Development of a thermostable spray dried outer membrane vesicle pertussis vaccine for pulmonary immunization

Gaurav Kanojia\textsuperscript{a,b,1}, René H.M. Raeven\textsuperscript{a,⁎,1}, Larissa van der Maas\textsuperscript{a}, Tim H.E. Bindels\textsuperscript{a}, Elly van Riet\textsuperscript{a}, Bernard Metz\textsuperscript{a}, Peter C. Soma\textsuperscript{a}, Rimko ten Have\textsuperscript{a}, Henderik W. Frijlink\textsuperscript{b}, Jean-Pierre Amorji\textsuperscript{b}, Gideon F.A. Kersten\textsuperscript{b,c}

\textsuperscript{a} Intravacc (Institute for Translational Vaccinology), Bilthoven, The Netherlands
\textsuperscript{b} University of Groningen, Department of Pharmaceutical Technology and Biopharmacy, Groningen, The Netherlands
\textsuperscript{c} Division of Biotherapeutics, Leiden Academic Center for Drug Research, Leiden University, Leiden, The Netherlands

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\textbf{A B S T R A C T}

Worldwide resurgence of whooping cough calls for improved, next-generation pertussis vaccines that induce broad and long-lasting immunity. A mucosal pertussis vaccine based on outer membrane vesicles (omvPV) is a promising candidate. Further, a vaccine that is stable outside the cold chain would be of substantial advantage for worldwide distribution and application. A vaccine formulated as a powder could both stabilize the vaccine as well as make it suitable for pulmonary vaccination. To that end, we developed a spray dried omvPV with improved stability compared to the liquid omvPV formulation. Spray drying did not affect the structural integrity of the omvPV. The antigenicity of Vag8, a major antigen in omvPV was diminished slightly and an altered tryptophan fluorescence indicated some changes in protein structure. However, when administered via the pulmonary route in mice after reconstitution, spray dried omvPV showed comparable immune responses and protection against challenge with live \textit{B. pertussis} as liquid omvPV. Mucosal IgA and Th17 responses were established in addition to broad systemic IgG and Th1/Th17 responses, indicating the induction of an effective immunity profile. Overall, a spray dried omvPV that was developed that maintained effective immunogenic properties and has an improved storage stability.

1. Introduction

Pertussis or whooping cough, caused by the gram-negative bacteria \textit{Bordetella pertussis}, is a highly contagious airway infection. Young, unvaccinated infants represent the most vulnerable group with the highest rate of respiratory complication and death following \textit{B. pertussis} infection \cite{1, 2}. Immunization against whooping cough is recognized as a cost-effective method for controlling the disease. Currently two types of pertussis vaccines are used in vaccination programs: whole-cell inactivated pertussis vaccine (wPV) and acellular pertussis vaccine (aPV) \cite{3, 4}. However, the recent resurgence of whooping cough due to strain adaptation and waning immunity, has brought the efficacy of current vaccines under scrutiny \cite{5, 6}. Therefore, the call for improved pertussis vaccines remains pertinent.

A novel pertussis vaccine based on outer membrane vesicles (omvPV) is a potential vaccine candidate. OMVs are naturally produced by \textit{B. pertussis} \cite{7, 8} and consist of a broad range of immunogenic antigens in their native conformation \cite{9, 10}. After subcutaneous immunization, omvPV protects mice to a comparable degree as aPV and wPV, based on lung colonization data obtained after \textit{B. pertussis} challenge \cite{9, 11, 12}. Moreover, omvPV elicited a broader humoral immunity against more antigens as well as more different antibody subtypes compared to aPV \cite{10} and mixed systemic T-helper 1, 2 and 17 responses \cite{12–14}, which are also found in responses to a \textit{B. pertussis} infection \cite{13, 15, 16}. Importantly, we recently demonstrated that administration of omvPV at mucosal membranes, the port of entry for \textit{B. pertussis}, conferred superior immunity over subcutaneous administration. The response was characterized by induction of mucosal IgA, and improved B-cell and Th17 responses \cite{13}.

Maintenance of the cold chain is challenging and complicating for the widespread vaccine distribution, especially in developing countries \cite{17, 18}. During cold chain transport, accidental exposure to higher or freezing temperatures could occur, which can be detrimental for the potency of traditional vaccines \cite{18}. The cold chain also adds up to the...
financial burden of the vaccination programs. A future pertussis vaccine should therefore not only be more effective, but ideally also stable at tropical temperatures and affordable.

A way to stabilize vaccines is to spray dry them in the presence of stabilizing excipients. Removal of water and incorporation of excipients can improve the vaccine stability due to decreased mobility and prevention of degradation pathways that are facilitated by water [19]. Unlike lyophilization, the potential damage to antigens caused by freezing is avoided when spray dried. In addition, the spray drying process is fast (taking only several minutes) and consumes less energy compared to freeze-drying (which can take several days). Faster drying will result in lower operating costs. Previous studies have indicated the potential of spray dried vaccines to overcome requirements for cold chain, as they possess a longer shelf life at elevated temperatures [20–24]. The edge of spray drying resides in its ability for dry particles to be engineered to desired requirements [25–27], which can be used for pulmonary, intranasal or oral delivery [28–33]. To the best of our knowledge, no spray dried formulation of omvPV has been developed so far.

The purpose of this study was to design a dry powder formulation of omvPV with extended storage stability. Firstly, we formulated a spray dried omvPV using trehalose as a stabilizer. Besides physical characteristics of the powder, we investigated the preservation of the structural integrity and biological activity of a spray dried omvPV in comparison to the (non-processed) liquid omvPV formulation. Secondly, a stability study was performed by storage of spray dried omvPV at elevated temperatures (4 weeks at 40 °C and 65 °C). Finally, the effects of drying on the immunogenicity were tested in an in vivo model in which mice were pulmonary immunized with reconstituted spray dried omvPV or with subcutaneous liquid omvPV that resembles the conventional route of intramuscular administration for pertussis vaccines in humans. The induction of protective immunity markers such as antibody, B-cell and T-cell responses next to the protection efficacy after intranasal B. pertussis challenge were compared.

2. Materials and methods

2.1. Materials

The excipient D-( + ) trehalose dihydrate was purchased from Sigma (USA) and PBS (0.01 M, pH 7.2) from Gibco life technology (USA).

2.2. Vaccine and antigens

The omvPV from B. pertussis B1917 was produced as previously described [12]. Pertussis monoclonal antibodies directed towards Virulence associated gene 8 (Vag8) and Pertactin (Prn) were kindly provided by the Dutch National Institute for Public Health and the Environment (Bilthoven, the Netherlands).

2.3. Formulation preparation

For spray drying, 2 mL omvPV stock solution (890 μg/mL total protein) was mixed with 18 mL D-( + )-trehalose dihydrate (100 mg/mL) solution in PBS (matrix) to a final volume of 20 mL (89 μg/mL total protein concentration). The trehalose solution was filtered using a 0.45 mm Millex-HV filter (Millipore) prior to mixing with the vaccine.

2.4. Spray drying process

The spray dried omvPV was produced using a Büchi mini spray-drier B-290 in conjunction with a high performance cyclone and a B-296 dehumidifier (both from Büchi Labortechnik AG). All the experiments were performed in a closed loop configuration using nitrogen as drying medium. A two-way nozzle with orifice diameter of 0.7 mm was used in a co-current mode with nitrogen as atomizing gas. The nitrogen pressure was set constant at 5 bar. The spray drying parameters were adapted from a study described previously [20]. The inlet air temperature was set at 135 °C. The feed flow rate was displayed in percentage (%) on the equipment and feed flow rate of 10% corresponding to 3.4 mL/min. An atomizing airflow of 12.4 L/min. The aspirator rates were set at 22 m³/h in all experiments.

After spray drying, the spray dried product was collected, aliquoted (100 mg) in vials (3 mL vial, Nuova Ompi) in a N₂-flushed glove box (Terra Universal Inc., Series 100) at a relative humidity of < 3% and sealed. The powder yield was determined as described previously [20].

2.5. Thermostability study

The sealed spray dried (100 mg per vial) and liquid omvPV (100 μL per vial) were stored for 4 weeks at 40 °C and 65 °C. Samples were collected at day 0 (after drying), 1, 7, 15 and 30 (after storage) for further analysis.

2.6. Geometric particle size

The particle size of the spray dried powder product was analyzed by laser diffraction with a Helos system (Sympatec GmbH) and distribution data of the spray dried product was reported as cumulative undersize distribution. The particle sizes at its undersize values of 10%, 50% and 90% (denoted as X₁₀, X₅₀ and X₉₀ respectively). The geometric particle size (X₅₀ defined as the median particle size). The powder was dispersed into the Helos system using an Aspiros dispersing system operated at a dispersing pressure of 1.0 bar. The vaccine powder was measured with a lens having a measuring range of 0.1/0.18–35 mm. Furthermore, to check for any aggregates, the analysis was repeated by increasing the dispersing pressure (between 1 and 5 bar). Results are presented as the mean of three measurements.

2.7. Scanning electron microscopy (SEM)

The morphology of the particles was visualized using a JSM 6301F scanning electron microscope (JEOL). Spray dried omvPV samples were prepared by placing the powders on double-sided sticky carbon tape on a metal disk. Subsequently, the particles were coated with a gold layer with a thickness of approximately 10 nm using a Balzers 120 B sputtering device (Balzer UNION). The voltage used for the analysis was 10 kV with a spot size of 7–8. Images were taken at a magnification of 1000 × and 5000 ×.

2.8. Cryo-Transmission Electron Microscopy (Cryo-TEM)

A few microliters of sample was deposited on a holey carbon-coated copper grid (Quantifoil 3.5/1). After blotting the excess of the sample with filter paper, the grids were plunged into liquid ethane (FEI vitrobot). Frozen-hydrated specimens were mounted in a cryo-stage (Gatan, model 626) and observed in a FEI Tecnai T20 electron microscope, operating at 200 kV. Micrographs were recorded under low-dose conditions on a slow-scan CCD camera (Gatan, model 794).

2.9. Prn and Vag8 antigen ELISA

The reconstituted spray dried omvPV (in PBS) and liquid omvPV control were coated 100 μL/well (serially diluted between 6 and 0.375 μg/mL), on flat bottomed 96 well plates (Immulon, 2 HB, Fisher Scientific). The plates were sealed and incubated overnight at room temperature. The next day, the coating solution was discarded and washed 6 times with wash buffer (0.05% Tween80 in demineralized water), plates were blocked with blocking buffer (0.5% Protipar in PBS) for 30 min at 37 °C. Plates were washed 6 times, and subsequently incubated for 1.5 h at 37 °C with specific monoclonal antibodies against Prn (1:20) or Vag8 (1:50) diluted in reagent buffer (0.1% Tween80 in
2.10. Residual moisture content (RMC)

The RMC of spray dried omvPV samples was determined using a C30 Compact Karl Fischer Coulometer (Mettler-Toledo) as previously described [20].

2.11. Differential scanning calorimetry

Modulated differential scanning calorimetry (mDSC) was conducted using a TA DSC Q100 (TA instruments) as described previously [20].

2.12. Protein digestion and peptide identification with LC – MS/MS analysis

Liquid omvPV or reconstituted spray dried omvPV samples were diluted in a denaturation buffer containing 1 M guanidine hydrochloride (Sigma) and 50 mM triethylammonium bicarbonate (Sigma), pH 8.5, to a final concentration of 0.2 mg/mL protein. The digestion was carried out as described previously [10]. Samples were analyzed by nanoscale reversed-phase liquid chromatography electrospray mass spectrometry and the analysis was performed on LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Germany) as described previously [10].

2.13. Fluorescence spectroscopy

2.13.1. Intrinsic fluorescence

Liquid omvPV and reconstituted spray dried omvPV containing 80 µg/mL of total protein were analyzed. Spectra (3 averaged scans, excitation 295 nm, emission 300–450 nm, 50 nm/min and band width 2.5 nm for both excitation and emission) were recorded on a LS55 fluorescence spectrometer (PerkinElmer, USA). All fluorescence spectra were corrected for matrix background. The measurements were performed in triplicate.

2.13.2. Extrinsic fluorescence

Liquid omvPV and reconstituted spray dried omvPV containing 80 µg/mL of total protein were analyzed. Bis-ANS (Sigma) was dissolved in MilliQ water and added to the samples at a final concentration of 10 µM. Samples were incubated for 20 min (dark, room temperature). Emission spectra (3 averaged scans, 430–600 nm, 50 nm/min) were recorded using an excitation wavelength of 395 nm (band width 2.5 nm). All spectra were corrected by subtracting the matrix background. The measurements were performed in triplicate.

2.14. Dynamic light scattering (DLS)

OmvPV particle size was measured using a Zetasizer Nano-ZS system (Malvern Instruments, UK). DLS measurements were done in triplicate with 0.1 mL liquid vaccine (in matrix) and reconstituted spray dried powder omvPV at an operating temperature of 25 °C. Homogeneity of the size distribution was reflected in the polydispersity index (Pdi), which ranges between 0.0 (monodisperse) and 1.0 (random size distribution).

2.15. Powder X-ray diffraactometry (XRD)

Spray dried omvPV samples were analyzed by a D2 Phaser desktop X-ray diffractometer equipped with a LynxEye Si strip one-dimensional detector (both from Bruker AXS). The samples were exposed to Cu Kα (X-rays) at an angular range from 5° to 30° 2θ. Theta, with a step size of 0.01° and a dwell time of 0.5 s. The crystalline status of the powder was assessed qualitatively by examination of the resulting diffraction patterns.

2.16. Immunization and challenge of mice

Female, 8-week old BALB/c mice (Harlan, The Netherlands) were immunized twice (day 0 and 28) with 4 µg total protein omvPV either administered pulmonary as a liquid; 50 µL), pulmonary (as a reconstituted powder; 50 µL), or subcutaneously (as a liquid; 300 µL). Pulmonary administration was performed using a MicroSprayer aerosolizer supplied with a high-pressure syringe (IA-1C and FMJ-250; Penn-Century, Philadelphia, USA), as described previously [13]. The B. pertussis challenge with 2x10E5 colony forming units (CFU) of strain B1917 was performed as described previously [34].

2.17. Sample collection

Mice were anesthetized (isoflurane/oxygen) for orbital blood collection and sacrificed at day 56, by cervical dislocation. Serum was obtained by collecting whole blood in a serum collection tube (MiniCollect 0.8 mL Z Serum Sep GOLD, Greiner). After coagulation (10 min., room temperature) and centrifugation (10 min., 3000 g, sera) were collected, aliquoted and stored at −80 °C. For antibody detection and colonization assays, the lungs and trachea were homogenized in THIJS medium [35] using a Bio-Gen PRO2000 Homogenizer (Pro Scientific Inc., Oxford, USA) while nose lavages were obtained by flushing the nose with 1 mL THIJS medium. For B- and/or T-cell assays, complete lungs and spleens were collected in RPMI complete medium [RPMI-1640 medium (Gibco) supplemented with 10% PCS (Hyclone), 100 units penicillin, 100 units streptomycin and 2.92 mg/mL-l-glutamine (Invitrogen)] and homogenized using a 70-µm cell strainer (BD Falcon, BD Biosciences). Erythrocytes in spleen and lung samples were lysed using home-made lysis buffer (10 g/L NH₄Cl, 1.25 g/L NaHCO₃, 0.125 mM EDTA in dH₂O; pH 7.4). Whole blood for B-cell assays was collected in heparinized tubes (MiniCollect 1 mL LH, Greiner, Austria) after which erythrocytes were lysed using RBC lysis buffer (Pharm Lyse, BD).

2.18. Colonization assays

Lung and trachea lysates, and nose lavages were serially diluted (undiluted, 1:10, 1:100, and 1:1000) in THIJS medium. Subsequently, samples were plated on Bordet-Gengou agar plates and incubated for 5 days at 35 °C. The number of CFU/mL was determined using a colony counter (ProtoCOL, Symbiosis, Cambridge, UK).

2.19. Antibody measurements

Antibodies specific for BrkA (Bordetella resistance to killing), Fim2/3, Filamentous hemagglutinin (FHA), OMV, Pmr, Pertussis toxin (Ptx), and Vag8 were measured using a previously described mouse multiplex immunoassay (MIA) [13]. Serum samples were diluted 1:2000 for anti-OMV IgG and 1:100 for IgG (subclass) and IgA measurements. Lung lysates and nasal washes were diluted 1:10 and undiluted, respectively, for measuring IgA levels. Data were acquired with a Bio-Plex 200, processed using Bio-Plex Manager software (v5.0, Bio-Rad Laboratories), and presented as fluorescence intensities (FI).
2.20. Immunoproteomic profiling

To identify immunogenic proteins in omvPV, SDS-PAGE and Western blotting was used to analyze pulmonary IgA and serum IgG antibody responses as described with a little modification [10, 13]. In this study, nitrocellulose membranes (Thermo Scientific) were used. Western blotting was used for analysis of the epitope availability of proteins present in omvPV samples that were included in the stability study [10]. In short, proteins in the omvPV samples (5 μg/sample) were separated with SDS-PAGE and blotted on nitrocellulose membranes (Thermo Scientific). Subsequently, the blots were incubated (1:100 dilution) with an equal mixture of murine sera from three omvPV-immunized groups (pulmonary liquid, pulmonary reconstituted powder and subcutaneous liquid) derived from the animal experiment used in this study that was described earlier.

2.21. B-cell ELISpot

Analysis of OMV-specific IgG- and IgA-producing plasma cells in blood, spleen and lungs and analysis of OMV-specific memory B-cells in spleen and lungs were determined by ELISpot, as described before [13]. Plates were coated with 10 μg/mL wildtype B1917 OMV.

2.22. Multiplex cytokine analysis

The concentration of T-helper subset cytokines (IL-4, IL-5, IL-10, IL-13, IL-17A, TNFα and IFNγ) was determined in splenic culture supernatant using a ProcartaPlex Mix&Match Mouse 7-plex (Thermo-Fisher). Data acquisition and analysis were executed with a Bio-Plex 200 (Bio-Rad) and using Bio-Plex Manager software (v5.0, Bio-Rad). Results were corrected for the background (IMDM complete medium control) per mouse per stimulation per cytokine, and calculated in pg/mL.

2.23. Statistics

Significance of differences between spray dried and liquid omvPV in the Prn and Vag8 ELISA were determined using a 2-way ANOVA. Significance of inter-group differences for B-cell ELISpot analysis was determined using a Mann-Whitney t-test. Data from antibody, cytokine, and colonization assays were log-transformed and statistically tested using a t-test. P-values ≤ 0.05 were considered as significant differences.

3. Results

3.1. Physical characterization of spray dried omvPV

3.1.1. Particle size and morphology

A spray dried vaccine was prepared from OMVs of B. pertussis (omvPV). The cumulative geometric mean \( X_{50} \) particle size of powder vaccine as determined by laser diffraction analysis was 5.1 ± 0.1 μm (Table 1). No visible aggregates could be observed in the powders by eye. Scanning electron micrographs (SEM) (Fig. 1A) revealed spherical particles. Due to a preparation artifact some particles did stick to the vaccine as determined by laser diffraction analysis.

Table 1: Particle size of the spray dried pertussis outer membrane vesicles vaccine as determined by laser diffraction using aspiros dispersing system.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Particle size freshly prepared powder vaccine (μm)</th>
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<tr>
<td></td>
<td>( X_{50} )</td>
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<tr>
<td>Spray dried omvPV</td>
<td>0.93 ± 0.02</td>
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3.1.2. Amorphous status of the spray dried formulation and residual moisture content

Analysis of spray dried vaccine powder by DSC revealed a glass transition temperature (Tg) of 73 ± 2°C. The residual moisture content of the spray dried omvPV was 2.3 ± 0.1% as determined by Karl Fischer analysis. X-ray diffraction (XRD) was used to study the extent of crystallinity. The expected peaks in the XRD profile indicated that solid trehalose dihydrate (starting material) was crystalline. The absence of peaks in the XRD profile of spray dried vaccine powder (Fig. 1B) indicates that drying rendered an amorphous powder.

3.2. OmvPV stabilization during drying

The spray dried omvPV was reconstituted and then analyzed with TEM (Fig. 2A) to evaluate whether the vesicle structure of omvPV was preserved during the drying process. TEM analysis of liquid omvPV and reconstituted powder omvPV revealed that spray drying did not affect the particulate nature of omvPV. Both samples revealed an intact lipid bilayer. The particle size of both products was found to be 100 to 150 nm. The particle size and structural integrity of both liquid and reconstituted omvPV was also confirmed by DLS (Table 2). The average size of omvPV particles (formulated with trehalose) was 129.7 ± 1.7 nm before and 133.3 ± 1.2 nm after drying, with a low polydispersity index (0.22 ± 0.01). The particle size and morphology were not affected by the drying process.

The total protein composition of the liquid omvPV and the reconstituted spray dried omvPV were analyzed using LC-MS and compared. In total 195 proteins were identified and quantified in both samples. For both samples, the top 25 most abundant proteins comprised 92% of the total omvPV protein content. The two sets of samples were not different from each other.

Pertactin (Prn) is a major antigen in the current vaccine and was also detected in abundance in omvPV (Supplementary Fig. 1). Therefore, Prn antigenicity was also confirmed (Fig. 2C). No differences were observed between spray dried and the liquid omvPV whereas heat treated negative control (liquid omvPV sample stored at 65°C) showed complete loss of binding was observed. However, a 16% decrease in antibody binding intensity was observed in the spray dried omvPV compared to the liquid omvPV. Since Vag8 is the most abundant protein present in omvPV [10], the Vag8 antigenicity was determined by ELISA (Fig. 2B). Binding intensity of an anti-Vag8 monoclonal antibody in the liquid omvPV and spray dried omvPV was significantly better than the heat treated control (liquid omvPV sample stored at 65°C), where nearly complete loss of binding was observed. However, a 16% decrease in antibody binding intensity was observed in the spray dried omvPV compared to the liquid omvPV.

To investigate changes in tertiary structure of proteins in the omvPV after spray drying, intrinsic fluorescence (tryptophan residues) and extrinsic fluorescence (Bis-ANS) were measured to determine the changes in the environment of tryptophan [36] and the exposure of hydrophobic sites [37], respectively. Liquid omvPV and reconstituted spray dried omvPV showed similar emission spectra tryptophan maxima, 339 ± 1 nm and 340 ± 1 nm, respectively, but with a decreased intensity for the spray dried product (Supplementary Fig. 2A). For both products, the Bis-ANS maximum was 490 ± 2 nm with the spray dried omvPV showing increased intensity for the spray dried product (Supplementary Fig. 2A).

Since Vag8 is the most abundant protein present in omvPV, the Vag8 antigenicity was determined by ELISA (Fig. 2B). Binding intensity of an anti-Vag8 monoclonal antibody in the liquid omvPV and spray dried omvPV was significantly better than the heat treated control (liquid omvPV sample stored at 65°C), where nearly complete loss of binding was observed. However, a 16% decrease in antibody binding intensity was observed in the spray dried omvPV compared to the liquid omvPV.

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3.3. Thermostability of spray dried omvPV

To investigate the thermostability of the spray dried product, an accelerated stability study (40 and 65°C) was performed with liquid and spray dried omvPV. Liquid omvPV (unformulated) stored at 2–8°C...
was used as a reference. The vesicle size in spray dried and liquid omvPV was determined by DLS after storage. There was no change in vesicle size of omvPV in liquid or spray dried product at 40 °C. No change in vesicle size was noted at day 7 and 15 at 65 °C storage and on day 30 an apparent increase in vesicle size (~163 nm) was observed for spray dried omvPV (Supplementary Table 1). It is important to note that the addition of trehalose in liquid omvPV formulation influenced the vesicle size [formulated liquid omvPV (130 nm, Table 2) vs unformulated liquid omvPV (115 nm, Supplementary Table 1)].

The binding intensity of an anti-Vag8 monoclonal antibody to the omvPV did not change for the liquid and spray dried omvPV after prolonged storage at 40 °C (Fig. 3A). However, the binding decreased by 80% in the liquid vaccine after 1 day at 65 °C, followed by complete loss of antigenicity during further storage (Fig. 3A). On the other hand, the Vag8 epitope was maintained in the spray dried omvPV during the entire storage period.

To identify whether the epitope availability of other immunogenic antigens was also affected by the applied storage conditions, SDS-PAGE and Western blotting was performed on the liquid and spray dried omvPV. When comparing the total protein composition in both spray dried and liquid omvPV, a reduction in the intensity of an unknown protein (20 kDa) due to spray drying was observed (Supplementary Fig. 3). However, SDS-PAGE showed that the storage at 40 °C and 65 °C had no further effect on both products. Subsequently, these samples were analyzed in an immunoblot with a mixture of murine sera from three omvPV-immunized groups (pulmonary liquid, pulmonary reconstituted powder and subcutaneous liquid). The antigenicity of the omvPV components remained similar for both liquid and powder omvPV stored at 4 °C and 40 °C (Fig. 3B). However, while the spray dried omvPV stored at 65 °C remained stable, the liquid omvPV lost antigenicity; epitopes on several antigens including Vag8 and BrkA next to unknown proteins U1 and U2 disappeared after the heat treatment. Several new bands were formed against unknown proteins U3, U4, and U6 of respectively 38, 28 and 22 kDa, which may be degradation products. Not all antigens were affected, as the antibody binding to unknown proteins U5, U7 and, as expected, LPS, remained intact.

To investigate changes in the tertiary structure of omvPV during storage, both intrinsic and extrinsic fluorescence were analyzed. The intensity of the tryptophan fluorescence of the liquid omvPV stored at 65 °C decreased by 306 F.I. units compared to the product stored at 4 °C (Fig. 3C, i), whereas the spray dried omvPV only decreased by 114 F.I. units (Fig. 3C, ii). The emission maximum for both products stored at 4 °C was 339 ± 2 nm. During storage at 65 °C, a shift of 8 nm was observed for the liquid omvPV, whereas there was no shift in the
emission maximum of the spray dried omvPV. Bis-ANS fluorescence spectrum revealed an increased fluorescence intensity during elevated temperature storage, for both liquid (125 F.I. units) (Fig. 3D, i) and reconstituted spray dried (37 F.I. units) (Fig. 3D, ii) omvPV, indicating protein unfolding during storage. Overall, storage at elevated temperatures resulted in changes in the tertiary structure of both liquid and spray dried omvPV, however these changes were more pronounced in the liquid omvPV.

3.4. Immunogenicity of spray dried omvPV

3.4.1. Bacterial clearance from respiratory tract

In order to investigate the immunogenicity of the spray dried omvPV, mice were immunized twice with a four week interval (day 0 and 28) by either pulmonary administration of liquid omvPV (PM-mice), reconstituted spray dried omvPV powder (PMp-mice), or subcutaneous administration of liquid omvPV (SC-mice) (Fig. 4). First, the efficacy of the immunization was tested by investigating the number of colony-forming units (cfu) in the lungs, trachea and nose of naive and immunized mice following a B. pertussis challenge, four weeks after the second immunization (day 56) (Fig. 5). The cfu were determined in the respiratory tract on day 1 and day 6 post-challenge. In lungs and trachea of omvPV-immunized mice, no B. pertussis bacteria could be detected, except for a few bacteria in the lungs of SC-mice, 1 day post challenge, whereas naive mice were heavily colonized after a B. pertussis challenge (Fig. 5A and B). With respect to the nose, only PMp-mice were free of viable B. pertussis bacteria, whereas the mice in other groups showed presence of bacteria in the nasal wash. However, the number of bacteria in the noses of PM- and SC-mice were lower compared to naive mice (Fig. 5C). These results indicate that immunization with omvPV does protect mice upon challenge. Moreover, spray drying of the omvPV did not result in any loss in immunogenic efficacy.

3.4.2. IgA responses

To determine whether the (spray dried) omvPV induced mucosal immune responses after pulmonary immunization, anti-OMV IgA-secreting cells, IgA-producing memory B-cells and IgA antibody responses were assessed before challenge. Similar numbers of anti-OMV IgA-secreting cells were detected in the lungs, spleen and blood of both groups that received pulmonary immunization while these numbers were significantly higher than the numbers found in subcutaneously immunized and naive control mice (Fig. 6A). With respect to IgA-producing memory B-cells, significantly higher levels were observed in PM-mice.
compared to the other groups. Despite the presence of IgA-producing memory B-cells in the lungs of some PMp-mice, no significant differences were detected when compared to SC-mice or naive mice (Fig. 6B). Moreover, there were no significant differences detected between the groups in the levels of anti-OMV IgA-producing memory B-cells in the spleen (Fig. 6B). In accordance with the findings on IgA producing B-cells, IgA antibodies were exclusively detected in PM-mice and PMp-mice, in lungs (Fig. 6C), nose (Fig. 6E) and serum (Supplementary Fig. 4A). In lungs of PM-mice, IgA antibodies were found to be directed against BrkA, FHA, Fim2/3, Prn, Ptx, OMV and Vag8. With the exception for anti-FHA, these levels were all significantly higher in PM mice when compared to PMp-mice (Fig. 6C). The anti-OMV IgA detected in the lungs of PM-mice and PMp-mice was clearly directed against the vaccine constituents Vag8 and LPS (Fig. 6D). In the nose lavages of all pulmonary immunized mice, IgA antibodies directed against all measured antigens were found significantly enhanced compared to naive and SC-mice (Fig. 6E). The levels of anti-Ptx and anti-Prn IgA were significantly higher in PM-mice compared to PMp-mice. In SC-mice, a small detectable amount of anti-OMV IgA was observed (Fig. 6E). In serum, only a modest induction of IgA antibodies directed against OMV was found in both pulmonary immunized groups and for PM-mice also against Prn compared to naive and SC-mice (Supplementary Fig. 3A). In addition, both pulmonary immunized groups exhibited increased levels of anti-Vag8 IgA in the serum compared to naive mice. Thus, mucosal IgA-related responses were almost solely induced after pulmonary immunization of omvPV.

3.4.3. IgG responses

To determine whether the spray dried omvPV was capable of inducing systemic immune responses after pulmonary immunization, anti-OMV IgG-secreting cells, IgG-producing memory B-cells and IgG (subclass) antibody responses were determined. Significantly increased numbers of OMV-specific IgG plasma cells were detected in spleen, lungs and blood of all omvPV-immunized mice compared to naive mice,
7 days after booster immunization (Fig. 7A). In the spleen, numbers of plasma cells were significantly lower in PMp-mice when compared to SC-mice. On the other hand, significantly higher numbers of these plasma cells were observed in the lungs of both mucosally immunized groups compared to the SC-mice. In the blood, no significant differences were observed between the immunized groups (Fig. 7A). Increased numbers of OMV-specific IgG memory B-cells were found in the spleens of all immunized mice (Fig. 7B). In the lungs, numbers of IgG memory B-cells were significantly enhanced in both groups receiving pulmonary immunization compared to naive mice. Moreover, the PM-mice demonstrated significantly higher numbers of IgG memory B-cells in the lungs compared to both PMp- and SC-mice (Fig. 7B).

In serum, the levels of IgG antibodies directed against BrkA, FHA, Ptn, OMV, and Vag8 were significantly enhanced in the omvPV-immunized groups compared to naive mice (Fig. 7B). Anti-Ptn IgG levels were significantly lower in PMp-mice compared to PM- and SC-mice. Moreover, the levels of IgG antibodies directed against BrkA, FHA and Vag8 were slightly higher in SC-mice compared to mice receiving pulmonary immunization (Fig. 7C). The immunoproteomic profile of serum IgG revealed no differences between both PM- and PMp-mice, whereas the SC-mice induced additional antibodies against a few other antigens such as GroEL (Fig. 7D). All omvPV-immunized mice demonstrated the induction of a broad IgG subclass response as all mice induced significant levels of IgG1, IgG2a, IgG2b and IgG3 antibodies against a variety of antigens compared to naive mice (Supplementary Fig. 4B). Of note, the anti-Ptn IgG1 and IgG2b levels were significantly lower in PMp-mice when compared to PM- and SC-mice. IgG3 levels were slightly higher in SC-mice when compared to pulmonary immunized mice, especially PM-mice.

These results indicate that IgG immune responses were induced by omvPV immunization irrespective of the route of administration. However, compared to non-spray dried material, immunization with spray dried omvPV resulted in lower numbers of IgG-producing memory cells in the lungs and lower levels of serum antibodies against Ptn. This may indicate an effect due to spray drying.

3.4.4. T-cell responses

To determine the effect of omvPV immunization on T-cell responses,
levels of seven T-cell related cytokines in supernatants of OMV-stimulated splenocytes and lung cells were analyzed. The presence of IFN-$\gamma$ and IL-17A, indicative for Th1 and Th17 responses, respectively, was observed in the supernatant of OMV-stimulated splenocytes for all administration routes. As expected, the IL-17A response was higher in the mice receiving mucosal administration (Fig. 8A). IL-10 levels were increased in all immunized groups. Levels of IL-4 and IL-13, indicative of a Th2 response, were enhanced in all immunized groups but were slightly higher in SC-mice. A significantly higher IL-5 production (also a Th2 marker) was observed in all omvPV-immunized mice, but these levels were more pronounced for the subcutaneous administration compared to the mucosal administration.

Stimulation of lungs cells with OMVs led to increased levels of IFN-$\gamma$ (Th1) and IL-17A (Th17) in all immunized groups, but these levels were significantly higher in both mucosal administered groups compared to the subcutaneously administrated group (Fig. 8A). IL-10 levels were increased in all immunized groups but were slightly higher in SC-mice. A significantly higher IL-5 production (also a Th2 marker) was observed in all omvPV-immunized mice, but these levels were more pronounced for the subcutaneous administration compared to the mucosal administration.

Fig. 6. - Mucosal humoral responses. (A-B) Numbers of (A) OMV-specific IgA-secreting plasma cells in lungs, blood, and spleens and (B) numbers of IgA-producing memory cells in spleens and lungs were determined by B-cell ELISpot of 6 mice per group at day 35 and day 56, respectively. Results are indicated as antibody-secreting cells (ASCs) per $5 \times 10^5$ cells. (C-E) Levels of immunoglobulin A (IgA) antibodies directed against Ptx, Prn, FHA, Fim2/3, BrkA, Vag8 and OMV were determined in (C) the lungs and (E) the nose. Results are expressed as fluorescence intensities (F.I.) of 6 mice per group. (D) Immunoproteomic profile of pooled ($n = 6$) pulmonary IgA was determined using Western blotting. Significant differences are indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$ obtained using a Mann-Whitney $t$-test (B-cells) and $t$-test (antibodies) after log-transformation of data.

4. Discussion

The present study reports the development of a novel spray dried pertussis vaccine formulation, based on OMVs, with increased thermostability compared to the liquid formulation. Results show that spray dried omvPV could be prepared without aggregation or decomposition of the vesicles. Although a decrease in Vag8 antigenicity after drying suggested minor epitope loss. However, when administered via the pulmonary route after reconstitution, the spray dried omvPV exhibited comparable immune responses as well as similar protection (upon challenge with live bacteria) as compared to the liquid vaccine when administered via the same route. Moreover, in contrast to the liquid omvPV the Vag8 antigenicity of the spray dried omvPV was maintained
During storage at 65 °C for 4 weeks as depicted by the antibody binding assays, providing a promising prospect for the design of a more stable solid vaccine dosage form.

Not much is known about spray dried OMVs. Arigita et al. [38] reported the use of freeze drying for an OMV of Neisseria meningitidis in the presence of sucrose (3% w/v), but this resulted in aggregation possibly due to rupture of the vesicular structure during the freezing step. Much more is known on drying of liposomes. Although there are many differences between liposomes and OMVs, some physical and biochemical aspects are similar. Ohtake et al. [39] successfully used trehalose for stabilization of DPPC-cholesterol vesicles during freeze drying. Trehalose (5% w/v) provided stability to the phospholipid membrane by direct interaction with it, possibly due to hydrogen bonding. In the current study, spray drying of omvPV using trehalose (10% w/v) preserved the vesicular structure of the vaccine (as observed by DLS and TEM), further no aggregation occurred (as concluded from DLS data). Trehalose is believed to exert its protective mechanism by immobilization of the vaccine in an amorphous matrix portrayed by the vitrification theory [40]. Thus, based on the ability of trehalose to interact with protein surfaces [41], it proved to be a good choice for stabilizing omvPV.

Storage at elevated temperatures, but also the spray drying process itself may affect the conformation of antigens, thereby resulting in a change in antigenicity [42] and possibly as a result the immunogenicity. Spray drying of omvPV revealed some changes in the vesicular structure as shown by changes in protein immunogenicity. Spray drying of omvPV using trehalose for stabilization of DPPC-cholesterol vesicles during freeze drying. Trehalose (5% w/v) provided stability to the phospholipid membrane by direct interaction with it, possibly due to hydrogen bonding. In the current study, spray drying of omvPV using trehalose (10% w/v) preserved the vesicular structure of the vaccine (as observed by DLS and TEM), further no aggregation occurred (as concluded from DLS data). Trehalose is believed to exert its protective mechanism by immobilization of the vaccine in an amorphous matrix portrayed by the vitrification theory [40]. Thus, based on the ability of trehalose to interact with protein surfaces [41], it proved to be a good choice for stabilizing omvPV.

An immunization study demonstrated immunogenicity and protective capacity of the omvPV after spray drying. In comparison to the liquid omvPV, the spray-dried omvPV maintained its capability to induce strong and broad IgG antibody responses [10, 46], systemic plasma and memory B-cells [13] and a mixed systemic Th1 and Th17 response [10, 12–14]. Moreover, mucosal immunity was attained by pulmonary administration [13], given the significant amounts of IgA plasma and memory B-cells, pulmonary and nasal IgA responses and Th17 responses in the lungs. Pulmonary administration of spray dried omvPV provided protection against Bordetella pertussis in a similar manner as the liquid omvPV in our mouse challenge model. These mucosal responses and improved level of protection induced by pulmonary omvPV immunization were comparable with infection-induced responses by B. pertussis [13, 15, 16, 34]. Moreover, the protection was similar to subcutaneously administered vaccine. This indicates that spray drying, despite antigenic changes, did not affect vital components of the vaccine that are required for obtaining immunity.

A few differences in antibody profiles were observed, that were perhaps due to spray drying. The spray dried omvPV evoked lower
number of pulmonary IgG- and IgA memory B-cells as well as lower levels of pulmonary IgA antibodies against BrkA, Fim, OMV, Prn, Ptx and Vag8. As the nasal IgA antibody responses against these antigens were not affected, this seemed not an effect due to spray drying but a possible effect of the distribution in the respiratory tract. Pertactin did seem to be slightly affected by spray drying. However, no change in epitope availability was demonstrated during product characterization with a Prn-specific monoclonal antibody, the in vivo systemic anti-Prn IgG antibody induction was lower. On the contrary, the product characterization did demonstrate a moderately affected Vag8 antigenicity after drying. However, this effect did not result in an altered anti-Vag8 antibody response. Of note, the in vivo-induced polyclonal antibodies cover a broader range of epitopes on antigens as compared to the included monoclonal antibodies used in vitro.

In the current study, we demonstrated that the administration of reconstituted spray dried omvPV powder induced protective immune responses in a mouse challenge model [13]. At this point, the delivery with either reconstituted or powder would most likely give a similar indication whether changes have occurred during drying. Direct inhalation of the spray dried powder itself could even enhance better dispersion in the respiratory tract and circumvent the logistics of reconstitution [42]. However, Tonnis et al. [47] have previously shown that the Penn-Century dry powder insufflator, deposits spray dried powder (X50 4.5 μm) predominantly in the trachea of mice, leaving a relatively small amount of powder to reach the lungs. Moreover, spray dried trehalose is hygroscopic and would likely agglomerate in the device so such an approach would require further formulation studies to mitigate that. In a clinical situation where the vaccine is administered to humans, devices like Twincer® can be used for pulmonary delivery of spray dried powders [48]. A study from Saluja et al. [49] showed that agglomeration of spray dried powders and their retention in the delivery devices like Twincer® can be reduced by addition of so-called sweeper crystals like mannitol. Further work is required to establish the pulmonary delivery of pertussis vaccine as a powder, preferably in more suitable animals like baboons [50] or even in clinical studies.

In conclusion, the study demonstrates that spray drying can be used to produce an omvPV powder. The powder has an improved stability at
increased temperatures compared to the liquid formulation. Despite small changes in antigenicity during drying, when pulmonary im-
munized in mice, the immunogenecity and protection induced by re-
constituted spray dried omvPV was very similar to the responses in-
duced by the liquid omvPV. Thus, the spray drying of omvPV could
facilitates extended storage outside the cold chain and is a promising
technology for future powder immunization.

Supplementary data to this article can be found online at https://

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