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Suitability of differently formulated dry powder Newcastle disease vaccines for mass vaccination of poultry

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Key words: Spray drying; Dry powder vaccine; Mass poultry vaccination; Live-attenuated vaccine; Newcastle disease; Mannitol

Abbreviations¹

¹ BSA: Bovine Serum Albumin; DPBS: Dulbecco's Phosphate Buffered Saline; DSC: Differential Scanning Calorimetry; EID₅₀: 50% Egg Infectious Dose; ND: Newcastle Disease; PVP: Polyvinylpyrrolidone; RH: Relative Humidity; SEM: Scanning Electron Microscopy; SPF: Specific Pathogen Free; T_g: Glass transition temperature; T_m: Melting temperature.

Abstract

Dry powders containing a live-attenuated Newcastle disease vaccine (LZ58 strain) and intended for mass vaccination of poultry were prepared by spray drying using mannitol in combination with trehalose or inositol, polyvinylpyrrolidone (PVP) and/or bovine serum albumin (BSA) as stabilizers. These powders were evaluated for vaccine stabilizing capacity during production and storage (at 6°C and 25°C), moisture content, hygroscopicity and dry powder dispersibility. A mixture design, varying the ratio of mannitol, inositol and BSA, was used to select the stabilizer combination which resulted in the desired powder properties (i.e. good vaccine stability during production and storage, low moisture content and hygroscopicity and good dry-dispersibility). Inositol-containing powders had the same vaccine stabilizing capacity as trehalose powders, but were less hygroscopic. Incorporation of BSA enhanced the vaccine stability in the powders compared to PVP-containing formulations. However, increasing the BSA concentration increased the hygroscopicity and reduced the dry dispersibility of the powder. No valid mathematical model could be calculated for vaccine stability during production or storage, but the individual experiments indicated that a formulation combining mannitol, inositol and BSA in a ratio of 73.3:13.3:13.3 (wt/wt) resulted in the lowest vaccine titre loss during production (1.6-2.0 \log_{10} 50% egg infectious dose (EID₅₀) and storage at 6°C (max. 0.8 \log_{10} EID₅₀ after 6 months) in combination with a low moisture content (1.1-1.4%), low hygroscopicity (1.9-2.1% water uptake at 60% relative humidity) and good dry-dispersibility properties.

1. INTRODUCTION

In poultry farming spray- and aerosol vaccinations are frequently used as mass vaccination methods against various viral respiratory diseases amongst which Newcastle disease (ND) is most significant [1, 2]. After reconstitution of the freeze-dried vaccine cake in water, the vaccine dispersion is nebulized throughout the poultry house followed by inhalation of vaccine particles by the birds. The efficiency of this method can be greatly reduced by a number of factors: 1) the currently used liquid vaccine nebulizers generate sprays or aerosols with wide droplet size distributions, reducing the inhalable fraction of vaccine particles. Moreover, in case of primary vaccination of young birds, fine respirable vaccine particles produced during spraying may cause severe post-vaccination reactions [3, 4]; 2) after nebulization the airborne vaccine droplets evaporate, leading to vaccine inactivation and uncontrolled vaccine particle size spectra [5-7] and 3) the tap water used for vaccine reconstitution cannot stabilize the vaccine sufficiently [6, 8] and is often contaminated with viricidal agents such as chlorines.

In order to circumvent mentioned drawbacks monodisperse dry powder vaccines may represent a better alternative for current liquid spray and aerosol vaccines in which case the ideal powder vaccine should meet the following requirements: 1) show no or only limited virus loss during production and storage; 2) be monodisperse; 3) have a size that will enable exclusive targeting of the high respiratory tract for primary vaccinations or the lower respiratory airways for secondary (booster) vaccinations; 4) be easy to disperse into their primary particle size; 5) be non-hygroscopic to prevent hygroscopic growth of the vaccine virus particles in the airways during respiration [9] and 6) is non-toxic for man, animals and environment.

Milling of freeze-dried ND vaccines enabled Fournier and Gaudry to successfully produce powder ND vaccines [10, 11]. However, this technique did not allow the manufacturing of

powder vaccines with narrow-sized particle spectra. In contrast, Corbanie and others were able to improve the monodispersity of ND powder vaccines by means of spray drying [12]. Although the newly produced spray dried powder vaccines consisting of trehalose with polyvinylpyrrolidone (PVP) and/or bovine serum albumin (BSA) stabilized the ND vaccine during spray drying, they were highly hygroscopic and significant virus losses were observed during storage. An exception was the low hygroscopic mannitol powder vaccine which was stable during storage, but suffered from severe vaccine virus titre loss during spray drying [12].

Therefore, further improvement of spray dried powder vaccines regarding vaccine stability, hygroscopicity and dispersibility, was attempted in a series of studies. At this stage it was not the aim to produce powder vaccines with defined particle sizes and a narrow span.

To combine the stabilizing properties of the carriers used by Corbanie during spray drying and storage, in this study mannitol was spray dried with trehalose and PVP and/or BSA. In order to minimize hygroscopicity, the formulation mainly consisted of mannitol. Inositol, a low hygroscopic sugar, was introduced as a new stabilizer as substitute for trehalose in the powder formulation. After preliminary experiments focusing on vaccine stability, dry-dispersibility and hygroscopicity, the optimization of a selected ND vaccine formulation was investigated in the main experiments.

2. MATERIALS AND METHODS

2.1. Preliminary experiments

2.1.1 Powder formulations and spray drying

Combinations of mannitol (C*Mannidex, Cargill, Krefeld, Germany), trehalose (Treha 16400, Cargill, Krefeld, Germany), inositol (myo-inositol, Sigma-aldrich, Steinheim, Germany), PVP (Plasdone[®] K-25, ISP, Switzerland) and BSA (Sigma-aldrich, Steinheim, Germany) were prepared as 5% (wt/wt) aqueous solutions, using the ratios as shown in Table 1. The feed solutions supplied at a rate of 7.5 ml/min were spray dried in a Büchi B-290 spray dryer (Büchi Labortechnik AG, Flawil, Switzerland) with an inlet temperature set at 150°C and an outlet temperature at 70-75°C. Immediately before spray drying ND vaccine virus (LZ58 strain, kindly provided by Intervet, Boxmeer, the Netherlands) was added to the feed solution aiming at a virus titre of 10^{10} 50% egg infectious dose (EID₅₀) per 100 ml feed solution. A sample of 1 ml feed suspension was taken immediately after preparation and stored on ice until virus titration was performed, which was done within 3 hours. During the spray drying process the feedstock was kept on ice to minimize virus loss. The spray dried powder samples were stored in sealed glass vials at 6°C (conditioned room) or 25°C (incubator; Memmert INP 600, Memmert GmbH, Schwabach, Germany) packed in airtight aluminium bags.

Powder vaccines without polymer or protein were prepared once while those containing polymers were spray dried in duplicate. Placebo powders used to assess the powder characteristics were spray dried in triplicate.

2.1.2 Assessment of virus concentrations

Samples of the feed solution (1:10 dilution in Dulbecco's phosphate buffered saline (DPBS, Gibco, Invitrogen, Paisley, UK)) and powders (100 mg dissolved in 1 ml DPBS) were titrated in specified pathogen free (SPF) chicken eggs in order to determine the loss of vaccine virus infectivity during spray drying. The vaccine virus titre of the powders was also determined after 1, 6 and 12 months storage at 6°C or 25°C to investigate the stabilizing properties of the powders. Tenfold dilution series of the samples were prepared in sterile DPBS containing 1% penicillin-streptomycin solution (Penicillin G (10000 IU/ml)-streptomycin sulphate (10000 µg/ml) solution, Invitrogen, Paisley, UK). Of each dilution 100 µL was injected into the allantoic cavity of 10-days-old embryonated SPF-eggs (4 eggs per dilution). After incubating the eggs during 72 hours at 37°C the allantoic fluid of the eggs was tested for haemagglutinin activity [13]. Virus titres were calculated as EID₅₀ according to Reed and Muench [14]. The detection limit was calculated through assumption of the minimal requirements for applying the formula of Reed and Muench (i.e. 3/4 infected eggs using the undiluted suspension and no infected eggs injected with diluted suspensions) leading to a detection limit of 10^{2.3} EID₅₀ per g powder.

2.1.3. Characterization of powders

2.1.3.1 Moisture-related powder properties

To determine the residual moisture content Karl Fischer titrations (Mettler DL35, Mettler Toledo, Zaventem, Belgium) were performed on the powder samples immediately after production. Powder (100-200 mg) was added to an airtight beaker containing absolute dry methanol (Biosolve, Valkenswaard, the Netherlands). Titration of the samples was performed using Karl Fischer reagent (Hydranal[®] Composite 2, Sigma-Aldrich, Munich, Germany). The mixture was stirred for 5 minutes before actual titration. All titrations were carried out in triplicate.

The hygroscopic nature of the powders was investigated by dynamic vapor sorption (DVS Advantage, Surface Measurement Systems, Middlesex, UK). Using this technique, water sorption isotherm curves were recorded for each powder sample. Powder (10-20 mg) was weighed in the sample cup. Prior to exposure to various relative humidities (RH) the samples were dried under a stream of dry nitrogen at 25°C until equilibrium (i.e. a weight change of less than 0.002% per minute during at least 15 minutes). Subsequently, the RH was increased from 0 to 90% by steps of 10% at 25°C allowing equilibration at each interval.

2.1.3.2 Physical state of powders

The occurrence of amorphism and crystallinity in the spray dried powders was investigated using differential scanning calorimetry (DSC) (DSC Q2000, TA Instruments, Zellik, Belgium). Samples of spray dried powders (5-10 mg) were transferred into a hermetically sealed aluminium cup, and after 1 min equilibration at 10°C the temperature was increased at a rate of 20°C/min to 250°C.

2.1.3.3 Dry-dispersion properties of the spray dried powders

Particle size distributions of the powders were measured within 24 hours after production by laser diffraction using a Malvern Mastersizer-S long bench (Malvern Instruments, Malvern, UK). Primary particle sizes were compared to the particle sizes measured during dry dispersion of

the powder to evaluate the dry dispersion properties of the powder. The obtained particle size parameters from the measurements were volume diameters $D(v, 0.1)$, $D(v, 0.5)$, $D(v, 0.9)$. The results were expressed as the ratio of the particle size parameters measured by dry and wet dispersion (dry/wet ratio). Powders with a significant increase ($P < 0.05$) in $D(v, 0.1)$, $D(v, 0.5)$ and $D(v, 0.9)$ parameters upon dry dispersion (dry/wet ratio > 1) were categorized as poorly dry dispersible. When only $D(v, 0.1)$ significantly increased upon dry dispersion the dry-dispersibility of the powder was classified as 'incomplete'.

Measurement of the primary particle size distributions was done by the wet dispersion method using the 300 RF lens (Malvern Instruments, Malvern, UK). Powder samples were dispersed in Miglyol[®] 812N (capric triglyceride; Sasol, Hamburg, Germany) with 0.2% Tween[®] 80 (polysorbate 80; Fagron, Waregem, Belgium) and subsequently sonicated to remove visible agglomerates.

Dry dispersion of the powders was performed using an experimental nebulizer consisting of a small compressor (OMRON CX3, Hoofddorp, the Netherlands) generating an airflow of 10 L/min at 2.5 bar coupled to a glass Büchner flask (Schott Duran 100 ml, Mainz, Germany) and expelling the powder particles via a 21 cm long plastic tube (Tygon[®] Laboratory Tubing R3603, Saint-Gobain Performance Plastics, Akron (OH), USA). Powder samples were dispersed directly into the laser beam of the laser diffractor. During the measurements the orifice (2x10 mm) of the disperser was held 4 cm from the laser beam and 4 cm from the lens (300 mm lens, Malvern Instruments, Malvern, UK). All measurements were performed in triplicate.

2.2. Main experiments

2.2.1 Powder formulations and spray drying

Based on the stabilizing properties of the powders observed in the preliminary experiments, mannitol, inositol and BSA were selected as powder compounds in the main experiments. A modified simplex centroid mixture design [15] was carried out to investigate the influence of each stabilizer on vaccine stability (i.e. remaining vaccine titre), the residual moisture content and the hygroscopicity after spray drying. To assure sufficient powder yield after spray drying, each formulation contained a minimum of 60% mannitol as concluded from other experiments (unpublished results). The ratios between mannitol, inositol and BSA were varied as shown in Table 3. Three centre points were included in the design to allow random error estimation. The design was generated and analyzed using MODDE software (MODDE 9.0, Umetrics, Sweden) [15].

Formulations were prepared as 10% (w/w) aqueous solutions with approximately 10^{10} EID₅₀ of ND vaccine virus (LZ58) per 100 ml and subsequently spray dried using a Büchi B-290 lab scale spray dryer at the same settings as for the powders of the preliminary experiments. Placebo powders were spray dried in triplicate to determine the powder characteristics.

2.2.2. Assessment of virus concentrations and powder characteristics

Virus titres of the feed solutions and spray dried powders were determined by virus titrations in chicken SPF-eggs immediately after production and after 1 and 6 months storage at 6°C or 25°C. Titrations were performed as described in 'Assessment of virus concentrations' (2.1.2). The detection limit was $10^{2.3}$ EID₁₀ per g powder.

Placebo powders were characterized immediately after production for moisture content, hygroscopicity at 60% RH and dry dispersion properties as described previously (2.1.3). The morphology of the spray dried samples was examined by scanning electron microscopy (SEM) (SEM Quanta 200F, FEI, Hillsboro (OR), USA). To improve the electron conductivity of the samples a gold coating of approximately 40 nm was sputtered onto the samples.

2.3. Statistical analysis

The moisture content data and particle size parameters of the preliminary experiments obtained by wet and dry dispersion were statistically evaluated by a one-way ANOVA (SPSS 16.0; SPSS, Chicago (IL), USA) and checked for homogeneity of variances using the Levene test. Log-transformation of data was performed when needed to obtain good homogeneity of variances.

The dry and wet dispersion data of the powders from the main experiments were analysed in the same way. The raw data of the remaining vaccine titre (%) after spray drying and storage were evaluated by means of a replicate plot (MODDE 9.0, Umetrics, Sweden). A replicate plot evaluates the random variability of the data *versus* the variability obtained by changing factors. The data of residual moisture content and hygroscopicity of the samples were also analysed using this software and plots of the scaled and centred model coefficients were generated.

3. RESULTS

3.1. Preliminary experiments

3.1.1. ND vaccine virus concentrations after spray drying and storage

The virus titre of the feeds ranged from $10^{9.0-10.5}$ EID₅₀ per 100 ml for both the preliminary and the main experiments. The loss of virus titre after spray drying of the ND vaccine containing feeds and after storage of the powders at 6°C or 25°C is shown in Table 1. The mannitol/trehalose combination led to the highest titre loss after spray drying (2.9 log₁₀ EID₅₀), while a loss of 1 log₁₀ EID₅₀ was observed using the combination of mannitol with inositol. The virus titres in the powder formulations containing also PVP and/or BSA were reduced by 1.3-2.6 log₁₀ EID₅₀ after spray drying.

After 12 months storage the powder vaccine titres declined more severely when stored at 25°C than at 6°C, resulting in final vaccine titres below the detection limit ($10^{2.3}$ log₁₀ EID₅₀/g) for most powder formulations. The vaccine stability in the powders did not depend on the use of trehalose or inositol as similar results were obtained for both powder series. However, the presence of BSA in the powder formulation enhanced the vaccine stability when powders were stored during 12 months at 6°C, losing between 0.7 – 1.7 log₁₀ EID₅₀, regardless whether BSA was used together with PVP or not.

3.1.2. Moisture-related powder properties

Although the moisture contents of all powders were low (max. 1.5%), adding PVP and/or BSA significantly ($P < 0.05$) increased the moisture content of powders (Table 2). This effect was more pronounced for the trehalose powders, as shown also by the water sorption experiments (Fig. 1). The water uptake by trehalose powders was generally higher than that of inositol powders: a maximal water uptake of 8.7% was observed at 90% RH in case of the

mannitol/trehalose/PVP/BSA formulation, while the mannitol/inositol/PVP/BSA powder contained 3.2% water at 90% RH.

3.1.3. Physical state of powders

According to the DSC-results presented in Table 2, all powders were crystalline with a melting peak around 160°C due to the presence of mannitol. However, except for the mannitol/inositol formulation, glass transition temperatures were observed in the powders, indicating that these powder matrices are not purely crystalline, but are to a certain degree also amorphous. PVP-containing powders showed the highest glass transition temperatures ($T_g = 88-90^\circ\text{C}$), followed by the mannitol/trehalose powder ($T_g = 73^\circ\text{C}$).

3.1.4. Dry-dispersion properties of the spray dried powders

The $D(v, 0.1)$, $D(v, 0.5)$ and $D(v, 0.9)$ of the powders when measured in wet dispersion mode ranged from 1.7 to 13.3 μm , 7.5 to 62.7 μm and 17.5 to 221.5 μm , respectively. Table 2 shows the dry/wet ratio of the particle size parameters when measured by wet dispersion (primary particle size) and by dry dispersion method immediately after production. All powders had a significantly higher $D(v, 0.1)$ after dry dispersion, while $D(v, 0.5)$ and $D(v, 0.9)$ were similar upon wet and dry dispersion. This indicated that only the smallest particles could not be deagglomerated via the dry dispersion method and the dispersibility of the powders was categorized as incomplete via the dry-dispersion method.

3.2. Main experiments

No valid model could be calculated for vaccine loss after spray drying and storage, however some trends could be observed from the individual experiments (Table 3).

The highest titre loss, being 4.5 \log_{10} EID₅₀, occurred when only mannitol was used during spray drying. Integrating inositol and/or BSA in the mannitol matrix limited the titre loss to 1.4-3.7 \log_{10} EID₅₀, depending on the ratio of the stabilizer compounds. In general, inositol concentrations $\geq 20\%$ in the mannitol-matrix resulted in higher titre loss after spray drying (2.9-3.7 \log_{10} EID₅₀) in comparison with BSA-containing powders ($\pm 2 \log_{10}$ EID₅₀), with the exception of mannitol/inositol/BSA (60:20:20) which lost only 1.4 \log_{10} EID₅₀.

When stored for 1 month at 6°C notable titre losses were observed for only 2 formulations: formulation 2 (mannitol/inositol (60:40)) with 1.1 \log_{10} EID₅₀ and formulation 10 (mannitol/inositol/BSA (73.3:13.3:13.3)) with 0.8 \log_{10} EID₅₀. The decline in vaccine titre was more pronounced when the powder vaccines were stored at 25°C during the same period. Exceptions were formulations 11 and 12 (a replicate of 11) in which no loss of vaccine was observed. In contrast formulation 10, having identical composition as formulation 11 and 12, did show vaccine titre loss after 1 month storage.

Titre losses after storage during 6 months at 6°C were relatively low (max. 1.6 \log_{10} EID₅₀ for mannitol/inositol (60:40)), while stored at 25°C the titre of mannitol (100%) and mannitol/inositol/BSA (60:20:20) dropped below the detection limit ($10^{2.3}$ EID₅₀/g).

Higher BSA concentrations in the powder increased moisture content and hygroscopicity of the formulations as indicated by the coefficient plots in Fig. 2. The highest moisture content (3.5%)

and water uptake at 60% RH (5.6%) was found for the powder formulation with the highest BSA content (mannitol/BSA (60:40); Table 3).

When measured in wet dispersion the $D(v, 0.1)$, $D(v, 0.5)$ and $D(v, 0.9)$ of the powders ranged from 2.1 to 15.4 μm , 7.7 to 193.0 μm and 15.8 to 449.0 μm , respectively. The dry dispersibility of the powders is outlined in Table 3. Powders with 20% BSA showed incomplete to poor deagglomeration of the particles upon dry dispersion.

The powder particle morphology (Fig. 3) was mainly influenced when 40% of inositol or BSA was present in the powders: spray drying 100% mannitol resulted in spherical particles (Fig. 3A), while the spray dried powder mannitol/inositol (60:40) consisted of large agglomerates (Fig. 3B) and mannitol/BSA (60:40) of strongly corrugated particles (Fig. 3C). In other powders such as the mannitol/inositol/BSA formulation (73.3:13.3:13.3) in Fig. 3 J, K and L (clusters of) spherical particles were observed.

4. DISCUSSION

Immediately after spray drying the ND powder vaccines were evaluated on several aspects: 1) ND vaccine stability in the powder during production and storage; 2) dry dispersibility of the powder into its primary particles and 3) moisture content and hygroscopicity.

To limit the ND vaccine titre loss in the mannitol-matrix during spray drying the incorporation of trehalose, inositol or polymers such as PVP and/or BSA was required: a reduction in titre loss from 4.5 \log_{10} EID₅₀ in the mannitol powder (Table 3) to 1.3-2.9 \log_{10} EID₅₀ (Table 1) in the preliminary experiments was achieved. The amorphous fractions in the crystalline mannitol-matrix induced by trehalose, BSA or PVP could have added to the vaccine stability during spray drying by preserving its structural integrity through immobilization in a glassy matrix (vitrification) [16]. However, in the mannitol/inositol powder of the preliminary experiments no glass transition temperature was measured, while only 1 \log_{10} EID₅₀ was lost after spray drying. It is possible that the amorphous phase induced by inositol remained undetected by DSC [17] or that vaccine virus stabilization mainly occurred due to hydrogen bond interaction between virus and inositol during drying (water replacement theory) [18]. The latter theory seems unlikely as the enveloped ND vaccine virus is hydrophobic [19].

A low storage temperature was beneficial for the vaccine integrity in the powders, as derived from the experiments, probably because the amorphous powder structure remains intact when stored at temperatures well below the glass transition temperature [20] and vaccine degradation reactions occur slower [21]. Although high glass transition temperatures would keep the vaccine more stable during storage, replacing PVP by BSA enhanced vaccine virus stability (Table 1). Probably other stabilizing mechanisms such as hydrophobic interaction of BSA [22-24] with the enveloped ND vaccine were responsible for the vaccine storage stability, while PVP can only engage in hydrophilic interactions [25].

In the main experiments mannitol was combined at varied ratios with inositol and BSA using a design of experiments, as inositol occurred less hygroscopic than trehalose in the preliminary experiments and BSA resulted in better vaccine stabilization during storage. Nevertheless, no valid model could be calculated for vaccine loss after spray drying and storage because the random variability of vaccine titre loss between the replicate samples (formulations 10, 11 and 12) was higher than the response variability obtained by changing the ratio of the

compounds (formulations 1-9). This was partially due to the assay-inherent variability of 0.5-1.0 \log_{10} units of the virus titration [26].

Based on the individual experiments the mannitol/inositol/BSA (73.3:13.3:13.3) formulation was selected as the most suitable combination for stabilizing the ND vaccine virus during production (virus titre loss of 1.6-2.0 \log_{10} units) and storage (virus titre loss of 0.0-0.8 \log_{10} units after 6 months at 6°C). Considering its low hygroscopicity and good dry-dispersibility (Table 3), the combination is qualified as a powder for inhalation. Mannitol is a well-tolerated sugar upon inhalation [27, 28] and is widely used in the pharmaceutical field [29] as well as in food industry [30]. Inositol is commonly present in food [31] and has no adverse effects after human oral consumption [32], however its effects upon inhalation by man or animal have not yet been reported. As BSA is a protein from animal origin, present in beef and cow milk, its consumption can in some cases provoke allergic reactions in humans [33]. Nevertheless, this powder combination is generally considered safe, although inhalation experiments in both man and animal should be performed to confirm this.

This study did not focus on the production of powder vaccines with suitable particle size parameters (>20 μm for primary vaccination of young chicks, <5 μm and <10 μm for secondary vaccination of 2- and 4-weeks old chickens [34]) as this can be achieved by adapting the spray dry equipment and process. However, for the production of powders consisting of particle sizes exceeding 20 μm intended for primary vaccination of chickens, a higher polymer content (BSA) could be needed as this will favor the formation of larger droplets upon feed atomization by increasing the feed viscosity and so larger particles will be formed [35].

Although these powder ND vaccines lose more vaccine titre during spray drying (average of 2 \log_{10} EID₅₀) compared to their commercially freeze-dried counterparts (up to 0.5 \log_{10} EID₅₀ [36]), the titre loss of the spray dried powder vaccines after 1 year storage at 6°C (1-1.5 \log_{10} EID₅₀) approximates to that of the commercially freeze-dried ND vaccines (1-year titre loss of max. 1 \log_{10} EID₅₀ [37]). It should be considered that in this study all powders were spray dried at the same process parameters and the titre loss during spray drying could be decreased further by optimizing the spray dry process. Furthermore, the used spray dryer was a lab-scale model in which case the powder recipient and powder remained into contact with the exhaust air temperature throughout the entire process. The powder is therefore exposed to higher temperatures than would be achieved in larger models in which powder discharge occurs during spray drying [35]. The feasibility of preparing ND powder vaccines on a larger spray dryer model (Mobile Minor D-2000, GEA Niro, Soeborg, Denmark) was previously demonstrated [7].

The potential advantages of dry powder ND mass vaccination i.e. improved airborne vaccine stability, reduction of negative vaccinal reactions and improvement of immune response in the birds might overcome the higher titre loss during spray drying. This will be examined in future experiments in which the effects of powder ND vaccines will be compared with those of the currently applied liquid spray and aerosol vaccination methods in young broiler chicks.

In conclusion, combining mannitol, inositol and BSA (in a ratio of 73.3:13.3:13.3) resulted in a storage-stable spray dried powder ND vaccine with a low moisture content, low hygroscopicity and good dry powder dispersibility.

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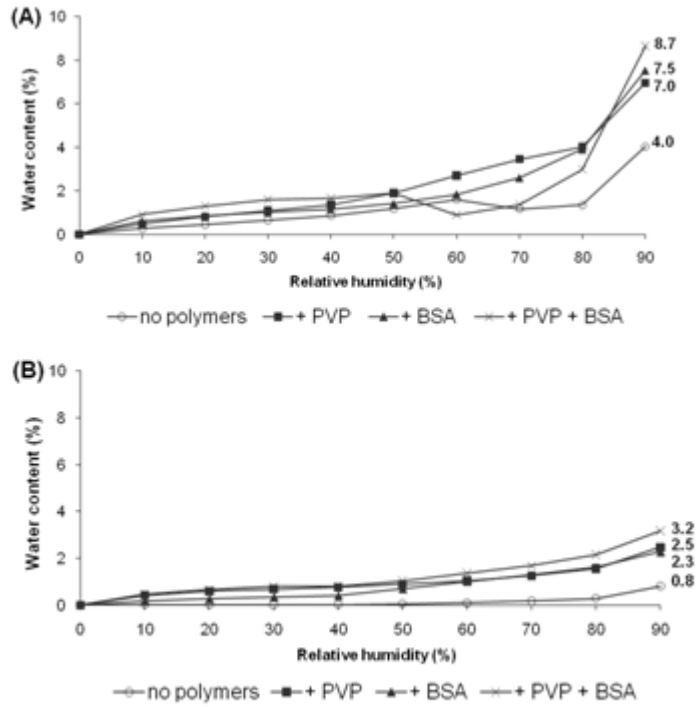


Fig. 1. Huyge Preliminary experiments: Water sorption isotherms (25°C) obtained from dynamic vapor sorption experiments (A) mannitol/trehalose series; (B) mannitol/inositol series.

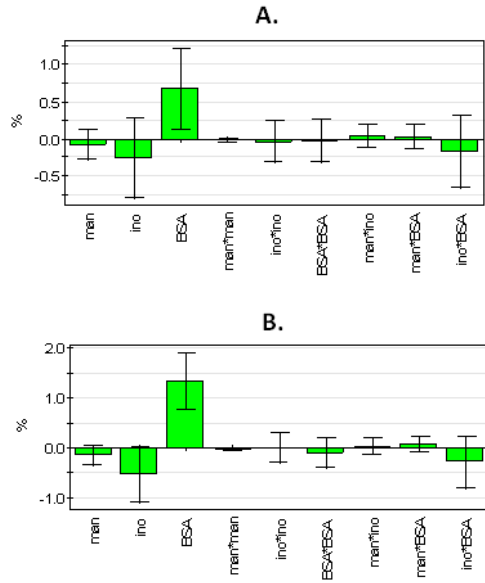


Fig. 2. Huyge Main experiments: coefficient plots of residual moisture content (A) and hygroscopicity (B) responses (man = mannitol; ino= inositol; BSA = Bovine Serum Albumin). Note the significant positive effect of BSA on the residual moisture content and hygroscopicity.

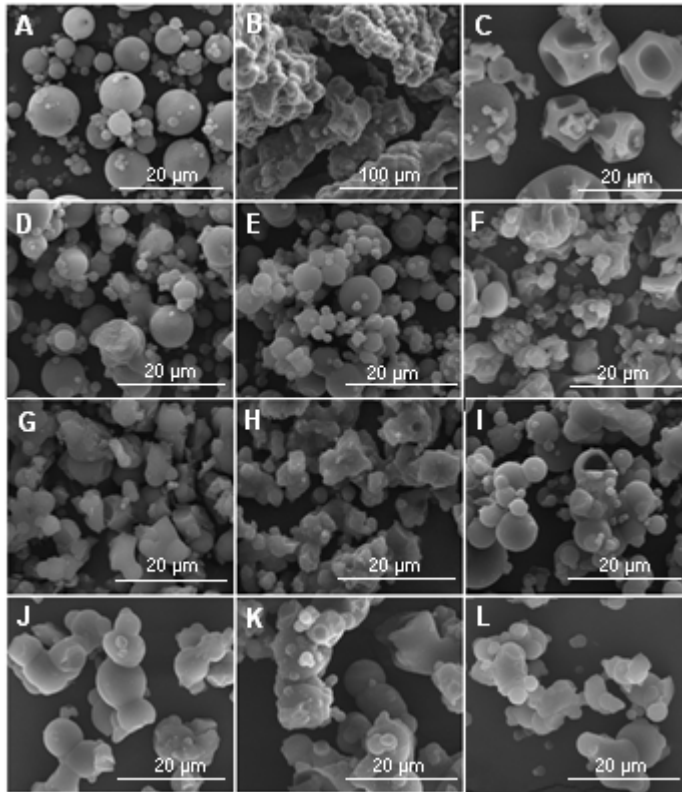


Fig. 3. Huyge

Main experiments: scanning electron microscopy images of formulations 1-12 (A-L) of which compositions are given in Table 3.

Table 1. Preliminary experiments: composition of feed solutions¹ for preparation of ND powder vaccines and loss of virus titre after spray drying and storage ($n = 2$)

	Content (wt/wt %)					Loss of virus titre (10^x EID ₅₀)						
	Mannitol	Trehalose	Inositol	PVP	BSA	after spray drying	1 month		6 months		12 months	
							6°C	25°C	6°C	25°C	6°C	25°C
Mannitol trehalose series	90	10	-	-	-	2.9	1.9	2.5	- ²	-	-	-
	80	10	-	10	-	1.3/2.3	0.5/0.8	3.2/-	2.5/1.8	>5.7 ³ /-	>5.7/3.5	>5.7/-
	80	10	-	-	10	1.8/2.3	0.5/0.3	0.8/1.2	1.5/1.0	3.5/2.2	1.7/1.3	4.8/3.5
	60	20	-	10	10	2.5/1.5	0.0/0.0	1.0/0.7	0.3/0.0	3.3/2.8	0.8/0.7	>4.2/3.3
Mannitol inositol series	90	-	10	-	-	1.0	0.3	3.8	1.1	3.8	2.3	>5.0
	80	-	10	10	-	2.0/2.0	1.0/0.0	2.2/2.1	1.5/-	>5.2/-	2.0/-	>5.2/-
	80	-	10	-	10	2.1/1.8	1.0/0.5	1.0/0.2	1.0/0.0	2.0/2.9	1.2/1.2	3.3/>4.9
	60	-	20	10	10	2.0/1.8	0.0/0.0	0.5/0.5	0.5/1.7	2.3/3.3	1.5/0.8	>4.7/4.2

¹ 5% wt/wt aqueous solutions.² Not done.³ > Symbol indicates virus titre was below detection limit ($10^{2.3}$ EID₅₀/g).

Table 2. Preliminary experiments: mean moisture content ($n = 3$), mean melting and glass transition temperature ($n = 3$), ratio of dry/wet particle size ($n = 3$), dry powder dispersibility and reference to results of dynamic vapor sorption experiments ($n = 1$) of spray dried placebo powders

Powder formulation ¹	Mean moisture content \pm SD (%)	DSC ²		Ratio of dry/wet particle size			Dry powder dispersibility	DVS ³
		T _m \pm SD (°C)	T _g \pm SD (°C)	D(v, 0.1)	D(v, 0.5)	D(v, 0.9)		
Mannitol trehalose	0.5 \pm 0.1 ^a	164 \pm 3	73 \pm 10	3.8 ^f	0.9	0.7	incomplete	Fig. 1A
Mannitol trehalose PVP	0.8 \pm 0.0 ^b	160 \pm 2	88 ⁴	1.7 ^f	1.0	1.5	incomplete	
Mannitol trehalose BSA	1.1 \pm 0.1 ^c	160 \pm 3	59 \pm 3	1.4 ^f	1.1	1.1	incomplete	
Mannitol trehalose PVP BSA	1.5 \pm 0.1 ^d	161 \pm 0	58 \pm 7 89 \pm 1	2.7 ^f	0.9	1.1	incomplete	
Mannitol inositol	0.3 \pm 0.1 ^e	160 \pm 1	-	3.6 ^f	1.0	0.9	incomplete	Fig. 1B
Mannitol inositol PVP	0.5 \pm 0.0 ^a	157 \pm 3	86 \pm 2	3.0 ^f	0.9	0.7 ^f	incomplete	
Mannitol inositol BSA	0.5 \pm 0.0 ^a	156 \pm 2	62 \pm 3	3.1 ^f	1.1	1.2	incomplete	
Mannitol inositol PVP BSA	0.5 \pm 0.1 ^a	157 \pm 3	90 ⁴	3.6 ^f	1.1	0.9	incomplete	

¹ Ratios of powder compounds are given in Table 1.

² DSC = differential scanning calorimetry experiments: T_m = melting temperature; T_g = glass transition temperature.

³ DVS = dynamic vapor sorption experiments.

⁴ Measured once.

^{a-e} Values with different superscript were significantly different ($P < 0.05$).

^f Particle sizes measured by dry and wet dispersion method were significantly different ($P < 0.05$).

Table 3. Main experiments: composition of feed solutions¹ for preparation of ND powder vaccines and loss of virus titre after spray drying and storage ($n = 1$), mean moisture content ($n = 3$), water sorption at 60% RH ($n = 1$), ratio of dry/wet particle size ($n = 3$), dry powder dispersibility and reference to scanning electron microscopy images ($n = 1$)

Formulation	Content (wt/wt %)			Powder vaccine					Placebo powder			SEM ²			
				Loss of virus titre (10^xEID_{50})					Mean moisture content \pm SD (%)	Water sorption at 60% RH (%)	Ratio of dry/wet particle size			Dry powder dispersibility	
				after spray drying	1 month		6 months				$D(v, 0.1)$		$D(v, 0.5)$		$D(v, 0.9)$
Mannitol	Inositol	BSA	6°C	25°C	6°C	25°C	(%)	(%)							
1	100	-	-	4.5	0.2	1.0	0.5	>1.7 ³	0.2 \pm 0.0	0.2	0.8	1.1	1.9	good	Fig. 3A
2	60	40	-	2.9	1.1	2.1	1.6	2.8	0.1 \pm 0.0	0.1	1.1	0.7 ^a	0.8 ^a	good	Fig. 3B
3	60	-	40	2.8	0.3	0.7	0.5	3.5	3.5 \pm 0.1	5.6	3.2 ^a	0.9	1.1	incomplete	Fig. 3C
4	80	20	-	3.7	0.0	1.1	0.3	1.3	0.1 \pm 0.0	0.1	1.1	0.7	1.3	good	Fig. 3D
5	80	-	20	2.2	0.0	1.3	1.0	3.3	2.0 \pm 0.2	2.9	2.1 ^a	1.1	1.0	incomplete	Fig. 3E
6	60	20	20	1.4	0.2	2.3	1.3	>5.0	0.5 \pm 0.2	3.1	2.9 ^a	1.9 ^a	3.7 ^a	poor	Fig. 3F
7	86.7	6.7	6.7	2.1	0.2	1.0	1.2	3.7	1.0 \pm 0.3	0.4	0.8	0.5 ^a	0.5 ^a	good	Fig. 3G
8	66.7	26.7	6.7	2.9	0.3	1.3	- ⁴	-	1.1 \pm 0.0	1.0	0.4 ^a	0.2 ^a	0.3 ^a	good	Fig. 3H
9	66.7	6.7	26.7	2.1	0.0	1.0	1.3	4.2	2.1 \pm 0.1	4.2	2.4 ^a	1.2	1.1	incomplete	Fig. 3I
10	73.3	13.3	13.3	1.7	0.8	1.6	0.8	3.1	1.4 \pm 0.2	2.1	1.1	0.8 ^a	0.8 ^a	good	Fig. 3J
11	73.3	13.3	13.3	1.6	0.0	0.0	-	-	1.1 \pm 0.2	2.0	1.1	0.9 ^a	0.9 ^a	good	Fig. 3K
12	73.3	13.3	13.3	2.0	0.0	0.0	-	-	1.3 \pm 0.0	1.9	1.2 ^a	1.0	1.0	incomplete	Fig. 3L

¹ 10% wt/wt aqueous solutions.

² SEM = scanning electron microscopy.

³ > Symbol indicates virus titre was below detection limit ($10^{2.3} \text{EID}_{50}/\text{g}$).

⁴ Not done.

^a Particle sizes measured by dry and wet dispersion method were significantly different ($P < 0.05$).