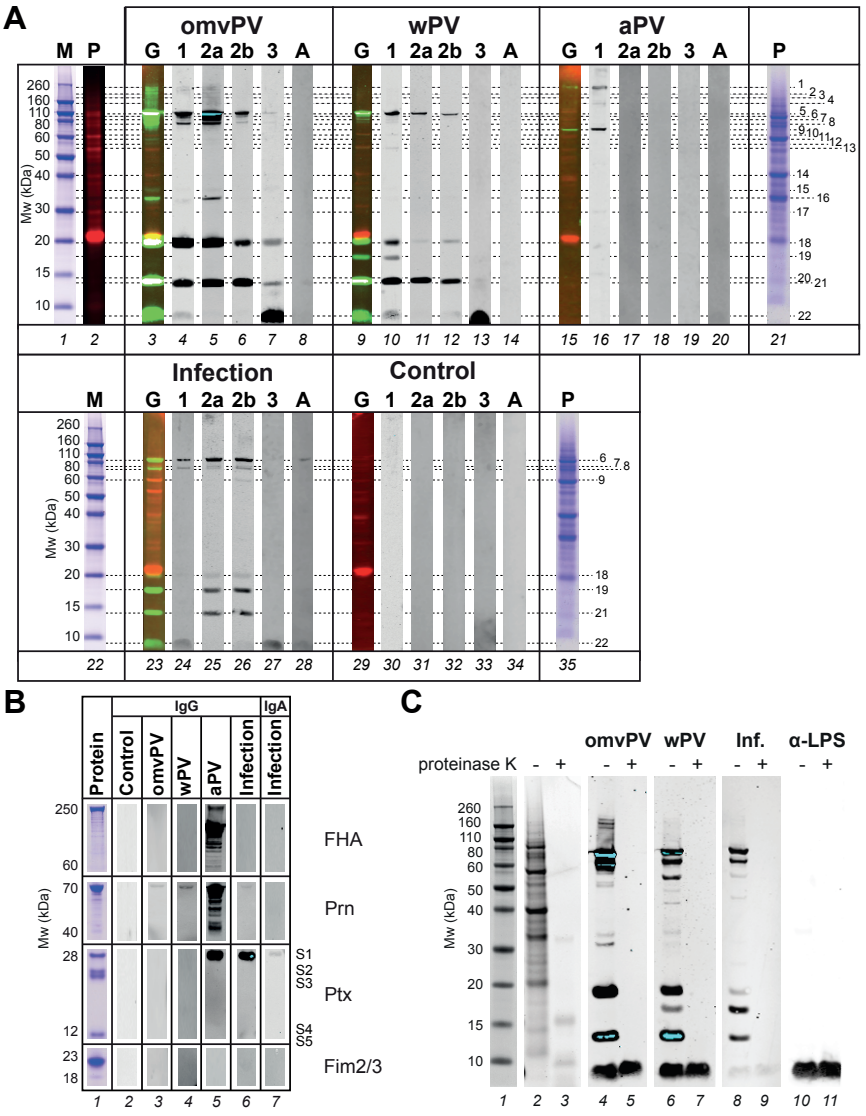


Figure 3 - Immunoproteomic profiling by multiplex immunoassay of different pertussis vaccines. **(A-G)** Antibody profiles against OMV, Prn, Ptx, FHA and Fim2/3 antigens (indicated at x-axes) were analyzed in serum of mice (n = 10). Each group of mice was either untreated (naive), received a different pertussis vaccine (omvPV, wPV, aPV), or underwent an intranasal infection. The intensity of all antibody concentrations is expressed in fluorescence intensity (F.I.). **(A)** The antibody levels were determined in 1:100 diluted sera. **(B)** Samples exceeding the detection limit were further analyzed by using a 1:1250 serum dilution. In addition, the antibody concentrations of IgG-subclasses **(C)** IgG1, **(D)** IgG2a, **(E)** IgG2b and **(F)** IgG3, as well as **(G)** IgA antibodies were determined in 1:100 diluted serum samples.

and 3B). As expected, vaccination with aPV induced antibodies against all components present in the vaccine: Prn, Ptx and FHA. Anti-Prn IgG antibodies were also detected upon omvPV and wPV vaccination. Anti-FHA IgG was measured after immunization with wPV, but not after vaccination with omvPV. *B. pertussis* infection resulted in high anti-Ptx IgG and moderate IgG levels against Prn and FHA. In addition, upon infection antibodies against Fim2/3, antigens that are present in certain other aPVs, were present in serum (Figure 3A).

Subsequently, IgG subclass (IgG1, IgG2a, IgG2b and IgG3) and IgA serum levels were determined (Figure 3C-G). Vaccination with omvPV and wPV generated antibodies of all IgG subclasses (IgG1, IgG2a, IgG2b and IgG3). However, the magnitude of the responses was lower in case



of wPV vaccination with the exception of IgG1. Vaccination with aPV induced antibodies solely of the IgG1 subclass. An intranasal infection induced predominantly IgG2a and IgG2b antibodies and was the only condition inducing measurable serum IgA antibodies (**Figure 3G**). After the infection of mice, high levels of serum IgA antibodies were detected binding to pertussis OMVs. In addition, IgA against Fim2/3, Prn or Ptx could be measured in some mice. No pertussis-specific antibodies were measured in serum of naive mice (**Figure 3A-G**).

Heterogeneity of antigen specificity and anti-LPS antibodies identified by using immunoblotting

A combination of 1-dimensional gel electrophoresis and western blotting (1DEWB) was used in order to identify the antigen-specificity of the antibody isotypes or subclasses elicited by the different vaccines (**Figure 4**). 1DEWB unraveled staining against 22 bands in a *B. pertussis* lysate, as marked in lane 21 and 35 (**Figure 4A**). The most intense staining was obtained with sera from omvPV-vaccinated mice. In addition, it was observed that antibody subclass formation is antigen specific in some cases. For instance, antibodies against band 16 were only formed upon omvPV vaccination and were exclusively of the IgG2a subclass. A broad subclass induction was observed in these sera for the other antigens (**Figure 4A, lane 3-8**). These results for IgG subclasses are in line with those of the MIA measurements, as are the results found for wPV (**Figure 4A, lane 9-14**), aPV (**Figure 4A, lane 15-20**), and infection (**Figure 4A, lane 23-28**). Untreated mice showed no staining on the Western blots (**Figure 4A, lane 29-34**).

Figure 4 (Left) - 1-Dimensional immunoproteomic profiles of pertussis vaccines. **(A)** *B. pertussis* B1917 proteins labeled with IR-680 were separated by SDS-PAGE (lane 2, P) (680 nm, Int. 5.0). Subsequently, blots were incubated with pooled serum of ten mice either vaccinated with omvPV (lane 3-8), wPV (lane 9-14), or aPV (lane 15-20), mice that underwent an intranasal infection (lane 23-28) or untreated mice (lane 29-34). Immunostaining was performed with six different IR-800-labeled goat-anti-mouse secondary antibodies (IgG, IgG1, IgG2a, IgG2b, IgG3 or IgA). Results for total IgG (G) are shown as dual staining (680 nm, Int. 5.0, 800 nm, Int. 8.0) with *B. pertussis* proteins in red and IgG antibodies in green. Blots with IgG subclasses and IgA display single staining for antibodies in grey/black. The 22 unique bands detected on one or more blots are marked on a Coomassie stained gel with *B. pertussis* B1917 proteins (P, lane 21 & 35). **(B)** Purified pertussis antigens (FHA, Prn, Ptx, Fim2/3) (1 µg) were individually separated on SDS-PAGE (Lane 1). Blots were incubated with serum of naive mice (lane 2), mice vaccinated either with omvPV (lane 3), wPV (lane 4), or aPV (lane 5) or mice that underwent an intranasal infection (lane 6-7). Subsequent IR800-labeled goat anti-mouse IgG incubation (1:5000) indicated IgG formation against FHA, Prn and Ptx in aPV vaccinated mice. In addition, anti-Prn was detected in omvPV and wPV vaccinated mice as well as infected mice. Both IgG (Lane 6) and IgA (lane 7) formation against Ptx was observed in serum upon intranasal infection. No antibody formation was observed against Fim2/3. **(C)** Coomassie stained *B. pertussis* B1917 proteins were untreated (-) (lane 2) or digested (+) (lane 3) with proteinase K. Western blot was performed with both treated and untreated *B. pertussis* by incubation with serum (1:100) of mice vaccinated either with omvPV (lane 4-5) or wPV (lane 6-7), or mice that underwent an intranasal infection (lane 8-9). Treatment with IR800-labeled anti-IgG (1:5000) (800 nm, Int. 7.0) showed that proteinase K treatment leads to diminished immunostaining in all sera except for the band at approximately 7kD. Anti-LPS incubation (1:200) showed a similar band (lane 10-11) (800 nm, Int. 4.0). Blue color in lanes 4 and 6 indicates saturation of signal obtained by the Odyssey scanner. For each condition, experiments were performed three times with pooled serum of 10 mice resulting in similar outcomes, and one representative blot is presented.

Since concentrations of Ptx, Prn, and Fim2/3 were low in the *B. pertussis* B1917 lysate used on the blot, the pools of serum of vaccinated mice were analyzed on blotted purified antigens (i.e. Ptx, Prn, Fim2/3 and FHA) (Figure 4B). This analysis revealed that the anti-Ptx antibodies detected upon both aPV vaccination (IgG) and infection with *B. pertussis* (IgG and IgA) were only directed against Ptx subunit 1 (Ptx S1) (Figure 4B). In addition, results revealed high anti-Prn, anti-FHA and anti-Ptx antibody formation after aPV vaccination, whereas these antibodies were not detectable or low in serum of mice vaccinated with omvPV or wPV. These results are in line with the observations made by MIA.

Since western blot analysis was performed on both untreated *B. pertussis* proteins and proteins digested with proteinase K, one non-proteinaceous band could be identified as LPS by using an anti-LPS monoclonal antibody (Figure 4C). Thus, a substantial fraction of the IgG

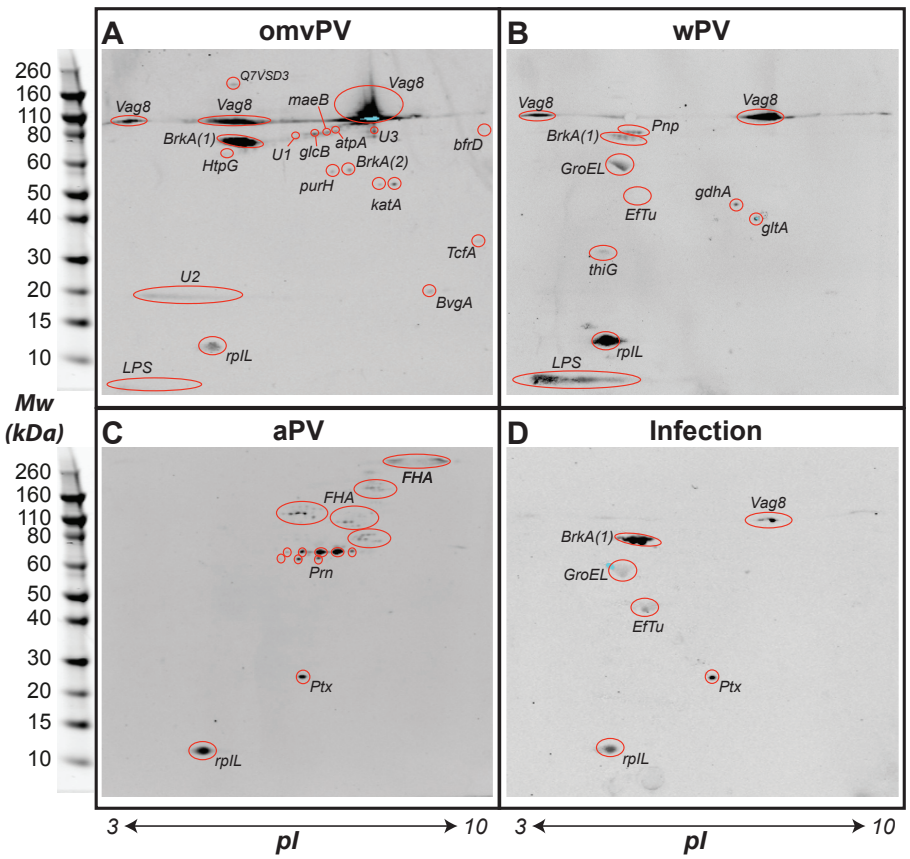


Figure 5 - 2-Dimensional immunoproteomic profiling. *B. pertussis* B1917 proteins were separated by 2-dimensional gel electrophoresis followed by western blot analysis with serum of mice that underwent vaccination with **(A)** omvPV, **(B)** wPV, or **(C)** aPV, or **(D)** a *B. pertussis* infection. Immunogenic proteins were detected (800 nm, Int. 8.0) with IR800-labeled anti-IgG and identified by LC-MS-analysis. For each condition, experiments were performed three times with pooled serum of 10 mice resulting in similar outcomes. One representative total IgG blot is presented.

elicited by omvPV and wPV was directed against LPS (Figure 4C). Antibodies against LPS were mainly of the IgG3 subclass (Figure 4A, lane 7, 13 and 27), but were also of the IgG1 subclass (Figure 4A, lane 4, 10 and 24). After a *B. pertussis* infection, less anti-LPS IgG antibodies were found but in addition IgA antibodies were formed against LPS (Figure 4A, lane 28).

Identification of the antibody-inducing antigens

A combination of 2-dimensional gel electrophoresis, western blot analysis (2DEWB) and LC-MS was performed on a *B. pertussis* lysate (Figure 2B) to identify the immunogenic proteins and the subclass specificity of the serum antibodies from vaccinated or infected mice (Figure 5A-D). We detected 16, 9, 3, and 5 antibody-inducing proteins in serum from omvPV, wPV and aPV-vaccinated mice, or infected mice, respectively. The immunogenic proteins were identified and quantified by LC-MS (Table 2). Highest staining intensities for IgG after omvPV vaccination were found directed against Vag8 and BrkA. Moderate antibody binding was measured against atpA, bfrD, BvgA, glcB, HtpG, katA, maeB, purH, Tcf and three unidentified proteins (U1-3). Anti-Tcf antibodies were only detected upon omvPV vaccination (Figure 5A), although similar concentrations of Tcf are present in wPV (Figure 6). The humoral response induced by wPV was mainly characterized by antibodies against BrkA, GroEL and Vag8 (Figure 5B). In addition, immunostaining of EfTu, gdhA, gltA, Pnp, and thiG was observed. It was confirmed that vaccination with aPV induced antibodies directed against FHA, Prn and Ptx (Figure 5C). Anti-Ptx antibodies were directed against a single spot, whereas anti-Prn and anti-FHA were detected as multiple spots, most likely indicating degradation (change in size), deamination or post-translational modifications (change in pI). Although FHA was present in both wPV and omvPV, only aPV elicited detectable anti-FHA antibodies. *B. pertussis* infection resulted in moderate antibody levels against BrkA, EfTu, GroEL, Ptx and Vag8 (Figure 5D). Western blot analysis of serum of naive mice revealed the presence of antibodies against the 50S ribosomal protein L7/L12 (Q7W0S0) (Figure 6).

The results of 2DEWB analyses are summarized in heatmaps (Figure 6). Vaccination with omvPV induced antibodies of all IgG subclasses against Vag8 and BrkA. The formation of IgG3 against Vag8 and BrkA and IgG2a against Tcf was only detected upon omvPV vaccination. All vaccines generated IgG1 against multiple antigens. However, the staining intensities were the strongest upon aPV and wPV vaccination. The aPV elicited only antibodies of the IgG1 subclass against FHA, Prn, and Ptx. The wPV-induced antibodies against BrkA, Pnp and EfTu were only of the IgG1 subclass, while antibodies against Vag8 consisted of both IgG1 and IgG2a subclasses. Western blots revealed that anti-GroEL antibodies formed upon wPV vaccination and after pertussis infection were predominantly IgG1 and to a lesser extent IgG2b. The infection induced IgG2a and IgG2b antibodies against Vag8 and BrkA. However, the highest staining intensities of BrkA were found to be IgG1. Finally, serum IgA was exclusively measured after infection and was directed against Vag8 and BrkA.

Table 2. Immunogenic proteins determined by LC/MS of excised spots found by immunoblotting on 2DE gels.

Protein Description[1]	Accession number[2]	ID[2]	Mw (kDa)[3]	pI[3]	Coverage (%)[4]	Number of peptides assigned with high confidence	Estimated amount in omvPV (µg)[5]	Estimated amount in wPV (µg)[5]
50S ribosomal protein L7/L12	Q7W0S0	rplI	12.8	4.9	68	5	0.001	0.006
60 kDa chaperonin	P48210	GroEL	57.4	5.2	80	45	0.2	0.7
Autotransporter	Q79GN7	Vag8	94.8	6.8	42	26	1.4	0.2
Bifunctional purine biosynthesis protein PurH	Q7VTU1	purH	55.8	6.3	46	16	N/D	N/D
BrkA (1)	Q45340	BrkA	103.3	7.1	41	25	1.0	0.1
BrkA (2)	Q45340	BrkA	103.3	7.1	18	11	1.0	0.1
Catalase	P0A323	katA	54.5	7.1	66	23	0.02	0.004
Chaperone protein HtpG	Q7W0M8	htpG	71.1	5.2	55	33	0.001	0.01
Citrate synthase	Q7VWA4	gltA	48.4	6.4	40	14	N/D	N/D
Elongation factor Tu	Q7TT91	tuf1	42.9	5.5	78	22	0.02	0.02
Filamentous hemagglutinin	P12255	fhaB	367.3	8.8	41	93	0.02	0.5
Glutamate dehydrogenase	Q7VXC5	gdhA	46.3	6.4	31	10	N/D	0.003
Malate synthase G	Q7VT71	glcB	78.5	6.3	40	22	0.002	N/D
NADH-ubiquinone oxidoreductase, 75 kDa subunit	Q7VU46	atpA	81.1	6.3	25	16	0.002	0.01
NADP-dependent malic enzyme	Q7VZ22	maeB	82.2	6.1	37	22	N/D	0.002
Pertussis toxin subunit 1	P04977	ptxA	30.0	7.5	47	11	N/D	N/D
Polyribonucleotide nucleotidyltransferase	Q7VZU0	pnp	77.3	5.4	56	26	N/D	0.002
Probable TonB-dependent receptor BfrD	P81549	bfrD	81.5	9.2	59	48	0.004	N/D
Putative uncharacterized protein	Q7VSD3	BP0500	68.6	5.3	49	21	0.002	0.01
Thiazole synthase	Q7VTE5	thiG	28.1	5.0	31	7	0.001	N/D
Tracheal colonization factor	Q79GX8	Tcf	66.3	6.0	35	22	0.2	0.2
Virulence factors putative positive transcription regulator BvgA	P0A4H2	bvga	22.9	8.4	77	14	N/D	0.02

[1] Proteins in excised gel spots identified by LC-MS analysis
[2] Accession numbers of proteins and corresponding ID provided by uniprot (http://www.uniprot.org)
[3] Molecular weight (Mw) and iso-electric point (pI) are calculated based on protein sequence
[4] Percentage of a protein sequence covered by identified peptides from the corresponding protein
[5] Estimated amount (µg) of protein present in omvPV and wPV based on the vaccine dose of 4 µg total protein (N/D = Not detected).

Figure 6 - Immunoproteomic profiles elicited by pertussis vaccines and pertussis infection. In total, against 26 immunogenic proteins antibodies were detected that were induced by one or more vaccination schemes. For each protein, the estimated amount (0 – 1.4 µg) present in one vaccination dose of omvPV, wPV (vaccine dose is 4 µg total protein) (analyzed by LC-MS) and aPV (0.25 µg Ptx, 0.25 µg FHA, and 0.08 µg Prn) is listed. The staining intensities of the immunogenic spots were determined and are presented as a heatmap for total IgG (average of 3 blots with pooled serum of 10 mice), IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) and IgA (1 blot with pooled serum of 10 mice). For immunogenic proteins that were detected in multiple spots (i.e. Vag8), the signal of these spots were averaged and summarized as one antigen in the heatmap.

Discussion

This study demonstrates that vaccination with three different pertussis vaccines (omvPV, wPV or aPV) elicited distinct differences in humoral responses, not only with respect to antigen–specificity and antibody levels, but also with respect to antibody isotypes and IgG subclasses. Characterization of complex vaccines by immunoproteomic profiling enabled us to identify and quantify immunogenic proteins in omvPV and wPV. Vaccine protein composition was determined by quantitative proteomics using LC-MS. The immunogenic proteins of the *B. pertussis* B1917 proteome were identified by 2-dimensional immunoblotting and subsequent LC-MS analysis.

To our best knowledge, this is the first time that immunoproteomic profiles of different pertussis vaccines were extensively compared. This study will be useful for understanding host-pathogen interaction and for vaccine design, since complete subclass profiling, especially regarding IgG2b and IgG3, is often absent [15, 43]. Distinct variances in IgG subclass response were seen in mice receiving different pertussis vaccines or *B. pertussis* infection (**Figure 3, 4 and 6**). Vaccination with omvPV and wPV resulted in antibodies of all IgG subclasses, although the wPV elicited a subclass response that was dominated by IgG1. Vaccination with aPV solely induced IgG1 antibodies, while a *B. pertussis* infection led to the formation of mainly IgG2a and IgG2b antibodies. The formation of antibody-producing B-cells can occur either through a T-cell dependent or independent way. In the case of T-cell involvement, the type of cytokines secreted, e.g. IFNγ, IL-4, IL-5, and IL-17, determines the IgG subclass production by B-cells [19, 20, 44]. In mice, Th1 type responses promote the production of IgG2a, whereas Th2 responses stimulate the formation of IgG1 [19, 44]. In addition, production of IgG2b seems to be linked to a Th17 type response [20]. Whereas all types of T-cell help stimulate the secretion of IgG3 [19, 44], the highest IgG3 formation is achieved by T-cell independent antibody responses by B-cells, i.e. against LPS [45].

The differences in IgG subclass responses observed in the mice upon vaccination with different pertussis vaccines are likely influenced by the vaccine characteristics, such as (i) particle size, (ii) vaccine composition or (iii) presence of adjuvants, along with (iv) the route of administration. For instance, OMVs are ten times smaller in diameter than whole bacteria. This may increase the exposure to different cell types because there are more particles present in the omvPV or affect the uptake by different cell types with respect to efficiency in uptake and routing within the cells [46]. In addition, OMVs may reach immune cells deeper in the tissue, which are less accessible for the whole bacteria or the large alum particles [47], or drain faster (unprocessed) to the lymph nodes [46], thereby affecting the immune responses induced.

Further, the presence of natural immune potentiators or co-administered adjuvants is known to influence the type of immune response. No adjuvants were added to the omvPV and wPV vaccines. However, these vaccine formulations do contain LPS and bacterial DNA, which are agonists for TLR4 and TLR9, respectively [48]. Activation of these pathogen recognition receptors stimulates and directs the humoral immune response in a T-cell dependent or independent way [49]. The higher concentrations of LPS and especially DNA detected in wPV, when compared to omvPV, might partly explain the different subclass responses. In addition, LPS-recognition by TLR4 in synergy with the B-cell receptor may also lead to direct T-cell independent anti-LPS antibody formation, especially IgG3, by B-cells [45]. In the case of aPV, the presence of aluminum hydroxide leads to a Th2 biased response [50] and subsequently the formation of IgG1 against purified pertussis antigens [22], as was also found in this study. Interestingly, it was shown that this Th2 response caused by aluminum-containing vaccines could be steered towards a Th1 response by addition of TLR ligands that induce strong Th1 responses, such as an LPS derivate (TLR4 ligand) [51] or CpG (TLR9-agonist) [52]. This indicates that the sum of all interactions within and between activated pathways determines the environmental conditions in which the B-cells reside, and consequently the outcome of the humoral response.

The intranasal infection resulted in production of IgG2a and IgG2b, which is thought to be related to a Th1/Th17 response [18, 20]. An enhanced IgG2a antibody formation, relative to IgG1, was also found upon intranasal administration of live attenuated pertussis vaccine [5]. A bacterial infection results in colonization and protein secretion of the bacteria leading to prolonged antigen exposure [18] when compared to vaccination. The intranasal route of administration of the infection, compared to subcutaneous vaccination with omvPV, wPV or aPV, at least partly explains the exclusive presence of serum IgA antibodies in infected mice [18].

Antibody subclasses determine the functionality of antibodies, since IgG subclasses may have a different bactericidal and opsonophagocytic activity [53]. Recently, it was suggested that for *B. pertussis*, antibodies with opsonophagocytic killing functionality might be more important compared to direct serum bactericidal killing [54]. For *B. pertussis*, Fc-receptors play an important role in opsonophagocytic killing in the respiratory tract in mice [24, 25]. IgG subclasses have a different affinity and selectivity towards FcRs [55]. In mice, IgG1 predominantly targets FcγRIII (CD16) [56]. FcγRIV (CD16-2) exclusively binds IgG2a and IgG2b that both have the highest affinity for this receptor but are able to bind all FcRs [57]. Finally, murine IgG3 has a high affinity for FcRn and low affinity for FcγRI (CD64). Fc-receptors are present on several cell types such as B-cells and NK-cells, but predominantly on phagocytes, such as neutrophils and macrophages [55]. The neutrophils seem essential to protect against *B. pertussis*, since neutrophil depletion resulted in impaired lung clearance, especially in

immunized mice [58]. Because multiple immune cells, such as neutrophils, B-cells and macrophages, express different FcγRs [55], a certain IgG subclass cannot predict the involved type of FcγR-expressing cells. However, the humoral response with multiple antibody subclasses after omvPV vaccination might result in the involvement of different FcγR-expressing cells resulting in a broad immune response. In contrast, selective IgG1 induction by aPV suggests mainly involvement of FcγRIII-expressing cells. A broad subclass distribution against surface exposed antigens, such as Vag8, BrkA, Prn and FHA should therefore lead to addressing a variety of FcγR-containing cells. The involvement of multiple FcγR-containing cells might result in increased phagocytosis. Antibodies against secreted antigens, such as Ptx, are less involved in direct phagocytosis of the bacteria. However, Ptx inhibits neutrophil recruitment [59]. Therefore, antibody-mediated neutralization of Ptx might prevent the impaired recruitment of neutrophils, increasing phagocytosis of bacteria in the respiratory tract.

The present study showed the essence of characterization of complex vaccines for understanding antibody profiles. In another study describing the antigenic composition of an omvPV, Ptx and Fim2 were reported to be present in that vaccine [30]. However, this was not observed in our study using a different omvPV. The differences in vaccine composition might be explained by the use of different *B. pertussis* strains, culture conditions, OMV extraction methods [38], vaccine dose, and methods of analysis. Though some antigens that were most abundant induced the highest antibody responses (such as Vag8 and BrkA in omvPV), other abundant proteins (such as FHA and Prn in omvPV and wPV) did not induce that high antibody responses, whereas some less abundant proteins (Pnp, BvgA) induced higher responses than expected based on the quantity present. In addition, some proteins present in equal amounts in omvPV and wPV (Tcf, EfTu) induced different antibody levels.
While many immunogenic proteins are present in omvPV and wPV, little is known about their protective capacity. Some indications have been reported, for example, using a mouse intracerebral protection assay it was shown that in combination with non-protective levels of anti-Ptx, Vag8 induced protective activity [60]. In addition, when administered as a single component vaccine, recombinant Vag8 could induce a protective response in the lung [16]. For another autotransporter, BrkA, human antibodies against this protein were shown to have bactericidal activity [61]. In addition, a three-component acellular vaccine with BrkA, Ptx and FHA was shown to induce a protective immune response in mice [62]. However, vaccination with recombinant BrkA as a single component did not protect against *B. pertussis* infection [62]. The tracheal colonization factor (Tcf) is a virulence factor and aerosol challenge of mice with a Tcf-deficient *B. pertussis* strain resulted in a 10-fold decrease in trachea colonization compared to a WT strain, indicating an important role for Tcf during colonization [63]. This might suggest a protective function for anti-Tcf antibodies in the respiratory tract. Indeed, intranasal administration of Tcf, carried by an attenuated strain of *Vibrio cholerae*,

resulted in clearance of *B. pertussis* in the trachea, but not in the lungs of vaccinated mice [64]. The GroEL protein is a conserved protein, found in many species of bacteria as well as in humans and therefore probably less suitable as a vaccine antigen [65]. In this study, anti-GroEL antibody formation was found upon wPV vaccination and *B. pertussis* infection. Active immunization with GroEL induced only little protection against a *B. pertussis* infection [65]. In the present study, anti-LPS antibodies were detected at high levels in mice vaccinated with omvPV or wPV and to a lesser extent in infected mice. Others have demonstrated that anti-LPS antibodies in mice [66], rats [67], and humans [68] have bactericidal activity and reduce bacterial colonization in the respiratory tract. In addition, anti-LPS antibodies might lead to diminished pro-inflammatory cytokine production by binding LPS, leading to reduced endotoxic effects of an infection. Immunization with LPS-based oligosaccharide conjugates induced bactericidal antibodies, indicating the potential for improving immunity by implementing pertussis LPS in a vaccine [69].

The question remains whether omvPV provides protective immunity by eliciting a humoral immune response. Based on the protective capacity of antibody induction against antigens, such as BrkA, LPS, Tcf and Vag8 described in literature [61, 64, 69, 70], the omvPV antibody profile in our study suggests potential induction of protective humoral immunity in mice. Other studies have shown that omvPV confers protection similar to aPV when studying the short-term responses [6, 31]. More importantly, it was shown that omvPV could also provide long-lasting immunity. This was also achieved by a commercial aPV but only at a high dose (1/10 Human Dose) in mice [31]. In addition, omvPV protects against different strains [31]. The broader response against multiple relevant antigens by omvPV, instead of selective antigens by aPV, may prevent impaired immunity due to strain adaptation. Future studies should demonstrate the relationship between the immunoproteomic profiles determined for different pertussis vaccines and the presence of long lasting-immunity. These studies should provide us with important new insights into the role of the humoral response and protection.

In order to maintain high vaccine compliance after introducing an improved pertussis vaccine, first a better efficacy but not more reactogenicity of omvPV compared to aPV should be established.

In conclusion, the humoral immune response elicited by omvPV is distinct from that induced by classic pertussis vaccines, such as wPV and aPV. The omvPV elicits high serum antibody levels that consist of a broad subclass response in mice against multiple antigens. The study supports wider exploration of omvPV as an improved pertussis vaccine.

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Supplementary information

Supplementary Table 1 - Protein composition of omvPV and wPV determined by LC-MS. This table is available at http://pubs.acs.org/doi/abs/10.1021/acs.jproteome.5b00258.

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About the cover: Temples and pagodas of the ancient city of Bagan in Myanmar. Capital of the kingdom of Pagan between the 9th and 13th century. Today, the remains of approximately 2,200 temples and pagodas are still present. Part of the UNESCO World Heritage list.