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Efficacy and safety of an attenuated live QX-like infectious bronchitis virus strain as a vaccine for chickens

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The attenuation of infectious bronchitis (IB) QX-like virus strain L1148 is described. The virus was passaged multiple times in embryonated specific pathogen free (SPF) chicken eggs, and at different passage levels samples were tested for safety for the respiratory tract and kidneys in 1-day-old SPF chickens. There was a clear decrease in pathogenicity for the respiratory tract and kidneys when the virus had undergone a large number of passages. Passage level 80 was investigated for safety for the reproductive tract in 1-day-old and 7-day-old SPF chickens. In 1-day-old chickens, 12.5% of the vaccinated birds had macroscopic lesions. No lesions were observed if the chickens had been vaccinated at 7 days of age. Passage level 80 was investigated for its ability to spread from vaccinated to non-vaccinated chickens and for dissemination in the body. The virus was able to spread from vaccinated chickens to groups of non-vaccinated chickens, and in the vaccinated birds the virus was found frequently in oro-pharyngeal and cloacal swabs. A fragment of the hypervariable region of the S1 protein of passage level 80 was sequenced and revealed nucleotide changes resulting in two amino acid substitutions. Passage level 80 was given additional passages to levels 82 and 85. Both passage levels were tested for efficacy in SPF chickens and passage level 85 was tested for efficacy in commercial chickens with maternally derived antibodies (MDA) against a challenge with QX-like strain IB D388. In both SPF chickens and chickens with MDA, the vaccines based on strain IB L1148 were efficacious against challenge.

Introduction

Infectious bronchitis virus (IBV) is a coronavirus that causes respiratory disease in chickens. Infectious bronchitis (IB) disease signs include respiratory distress, reduced weight, reduced egg production, increased frequency of abnormal eggs and increased rates of mortality (Cavanagh & Gelb, 2008). Several different serotypes and genotypes of IBVs have been identified and new variants are still emerging. One of these new variants is QX-like IBV. Wang *et al*. (1998) were the first to isolate IBV QX-like virus from a flock of chickens suffering with proventriculitis in China. Later on, the virus was reported more frequently in relation to cases of nephritis and false layer syndrome (Benyeda *et al.,* 2009). QX-like IBVs were detected in East Russia in 2001 and in 2002 in West Russia (Bochkov *et al*., 2006). From 2003 onwards, IB QX-like viruses were detected in many countries in Europe: Germany, Holland, Belgium, France, Italy (Monne *et al*., 2008; Worthington *et al.*, 2008), Poland (Domanska-Blicharz *et al*., 2006), the UK (Gough *et al*., 2008) and Spain (Dolz *et al*., 2009). Various sources report that IB QX-like viruses are currently present in all European countries. Clearly, IB QX-like viruses pose a serious threat to the poultry industry. No vaccines specific for IB QX-like viruses are yet available. In the present paper the development of a live attenuated IB QX-like vaccine virus based on strain IB L1148 is described and the results of studies on safety and efficacy are discussed. Most of the tests described here were performed in accordance with the requirements of the European Pharmacopoeia (EP) monograph 01/2008:442, to which reference should be made for more detail of specific requirements.

Materials and Methods

Viruses. IB QX-like virus strain L1148, which was used for vaccine development, was isolated from broilers with respiratory disease. Allantoic fluid from the seventh passage of strain IB L1148 in eggs was kindly provided by Mrs K.J. Worthington and Prof. R.C. Jones, Department of Veterinary Pathology, University of Liverpool, UK (Worthington *et al*., 2008). IB QX-like challenge strain D388 was kindly provided by the Dr J.J. de Wit of the Animal Health Service, Deventer, The Netherlands (Landman *et al*., 2005).

Specific pathogen free chickens and chicken eggs. Specific pathogen free (SPF) eggs were obtained from Lohmann Tierzucht GmbH (Cuxhaven,

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Germany) or from SPAFAS (North Franklin, Connecticut, USA). For animal experiments performed at the Animal Health Service (Deventer, The Netherlands), SPF chickens were used from the in-house SPF layer flock. For production of virus and for titrations, eggs were incubated at 37°C in a relative humidity of 55%. For hatching, eggs were incubated under similar conditions but with turning.

Passaging of virus in SPF eggs. Virus passages were made, from passage 8 up to passage 101, by inoculation of 9-day-old to 11-day-embryonated SPF chicken eggs into the allantoic cavity, each with a quantity of 0.1 ml virus. After 1 day of incubation, dead eggs were discarded; and after 2 to 3 days of incubation, the allantoic fluid was harvested from the surviving eggs and pooled. The pooled allantoic fluid was centrifuged at $1000 \times g$ to remove debris. The supernatant was dispensed in small portions. The portions were partly used for further passages and partly frozen and stored at −70°C for further use. For subsequent passages the virus was diluted 1:1000 in phosphate-buffered saline. If considered necessary, samples of allantoic fluid were mixed with a stabilizer in a 3:1 allantoic fluid:stabilizer ratio. The stabilizer was an autoclaved solution containing 65 g peptone, 68 g gelatin, 50 g d-mannitol and 50 g inositol per litre in distilled water. The mixture was dispensed in 3 ml glass vials, 1 to 1.5 ml per vial, and the vials were lyophilized according to standard manufacturing procedures. Lyophilized samples were stored at −20°C.

Virus titration. Dilutions of the sample to be tested were inoculated into the allantoic cavity of 10-day-old embryonated SPF chicken eggs, six eggs per dilution. After 1 day of incubation, dead embryos were considered non-specific deaths and discarded. After an incubation period of 7 days, the embryos were examined for the presence of specific lesions caused by the virus. Dead embryos were considered positive for IBV. Live embryos were examined for signs of IBV infection; for example, dwarfing, curling and stunting. The titre, expressed as the median embryo infectious dose (EID_{50}) per millilitre, was calculated according to the method of Spearmann-Karber (Finney, 1964).

Identity testing by polymerase chain reaction. To distinguish or verify the IB strain, RNA was isolated using the High Pure Viral RNA isolation kit from Roche, according to the manufacturer's instructions. The RNA was used in a reverse transcriptase (RT)-polymerase chain reaction (PCR) using a one-step RT-PCR kit from Invitrogen, in which the RNA underwent a cDNA synthesis step for 30 min at 50°C, followed by an initial denaturation step for 10 min at 95°C. Forty repeat steps of denaturation for 30 sec at 95°C, annealing for 30 sec at 50°C and extension for 45 sec at 72°C were performed. The final extension step had a duration of 7 min at 72°C and the reaction was stopped by incubation of the PCR mixture for 5 min at 4°C. The IB L1148-specific primer sequences used were 5′-GCTTATGCAGTAGTCAAT-3′ as forward primer and 5′-CACGTGGAATCATGCCTGTTAT-3′ as reverse primer. These primers were designed from the nucleotide sequence of the hypervariable region of the S1 protein of strain IB L1148, NCBI Genbank accession number DQ431199 (Worthington *et al*., 2008). The specificity of the primers had been tested in a PCR with RNA from IB strains H120, D274 and M41. No PCR product was formed from the RNA of these IB strains. After completion of the RT-PCR reaction, the size of the PCR product was determined by agarose-gel electrophoresis. The fragments were stained with ethidium bromide and their position viewed under ultraviolet light. Their size was calculated by comparing the position of the bands with base pair markers that had also been loaded on the gel.

Sequence analysis. The nucleotide sequence coding for a part of the S1 protein of the attenuated IB L1148 strain was determined. RNA was isolated using the High Pure Viral RNA isolation kit from Roche, according to the manufacturer's instructions. The RNA was used in an RT-PCR reaction using a one step RT-PCR kit from Invitrogen, in which the RNA underwent a cDNA synthesis step for 30 min at 50°C, followed by an initial denaturation step for 10 min at 95°C. Forty repeating steps of denaturation for 60 sec at 95°C, annealing for 45 sec at 51.9°C, and extension for 120 sec at 72°C were performed. The final extension step had a duration of 10 min at 72°C, and the reaction was stopped by incubation of the PCR mixture for 5 min at 4°C. The IB L1148-specific primer sequences used for the test on the presence of the virus were 5′-CTTCAGGGTATGGCTTGGTCT-3′ as forward primer and 5′-AATTCTTCTGGCACTATCATCTTT-3′ as reverse primer. These primers were designed from the nucleotide sequence flanking the hypervariable region of the S1 protein of strain IB L1148, NCBI Genbank accession number DQ431199 (Worthington *et al*., 2008). The PCR product was purified with the Qiaquick PCR purification kit from Qiagen, according to the instructions of the manufacturer. After completion of the RT-PCR reaction, the size of the PCR product was determined by agarose-gel electrophoresis as described above. The nucleotide sequence was determined by Baseclear B.V. (Leiden, The Netherlands). The obtained sequence was compared with sequences of other QX-like IBVs using BLAST analysis.

Safety testing for respiratory tract and kidneys in chickens. The degree of virulence of virus at the various egg passage levels was determined by administering the virus to chicks, just after hatching, by eye drop at a titre of $10^{6.0}$ EID_{50} per dose in 0.1 to 0.2 ml. A dose of $10^{6.0}$ EID_{50} was used in the safety tests for the respiratory tract and kidneys, because the EP requires testing 10 times the maximum release titre for safety of the respiratory tract, and testing the maximal release titre in the test for safety of the reproductive tract. It was anticipated that a dose of $10^{5.0}$ EID_{50} would be the maximal titre for the vaccine. In Experiment A we tested egg passage levels 8, 25, 40 and 50, and in Experiment B we tested egg passage levels 65 and 80. In each test, a non-infected control group was included. Five chickens were killed humanely 5 days after inoculation, by inhalation of 100% carbon dioxide, another five birds at 7 days after inoculation and the remaining five birds at 10 days after inoculation. The ciliostasis test was performed in accordance with the EP. Briefly, the trachea was removed, rinsed and stored in physiological saline at 37°C. Transverse sections of 0.6 mm of the trachea were made using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd, Surrey, UK). The ciliary activity of three, four and three sections of, respectively, the upper, middle and lower part of the trachea was determined by light microscopy at a magnification of $400\times$. A score of 0 was given if the cilia in the complete tracheal section showed movement; a score of 1 was given if the cilia of 67% to 100% of the tracheal section showed movement; a score of 2 if the cilia of 33% to 67% of the trachea showed movement; a score of 3 if the cilia of less than 33% but not 0% of the tracheal section showed movement; and a score of 4 if the cilia in the complete tracheal section showed no movement at all. For each group, the average ciliostasis score was calculated. Gross pathological examination was done on the kidneys of each chicken used for the ciliostasis test. During the examination, some samples of the kidneys were taken for further histological examination and immunohistochemistry (IHC) staining. In some instances it was considered necessary to take additional samples for histology and IHC staining. The samples were preserved by fixation in formalin. Kidney lesions were scored as follows: −, no lesions; +, some infiltration of lymphocytes locally; ++, strong infiltration of lymphocytes through whole kidney; +++, strong infiltration of lymphocytes, some necrosis of epithelia in tubuli, atrophic foci. Histological examination was performed by the Animal Health Service.

Immunohistochemical staining. Sections of the tracheas and kidneys were deparaffinized. Endogenous peroxidase activity was blocked by incubation with 1% H_2O_2 containing 0.1% NaN_3 for 20 min at room temperature and subsequently boiled in Tris (0.01 M)–ethylenediamine tetraacetic acid (0.001 M), pH 9.0, for 10 min. The binding of Fc receptors was blocked by incubation with 10% foetal bovine serum for 20 min at room temperature. Immunohistochemical staining was performed using MAb 48.4 (Prionics, Lelystad, The Netherlands) directed against the IBV nucleocapsid protein (Koch *et al*., 1991) for 30 min at room temperature (dilution 1:20,000) in Normal Antibody Diluent (Klinipath, Duiven, The Netherlands). After three subsequent wash steps with PBS containing 0.05% Tween, the sections were treated with anti-mouse Dako EnVision+ (Dako UK Ltd, Cambridgeshire, UK) for 30 min at RT. Again, sections were washed three times with PBS Tween and then treated with diaminobenzidine (Dako UK Ltd) for 5 min at room temperature. This was followed by counterstaining using haematoxylin. Sections incubated in the absence of primary antibody

were included as negative controls. Immunohistochemical staining was performed by the Animal Health Service.

Testing safety for the reproductive tract of chickens. The safety of IB strain L1148 (passage 80) for the reproductive tract was assessed by inoculating groups of 50 female SPF chicks at the day of hatching or at 7 days of age, with $10^{5.0}$ EID_{50} per dose by eye-drop. This dose was used because the EP requires the maximum release titre of the vaccine to be tested for safety of the reproductive tract and it was anticipated that this would be a titre of $10^{5.0}$ EID_{50}. During the study, the birds were observed daily for clinical signs. Eleven weeks after vaccination, the birds were killed humanely, the coelomic cavity was opened and the complete oviduct examined macroscopically, externally and internally for the presence of cysts, strictures, deformation or aplasia. This test was performed in accordance with the EP monograph.

Dissemination in the body of chickens and spread of the virus strain. On the day of hatching, SPF chickens were vaccinated with $10^{5.0}$ EID_{50} IB L1148 passage 80 in a volume of 0.1 ml by eye-drop; this dose being selected to comply with the EP monograph. Three days after vaccination, 30 of the vaccinated chickens were added to a containment unit in which a group of 35 non-vaccinated chickens of the same age and of the same origin was housed. Seven days after vaccination, a group of 30 non-vaccinated 1-day-old chickens was added to this containment unit. Fourteen days after vaccination, 35 non-vaccinated chickens of the same age and origin as the vaccinated chickens were added to the containment unit. At regular intervals, two chickens of each group were removed and killed by inhalation of 100% carbon dioxide. Samples of the bursa, duodenum, lung, kidney, pancreas and trachea were taken in order to determine the presence of IBV by IHC staining as described above. The cloaca and larynx of these chickens were also swabbed after killing. The presence of IB QX-like virus in the swabs was determined by PCR.

Testing efficacy of the experimental vaccine strain against IB QX-like challenge in SPF chickens. On the day of hatching, SPF chickens, group housed in isolators, were vaccinated with doses of $10^{3.0}$ or $10^{3.3}$ EID_{50} IB L1148 passage levels 82 and 85, and with a dose of $10^{3.3}$ EID_{50} IB L1148 passage level 101. The samples of the passage levels 82 and 85 were lyophilized; the samples of passage level 101 were frozen allantoic fluid. IB strain L1148 at passage levels 82 and 85 was chosen to test its efficacy as a vaccine against challenge with a virulent IB QX-like virus, because we took into account that passage level 80 would most probably be the passage level of the master seed virus. In order to be able to produce a vaccine from the master seed virus, a few passages are required to scale up the virus. Passage 101 was tested to investigate whether higher passage levels would also be efficacious. Vaccine titres per dose of $10^{3.0}$ and $10^{3.3}$ EID_{50} were chosen because it was anticipated that a vaccine based on the attenuated IB L1148 strain would have a minimal titre of $10^{3.0}$ or $10^{3.3}$ EID_{50} per dose. The freeze-dried vaccines were reconstituted in distilled water. Reconstituted virus and the allantoic fluid of passage 101 were further diluted to the appropriate titre in distilled water. The chickens were vaccinated by coarse spray using a Hozelock flower sprayer. Generation of pressure and flow had been determined before vaccination began. The sprayer had a flow rate of approx. 2.5 ml/sec. Before vaccination, the exact vaccination time for the applicable number of birds was determined (0.5 ml per bird) and the sprayer was disinfected with 70% ethanol, followed by three rinses with distilled water. After preparation, the container with the diluted vaccine was placed in a water bath with melting ice. Just before vaccination the vaccine was put into the sprayer and the sprayer was put under pressure by moving the pump up and down 40 times. Prior to vaccination, it was tested for flow. The chicks, which had been placed in a small box, were vaccinated by spraying the vaccine from a height of 40 to 50 cm for the predetermined period. After spraying, the chicks were kept for at least 15 min inside the box. Subsequently, the chicks were released into their housing facilities. A non-vaccinated group served as control. At 21 days of age, 20 chickens taken from each group were challenged with $10^{4.0}$ EID_{50} IB D388 per dose of 0.1 ml. The challenge virus was applied by eye-drop. Of the non-vaccinated group, one-half was challenged and the other half was not challenged. During the whole study period the chickens were observed daily for clinical signs. Five days after challenge, the ciliary activity of tracheal explants was examined in 20 chickens per vaccine group and in 10 chickens per control group. Chickens were killed by injecting sodium pentobarbital (T61) intravenously. Immediately after death the trachea was removed and rinsed with, and stored in, physiological saline at 37°C. Small transverse sections of the trachea were cut by hand. Ciliary activity of three sections of the upper part, four sections of the middle part and three sections of the lower part of the trachea were examined by light microscopy at a magnification of $400\times$. The ciliary activity was scored as described in the EP monograph using the following classification criteria: 1 if $\geq 50\%$ of the tracheal section showed ciliary activity, and 0 if $<50\%$ of the tracheal section showed ciliary activity. For a given tracheal section, ciliary activity was considered normal when at least 50% (score 0) of the internal ring showed vigorous ciliary movement. A chicken was considered protected if no fewer than nine out of 10 rings showed normal ciliary activity. Significance of differences between groups was evaluated by chi-square analysis.

Testing efficacy of the experimental vaccine strain against IB QX-like challenge in chickens with maternally derived antibodies. We used commercial layer-type chickens on the day of hatching. The parent chickens had been vaccinated against, amongst other pathogens, IB at 1 day of age with a live IB Massachusetts type strain, at 5 weeks of age with a live IBMM vaccine, at 9 weeks of age with a live IB 4/91, and at 16 weeks of age with an inactivated combination vaccine containing IB M41 and IB D274. Five chickens were killed and blood samples were collected to test for the presence of IB maternally derived antibodies (MDA). This test was performed using the enzyme-linked immunosorbent assay test kit (Flock check, catalogue no. 04-01103-07; IDEXX Laboratories, Westbrook, Maine, USA). The chickens were housed on wood shavings in containment facilities. Each group was housed in a separate unit. The chickens were vaccinated with $10^{3.0}$ EID_{50} or $10^{3.3}$ EID_{50} per dose of IB strain L1148 passage level 85 as described above. A third group of MDA-positive chickens was not vaccinated. A group of 1-day-old SPF chickens was vaccinated with $10^{3.0}$ EID_{50} per dose. The vaccine virus was reconstituted and further diluted to the appropriate titre in distilled water. The chickens were vaccinated by coarse spray. At 21 days of age, 20 chickens were taken from each group and challenged with $10^{4.0}$ EID_{50} IB D388 per dose of 0.1 ml. The challenge virus was applied by eye-drop. During the whole study period the chickens were observed for clinical signs. Five days after challenge, the ciliary activity of tracheal explants was examined in the challenged chickens. All challenged chickens were killed by injecting sodium pentobarbital (T61) intravenously. The ciliostasis test was performed as described above for the SPF chickens.

Results

Passaging of the virus. During passaging, allantoic fluid was harvested 2 to 3 days after inoculation. The titres of the allantoic fluids ranged between $10^{8.0}$ and $10^{8.8}$ EID_{50}/ml. The virus titres did not change during the passages until passage level 85. After lyophilization, passage 80 had a titre of $10^{8.2}$ EID_{50} per vial, containing 1 ml allantoic fluid plus stabilizer mixture. Lyophilized preparations of passage 82 and passage 85 were also prepared. The titres per millilitre were $10^{8.41}$ EID_{50} and $10^{8.20}$ EID_{50}, respectively. Surprisingly, passage 101 had a much higher titre (i.e. $10^{9.51}$ EID_{50}/ml). All samples, including passage 101, which were tested for identity, reacted positively in the PCR with the IB L1148-specific primers (data not shown); that is, a clearly visible band was formed in the agarose gel with the expected size.

Sequence analysis. The nucleotide sequence of a 1456 bp fragment, between nucleotide positions 262 and 1718, of the region coding for the hypervariable region of the S1 protein of the vaccine virus was determined, and the

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DQ341199        285 GTCACAATTCTGTAGTGCACACTGTAATTTTTCTGAAATTACAGTTTTTGTCACACATTGTTATAGTAGTGGTAGTGGGTCTTGTCCTATAACAGGCATGATTCCAC
IB L1148 P80        ------------------------------------------------------------------------------------------------------------
DQ341199        392 GTGATCATATTCGTATTTCTGCAATGAAAAATGGTTCTTTATTTTATAATTTAACAGTTAGCGTATCTAAATACCCTAATTTTAAATCTTTTCAATGTGTTAACAAC
IB L1148 P80        ------------------------------------------------------------------------------------------------------------
DQ341199        499 TTCACATCTGTTTATTTAAATGGTGATCTTGTTTTTACTTCCAATAAAACTACTGATGTTACGTCAGCAGGTGTGTATTTTAAAGCAGGTGGACCTGTAAATTATAG
IB L1148 P80        ------------------------------------------------------------------------------------------------------------
DQ341199        606 TATTATGAAAGAATTTAAGGTTCTTGCTTACTTTGTTAATGGTACAGCACAAGATGTAGTTTTGTGCGACAATTCCCCCAAGGGTTTGCTAGCTTGTCAATATAACA
IB L1148 P80        ------------------------------------------------------------------------------------------------------------
DQ341199        713 CTGGCAATTTTTCAGATGGCTTTTATCCTTTTACTAATAGTACTTTGGTTAGGGAAAAGTTCATCGTCTATCGCGAAAGTAGTGTTAATACTACTCTGGCGTTAACT
IB L1148 P80        ----------------------------------------------------------------------------------------T-------------
DQ674739                                        ------------------------------------------------------------------------
DQ341199        820 AATTTCACTTTTACTAATGTAAGTAATGCACAGCCTAATAGTGGTGGTGTTAATACTTTTCATTTATACCAAACACAAACAGCTCAGAGTGGTTATTATAATTTTAA
IB L1148 P80        ------------------------------------------------------------------------------------------------------------
DQ674739            ------------------------------------------------------------------------------------------------------------
DQ341199        927 TTTGTCATTTCTGAGTCAGTTTGTGTATAAGGCAAGTGATTTTATGTATGGGTCCTACCACCCTAGTTGTTCTTTTAGACCAGAAACCATTAATAGTGGTTTATGGT
IB L1148 P80        ------------------------------------------------------------------------------------------------------------
DQ674739            ------------------------------------------------------------------------------------------------------------
DQ341199       1034 TTAATTCCTTGTCAGTTTCTCTTACTTATGGACCCCTACAGGGAGGGTGTAAGCAATCTGTTTTTAGTGGTAAGGCAACGTGTTGTTACGCCTACTCTTATAAAGGC
IB L1148 P80        ------------------------------------------------------------------------------------------------------------
DQ674739            ---------------------------------------------T--------
DQ341199       1141 CCAATGGCATGTAAAGGTGTTTATTCAGGTGAATTAAGCACGAATTTTGAATGTGGATTGCTGGTTTATGTTACTAAGAGTGATGGCTCTCGTATACAGACTAGAAC
IB L1148 P80        ----------------------------TT-C------------------------------------------------------------------------
DQ341199       1248 AGAGCCCTTAGTATTAACGCAATACAATTATAATAATATTACTTTAGATAAGTGTGTTGCCTATAATATATATGGCAGAGTAGGCCAAGGTTTTATTACTAATGTGA
IB L1148 P80        ------------------------------------------------------------------------------------------------------------
DQ341199       1355 CTGATTCTGCTGCTAATTTTAGTTATTTAGCAGATGGTGGGTTAGCTATTTTAGATACGTCGGGTGCCATAGATGTTTTTGTTGTACAGGGCATCTATGGTCTTAAT
IB L1148 P80        ------------------------------------------------------------------------------------------------------------
DQ341199       1462 TATTACAAGGTTAATCCTTGTGAAGATGTTAATCAACAATTTGTAGTGTCTGGTGGCAATATAGTTGGCATTCTTACTTCTAGAAATGAAACAGGTTCTGAACAGGT
IB L1148 P80        ------------------------------------------------------------------------------------------------------------
DQ341199       1569 TGAGAACCAGTTTTATGTTAAGTTAACCAATAGCTCACATCGTCGTAGGCGTTCTATTGGCCAAAATGTAACAAGTTGTCCTTATGTTAGTTATGGCAGATTTTGTA
IB L1148 P80        ------------------------------------------------------------------------------------------------------------
DQ341199       1676 TTGAACCAGATGGTTCGTTAAAGATGATAGTGCCAGAAGAATTGAAACAGTTTGTGGCACCTTTACTTAATATTACTGAAAGTGTACTCATACCTAACAGTTTTAAC
IB L1148 P80        ---------------------------------
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Figure 1. *Nucleotide sequence of the genomic fragment coding for the hypervariable sequence of the S1 protein of the master seed virus of IB L1148 at passage level 80; nucleotide positions 285 to 1708. DQ341199 is GenBank reference for low egg passage IB strain L-1148A. DQ674739 is GenBank reference for IB strain D388. IB L1148 p80 is the attenuated QX-like IBV at passage level 80.*

Table 1. *Results of safety tests with different passage levels of IB L1148 at a dose of $10^{6.0}$ EID_{50} for the respiratory tract of SPF chickens.*

		Ciliostasis scores						
		Day 5 post vaccination		Day 7 post vaccination		Day 10 post vaccination		
Study	Passage level inoculated	Individual	Mean	Individual	Mean	Individual	Mean	Total mean
A	8	40[a], 40, 40, 40, 40	40	40, 40, 40, 40, 40	40	40, 40, 31, 38, 39	38	39
	25	0, 38, 30, 0, 39	21	0, 40, 0, 0, 0	8	40, 0, 37, 3, 37	23	18
	40	30, 0, 0, 40, 40	22	40, 40, 40, 40, 39	40	0, 0, 0, 0, 0	0	21
	50	0, 39, 40, 40, 39	32	40, 40, 40, 40, 40	40	40, 3, 27, 0, 36	21	31
	None	0, 0, 0, 0, 0	0	0, 0, 0, 0, 0	0	0, 0, 0, 0, 0	0	0
B	65	0, 1, 40, 40, 2	17	38, 40, 39, 40, 38	39	31, 0, 0, 1, 0	6	21
	80	0, 0, 0, 0, 0	0	39, 40, 1, 0, 38	24	35, 0, 2, 3, 0	8	11
	None	0, 0, 0, 0, 0	0	0, 0, 0, 0, 0	0	0, 0, 0, 0, 0	0	0

[a]Ten tracheal rings were examined/bird as detailed in the Materials and Methods section. Each was scored as follows: 0, cilia in whole section showed movement; 1, 67% to 100% of the cilia showed movement; 2, 33% to 67% of the cilia showed movement; 3, less than 33% but more than 0% of the cilia showed movement; and 4, no ciliary movement. For each group, the average ciliostasis score was calculated.

results are shown in Figure 1. The nucleotide sequence of the original isolate was available from GenBank (DQ431199). Of the overlapping 1424 bp fragment, there were substitutions at four positions (i.e. positions 913, 1275, 1276 and 1278). The substitution of C by G is silent. The substitutions at the other positions result in substitutions of glycine and glutamate into phenylalanine and glutamine. The nucleotide sequence of a 340 bp fragment of the region coding for a portion of hypervariable region of the S1 protein QX-like strain IB D388 was also available from GenBank (DQ674739) (Figure 1). In comparison with the original IB L1148 strain, there was one silent substitution from A to T at position 1485 in this 340 bp fragment.

Safety for respiratory tract and kidneys in chickens. No clinical signs were observed during the safety tests. Passage 8 of strain IB L1148 had a maximum average ciliostasis score at 5 and 7 days after vaccination, and a score of 38 at 10 days, which gives a total average score of 39 (Table 1). There was a strong decrease in average score to 18 at passage level 25. Surprisingly, there were increases in ciliostasis score at passage levels 40 and 50. Passage 50 had an average score of 31. At passage levels 65 and 80 there was a decrease in ciliostasis scores to 21 and 11, respectively. The highest average ciliostasis scores were observed at 7 days after vaccination, with the exception of passage level 25 that had the highest value at day 10. In a number of instances there were great differences between individual values in one group; for example, with passage 25 at day 5, three chickens had a high ciliostasis score whereas the other two had scores of 0. No ciliostasis at all was observed in the uninoculated groups. The results presented in Table 2 show that in Study A in which passage levels 8, 25, 40 and 50 were tested, some kidneys were affected macroscopically; one at 5 days after vaccination with passage level 50, and several at day 10 in the groups inoculated with each virus. The kidneys were swollen and pale. No gross pathological lesions were recorded in study B in which passage levels 65 and 80 were tested. Passage levels 8, 25, 40 and 50 of IB L1148 affected the kidneys histologically, but there was a decrease in the severity of the lesions in the kidneys with increasing passage level. Passage levels 8 and 25 caused quite severe lesions; scores being $++$ until 10 days after vaccination in both groups, and likewise at 5 days after vaccination with passage level 40. At higher passage levels, lesion scores were never higher than $+$. Lesion scores of $+++$ were not observed in any group. Passage level 65 hardly caused any effect on the kidneys histologically, and passage level 80 had no effect. The kidneys in the uninoculated group were normal. In the IHC test, IB antigen could be detected frequently in the kidneys from chickens inoculated with lower passage 8 (i.e. in total, in nine of the 15 samples tested). In the kidneys from the chickens with the higher passage levels, IBV could be detected less frequently. In both passages 65 and 80, IB was detected in only one sample.

Safety for the reproductive tract in chickens. No clinical signs were observed during the whole 11-week observation period. In the group vaccinated on the day of hatching two chickens died because of cannibalism, whereas in the group vaccinated at 7 days one chicken died because it was trapped in the feeding system. When passage 80 was administered on the day of hatching, there was a damaging effect on the oviducts in six of the 48 chickens. These chickens had cystic oviducts and aplasia of the upper segment. The tubular structure of the oviducts was affected because of the presence of cysts in the interior. These cysts were present especially in the middle section of the oviducts. In the birds vaccinated at 7 days of age, no effect on the oviducts was observed. In the control group, all oviducts were in good condition.

Dissemination in the body of chickens and spread of the virus strain. These results are presented in Table 3. The vaccine virus was not found in the bursa, duodenum, lung or kidney of any of the chickens, therefore these organs are not included in Table 3. In the inoculated chickens the vaccine virus was detected with IHC staining in tracheas from 4 to 11 days and in the

Table 2. *Results of safety tests with different passages of IB L1148 at a dose of $10^{6.0}$ EID_{50} for the kidneys of SPF chickens.*

Study	Passage level inoculated	Macroscopic examination						Histological examination[a]						IHC staining for IB					
		Day 5		Day 7		Day 10		Day 5			Day 7			Day 10			Day 5			Day 7			Day 10	
		−	+	−	+	−	+	−	+	+ +	−	+	+ +	−	+	+ +	−	+	−	+	−	+	−	+
A	8	5	0	5	0	3	2	3	1	1	2	1	2	0	4	1	1	4	1	4	4	1
	25	5	0	5	0	4	1	5[b]	2[b]	0	0	5	0	0	3	2	7[b]	0	6[b]	0	3	2
	40	5	0	5	0	2	3	5[b]	1[b]	1[b]	5[b]	2[b]	0	2[b]	4[b]	0	6[b]	1[b]	3[b]	3[b]	6[b]	0
	50	4	1	5	0	2	3	3[c]	1[c]	0	7[b]	2[b]	0	4[b]	3[b]	0	4[c]	0	6[b]	3[b]	6[b]	1[b]
	None	5	0	5	0	5	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B	65	5	0	5	0	5	0	5[b]	1[b]	0	3	2	0	5	0	0	6[b]	0	5	0	4	1
	80	5	0	5	0	5	0	5	0	0	5	0	0	5	0	0	5	0	4	1	5	0
	None	5	0	5	0	5	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a]Kidney lesions were scored as follows: −, no lesions; +, some infiltration of lymphocytes locally; + +, strong infiltration of lymphocytes through whole kidney. ND, not done. [b]More than five samples tested. [c]One of five samples was lost.

pancreas on day 4. The virus was found in one cloacal swab on day 14 and in oro-pharyngeal swabs from days 2 to 11. In the three groups of contact chickens, the virus could be detected frequently in oro-pharyngeal swabs, and was found also in cloacal swabs, trachea and once in the pancreas. There were no great differences in susceptibility of the contact chickens for the virus, in the 1-day-old chickens introduced 7 days after vaccination, virus was detected in the swabs and in the trachea, but not in the pancreas. Chickens introduced 2 weeks after vaccination became infected, because some swabs from these chickens were also positive.

Efficacy of the experimental vaccine strain against IB QX-like challenge in SPF chickens. No clinical signs were observed after vaccination. The results of examining tracheas for damage after challenge using the ciliostasis test are given in Table 4. The results show that maximum damage (highest possible ciliostasis score) was seen in the non-vaccinated chickens, whereas the tracheas of the non-vaccinated, non-challenged chickens appeared normal. After challenge the chickens vaccinated with passage levels 82 and 85 of IB L1148 showed hardly any damage in the tracheas. The results complied with the requirements of the EP, which prescribe that at least 80% of the vaccinated chickens should be protected from ciliostasis. Chi-square analysis of the data showed that the differences between the groups vaccinated with passage levels 82 and 85 and the non-vaccinated controls were statistically significant ($P \leq 0.05$). Passage level 101 also was efficacious and the difference between the vaccinated group and the non-vaccinated control group was statistically significant. Passage level 101 was less efficacious than passages 82 and 85, but the difference in levels of protection by passages 82 and 85 and passage level 101 was not statistically significant. Passage level 101 did not comply with the EP requirements.

Efficacy of the experimental vaccine strain against IB QX-like challenge in commercial chickens with MDA. Blood samples taken from five chicks of the MDA-positive flock were tested for the level of IB antibodies by enzyme-linked immunosorbent assay. The chickens had antibody titres of $\log_2$ 9.9, 11.5, 11.5, 12.1 and 9.7, respectively. The average titre was $\log_2$ 10.9, which is normal for MDA against IB. After vaccination as well as after challenge no clinical signs were observed. The results in Table 5 show that the maximal damage (the highest possible ciliostasis score) to the tracheas after challenge was seen in the non-vaccinated group. In the group vaccinated with $10^{3.0}$ EID_{50} of IB L1148, passage level 85, 70% of the birds were protected against tracheal damage after challenge, whereas 80% of the group vaccinated with $10^{3.3}$ EID_{50} IB L1148, passage level 85, was protected. The difference in level of protection observed in the vaccinated group compared with the non-vaccinated controls was statistically significant by chi-square analysis. In this study, a group of SPF chickens was included, because this is a recommendation in the EP monograph for efficacy studies using birds with MDA. Ninety-five per cent of the SPF chickens were protected against damage to the tracheal ciliated epithelium after challenge, which is in line with the results of the study in SPF chickens. The protection level observed in the SPF chickens was not significantly

Table 3. *Results of study on dissemination of IBV in the chicken and spread of the vaccine strain.*

Group	Test	Organ	Days after vaccination of the vaccinated group										
			2	4	7	9	11	14	16	18	21	25	28
Vaccinated chickens (vaccine group)	IHC	Pancreas	− −	+ −	− −	− −	− −	− −	− −				
		Trachea	− −	+ +	+ −	+ −	+ −	− −	− −				
	PCR	Cloacal swabs	− −	− −	− −	− −	− −	+ −	− −	− −	− −		
		Oro-pharyngeal swabs	+ +	+ +	+ +	− −	+ −	− −	− −	− −	− −		
Non-vaccinated chickens of the same age in contact with vaccinated chickens 3 days after vaccination	IHC	Pancreas		− −	− −	− −	+ −	− −	− −				
		Trachea		− −	− −	+ −	−	− −	− −				
	PCR	Cloacal swabs		− −	− −	− −	− −	+ −	− −	+ −	−[a]		
		Oro-pharyngeal swabs		− −	+ −	+ −	− −	− −	− −	+ −	−[a]		
Non-vaccinated chickens of the same age in contact with vaccinated chickens 14 days after vaccination	IHC	Pancreas							− −				
		Trachea							− −				
	PCR	Cloacal swabs							− −	− −	− −	− −	− −
		Oro-pharyngeal swabs							− −	+ −	+ +	+ +	+ −
Non-vaccinated chickens of one day of age in contact with vaccinated chickens 7 days after vaccination	IHC	Pancreas				− −	− −	− −	− −				
		Trachea				− −	− −	+ −	− −				
	PCR	Cloacal swabs				+ −	− −	− −	− −	− −	− −		
		Oro-pharyngeal swabs				− −	− −	− −	+ −	− −	+ − −[b]		

− −, no IBV detected; + −, IBV detected in one of the two chickens; + + IBV detected in both chickens. [a]One chicken was swabbed instead of two. [b]Three chickens were swabbed instead of two.

Table 4. *Results of ciliary movement test following challenge of SPF chickens vaccinated on day of hatching with different doses of IB L1148 at different passage levels.*

Vaccination	Titre per dose (EID_{50})	Challenge at day 21	Protected[a]/ total (%)
Passage 101	$10^{3.3}$	Yes	13/20 (65)
Passage 85	$10^{3.0}$	Yes	17/20 (85)
Passage 85	$10^{3.3}$	Yes	17/20 (85)
Passage 82	$10^{3.0}$	Yes	18/19 (95)
Passage 82	$10^{3.3}$	Yes	18/20 (90)
None	NA	Yes	0/20 (0)
None	NA	No	20/20 (100)

NA, Not applicable. [a]A chicken was considered protected if no fewer than nine out of 10 tracheal rings/bird showed normal ciliary activity.

different from that in the MDA-positive chickens as evaluated by chi-square analysis.

Discussion

Many poultry vaccines are based on live-attenuated vaccine strains. Examples of live attenuated IB vaccines that are common in Europe are D274, H52, H120, 4/91, MM and CR88. The classical procedure for attenuation of IBVs is by passage in embryonated eggs (MacDonald & McMartin, 1976; Klieve & Cummings, 1988; Jackwood *et al*., 2003; Huang & Wang, 2006, 2007; Liu *et al*., 2007). In our studies, 80 passages were required to achieve a virus strain that was attenuated sufficiently to comply with safety requirements for live IB vaccines. This is in line with the number of passages required to attenuate other IB strains. It is not known what type of mechanism causes a virus to attenuate. Huang & Wang (2007) sequenced the 3' 7.3 kb of the genome of an attenuated and parent virus strain from which an attenuated strain was derived. They found that there were amino acid substitutions in the spike 1 and 2 subunits, the small membrane protein and the membrane protein. No substitutions were detected in the nucleoprotein. We sequenced a substantial part of the genomic region coding for the hypervariable region of the S1 protein or our strain (Worthington *et al*., 2008). In comparison with the parent field isolate there were no more than four substitutions in 1424 nucleotides, resulting in two amino acid substitutions. The substitutions we detected were different from those found by Huang & Wang (2007). The role of these substitutions remains unclear.

We found that there was a clear effect of increased egg passage level on the results of ciliostasis scores. The effect was not gradual. There was a clear decrease in ciliostasis scores after 25 passages, followed by an increase until passage level 50. After 50 passages, ciliostasis scores decreased again. To our knowledge this effect has not been reported before. At passage level 80 a ciliostasis score of 11 was determined, which is much lower than the maximum score of 40, often found with virulent IB strains, therefore the virulence of the virus strain for the respiratory tract has been decreased significantly. There is no threshold defined to distinguish safe IB strains from an unsafe one. In the EP monograph, an average value of 25 has been indicated for many years as the threshold, but nowadays the requirement is that a risk–benefit analysis should be made, taking into account the average ciliostasis score and the benefits expected from the use of the vaccine. The average ciliostasis score for passage level 80 was well below the threshold of 25. Kidney lesions also decreased to very low levels at passage 80, and virus could be detected in the kidneys on only one occasion. These results clearly demonstrate that the virus was attenuated during passaging and the data for passage level 80 can be considered in compliance with requirements in the EP, with respect to safety for the respiratory tract and kidneys.

Following vaccination of female chickens on the day of hatching with passage 80, 12.5% developed lesions in the oviducts. IB has been described as an agent causing such lesions by Crinion & Hofstad (1971) who found that at egg passage level 55, IBV caused lesions only in chickens vaccinated at 1 day of age, but no lesions were found in chickens vaccinated at 8 days of age. These findings are in line with our results.

IBV replicates at many epithelial surfaces. Cavanagh (2005) mentioned the Harderian gland, trachea, oesophagus, lung, kidney, oviduct, duodenum, caecum, rectum, ileum and bursa as potential sites of replication of IBV. We recovered the virus from pancreas and trachea, and from cloacal and oro-pharyngeal swabs. In the contact birds that were infected by spreading of the virus from the vaccinated chickens, we found that oro-pharyngeal swabs were often virus-positive, whereas in the tracheal tissues the virus could no longer be detected. This may be because the cells producing the virus had been destroyed, whereas the virus that was produced from these cells was still present. It is possible also that the PCR test was more sensitive than the IHC staining method. Furthermore, it has to be said that the PCR test detects genomic material of the virus—that is, a part of the genomic region coding for the hypervariable region of the S1 protein—whereas the IHC staining method is based on the reactivity of a monoclonal antibody with the nucleoprotein of the virus. The observed presence of the virus in trachea and pancreas suggest that this attenuated IB L1148 strain at passage

Table 5. *Results of ciliary movement test following challenge of chickens with maternally derived antibodies vaccinated at day of hatching with IB L1148 at passage level 85.*

Chickens	Vaccination at day 0	Titre per dose (EID_{50})	Challenge at day 21	Protected[a]/total (%)
Broilers with MDA	Passage 85	$10^{3.0}$	Yes	14/20 (70)
Broilers with MDA	Passage 85	$10^{3.3}$	Yes	17/20 (85)
SPF chickens	Passage 85	$10^{3.0}$	Yes	18/19 (95)
Broilers with MDA	None	NA	Yes	0/20 (0)

NA, Not applicable. [a]A chicken was considered protected if no fewer than nine out of 10 tracheal rings/bird showed normal ciliary activity.

level 80 has the trachea and the alimentary tract as main sites of replication. Replication of IBV in the trachea is a common finding and replication in the alimentary tract has also been described (Wang *et al*., 1998), but this is not the case for replication of IBV in pancreatic tissue. Qian *et al*. (2006) isolated an IBV from sick pigeons; and when the isolated virus was inoculated into SPF chickens, pancreatitis developed. We did not observe any abnormalities in the pancreatic tissue in the dissemination study. In the other safety studies, we likewise did not observe any abnormalities that could be attributed to IBV infection in the pancreas. Thus, there is no evidence that the attenuated IB L1148 strain has a damaging effect on the pancreas. It has to be mentioned that the IHC test detects the presence of epitope 48.4 of the nucleoprotein of the IBV (Koch *et al*., 1991). More research will need to be done to confirm that the virus replicates in the pancreas.

In the dissemination study we introduced three groups of non-vaccinated contact chickens to the vaccinated chickens. Two groups were chickens of the same age as the vaccinated chickens; the other group consisted of 1-day-old chickens. In these younger chickens, the vaccine virus was present in the same tissues as in the chickens of the same age as the vaccinated chickens. No signs of disease were observed in the contact chickens of the same age as the vaccinated chickens or in the younger contact chickens. It is common knowledge that IB vaccine viruses can spread from vaccinated to non-vaccinated chickens (Hopkins & Yoder, 1984), so the results described in this paper are not surprising. Just as for other IB vaccine viruses, it is strongly recommended to ensure that if a flock is vaccinated then all birds are vaccinated directly to avoid rolling reactions.

Our results show that chickens with MDA and vaccinated with IB strain L1148 passage level 85 were protected against challenge with IB D388. The degree of protection in the SPF chickens was higher than that in the MDA-positive chickens. This can be a consequence of the presence of MDA, which may have had a negative impact on vaccine efficacy. However, a direct comparison between the SPF chickens and the MDA-positive chickens is difficult, because genetic differences between the chicken lines may also have had an effect on the susceptibility of the chickens to the IB challenge (Ignjatovic *et al*., 2003). The MDA will have been directed against the Massachusetts, 4/91 and D274 types of IB, because the parent birds had been vaccinated with these IB vaccines, but it cannot be excluded that the MDA was directed against IB QX-like viruses also, because of possible field infections. It has to be said that the MDA-positive chickens came from France, where IB QX-like viruses are prevalent (Worthington *et al*., 2008).

A vaccine titre of 10^3 EID_{50} per dose was sufficient to generate protection in SPF chickens as well as chickens with MDA. If it is taken into account that virus titres of higher than 10^8 EID_{50}/ml could be produced easily in SPF chicken eggs, it is obvious that a commercial vaccine based on IB L1148 passage level 85 is achievable.

Vaccines based on QX-like strains do not yet exist. In order to find ways to protect poultry against IB QX-like viruses, combinations of existing vaccine strains have been tested. For example, Terregino *et al.* (2008) vaccinated chickens with the IB Ma5 vaccine strain at 1 day of age and with IB 4/91 vaccine strain at 14 days of age. At 5 weeks of age the chickens were challenged with an IB QX-like virus, isolated in northern Italy in 2005 (IBV QX-like virus 1997/V05). The results of that study show that combined vaccination with IB Ma5 and IB 4/91 conferred protection against IB QX-like virus challenge. It is not unusual for chickens to be vaccinated with different combinations of vaccines to generate broad protection. A consequence of the use of combinations of vaccine viruses, not related to the virus that has to be controlled, may result in a situation in which new virus strains (i.e. the vaccine strains) are introduced in the environment. This is not the case if a vaccine derived directly from the field virus is used. In this respect, the use of such vaccine virus strains is advantageous. However, other aspects, such as degree of attenuation and interactions and compatibilities with other vaccines, also should be taken into account.

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