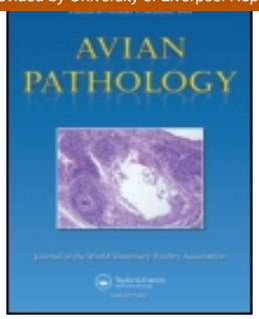

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Infectious bronchitis vaccine virus detection and part-S1 genetic variation following single or dual inoculation in broiler chicks

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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 $Mass_1$ and $Mass_2$, and the 793B vaccines are referred to as $793B_1$ and $793B_2$. These monovalent vaccines were produced by different manufacturers. Mixtures of $Mass_1$+$793B_1$ and of $Mass_2$+$793B_2$ were prepared as previously described (Awad*, et al.*, 2016). In addition, commercially combined live vaccines of $Mass_1$+D274 and $Mass_3$+Ark were also used (Awad*, et al.*, 2016). Vaccine titres (EID_{50}/chick) were as follows: $Mass_1$ (log_{10} 4.0), $Mass_2$ (log_{10} 4.1), $793B_1$ (log_{10} 4.75), $793B_2$ (log_{10} 4.4 , $Mass_1$+D274 (log_{10} 4.3 [3]) and $Mass_3$+Ark (log_{10} 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 oculo (50 µl) and nasal (50 µl) routes with one of the following: $Mass_1$ (Group 1), $Mass_2$ (Group 2), $Mass_1$+D274 (Group 3), $Mass_1$+$793B_1$ (Group 4) $Mass_2$+$793B_2$ (Group 5) $Mass_3$+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^{o}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^{o}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 AATTTTTCAGA), SX4− (AATACAGATTGCTTACAACCACC) [PCR 2]. Reaction products were visualised on a 1.5% agarose gel using electrophoresis.

Positive amplicons were purified using 0.15 µl Exonuclease 1 (EXO) and 0.99 µl 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 $TCID_{50}$/ml to $\log_{10}$ 2.75 $TCID_{50}$/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 $Mass_1$ 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 $Mass_1$+$793B_1$ vaccine was given (Group 4), $Mass_1$ was only recovered in the trachea on one occasion (10 dpv), whereas the 793B-type strain had distribution within all tissues. For Group 3 ($Mass_1$+D274), $Mass_1$ 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 $Mass_2$ vaccination, the vaccine strain was detected at 3 dpv in the duodenum and from 10 dpv in other tissues. In Group 5, where $Mass_2$ was given in combination with $793B_2$, 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 ($Mass_3$+Ark), the $Mass_3$ 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 $Mass_1$ group, the OP swabs were positive from 3 to 18 dpv compared to 6-14 dpv for the CL swabs. In the $Mass_1$+$793B_1$ group, $Mass_1$ was only detected up to 10 dpv in the OP and CL swabs and thereafter only 793B was detected. In the $Mass_1$+D274 group, only $Mass_1$ was detected in the OP and CL swabs, with D274 not being detected at any sampling time. For the single $Mass_2$ 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 $Mass_2$ was simultaneously given alongside $793B_2$, it was only detected at 3 dpv in the OP and CL swabs, and at the remaining sampling intervals only $793B_2$ was detected. In the birds where $Mass_3$+Ark was given, the $Mass_3$ 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 ($Mass_3$+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 $Mass_2$-like, the changes occurred at different locations when compared to the single $Mass_2$ 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 ($Mass_1$ or $Mass_2$) vaccination were included to allow for comparison to those groups that received co-vaccination with $Mass_1$+$793B_1$ or $Mass_2$+$793B_2$. 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 ($Mass_2$+$793B_2$) 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 $Mass_2$ 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 $Mass_1$+D274. This could again be explained by a higher replication of the $Mass_1$ 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 ($Mass_3$+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 ($Mass_1$+D274) or 4 ($Mass_1$+$793B_1$). It was only those identified as $Mass_1$-like that contained synonymous SNPs. In all twelve strains that were identified as $793B_1$-like, SNPs were entirely absent, suggesting a somewhat stable genome for this $793B_1$-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 haemagluttination inhibiting antibodies. Despite all hydrophobicity changes occurring between positions 293-334, those present in the $Mass_2$ samples in Group 5 were at a separate location from the change in $Mass_2$ given alone (Group 2). As the only difference was the presence of a second strain at the time of inoculation, the additional vaccine strain ($793B_2$) may have influenced the mutation rate of the $Mass_2$ virus (Jia*, et al.*, 1995; McKinley*, et al.*, 2008). As no $Mass_2$ 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.

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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 [a]*TR -Trachea;*

210 *DU -Duodenum*

211 *CT -Caecal tonsil*

212 *KID - Kidney*

213 *OP - Oropharyngeal swab*

214 *CL - Cloacal swab*

215

216

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

			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

A Tissue samples

B Swab samples

[a]TR -Trachea

[b]DU - Duodenum

[c]CT - Caecal tonsil

[d]KID - Kidney

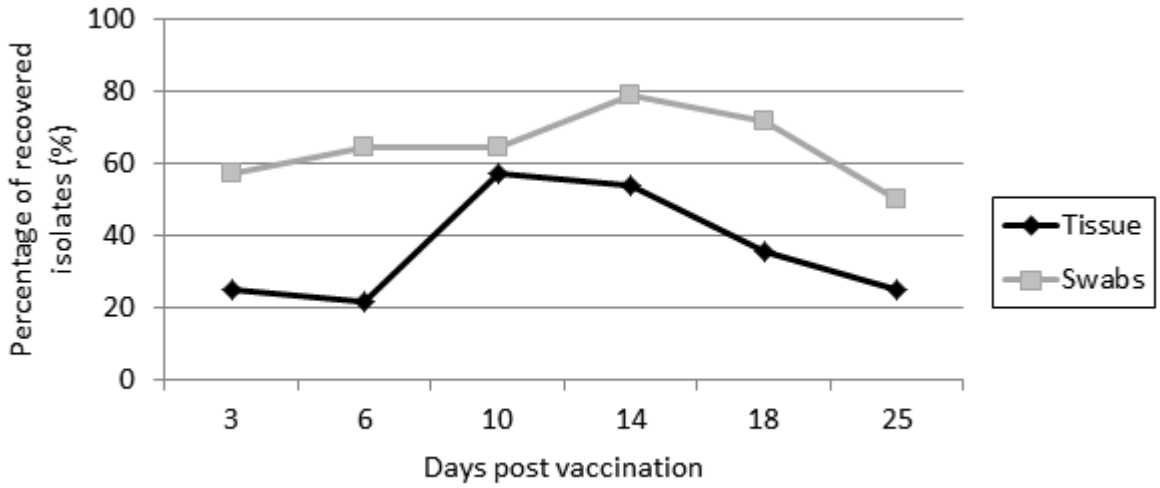
[e]OP - Oropharyngeal swab

[f]CL - Cloacal swab

FIGURE LEGENDS

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

Figure 2. Hydrophobicity changes occurring within each vaccinated group. Each sample is identified by swab location, either oropharyngeal of cloacal and days post vaccination. Amino acids coloured red are hydrophobic and blue are hydrophilic. Asterisks indicate the position of a change in hydrophobicity. **A:** $Mass_2$-type strain used for vaccination, **B:** $Mass_2$+$793B_2$ vaccine group ($Mass_2$-type samples) and **C:** $Mass_2$+$793B_2$ vaccine group ($793B_2$-type samples).



241

A: Mass$_2$

```
                          263                                                 *
Mass-type GenBank         VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
Mass$_2$ Vaccine          VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
OP 3 dpv                  VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
OP 6 dpv                  VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
CL 6 dpv                  VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
OP 10 dpv                 VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
CL 10 dpv                 VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
OP 14 dpv                 VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
CL 14 dpv                 VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRQKTINKGLGFNSLSVSIAYGP
OP 18 dpv                 VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
CL 18 dpv                 VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
CL 25 dpv                 VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
```

B: Mass$_2$+793B$_2$ (Mass$_2$)

```
                          263                 *                   *
Mass-type GenBank         VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
Mass$_2$ Vaccine          VNTTFTLHNFTFHNETGANPNPSGVQNIQTYQTQTAQSGYYNFNFSFLSSFVYKESNFMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
OP 3 dpv                  VNTTLKLPNFTFSNETDATPNPRGVQTIQIYQTQTAQSGYYNFNFSFLSSFVYKESNYMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
CL 3 dpv                  VNTTLKLPNFTFSNETDATPNPRGVQTIQLYQTQTAQSGYYNFNFSFLSSFVYKESNYMYGSYHPSCNFRLETINNGLWFNSLSVSIAYGP
```

C: Mass$_2$+793B$_2$ (793B$_2$)

```
                          262                         *
793B-type GenBank         ESSTNTTLELTNFTFTNVSNASPNSGGVDTFQLYQTHTAQDGYYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
Mass$_2$+793B$_2$ Vaccine ESSTNTTLELTNFTFTNVSNASPNSGGVDTFQLYQTHTAQDGYYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
CL 6 dpv                  ESSTNTTLELTNFTFTNVSNASPNSGGVDTFQLYQTHTAQDGYYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
CL 10 dpv                 ESSTNTTLELTNFTFTNVSNASPNSGGVDTFQLYQTHTAQDGYYNFNLSFLSIFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
OP 14 dpv                 ESSTNTTLELTNFTFTNVSNASPNSSGVDTFQLYQTHTAQDGYYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
CL 14 dpv                 ESSTNTTLELTNFTFTNVSNASPNSGGVDTFQLYQTHTAQDGYYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
CL 18 dpv                 ESSTNTTLELTNFTFTNVSNASPNSGGVDTFQLYQTHTAQDGYYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
OP 25 dpv                 ESSTNTTLELTNFTFTNVSNASPNSGGVDTFQLYQTHTAQDGYYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
CL 25 dpv                 ESSTNTTLELTNFTFTNVSNASPNSGGVDTFQLYQTHTAQDGYYNFNLSFLSSFVYKPSDFMYGSYHPNCNFRPENINNGLWFNSLSVSLTYGPIQGGC
```

242