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The presence of viral subpopulations in an infectious bronchitis virus vaccine with differing pathogenicity – A preliminary study

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ABSTRACT

There are currently four commercially available vaccines in Australia to protect chickens against infectious bronchitis virus (IBV). Predominantly, IBV causes clinical signs associated with respiratory or kidney disease, which subsequently cause an increase in mortality rate. Three of the current vaccines belong to the same subgroup (subgroup 1), however, the VicS vaccine has been reported to cause an increased vaccinal reaction compared to the other subgroup 1 vaccines. Molecular anomalies detected in VicS suggested the presence of two major subspecies, VicS-v and VicS-del, present in the commercial preparation of VicS. The most notable anomaly is the absence of a 40 bp sequence in the 3′UTR of VicS-del. In this investigation, the two subspecies were isolated and shown to grow independently and to similar titres in embryonated chicken eggs. An *in vivo* investigation involved 5 groups of 20 chickens each and found that VicS-del grew to a significantly lesser extent in the chicken tissues collected than did VicS-v. The group inoculated with an even ratio of the isolated subspecies scored the most severe clinical signs, with the longest duration. These results indicate the potential for a cooperative, instead of an expected competitive, relationship between VicS-v and VicS-del to infect a host, which is reminiscent of RNA viral quasi-species.

1. Introduction

Infectious bronchitis virus (IBV) causes respiratory, renal and/or reproductive disease in chicken flocks worldwide and causes major negative economic impacts. Depending on the strain of IBV involved, clinical signs may range from minor snicking and head shaking to coughing and gasping. Nephropathogenic strains of IBV can cause accumulation of urates in the tubules which causes polyurea and severe weight loss in affected chickens. In layer chickens, thin- and pale-shelled eggs with watery albumen are suspected to be related to IBV infection, however direct correlation of IBV with the presence of these eggs has been difficult to establish [1,2]. The differences in tropism and pathogenicity displayed by strains of IBV [3,4] have major implications for the effective control of IBV infection and development of vaccines with broad cross-protection.

Since the first description of IBV in the 1930s [5], the majority of efforts to curb the spread of IBVs have focused on the production of vaccines and the implementation of vaccination strategies [6–11]. Both live and inactivated vaccines have been produced and used successfully, with the strain of IBV used for vaccination reflecting the IBV strains prevalent in a particular country or region [12–14]. Inactivated vaccines have been shown to be effective as booster vaccines in layers and breeders only [15]. Live virus vaccines are effective, although their use carries the risk of reversion to virulence after repeated passage through chickens [16] and/or the acquisition of mutations during passage through embryonated chicken eggs [17]. Selection of an IBV strain for the production of an effective vaccine that induces broad cross-protection is complicated further by the differences in tropism and pathogenicity displayed by IBV strains [3,4].

In Australia, there are three prominent subgroups of IBV viruses, subgroups 1, 2, and 3, circulating in chicken flocks. Subgroup 1 strains comprise a large group of strains isolated between 1962 and 1984, including the subtype B vaccine strains [18], while subgroup 2 and 3 strains comprise variant IBVs that have emerged over the last 30 years [19,20]. There are four live virus vaccines available, namely vaccines A, VicS, I and S, and all but vaccine A are the same serotype (subtype B) [18]. The first Australian vaccine produced and widely used was the VicS vaccine [21], which has been used nationally since recognition of IBV in Australian chicken flocks in the 1960s [22–24]. VicS was developed from an Australian field strain of IBV that caused renal and respiratory disease in young chickens [20,25,26] and its structural protein gene region has been sequenced [27]. The pathogenicity of the

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VicS vaccine has previously been assessed in two-week-old chickens and it was found to cause a slight increase in mortalities as well as histopathological lesions in both the tracheal mucosa and kidneys [3]. It is also believed that under field conditions VicS causes a rather more severe respiratory vaccinal reaction than the other subtype B vaccines in young (less than two-weeks-old) chickens (Dr. Peter Groves; Dr Ben Wells; Dr Roger Chubb; Dr Peter Scott; personal communications). Although this perception has not been investigated as part of a published study, it has led to the limited use of the VicS vaccine in broiler chickens [21], which are not commonly grown past six weeks of age. However, there is widespread use of the VicS vaccine for older chickens, predominantly pullets and breeders during rear to optimise protection against IBV infection during lay, as well as a booster vaccine during the laying period.

Infectious bronchitis virus is a group III Coronavirus with a 27–32 kb single-stranded positive sense RNA genome which is arranged as 5′untranslated region (UTR) – 1a/1b – S1/S2 – 3 – M – 5 – N – 3′UTR and is transcribed *via* a nested set of subgenomic mRNAs. The structural protein gene region comprises all genes from the spike gene (S1/S2) downstream. The IBV S gene product is post-translationally cleaved into the S1 and S2 proteins, which provide mature IBV virions with a receptor binding subunit and a structural transmembrane domain, respectively [28,29]. One of the most antigenic proteins produced by IBV is the S1 protein, probably because of its location on the surface of the virion. The section of the S gene that encodes S1 is the most variable region of the entire IBV genome [30–33]. The M and N genes code for the membrane and nucleocapsid proteins respectively, and are each preceeded by genes 3 and 5, respectively, which code for non-structural accessory proteins [34]. Genes 3 and 5 are polycistronic and encode proteins 3a, 3b and E, and 5a and 5b respectively [35,36]. There was previously reported to be an 'intergenic' region located between the genes M and 5 in the group 3 coronaviruses, IBV and TCoV, however recent reports have demonstrated the presence of two ORFs, 4b and 4c, in this region [37–39]. Another ORF, 6b, was also detected in these studies immediately downstream of the N gene. As with the known non-structural accessory proteins, the functions of the putative proteins translated from these three ORFs have yet to be fully elucidated.

An inherent characteristic of RNA viruses is their ability to quickly adapt to environmental changes by introducing mutations during replication. This results in a heterogeneous population of viruses transcribed from a single parent genome [40,41], which is referred to as a 'quasi-species' [42]. Coronaviruses contain the largest known single-stranded RNA genome [43,44], so it is not surprising that IBV quasi-species have been reported [45,46]. Further investigation of the dynamics of quasi-species has shown that complementation between individual variants produces the viral phenotype, instead of dominance by a single variant virus in a given environment [47]. Previously, a reverse transcriptase-PCR (RT-PCR) method designed to amplify a section of the IBV 3′ UTR for diagnosis and differentiation of vaccine and field IBVs found that all IBV vaccine strains tested produced a single amplicon, except for the VicS vaccine, which produced two [48]. One of the amplicons was the same size as the single amplicon produced by other subgroup 1 vaccines and the other was slightly smaller. High resolution melt (HRM) curve analysis of the PCR products amplified from the 3′ UTR of the vaccine and field IBVs concluded that the VicS vaccine did not produce the characteristic HRM curve produced by other subgroup 1 vaccine strains [49].

Although the VicS vaccine is currently available only in Australia, the presence of viral subpopulations has been reported in variety of viral vaccines [41,50] including IBV vaccines [51] in other countries. The purpose of this investigation was to use the VicS vaccine as a model to elucidate the origin of two different amplicons produced during RT-PCR and to determine whether the two amplicons represent the presence of two subpopulations. If two subpopulations were present, this investigation then sought to further determine whether the two different IBVs differed in pathogenicity and if this might explain the more severe vaccine reaction to VicS observed in young chickens.

2. Materials and methods

2.1. Purification of two IBV subpopulations from the VicS vaccine

A commercial preparation of the VicS vaccine (Pfizer Animal Health Australia, Victoria) was reconstituted to one dose/μl using sterile phosphate buffered saline (PBS). The vaccine was serially diluted in tenfold steps to 10^{-6} and 200 μl of each dilution was inoculated into 9 to 11-day-old embryonated specific pathogen free (SPF) (SPAFAS Australia Pty Ltd, Victoria, Australia) or commercial (flocks maintained for human commercial vaccine production) (Research Poultry Farm, Victoria, Australia) chicken eggs. These flocks are unexposed and regularly monitored for, and determined to be free of, various pathogens including IBV. At 48 h post inoculation (PI) the allantoic fluid (AF) was collected and subjected to RT-PCR followed by PCR/HRM curve analysis and gel electrophoresis, as previously described [49] to assess the ratio of the two different amplicons in each dilution. At each passage, preparations of AF that showed a dominance of either amplicon were selected and serial dilutions of the AF passaged further. This process was continued until the preparation produced a single amplicon when analysed on an agarose gel. This virus was subsequently passaged an additional two times. The virus concentration (determined as the Egg Infectious Dose – EID_{50}) was determined by inoculating ten-fold dilution series of the AF containing each viral subpopulation into five, 9–11-day-old embryonated commercial chicken eggs, with the embryos assessed at 48 h PI for stunting and curling, as compared to un-inoculated controls [52]. The Australian vaccine strain I was similarly titrated and inoculated simultaneously as a control.

2.2. Nucleotide sequence analysis

The two amplicons produced by 3′ UTR RT-PCR were subjected to gel electrophoresis, separately excised and purified using a QIAquick Gel Extraction Spin Kit (Qiagen) and subjected to nucleotide sequencing using 5 μM of each PCR primer (Applied Genetic Diagnostics).

The nucleotide sequence of the structural protein gene region of the virus subpopulation that produced the smaller band in the 3′ UTR RT-PCR was obtained by primer walking. Multiple primer pairs were designed and used to amplify sections of the genome. Each RT-PCR reaction was subjected to gel electrophoresis and the nucleotide consensus sequence obtained and aligned to the VicS nucleotide sequence deposited in GenBank [53] (ID: DQ490221) using ClustalW2 (http://www.ebi.ac.uk). The 3′ UTR only of the subpopulation that produced the larger band during 3′ UTR PCR was sequenced as described above using previously described primers [49].

The putative amino acid sequences were aligned to the putative amino acid sequences for the VicS sequence deposited in GenBank using ClustalW2 as above to identify non-silent mutations.

2.3. Growth characteristics of the two viral subpopulations present in the VicS vaccine in embryonated eggs

To determine and compare the growth characteristics of the two viral subpopulations in eggs to each other, and to vaccine I (Pfizer Animal Health Australia), AF was collected from two embryonated

commercial eggs inoculated with each virus over a period of 48 h, and a comparative quantitative analysis performed to determine the amount of virus present in the AF. Briefly, 100 μl of AF containing each purified subpopulation was inoculated into four eggs. Fifty microliters of AF was collected from each egg, using a 26 gauge needle, through a small hole created in the shell near the airsac. For each subpopulation, two eggs were used for collection of AF at 4, 8, 12, 24, 32, 36 and 48 h PI, whilst the remaining two eggs were used for collection of AF at 16, 20, 40 and 44 h PI. Two eggs were left un-inoculated and AF was sampled as above to provide negative controls. This experiment was performed in duplicate to confirm results.

Viral RNA was extracted from the AF and subjected to RT-PCR/HRM curve analysis as described previously [49]. The 30 – Ct value (number of PCR cycles minus the cycle that amplification exceeded a specified threshold) was used for comparison of the quantity of virus and this was plotted against time to compare growth profiles.

2.4. In vivo pathogenicity

To identify any *in vivo* differences in pathogenicity and/or replication between the two viral subpopulations, an experimental infection study was performed in SPF chickens. Ethics approval was granted by The University of Melbourne Animal Ethics Committee (ID: 0911360). One hundred SPF chickens (Australian SPF Pty Ltd, Victoria, Australia) were hatched and transferred to negative pressure HEPA-filtered isolators. At one week of age, the chickens were separated into five groups of twenty chickens each and transferred into five separate isolators.

Using a micropipette, chickens in each group were inoculated by eye drop with 30 μl of AF containing commercial VicS vaccine, either subpopulation of virus, a mixture of an equal ratio of each viral subpopulation, or sterile PBS. All inocula, excluding the PBS, were diluted to the same concentration as the commercial preparation of the vaccine (10^6 EID_{50}/ml).

To monitor the dissemination of virus throughout the body of the chickens, three chickens from each group were randomly selected and euthanised at days 1, 3, 5, 7 and 9 PI and tissues collected and examined by RT-PCR and histopathology. The upper and lower trachea, kidneys and caecal tonsils were collected for virus detection and the tracheal tissues and kidneys were processed for histopathology. The remaining five chickens from each group were euthanised at day 20 PI. All chickens were euthanised by exposure to halothane.

All chickens were monitored daily for clinical signs of IBV infection, such as coughing, snicking, lethargy and conjunctivitis. A scoring system was established based on the clinical signs associated with infection of respiratory tissues by IBV and the minimal reaction expected of a vaccine strain (*i.e.* mortalities should not occur). Clinical signs were scored on a scale of 0–3, with a single score given to each group for each day. No observable signs was scored as 0, eye irritation/scratching was scored as 1, eye irritation/scratching with lethargy, minor conjunctivitis, coughing and snicking was scored as 2, and signs of lethargy/depression, excessive production of mucus from the nares and conjunctiva and coughing or gasping was scored as 3. A clinical sign score that represented half or more of the chickens in each group, on each day, was then used as the general clinical sign score for that group. Clinical signs were scored by two independent observers to enable objective characterisation of the clinical signs. In the case of disagreement between observers, a third observer was asked to score the clinical signs based on the above scoring system, and the majority score was recorded.

2.5. Histopathology

Kidney and tracheal tissues collected at each time point were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin. Tracheal sections were examined microscopically for lesions associated with IBV, such as epithelial cell necrosis and/or sloughing [2]. Lesions were scored as 0 when there was no apparent sloughing of the epithelial cell layer, 1 when lesions included sloughing of 10–40%, 2 when lesions included sloughing of 40–80%, and 3 where sloughing of greater than 80% of the epithelial cell layer was observed.

Kidney sections were examined microscopically for lesions such as damage to the kidney epithelial cells/tubules.

2.6. RT-PCR and high resolution melt curve analysis of collected tissues

Kidney tissue, approximately 0.5 cm^3 in size, and 0.5 cm of the upper and lower trachea were obtained from each chicken and placed separately in 400 μl RLT buffer (Qiagen) containing 1% β-mercaptoethanol and stored at 4 °C overnight.

Tissues were classified as IBV negative, IBV positive, or IBV trace positive (enough virus present for detection by RT-PCR, but not for reliable HRM curve analysis) using the 3′ UTR RT-PCR and HRM curve analysis, as previously described [49]. The 30 – Ct value for each tissue was calculated as described above. Amplicons were subjected to electrophoresis through a 12% Mini-Protean TGX precast polyacrylamide gel (BioRad, Victoria, Australia) to determine which amplicon/s were present in tissues collected from chickens inoculated simultaneously with both subpopulations of virus.

An arbitrary value of 0.5 was assigned to tissues that were classified as trace positive, and an arbitrary value of 0.1 assigned to tissues which were confirmed to have virus present by agarose gel electrophoresis only as the virus concentrations in these tissues were not sufficiently high to calculate a 30 – Ct value. These values and the 30 – Ct values were plotted over time for each tissue, from each chicken, in each group.

2.7. Immunofluorescence

Allantoic fluid containing virus was centrifuged and the cells spotted onto coverslips (22 × 22 mm) in 6-well plates. After adsorption, 3 ml medium 199 with 1% newborn bovine serum (Gibco, Victoria, Australia) was added to each well and coverslips incubated at 37 °C with 5% CO_2 for 1 day. They were then removed, washed in sterile PBS, drained and fixed by dipping in acetone.

Each well was inoculated with anti-sera against Australian subtype B vaccine strain S, subgroup 2 strains Q3/88 and N1/88 and field strain Q1/76. Strains N1/88, Q3/88 and vaccine B are different serotypes [20], whereas Q1/76 anti-sera has been shown to cross-react with IBV vaccine strains that are related to VicS (personal communication, Denise O'Rourke).

Coverslips were then incubated at room temperature for 30 min, washed three times for 2 min in sterile PBS and rabbit anti chicken IgG (conjugated with fluorescein (Zymed, California, USA)) diluted 1 in 50 in PBS applied to each coverslip. The coverslips were washed as before after a 30 min incubation, then mounted on glass microscope slides using a 1:1 mixture of PBS and glycerol. The coverslips were examined under a UV microscope.

2.8. Statistical analysis

A 'comparison of proportions' test was performed (WinPepi Version 11.4) to determine which of the two subpopulations of virus grew preferentially in each tissue in groups into which both subpopulations were inoculated simultaneously.

```
Vaccine I       CAGCGCCAAAACAACAGCGCCCTAAGAAGGAGAAAAAGCCAAAGAAGCAGGATGATGAAG 60
VicS_higher     CAGCGCCAAAACAACAGCGCCCTAAGAAGGAGAAAAAGCCAAAGAAGCAGGATGATGAAG 60
VicS_lower      CAGCGCCAAAACAACAGCGCCCTAAGAAGGAGAAAAAGCCAAAGAAGCAGGATGATGAAG 60
                ************************************************************

Vaccine I       TAGATAAAGCATTAACCTCAGATGAGGAGAGGAACAATGCACAGCTGGAATTTGATGATG 120
VicS_higher     TAGATAAAGCATTAACCTCAGATGAGGAGAGGAACAATGCACAGCTGGAATTTGATGATG 120
VicS_lower      TAGATAAAGCATTAACCTCAGATGAGGAGAGGAACAATGCACAGCTGGAATTTGATGATG 120
                ************************************************************

Vaccine I       AACCCAAAGTAATTAACTGGGGGGATTCAGCACTAGGAGAGAATGAACTTTGAACATAAT 180
VicS_higher     AACCCAAAGTAATTAACTGGGGGGATTCAGCACTAGGAGAGAATGAACTTTGAACATAAT 180
VicS_lower      AACCCAAAGTAATTAACTGGGGGGATTCAGCACTAGGAGAGAATGAACTTTGAACA---- 176
                ************************************************************

Vaccine I       GGACTTGCCGCATTTGCTGGCACATTTTGTTAAACACTAAGTTATTTTTGGTTGCTCTTT 240
VicS_higher     GGACTTGCCGCATTTGCTGGCACATTTTGTTAAACACTAAGTTATTTTTGGTTGCTCTTT 240
VicS_lower      ------------------------------------CTAAGTTATTTTTGGTTGCTCTTT 200
                                                     ************************
Vaccine I       GCTTGTTGTGTTGTAGCTGTGCTCTTTATTATTGTTGTTCTCCTTTCTTTGCTTTATAGA 300
VicS_higher     GCTTGTTGTGTTGTAGCTGTGCTCTTTATTATTGTTGTTCTCCTTTCTTTGCTTTATAGA 300
VicS_lower      GCTTGTTGTGTTGTAGCTGTGCTCTTTATTATTGTTGTTCTCCTTTCTTTGCTTTATAGA 260
                ************************************************************

Vaccine I       AAAAGTTCAATAGTAAGAGTTAAGGCAGATAGGCATGTAGCTTGATTACCTACATGTCTA 360
VicS_higher     AAAAGTTCAATAGTAAGAGTTAAGGCAGATAGGCATGTAGCTTGATTACCTACATGTCTA 360
VicS_lower      AAAAGTTCAATAGTAAGAGTTAAGGCAGATAGGCATGTAGCTTGATTACCTACATGTCTA 320
                ************************************************************

Vaccine I       TCGCCAGGGAAATG 374
VicS_higher     TCGCCAGGGAAATG 374
VicS_lower      TCGCCAGGGAAATG 334
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Fig. 1. Alignment of the partial nucleotide sequences of the VicS vaccine 3′ UTR with those of the two subpopulations of virus purified from this vaccine. The 40 bp deletion detected in the lower amplicon produced during 3′ UTR RT-PCR of the VicS vaccine is represented by dashes. Sequence alignment performed using ClustalW2 (http://www.ebi.ac.uk/).

A Spearman's rank correlation and a Mann–Whitney independent samples test were performed (PASW Statistics 18 package) to determine if there was a correlation between the scores for the upper and lower trachea within groups, and to determine if there were significant differences between the upper and lower tracheal lesion scores between the groups, respectively.

3. Results

3.1. The nucleotide sequence of the two PCR bands were identical except that the lower band contained a 40 bp deletion

In order to examine if the two bands generated by the 3′ UTR RT-PCR of the commercial VicS vaccine resulted from the presence of two different viruses, the amplicons were gel purified and subjected to nucleotide sequencing. Nucleotide sequence analysis showed that the larger amplicon was 374 bp and had 99% identity in this region with other Australian subgroup 1 vaccines. The smaller amplicon was 334 bp and had an identity of 99% with Australian subgroup 1 vaccines. This smaller amplicon contained a 40 bp deletion compared to the larger amplicon (Fig. 1).

3.2. Two different populations of IBV exist in the commercial IBV VicS vaccine

Previous reports of RT-PCR of the 3′ UTR of the VicS vaccine demonstrated a greater quantity of the 374 bp amplicon compared to the 334 bp amplicon, as determined by fluorescence on an agarose gel [48,54]. This ratio was not maintained during separation of the subpopulations from the commercial preparation of the vaccine, as in some cases the 334 bp amplicon produced the more intense band after 3′ UTR RT-PCR of the AF collected from embryonated eggs inoculated with various dilutions of the vaccine (results not shown).

After four passages through embryonated eggs, a virus stock was obtained which yielded only the 374 bp amplicon following 3′ UTR RT-PCR and gel electrophoresis (viral stock named VicS-v). The virus stock that contained the subpopulation with only the 334 bp amplicon (viral stock named VicS-del) was purified after 9 passages. Both subpopulations were passaged an additional two times prior to *in vivo* inoculation. VicS-v passage 6 and VicS-del passage 11, along with vaccine I, consistently grew to a titre of 10^6 EID_{50}/ml. The 3′ UTR sequences obtained for the VicS-v and VicS-del stocks were identical to the 3′ UTR sequences of the 374 bp and 334 bp amplicons respectively. The VicS nucleotide sequence deposited in GenBank was found to represent the VicS-v sequence, as it did not include the 40 bp deletion found in the 3′ UTR of VicS-del.

3.3. HRM curve analysis of the VicS-v and VicS-del 3′ UTR

Melt curves produced by the amplicons from the 3′ UTR of VicS-v, VicS-del, VicS vaccine and vaccine I are shown in Fig. 2. The HRM curve for the VicS vaccine had four small peaks, which was consistent with a previous report [49], while the VicS-v subpopulation and vaccine I had two distinct high peaks produced at the same temperatures. The curve for VicS-del was similar in shape to that of VicS-v and vaccine I, but its second peak occurred at a temperature approximately 0.5 °C lower than that of the second peak for VicS-v and vaccine I.

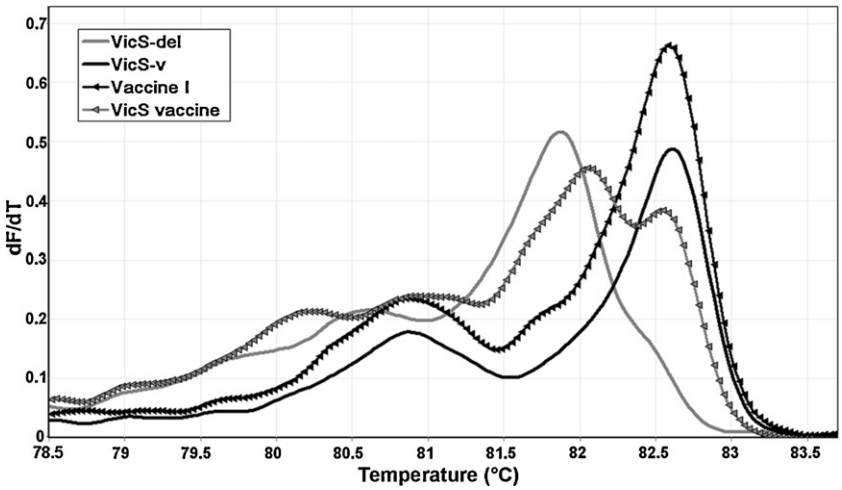

Fig. 2. High resolution melt curves produced by the VicS vaccine, the two viral subpopulations VicS-v and VicS-del, and vaccine I. The d*f*/d*t* value on the *y*-axis indicates the extent of the release of fluorescent dye during high resolution melting of the 3′ UTR amplicons.

3.4. Non-silent mutations were found in all VicS-del genes sequenced except the 3a, 3b, E and 5b genes

The VicS-del structural protein gene region nucleotide sequence was 7095 bp long from the start of the S1 gene to 295 bp into the 3′ UTR. This was 41 bp shorter than the VicS nucleotide sequence across this same region. The VicS-del structural protein gene region nucleotide sequence was deposited in GenBank (ID: JN983807).

Complete ORFs were found for all genes sequenced in the VicS-del genome except ORFs 4b and 6b. Six mutations were found in the S1 gene, five of which resulted in amino acid changes. Two mutations were detected in the S2 gene and both resulted in amino acid changes. The single mutation detected in the M gene resulted in an amino acid change. Two mutations, including a single base deletion, were found in ORF 4b. This deletion introduced a premature termination codon 29 bp downstream of the ORF 4b initiation codon. Five mutations were detected in ORF 4c, two of which resulted in amino acid changes. One of these mutations removed the predicted ORF 4c termination codon, increasing the size of this ORF by 30 bp. Four of the mutations present in ORF 4c were also located in the ORF coding for gene 5a, three of which resulted in amino acid changes. Three mutations were detected in the N gene, two of which resulted in amino acid changes. One mutation was found in the 3′ UTR at position 6794 (40 bp deletion) which removed the initiation codon, and subsequent codons, for ORF 6b. Non-silent mutations are summarised in Table 1.

Table 1
Non-silent mutations found in the VicS-del structural protein gene region compared to VicS-v.

Genome region	Location[a]	Nucleotide change	Predicted amino acid change (location[b])
S1	287	A to C	Glu[c] to Ala (96)
	408	A to G	Ile to Met (136)
	1029	A to T	Lys to Asn (343)
	1082	A to G	Asp to Gly (361)
	1237	C to T	Arg to Cys (413)
S2	2066	C to A	Pro to His (149)
	3102	A to T	Leu to Phe (494)
M	4718	G to A	Arg to Gln (189)
ORF 4b	4853	T deletion	frameshift/truncation
ORF 4c	5050	G to A	Arg to Gln (10)
	5192	G to T	termination to Tyr (57)
5a	5192	G to T	Ser to Ile (6)
	5200	G to A	Gly to Ser (9)
	5219	A to G	Tyr to Cys (15)
N	5697	T to C	Val to Ala (46)
	6372	C to T	Thr to Met (271)
3′ UTR	6794	40 bp deletion	truncation

[a] Location of nucleotide change relative to the S gene initiation codon.
[b] Location of the amino acid change relative to the initiation codon of each gene/ORF.
[c] Three letter amino acid code.

3.5. The VicS-del and VicS-v subpopulations had similar growth profiles to vaccine I in embryonated chicken eggs

Fig. 3 shows the average 30 – Ct values for AF collected from embryonated chicken eggs inoculated with VicS-v, VicS-del and vaccine I. The two virus subpopulations had a very similar growth curve to that of vaccine I. Samples were not collected from eggs inoculated with VicS-del at 40 h PI or from those inoculated with VicS-v, VicS-del and vaccine I at 44 h PI as the embryos that were to be used for collection at these time points died. The peak of viral growth for VicS-v, VicS-del and vaccine I was at 28, 20 and 24 h PI, respectively.

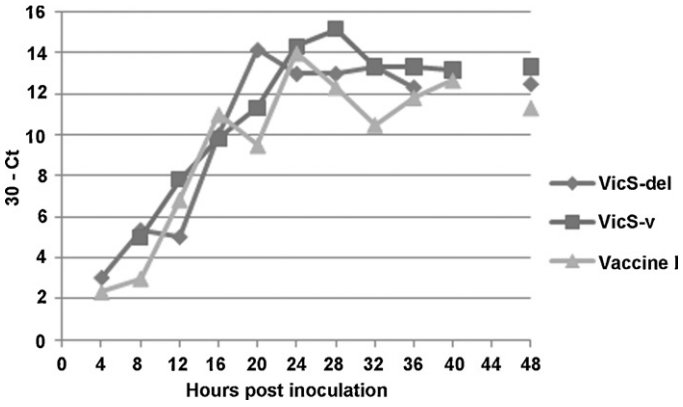

Fig. 3. Comparative growth curves of VicS-v, VicS-del and vaccine I in embryonated SPF chicken eggs. AF was sampled every 4 h for 48 h from embryonated eggs after inoculation with either VicS-v, VicS-del or vaccine I. No samples were collected from eggs inoculated with VicS-del at 40 h PI or those inoculated with VicS-v, VicS-del or vaccine I at 44 h PI as the embryos to be used for collection at this time point died prior to collection of the samples.

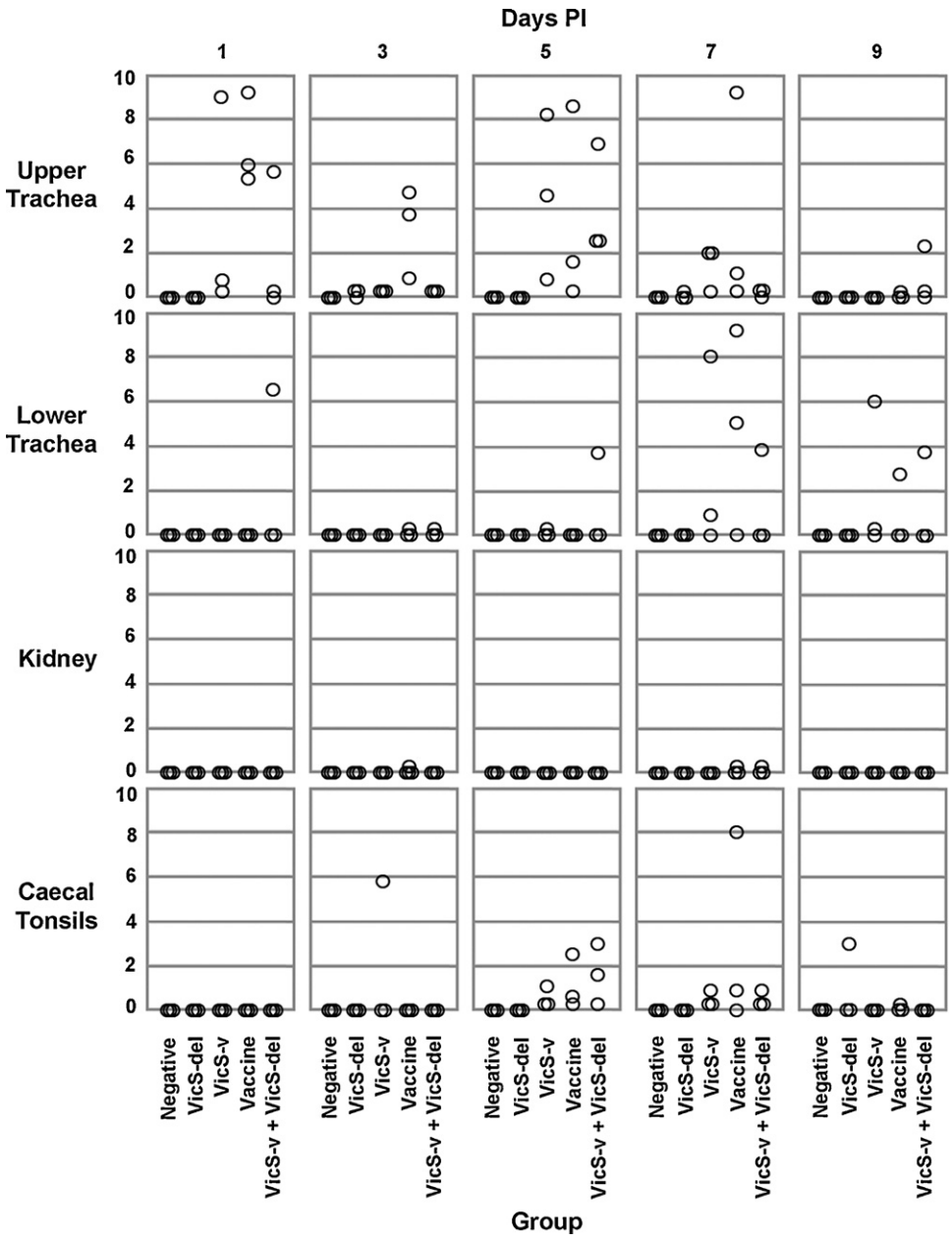

Fig. 4. Comparative analysis of the concentration of virus detected in the upper and lower trachea, the kidney and the caecal tonsils at each time point for chickens inoculated with VicS-v, VicS-del, VicS vaccine, or VicS-v + VicS-del. The 30 – Ct value (y-axis) was used as an approximate quantitative measure of viral concentration in the upper and lower trachea, kidney and caecal tonsils at days 1, 3, 5, 7 and 9 PI. The Ct value is the first cycle in which PCR amplification exceeds a specified threshold. Each value represents the average of the triplicate assays performed on each sample. Where two or more points overlapped, horizontal jittering was employed.

3.6. Concurrent in vivo infection with VicS-v and VicS-del causes comparatively more severe clinical signs than infection with either subpopulation alone

In order to examine the pathogenicity of VicS-v and VicS-del *in vivo*, five groups of chickens were inoculated with commercial VicS vaccine ('Vaccine'), either subpopulation ('VicS-v' or 'VicS-del'), a mixture of an equal ratio of each subpopulation ('VicS-v + VicS-del'), or sterile PBS ('negative').

Chickens were observed for clinical signs throughout the experiment and scored for each group over days 1–9 PI. For logistical reasons, clinical signs could not be scored on day 4 PI. No clinical signs were noted in the negative control group at any stage.

For the VicS-v group, clinical signs included head shaking and eye scratching/irritation, and were observed from day 2 PI and persisted until day 6 PI. At day 7 PI only one out of 11 chickens displayed signs, while at day 8 PI clinical signs were evident in a majority of the chickens, and at day 9 PI no clinical signs were evident in any chicken. All scores for the clinical signs for this group were $\leq$1. Chickens in the Vaccine group displayed a pattern of clinical signs similar to that seen in the VicS-v group, however the peak score for the clinical signs was 1.5 at day 3 PI and clinical signs were still evident at days 9 PI. For the VicS-del group, clinical signs included head shaking and swollen eyes and were observed only on days 6, 7 and 8 PI. All scores for the clinical signs for this group were $\leq$1. For the VicS-v + VicS-del group, clinical signs included head shaking and eye irritation/scratching and were observed from day 1 PI. Clinical signs were scored as 1 for the first 5 days PI. At days 6 and 7 days PI lethargy/depression and mild conjunctivitis were observed in a majority of the chickens (scored as 2). The clinical

signs reduced to <1 at days 8 and 9 PI. No clinical signs were noted in the negative control chickens at any stage.

3.7. The lowest quantity of virus was detected in the VicS-del group for each tissue

The Ct values generated from the RT-PCR assay allowed a comparative quantitative analysis of viral concentration in each tissue sample collected at each time point after infection with the VicS vaccine, VicS-v, VicS-del or VicS-v + VicS-del. Fig. 4 shows the average Ct value for each tissue at each time point from each chicken. No virus was detected in tissues of the negative control chickens at any time point. The 30 – Ct values were ≤3 for the VicS-del group, ≤8 for the VicS-v group, ≤9.5 for the Vaccine group and ≤7 for the VicS-v + VicS-del group.

In the VicS-del group, virus was only detected in the upper trachea of two chickens on days 3 PI and one chicken on day 7 PI and in the caecal tonsils of one chicken at day 9 PI.

Virus was detected in the upper trachea of every chicken on days 1, 3, 5, 7 and 9 in the VicS-v, Vaccine and VicS-v + VicS-del groups, except for all chickens from the VicS-v group and two chickens from the Vaccine group on day 9 PI, and one chicken each from the VicS-v + VicS-del group on days 1, 7 and 9 PI.

Virus was detected in the lower trachea of one, two and two chickens from the VicS-v group on days 5, 7 and 9 PI, respectively, one, two and one chickens from the Vaccine group on days 3, 7 and 9 PI, respectively, and one chicken each at days 1, 3, 5, 7 and 9 PI in the VicS-v + VicS-del group.

Virus was detected in the kidneys of one chicken on day 3 PI from the Vaccine group and one chicken each from the Vaccine and VicS-v + VicS-del groups on day 7 PI only.

Virus was detected in the caecal tonsils of one chicken from the VicS-v group only at day 3 PI, but virus was detected in all chickens from the VicS-v, Vaccine and VicS-v + VicS-del groups on days 5 and 7 PI, except for one chicken from the VicS-v group on day 5 PI and one chicken each from the VicS-v and Vaccine groups on day 7 PI. Virus was detected in the caecal tonsils of one chicken only from the Vaccine group on day 9 PI.

At day 20 PI, no virus was detected in the trachea or kidney of any chicken. Virus was detected in the caecal tonsils of one, five, four and four chickens from chickens in the VicS-del, VicS-v, Vaccine and VicS-v + VicS-del groups respectively (results not included in Fig. 4).

A Cochran's q-test found that overall virus was detected in the upper trachea more frequently than in other tissues ($P < 0.0001$).

3.8. The growth of VicS-v was dominant in tissues from chickens inoculated simultaneously with VicS-v and VicS-del

The RT-PCR products from tissues collected from the Vaccine and VicS-v + VicS-del groups were subjected to gel electrophoresis to determine which subpopulations were present, as the inoculum for both of these groups contained both VicS-v and VicS-del.

VicS-v was detected alone in 39 of the 49 samples of tissue containing detectable viral RNA collected from chickens inoculated with Vaccine or VicS-v + VicS-del on days 1–9 PI. This included all the kidney tissue samples, the majority of the caecal tonsils and upper and lower tracheal samples containing detectable viral RNA.

VicS-v and VicS-del were detected simultaneously in very few samples. All virus detected in the caecal tonsils on day 20 PI was VicS-v alone, except in the caecal tonsils in one chicken from the VicS-v + VicS-del group which was VicS-del alone.

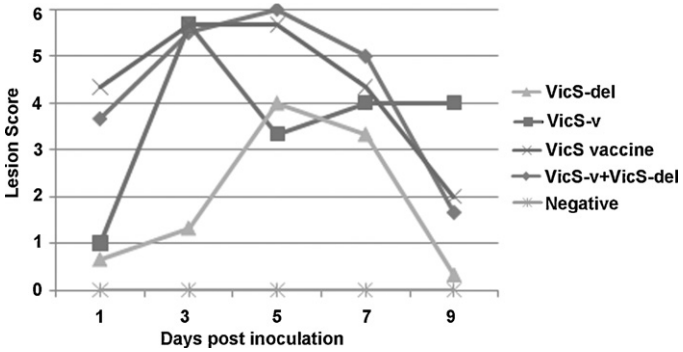

Fig. 5. Comparative analysis of the combined upper and lower tracheal lesion score for chickens in each group at each time point. The average combined upper and lower tracheal lesion score was plotted. Lesions were scored as 0 when there was no apparent sloughing of the epithelial cell layer, 1 when lesions included sloughing of 10–40%, 2 when lesions included sloughing of 40–80%, and 3 where sloughing of greater than 80% of the epithelial cell layer was observed.

3.9. Both VicS-v and VicS-del cross-react with subgroup 1 vaccines

The results of the immunofluorescent assay indicated that the cells from AF containing both VicS-v and VicS-del were of similar serotypes. Importantly, both VicS-v and VicS-del reacted the same to antisera against known subtype B vaccines and a subtype B-related field strain (Q1/76).

3.10. Histopathological lesions were observed only in the upper and lower trachea of inoculated chickens

No lesions of any type were observed in the trachea of the negative control chickens at any time, or in the kidney tissue from chickens in any group. In very few cases epithelial cell necrosis and deciliation accompanied by inflammatory cell infiltration was evident in the upper and lower trachea of chickens in the VicS-v, Vaccine and VicS-v + VicS-del groups. The most common lesion observed in the trachea consisted of complete or partial removal of the epithelial cell layer from the mucosa.

There was a strong correlation between the scores for the upper and lower trachea within groups using a Spearman's Rank test ($P < 0.0001$). Subsequently, the upper and lower tracheal lesion score for each chicken in each group was combined and the average value for the three chickens at each time point in each group was plotted against time and is shown in Fig. 5. Significant differences were found between the lesion scores for the upper ($P < 0.01$) and lower (P-value < 0.05) trachea of chickens in the VicS-del group with the lesion scores for the upper and lower trachea of chickens in the VicS-v, Vaccine and VicS-v + VicS-del groups. No significant differences were found between the lesion scores for the upper or lower trachea respectively of chickens in the VicS-v, Vaccine and VicS-v + VicS-del groups.

4. Discussion

This investigation confirmed the presence of two IBV subpopulations, VicS-v and VicS-del, in the commercial preparation of the IBV VicS vaccine and demonstrated their abilities for independent growth in embryonated chicken eggs. The results also provided an explanation for the HRM curve anomalies observed for VicS in our previous study [49]. The subsequent *in vivo* experiment demonstrated differences in the pathogenicity of these two virus subpopulations in chickens.

The 3′ UTR RT-PCR, when applied to the VicS vaccine, consistently yielded a greater quantity of the VicS-v (374 bp) amplicon

than the VicS-del (334 bp) amplicon, as determined by fluorescence intensity on an agarose gel [48,54]. This ratio was not maintained during separation of the subpopulations from the VicS vaccine, and these two distinct subpopulations had comparable growth rates in eggs. However, growth of VicS-v and VicS-del varied considerably *in vivo*. VicS-del alone caused minimal clinical signs and was less frequently detected in the tissues analysed in this study than VicS-v alone. Interestingly, the differing severity of clinical signs seen in groups inoculated with one or both virus subpopulations suggested that possibly some synergism existed between VicS-v and VicS-del *in vivo*. Chickens simultaneously inoculated with an even ratio of both subpopulations of virus appeared to display more severe clinical signs for a longer period, and viral RNA was detectable for a longer period, than in chickens that were inoculated with one virus subpopulation alone. This was despite the fact that the quantity of VicS-v inoculated into the VicS-v alone group was twice that inoculated into the VicS-v + VicS-del group (considering this group was inoculated with an even ratio of VicS-v and VicS-del at the same final concentration as the VicS-v alone group). The rate of detection and concentrations of VicS-v RNA were comparable between the VicS-v and VicS-v + VicS-del groups (VicS-v was detected significantly more frequently than VicS-del in the VicS-v + VicS-del group).

All of these findings suggest that VicS-del aided in the growth and pathogenicity of VicS-v. It appeared that an increase in the number of VicS-del particles in the inoculum was associated with an increase in the severity of clinical signs and infectivity (the only cases of renal infection were in the Vaccine and VicS-v + VicS-del groups), although VicS-v remained the predominant subpopulation detected by RT-PCR. This suggests that the presence of the substantially mutated VicS-del subpopulation potentially boosts the capacity VicS-v (the predominant subpopulation and original vaccine strain) to spread and cause disease in the host; *i.e.* a synergistic relationship exists between VicS-v and VicS-del *in vivo*. This is reminiscent of the RNA viral quasi-species theory, which proposes that all variant progeny produced during viral replication comprise the quasi-species and act as a single entity [41]. Interestingly, the coronavirus leader sequence, located at the 5′ end of the genomic RNA, is able to regulate the transcription of other coronavirus genomes infecting the same cell during the template switch process (*i.e.* in cis or trans) [55,56]. Therefore, a possibility to be investigated further is whether the molecular machinery produced by VicS-del can be used for the replication of VicS-v *in vivo*, assuming both viruses can co-infect cells, which would go some way to explaining the concurrence of reduced growth of VicS-del and an increased growth of VicS-v *in vivo*, in cases when both subpopulations were present simultaneously.

The apparent reduced ability of VicS-del to infect host cells does not necessarily indicate a loss of pathogenicity, but could indicate an altered capacity to bind to different cell receptors. It has been suggested that changes near the receptor binding domain of the S1 protein could modify the affinity of coronaviruses for different cell surface glycoproteins [57–59]. Potentially, the variations found in the S1 gene might have reduced the ability of VicS-del to infect respiratory tract cells, but not its ability to infect the cells of the chorioallantoic membrane (CAM) (where replication of IBV initially occurs in embryonated chicken eggs). As no genome sequence is available upstream of the S1 gene for any Australian strain (including the gene for the RdRP), it is possible that other, unknown genomic differences may have affected the pathogenicity of VicS-del and/or VicS-v. It was not expected that the additional passages through embryonated chickens eggs required to isolate the VicS-del subpopulation would contribute to any differences in the pathogenicity between VicS-v and VicS-del *in vivo*. It is notable that vaccine viruses are propagated by an already large number of passages in eggs. Passage of IBV *in ovo* adapts the virus to growth *in ovo* and reduces the virulence of IBV *in vivo* [60,61]. Embryo mortalities were observed during the process of separating the subpopulations, therefore the mortalities observed during the comparative growth experiment were not unexpected.

In this investigation, the entire structural protein gene region of VicS-del was obtained and compared to the structural protein gene region of VicS. After the S gene, most mutations were detected in ORFs 4b and 4c and gene 5a (all mutations identified in gene 5a were present in ORF 4c as these two ORFs overlap). The mutations in ORFs 4b and 4c resulted in substantial changes to the predicted proteins, as 4b would be severely truncated, while 4c would be lengthened, compared to ORFs 4b and 4c in other strains [39]. These ORFs are located in a region of the IBV genome that, until recently, was considered an 'intergenic region'. Consequently, no study has been conducted to investigate whether ORFs 4b and 4c are in fact transcribed and/or functional and, if so, what their potential functions might be. However, using the reverse genetics technique, this 'intergenic region' has been removed from an apathogenic IBV (suspected to be a result of adaptation to cell culture) with no obvious effects on the functionality of the virus (Dr. Paul Britton, Institute for Animal Health, personal communication). A recent study demonstrated that replacement of the accessory proteins, including the 'intergenic region' in an apathogenic strain of IBV with those from a pathogenic strain did not restore pathogenicity [62]. This suggests that the IBV non-structural accessory proteins have a limited or non-existent role in strain virulence and tissue tropism and are involved in modulation of the host cell during infection/viral replication. For this reason, it would not be appropriate to investigate the roles of the IBV non-structural accessory proteins *in vitro*.

It is possible that the other amino acid changes detected could play a role in pathogenicity by affecting the secondary structure of the respective translated proteins. For example, the 40 bp deletion found in the 3′ UTR of VicS-del may affect the efficacy of its replication, as the IBV 3′ UTR contains cis-acting elements involved in virus replication [63]. This could also explain the substantially reduced concentrations of VicS-del detected in chickens inoculated with VicS-del only. It has been shown that host specific variation (variations specific for adaptation to growth in different hosts, *i.e.* chickens or embryonated chicken eggs) occurs in the coronavirus S1 gene during passage of isolates from clinical specimens in embryonated chicken eggs [59,64]. The authors of one of these studies [59] describe a single amino acid substitution of a serine with an alanine at position 95 in the IBV S1 protein that is linked to adaptation of viral growth in chickens and eggs respectively. It was concluded that this change did not affect viral pathogenicity. Interestingly, in both VicS-del and VicS there is a serine at position 95, but at position 96 in VicS-del a glutamic acid has been substituted with an alanine. Another study that investigated amino acid differences between the S1 gene of IBVs before and after adaptation to growth in embryonated eggs also found host specific variations in the structural protein gene region [65]. In that study, the authors found an alanine to serine substitution at position 94 in the IBV S1 gene that resulted in attenuation in embryonated eggs, in contrast to the study of Cavanagh et al. (2005) [59]. Based on the evidence in these reports, a single mutation in the S1 protein of VicS-del alone could potentially explain the reduced capacity of VicS-del to grow in chickens, even though it retained its ability to grow efficiently in embryonated eggs.

An important aim of this study was to provide an explanation for the observations by poultry veterinarians and producers regarding the vaccinal reaction of young chickens (<two-weeks-old) to vaccination with a commercial, live, IBV vaccine. Importantly, clinical signs were observed in the group inoculated with the commercial preparation of the VicS vaccine, which were accompanied by lesions (in some cases this lesion comprised of complete removal of the epithelial cell layer) in both the upper and lower trachea of

most chickens in this group. It is of concern that these clinical signs may contribute to the onset of secondary infections specifically due to the damaged tracheal mucosa.

This study provides a basis for future studies investigating the possible reasons for the increased vaccinal reaction seen in young chickens vaccinated with VicS, and suggests *in vivo* synergism between the two virus subpopulations present in the commercial preparation of the VicS vaccine.

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