



**HAL**  
open science

# EFFICACY AND SAFETY OF ATTENUATED LIVE INFECTIOUS BRONCHITIS VIRUS STRAIN QX-LIKE IN CHICKENS

Harm Geerligs, Gert Jan Boelm, Cindy Meinders, Diana Stuurman, Jori Symons, Jordi Tarres-Call, Tura Bru, Rut Vila, Mark Mombarg, Kemal Karaca, et al.

► **To cite this version:**

Harm Geerligs, Gert Jan Boelm, Cindy Meinders, Diana Stuurman, Jori Symons, et al.. EFFICACY AND SAFETY OF ATTENUATED LIVE INFECTIOUS BRONCHITIS VIRUS STRAIN QX-LIKE IN CHICKENS. *Avian Pathology*, 2011, 40 (01), pp.93-102. 10.1080/03079457.2010.542742. hal-00670245

**HAL Id: hal-00670245**

**<https://hal.science/hal-00670245>**

Submitted on 15 Feb 2012

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



**EFFICACY AND SAFETY OF ATTENUATED LIVE INFECTIOUS BRONCHITIS VIRUS STRAIN QX-LIKE IN CHICKENS**

Journal:	<i>Avian Pathology</i>
Manuscript ID:	CAVP-2010-0148.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	14-Oct-2010
Complete List of Authors:	Geerligs, Harm; Pfizer Animal Health, VMRD Bios Boelm, Gert Jan; Pfizer Animal Health, Quality Control Dept. Meinders, Cindy; Pfizer Animal Health, Pfizer Global Manufacturing Stuurman, Diana; Pfizer Animal Health, Veterinary Medicine Research & Development Bios Symons, Jori; University of Utrecht, University Medical Center Tarres-Call, Jordi; European Food Safety Authority, Animal health and animal welfare Bru, Tura; Pfizer Animal Health, Veterinary Medicine Research & Development Bios Vila, Rut; Pfizer Animal Health, Veterinary Medicine Research & Development Bios Mombarg, Mark; Pfizer Animal Health, Veterinary Medicine Research & Development Bios Karaca, Kemal; Pfizer Animal Health, Veterinary Medicine Research & Development Bios Wijmenga, Willem; Pfizer Animal health, Poultry Business Kumar, Mahesh; Pfizer Animal Health, Veterinary Medicine Research & Development Bios
Keywords:	QX-like IB, attenuated, safety, efficacy

SCHOLARONE™  
Manuscripts

For Peer Review Only

CAVP-2010-0148

Efficacy and safety of attenuated live QX-like infectious bronchitis virus strain in chickens

**H.J. Geerlig<sup>1\*</sup>, G.- J. Boelm<sup>2</sup>, C.A.M. Meinders<sup>3</sup>, B.G.M. Stuurman<sup>1</sup>, J. Symons<sup>1</sup>, J. Tarres-Call<sup>4</sup>, T. Bru<sup>4</sup>, R. Vila<sup>4</sup>, M. Mombarg<sup>4</sup>, K. Karaca<sup>5</sup>, W. Wijmenga<sup>6</sup> and M. Kumar<sup>5</sup>**

*<sup>1</sup>Pfizer Animal Health, Veterinary Medicine Research & Development Bios, Pfizer Global Manufacturing, C.J. Van Houtenlaan 36, 1381CP, Weesp, The Netherlands. <sup>2</sup>Pfizer Animal Health, Quality Control Department, Weesp, The Netherlands. <sup>3</sup>Pfizer Animal Health, Pfizer Global Manufacturing, Weesp, The Netherlands. <sup>4</sup>Pfizer Animal Health, Veterinary Medicine Research & Development Bios, Vall de Bianya, Spain. <sup>5</sup>Pfizer Animal Health, Veterinary Medicine Research & Development Bios, Kalamazoo, Michigan, USA. <sup>6</sup>Pfizer Animal Health, Poultry Business, Paris, France.*

*Running title: Attenuated QX-like IB virus*

\*To whom correspondence should be addressed. Tel: +31 294 478050.

E-mail: [harm.geerlig@pfizer.com](mailto:harm.geerlig@pfizer.com)

*Received: 14 October 2010*

## Abstract

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) chickens eggs and at different passage levels samples were tested for safety for the respiratory tract and kidneys in one-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 one-day-old and 7 day-old SPF chickens. In one-day-old chickens 12.5% of the vaccinated animals had macroscopical 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 tissues. The virus was able to spread from vaccinated chickens to groups of non-vaccinated chickens, and in the vaccinated animals 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 there were nucleotide changes resulting in 2 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 in commercial chickens with 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 (IB) virus is a coronavirus which causes respiratory disease in chickens. 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 IB viruses have been identified and new variants are still emerging. One of these new variants is QX-like IB virus. 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 IB viruses 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 (Worthington *et al.*, 2008; Monne *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 available yet. In this 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 7<sup>th</sup> 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 (NL) (Landman *et al.*, 2005).

**Specific pathogen free (SPF) chickens and chicken eggs.** SPF eggs were obtained from Lohmann Tierzucht GmbH, Cuxhaven, Germany, or from SPAFAS, North Franklin, Connecticut, USA. For animal experiments performed at Animal Health Service, Deventer, NL, 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 to 11 day embryonated SPF chicken eggs into the allantoic cavity, each with a quantity of 0.1 ml of the virus. After 1 days incubation, dead eggs were discarded and after 2 to 3 days incubation, the allantoic fluid was harvested from the surviving eggs and pooled. The pooled allantoic fluid was centrifuged at 1000g to remove debris. The supernatant was filled out 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 65g peptone, 68g gelatin, 50g d-mannitol and 50g inositol per litre in distilled water. The mixture was filled in 3ml glass vials, 1 to 1.5ml 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, 6 eggs per dilution. After 1 days incubation dead embryos were considered nonspecific 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<sub>50</sub>)/ml, was calculated according to the method of Spearman-Kärber (Finney, 1964).

**Identity testing by PCR.** In order to distinguish or verify the IB strain, RNA was isolated using the High Pure Viral RNA isolation kit of 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 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 for the test on the presence of the virus 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 nr. DQ431199 (Worthington *et al.*, 2008). The specificity of the primers had been tested in a PCR test in which RNA from IB strains H120, D274 and M41 was tested. 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 made visible with Ethidium Bromide. The position of the fragments was shown as orange bands by illumination with UV light. The size of the fragments was calculated by comparing the position of the bands with base pair markers which 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 of 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 nr. 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. The fragments were made visible with Ethidium Bromide. The position of the fragments was shown as orange bands by illumination with UV light. The size of the fragments was calculated by comparing the position of the bands with base pair markers which had also been loaded on the gel. The nucleotide sequence was determined by Baseclear B.V., Leiden, NL. The obtained sequence was compared with sequences of other QX-like IB viruses 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<sub>50</sub> per dose in 0.1 to 0.2 ml. A dose of  $10^{6.0}$  EID<sub>50</sub> 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<sub>50</sub> 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% CO<sub>2</sub>, another 5 at 7 days after inoculation and the remaining 5 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, U K). The ciliary activity of 3, 4 and 3 sections of respectively, the upper, middle and lower part of the trachea was determined by light microscopy at a magnification of 400x. 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 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 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, Deventer, NL.

**Immunohistochemical staining.** Sections of the tracheas and kidneys were deparaffinised. Endogenous peroxidase activity was blocked by incubation with 1% H<sub>2</sub>O<sub>2</sub> containing 0.1% NaN<sub>3</sub> for 20 min at room temperature (RT) and subsequently boiled in Tris (0.01 M) - EDTA (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 RT. Immunohistochemical staining was performed using MAb 48.4 (Prionics, Lelystad, NL) directed against the IBV nucleocapsid protein

(Koch *et al.*, 1991) for 30 min at RT (dilution 1:20,000) in Normal Antibody Diluent (Klinipath, Duiven, NL). After 3 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 3 times with PBS Tween and then treated with diaminobenzidine (Dako UK Ltd, Cambridgeshire, UK) for 5 min at RT. This was followed by counterstaining using haematoxylin. Sections incubated in the absence of primary antibody were included as negative controls. Immunohistochemical (IH) staining was performed by the Animal Health Service, Deventer, NL.

**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<sub>50</sub> 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<sub>50</sub>. 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 day of hatching, SPF chickens were vaccinated with  $10^{5.0}$  EID<sub>50</sub> of 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 one-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, 2 chickens of each group were removed and killed by inhalation of 100% CO<sub>2</sub>. Samples of 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 the 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<sup>3.0</sup> or 10<sup>3.3</sup> EID<sub>50</sub> of IB L1148 passage levels 82 and 85, and with a dose of 10<sup>3.3</sup> EID<sub>50</sub> of 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 likely 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<sup>3.0</sup> and 10<sup>3.3</sup> EID<sub>50</sub> were chosen because it was anticipated that a vaccine based on the attenuated IB L1148 strain would have a minimal titre of 10<sup>3.0</sup> or 10<sup>3.3</sup> EID<sub>50</sub> 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 of approx. 2.5 ml per sec. Before vaccination the exact vaccination time for the applicable number of animals was determined (0.5 ml per animal) and the sprayer was disinfected with 70% ethanol, followed by 3 rinses with distilled water. After preparation, the container with the diluted vaccine was placed in a 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 animals, 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 animals were kept for at least 15 min inside the box. Subsequently, the animals 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<sub>50</sub> of IB D388 per dose of 0.1 ml. The challenge virus was applied by eye-drop. Of the non-vaccinated group, half was challenged; 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, rinsed with, and stored in, physiological saline at 37°C. Small transverse sections of the trachea were cut by hand. Ciliary activity of 3 sections of the upper part, 4 sections of the middle part and 3 sections of the lower part of the trachea were examined by light microscopy at a magnification of 400x. 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, 0 if  $< 50\%$  of the tracheal section showed ciliary activity. For a given tracheal section, ciliary activity was considered as normal when at least 50% (score 0) of the internal ring showed vigorous ciliary movement. A chicken was considered protected if not fewer than 9 out of 10 rings showed normal ciliary activity. Significance of differences between groups was evaluated by Chi-square analysis.

**Testing the efficacy of the experimental vaccine strain against IB QX-like challenge in chickens with maternally derived antibodies (MDA).** We used commercial layer type chickens on the day of hatching. The parent chickens had been vaccinated against amongst other pathogens, IB at one 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 MDA. This test was performed using the ELISA test kit, Flock check, cat. 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<sub>50</sub> or  $10^{3.3}$  EID<sub>50</sub> 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 day-old SPF chickens was vaccinated with  $10^{3.0}$  EID<sub>50</sub> 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<sub>50</sub> of 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<sub>50</sub> per ml. The virus titres did not change during the passages until passage level 85. After lyophilisation passage 80 had a titre of  $10^{8.2}$  EID<sub>50</sub> per vial, containing 1 ml of allantoic fluid plus stabiliser mixture. Lyophilised preparations of passage 82 and passage 85 were also prepared. The titres per ml were  $10^{8.41}$  EID<sub>50</sub> and  $10^{8.20}$  EID<sub>50</sub>, respectively. Surprisingly, passage 101 had a much higher titre, i.e.  $10^{9.51}$  EID<sub>50</sub> per 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), i.e. 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 position 262 and 1718, of the region coding for the hypervariable region of the S1 protein of the vaccine virus was determined, and the results are given in Figure 1. The nucleotide sequence of the original isolate was available from the GenBank (DQ431199). Of the overlapping 1424 BP fragment, there were substitutions at 4 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 340BP fragment of the region coding for a portion of hypervariable region of the S1 protein QX-like strain IB D388 was also available from the GenBank (DQ674739) (Figure 1). In comparison to 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 (Table1). 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, which 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, 3 chickens had a high ciliostasis score, whereas the other 2 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 9 of the 15 samples tested. In the kidneys from the chickens with the higher passage levels, IB virus could be detected less frequently. In both passages 65 and 80, IB was detected in only 1 sample.

**Safety for the reproductive tract in chickens.** No clinical signs were observed during the whole 11 week observation period. In the group vaccinated at day of hatching 2 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 at day of hatching, there was a damaging effect on the oviducts in 6 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.** The results are given 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 IH staining in tracheas from 4 to 11 days and in the 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 3 groups of contact animals, 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 one-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 on one side, and the non-vaccinated controls on the other, 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 on one side, and passage level 101 on the other, 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 5 chicks of the MDA positive flock were tested for the level of IB antibodies by ELISA. 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<sub>50</sub> 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<sub>50</sub> of IB L1148, passage level 85 was protected. The difference in level of protection observed in the vaccinated group compared to 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. 95% 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 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 which are common in Europe are D274, H52, H120, 4/91, MM and CR88. The classical procedure for attenuation of IB viruses is by passage in embryonated eggs (MacDonald & McMartin, 1976; Klieve & Cummings, 1988; Jackwood *et al.*, 2003; Huang & Wang, 2006; Huang & Wang, 2007; Liu *et al.*, 2007). In our studies, 80 passages were required to achieve a virus strain which was attenuated sufficiently to comply with safety requirements for live IB vaccines. This is in line with 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 of our strain (Worthington *et al.*, 2008). In comparison to the parent field isolate there were not more than 4 substitutions in 1424 nucleotides, resulting in 2 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 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 in 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 (Crinion & Hofstad, 1971). They found that at egg passage level 55, IBV caused lesions only in chickens vaccinated at one 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 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 which 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 which 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, i.e. 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 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 been described also (Wang *et al.*, 1998), but this is not the case for replication of IBV in pancreatic tissue. Qian *et al.* (2006) isolated an IB virus 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 likewise we did not observe any abnormalities which 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 IB virus (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 3 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, 1983), 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; 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.*, 2002). The MDA will have been directed against the Massachusetts, 4/91 and D274 types of IB, because the parent animals had been vaccinated with these IB vaccines, but it can not 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<sub>50</sub> 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<sub>50</sub> per 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 which 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.

#### **Acknowledgement.**

The efficacy tests were performed by Dr. J.J. de Wit, Dr. W. Landman, Dr. A. Feberwee, H. van de Sande and coworkers at the Animal Health Service, Deventer, NL. The description of the immunohistochemistry was kindly provided by Dr. J.J. de Wit.

## References

- Benyeda, Z., Mato, T., Süveges, T., Szabo, E., Kardi, V., Abonyi-Toth, Z., Rusvai, M. & Palya, V. (2009). Comparison of the pathogenicity of QX-like, M41 and 793/B infectious bronchitis strains from different pathological conditions. *Avian Pathology*, 38, 449-456.
- Bochkov, Y.A., Batchenko, G.V., Shcherbakova, L.A., Borisov, A.V. & Drygin V.V. (2006). Molecular epizootiology of avian infectious bronchitis in Russia. *Avian Pathology*, 35, 379-393.
- Cavanagh, D. (2005). Coronaviruses in poultry and other birds. *Avian Pathology*, 34, 439-448.
- Cavanagh, D. & Gelb, J. (2008). Infectious bronchitis. In Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan & D.E. Swayne. (2008). *Diseases of Poultry* 12<sup>th</sup> edn (pp.117-137). Ames, Oxford, Victoria: Blackwell Publishing.
- Crinion, R.A.P. & Hofstad, M.S. (1971). Pathogenicity of two embryo-passage levels of avian infectious bronchitis virus for the oviduct of young chickens of various ages. *Avian Diseases*, 16, 967-973.
- Dolz, R., Bertran, K. & Majó, N. (2009). First report of IBV QX-like strains in Spain. In U. Heffels-Redmann, D. Sommer & E.F. Kaleta. (2009). VI. *International Symposium on avian corona- and pneumoviruses and complicating pathogens* (pp. 27-33). Giessen: VVB Laufersweiler Verlag.
- Domanska-Blicharz, Z., Minta, Z. & Smientanka, K. (2006). New variant of IBV in Poland. *The Veterinary Record*, 158, 808.
- Finney, D.J. (1964). The Spearman-Kärber method. In D.J. Finney. (1964). *Statistical methods in biological assays* (pp.524-530). Charles Griffin, London, UK.
- Gough, R.E., Cox, W.J., de Welchman, B., Worthington, K.J. & Jones, R.C. (2008). Chinese QX strain of infectious bronchitis virus was isolated in the UK. *The Veterinary Record*, 162, 99-100.

- Hopkins, S.R. & Yoder, H.W. (1983). Increased incidence of airsacculitis in broilers infected with *Mycoplasma synoviae* and chicken-passaged infectious bronchitis vaccine virus. *Avian Diseases*, 28, 386-396.
- Huang, Y.-P. & Wang, C.-H. (2006). Development of attenuated vaccines from Taiwanese infectious bronchitis virus strains. *Vaccine*, 24, 785-791.
- Huang, Y.-P. & Wang, C.-H. (2007). Sequence changes of infectious bronchitis virus isolates in the 3' 7.3 kb of the genome after attenuating passage in embryonated eggs. *Avian Pathology*, 36, 59-67.
- Ignjatovic, J., Reece, R. & Ashton, F. (2002) Susceptibility of three genetic lines of chicks to infection with a nephropathogenic T strain of avian infectious bronchitis virus. *Journal of Comparative Pathology*, 128, 92-98.
- Jackwood, M.W. Hilt, D.B. & Thomas, T.P. (2003). Attenuation, safety, and efficacy of in infectious bronchitis virus GA98 serotype vaccine. *Avian Diseases*, 47, 627-632.
- Klieve, A.V. & Cummings, R.B. (1988). Immunity and cross-protection to nephritis produced by Australian infectious bronchitis viruses used as vaccines. *Avian Pathology*, 17, 829-839.
- Koch, G., Kant, A., Vos, J.G. & de Boer, G.F. (1991) Rapid detection of infectious bronchitis virus by nucleoprotein specific antigen capture assay. In: *Proceedings of 2nd International Symposium on Infectious bronchitis Rauischholzhausen*, pp. 168-172.
- Landman, W.J.M., Dwars, R.M. & de Wit J.J. (2005). High incidence of false layers in (re)production hens supposedly attributed to a juvenile infectious bronchitis virus infection. In *Proceedings of the 14<sup>th</sup> World Veterinary Poultry Congress* (p. 369). 22-26 August 2005, Istanbul, Turkey.
- Liu, S., Han, Z., Chen, J., Liu, X., Shao, Y., Kong, X., Tong, G. & Rong, J. (2007). S1 gene sequence heterogeneity of a pathogenic infectious bronchitis virus strain and its embryo-passaged, attenuated derivatives. *Avian Pathology*, 36, 231-234.



- MacDonald, J.W. & McMartin, D.A. (1976). Observations on the effects of the H52 and H120 vaccine strains of the infectious bronchitis virus in the domestic fowl. *Avian Pathology*, 5, 157-173.
- Monne, I., Cattoli, G., Jones, R., Worthington, K. & Wijnenga, W. (2008). QX genotypes of infectious bronchitis virus circulating in Europe. *The Veterinary Record*, 163, 606-607.
- Qian, D.H., Zhu, G.J., Wu, L.Z., Hua, G.X. (2006) Isolation and characterization of a coronavirus from pigeons with pancreatitis. *American Journal of Veterinary Research*, 67, 1575-1579.
- Terregino, C., Toffan, A., Beato, M.S., de Nardi, R., Vascellari, M., Meini, A., Ortali, G., Mancin, M. & Capua, I. (2008). Pathogenicity of a QX strain of infectious bronchitis virus in specific pathogen free and commercial boiler chickens, and evaluation of protection induced by a vaccination program based on the Ma5 and 4/91 serotypes. *Avian Pathology*, 37, 487-493.
- Wang, Y.D., Wang, Y.L., Zhang, Z., Fan, G., Jiang, Y., Liu, X., Ding, J. & Wang, S. (1998). Isolation and identification of glandular stomach type IBV (QX IBV) in chickens. *Chinese Journal of Animal Quarantine*, 15, 1-3.
- Worthington, K.J., Currie, R.J.W. & Jones, R.C. (2008). A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to 2006. *Avian Pathology*, 37, 247-257.
- Zhang, Z., Schwartz, S., Wagner, L. & Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology*, 7, 203-214.

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

Study	Passage level inoculated	Ciliostasis scores at 5, 7, 10 days post vaccination							
		Day 5		Day 7		Day 10		Total mean	
		Individual	Mean	Individual	Mean	Individual	Mean		
A	8	40 <sup>a</sup> , 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	

<sup>a</sup> Ten tracheal rings were examined/ bird as detail in the 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; 4: no ciliary movement. For each group the average ciliostasis score was calculated.

**Table 2.** Results of safety tests with different passages of IB L1148 at a dose of 10<sup>6.0</sup> EID<sub>50</sub> for the kidneys of SPF chickens.

Study	Passage level inoculated	Macroscopic examination						Histological examination									IHC staining for IB					
		Day 5		Day 7		Day 10		Day 5			Day 7			Day 10			Day 5		Day 7		Day 10	
		- <sup>b</sup>	+	-	+	-	+	-	+	++	-	+	++	-	+	++	-	+	-	+	-	+
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 <sup>c</sup>	2 <sup>c</sup>	0	0	5	0	0	3	2	7 <sup>c</sup>	0	6 <sup>c</sup>	0	3	2
	40	5	0	5	0	2	3	5 <sup>c</sup>	1 <sup>c</sup>	1 <sup>c</sup>	5 <sup>c</sup>	2 <sup>c</sup>	0	2 <sup>c</sup>	4 <sup>c</sup>	0	6 <sup>c</sup>	1 <sup>c</sup>	3 <sup>c</sup>	3 <sup>c</sup>	6 <sup>c</sup>	0
	50	4	1	5	0	2	3	3 <sup>d</sup>	1 <sup>d</sup>	0	7 <sup>c</sup>	2 <sup>c</sup>	0	4 <sup>c</sup>	3 <sup>c</sup>	0	4 <sup>d</sup>	0	6 <sup>c</sup>	3 <sup>c</sup>	6 <sup>c</sup>	1 <sup>c</sup>
	None	5	0	5	0	5	0	ND <sup>e</sup>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B	65	5	0	5	0	5	0	5 <sup>c</sup>	1 <sup>c</sup>	0	3	2	0	5	0	0	6 <sup>c</sup>	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

<sup>a</sup> Immunohistochemistry.

<sup>b</sup> Kidney lesions were scored as follows: - no lesions, + some infiltration of lymphocytes locally, ++ strong infiltration of lymphocytes through whole kidney.

<sup>c</sup> More than 5 samples tested.

<sup>d</sup> One of 5 samples was lost.

<sup>e</sup> ND, not done.

**Table 3.** Results of study on dissemination of IB virus 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 <sup>a</sup>	Pancreas	-- <sup>c</sup>	+ <sup>d</sup>	--	--	--	--	--	--	--	--	--
		Trachea	--	++ <sup>e</sup>	+ <sup>-</sup>	+ <sup>-</sup>	+ <sup>-</sup>	--	--	--	--	--	--
	PCR <sup>b</sup>	Cloacal swabs	--	--	--	--	--	+ <sup>-</sup>	--	--	--	--	--
		Oro-pharyngeal swabs	++	++	++	--	+ <sup>-</sup>	--	--	--	--	--	--
Non-vaccinated chickens of the same age in contact with vaccinated chickens 3 days after vaccination	IHC	Pancreas	--	--	--	--	+ <sup>-</sup>	--	--	--	--	--	--
		Trachea	--	--	--	+ <sup>-</sup>	-	--	--	--	--	--	--
	PCR	Cloacal swabs	--	--	--	--	--	+ <sup>-</sup>	--	--	+ <sup>-</sup>	--	- <sup>f</sup>
Oro-pharyngeal swabs		--	--	+ <sup>-</sup>	+ <sup>-</sup>	--	--	--	--	+ <sup>-</sup>	--	- <sup>f</sup>	
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		--	--	--	--	--	--	--	--	+ <sup>-</sup>	++	++	+ <sup>-</sup>
Non-vaccinated chickens of one day of age in contact with vaccinated chickens 7 days after vaccination	IHC	Pancreas	--	--	--	--	--	--	--	--	--	--	--
		Trachea	--	--	--	--	--	+ <sup>-</sup>	--	--	--	--	--
	PCR	Cloacal swabs	--	--	--	--	--	--	--	--	--	--	--
		Oro-pharyngeal swabs	--	--	--	--	--	--	--	--	+ <sup>-</sup>	--	++ <sup>g</sup>

<sup>a</sup> Immunohistochemistry.

<sup>b</sup> Polymerase chain reaction.

<sup>c</sup> No IBV detected.

<sup>d</sup> IBV detected in one of the 2 chickens.

<sup>e</sup> IB virus detected in both chickens.

<sup>f</sup> One chickens was swabbed instead of 2.

<sup>g</sup> 3 chickens were swabbed instead of 2.

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

Vaccinated with	Titre per dose (EID <sub>50</sub> )	Challenge at day 21	Protected <sup>a</sup> / total (%)
Passage 101	10 <sup>3.3</sup>	Yes	13/20 (65)
Passage 85	10 <sup>3.0</sup>	Yes	17/20 (85)
Passage 85	10 <sup>3.3</sup>	Yes	17/20 (85)
Passage 82	10 <sup>3.0</sup>	Yes	18/19 (95)
Passage 82	10 <sup>3.3</sup>	Yes	18/20 (90)
None	NA <sup>b</sup>	Yes	0/20 (0)
None	NA	No	20/20 (100)

<sup>a</sup> A chicken was considered protected if not fewer than 9 out of 10 trachea rings/bird showed normal ciliary activity.

<sup>b</sup> Not applicable.

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

Chickens	Vaccinated at day 0 with	Titre per dose (EID <sub>50</sub> )	Challenge at day 21	Protected <sup>b</sup> / total (%)
Broilers with MDA <sup>a</sup>	Passage 85	10 <sup>3.0</sup>	Yes	14/20 (70)
Broilers with MDA	Passage 85	10 <sup>3.3</sup>	Yes	17/20 (85)
SPF chickens	Passage 85	10 <sup>3.0</sup>	Yes	18/19 (95)
Broilers with MDA	None	NA <sup>c</sup>	Yes	0/20 (0)

<sup>a</sup> Maternally derived antibody.

<sup>b</sup> A chicken was considered protected if not fewer than 9 out of 10 trachea rings/bird showed normal ciliary activity.

<sup>c</sup> Not applicable.

```

DQ341199      285  GTCACAATTCTGTAGTGCACACTGTAATTTTTCTGAAATTACAGTTTTTGTCCACACATTGTTATAGTAGTGGTAGTGGGTCTTGTCCCTATAACAGGCATGATTCCAC
IB L1148 P80  -----
DQ341199      392  GTGATCATATTTCGTATTTCTGCAATGAAAAATGGTTCTTTATTTTATAAATTTAACAGTTAGCGTATCTAAATACCCTAATTTTAAATCTTTTCAATGTGTTAAACAAC
IB L1148 P80  -----
DQ341199      499  TTCACATCTGTTTATTTAAATGGTGATCTTGTTTTTACTTCCAATAAACTACTGATGTTACGTCAGCAGGTGTGTATTTAAAGCAGGTGGACCTGTAAATTATAG
IB L1148 P80  -----
DQ341199      606  TATTATGAAAGAATTTAAGGTTCTTGCTTACTTTGTTAATGGTACAGCACAAGATGTAGTTTTGTGCGACAATCCCCCAAGGGTTTGCTAGCTTGCAATATAACA
IB L1148 P80  -----
DQ341199      713  CTGGCAATTTTTCAGATGGCTTTTATCCTTTTACTAATAGTACTTTGGTTAGGGAAAAGTTCATCGTCTATCGCGAAAGTAGTGTTAATACTACTCTGGCGTTAACT
IB L1148 P80  -----
DQ674739      -----
DQ341199      820  AATTTCACTTTTACTAATGTAAGTAATGCACAGCCTAATAGTGGTGGTGAATACTTTTCATTTATACCAAACACAAACAGCTCAGAGTGGTTATTATAATTTTAA
IB L1148 P80  -----
DQ674739      -----
DQ341199      927  TTTGTCATTTCTGAGTCAGTTTGTGTATAAGGCAAGTGATTTTATGTATGGGTCTACCACCCTAGTTGTTCTTTTAGACCAGAAACCATTAATAGTGGTTTATGGT
IB L1148 P80  -----
DQ674739      -----
DQ341199     1034  TTAATTCCTTGTTCAGTTTCTCTTACTTATGGACCCCTACAGGGAGGGTGAAGCAATCTGTTTTTAGTGGTAAGGCAACGTGTTGTTACGCCTACTCTTATAAAGGC
IB L1148 P80  -----
DQ674739      -----
DQ341199     1141  CCAATGGCATGTAAAGGTGTTTATTCAGGTGAATTAAGCACGAATTTGAATGTGGATTGCTGGTTTATGTTACTAAGAGTGATGGCTCTCGTATACAGACTAGAAC
IB L1148 P80  -----
DQ341199     1248  AGAGCCCTTAGTATTAACGCAATACAATTATAATAATATTACTTTAGATAAGTGTGTTCCTATAATATATATGGCAGAGTAGGCCAAGGTTTTTACTAATGTGA
IB L1148 P80  -----
DQ341199     1355  CTGATTCTGCTGCTAATTTTAGTTATTTAGCAGATGGTGGGTTAGCTATTTTAGATACGTCGGGTGCCATAGATGTTTTGTTGTACAGGGCATCTATGGTCTTAAT
IB L1148 P80  -----
DQ341199     1462  TATTACAAGGTTAATCCTTGTGAAGATGTTAATCAACAATTTGTAGTGTCTGGTGGCAATATAGTTGGCATTCTTACTTCTAGAAATGAAACAGGTTCTGAACAGGT
IB L1148 P80  -----
DQ341199     1569  TGAGAACCAGTTTTATGTTAAGTTAACCAATAGCTCACATCGTCGTAGGCGTTCTATTGGCCAAAATGTAACAAGTTGTCCTTATGTTAGTTATGGCAGATTTTGTA
IB L1148 P80  -----
DQ341199     1676  TTGAACCAGATGGTTCGTTAAAGATGATAGTGCCAGAAGAATTGAAACAGTTTGTGGCACCTTTACTTAATATTACTGAAAGTGTACTCATACTAACAGTTTTTAAC
IB L1148 P80  -----

```

**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 IB virus at passage level 80.