

Infectious bronchitis vaccine virus detection and part-S1 genetic variation following single or dual inoculation in broiler chicks

Christopher Ball, Faez Awad, Sally Hutton, Anne Forrester, Matthew Baylis & Kannan Ganapathy

To cite this article: Christopher Ball, Faez Awad, Sally Hutton, Anne Forrester, Matthew Baylis & Kannan Ganapathy (2016): Infectious bronchitis vaccine virus detection and part-S1 genetic variation following single or dual inoculation in broiler chicks, Avian Pathology, DOI: [10.1080/03079457.2016.1268675](https://doi.org/10.1080/03079457.2016.1268675)

To link to this article: <http://dx.doi.org/10.1080/03079457.2016.1268675>



Accepted author version posted online: 05 Dec 2016.



Submit your article to this journal [↗](#)



Article views: 29



View related articles [↗](#)



View Crossmark data [↗](#)

Publisher: Taylor & Francis & Houghton Trust Ltd

Journal: *Avian Pathology*

DOI: 10.1080/03079457.2016.1268675

**Infectious bronchitis vaccine virus detection and part-S1 genetic variation
following single or dual inoculation in broiler chicks**

Christopher Ball^{1*}, Faez Awad^{1,2*}, Sally Hutton¹, Anne Forrester¹,
Matthew Baylis^{1, 3} & Kannan Ganapathy^{1**}

¹ *University of Liverpool, Institute of Infection and Global Health, Leahurst Campus, Neston, Cheshire, CH64 7TE, UK;* ² *University of Omar Al-Mukhtar, Faculty of Veterinary Medicine, Al-Bayda, Libya;* ³ *NIHR Health Protection Research Unit in Emerging and Zoonotic Infections, University of Liverpool, UK.*

RUNNING TITLE: *In vivo* IBV vaccine genetic variation

*Co-first authors

**Corresponding author Tel.: +44 151 7946019; fax: +44 151 7946005.

E-mail address: gana@liverpool.ac.uk

ABSTRACT

An investigation was undertaken of the extent of genetic variation occurring within infectious bronchitis virus (IBV) vaccine strains following vaccination of day-old broiler chicks. Chicks were divided into seven groups, with two groups receiving single Massachusetts (Mass) vaccinations and the other four were inoculated with combinations of different IBV serotypes; Mass, 793B, D274, and Arkansas (Ark). The remaining group was maintained as an unvaccinated control. Following vaccination, swabs and tissues collected at intervals were pooled and RNA was extracted for detection of IBV by reverse transcription polymerase chain reaction (RT PCR). Positive amplicons were sequenced for the part-S1 gene and compared to the original vaccine strain sequences. Single nucleotide polymorphisms (SNPs), amino acid variations and hydrophobicity changes were identified and recorded for each sampling point. A total of 106 SNPs were detected within 28 isolates. The average SNP counts of swab isolates were greater than those found in tissue samples. This translated into 64 amino acid changes, however only six resulted in a change to the hydrophobicity properties. All hydrophobic alterations occurred within swab isolates and the majority were recovered at 3 days post vaccination suggesting such changes to be detrimental to early virus survival. Nucleotide deletions were seen only in the group given the combination of Mass and Ark. Of the 16 sequenced samples in this group, 13 contained the same AAT deletion at position 1033 1035 in the Ark strains. Findings presented in this study demonstrate alteration in the S1 nucleotide sequence following co-administration of live IBV vaccines.

Keywords: IBV, live vaccine, PCR and sequencing, SNP, broiler chicks

INTRODUCTION

Infectious bronchitis virus (IBV) is a positive sense single stranded RNA virus and a member of the *Coronaviridae* family (Gonzalez *et al.*, 2003; Cavanagh, 2005). The complete IBV genome is around 27.61kb (Cavanagh, 2005) with a GC content of 37.9%. There are a total of ten open reading frames (ORFs) that encode ten proteins, four of which are main structural proteins. These are the spike (S) glycoprotein, the membrane (M) glycoprotein, the nucleocapsid (N) protein and the small envelope (E) protein (Sutou *et al.*, 1988; Ammayappan & Vakharia, 2009).

It has been previously documented that new IBV genotypes can arise as a result of genetic alterations (Zhang *et al.*, 2010; Han *et al.*, 2011). This is in part due to the highly variable S1 protein, a 1.7kb subunit of the S glycoprotein (Cavanagh *et al.*, 1992). S1 is important for attachment of the virus to host cell receptors and induces virus neutralizing and haemagglutination inhibition antibodies (Cavanagh *et al.*, 1988; Ignjatovic & Galli, 1994; Cavanagh & Gelb, 2008).

Changes in nucleotide sequences, such as point mutations and insertions or deletions (indels), may have a downstream effect on the protein structure and functional consequences (Cavanagh, *et al.*, 1992; Studer *et al.*, 2013). However it seems that with S1 variations, the majority of the remaining virus genome remains unchanged (Cavanagh, *et al.*, 1992). This has led to suggestions that the currently available vaccines may still offer partial protection against challenge from particular variant strains (Cook *et al.*, 1999). Under experimental conditions, the extent of genomic variation within the Arkansas (Ark) vaccine strain has been previously investigated in SPF chicks (van Santen & Toro, 2008).

Additionally, the subpopulations present in a number of strains (Mass [M41], Connecticut, DE, GA98 and Ark) were examined (McKinley *et al.*, 2008). However, little or no data are available for other serotypes of live vaccine viruses such as 793B (eg. 4/91, CR88), D274 and Mass (e.g. H120 and Ma5).

Mass was the first IBV serotype to be identified and subsequent work outlined the poor cross-protective nature when compared to other strains at the time, such as the Connecticut isolate (de Wit *et al.*, 2011). It is well known that the most widely used live IBV vaccines, for example Mass/Mass41/H52/H120/Ma5 are derived from the Massachusetts serotype (Bijlenga *et al.*, 2004; Jackwood & de Wit, 2013). There are now a number of recognised live Mass vaccine strains (e.g. H52, H120, MM and Ma5). Recent field studies utilising molecular approaches have reported the identification of a number of Mass-like variants (Villarreal *et al.*, 2010; Han, *et al.*, 2011; Sumi *et al.*, 2012; Fraga *et al.*, 2013). The D274 and Ark serotypes were first reported in the 1970s following studies into 15 field strains from the UK (Dawson & Gough, 1971) and 40 broiler flocks in Arkansas (Fields, 1973) respectively. Both strains are available as commercial vaccines (Gelb & Cloud, 1983; de Wit, *et al.*, 2011). Likewise, since its emergence in the 1990s (Gough *et al.*, 1992), 793B has been reported in most poultry producing countries leading to the widespread use of homologous live vaccines (e.g. 4/91, CR88, 1/96) (Jones, 2010).

Nucleotide variation may affect the antigenic properties of an infecting field strain (Cavanagh, *et al.*, 1992), and this could potentially circumvent the protection conferred by existing vaccines. Furthermore, should a host be immunosuppressed and have a reduced ability to clear an IBV infection, virulent strains can establish within flocks with greater ease (Winterfield *et al.*, 1978; Pejkovski *et al.*, 1979).

In recent years, Sareung *et al.* (2014) demonstrated the benefit of heterologous live vaccination programmes against heterologous IBV challenges (Sarueng *et al.*, 2014).

Chhabra *et al.* (2015) and Awad *et al.* (2016) have reported on concurrent vaccination of Mass+793B vaccines and their ability to provide protection against a number of virulent variant IBVs. Such simultaneous heterologous IBV vaccination programmes are widely practiced, as they are often administered in the hatchery or upon the chick's arrival at the farm. The clinical signs, lesions and immune responses following experimental co-vaccination have been previously reported (Chhabra *et al.*, 2015; Awad *et al.*, 2016).

We report the genomic variations occurring in seven IBV strains from four serotypes following simultaneous *in vivo* inoculation in IBV maternal-antibody positive commercial day-old chicks (Awad, *et al.*, 2016). Swabs and tissues collected at intervals from groups of chicks that were single or dual vaccinated, were subjected to IBV RT-PCR. Positive samples were sequenced and analysed for single nucleotide polymorphisms (SNPs), amino acid changes and hydrophobicity alterations.

MATERIALS AND METHODS

Chicks and ethical statement. Day-old commercial broiler chicks with maternally-derived IBV antibodies (MDA) were obtained from a commercial hatchery. Chicks were kept in an isolation unit (University of Liverpool) throughout the experiment and reared on deep litter with water and feed provided *ad libitum*. All experimental procedures were undertaken after approval of the University of Liverpool ethical review committee and according to the UK legislation on the use of animals for experiments.

IBV vaccines. Seven commercially available live IBV vaccine viruses were reconstituted in sterile distilled water and used either singly or in combination. For this study, the Mass vaccines are referred to as Mass₁ and Mass₂, and the 793B vaccines are referred to as 793B₁ and 793B₂. These monovalent vaccines were produced by different manufacturers. Mixtures of Mass₁+793B₁ and of Mass₂+793B₂ were prepared as previously described (Awad, *et al.*, 2016). In addition, commercially combined live vaccines of Mass₁+D274 and Mass₃+Ark were also used (Awad, *et al.*, 2016). Vaccine titres (EID₅₀/chick) were as follows: Mass₁ (log₁₀ 4.0), Mass₂ (log₁₀ 4.1), 793B₁ (log₁₀ 4.75), 793B₂ (log₁₀ 4.4), Mass₁+D274 (log₁₀ 4.3³) and Mass₃+Ark (log₁₀ 4.0). All vaccines were prepared prior to administration to provide the dosage per chick as recommended by the manufacturers.

Experimental design. Protocols were as previously described (Awad, *et al.*, 2016). Briefly, two hundred and ten day-old chicks were randomly divided into seven groups and kept in

separate isolation units, with 30 chicks per group. Each chick was inoculated via the ocular (50 µl) and nasal (50 µl) routes with one of the following: Mass₁ (Group 1), Mass₂ (Group 2), Mass₁+D274 (Group 3), Mass₁+793B₁ (Group 4) Mass₂+793B₂ (Group 5) Mass₃+Ark (Group 6), and sterile water (Group 7, control). At 3, 6, 10, 14, 18 and 25 days post vaccination (dpv), oropharyngeal (OP) and cloacal (CL) swabs, with tissue samples of the trachea (TR), duodenum (DU), caecal tonsil (CT) and kidney (KID) were collected from five chicks in each group.

Processing of swab and tissue samples. Both OP and CL swabs from each sampling point were pooled. The swabs were dipped into a sterile tube containing 1.5 ml of Eagles serum-free minimum essential medium with glutamine, streptomycin [50 mg/ml] and penicillin [50 IU/ml] and stored at -70°C until required.

Like-tissue samples collected at respective sampling points from the same group were pooled and ground up with sterile sand and 1.5 ml of tracheal organ culture medium (Minimum Essential Medium, sodium bicarbonate and 0.4% pen/strep) using a pestle and mortar. Homogenates were subjected to freeze-thaw three times then centrifuged at 3000 g for 15 min (Awad *et al.*, 2014). Supernatants were placed in sterile universal bottles and kept at -70°C until required for RT-PCR.

Detection of IBV by RT-PCR. RNA was extracted from both the swab and tissue samples using the phenol chloroform method (Chomczynski & Sacchi, 2006) and subjected to RT-PCR (Worthington *et al.*, 2008; Ganapathy *et al.*, 2015). In brief, 0.5 µl of extracted RNA was used to create cDNA during the reverse-transcriptase (RT) stage with primer SX2- (TCCACCTCTAAACACCC/T TT). Created cDNA was then amplified using a nested PCR with the following primers SX1+ (CACCTAGAGGTTTGT/CT A/T GCAT), SX2- [PCR 1] and SX3+ (TAATACTGGC/T AATTTTTTCAGA), SX4- (AATACAGATTGCTTACAACCACC) [PCR 2]. Reaction products were visualised on a 1.5% agarose gel using electrophoresis.

Positive amplicons were purified using 0.15 μ l Exonuclease 1 (EXO) and 0.99 μ l shrimp alkaline phosphatase (SAP) (Affymetrix Ltd, UK) at 37°C for 30 min, then at 80°C for 10 min to remove extraneous material. Purified products were sent for commercial sequencing (Source BioScience, Nottingham, UK) using the positive sense (SX3+) primer for analysis of the partial S1 gene.

All IBV vaccine strains used during the study were extracted, amplified and sequenced using the above methodology to allow for comparison of redetected strains to the original vaccination strain. Our RT-PCR assay detection limits of the vaccines used in this study ranged from \log_{10} 1.4 TCID₅₀/ml to \log_{10} 2.75 TCID₅₀/ml.

Nucleotide comparison of partial S1 gene sequences within detected vaccine strains.

Sequence data was initially analysed in ChromasPRO v1.7.3 (<http://technelysium.com.au/>) and BLAST searches were conducted to confirm isolate identification. Alignments were carried out in MEGA6 (Tamura *et al.*, 2013), using Clustal W (Thompson *et al.*, 1994). Retrieved IBV sequences were identified by comparison against the original vaccine sequence and BLAST (National Centre of Biotechnology Information).

Single nucleotide polymorphism (SNP) and indel detection was carried out in MEGA6 following alignment to sequenced vaccine strains. SNPs were defined as a nucleotide variation when compared to the original vaccine strain which did not alter the sequence length. SNPs were characterised as non-synonymous (ns) if they led to an amino acid change and synonymous (if they led to no amino acid changes). Indels were defined as the insertion or deletion of a nucleotide that altered the sequence length. The d_s/d_{ns} ratio was calculated for each group using the Nei-Gojobori method (Nei & Kumar, 2000), where <1.0 indicates the recovered strains to be under positive selective pressure (Fitch *et al.*, 1991). Positive selection was considered significant at $p < 0.05$. Translated amino acid variations

were also identified and variations that resulted in a change in hydrophobicity were noted according to the Kyte and Doolittle scale (Kyte & Doolittle, 1982).

RESULTS

RNA from a total of 138 isolates was extracted from tissue samples (n=74) and swabs (n=64). Following RT-PCR, interpretable sequence data for the partial S1 gene was obtained for 118 (85.51%) of them. Results from BLAST searches confirmed strain identity as the same strains as were used for initial vaccination of the birds, with homology ranging from 89% to 100% (Table 1). IBV strains were recovered from a greater percentage of swab samples compared to the tissue samples at each of the sampling points (Figure 1).

Tissues. The single Mass₁ vaccine strain was detected at later stages (10 dpv onwards) and persisted in both the caecal tonsil and kidney up to 25 dpv. However, when the combined Mass₁+793B₁ vaccine was given (Group 4), Mass₁ was only recovered in the trachea on one occasion (10 dpv), whereas the 793B-type strain had distribution within all tissues. For Group 3 (Mass₁+D274), Mass₁ was only detected in the trachea and caecal tonsils from 10 dpv. The D274-type strain was not detected at any time point. For the single Mass₂ vaccination, the vaccine strain was detected at 3 dpv in the duodenum and from 10 dpv in other tissues. In Group 5, where Mass₂ was given in combination with 793B₂, it was not detected at any time points. In contrast, the 793B vaccine strain was detected from 3 to 18 dpv in all sampled tissues, and only in the trachea at 25 dpv. For Group 6 (Mass₃+Ark), the Mass₃ strain was only detected on one occasion at 3 dpv in the trachea, however the Ark-type strain persisted for longer in the caecal tonsils and kidney (up to 14 dpv) (Table 1).

Swabs. In the single Mass₁ group, the OP swabs were positive from 3 to 18 dpv compared to 6-14 dpv for the CL swabs. In the Mass₁+793B₁ group, Mass₁ was only detected up to 10 dpv in the OP and CL swabs and thereafter only 793B was detected. In the Mass₁+D274 group, only Mass₁ was detected in the OP and CL swabs, with D274 not being detected at any sampling time. For the single Mass₂ inoculated birds, other than 24 dpv for OP and 3

dpv for CL, the strain was consistently detected in both swab locations. In contrast, when Mass₂ was simultaneously given alongside 793B₂, it was only detected at 3 dpv in the OP and CL swabs, and at the remaining sampling intervals only 793B₂ was detected. In the birds where Mass₃+Ark was given, the Mass₃ strain was only detected at 3 dpv in the OP swab and Ark was detected from OP and CL from 10 and 14 dpv respectively (Table 1).

SNP identification. Following alignment to the relevant vaccine sequence, SNPs were identified within the 118 isolates. A total of 28 isolates (tissue = 15 and swabs = 13) contained at least one SNP present within their sequence, with the swab isolates having a greater number of SNPs overall (83 compared to 23 in the tissue isolates) (Table 2). Average SNP counts were higher in the swab isolates with 1.57 per sample compared to 0.35 per sample in the tissue.

SNP and amino acid variation analysis. We identified 114 nucleotide variations from the 36 isolates containing at least one SNP (3.12 per sample). This translated into a total of 64 amino acid changes (1.78 per sample) from the original vaccine strain. The majority of amino acid changes (n=56) occurred in Groups 5 and 6, with the remaining eight in Groups 1 and 2 (three and five samples respectively). Groups 3 contained only a single SNP and no amino acid variations. Group 4 contained no SNPs when compared to the original inoculated strain.

Virus strains recovered from cloacal and kidney tissue demonstrated a greater number of non-synonymous SNPs (7 and 5 respectively) when compared to trachea and duodenum tissue isolates (one in both). From the same tissue samples, a greater number of SNPs were witnessed after 10 dpv. Interestingly, within Group 6 (Ark-type), all SNPs were non-synonymous (n=10) and at the same position (976; thymine to adenine) (Figure 2). This resulted in an amino acid change of tyrosine to asparagine; however this did not cause a change in hydrophobicity.

On average, OP swab samples contained a lower total number of SNPs than the CL swabs (37 compared to 46). However, OP samples had a d_s/d_{ns} ratio of 1.42, whereas CL

sequences had a ratio of 0.92. In contrast, the d_s/d_{ns} ratio for SNPs detected within tissue samples was 1.03. Overall, both swab and tissue samples showed no evidence of selective pressures ($p > 0.05$).

Group 6 (Mass₃+Ark) was the only group to contain a deletion. Of the 17 sequenced samples within the vaccine group (both tissue and swabs), 14 contained the same codon deletion (AAT) at position 1033-1035. This deletion removed a single amino acid (asparagine), and with no insertions, resulted in an overall smaller S1 protein.

Two isolates from Group 5 at 3 dpv contained a much higher SNP count of 27 and 30 (15 and 17 non-synonymous variations respectively). Furthermore, both isolates shared a high sequence similarity with each other, with only three nucleotide variations.

Hydrophobicity changes. Despite there being 64 amino acid changes, only six (9.38%) of them resulted in a change of hydrophobicity, at four locations within four isolates (Figure 2). All hydrophobicity changes occurred within OP (n=2) and cloacal (n=4) swab samples and between amino acid positions 293-334. Of the four locations, two resulted in a switch from hydrophilic to hydrophobic whilst the other two were hydrophobic to hydrophilic. Five changes occurred in Group 5; the other in Group 2. However, despite the two samples within Group 5 being Mass₂-like, the changes occurred at different locations when compared to the single Mass₂ strain (Group 2).

DISCUSSION

In a previous publication, based on findings from the same experimental birds, we have reported on the clinical signs, ciliary health, immune responses and protection against virulent IBVs (Awad, *et al.*, 2016). Due to the increasing use of heterologous vaccination of day-old chicks, either in hatcheries or farms, we set out to identify the presence of nucleotide variation within the hyper-variable region of S1 in IBV vaccine virus strains following

vaccination of day-old commercial broiler chicks. After the live vaccination, samples collected at intervals were analysed for the distribution of vaccine viruses in swabs and tissues. IBV positive samples were sequenced and analysed for SNPs, and we also analysed the downstream effects on the hydrophobic properties of amino acid variations. It has previously been suggested that genomic changes within viruses during infection can aid with evading the host immune system (Alcami & Koszinowski, 2000), particularly those resulting in a change to the antigenic properties of a protein. Further to this, the hyper-variable genetic regions of the S1 subunit have previously been well characterised (Cavanagh, *et al.*, 1988; Cavanagh *et al.*, 1997).

In comparison to Awad *et al.* (2016), additional groups of chickens given a single Mass (Mass₁ or Mass₂) vaccination were included to allow for comparison to those groups that received co-vaccination with Mass₁+793B₁ or Mass₂+793B₂. Our results indicate that Mass strains can be detected in tissues at an early stage (3 dpv), but only establish themselves by 10 dpv when given individually. Redetection can persist to at least 25 dpv. However, when given in combination, only the 793B strains were identified at later time points (from 14 dpv), suggesting an increased ability for the 793B strains to replicate at a greater rate over the Mass strains within the same host. It was interesting to note that both 793B vaccine strains were detected at all sampling times throughout the experiment, demonstrating the persistence of this genotype. Though a single 793B vaccination group was not included due to space limitation, previous studies have demonstrated the ability of this serotype to persist in broiler chicks until at least 20 days post infection when given alone (Boroomand *et al.*, 2012). In a separate experiment at Liverpool, we recovered 793B vaccine strains from the trachea, lungs, caecal tonsil and kidneys of broiler chicks up to the end of the experiment (42 days old) (K Ganapathy, unpublished data).

Strain persistence over time may also increase the likelihood of mutations occurring, potentially leading to substantial antigenic alterations over time (McKinley *et al.*, 2011). Furthermore, inoculation of multiple IBV serotypes within the same host or system increases

the chances of recombination events occurring between the strains (Jia *et al.*, 1995). Despite this, we witnessed between 99-100% homology with the original vaccine strain for all recovered 793B strains. This limited variation is in contrast with strains previously identified in commercial poultry samples, where greater variations in vaccine strains were witnessed (Ganapathy, *et al.*, 2015). The reason for such differences is not known, however previous reports have highlighted that the complex interactions between IBV, host, environment and co-infections in the field could have contributed to these differences (Wickramasinghe *et al.*, 2011; Sid *et al.*, 2015).

Strains recovered from swab samples in all groups (n=53) contained an average of 1.57 SNPs per sample. This was mainly the result of the presence of two Mass-like strains in Group 5 (Mass₂+793B₂) which contained high SNP counts (57 in total). Interestingly, both recovered Mass-like strains were from swab samples collected at the same time (3 dpv) and shared a high homology (98.91%), despite both only being around 90% similar to the original Mass₂ vaccine strain. Furthermore, no similar strains were recovered from tissue samples or at later time points, suggesting that the changes were limited to a subpopulation present at an early stage of vaccination. Such findings agree with previous work which demonstrated that when variant subpopulations appear in experimentally infected chickens, it is the original infected strain that persists over time, rather than the variations (Toro *et al.*, 2012).

At no time did we detect the D274 strain in the birds vaccinated with Mass₁+D274. This could again be explained by a higher replication of the Mass₁ strain compared to D274, as growth rates have previously been shown to differ between IBV serotypes (Darbyshire *et al.*, 1978; Mardani *et al.*, 2008). In addition, viral titres of Mass and D274 within a commercial combined vaccine have been shown to differ following co-inoculation of embryonated eggs (Geerligs *et al.*, 2013). Alternatively, it may be due to a greater presence and neutralization of MDA specific for the D274 strain (Awad, *et al.*, 2016). Given that D274 was not detected by RT-PCR, haemagglutination inhibition testing was carried out on all serum samples, including those collected at day 0, to confirm the presence of D274-specific antibodies

following vaccination (data not shown). An antibody response to the D274-specific antigen (average titre $<2.0 \log_2$) was seen at all times tested.

All tissue and swab samples from Group 6 (Mass₃+Ark) were identified as Ark-like (n=16) and had the same SNP change at position 976 (thymine to adenine), altering the amino acid from tyrosine to asparagine. With such a variation being common place within Ark-type isolates, it would seem to confer a selective advantage to this serotype (Lauring *et al.*, 2013). Fourteen of the samples (87.5%) also contained the same three nucleotide deletion (AAT) at position 1033-1035. Previous studies have also witnessed the same variations within Ark-type vaccine strains from SPF chicks (McKinley, *et al.*, 2008; van Santen & Toro, 2008). As the deletion was identified in the caecal tonsil as early as 3 dpv, it seems that a subpopulation within the Arkansas vaccine was able to survive and propagate following vaccination more readily, when compared to the major population as initially detected by Sanger sequencing. While other studies focused on genetic changes in the trachea and Harderian gland (McKinley, *et al.*, 2008; van Santen & Toro, 2008), interestingly, we discovered that the codon deletion did not occur in Ark-like samples recovered from the duodenum. However the reason for this remains unknown. A thorough analysis of Ark-type vaccine viruses in oropharyngeal and cloacal swabs, and in various tissues over a longer period of time may provide further information for this variable genetic change.

Interestingly, there were no amino acid variations within recovered strains from either Group 3 (Mass₁+D274) or 4 (Mass₁+793B₁). It was only those identified as Mass₁-like that contained synonymous SNPs. In all twelve strains that were identified as 793B₁-like, SNPs were entirely absent, suggesting a somewhat stable genome for this 793B₁-like strain.

Previous work by Cavanagh *et al.*, (2005) reported that the majority of SNPs occurring in a number of 793B strains were synonymous following passage in embryonated eggs and chickens, and did not cause a change in strain pathogenicity (Cavanagh *et al.*, 2005). These findings echo the results from this study where the birds were kept under experimental conditions. Conversely, Ganapathy *et al.* (2015) reported that extensive nucleotide changes

were witnessed within the same S1 portion for 793B-like viruses detected within commercial chickens. However it is not known if such changes altered pathogenicity (Ganapathy, *et al.*, 2015).

Previous work analysed the d_s/d_{ns} ratios for partial S1 sequences of 793B, Mass and D274 (Cavanagh, *et al.*, 2005) and identified that the nucleotide variations were not subject to selection pressures. Our experimental vaccination data shows an agreement, as even though the overall majority (64/106; 60.38%) of SNPs within recovered IBV strains resulted in an amino acid sequence change, the average d_s/d_{ns} ratio was 1.12. Previous studies have indicated that IBV strains isolated from commercial birds may be under selective pressures (Mo *et al.*, 2013; Moreno *et al.*, 2016). Positive selection pressure may go some way to further our understanding of emerging strain variants. Should the variant strains persist, this nucleotide variability also demonstrates the potential for greater hydrophobicity changes in the IBV genome over time.

As the hydrophobic properties of a peptide chain will affect the folding of the translated protein structure (Dill, 1990), we investigated hypothetical changes in hydrophobic properties arising from nucleotide (and subsequently amino acid) alterations in the partial S1 sequence. Since the spike glycoprotein is involved with host cell attachment and thought to be a causative reasoning behind strain variants (Jackwood *et al.*, 2012), it is possible that minor changes could affect the overall function, including the induction of neutralizing and haemagglutination inhibiting antibodies. Despite all hydrophobicity changes occurring between positions 293-334, those present in the Mass₂ samples in Group 5 were at a separate location from the change in Mass₂ given alone (Group 2). As the only difference was the presence of a second strain at the time of inoculation, the additional vaccine strain (793B₂) may have influenced the mutation rate of the Mass₂ virus (Jia, *et al.*, 1995; McKinley, *et al.*, 2008). As no Mass₂ hydrophobic changes persisted past 14 dpv, it appears that this could have provided some advantages for replication and persistence of this virus up to, but not beyond, 14 dpv.

The Sanger based sequencing approach utilised for this study generates sequence data based on the majority population within recovered isolates. As a result minor populations of either the same serotype, or different serotypes, would be neglected. During analysis minor peaks were only observed for a small number of recovered strains, however as the study included combined vaccine groups, a sequencing approach which takes this factor into consideration (such as next generation sequencing) may have identified the presence of other sub-populations.

Findings from this first comprehensive study on the effects of live IBV vaccine viruses, administered either singly or dually in day-old commercial broiler chicks, demonstrated that the majority of SNPs detected within recovered IBV vaccine strains lead to amino acid changes. Most such changes occur at an early stage of vaccination (less than 7 dpv), rather than at later ages. All detected hydrophobic changes occurred solely in OP and CL swabs. In IBV-vaccinated commercial broiler chicks with IBV maternal antibodies, it appears that the extent of part-S1 amino acid, and resulting hydrophobic changes, were limited.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

1 REFERENCES

2 Alcami, A. & Koszinowski, U. H. (2000). Viral mechanisms of immune evasion. *Immunology Today*, 21,
3 447-455.

4
5 Ammayappan, A. & Vakharia, V. N. (2009). Complete nucleotide analysis of the structural genome of
6 the infectious bronchitis virus strain md27 reveals its mosaic nature. *Viruses*, 1, 1166-1177.

7
8 Awad, F., Baylis, M., Jones, R. C. & Ganapathy, K. (2014). Evaluation of Flinders Technology
9 Associates cards for storage and molecular detection of avian metapneumoviruses. *Avian*
10 *Pathology*, 43, 125-129.

11
12 Awad, F., Hutton, S., Forrester, A., Baylis, M. & Ganapathy, K. (2016). Heterologous Live IBV
13 Vaccination in Day-Old Commercial Broiler Chicks: clinical Signs, Ciliary Health, Immune
14 Responses and Protection Against Variant IBVS. *Avian Pathology*, 1-29.

15
16 Bijlenga, G., Cook, J. K. A., Gelb, J., Jr. & de Wit, J. J. (2004). Development and use of the H strain of
17 avian infectious bronchitis virus from the Netherlands as a vaccine: a review. *Avian*
18 *Pathology*, 33, 550-557.

19
20 Boroomand, Z., Asasi, K. & Mohammadi, A. (2012). Pathogenesis and tissue distribution of avian
21 infectious bronchitis virus isolate IRFIBV32 (793/B serotype) in experimentally infected
22 broiler chickens. *Scientific World Journal*.

23
24 Cavanagh, D. (2005). Coronaviruses in poultry and other birds. *Avian Pathology*, 34, 439-448.

25
26 Cavanagh, D., Davis, P. J., Cook, J. K. A., Li, D., Kant, A. & Koch, G. (1992). Location of the amino acid
27 differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious
28 bronchitis virus. *Avian Pathology*, 21, 33-43.

29
30 Cavanagh, D., Davis, P. J. & Mockett, A. P. A. (1988). Amino acids within hypervariable region 1 of
31 avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with
32 neutralization epitopes. *Virus Research*, 11, 141-150.

33
34 Cavanagh, D., Ellis, M. M. & Cook, J. K. A. (1997). Relationship between sequence variation in the S1
35 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. *Avian*
36 *Pathology*, 26, 63-74.

37
38 Cavanagh, D. & Gelb, J., Jr. (2008). Infectious bronchitis. In Y. M. Saif, A.M. Fadly, J.R. Glisson , L.R.
39 McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry* (12th ed., pp. 117-133).
40 Ames: Blackwell Publishing.

41

42 Cavanagh, D., Picault, J. P., Gough, R., Hess, M., Mawditt, K. & Britton, P. (2005). Variation in the
43 spike protein of the 793/B type of infectious bronchitis virus, in the field and during
44 alternate passage in chickens and embryonated eggs. *Avian Pathology*, 34, 20-25.

45

46 Chhabra, R., Forrester, A., Lemiere, S., Awad, F., Chantrey, J. & Ganapathy, K. (2015). Mucosal,
47 Cellular, and Humoral Immune Responses Induced by Different Live Infectious Bronchitis
48 Virus Vaccination Regimes and Protection Conferred against Infectious Bronchitis Virus Q1
49 Strain. *Clinical and Vaccine Immunology*, 22, 1050-1059.

50

51 Chomczynski, P. & Sacchi, N. (2006). The single-step method of RNA isolation by acid guanidinium
52 thiocyanate-phenol-chloroform extraction: twenty-something years on.
53 [10.1038/nprot.2006.83]. *Nature Protocols*, 1, 581-585.

54

55 Cook, J. K. A., Orbell, S. J., Woods, M. A. & Huggins, M. B. (1999). Breadth of protection of the
56 respiratory tract provided by different live-attenuated infectious bronchitis vaccines against
57 challenge with infectious bronchitis viruses of heterologous serotypes. *Avian Pathology*, 28,
58 477 - 485.

59

60 Darbyshire, J. H., Cook, J. K. A. & Peters, R. W. (1978). Growth comparisons of avian infectious
61 bronchitis virus strains in organ cultures of chicken tissues. *Archives of Virology*, 56, 317-325.

62

63 Dawson, P. S. & Gough, R. E. (1971). Antigenic variation in strains of avian infectious bronchitis virus.
64 *Archiv fur die Gesamte Virusforsch*, 34, 32-39.

65

66 de Wit, J. J., Cook, J. K. A. & van der Heijden, H. M. (2011). Infectious bronchitis virus variants: a
67 review of the history, current situation and control measures. *Avian Pathology*, 40, 223-235.

68

69 Dill, K. A. (1990). Dominant forces in protein folding. *Biochemistry*, 29, 7133-7155.

70

71 Fields, D. B. (1973). Arkansas 99, a new infectious bronchitis serotype. *Avian Diseases*, 17, 659-661.

72

73 Fitch, W. M., Leiter, J. M., Li, X. Q. & Palese, P. (1991). Positive Darwinian evolution in human
74 influenza A viruses. *Proceedings of the National Academy of Sciences of the United States of*
75 *America*, 88, 4270-4274.

76

77 Fraga, A. P., Balestrin, E., Ikuta, N., Fonseca, A. S., Spilki, F. R., Canal, C. W. & Lunge, V. R. (2013).
78 Emergence of a new genotype of avian infectious bronchitis virus in Brazil. *Avian Diseases*,
79 57, 225-232.

80

81 Ganapathy, K., Ball, C. & Forrester, A. (2015). Genotypes of infectious bronchitis viruses circulating in
82 the Middle East between 2009 and 2014. *Virus Research*, 210, 198-204.

83

84 Geerligs, H. J., Meinders, C. A., Snel, J. & Duyves, W. (2013). The use of RT-PCR for determination of
85 separate end-points for the strains IB H120 and IB D274 in titration of the combination
86 vaccine Poulvac IB(R) primer. *Journal of Virological Methods*, 193, 508-511.

87

88 Gelb, J., Jr. & Cloud, S. S. (1983). Effect of serial embryo passage of an Arkansas-type avian infectious
89 bronchitis virus isolate on clinical response, virus recovery, and immunity. *Avian Diseases*,
90 27, 679-687.

91

92 Gonzalez, J. M., Gomez-Puertas, P., Cavanagh, D., Gorbalenya, A. E. & Enjuanes, L. (2003). A
93 comparative sequence analysis to revise the current taxonomy of the family Coronaviridae.
94 *Archives of Virology*, 148, 2207-2235.

95

96 Gough, R. E., Randall, C. J., Dagless, M., Alexander, D. J., Cox, W. J. & Pearson, D. (1992). A new strain
97 of infectious bronchitis virus infecting domestic fowl in Great Britain. *Veterinary Record*, 130,
98 493 - 494.

99

100 Han, Z., Sun, C., Yan, B., Zhang, X., Wang, Y., Li, C., Zhang, Q., Ma, Y., Shao, Y., Liu, Q., Kong, X. & Liu,
101 S. (2011). A 15-year analysis of molecular epidemiology of avian infectious bronchitis
102 coronavirus in China. *Infection, Genetics and Evolution*, 11, 190-200.

103

104 Ignjatovic, J. & Galli, L. (1994). The S1 glycoprotein but not the N or M proteins of avian infectious
105 bronchitis virus induces protection in vaccinated chickens. *Archives of Virology*, 138, 117-
106 134.

107

108 Jackwood, M. W. & de Wit, J. J. (2013). *Infectious Bronchitis* (13th ed.). Ames: Wiley-Blackwell.

109

110 Jackwood, M. W., Hall, D. & Handel, A. (2012). Molecular evolution and emergence of avian
111 gammacoronaviruses. *Infection, Genetics and Evolution*, 12, 1305-1311.

112

113 Jia, W., Karaca, K., Parrish, C. R. & Naqi, S. A. (1995). A novel variant of avian infectious bronchitis
114 virus resulting from recombination among three different strains. *Archives of Virology*, 140,
115 259-271.

116

117 Jones, R. C. (2010). Europe: History, Current Situation and Control Measures for Infectious
118 Bronchitis. *Brazilian Journal of Poultry Science*, 12, 125-128.

119

120 Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a
121 protein. *Journal of Molecular Biology*, 157, 105-132.

122

123 Lauring, A. S., Frydman, J. & Andino, R. (2013). The role of mutational robustness in RNA virus
124 evolution. *Nature Reviews. Microbiology*, 11, 327-336.

125

126 Mardani, K., Noormohammadi, A. H., Hooper, P., Ignjatovic, J. & Browning, G. F. (2008). Infectious
127 bronchitis viruses with a novel genomic organization. *Journal of Virology*, 82, 2013-2024.

128

129 McKinley, E. T., Hilt, D. A. & Jackwood, M. W. (2008). Avian coronavirus infectious bronchitis
130 attenuated live vaccines undergo selection of subpopulations and mutations following
131 vaccination. *Vaccine*, 26, 1274-1284.

132

133 McKinley, E. T., Jackwood, M. W., Hilt, D. A., Kissinger, J. C., Robertson, J. S., Lemke, C. & Paterson, A.
134 H. (2011). Attenuated live vaccine usage affects accurate measures of virus diversity and
135 mutation rates in avian coronavirus infectious bronchitis virus. *Virus Research*, 158, 225-234.

136

137 Mo, M. L., Li, M., Huang, B. C., Fan, W. S., Wei, P., Wei, T. C., Cheng, Q. Y., Wei, Z. J. & Lang, Y. H.
138 (2013). Molecular characterization of major structural protein genes of avian coronavirus
139 infectious bronchitis virus isolates in southern china. *Viruses*, 5, 3007-3020.

140

141 Moreno, A., Franzo, G., Massi, P., Tosi, G., Blanco, A., Antilles, N., Biarnes, M., Majo, N., Nofrarias,
142 M., Dolz, R., Lelli, D., Sozzi, E., Lavazza, A. & Cecchinato, M. (2016). A novel variant of the
143 infectious bronchitis virus resulting from recombination events in Italy and Spain. *Avian
144 Pathology*, 1-8.

145

146 Nei, M. & Kumar, S. (2000). *Molecular evolution and phylogenetics*: Oxford University Press.

147

148 Pejkovski, C., Davelaar, F. G. & Kouwenhoven, B. (1979). Immunosuppressive effect of infectious
149 bursal disease virus on vaccination against infectious bronchitis. *Avian Pathology*, 8, 95-106.

150

151 Sarueng, E., Wanasawaeng, W., Sasipreeyajan, J. & Chansiripornchai, N. (2014). Efficacy of Live
152 Infectious Bronchitis Vaccine Programs against Infection by QX-Like Strain of Infectious
153 Bronchitis Virus. *The Thai Journal of Veterinary Medicine*, 44, 187.

154

155 Sid, H., Benachour, K. & Rautenschlein, S. (2015). Co-infection with Multiple Respiratory Pathogens
156 Contributes to Increased Mortality Rates in Algerian Poultry Flocks. *Avian Diseases*, 59, 440-
157 446.

158

159 Studer, R. A., Dessailly, B. H. & Orengo, C. A. (2013). Residue mutations and their impact on protein
160 structure and function: detecting beneficial and pathogenic changes. *The Biochemical
161 Journal*, 449, 581-594.

162

163 Sumi, V., Singh, S. D., Dhama, K., Gowthaman, V., Barathidasan, R. & Sukumar, K. (2012). Isolation
164 and molecular characterization of infectious bronchitis virus from recent outbreaks in broiler
165 flocks reveals emergence of novel strain in India. *Tropical Animal Health and Production*, 44,
166 1791-1795.

167

168 Sutou, S., Sato, S., Okabe, T., Nakai, M. & Sasaki, N. (1988). Cloning and sequencing of genes
169 encoding structural proteins of avian infectious bronchitis virus. *Virology*, 165, 589-595.

170

171 Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: Molecular Evolutionary
172 Genetics Analysis Version 6.0. *Molecular Biology and Evolution*, 30, 2725-2729.

173

174 Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of
175 progressive multiple sequence alignment through sequence weighting, position-specific gap
176 penalties and weight matrix choice. [Comparative Study]. *Nucleic Acids Research*, 22, 4673-
177 4680.

178

179 Toro, H., Pennington, D., Gallardo, R. A., van Santen, V. L., van Ginkel, F. W., Zhang, J. & Joiner, K. S.
180 (2012). Infectious bronchitis virus subpopulations in vaccinated chickens after challenge.
181 *Avian Diseases*, 56, 501-508.

182

183 van Santen, V. L. & Toro, H. (2008). Rapid selection in chickens of subpopulations within ArkDPI-
184 derived infectious bronchitis virus vaccines. *Avian Pathology*, 37, 293-306.

185

186 Villarreal, L. Y., Sandri, T. L., Souza, S. P., Richtzenhain, L. J., de Wit, J. J. & Brandao, P. E. (2010).
187 Molecular epidemiology of avian infectious bronchitis in Brazil from 2007 to 2008 in
188 breeders, broilers, and layers. *Avian Diseases*, 54, 894-898.

189

190 Wickramasinghe, I. N., de Vries, R. P., Grone, A., de Haan, C. A. & Verheije, M. H. (2011). Binding of
191 avian coronavirus spike proteins to host factors reflects virus tropism and pathogenicity.
192 *Journal of Virology*, 85, 8903-8912.

193

194 Winterfield, R. W., Hoerr, F. J. & Fadly, A. M. (1978). Vaccination against infectious bronchitis and
195 the immunosuppressive effects of infectious bursal disease. *Poultry Science*, 57, 386-391.

196

197 Worthington, K. J., Currie, R. J. W. & Jones, R. C. (2008). A reverse transcriptase polymerase chain
198 reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to
199 2006. *Avian Pathology*, 37, 247 - 257.

200

201 Zhang, Y., Wang, H. N., Wang, T., Fan, W. Q., Zhang, A. Y., Wei, K., Tian, G. B. & Yang, X. (2010).
202 Complete genome sequence and recombination analysis of infectious bronchitis virus
203 attenuated vaccine strain H120. *Virus Genes*, 41, 377-388.

206 **Table 1.** Genotype identity of recovered IBV strains as determined by BLAST.

Vaccine group	Sample from ^a	Day post vaccination					
		3	6	10	14	18	25
Group 1 (Mass ₁)	TR	-	-	-	Mass ₁ (99%)	Mass ₁ (99%)	-
	DU	-	-	-	Mass ₁ (99%)	-	-
	CT	-	-	Mass ₁ (99%)	Mass ₁ (99%)	Mass ₁ (99%)	Mass ₁ (98%)
	KID	-	-	-	-	-	-
	OP	Mass ₁ (100%)	Mass ₁ (100%)	Mass ₁ (100%)	Mass ₁ (100%)	Mass ₁ (99%)	-
	CL	-	Mass ₁ (99%)	Mass ₁ (100%)	Mass ₁ (100%)	-	-
Group 2 (Mass ₂)	TR	-	-	Mass ₂ (99%)	-	-	-
	DU	Mass ₂ (99%)	-	Mass ₂ (99%)	-	-	-
	CT	-	-	Mass ₂ (99%)	Mass ₂ (99%)	Mass ₂ (99%)	Mass ₂ (99%)
	KID	-	-	-	Mass ₂ (99%)	-	Mass ₂ (99%)
	OP	Mass ₂ (100%)	Mass ₂ (100%)	Mass ₂ (100%)	Mass ₂ (100%)	Mass ₂ (100%)	-
	CL	-	Mass ₂ (100%)	Mass ₂ (100%)	Mass ₂ (98%)	Mass ₂ (100%)	Mass ₂ (99%)
Group 3 (Mass ₁ +D274)	TR	-	-	Mass ₁ (99%)	Mass ₁ (100%)	Mass ₁ (99%)	-
	DU	-	-	-	-	-	-
	CT	-	-	-	Mass ₁ (99%)	-	Mass ₁ (99%)
	KID	-	-	-	-	-	-
	OP	Mass ₁ (100%)	Mass ₁ (100%)	Mass ₁ (100%)	Mass ₁ (100%)	Mass ₁ (100%)	-
	CL	-	Mass ₁ (100%)	Mass ₁ (100%)	-	Mass ₁ (100%)	-
Group 4 (Mass ₁ +793B ₁)	TR	-	-	Mass ₁ (99%)	-	793B ₁ (100%)	793B ₁ (100%)
	DU	-	793B ₁ (100%)	-	793B ₁ (99%)	793B ₁ (100%)	-
	CT	-	793B ₁ (100%)	793B ₁ (100%)	793B ₁ (100%)	793B ₁ (100%)	793B ₁ (100%)
	KID	-	-	793B ₁ (99%)	-	-	-
	OP	Mass ₁ (100%)	Mass ₁ (100%)	Mass ₁ (100%)	793B ₁ (100%)	793B ₁ (100%)	793B ₁ (100%)
	CL	Mass ₁ (100%)	793B ₁ (100%)	-	793B ₁ (100%)	793B ₁ (100%)	793B ₁ (100%)
Group 5 (Mass ₂ +793B ₂)	TR	793B ₂ (100%)	793B ₂ (100%)	793B ₂ (100%)	793B ₂ (100%)	-	793B ₂ (100%)
	DU	793B ₂ (100%)	793B ₂ (100%)	793B ₂ (100%)	793B ₂ (100%)	793B ₂ (100%)	-
	CT	793B ₂ (100%)	793B ₂ (100%)	793B ₂ (100%)	793B ₂ (100%)	793B ₂ (100%)	-
	KID	793B ₂ (100%)	793B ₂ (100%)	793B ₂ (100%)	793B ₂ (100%)	793B ₂ (100%)	-
	OP	Mass ₂ (90%)	-	-	793B ₂ (100%)	-	793B ₂ (100%)
	CL	Mass ₂ (89%)	793B ₂ (100%)	793B ₂ (99%)	793B ₂ (100%)	793B ₂ (100%)	793B ₂ (100%)
Group 6 (Mass ₃ +Ark)	TR	Mass ₃ (100%)	-	Ark (100%)	-	-	-
	DU	-	-	Ark (99%)	-	-	-
	CT	Ark (99%)	-	Ark (100%)	Ark (99%)	-	-
	KID	-	Ark (99%)	Ark (99%)	Ark (99%)	-	-
	OP	Mass ₃ (100%)	-	Ark (99%)	Ark (99%)	Ark (99%)	Ark (99%)
	CL	-	-	-	Ark (99%)	Ark (97%)	Ark (95%)

207

208 Brackets indicate homology to vaccine strain.

209 ^aTR -Trachea;

210 DU -Duodenum

- 211 *CT -Caecal tonsil*
- 212 *KID - Kidney*
- 213 *OP - Oropharyngeal swab*
- 214 *CL - Cloacal swab*
- 215
- 216

ACCEPTED MANUSCRIPT

217 **Table 2.** Summary of SNP data from recovered isolates, including non-synonymous SNPs and
 218 relevant hydrophobicity changes.

219

		Total Sample Number	Samples Containing ≥ 1 SNP	Number of SNPs		Deletions	Total Amino Acid Changes	Hydrophobicity Changes	
				Synonymous	Non-synonymous				
A	Location	TR ^a	15	3	4	1	3	2	0
		DU ^b	13	2	1	1	0	1	0
		CT ^c	23	5	1	7	12	11	0
		KID ^d	14	5	3	5	12	9	0
	Days post vaccination	3	6	1	0	1	3	2	0
		6	7	1	0	1	3	2	0
		10	16	5	1	4	9	7	0
		14	16	4	4	3	6	5	0
		18	11	3	3	2	6	4	0
		25	9	1	1	3	0	3	0
	Vaccine group	Mass ₁	7	2	2	3	0	3	0
		Mass ₂	9	1	3	1	0	1	0
		Mass ₁ +D274	5+0	1+0	3+0	0+0	0+0	0+0	0+0
		Mass ₁ +793B ₁	1+12	1+0	1+0	0+0	0+0	0+0	0+0
		Mass ₂ +793B ₂	0+21	0+0	0+0	0+0	0+0	0+0	0+0
	Mass ₃ +Ark	0+10	0+10	0+0	0+10	0+27	0+19	0+0	
B	Location	OP ^e	29	7	14	23	12	22	2
		CL ^f	24	6	19	27	3	21	4
	Days post vaccination	3	8	3	27	35	0	24	4
		6	9	1	2	0	0	0	0
		10	9	2	1	2	3	3	1
		14	11	4	1	7	6	9	1
		18	10	2	2	5	3	5	0
		25	6	1	0	1	3	2	0
	Vaccine group	Mass ₁	8	1	2	0	0	0	0
		Mass ₂	10	1	1	4	0	4	1
		Mass ₁ +D274	8+0	0+0	0+0	0+0	0+0	0+0	0+0
		Mass ₁ +793B ₁	4+7	0+0	0+0	0+0	0+0	0+0	0+0
		Mass ₂ +793B ₂	2+7	2+2	25+1	32+2	0+0	24+3	4+1
		Mass ₃ +Ark	1+6	1+6	1+3	3+9	0+15	3+9	0+0

220 **A** Tissue samples

221 **B** Swab samples

222 ^aTR -Trachea

223 ^bDU - Duodenum

224 ^cCT - Caecal tonsil

225 ^dKID - Kidney
226 ^eOP - Oropharyngeal swab
227 ^fCL - Cloacal swab

228

229 **FIGURE LEGENDS**

230 **Figure 1.** IBV strain recovery rates given as a percentage for both swab (14 per time point)
231 and tissue (28 per time point) samples up to 25 days post vaccination.

232

233

234 **Figure 2.** Hydrophobicity changes occurring within each vaccinated group. Each sample is
235 identified by swab location, either oropharyngeal or cloacal and days post vaccination.

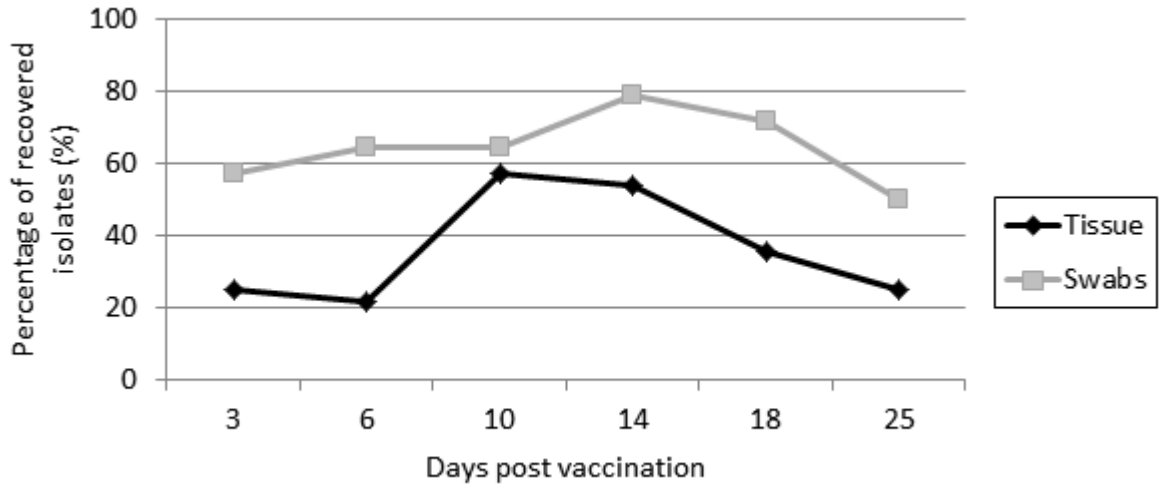
236 Amino acids coloured red are hydrophobic and blue are hydrophilic. Asterisks indicate the

237 position of a change in hydrophobicity. **A:** Mass₂-type strain used for vaccination, **B:**

238 Mass₂+793B₂ vaccine group (Mass₂-type samples) and **C:** Mass₂+793B₂ vaccine group

239 (793B₂-type samples).

240



241

A: Mass₂

Mass-type [GenBank](#)
Mass₂ Vaccine
OP 3 dpv
OP 6 dpv
CL 6 dpv
OP 10 dpv
CL 10 dpv
OP 14 dpv
CL 14 dpv
OP 18 dpv
CL 18 dpv
CL 25 dpv

263 *
VNTTFTLHNFTHHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
VNTTFTLHNFTHHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
VNTTFTLHNFTHHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
VNTTFTLHNFTHHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
VNTTFTLHNFTHHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
VNTTFTLHNFTHHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
VNTTFTLHNFTHHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
VNTTFTLHNFTHHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
VNTTFTLHNFTHHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
VNTTFTLHNFTHHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP

B: Mass₂+793B₂ (Mass₂)

Mass-type [GenBank](#)
Mass₂ Vaccine
OP 3 dpv
CL 3 dpv

263 * *
VNTTFTLHNFTHHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
VNTTFTLHNFTHHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
VNTTFLKLPNFTESNETDATPNPRGVTIQIYQYQTQTAQSGYYNFNFSFLSSFVYKESNYMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
VNTTFLKLPNFTESNETDATPNPRGVTIQIYQYQTQTAQSGYYNFNFSFLSSFVYKESNYMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP

C: Mass₂+793B₂ (793B₂)

793B-type [GenBank](#)
Mass₂+793B₂ Vaccine
CL 6 dpv
CL 10 dpv
OP 14 dpv
CL 14 dpv
CL 18 dpv
OP 25 dpv
CL 25 dpv

262 *
ESSTNTTLEL TNFTFTNVSASPNSGGVDTFQLYQHTAQDGYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
ESSTNTTLEL TNFTFTNVSASPNSGGVDTFQLYQHTAQDGYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
ESSTNTTLEL TNFTFTNVSASPNSGGVDTFQLYQHTAQDGYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
ESSTNTTLEL TNFTFTNVSASPNSGGVDTFQLYQHTAQDGYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
ESSTNTTLEL TNFTFTNVSASPNSGGVDTFQLYQHTAQDGYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
ESSTNTTLEL TNFTFTNVSASPNSGGVDTFQLYQHTAQDGYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
ESSTNTTLEL TNFTFTNVSASPNSGGVDTFQLYQHTAQDGYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
ESSTNTTLEL TNFTFTNVSASPNSGGVDTFQLYQHTAQDGYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
ESSTNTTLEL TNFTFTNVSASPNSGGVDTFQLYQHTAQDGYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC