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**ABSTRACT**
In the period from July 2008 to 2010, a disease episode resulting in serious economic losses in the major production area of the Chilean poultry industry was reported. These losses were associated with respiratory problems, increase of condemnations, drops in egg production and nephritis in breeders, laying hens and broilers due to infections with infectious bronchitis virus (IBV). Twenty-five IBV isolates were genotyped and four strains were selected for further testing by pathotyping and protectotyping. Twenty-four IBV isolates were of the Q1 genotype. The experiments also included comparing the ability of six vaccination programmes to induce virus neutralizing antibodies (VNA) in layers against four selected Chilean strains. Despite the high genetic homology in the S1 gene between the four strains, the heterogeneity in biological behaviour of these different Q1 strains was substantial. These differences were seen in embryonated eggs, in cell culture, in pathogenicity and in level of cross-protection by IBV Massachusetts (Mass) vaccination. This variability underlines the importance of testing more than one strain per serotype or genotype to determine the characteristics of a certain serotype of genotype. The combination of Mass and 793B vaccine provided a high level of protection to the respiratory tract and the kidney for each strain tested in the young birds. The combination of broad live priming using Mass and 793B vaccines and boosting with multiple inactivated IBV antigens induced the highest level of VNA against Q1 strains, which might be indicative for higher levels of protection against Q1 challenge in laying birds.

## Introduction

In chickens, infections with infectious bronchitis virus (IBV) are a known cause of respiratory problems, drops in egg production, poor egg shell quality, drops in hatchability, nephritis and sometimes false layers (Jackwood & de Wit, 2013). IBV is a ubiquitous virus with a high mutation rate and the number of types (serotypes or genotypes) of IBV strains that have been detected and reported worldwide is increasing. At this moment, more and more countries have shown that multiple variant strains are circulating in their poultry (De Wit *et al.*, 2011a; Jackwood, 2012).

In Chile, since 1967 presumptive diagnoses of infectious bronchitis (IB) in chicken flocks have been made on the basis of clinical and pathological signs and serological results (Garcia & Norambuena, 1969). In 1975, an IB-like virus was isolated from IBV unvaccinated 6-week-old broiler chickens. The isolated virus showed some antigenic relationship to the Massachusetts (Mass) serotype (Hidalgo *et al.*, 1976). By the mid-1980s, IB was reported as a serious problem in commercial chicken flocks as the Mass and Conn serotypes as well as novel variants were isolated from broiler and

layer flocks. H120 vaccine was found to protect poorly against challenge with these variants (Hidalgo *et al.*, 1986; Cubillos *et al.*, 1991; Lopez *et al.*, 2006). This situation was controlled by modifying the vaccination scheme and the use of autogenous vaccines in some cases.

In the period of July 2008–2010, a new episode of serious economic losses in the major production area of the Chilean poultry industry was reported, associated with respiratory problems, increase of condemnations, drops in egg production and nephritis. In breeders, drops in egg production of between 14% and 40% were seen in laying hens; the infection resulted in a 5% drop in annual production of table eggs. In broilers there were cases with up to 10% mortality and condemnation rates up to 18%. From these flocks, IBV strains could be isolated by the Poultry Research and Diagnostic Laboratory (College of Veterinary Medicine, University of Chile). As these problems also occurred in well-vaccinated flocks using vaccines of the Mass serotype, it raised the question as to whether new IBV variants, against which Mass vaccines induced an insufficient level of the cross-

protection, were involved. The producer association "Asociacion de Productores Avicolas de Chile A.G (APA)" and "Servicio Agricola y Ganadero (SAG)" decided to establish a joint effort to obtain the required knowledge to control the situation again and sent 17 IBV isolated strains to GD (GD Animal Health, Deventer, the Netherlands) for genotyping (all strains), pathotyping and protectotyping studies. The experiments also included comparing the ability of six vaccination programmes to induce virus neutralizing antibodies (VNA) against four selected Chilean strains, as a higher level of VNA is correlated with a higher level of protection against a drop in egg production post challenge of breeders and layers (Box et al., 1973, 1980, 1988; Box & Ellis, 1985).

## Materials and methods

### Origin of the virus isolates

Sixteen IBV isolates that had been isolated by the Poultry Research and Diagnostic Laboratory (College of Veterinary Medicine, University of Chile) between October 2008 and October 2009 were sent to GD in early 2010. Another isolate from 2010 was sent later that year and eight samples followed in 2011 on FTA cards. The isolates originated from broiler, broiler breeder, grand parent or layer flocks with respiratory signs (all flocks), kidney lesions (4), egg drops and egg shell disorders. More detailed information of the isolates is listed in Table 1. All isolates were tested by Reverse Transcription Polymerase Chain Reaction (RT-PCR) and sequenced. A selected number of strains was passaged 1–2 times using embryonated Specified Pathogen Free (SPF) eggs and adapted to primary chicken kidney cells for use in virus neutralizations tests.

### RT-PCR and sequencing

The viral RNA was extracted from 200 µl of allantoic fluid from infected embryonated SPF eggs using the High Pure Viral Nucleic Acid kit (Roche Diagnostics) according to the manufacturer's instructions. By RT-PCR a fragment of 336 base pairs of the S1 gene was amplified using primers XCE1+ and XCE3− (Cavanagh et al., 1999). The S1 PCR amplicons were separated on a 1% agarose gel, and visualized by ethidium bromide staining on an ultraviolet light transilluminator.

PCR products were purified and subsequently subjected to Sanger sequencing (BaseClear, Leiden, the Netherlands) using both the forward (XC1+) and reverse (XCE3−) primers. Consensus sequences were assembled from both forward and reverse sequences using MEGA6.0 (Tamura et al., 2013) and aligned using BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium).

In addition, by RT-PCR a fragment of 1565 base pairs of the S1 gene of seven strains (12.193, D100-272, 12.078, 12.080, 12.216, 12.185 and 12.124) that were used in the experiments and/or virus neutralization test were amplified using primers IBV-Q1-F1: 5′– ATGTTGGG-GAAGTCACTGTTTATAG – 3′ and IBV-Q1-R1: 5′– TGAGAACCAGTYTCATTACGAGA – 3′. PCR products were purified and subsequently subjected to Sanger sequencing (BaseClear, Leiden, the Netherlands) using primers IBV-Q1-F1, IBV-Q1-R1, IBV-Q1-R2: 5′ – GAAGTGAAYACAAGATCACCATTT – 3′, IBV-Q1-F3; 5′ – TCTGCTATGAGGAAGGGAAGTA – 3′, IBV-Q1-R3: 5′ – CACTCTGAGYTGTTTGTGTTTG – 3′, IBV-Q1-F4: 5′ – CYAATACAGGTGGTGTYCATAG – 3′, IBV-Q1-R4: 5′– RTCTGCCARATAGCCATGATT – 3′, IBV-Q1-F5: 5′ – CCWCTACAGGGTGGTT-GYAAG – 3′. Consensus sequences were assembled from both forward and reverse sequences using MEGA6.0 (Tamura et al., 2013) and aligned using BioNumerics (Applied Maths). Consensus sequences were deposited in Genbank (MF043932-MF043938).

### Phylogenetic analysis

Two phylogenetic trees were constructed from the obtained consensus sequences using MEGA6.0. One tree was based on the 336 bp partial sequence of the S1 and a second tree was based on an almost full-length (1565 bp) sequence of the S1. Sequences of different IBV genotypes were downloaded from Genbank and included in the trees for comparison purposes. The following strains were included: Genogroup GI-16, T3 (AF227438), Ck/CH/LDL/97I (|EF030995), 2992/02 (AY606323), CK/CH/Chongqing (GU938413), Q1 China (AF286302), CL/12020/08 (HM446006), Q1 Italy (KJ941019); Genogroup GI-12, D274 (X15832); Genogroup GI-23, Variant 2 (AF093796); Genogroup GI-9, Ark99 (M99482); Genogroup GI-1, M41 (AY561711); Genogroup GI-19, QX IBV (AF193423); Genogroup GI-13, 7/91 (Z83975) and Genogroup GI-21, Italy02 (AJ457137) (Valastro et al., 2016). Trees were constructed using the maximum likelihood method based on the Jukes Cantor model (Jukes & Cantor, 1969) and the tree with the highest log likelihood was shown. Sequence gaps were included in the analysis. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site. The tree based on the 336 bp fragment included 38 nucleotide sequences, and the tree based on the 1565 bp fragment included 20 nucleotide sequences.

### Vaccines

Five commercially available vaccines (coded Mass, 793B, IB1, IB2 and IB3) were used in the studies. Vaccine "Mass" was a live attenuated monovalent vaccine

**Table 1.** Sample identification information on 17 IBV Q1 strains from Chile used in this study.

| Sample identification | Type of bird | Age (days or weeks) | Clinical signs | Abnormalities in embryonated eggs | Adapted to CEKC[a] in passage 1–8 | Used for VNT[b] | Pathogenicity and cross-protection |
|---|---|---|---|---|---|---|---|
| 12.020 | Layer pullets | 12 W | Respiratory signs, conjunctivitis | | | | |
| 12.078 | Broilers | 32 D | Respiratory signs, sinusitis, airsacculitis, perihepatitis, pericarditis, cellulitis | High mortality | CPE | X | |
| 12.080 | Laying hens | 14 W | Respiratory signs, tracheitis | High mortality | CPE | X | |
| 12.124 | Layer breeder | 29 W | Respiratory signs, 30% drop in egg production | Abnormalities, no mortality | Not successful | | X |
| 12.185 | Broilers | 36 D | Respiratory signs, airsacculitis | Abnormalities, no mortality | Not successful | | X |
| 12.193 | Layers | 20 W | Respiratory signs, sinusitis, mild tracheitis | High mortality | CPE | X | |
| 12.216 | Broiler breeders | 42 W | Egg shell disorders, tracheitis, kidney lesions, salpingitis, peritonitis | Abnormalities, no mortality | CPE after 8 passages | X | X |
| D100-273-4 | Broilers | 5 W | Respiratory distress, renal problems, poor growth, more condemnations | Abnormalities, no mortality | | | x |
| 12.103(A) | Broilers | 37 D | Respiratory signs | | | | |
| 12.103(C) | Broiler breeders | 22 W | Respiratory signs | | | | |
| 12.103(B) | Broilers | 24 D | Respiratory signs | | | | |
| 12.021-6 | Broilers | 17 D | Respiratory signs, abundant sinuses mucous exudates, airsacculitis | | | | |
| 12.101 | Grand parents | 29 W | Respiratory signs, mild peritonitis and airsacculitis | | | | |
| 12.139 | Layers | 22 W | Respiratory signs, kidney lesions | | | | |
| 12.173 | Broilers | 31 D | Respiratory signs. airsacculitis, septicaemia | | | | |
| 12.215 | Broilers | 17 D | Respiratory signs, airsacculitis, arthritis, pericarditis, perihepatitis, kidney lesions | | | | |
| 12.229 | Broilers | 21 D | Respiratory signs | | | | |

[a]Chicken embryo kidney cells.
[b]Virus neutralization test.

of the Mass serotype (Nobilis IB MA5, Intervet International bv). Vaccine "793B" was a live attenuated monovalent vaccine of the 793B-4/91 serotype (Nobilis IB 4/91, Intervet International bv).

Three inactivated vaccines were used containing antigens to either 1, 2 or 3 different IB serotypes. Inactivated vaccine IB1 contained the M41 antigen (Nobilis M41 inac, Intervet SPAH, the Netherlands). Inactivated vaccine "IB2" contained the M41 and D274 antigens in combination with antigens of avian metapneumovirus, Newcastle disease virus and egg drop syndrome virus (Nobilis RT + IBmulti + ND + EDS, Intervet SPAH, the Netherlands). Inactivated vaccine "IB3" contained the M41, D274 and D1466 IBV antigens in combination with Newcastle disease virus antigen (Nobilis IB3 + ND, Intervet SPAH, the Netherlands).

## Vaccination

Vials of both live vaccines were reconstituted in sterile, cold demineralized water and stored on melting ice until use. Both live vaccines were applied by eye drop (two drops of 50 µl per bird). The actual vaccine dose per bird, as determined by egg-titration of inocula was $10^{2.7}$ EID$_{50}$/bird for MA5 and $10^{3.7}$ EID$_{50}$/bird for IB 4/91. The inactivated vaccines were

administered at the manufacturer's recommended dose of 0.5 ml by the intramuscular route.

## Challenge

Based on the result of the genotyping, origin of the strains (meat type or layers), year of isolation, type of lesions that were seen in the flocks of origin and differences in growth in embryonated eggs and cell culture, four strains were selected for further testing by pathotyping and protectotyping. These Q1 challenge strains 12.124, 12.185, 12.216 and D100-273-4 were applied by eyedrop (1 droplet in each eye), giving $10^4$ EID$_{50}$ per bird at 33 or 34 days of age (Experiment 1) or at 35 days of age (Experiment 2).

## Pathotyping and protectotyping

The experiments were conducted with the formal approval of the local animal welfare committee and registered according to the Dutch legislation.

## Experiment 1

Thirteen groups of twenty 1-day-old SPF layer chickens were placed in isolators and vaccinated and challenged according to Table 2. One group of 20 SPF

**Table 2.** Results of the vaccination-challenge experiment performed with Chilean IBV Q1-like strains.

| Q1 strain | Vaccination | | Challenge at day 33, 34 or 35) | Ciliostasis protection score (%) at 5 dpc[a] | Percentage of 10 birds with detectable IBV antigen in the kidney by IHC[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | Day of hatch | Day 14 | | | Kidney | | Proventriculus | |
| | | | | | 5 dpc | 8 dpc | 5 dpc | 8 dpc |
| Experiment 1 | | | | | | | | |
| Negative control | – | – | No | 100 | 0 | 0 | 0 | 0 |
| 12.124 | – | – | Yes | 0 | 50 | 40 | 0 | 0 |
| | Mass | – | Yes | 44 | 30 | 40 | 0 | 0 |
| | Mass | 793B | Yes | 100 | 0 | 10 | 0 | 0 |
| 12.185 | – | – | Yes | 6 | 10 | 20 | 0 | 0 |
| | Mass | – | Yes | 93 | 0 | 0 | 0 | 0 |
| | Mass | 793B | Yes | 99 | 0 | 0 | 0 | 0 |
| 12.216 | – | – | Yes | 4 | 20 | 0 | 0 | 0 |
| | Mass | – | Yes | 77 | 10 | 0 | 0 | 0 |
| | Mass | 793B | Yes | 100 | 0 | 0 | 0 | 0 |
| Experiment 2 | | | | | | | | |
| Negative control | | No | No | 98 | 0 | 0 | Nt[c] | nt |
| D100-273-4 | – | – | Yes | 0 | 20 | 20 | nt | nt |
| | Mass | – | Yes | 59 | 0 | 0 | nt | nt |
| | Mass | 793B | Yes | 100 | 0 | 0 | nt | nt |

[a]Days post challenge.
[b]Immunohistochemistry.
[c]nt = Not tested.

birds functioned as the negative control group. For each of the selected IBV strains ($n = 4$), three groups of birds were used. One group was not vaccinated to determine the pathogenicity of the challenge virus for the respiratory tract at 5 days post challenge (dpc.), and for the kidney and proventriculus at 5 and 8 dpc. Another group was only vaccinated at day 1 with Mass, to determine the level of protection by Mass vaccines (protectotyping). The third group for each virus was vaccinated at day 1 with Mass and at 14 days with 793B to test whether this vaccination schedule would induce an increased and higher level of protection against the challenge strain as has been shown for several other variant strains (Cook et al., 1999; 2001; Terregino et al., 2008; De Wit et al., 2011b).

### Experiment 2
Four groups of twenty 1-day-old SPF layer chickens were placed in isolators and vaccinated and challenged according to Table 2 with the IBV isolate from 2010.

### Production of antisera and virus neutralization test

Two groups of thirty 1-day-old SPF layers (individually marked) were housed in two isolators (A and B) and vaccinated (eyedrop) with Mass at day 1 only (group A) or with Mass at day 1 and 793B at day 14 (group B). At 12 weeks of age, birds 1–10 of groups A and B were boosted with vaccine IB1. Birds 11–20 were boosted with vaccine IB2, and birds 21–30 were boosted with vaccine IB3. At 18 weeks of age, all birds were bled. Blood was pooled per group (A-IB1, A-IB2, A-IB3, B-IB1, B-IB2 and B-IB3). Each pooled serum was tested fourfold for the presence and amount of VNA against a constant amount of 100 (30–300)

median tissue culture infectious dose ($TCID_{50}$) of chicken embryo kidney cell (CEKC) adapted Q1 field strains 12.078, 12.080, 12.193 and 12.216. Virus was mixed with twofold dilutions of the six antisera and incubated for 1 h at room temperature. Subsequently, the virus–serum mixtures were transferred in fourfold to wells of microtitre plates which contained monolayers of primary CEKC. The plates were placed in a $CO_2$ incubator at 38–39°C for 72 h. The VNA titres were expressed as the reciprocal of the highest dilution of serum that prevented cytopathic effects (CPE). The test was performed in the presence of appropriate controls.

### Ciliostasis

The level of protection of the trachea was determined using the ciliostasis test on five tracheal rings per chicken at 5 days post challenge (De Wit et al., 2013). The level of beating of the cilia in each ring (TOC score) was expressed as 4 (<25% beating of cilia), 3 (25–50% beating), 2 (50–75% beating), 1 (75–99% beating) or 0 (all beating). TOC scores per chicken were between 0 and 20 (five rings from each trachea; maximum score 4). An individual chicken was recorded as protected against challenge if the ciliostasis score was less than 10 (modified from Cook et al., 1999). For each group, a ciliostasis protection score (0–100%) was calculated by the formula:

Ciliostasis protection score (CPS) = 100% − (100 × total of the individual scores)/(number of individuals × 20).

### Immunohistochemistry

Immunochemistry (IHC) was used for the detection of IBV antigen in the kidney and proventriculus using

monoclonal antibody 48.4 directed against the nucleoprotein of IBV (De Wit et al., 2011b). The following scoring system was used: score 1 when a bird had IBV positive cells in the kidney, score 0.5 when the staining results were suspect, a score 0.25 was used when the ureter was IBV positive but the kidney was negative and a score 0 was used when the staining of both kidney and ureter were negative for that bird.

## Results

### Genotyping

The results of the genotyping are shown in a phylogenetic tree based on a part of S1 (Figure 1). One strain obtained in this study (12.229) was closely related (98.8% nucleotide similarity) to the M41 strain (AY561711). All other strains obtained in this study

clustered together and were genetically related (about 97.6–98.8% nucleotide similarity) to Chinese Q1 strains (acc. no. AF286302) belonging to the GI-16 lineage (Valastro et al., 2016). The differences in nucleotide similarity between the Chilean Q1 strains from this study varied from 0 to 2.3%. The level of similarity on amino acids deduced from the sequenced part of the S1 gene between these strains and the Mass genotype was 71.4–74.1%.

A separate phylogenetic tree was constructed and based on 1565 nucleotides of the S1 of the seven Chilean Q1 strains that were used for the animal experiments and/or virus neutralization test (Figure 2). The differences in nucleotide similarity between these Chilean Q1 strains and based on 1565 bp varied from 0.8 to 5.2%. The level of similarity on amino acids, deduced from the almost full-length S1 gene between these strains and the Mass genotype, was about 75%. Clustering of these seven strains based on partial S1
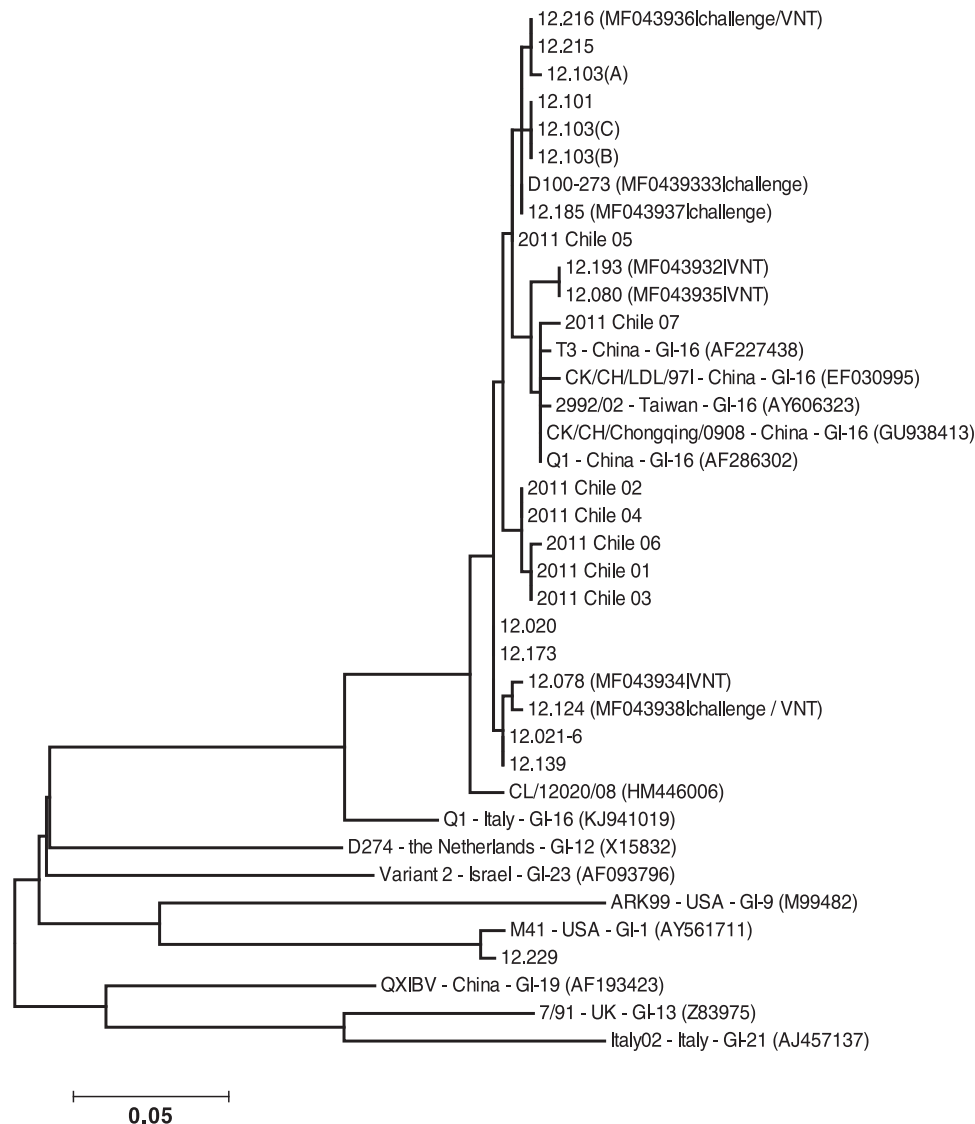


**Figure 1.** Maximum Likelihood tree (1000 bootstrap replicates) for the partial S1 subunit of the IBV spike protein (nucleotide sequences corresponding to amino acid 231–346). Shown are the Chilean strains typed in the present study and several IBV reference strains. The Q1 strains used for the pathotyping and vaccination/challenge experiment and/or VNT are identified with "challenge" or "VNT".
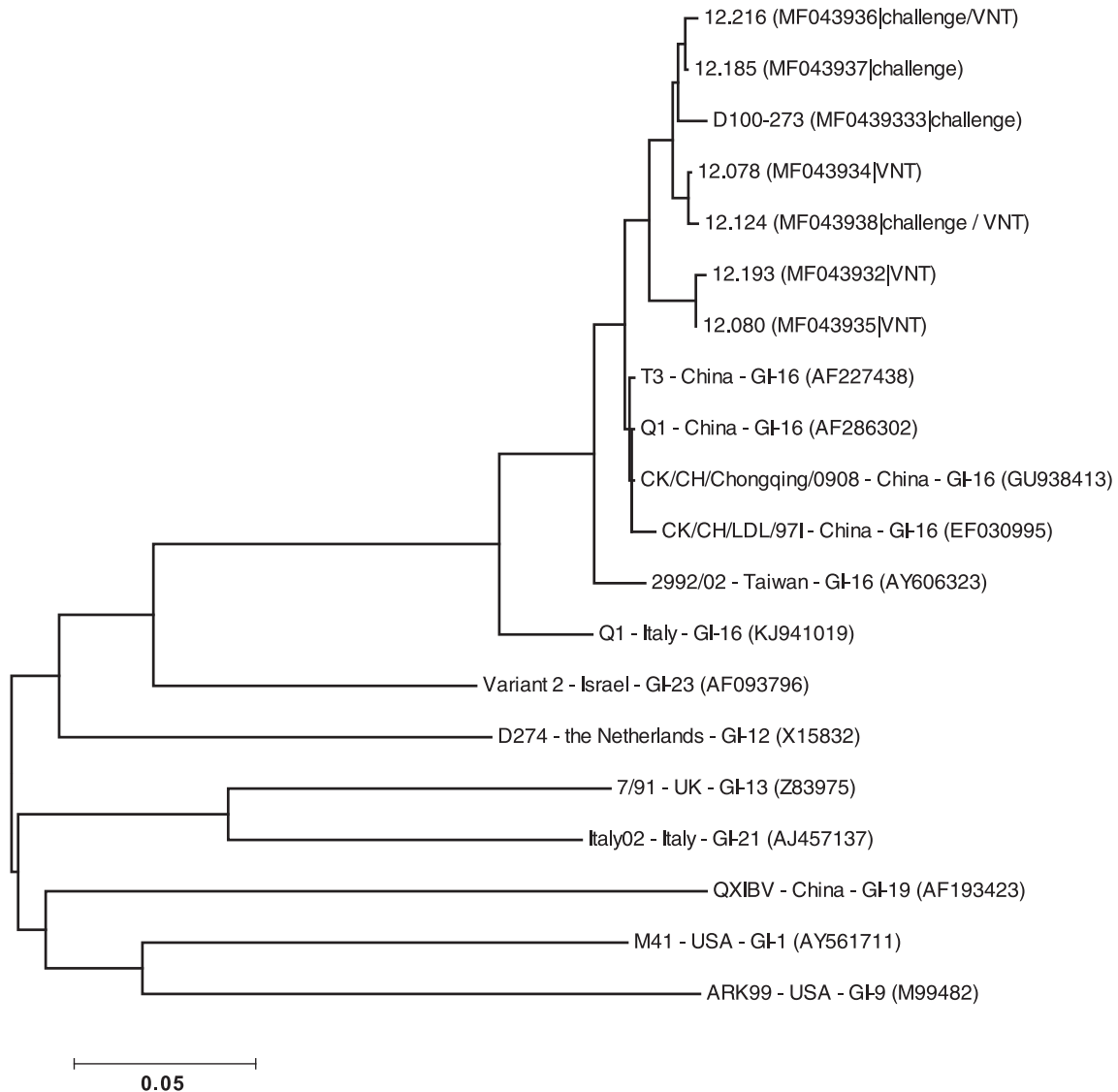
**Figure 2.** Maximum Likelihood tree (1000 bootstrap replicates) for the S1 subunit of the IBV spike protein (nucleotide sequences corresponding to amino acid 1–521). Shown are the Chilean strains typed in the present study and several IBV reference strains. The Q1 strains used for the pathotyping and vaccination/challenge experiment and/or VNT are identified with "challenge" or "VNT".

(336 bp) and almost full-length S1 (1565 bp) was very similar.

### Pathotyping and protectotyping

The results of the pathotyping and protectotyping are shown in Table 2. Based on the results of the genotyping and the paper of Yu *et al.* (2001), it was decided to test the proventriculus for the presence of detectable IBV replication also.

As can be expected for IBV strains, the four tested strains 12.124, 12.216, 12.185 and D100-273-4 caused ciliostasis in the trachea of the unvaccinated birds varying between 94% and 100% (Table 2). Replication in the kidney was also shown for all four strains in the unvaccinated birds, which identified them as nephropathogenic strains. The peak in percentage of birds with positive IHC staining on kidney at 5 and 8 days post challenge varied from 20% to 50%. IBV replication

could not be detected in the proventriculus of any of the challenged birds at 5 or 8 days post challenge.

The Mass vaccine protected against ciliostasis at a level of 44%, 93%, 77% and 59% by challenge strains 12.124, 12.216, 12.185 and D100-273-4, respectively. Vaccination with the Mass vaccine also resulted in lower rates of detectable renal replication of the challenge viruses compared to the unvaccinated groups. The peak percentage of birds with positive IHC staining of the kidney at 5 and 8 days post challenge for birds that were vaccinated with Mass only was 40%, 0%, 0% and 0% for IBV strains 12.124, 12.216, 12.185 and D100-273-4, respectively.

All five groups of birds that had been vaccinated with Mass and the 793B vaccine were protected at a level of 99% up to 100% against tracheal damage by the four different Q1 challenge Q1 strains. In only one of these 80 birds, were IB positive cells detected in the kidney post challenge.

### Embryo lesions and adaptation to primary chicken kidney cells

Seven strains were inoculated into 9–10 day embryonated SPF eggs for the production of titrated challenge virus. Three of these isolates caused high mortality in the embryos, whereas the four remaining strains did not kill the embryos but did induce intensive dwarfing and the formation of cloacal distention. In order to perform virus neutralization tests, virus strains had to be adapted to CEKC and had to induce a CPE. In collaboration with representatives from Chile, the adaptation of six strains was attempted for eight passages. Three strains (12.078, 12.080 and 12.193) that caused high mortality in the embryonated eggs could easily be adapted to CEKC within a few passages, showing a clear CPE, and could be used for the neutralization test (Table 1). Of the three strains that did not cause mortality in the embryos, strain 12.216 could be adapted more laboriously, as it started to produce a CPE in the 8th passage. For the two strains (12.124, 12.185) that did not cause mortality in embryonated

eggs, adaptation to CEK did not succeed within eight passages.

### Virus neutralization test

The pooled sera of the six vaccination programmes were tested in four-fold dilutions for the presence and amount of VNA against a constant amount of $100 - (30–300)$ median tissue culture infectious dose ($TCID_{50}$) of the adapted Q1 field strains 12.078, 12.080, 12.193 and 12.216. The results are listed in Table 3 and Figure 3.

The vaccination programme with live Mass priming only and a boost with the inactivated M41 antigen resulted in the lowest level of Q1 virus neutralizing antibodies. All other vaccination programmes (broad priming with Mass and 793B and/or use of multiple IBV antigens in the inactivated vaccine) resulted in a higher level of VNA against the Q1 strains from Chile. However, the level of VNA against each of the Q1 strains varied.

**Table 3.** Levels of virus neutralizing antibody titres ($log_2$) against four Chilean IBV Q1 viruses in the sera from chickens given six different vaccination programmes.

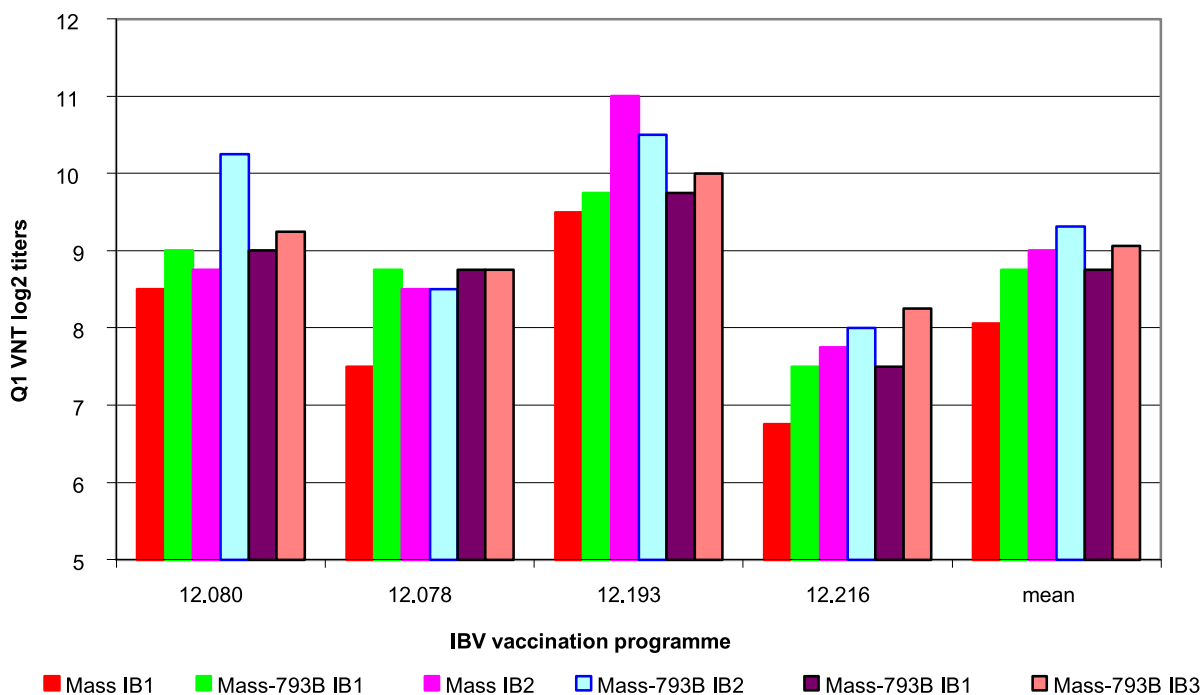| Vaccination programme | | Average virus neutralizing antibody titre (tested in four-fold dilutions) against Q1 strains | | | | |
|---|---|---|---|---|---|---|
| Live priming | Inactivated vaccine | 12.080 | 12.078 | 12.193 | 12.216 | All four strains |
| Mass | IB1 | 8.50 | 7.50 | 9.50 | 6.75 | 8.06 |
| Mass | IB2 | 8.75 | 8.50 | 11.00 | 7.75 | 9.00 |
| Mass | IB3 | 10.25 | 8.75 | 11.00 | 8.00 | 9.50 |
| Mass + 793B | IB1 | 9.00 | 8.75 | 9.75 | 7.50 | 8.75 |
| Mass + 793B | IB2 | 10.25 | 8.50 | 10.50 | 8.00 | 9.30 |
| Mass + 793B | IB3 | 9.25 | 8.75 | 10.00 | 8.25 | 9.06 |



**Figure 3.** Levels of IBV Q1 virus neutralizing antibody titres ($log_2$) induced by six different IBV vaccination programmes using live priming (Mass alone or Mass + 793B) and a boost using inactivated IBV vaccines containing only M41, M41 + D274 or M41 + D274 + D1466 inactivated antigens.

## Discussion

Sixteen out of 17 Chilean isolated field strains were, based on partial S1 sequencing, of the Chinese Q1 genotype. Also the samples on FTA cards that were tested in 2011 were positive for Q1. Three representatives of this genotype, being strains Q1, J2 and T3 have been described by Yu et al. (2001). Despite the high nucleotide similarity (on average 97%) for the partial S1 gene of the Chilean Q1-like isolates compared to Chinese Q1-like isolates, and the high nucleotide similarity within the Chilean isolates, major differences were seen in their biological behaviour. The Chinese Q1-like strains had been isolated from the proventriculus of different Chinese flocks of pullets that showed "an avian disease associated with the proventriculus", whereas the Chilean isolates were isolated from flocks with the traditional respiratory problems, drops in egg production or signs of nephritis. In an infection experiment with the three Chinese Q1-like strains (Q1, J2 and T3), the infected birds showed very high mortality, respiratory signs and diarrhoea with lesions of the proventriculus, whereas no macroscopically visible lesions were seen in the kidneys (Yu et al., 2001). Although the Chinese Q1-like strains were reisolated from the proventriculus during this experiment, local replication of IBV in proventriculus was not shown, which means it might have been a reisolation due to viraemia (De Wit & Cook, 2014). In our experiments, IHC staining of the proventriculi of all 60 birds at 5 of 8 days post challenge with one of the Chilean Q1-like strains gave negative results, suggesting that the replication of these Q1 strains was at a very low level or non-existent. In contrast to the, for IBV, extremely high mortality of 75–100%, that was seen post challenge with the Chinese Q1-like in 2-week-old SPF birds, no mortality was noticed post inoculation of the four Chilean Q1-like isolates in 1-day-old SPF birds despite the fact that they were pathogenic for the respiratory tract and kidney as shown by the ciliostasis test and IHC.

Nucleotide differences up to 22% in both the partial S1 sequence (336 bp) and almost full-length S1 sequence (1565 bp) were observed between the Chilean strains and Masss serotype, whereas small nucleotide differences were observed between different Chilean Q1-like strains. In several papers regarding the level of homology of the S1 gene (or a part of it) in relation to the level of cross-protection (Cavanagh et al., 1997; Cook et al., 2001; Meir et al., 2004; Gelb et al., 2005; Ladman et al., 2006), it was shown that there is an increased chance of a high level of cross-protection between strains with high nucleotide similarity when compared to strains with a low homology. However, this correlation is not very strong (De Wit et al., 2011a). Moreover, strains which differed only by a few per cent in nucleotide similarity showed a

significant drop in cross-protection (Meir et al., 2004; Abdel-Moneim et al., 2006), whereas there was a high level of cross-protection against other strains with a much lower homology (Meir et al., 2004). This phenomenon was also detected for the Chilean Q1 strains. Despite the high nucleotide similarity for the whole S1 gene, the strains that were investigated showed significant differences in embryonic lesions, the level of replication in the kidney, the ability to adapt to CEKC and induce CPE and the level of cross-protection by the Mass vaccination. A Mass vaccination was able to protect for 93% against respiratory damage and detectable replication on the kidney for Q1-like strain 12.185, which categorises this strain to the Mass protectotype. For the other three Q1-like strains, Mass vaccination did not result in a good level of protection against respiratory damage and/or replication in the kidney. Vaccination with Mass at day of hatch and subsequently with 793B vaccine at 14 days of age resulted in a very high level of cross-protection at both the respiratory tract and kidney level for all four strains that were tested. Protection by 793B vaccine alone has not been investigated in this study.

Regarding the induction of Q1 virus neutralizing antibodies in the laying birds, all six vaccination programmes induced detectable levels of virus neutralizing antibody against the four tested Q1 strains. The lowest level of VNA against the four Q1 strains was the group with only Mass vaccines (Mass live priming and boost with the inactivated M41 vaccine). As can be seen in Table 3 and Figure 3, all other vaccination programmes (broad priming with Mass and 793B and/or use of multiple IBV antigens in the inactivated vaccine) resulted in a higher level of VNA against the Q1 strains from Chile. Live priming with Mass and 793B resulted (on average) in more Q1 VNA compared to priming with Mass vaccine alone. Boosting with inactivated vaccines that contained multiple IBV antigens induced more VNA against the Q1 strains compared to boosting with inactivated M41 antigen alone. The combination of broad live priming and boosting with multiple inactivated IBV antigens induced the highest level of VNA against Q1. It has been reported before that chickens that have been in contact with only one type of IBV produce mainly antibodies against that type of IBV (Marquardt et al., 1981; Gelb & Killian, 1987; Karaca & Naqi, 1993; De Wit et al., 1997). However, chickens that been inoculated with two or more heterologous IBV serotypes tend to produce not only VNA against the inoculated strains but also types of IBV to which the birds have not been exposed (Gelb & Killian, 1987; De Wit et al., 1997). The true relevance for the detected differences in the ability of different vaccination programmes to induce Q1-virus neutralizing antibodies can only be tested with vaccination-challenge experiments with laying birds using Q1 challenge strains. These experiments are long-lasting and

therefore very expensive. In the experiments of Box and colleagues in the 1980s (Box *et al.*, 1973, 1980, 1988; Box & Ellis, 1985) using Mass and D274 challenge strains, a clear positive correlation was shown in laying birds between the level of serotype specific antibodies against the challenge strain at the day of challenge and the level of protection against a drop in egg production. Similar experiments have been performed at GD Animal Health for six serotypes of IBV (Mass, 793B, QX, D274, Italy-02 and D1466). In these experiments, on average, a 1 $\log_2$ higher level of VNA against the variant IBV challenge strain correlated with 9% less drop in egg production after challenge (De Wit, not published). It is therefore expected that the detected differences in levels of Q1 virus neutralizing antibodies by the different vaccination programmes are relevant.

The results of these experiments show heterogeneity in the biological behaviour of different Q1 strains despite the apparent high genetic homology in the S1 gene between these strains. Whole genome sequencing might elucidate the genetic background of these differences that were seen in the embryonated eggs, in cell culture, in pathogenicity and in level of cross-protection by a Mass vaccination. This underlines the importance of testing more than one strain per serotype or genotype to determine the characteristics of a certain serotype of genotype. The combination of Mass and a 793B vaccine provided a high level of protection of the respiratory tract and the kidney for each strain. The combination of broad live priming using Mass and 793B vaccines and boosting with multiple inactivated IBV antigens induced the highest level of VNA against Q1, which might be indicative for higher levels of protection against Q1 challenge in laying birds.

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