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Comparison of Newcastle disease vaccine administered as powder or liquid considering the serum antibody response and adverse vaccinal reaction in broilers

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Key words: ND, powder vaccine, liquid vaccine, vaccinal reaction, antibodies, broilers

24 **Abstract**

25

26 Liquid spray and aerosol mass vaccination of poultry has several drawbacks such as uncontrolled
27 deposition of vaccine particles in the respiratory tract and vaccine virus inactivation by formation
28 and evaporation of droplets, which may be addressed using dry powder vaccines with defined
29 particle size distributions targeting the upper (primary vaccination) or the entire respiratory tract
30 (booster vaccination). Therefore, a coarse ND (LZ58 strain) powder vaccine were administered to
31 SPF broiler hens to compare the seroresponse and adverse vaccinal reactions with those induced
32 by a coarse liquid spray and a fine liquid aerosol. Groups of 40 broilers each, housed in isolators
33 were vaccinated at 4 days of age and were intratracheally inoculated with *Escherichia coli* (strain
34 506) at 11 days of age. Adverse vaccinal reactions were evaluated by body weight gain and
35 mortality between 4 and 11 days of age and between 11 and 18 days of age, and by colibacillosis
36 lesions at 18 days of age. The antibody serum response was measured at 18 days of age by
37 haemagglutination inhibition test. Despite the relative low initial vaccine virus loss and narrow
38 particle size distribution of the powder vaccines in comparison with their liquid counter parts, no
39 significant differences (P >0.05) regarding adverse vaccinal reactions and serum response were
40 observed between broilers vaccinated with the powder vaccines or with their liquid counterparts.

41

INTRODUCTION

Vaccination of commercial poultry against Newcastle disease (ND) and other respiratory diseases is often performed by coarse liquid spray and fine liquid aerosol. Both, protection and adverse post vaccinal reactions (further referred to as vaccinal reactions) increase as vaccine loaded particles become smaller (Corbanie *et al.*, 2008; Gough & Allan, 1973; Meszaros *et al.*, 1992; Van Eck & Goren, 1991). To avoid severe vaccinal reactions coarse spray, intended to target the upper respiratory tract, is therefore used for primary vaccinations, while fine aerosol vaccinations, intended to reach the lower respiratory tract, are administrated as booster. Particles with a size >5 µm and >10 µm were hardly deposited in the lower respiratory tract (longs and thoracic airsacs) of 2- and 4-week-old broilers, respectively. These so-called cut-off particle sizes were defined as the smallest particle of which less than 5 volume percent (volume percentage(s) is further referred to as percentage(s) unless otherwise stated) is deposited in the lower respiratory tract. A reliable cut-off value for day-old chicks could not be assessed as these birds were breathing with open beaks (Corbanie *et al.*, 2006).

Spray and aerosol are relatively simple and cheap mass vaccination techniques, but have a number of drawbacks. Currently used spray and aerosol equipment generate broad droplet size distributions, which often results in severe vaccinal reactions when small droplets of a primary coarse spray vaccine are inhaled into the lower airways of young chickens (Giambrone, 1981, 1985; Van Eck & Goren, 1991), or in reduced deposition in the lower airways during secondary fine aerosol vaccination due to the presence of non-respirable droplets (e.g. droplet size spectra ranging between 10 and 1000 µm (Cargill, 1999)). Furthermore, the efficiency of vaccines administered by spray or aerosol might be jeopardized due to the use of tap water for reconstitution instead of distilled water (tap water often contains virucidal agents, such as chlorines) (Guittet *et*

al., 1997), by large shear forces, which are applied to the liquid in order to transform it into droplets (Swift, 1985) and most importantly, due to inactivation of the vaccine virus by evaporation of droplets after generation (Gough & Allan, 1973; Landman & Van Eck, 2001; Yadin & Orthel, 1978). It was hypothesized that these harmful effects might be overcome by formulating the vaccine in dry powder form with defined particle size, thereby improving vaccination efficiency. According to Huyge and others (Huyge *et al.*, 2012) 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) consist of particles with a size that will enable exclusive targeting of the upper 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 loaded particles in the airways during respiration (Morrow, 1986); and (6) is non-toxic for man, animals and environment. In previous research ND powder vaccines based on mannitol and bovine serum albumin (BSA), which approximated mentioned requirements, were prepared in a one-step spray-drying process (Corbanie *et al.*, 2007; Huyge *et al.*, 2012). A preliminary proof of principle experiment showed that the powder vaccine formulations induced high haemagglutination inhibiting (HI) antibody titres in 4-week-old broilers (Corbanie *et al.*, 2008).

In the present study, LZ58 ND vaccine was administered to 4-days-old SPF broiler hens either as powder or as liquid (further referred to as powder vaccine and liquid vaccine). Vaccinal reaction and immune response induced by coarse (particle size intended to be between 20 and approximately 50 µm) and fine (particle size intended to be <5 µm) ND powder vaccines were compared with those provoked by their liquid counterparts. Powder vaccines were based on mannitol-BSA formulations and had narrow-sized particle distributions. A third powder vaccine

consisting of 90 wt% coarse and 10 wt% fine particles (further referred to as mixed powder vaccine) was included in the experiment to examine the effect of fine particle contamination of a coarse powder vaccine on vaccinal reaction. Vaccinal reactions in the field are characterized by respiratory distress, growth retardation and most importantly enhanced susceptibility to secondary bacterial infections especially *Escherichia coli* infections. The latter may result in colibacillosis. Therefore, the vaccinal reactions in the present study were monitored using the following parameters: clinical signs, body weight gain, mortality and colibacillosis lesions following inoculation of the birds with a virulent *E. coli* strain (Dho-Moulin & Fairbrother, 1999; Goren, 1978, 1991; Gross, 1990; Van Eck & Goren, 1991). The immune response was determined by testing the sera of the birds two weeks after vaccination for HI ND antibodies.

SPF broiler hens were used in this study as maternal antibodies interfere with the serum antibody response (Van Eck *et al.*, 1991; Yadin, 1981). Further, broilers were chosen as they are more sensitive to colibacillosis than layers (Goren, 1991) and finally, birds of the same gender (hens) were used because body weight gain was one of the parameters for vaccinal reactions.

Although no longer commercially available the LZ58 ND vaccine virus strain was administered as it induces stronger vaccinal reactions compared to the Ulster 2C and Clone 30 ND virus strains (Van Eck & Goren, 1991). *E. coli* inoculation was performed intratracheally (i.t.) as the natural route of infection is likely airborne (Goren, 1978).

107 **MATERIALS AND METHODS**

108

109 **Experimental design and husbandry**

110 The experimental design is given in Table 1. Newly hatched (day of hatch is day 0) SPF broilers
111 obtained from and hatched at GD (Animal Health, Deventer, the Netherlands) were cloacally sexed
112 and subsequently eight groups of 40 hens each were formed. After individual tagging, groups were
113 placed in separated negative pressure HEPA isolators (Beyer & Eggelaar, Utrecht, the
114 Netherlands), with a volume of 1.38 m^3. At four days of age, broilers were vaccinated either with
115 powder vaccine (groups 4, 6 and 7) or with liquid vaccine (groups 5 and 8), further referred to as
116 vaccine groups. At 11 days of age all vaccinated birds were i.t. inoculated with *E. coli* strain 506.
117 Control groups (groups 1, 2 and 3) were included in the study. Group 1 served as negative control
118 group; further referred to as the negative group, while groups 2 and 3 were given powders without
119 vaccine virus (further referred to as placebo powders). Group 3 was *E. coli* inoculated and further
120 referred to as *E. coli* group, while Group 2 received phosphate buffered saline (PBS) instead,
121 further referred to as placebo group.

122 All broilers were individually weighed at four, 11 and 18 days of age and body weight gain
123 between day four and 11 and between day 11 and 18 was calculated. Groups of birds were
124 inspected daily for disease signs and mortality. Dead birds were stored at -20°C until gross
125 postmortem examination at the end of the experiment at 18 days of age at which time surviving
126 birds were stunned using a mixture of CO_2 and O_2. Thereafter they were debleeded by incision of
127 the *vena jugularis* and blood was collected for serology. Subsequently, birds were subjected to
128 postmortem examination to assess colibacillosis lesion scores. Bacteriological examination was

129 performed on all birds that had died during the study and of a number of surviving birds of all
130 groups (see post-mortem examination).

131 Commercial broiler feed and tap water were provided ad libitum during the whole
132 experiment. Up to eight days of age broilers were given 22 hours of light per day after which light
133 was reduced to 16 hours per day. Isolator temperature was 35°C on day 0 and was gradually
134 decreased to 25°C at day 18.

135

136 **Preparation of vaccines**

137 The coarse powder vaccine (intended particle size between 20 and approximately 50 µm)
138 was produced by spray drying using a 4M8-TriX Procept spray dryer, kindly supplied by Procept
139 (Zelzate, Belgium), equipped with a 25 kHz ultrasonic nozzle. The content of one vial with
140 approximately 10^{10} 50% egg infective dose (EID_{50}) of LZ58 ND vaccine virus, kindly provided
141 by Intervet-Schering Plough Animal Health (Boxmeer, the Netherlands), was suspended into 100
142 ml of a 15% (wt/wt) solution in demineralised water containing 60 wt% mannitol (C*Mannidex™,
143 Cargill, Krefeld, Germany) and 40 wt% BSA (Sigma-Aldrich, Steinheim, Germany). The vaccine
144 suspension was then spray dried at an inlet temperature of 100°C and a feed rate of 2 ml/min,
145 resulting in an outlet temperature of approximately 65°C. After production, the coarse powder
146 vaccine was sieved through a micro-sieve of 20 µm (W 0.02 MM, Hosokawa Alpine, Cheshire,
147 UK) using an air jet sieve (A200LS-N, Hosokawa Micron, Cheshire, UK) in order to minimize the
148 particle fraction below 20 µm.

149 The fine powder vaccine (intended particle size <5 µm) was produced by the Mobile Minor D-
150 2000 pilot plant spray dryer (GEA Niro, Soeborg, Denmark). Approximately, 10^{10} EID_{50} of LZ58
151 ND vaccine virus (content of one vial) was dispersed into 1500 ml of a 1% (wt/wt) solution in

152 demineralised water containing 80 wt% mannitol (C*Mannidex^{TM}, Cargill, Krefeld, Germany) and
153 20 wt% BSA (Sigma-Aldrich, Steinheim, Germany) and subsequently spray dried. The feed was
154 atomized by a two fluid nozzle (∅= 1 mm) operating at an air flow of 113.9 l/min and dried at an
155 inlet temperature of 120°C and a feed rate of 12 ml/min, resulting in an outlet temperature of
156 approximately 65°C.
157 During the production process, the powder recipients of the spray dryers were emptied every 10
158 minutes in order to minimize vaccine loss by heat stress. Coarse and fine placebo powders were
159 produced *via* the same processes as described earlier.
160 To obtain the mixed powder vaccine, 4.5 g of sieved coarse powder vaccine was physically mixed
161 with 0.5 g of fine powder vaccine. Placebo powders used in the experiment consisted of a mixture
162 of coarse and fine placebo powders in a 1:1 weight ratio.
163 Liquid vaccines used as coarse liquid spray and fine aerosol were prepared by suspending
164 approximately 10^{10} EID_{50} of LZ58 ND vaccine virus (content of one vial) in 500 ml sterile
165 demineralized water on the day of vaccination.
166
167 **Characterization of vaccines**
168 For virus titrations, 100 mg of each of the powder vaccines was dissolved into 1 ml
169 Dulbecco's phosphate buffered saline (DBPS, Invitrogen, Paisley, UK) with 1% penicillin-
170 streptomycin solution (penicillin G (10,000 IU/ml) – streptomycin sulphate (10,000 µg/ml)).
171 Samples of liquid vaccines, which had been taken immediately after vaccination and subsequently
172 frozen at -78°C by immersion of the sample containing test tubes (sterile polypropylene conical
173 test tube, 15 ml, MEUS, Piove di Sacco, Italy) into a mixture of dry ice and 70% ethanol (Chemlab,
174 Zedelgem, Belgium) and stored at -80°C until virus titration, were thawed at room temperature.

175 Ten-fold dilutions (10^{-3} – 10^{-9}) of each suspension were made in DPBS with penicillin-
176 streptomycin and 0.1 ml of each dilution was inoculated into the allantoic cavity of 10-day-old
177 embryonated SPF chicken eggs, using 4 eggs per dilution. After 72h of incubation at 37°C, the
178 allantoic fluid was tested for haemagglutinating (HA) activity (Grimes, 2002). Virus titres were
179 expressed as EID_{50}, calculated according to the formula of Reed and Muench (Reed & Muench,
180 1938).

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181 Particle and droplet size distributions were determined by a laser diffractor operating in
182 open mode (Mastersizer-S, long bench; Malvern Instruments, Malvern, UK) and equipped with a
183 300 mm lens using placebo powders and demineralized water, respectively. Dispersion was
184 performed with the same equipment used to vaccinate the broilers.

185 Powder samples were dispersed directly into the laser beam using an experimental air-assisted
186 device consisting of an air compressor (OMRON CX3, Hoofddorp, the Netherlands) forcing air
187 (10 l/min) into a glass Büchner flask (Schott Duran 100 ml, Mainz, Germany) and transporting the
188 powder particles to the outlet of the flask through a plastic tube (Tygon® Laboratory Tubing
189 R3606, Saint-Gobain Performance Plastics, Akron (OH), USA) of 21 cm with an orifice opening
190 of 2x10 mm.

191 The fine liquid aerosol was generated by the Walther Pilot I spray-head with a 0.5 mm nozzle
192 diameter (Walther Spritz-und Lackiersysteme, Wuppertal, Germany) attached to a compressor
193 (Mecha Concorde type 7SAX, 1001, SACIM, Verona, Italy) operating at 2 bar. The coarse liquid
194 vaccine was dispersed by manually actuating a hand-held spray bottle (Powerplus Garden POW
195 63868, Varo, Lier, Belgium) at a frequency of 1 actuation per second. During the measurements
196 with the hand-held spray bottle an air flow parallel to the lens protected it from moisture
197 deposition.

198 During analysis, orifices of the dispersion equipment were held 4 cm from the laser beam and 4
199 cm from the optic lens (Laboratory of Pharmaceutical Technology, Gent University, Gent,
200 Belgium; 'in house method'). All measurements were performed in triplicate and results were
201 expressed as mean volume diameters *D* (*v*, 0.1), *D* (*v*, 0.5), *D* (*v*, 0.9) and span. Span was calculated
202 as:

203 $$x = \frac{D\ (v, 0.9) - D\ (v, 0.1)}{D\ (v, 0.5)}$$

204 Also, percentages of particles and droplets with a size below 1, 5, 10 and 20 µm were determined.
205 Based on droplet size parameters *D* (*v*, 0.*x*), the size of dry particles obtained after complete
206 evaporation of the droplets was estimated using the following equation, taking into account the
207 content of dry matter in the droplets (1.25 mg/ml) and the density of the dry matter (assumed to be
208 1000 mg/cm^3):

209 $$D\ (v, 0.x)\ dry\ particle\ = D\ (v, 0.x)\ droplet\ \cdot \sqrt[3]{\frac{1.25}{1000}} = D\ (v, 0.x)\ droplet\ \cdot 0.108$$

210 The dry matter content of the liquid vaccine was determined in threefold by drying a sample of
211 known volume on a watch glass until constant weight (*i.e.* until ± 0.05 µg accuracy; analytical
212 balance AG245, Mettler Toledo, Zaventem, Belgium) in an incubator (Memmert INP 600,
213 Memmert, Schwabach, Germany) set at 40°C. Mean dry matter content was found to be 1.25
214 mg/ml.
215

216 **Vaccination**

217 Vaccination of the broilers was performed in the isolators in which they were housed.
218 During vaccination the isolator ventilation was switched-off until 30 min after the end of vaccine
219 dispersion. Temperature and relative humidity (RH) in the isolators were recorded every 2 seconds

220 by a temperature and humidity logger (Testostor 171-2, Testo, Ternat, Belgium) from the end of
221 vaccine dispersion until 30 minutes thereafter. During this period the RH raised from 50–70% to
222 65-80% at an average temperature ± SD of 30.4 ± 1.2°C.

223 Five grams of powder vaccine or 5 ml of liquid vaccine was dispersed in the corresponding
224 isolator aiming at a vaccine dose of approximately 10^8 EID_{50} per isolator. Powders and liquids
225 were dispersed as described under characterization of vaccines. Coarse vaccines were applied
226 beaming directly at the birds, while fine vaccines were dispersed in the air above the birds.

227

228 **Assessment of aerosol vaccine virus titres and estimation of inhaled vaccine dose per bird**

229 Aerosol sampling was performed using a Sartorius MD8 airscan (Sartorius B.V.,
230 Nieuwegein, the Netherlands) fitted with sterile gelatin filters with a pore size of 3.0 µm (17528-
231 80-ACD, Sartorius Stedim Biotech, Göttingen, Germany). The filters were held vertically at a
232 height of 15 cm above the isolator floor. Each time, a sample was taken during 2 min at 2000 l/h
233 resulting in a volume of 67 l. Samples were taken immediately after vaccine dispersion and 10 and
234 20 min thereafter. Directly after sampling gelatin filters were each dissolved in 10 ml sterile DPBS
235 (Invitrogen, Paisley, UK) kept at 37°C, frozen in a mixture of dry ice and 70% ethanol as described
236 under preparation of vaccines and subsequently stored at -80°C until virus titrations, which were
237 performed as described under characterization of vaccines with a few modifications. Samples
238 thawed at room temperature were inoculated as 10^0 to 10^{-6} dilutions into five 10-day-old
239 embryonated SPF eggs per dilution, using an inoculation volume of 0.3 ml per egg. Virus titres
240 were expressed per m^3 air, taking into account the dissolution volume of the gelatin filters (10 ml)
241 and the sample volume (67 l). The detection limit was calculated following the minimal conditions
242 necessary to be able to apply the formula of Reed and Muench (Reed & Muench, 1938) (i.e. 3 eggs

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243 with HA positive allantoic fluid out of 5 inoculated eggs with the lowest dilution (10^0)) and
244 appeared to be $10^{2.9}$ EID_{50} per m^3 air.

245 Initial vaccine virus loss defined as the difference between $\log_{10}$ vaccine dose per m^3 air
246 and $\log_{10}$ vaccine concentration of the aerosol per m^3 immediately after ending of the vaccine
247 dispersion, was calculated for each of the vaccine groups.

248 To assess vaccine virus loss during sample processing (freezing-thawing) 100 mg of coarse
249 powder vaccine was spread over a sterile gelatin filter. Subsequently, the filter was dissolved in
250 10 ml DPBS of 38°C and half of this volume was frozen and thawed as described above and kept
251 on melting ice together with the other non-frozen half until virus titration approximately two hours
252 later. Additionally, virus titration of 1 g powder vaccine without gelatin dissolved in 10 ml PBS
253 was performed. Vaccine virus titres per mg powder were $10^{4.8}$, $10^{5.0}$ and $10^{5.3}$ EID_{50} for the powder
254 vaccine without gelatin, the non-frozen and the frozen-thawed dissolved gelatin filter, respectively,
255 indicating that freezing and thawing of dissolved filters has no effect on vaccine virus titres.

256 The vaccine virus dose inhaled per broiler in the period from the start of vaccine dispersion
257 until 30 min after the end of dispersion was estimated based on a ventilation rate of 1932 ml/min
258 per kg body weight for 18-day-old broilers (Reeves *et al.*, 1991). The following assumptions were
259 made for the calculation of the vaccine virus-uptake: (1) all aerosol particles detected by air
260 sampling are inhalable; (2) the increase and decline of the aerosol vaccine concentration in the
261 isolator is linear; and (3) 30 min after the end of the vaccine dispersion the aerosol virus titres are
262 considered to be ‘0’; also all aerosol virus titres below the detection limit are considered to be ‘0’;
263 (4) vaccine retention by the birds is 100% (Hayter & Besch, 1974; Yadin, 1980).

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265 ***E. coli* inoculum**

266 *E. coli* strain 506 (serotype O78K80) used is a flumequine resistant and doxycycline
267 intermediary sensitive strain that was isolated from the inflamed pericardium of a broiler chick
268 suffering from natural colibacillosis. Frozen beads (-70°C) containing this strain were submersed
269 into 0.1% glucose broth (1000 ml purified water, 5 g Lab Lemco (Oxoid LP0029), 10 g
270 bacteriological peptone (Oxoid LP0-037), 5 g NaCl (VWR 1.06404.1000, Merck) and 1 g glucose
271 (VWR 1.08342.1000, Merck)), which was incubated during 17 hours at 37°C. This broth was
272 diluted 1:5000 (v/v) in PBS and kept on melting ice during the inoculation procedure. Every bird
273 received 0.3 ml i.t. One group (Group 2) was i.t. placebo inoculated with 0.3 ml PBS per broiler.
274 I.t. inoculations were performed using a 1 ml syringe coupled to a knobbed curved stainless steel
275 cannula of 1.5 X 45 mm (art.nr. 14186, AUV Group, Cuijk, the Netherlands).
276 Determination of the bacterial concentration of the inoculum was performed by means of
277 bacterial counting according to international standards using a sample taken at the start and at the
278 end of the inoculation procedure. The inoculum contained $10^{4.8}$ colony forming units (cfu) per ml
279 both, at the start and the end of the inoculation procedure, resulting in a dose of $10^{4.3}$ cfu per bird.
280

281 **Post-mortem examination**

282 Post-mortem examination was performed on all birds. The left and right thoracic air sacs,
283 the pericardium and the liver were macroscopically examined for colibacillosis lesions, which was
284 done blindly. The scoring (0 to 3 for each organ with a maximum score of 12 per bird) was
285 performed according to Van Eck and Goren (Van Eck & Goren, 1991). The mean lesion score
286 (MLS), the number and percentage of affected animals, and the number and percentage of birds
287 with generalized colibacillosis per group were assessed in surviving birds. A bird was considered

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288 affected when lesions were observed, while general colibacillosis was diagnosed when lesions
289 occurred on the pericardium and/or the liver.
290 The gender of each broiler was re-evaluated during post-mortem examination to correct the mean
291 body weights for sex faults on day 0.
292 Bacteriological examination of the bone marrow (femur) of all birds that died during the
293 experiment (except for one bird of group 7 from which the affected pericardium was examined),
294 supplemented with bacteriological examination of surviving birds to a maximum of 5 birds per
295 group was performed. Of the latter birds affected pericardia or airsacs were bacteriologically
296 examined. In case no colibacillosis lesions were observed (control groups), bone marrow was
297 examined of five birds per group.
298 Bone marrow from birds which died during the experiment was bacteriologically examined
299 to determine the specificity of mortality. Mortality was considered specific in case dead birds
300 showed generalized colibacillosis and/or *E. coli* was isolated from their bone marrow.
301 Bacteriological examination was performed using sheep-blood agar plates (K004P090;
302 Biotrading), which were incubated overnight at 37°C and thereafter visually inspected for purity.
303 Biochemical identification of colonies was performed using the indole and β-glucuronidase test,
304 which are both positive for *E. coli*.
305 All reisolates and the inoculum strain were stored at -80°C for Pulsed-Field Gel
306 Electrophoresis (PFGE).
307
308 **Serology**
309 Antibody titres to ND virus in sera of 24 surplus birds taken at day 0 and of all surviving
310 birds at day 18 were assessed by HI test as described by De Jong (De Jong, 1978) using 8 HA units

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311 of Ulster vaccine virus and expressed as $\log_2$ titres. Titrations were performed until endpoint and
312 all samples were analysed in one run. Titres <1 were given value 1 for the calculation of mean
313 titres and statistical analyses.
314 Log_2 antibody titres at day 0 were <1 confirming the SPF status of the broilers.
315
316 **PFGE**
317 The clonal relationship between reisolated *E. coli* bacteria and the inoculated *E. coli* strain
318 was analysed by PFGE.
319 The PFGE-technique of contour-clamped homogeneous electric fields (CHEF) was used
320 for genomic typing of the *E. coli* bacteria (one colony per positive sample). Genomic DNAs were
321 digested in agarose plugs with *Xba I* (10 U)(Roche Diagnostics, Mannheim, Germany). The
322 resulting fragments were resolved by CHEF-PFGE with a CHEF-DR® III apparatus (Bio-Rad
323 Laboratories, Richmond (CA), USA) at a constant voltage of 6 V/cm for 20 h at 14ºC, an included
324 angle of 120º and an initial and final switch time of 2.2 and 54.2 seconds, respectively. The
325 generated fingerprints were processed using BioNumerics software (Applied Maths NV, Sint-
326 Martens-Latem, Belgium). The similarity was calculated using the band-based DICE coefficient
327 with 1% optimization and 1% tolerance. Clustering was performed by the unweighted pair method
328 with arithmetic averages (UPGMA). Isolates were considered 'indistinguishable' if 100% of the
329 fragments were identical.
330
331 **Statistics**
332 Survival rates of groups were compared using the Mantel-Cox log-rank test. Body weights
333 at day 4 and body weight gain between 4 and 11, and between 11 and 18 days of age were compared

334 with one-way ANOVA followed by the post-hoc Scheffé's test after verifying the normality of
335 residuals with a Q-Q plot and homogenicity of variances with the Levene's test. Survival analysis
336 and body weight analysis were performed in SPSS version 20 (IBM, New York, USA).
337 Comparison of serum antibody titres and *E. coli* scores between the eight groups was performed
338 using the Kruskal-Wallis test. Pairwise comparisons were carried out using Wilcoxon rank sum
339 test, adjusting the p-values for the number of performed tests with the Bonferroni correction.
340 Numbers of birds affected by colibacillosis and with generalized colibacillosis were compared
341 between groups by fitting a logistic regression with infection as response and vaccination as
342 explanatory variable. All analyses were performed in R (version 2.14.1, the R Foundation for
343 Statistical Computing) unless specified differently. $P < 0.05$ was used as significance level.
344

345 **Ethics**

346 The study was approved by the Institutional Animal Experimental Committee, DEC-Consult
347 Foundation, according to Dutch law on experimental animals (Wet op de dierproeven).
348
349

350 **RESULTS**

351

352 **Vaccine characteristics**

353 Virus titres of the powder and liquid vaccines ranged from $10^{7.5}$ to $10^{7.7}$ EID_{50} per gram
354 and from $10^{7.8}$ to $10^{7.9}$ EID_{50} per ml, respectively (Table 2), resulting in a dosis per m^3 isolator air
355 (5 g or 5 ml vaccine was used per isolator) ranging from $10^{8.1}$ to $10^{8.5}$ EID_{50} (Table 3).
356 Average particle/droplet size distributions and distribution spans of the vaccines are presented in
357 Table 4. The percentages of particles and droplets with a diameter below 20, 10, 5 and 1 µm and
358 their corresponding vaccine virus titres per g or ml vaccine are presented in Table 2.

359 The median volume diameter (D(v,0.5)) of the coarse vaccine powder particles and of the
360 droplets of the coarse liquid vaccine were 37 and 106.4 µm, respectively. Dry particles originating
361 from droplets of the coarse liquid vaccine had a (D(v,0.5)) of 11.5 µm. Ninety percent of the
362 particles of the coarse vaccine powder had a size below 55 µm, while the same percentage of the
363 coarse liquid spray was present in droplets <300 µm. Spans of the coarse powder particle size
364 distribution, the droplet size distribution of the coarse liquid spray and of the dry particles
365 originating from droplets of the coarse spray were 0.8, 2.5 and 2.5, respectively (Table 4).

366 The coarse powder vaccine contained 4.4% particles smaller than 20 µm (virus titre in this
367 fraction $10^{6.3}$ EID_{50} g vaccine) and <0.01% particles smaller than 5 µm (virus titre in this fraction
368 <$10^{3.7}$ EID_{50} g vaccine). The coarse spray consisted of 0.52% of droplets below 5 µm; following
369 evaporation of the coarse spray droplets the percentage of dry particles below 5 µm was 17.8
370 (Table 2).

371 The fine powder vaccine particles and the droplets of the fine aerosol had a similar D(v,0.1)
372 (2.6 and 2.7 µm) and D(v,0.5) (6.5 and 10.0 µm), but a highly different D(v,0.9) (13.8 µm for the

fine powder vaccine and 237 µm for the fine liquid vaccine) resulting in substantially different spans (fine powder particles 1.7; fine aerosol droplets 23.5) (Table 4). The fine powder vaccine consisted of 35.3% of particles smaller than 5 µm (virus titre in this fraction $10^{7.0}$ EID_{50} g vaccine) and of 2.8% of particles below 1 µm (virus titre in this fraction $10^{5.9}$ EID_{50} g vaccine), while 83.7% and 49.6% of dry particles originating from fine aerosol droplets were below 5 and 1 µm, respectively (Table 2).

In general, particle size distribution of the mixed powder vaccine was in between that of the coarse and fine powder vaccine (tables 2 and 4). The percentages of particles smaller than 1, 5, 10 and 20 µm in the mixed powder vaccine were clearly below those of the fine powder (0.80, 5.0, 8.0 and 15.2% in the mixed powder vaccine, respectively versus 2.8, 35.3, 76.9 and 91.7% in the fine powder vaccine, respectively). However, differences in $\log_{10}EID_{50}$ vaccine virus titres in mentioned fractions of the vaccines were small (5.6, 6.4, 6.6 and 6.9 in the mixed powder vaccine, respectively versus 5.9, 7.0, 7.4 and 7.5 in the fine powder vaccine, respectively).

The percentage of particles below 1 µm in the mixed powder vaccine and the percentage of dry particles below 1 µm originating from the coarse vaccine droplets were almost equal (0.80 versus 0.81), while the percentages of particles below 5, 10 and 20 µm in the mixed vaccine powder (5.0, 8.0 and 15.2%, respectively) were below those of the dry particles originating from the coarse vaccine droplets (17.8, 44.7 and 76.1%, respectively) (Table 2).

Aerosol vaccine virus titres and estimated inhaled vaccine dose per bird

Vaccine doses per m^3 air, aerosol vaccine titres, initial virus losses and estimated vaccine doses inhaled per broiler are presented in Table 3. Vaccine virus was not detected in air samples taken after dispersion of placebo powders (groups 2 and 3). Although vaccine doses did not differ

396 substantially between vaccine groups ($10^{8.1}$ to $10^{8.5}$ EID_{50} per m^3 air) airborne virus titres did,
397 depending on the vaccine type. Lowest initial virus titre losses occurred after dispersion of fine
398 and mixed powder vaccines (1.0 $\log_{10}$ and 2.4 $\log_{10}$, respectively). These vaccines produced the
399 highest airborne virus concentrations: $10^{7.1}$ and $10^{5.9}$ EID_{50} per m^3 air for the fine and mixed
400 powder vaccine respectively immediately after dispersion, thereafter decreasing to $10^{4.4}$ and $10^{3.2}$
401 EID_{50} per m^3 air at 20 min after dispersion, respectively. Calculated inhaled doses of these
402 vaccines were $10^{4.3}$ and $10^{3.0}$ EID_{50} per broiler.
403 Coarse vaccines showed highest initial virus losses (4.1 $\log_{10}$ and 5.4 $\log_{10}$ for powder and liquid
404 vaccine, respectively) and lowest airborne concentrations ($10^{4.2}$ EID_{50} per m^3 air for the powder
405 vaccine and $10^{3.0}$ EID_{50} per m^3 air for the liquid vaccine, directly after nebulization), thereafter
406 decreasing to the detection limit ($10^{2.9}$ EID_{50} per m^3 air) or below resulting in the lowest inhaled
407 vaccine virus doses per bird ($10^{1.3}$ EID_{50} (= 20 EID_{50}) for the powder vaccine and $10^{-0.1}$ EID_{50} (=
408 0.8 EID_{50}) for the liquid vaccine).
409 The fine liquid vaccine gave an initial vaccine virus loss and airborne virus titres between the fine
410 and mixed powder vaccine on one side and the coarse vaccines on the other side.
411 The estimated inhaled vaccine dose per bird was $10^{2.5}$ EID_{50}.
412
413 **Vaccinal reactions and serum antibody response**
414 Clinical signs of disease were not observed in birds of the negative, placebo and *E. coli* group,
415 moreover antibodies to ND virus were not detected in these birds. Slight depression was observed
416 in all vaccine groups from 6 days after vaccination, being somewhat more severe in groups
417 vaccinated with the fine and mixed powder and the fine liquid vaccine (groups 6, 7 and 8).

Two to four broilers in each of the groups in which the fine vaccines or the mixed powder vaccine was administered (groups 6, 7 and 8) showed mouth breathing from 6 to 8 days following vaccination. This was not observed in the other vaccine groups (groups 4 and 5).

After *E. coli* inoculation clinical signs of depression increased in all vaccine groups (groups 4 to 8) and these signs were observed thereafter until the end of the experiment.

Values of parameters of vaccinal reaction other than clinical signs (mortality, body weight gain (BWG) and colibacillosis lesions) are presented in Table 1. No significant differences ($P > 0.05$) regarding these parameters were found between the control groups (groups 1, 2 and 3).

Mortality, which only occurred after *E. coli* inoculation, ranged from 2 to 4 broilers per group; differences between groups were not significant ($P > 0.05$). All mortality was specific, i.e. due to colibacillosis.

Mean body weight ± SD of broilers at day 4 (day of vaccination) ranged from 85 ± 6 to 91 ± 6 g between groups. Differences were not significant ($P > 0.05$).

All vaccine groups (groups 4 to 8) showed growth retardation and a significantly ($P < 0.05$) higher number of birds with colibacillosis, which was also more severe in comparison with birds of the *E. coli* group (Group 3). Growth retardation occurred in both, the week after vaccination (day 4 to day 11) and the week after *E. coli* inoculation (day 11 to day 18), but was more pronounced in the latter period.

BWG ± SD of vaccinated broilers (groups 4 to 8) between 4 and 11 days of age, and between 11 and 18 days of age ranged from 131 ± 27 g to 154 ± 18 g and from 126 ± 42 g to 160 ± 35 g, respectively. In the same periods BWG ± SD of the *E. coli* group (Group 3) was 161 ± 16± g and 189 ± 54 g, respectively.

440 In vaccine groups (groups 4 to 8) 68 to 97% of the birds showed colibacillosis lesions and the MLS
441 ranged from 1.7 to 4.9, while the values of these parameters in the *E. coli* group were 3% and 0.2.
442 Coarse vaccines (groups 4 and 5) induced less growth retardation, significantly ($P < 0.05$)
443 less colibacillosis and significantly ($P < 0.05$) lower HI ND virus titres compared to the fine
444 vaccines (groups 7 and 8).
445 Generalized colibacillosis hardly occurred in broilers vaccinated with the coarse vaccines (groups
446 4 and 5; 3 to 5%), while substantial numbers of birds with this condition were present in groups
447 vaccinated with the fine vaccines (groups 7 and 8; 15 to 28%).
448 Coarse vaccines (groups 4 and 5) induced mean $\log_2$ HI ND virus titres of 3.2 to 3.4; the fine
449 vaccines (groups 7 and 8) of 4.4. to 5.2. The highest mean HI titre (5.2) was found in broilers
450 vaccinated with the fine liquid vaccine (group 8).
451 No significant differences were seen between vaccinal reaction parameter values of birds
452 given the fine vaccines (groups 7 and 8) and those vaccinated with the mixed powder vaccine
453 (Group 6). The serum antibody response of broilers vaccinated with the mixed powder vaccine
454 (mean $\log_2$ HI ND virus titre is 4.3) was almost equal to that of broilers that received the fine
455 powder vaccine (Group 7: mean $\log_2$ HI ND virus titre is 4.4), but was significantly below the
456 response of birds supplied with the liquid aerosol (Group 8: mean $\log_2$ HI ND virus titre is 5.2).
457 Significant differences in values of vaccinal reaction parameters and seroresponse were
458 neither observed between broilers vaccinated with coarse powder (Group 4) and coarse liquid
459 vaccine (Group 5) nor between fine powder (Group7) and fine liquid vaccine (Group 8).
460
461 **Bacteriological analysis and PFGE**
462 Bacteria were not isolated from the bone marrow of birds of the negative and the placebo groups.

463 *E. coli* was isolated from the bone marrow of 16/17 broilers that died during the experiment, while
464 it was isolated from pericardium of one dead bird (group 7) of which the bone marrow was not
465 examined. *E. coli* was also isolated from 9/13 surviving birds of which affected organs
466 (pericardium or airsac) were bacteriologically examined.
467 PFGE of the 26 *E. coli* colonies revealed that, except for one colony from an airsac (group 5) all
468 colonies were clonal and showed 100% similarity to the parent strain used in this study. The
469 reference strains isolated from the bone marrow of layers with *E. coli* peritonitis syndrome (EPS)
470 were not genetically related to the parent strain and the reisolates (similarity $<85\%$).
471

472 **DISCUSSION**

473 In the present study, we hypothesized that the coarse powder vaccine aimed at targeting
474 exclusively the upper respiratory tract, would provoke less vaccinal reaction while retaining a
475 satisfactory immune response in comparison with coarse liquid vaccine and that the fine powder
476 vaccine would increase the seroresponse due to a lower loss of vaccine virus in the aerosol in
477 comparison with the fine liquid vaccine. The latter, likely due to the absence of virus inactivation
478 by formation and evaporation of droplets, was shown in previous research (Corbanie *et al.*, 2008).
479 However, neither a decreased vaccinal reaction using the coarse powder vaccine, nor an increased
480 seroresponse after vaccination with the fine powder vaccine was found.

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481 Although it was intended to produce a coarse powder vaccine with particles >20 µm to prevent
482 respirability, 4.4% had a diameter below this value. Regarding the fine powder vaccine our
483 intention was to manufacture a powder with particle sizes below 5 µm, which was achieved only
484 for 35.3% of the powder mass.

485 Estimated inhaled vaccine doses were 20 and 0.8 EID_{50} per bird for the coarse powder and
486 coarse liquid vaccine, respectively, which seems very low. If the calculated inhaled doses are real
487 and only a part of the afore mentioned doses is respirable, the amount of vaccine virus deposited
488 in the deeper airways will be even smaller. In this case there will be hardly any room to improve
489 the coarse powder vaccine regarding its exclusive deposition in the upper respiratory tract.
490 However, it is also possible that the inhaled doses have been underestimated. Plausible reasons for
491 underestimation are: 1. The ventilation volume used corresponded to 18-day-old broilers and may
492 have been relatively higher in younger birds. 2. The increase and decrease of airborne vaccine
493 virus concentrations is not linear and the virus concentration below the detection limit is not
494 necessarily zero. Even if underestimations resulting from both, point 1 and 2 would have been in

the order of magnitude of a factor 2 to 3, this would not result in substantial differences in virus inhalation. 3. Inefficient capturing of particles with a size below the pore size of that of the gelatin filters (<3 µm), however this is not of importance as the retaining efficiency of airborne particles of 0.01 to 0.9 µm proved to be >93% (Clark Burton *et al.*, 2007), while particles sized 0.5 to 3.0 µm were collected with an efficiency of 99.9% (Koller & Rotter, 1974). 4. Loss of virus in the gelatin filters and/or due to processing of the filters (snap freezing and thawing), however this does not play a significant role as shown in the present study. 5. The dispersion or spraying of coarse vaccines directly on the birds may have resulted in temporary relative high local concentrations of vaccine virus close to the birds, which were not or incompletely detected by air sampling. The latter seems the most plausible explanation for underestimation.

Whether coarse powder vaccines can be ameliorated regarding its vaccinal reaction inducing potency while retaining sufficient immunogenicity, is subject of current research.

The fine powder vaccine showed lower virus loss during dispersion compared to fine liquid vaccine) and persistence of the vaccine virus titre was highest in the powder aerosol (Table 3), which confirms earlier results (Corbanie *et al.*, 2008). Consequently, highest inhaled vaccine doses were estimated for the fine powder vaccine (Table 3). However, although not significantly different, highest HI ND titres were measured in broilers vaccinated with the fine liquid vaccine (Table 1). This is likely due to the relative high percentage of very small dry particles (<1 µm) in the liquid vaccine virus aerosol, which originate from droplets due to evaporation (Table 2). Obviously this high percentage of very small particles contained sufficient live vaccine virus to induce a strong serum response after penetrating into the deepest tissues of the lower respiratory tract (atria and air capillaries) (Corbanie *et al.*, 2006; Gough & Allan, 1973) despite virus inactivation due to formation and evaporation of droplets from which these particles emerge.

518 Therefore, it is concluded that the strongest immune response likely can be obtained by vaccine
519 particles of <1 μ, which is in agreement with the results of previous aerosol experiments with
520 inactivated ND vaccines (Van Eck, 1990). However, considering the current state of the art in
521 pharmaceutical technology, it seems almost impossible to manufacture a live vaccine virus
522 containing dry powder that (mainly) consists of particles <1 μm.

523 The mixed powder vaccine consisted of 90 wt % coarse powder vaccine and 10 wt % fine
524 powder vaccine, thus can be considered as a fine powder vaccine with a virus titre $1\log_{10}$ lower
525 than that of the vaccine that only contained fine particles. This difference in vaccine virus titres
526 was reflected in the estimated inhaled doses (Table 3), but did not result in significantly different
527 vaccinal reactions nor in significantly different seroresponses (Table 1). Obviously a dose of
528 $10^{3.0}$ EID_{50} vaccine virus per broiler already provoked the maximal reaction of the bird.

529 The mixed powder vaccine can also be considered as a coarse vaccine 'contaminated' by 10 wt%
530 fine powder vaccine. The foregoing illustrates that a contamination of mentioned magnitude is
531 detrimental for the desirable properties (exclusive deposition in the upper airways) of the coarse
532 powder vaccine.

533 The percentages of dry particles <1, <5, <10 and <20 μm, which originate from coarse spray
534 droplets by evaporation were equal or even greater than those of the mixed powder vaccine (Table
535 2). Nevertheless, the coarse liquid spray induced clearly less vaccinal reaction and a weaker
536 seroresponse compared to the mixed powder vaccine (Table 1). This is likely due the (almost)
537 complete absence of live vaccine virus in the small dry particles from the coarse liquid vaccine
538 due to inactivation by formation and evaporation of the droplets from which these small dry
539 particles emerge. Sufficient live vaccine virus to provoke a strong seroresponse and vaccinal
540 reaction was likely present in dry particles <1 and <5 μm originating from the fine liquid vaccine

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in contrast to the amount of live vaccine virus in dry particles of these sizes originating from the coarse liquid spray. An explanation for this may be the difference in mass in small dry particles between both vaccine types: high percentages from the fine liquid aerosol versus relative low percentages from the coarse liquid spray (Table 2).

It is common knowledge that ND vaccines induce vaccinal reactions characterized by respiratory distress, growth retardation and increased susceptibility to colibacillosis. It is also known that birds exposed to the vaccine per aerosol show more severe respiratory disease signs and growth depression compared to spray exposure (Bermudez & Stewart-Brown, 2008; Guittet *et al.*, 1997). Even mortality may occur after aerosol administration of ND vaccines to young chickens due to suffocation resulting from obstruction of the trachea bifurcation (Van Eck & Goren, 1991). The occurrence of respiratory distress and growth retardation inversely proportional to the size of vaccine particles was confirmed again in the present study. Moreover, it was also shown that vaccination with the fine vaccines increased the susceptibility to colibacillosis significantly more than vaccination with the coarse vaccines. Although this phenomenon was not unexpected, it is a novel finding. In comparison to broilers given the coarse vaccines, generalized colibacillosis occurred to a much greater extent after vaccination with the fine vaccines. From the present and other studies, it appears that generalized colibacillosis is very detrimental to broilers as it is associated with mortality and severe growth retardation (Matthijs *et al.*, 2005; Matthijs *et al.*, 2003; Peek *et al.*, 2013), while only a minority of birds recover (Peek *et al.*, 2013).

In the present study it was confirmed once more that an inhaled dose of about 100 to 1000 EID_{50} ND vaccine virus per bird administered via fine particles is able to induce excellent serum antibody responses in SPF broilers (Corbanie *et al.*, 2008; Kohn, 1955; Van Eck & Goren, 1991). However, it should be considered that if the same vaccine dose is used in broilers with maternal

564 antibodies a lower antibody response will likely be obtained (Davelaar & Kouwenhoven, 1977;
565 Gough & Allan, 1976; Van Eck *et al.*, 1991).

566 In conclusion, it can be stated that in the present laboratory experiment the coarse and fine
567 powder vaccines did not differ significantly from their liquid counterparts in terms of vaccinal
568 reaction and seroresponse. The room for improvement of the coarse powder vaccine is subject of
569 on-going research. Unfortunately, possibilities to ameliorate the fine powder vaccine at seem to be
570 out of reach at present.

571

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