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# Isolation of a variant infectious bronchitis virus in Australia that further illustrates diversity among emerging strains

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**Summary.** Australian infectious bronchitis viruses (IBV) have undergone a separate evolution due to geographic isolation. Consequently, changes occurring in Australian IBV illustrate, independently from other countries, types of variability that could occur in emerging IBV strains. Previously, we have identified two distinct genetic groups of IBV, designated subgroups 1 and 2. IBV strains of subgroup 1 have S1 and N proteins that share a high degree of amino acid identity, 81 to 98% in S1 and 91 to 99% in N. Subgroup 2 strains possess S1 and N proteins that share a low level of identity with subgroup 1 strains: 54 to 62% in S1 and 60 to 62% in N. This paper describes the isolation and characterisation of a third, previously undetected genetic group of IBV in Australia. The subgroup 3 strains, represented by isolate chicken/Australia/N2/04, had an S1 protein that shared a low level of identity with both subgroups 1 and 2: 61 to 63% and 56 to 59%, respectively. However, the N protein and the 3′ untranslated region were similar to subgroup 1: 90 to 97% identical with the N protein of subgroup 1 strains. This N4/02 subgroup 3 of IBV is reminiscent of two other strains, D1466 and DE072, isolated in the Netherlands and in the USA, respectively. The emergence of the subgroup 3 viruses in Australia, as well as the emergence of subgroup 2 in 1988, could not be explained by any of the mechanisms that are currently considered to be involved in generation of IBV variants.

## Introduction

Infectious bronchitis (IB) is one of the most economically significant diseases of the intensive poultry industry. In young chicks, respiratory disease or nephritis lead to mortalities, reduced weight gain and condemnation at processing, whereas in chickens of laying age, the disease is subclinical and results in reduced egg production and aberrant eggs [7]. Since the causative agent, infectious bronchitis virus

(IBV), is endemic on all commercial sites, most flocks are vaccinated with live attenuated vaccines. However, outbreaks of IB continue to occur, particularly at sites with high concentrations of poultry, and a great number of IBV variants have been isolated and characterised in an attempt to understand IBV epidemiology and improve its control [7, 13–15, 24, 27, 32, 35, 37].

IBV evolution is currently considered to be driven by three factors: (a) the inherent propensity of its RNA genome to mutate; (b) the continuing use of live, often multiple vaccines and (c) immunological pressure exerted on circulating viruses by the continuing presence of immune bird populations. These three factors probably act in concert and involve various mechanisms, such as point mutations, deletions, insertions and recombination to generate new variants [10, 24, 29, 30, 43].

IBV, together with turkey and pheasant coronaviruses, belongs to Group 3 of the genus *Coronavirus*, family *Coronaviridae* [6, 16]. All members of this genus have a linear, non-segmented, positive-sense, single-stranded RNA genome of approximately 27 kilo-bases (kb) in length. The first 20 kb encode the viral RNA-dependent RNA polymerase and proteases. The remainder of the genome encodes the five structural proteins, the spike (S) consisting of S1 and S2, envelope (E), membrane (M) and nucleocapsid (N) proteins, four small non-structural proteins, 3a, 3b, 5a and 5b, and a 3′ untranslated region (UTR). Variations in the S1 and N genes, in particular, are believed to be of critical importance for emergence of variants because of their role in virus replication and immunity, and hence S1 and N have been used most frequently to determine the relatedness of emerging IBV. The S1 glycoprotein is located on the surface of the virion and carries epitopes and determinants for virus-neutralising antibodies, protective immunity and cell tropism [5, 8, 19]. The N protein, located in the capsid of the virion is involved in RNA replication and carries group-specific antigenic determinants [20]. The 3′UTR region is involved in initiation of negative-strand RNA synthesis and has also been used to assess variation in emerging IBV strains and other members of coronavirus Group 3 [3, 38, 42].

In the majority of countries, with the exception of Australia and New Zealand, some of the most frequently used IB vaccines have been developed from IBV strains isolated either in Europe or in the USA [2, 7, 32, 35]. The S1 genes of IBV variants isolated from vaccinated flocks in Africa, Asia, Europe, Central and South America, the Middle East and the USA share between 75 and 99% amino acid identity [9, 10, 32, 39, 44], whereas the N genes share >94% amino acid identity [41, 45]. Based on the S1 gene sequences, strains within this tentatively named European/USA lineage belong to a number of phylogenetic clusters [14, 34, 38, 43]. However, some strains isolated in different countries may belong to the same phylogenetic cluster, having almost identical S1 genes [32, 35], reflecting some of the common features of IBV evolutionary direction. The phylogenetic analysis based on the N gene sequences usually follows closely the phylogenetic clustering based on the S1 gene [14, 41, 45]. In the European/USA lineage there have been only two exceptions to this commonly observed sequence conservation, strains D1466 and DE076, isolated in the Netherlands and the USA [15, 27, 28]. The S1 genes of both strains share a low level of amino acid identity, 46–53%, with the S1 genes of other IBV strains [15], however, the N sequence in both

strains is conserved, having >92% identity at the amino acid level with the N gene of other strains [30].

The IBVs occurring in Australia have evolved independently since early 1930 from strains of the European/USA lineage. The S1 genes of Australian and European/USA strains share >77% identity at the amino acid level [37], however, by phylogenetic analysis, Australian IBVs belong to a distinct lineage [15, 37, 39]. Within the Australian IBV lineage, there are two distinct genetic and antigenic groups of strains: IBV strains of one group, termed subgroup 1, have S1 and N proteins that share a level of identity found in strains of the European/USA linage: 81–98% identity in the S1 protein and 91–99% in the N protein [37, 38]. IBV strains of subgroup 2 have very different S1 and N proteins: amino acid identity with subgroup 1 is 54–62% in S1 and 60–62% in N [37, 38]. IBVs similar to the subgroup 2 strains have not been detected within the European/USA linage. Comparative sequence analysis of all members of the family *Coronaviridae* has indicated that the subgroup 1 strains cluster together with other strains of the European/USA lineage into one subgroup within Group 3, whereas the subgroup 2 strains belong to a separate cluster (subgroup) in the same Group 3 [16].

In this study, we characterised IBV stains from an outbreak that occurred over a period of six months on a large number of broiler farms in a poultry growing area in the state of New South Wales (NSW). Isolated IBVs were of a new genetic type and belonged to a separate, third genetic subgroup within the Australian IBV lineage.

## Material and methods

### *Origin of field samples*

Samples were collected from November 2002 to mid-February 2003 from 23 broiler farms located in areas west, southwest and north of Sydney, NSW. Flocks belonged to four poultry producers and were vaccinated at hatching with one of the four commercial IB vaccines. Some flocks received an additional vaccination between 7 and 10 days of age with a live IB vaccine different from that used for day-old vaccination. The ages of flocks at the time of the outbreak varied between 28 and 54 days. Disease observed was usually respiratory in nature: cough and secondary *Escherichia coli* infections, associated with slow growth rate and slightly elevated mortality. The following samples were received for virus isolation: trachea and kidney tissues collected from chicks from fourteen farms; tracheal mucus collected from chickens from five farms; allantoic fluid at egg passage three (tracheal mucus passaged in embryonated commercial eggs by the submitting laboratory) from five farms.

### *Processing of field samples*

From each submitted kidney, a small portion was taken, and 4 to 5 kidneys from the same farm were pooled and weighed, and medium was added to obtain a 20% tissue homogenate (medium 199 supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin and gentamycin). Tissues were homogenised using a mortar and pestle, and homogenate was frozen at −70 °C. From 4 to 5 tracheas, mucus was scraped using a sterile scalpel blade, placed in 5 ml of the above medium supplemented with 5% foetal calf serum and frozen at −70 °C. Tracheal mucus collected in the field was also treated in a similar manner. Suspensions of homogenized kidney and tracheal mucus were thawed and centrifuged at 1500 × *g* for 30 min, and the clarified supernatant was collected and passed through a 20 μm filter and stored at −70 °C.

*IBV isolation*

A 200-μl aliquot of each sample was inoculated into the allantoic cavity of 10-day-old embryonated chicken specific pathogen-free (SPF) eggs (SPAFAS Pty. Ltd., Woodend, Victoria) using 3 to 4 eggs per sample. Eggs were incubated at 37 °C, with daily inspection. Eggs where embryos died within 24 h of inoculation were discarded. At 2 to 3 days after inoculation, surviving eggs were placed at 4 °C overnight and the allantoic fluid collected. This allantoic fluid was then inoculated into another set of SPF eggs, 200 μl/egg, which were then incubated for 2 to 3 days, chilled, and the allantoic fluid collected in the same manner as described for the primary passage. This procedure was repeated for another time such that each pooled sample received three blind passages in embryonated eggs. At egg passage 3 (EP3), allantoic fluid was collected and clarified at 1500 × *g* for 30 min, and virus was pelleted at 40,000 × *g* for 1 hr. Pelleted virus was resuspended in 500 μl of PBS (1:60 of the original volume) and used in an antigen ELISA.

*Vaccines and reference strains*

VicS vaccine is a commercial preparation originally obtained from Arthur Webster Pty. Ltd., Sydney, NSW and has been described previously [21, 37]. Vaccines designated VacB2 and VacB3 (synonyms Inghams and Steggles strain, respectively) [18] are commercial vaccines obtained courtesy of one of the poultry companies that submitted samples for virus isolation. Vaccine strain VacC (synonym A3) was obtained from Fort Dodge, Castle Hill, Sydney, NSW. All three vaccines were propagated once in embryonated SPF eggs as described for field isolates, before being used in sequence analysis and as antigen in ELISA.

*Identification and characterisation of IBV by ELISA*

To determine if IBV was isolated from field samples, an antigen ELISA using monoclonal antibodies (Mabs) directed against the S, M an N proteins of IBV was performed as described previously [18, 21]. In brief, pelleted material from EP3 was diluted 1:20 in carbonate-bicarbonate buffer pH 9.5, and 100 μl was added to wells of a polystyrene micro-titre plate (Sarstedt, Adelaide, SA). Four vaccine strains, and reference IBV strains N1/62, N2/75, N1/88, Q3/88 and V18/91, concentrated in the same manner as described for the test samples, diluted 1:100 in PBS, were used as positive controls. After overnight incubation at 37 °C, plates were washed in PBS containing 0.05% Tween 20, and Mabs 16, 7, 9, 5, 2, 1, 51 and 24 added. After 1 hr at 37 °C and washing, anti mouse-IgG HRP (Sigma Chemical Company, Sydney, NSW) was added, followed by the addition of azino-bis 3-ethylbenz 2, 2′-thiazoline-6-sulfonic acid (ABTS) (Sigma). Absorbances at 405 nm were measured after 1 h.

*Identification of IBV in allantoic fluid by dwarfing test*

To confirm antigen ELISA results, the EP3 allantoic fluid of isolated IBV strains was diluted 1:50 in PBS and 200 μl inoculated into six 10-day-old embryonated SPF eggs. One group of eggs was inoculated with PBS. Inoculated eggs were examined daily for mortality; those that died at day 1 and 2 were considered as non-specific deaths. At 6 days post inoculation all surviving embryos were examined for embryo dwarfing and curling, signs typical of IBV [7].

*Confirmation of identity and purity of isolated IBV: SDS-PAGE, hemagglutination and hemagglutination inhibition test*

SDS-PAGE was performed in a discontinuous slab gel using 10% acrylamide in the separating gel as described previously [18, 19]. IBV concentrated from the EP3 allantoic fluid, as described above, was used, and proteins were detected by Coomassie Brilliant Blue staining.

Allantoic fluids of EP3 were also tested in hemagglutination and hemagglutination inhibition tests [18]. In the hemagglutination inhibition test sera specific for NDV, avian influenza and haemagglutinating avian adenoviruses were used.

### *cDNA synthesis*

Viral RNA was extracted from EP3 allantoic fluid using the "Rnaeasy" kit according to the manufacturer's recommendations (Qiagen) with the inclusion of Proteinase K. Randomly primed cDNA was produced using a cDNA synthesis kit (Promega) according to the manufacturer's recommendations.

### *Polymerase chain reaction (PCR) of S1 gene*

The entire S1 gene, containing approximately 1910 bp, was amplified for each of the isolates using primers pM74 (5′ AAAGCAACGCCAGTTGT, corresponding to a region 88 nucleotides upstream of the S1 gene) and pS507 (5′ TTGTATGTACTCATC, corresponding to a complementary region 114 nucleotides downstream of the S1 gene). Parameters used for PCR were 35 cycles consisting of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, with the last cycle containing an extension of 10 min at 72 °C. PCR products were purified using a PCR purification kit (Qiagen). A minimum of 2 to 3 independent PCR replicates were sequenced for each isolate, generating a consensus sequence.

### *PCR and cloning of N and 3′UTR*

The entire N gene and 3′UTR, containing approximately 1737 base pairs was amplified using primers pNuni (5′ CAAAGCAGGACAAGCAGAG, corresponding to a region 96 nucleotides upstream of the N gene) and pNUTR (5′ GCTCTAACTCTATACTAGCC, corresponding to a complementary region immediately upstream of the poly A tail). The same PCR parameters used for amplification of the S1 gene were also used for the amplification of N and the 3′UTR region. PCR products were cloned into the pGEM-Teasy vector (Promega). For each isolate, multiple clones were analysed, producing a consensus sequence.

### *Sequence analysis*

For each clone, sequencing was carried out on both strands of the DNA, using both internal and external primers, using the Applied Biosystems 3730S capillary sequencer (Micromon).

### *Sequence alignment and phylogenetic analysis*

Sequences were aligned using Clustal X [40]. The phylogenetic trees were obtained using the neighbour-joining method within Clustal X with 1,000 bootstrapping replicates, and TreeView [36] was used to display phylogenetic trees.

### *Accession numbers of nucleotide sequences*

The nucleotide sequence data reported in this paper have been submitted to the GenBank and have been assigned the following accession numbers: for the S1 DQ059618 (N4/02), DQ059619 (N5/03) and DQ059620 (N4/03) and for the N and 3′UTR DQ059621 (N4/02), DQ059622 (N5/03) and DQ059623 (N4/03). The accession numbers for the S1 nucleotide sequences of other IBV strains used for comparison were: VicS (U29519), N9/74 (U29452), N1/62 (U29522), N2/75 (U29523), N1/88 (U29450), Q3/88 (U29451), V18/91 (U29521), M41 (X04722), D1466 (M21971), 4/91 (AF093794), D274 (X15832), Ark99 (L10384), and DE072 (AF274435). The accession numbers for the N sequences were: VicS (U52594),

N1/62 (U52596), N2/75 (U52598), N1/88 (U52599), Q3/88 (U52600), V18/91 (U52601), M41 (M28566), D1466 (AF203006), Ark99 (M85244) and DE072 (AF203001).

## Results

### *IBV isolation and antigenic characterisation*

Following three passages of field samples in embryonated SPF eggs, a dwarfing test indicated that infectious virus was recovered from 12 out of 23 farms tested.

**Table 1.** Antigenic profile of isolated IBV strains as determined by monoclonal antibodies

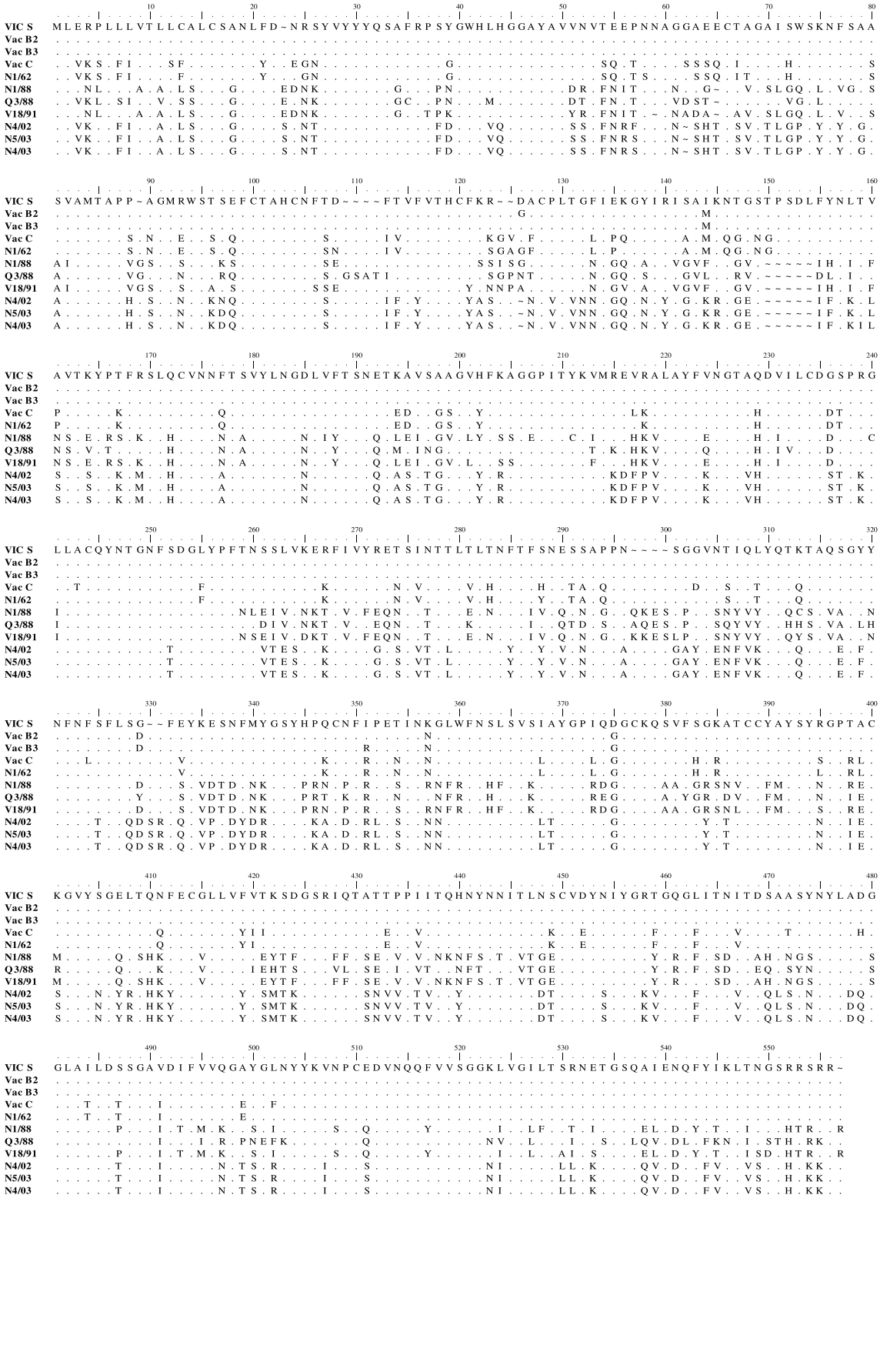
| IBV strain | Serotype | Monoclonal antibody[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | | | | | | M | S |
| | | 7 | 51 | 24 | 1 | 16 | 9 | 2 | 5 |
| *Vaccine strains* | | | | | | | | | |
| VicS | B | +[b] | + | + | + | + | − | + | + |
| VacB2 | B | + | + | + | + | + | − | + | + |
| VacB3 | B | + | + | + | + | + | − | + | + |
| VacC | C | − | − | + | − | + | − | + | + |
| *Reference subgroup 1 strains* | | | | | | | | | |
| N1/62 | C | − | − | + | + | + | − | + | + |
| N2/75 | I | − | − | + | + | + | − | + | + |
| *Reference subgroup 2 strains* | | | | | | | | | |
| N1/88 | L | − | − | − | − | − | + | − | + |
| Q3/88 | M | − | − | − | − | − | − | − | + |
| V18/91 | Q | − | − | − | − | − | − | − | + |
| *Isolated strains* | | | | | | | | | |
| N4/02 | ND[c] | − | + | + | + | + | − | + | + |
| N4/03 | ND | − | + | + | + | + | − | + | + |
| N5/03 | ND | − | + | + | + | + | − | + | + |

[a]Monoclonal antibodies directed against the peplomer S, membrane M and nucleocapsid N proteins

[b] +/− = Reaction or no reaction of monoclonal antibody with an IBV antigen in ELISA. Wells of micro-titre plates coated with partially purified, concentrated IBV and culture fluid of monoclonal antibodies added

[c]ND = Not determined

**Fig. 1.** Alignment of deduced amino acid sequences of the S1 protein of Australian IB vaccines, reference strains and strains isolated in this study. The complete deduced amino acid sequence of the VicS vaccine is shown from amino acid position 1 to 544 [37]. Below this sequence are aligned the deduced amino acid sequences for three other vaccines, VacB2, VacB3 and VacC, reference subgroup 1 strain N1/62, subgroup 2 strains N1/88, Q3/88 and V18/91 and IBV strains, N4/02, N5/03 and N4/03 isolated in this study. For all strains only those amino acid residues that differ from VicS are shown


VIC S MLERPLLLVTLLCALCSANLFD~NRSYVYYYQSAFRPSYGWHLHGGAYAVVNVTEEPNNAGGAEECTAGAISWSKNFSAA
Vac B2
Vac B3
Vac C ..VKS.FI...SF.....Y..EGN.....G.....SQ.T....SSSQ.I....H.....S
N1/62 ..VKS.FI....F.....Y...GN.....G.....SQ.TS....SSQ.IT...H.....S
N1/88 ...NL...A.A.LS...G....EDNK.....G...PN.....DR.FNIT...N..G~...V.SLGQ.L.VG.S
Q3/88 ..VKL.SI...V.SS...G....E.NK.....GC..PN...M.....DT.FN.T...VDST~.....VG.L.....
V18/91 ...NL...A.A.LS...G....EDNK.....G..TPK.....YR.FNIT.~.NADA~.AV.SLGQ.L.V..S
N4/02 ..VK..FI...A.LS...G....S.NT.....FD...VQ.....SS.FNRF...N~SHT.SV.TLGP.Y.Y.G.
N5/03 ..VK..FI...A.LS...G....S.NT.....FD...VQ.....SS.FNRS...N~SHT.SV.TLGP.Y.Y.G.
N4/03 ..VK..FI...A.LS...G....S.NT.....FD...VQ.....SS.FNRS...N~SHT.SV.TLGP.Y.Y.G.
VIC S SVAMTAPP~AGMRWST SEFCTAHCNFTD~~~~FTVFVTHCFKR~~DACPLTGFIEKGYIRISAIKNTGSTPSDLFYNLTV
Vac B2 .....G.....M.....
Vac B3 .....M.....
Vac C .....S.N..E..S.Q.....S.....IV.....KGV.F.....L.PQ.....A.M.QG.NG.....
N1/62 .....S.N..E..S.Q.....SN.....IV.....SGAGF.....L.P.....A.M.QG.NG.....
N1/88 AI.....VGS..S...KS.....SE.....SSISG.....N.GQ.A..VGVF..GV.~~~~~IH.I.F
Q3/88 A.....VG...N...RQ.....S.GSATI.....SGPNT.....N.GQ.S...GVL..RV.~~~~~DL.I..
V18/91 AI.....VGS..S..A.S.....SSE.....Y.NNPA.....N.GV.A..VGVF..GV.~~~~~IH.I.F
N4/02 A.....H.S..N..KNQ.....S.....IF.Y....YAS..~N.V.VNN.GQ.N.Y.G.KR.GE.~~~~~IF.K.L
N5/03 A.....H.S..N..KDQ.....S.....IF.Y....YAS..~N.V.VNN.GQ.N.Y.G.KR.GE.~~~~~IF.K.L
N4/03 A.....H.S..N..KDQ.....S.....IF.Y....YAS..~N.V.VNN.GQ.N.Y.G.KR.GE.~~~~~IF.KIL
VIC S AVTKYPTFRSLQCVNNFTSVYLNGDLVFTSNETKAVSAAGVHFKAGGPITYKVMREVRALAYFVNGTAQDVILCDGSPRG
Vac B2
Vac B3
Vac C P.....K.....Q.....ED..GS..Y.....LK.....H.....DT...
N1/62 P.....K.....Q.....ED..GS..Y.....K.....H.....DT...
N1/88 NS.E.RS.K..H....N.A.....N.IY...Q.LEI.GV.LY.SS.E...C.I...HKV....E....H.I....D...C
Q3/88 NS.V.T.....H....N.A.....N..Y...Q.M.ING.....T.K.HKV....Q....H.IV...D....
V18/91 NS.E.RS.K..H....N.A.....N..Y...Q.LEI.GV.L..SS.....F...HKV....E....H.I....D....
N4/02 S..S..K.M..H....A.....N.....Q.AS.TG...Y.R.....KDFPV....K...VH.....ST.K.
N5/03 S..S..K.M..H....A.....N.....Q.AS.TG...Y.R.....KDFPV....K...VH.....ST.K.
N4/03 S..S..K.M..H....A.....N.....Q.AS.TG...Y.R.....KDFPV....K...VH.....ST.K.
VIC S LLACQYNTGNFSDGLYPFTNSSLVKERFIVYRETSINTTLTLTNFTFSNESSAPPN~~~~SGGVNTIQLYQTKTAQSGYY
Vac B2
Vac B3
Vac C ..T.....F.....K.....N.V....V.H....H..TA.Q.....D..S..T...Q.....
N1/62 .....F.....K.....N.V....V.H....Y..TA.Q.....S..T...Q.....
N1/88 I.....NLEIV.NKT.V.FEQN..T...E.N...IV.Q.N.G..QKES.P..SNYVY..QCS.VA..N
Q3/88 I.....DIV.NKT.V..EQN..T...K.....I..QTD.S..AQES.P..SQYVY..HHS.VA..LH
V18/91 I.....NSEIV.DKT.V.FEQN..T...E.N...IV.Q.N.G..KKESLP..SNYVY..QYS.VA..N
N4/02 .....T.....VTES..K....G.S.VT.L.....Y..Y.V.N...A....GAY.ENFVK...Q...E.F.
N5/03 .....T.....VTES..K....G.S.VT.L.....Y..Y.V.N...A....GAY.ENFVK...Q...E.F.
N4/03 .....T.....VTES..K....G.S.VT.L.....Y..Y.V.N...A....GAY.ENFVK...Q...E.F.
VIC S NFNFSFLSG~~FEYKESNFMYGSYHPQCNFIPETINKGLWFNSLSVSIAYGPIQDGCKQSVFSGKATCCYAYSYRGPTAC
Vac B2 .....D.....N.....G.....
Vac B3 .....D.....R.....N.....G.....
Vac C ...L.....V.....K...R..N..N.....L....L.G.....H.R.....S..RL.
N1/62 .....V.....K...R..N..N.....L....L.G.....H.R.....L..RL.
N1/88 .....D...S.VDTD.NK...PRN.P.R...S..RNFR..HF..K.....RDG....AA..GRSNV..FM...N..RE.
Q3/88 .....Y...S.VDTD.NK...PRT.K.R...N...NFR..H...K.....REG....A.YGR.DV..FM...N..IE.
V18/91 .....D...S.VDTD.NK...PRN.P.R...S..RNFR..HF..K.....RDG....AA..GRSNL..FM...S..RE.
N4/02 ....T..QDSR.Q.VP.DYDR....KA.D.RL.S..NN.....LT.....G.....Y.T.....N..IE.
N5/03 ....T..QDSR.Q.VP.DYDR....KA.D.RL.S..NN.....LT.....G.....Y.T.....N..IE.
N4/03 ....T..QDSR.Q.VP.DYDR....KA.D.RL.S..NN.....LT.....G.....Y.T.....N..IE.
VIC S KGVYSGELTQNFECGLLVFVTKSDGSRIQTATTPPIITQHNYNNITLNSCVDYNIYGRTGQGLITNITDSAASYNYLADG
Vac B2
Vac B3
Vac C .....Q.....YII.....E..V.....K..E.....F...F...V....T.....H.
N1/62 .....Q.....YI.....E..V.....K..E.....F...F...V.....
N1/88 M.....Q.SHK...V.....EYTF...FF.SE.V.V.NKNFS.T.VTGE.....Y.R.F.SD..AH.NGS.....S
Q3/88 R.....Q...K...V.....IEHTS...VL.SE.I.VT..NFT...VTGE.....Y.R.F.SD..EQ.SYN.....S
V18/91 M.....Q.SHK...V.....EYTF...FF.SE.V.V.NKNFS.T.VTGE.....Y.R...SD..AH.NGS.....S
N4/02 S...N.YR.HKY.....Y.SMTK.....SNVV.TV..Y.....DT.....S...KV...F...V..QLS.N...DQ.
N5/03 S...N.YR.HKY.....Y.SMTK.....SNVV.TV..Y.....DT.....S...KV...F...V..QLS.N...DQ.
N4/03 S...N.YR.HKY.....Y.SMTK.....SNVV.TV..Y.....DT.....S...KV...F...V..QLS.N...DQ.
VIC S GLAILDSSGAVDIFVVQGAYGLNYYKVNPCEDVNQQFVVSGGKLVGILTSRNETGSQAIENQFYIKLTNGSRRSRR~
Vac B2
Vac B3
Vac C ...T..T...I.....E..F.....
N1/62 ...T..T...I.....E.....
N1/88 .....P...I.T.M.K..S.I.....S..Q.....Y.....I..LF..T.I....EL.D.Y.T..I...HTR..R
Q3/88 .....I...I.R.PNEFK.....Q.....NV..L...I..S..LQV.DL.FKN.I.STH.RK..
V18/91 .....P...I.T.M.K..S.I.....S..Q.....Y.....I..L..AI.S....EL.D.Y.T..ISD.HTR..R
N4/02 .....T...I.....N.TS.R....I...S.....NI.....LL.K....QV.D..FV..VS..H.KK..
N5/03 .....T...I.....N.TS.R....I...S.....NI.....LL.K....QV.D..FV..VS..H.KK..
N4/03 .....T...I.....N.TS.R....I...S.....NI.....LL.K....QV.D..FV..VS..H.KK..

Isolated IBVs were designated N1/02, N2/02, N3/02, N4/02, N5/02, N1/03, N2/03, N3/03, N4/03, N5/03, N6/03 and N7/03.

Antigenic typing of these 12 isolates using eight Mabs in ELISA indicated that all isolates were antigenically identical. Results for three representative strains, N4/02, N4/03 and N5/03, are presented in Table 1. As shown, isolated strains differed antigenically from subgroup 2 strains and resembled subgroup 1 strains. Although isolated strains resembled reference subgroup 1 and three vaccine viruses (VicS, VacB2 and VacB3, which are also subgroup 1 strains), they were not identical as they had the epitope recognised by Mab51 but lacked the epitope recognised by Mab7. Coomassie-brilliant-blue staining of samples run on SDS-PAGE gels and tested by haemagglutination inhibition indicated that seven isolates (N1/02, N2/02, N4/02, N2/03, N3/03, N4/03 and N5/03), were free of other avian pathogens; however, the other five (N3/02, N5/02, N1/03, N6/03 and N7/03) contained Newcastle disease virus (results not shown).

### *S1 gene sequencing*

Since broilers from which the 12 IBV strains were isolated had been vaccinated with four different vaccine strains, we initially determined the S1 gene sequence of VacB2, VacB3 and VacC as they were not available. Alignment of the S1 amino acid sequences (Fig. 1) showed that two vaccine strains, VacB2 and VacB3, were almost identical to each other and similar to the vaccine strain VicS, whereas VacC was similar to the N1/62 strain.

The complete S1 genes of isolates N1/02, N3/02, N4/02, N1/03, N2/03, N3/03, N4/03, N5/03, N6/03 and N7/03, originating from ten different farms, were sequenced. An alignment of the deduced S1 amino acid sequences for these ten NSW isolates showed that all shared a high degree of homology with each other (results not shown). The deduced S1 amino acid sequences for three representative isolates, N4/02, N5/03 and N4/03, are presented in Fig. 1, aligned with the vaccine strain VicS. Included in the comparison were subgroup 1 strain N1/62 and subgroup 2 strains N1/88, Q3/88 and V18/91. The S1 sequence of the newly isolated IBV strains differed significantly when compared with vaccine, subgroup 1 and subgroup 2 IBV strains. The new isolates had extensive amino acid changes through the entire length of S1, similar to changes observed in subgroup 2 strains. However, the amino acid changes within the S1 proteins of N4/02, N5/03 and N4/03, were different from those found in either N1/88, Q3/88 or V18/91. A deletion of five amino acids between residues 150 to 154, previously detected in subgroup 2 strains, was also present in N4/02, N5/03 and N4/03. There were only seven regions, between five and eight amino acid residues in length, that were conserved in all strains, the largest of eight amino acids, was at position 244 to 251. The percentages of identity at the nucleotide and amino acid levels between the S1 proteins of N4/02, N5/03, N4/03, subgroup 1 and subgroup 2 strains are shown in Table 2. The S1 proteins of N4/02, N5/03 and N4/03 shared 99–100% identity with each other, between 61–63% amino acid identity with subgroup 1, and 56–59% identity with subgroup 2 strains. Comparison of S1 proteins of

**Table 2.** Comparison of nucleotide and amino acid sequences of the S1 gene of Australian IBV strains

| | Nucleotide identity (%) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | VicS | VacB2 | VacB3 | VacC | N1/62 | N9/74 | N2/75 | N1/88 | Q3/88 | V18/91 | N4/02 | N5/03 | N4/03 |
| Amino acid identity (%) | | | | | | | | | | | | | |
| Subgroup 1 | | | | | | | | | | | | | |
| VicS | 100 | 99.6 | 99.6 | 83.3 | 84.1 | 83.1 | 90.2 | 62.8 | 64.7 | 62.9 | 67.0 | 67.1 | 67.0 |
| VacB2 | 99.0 | 100 | 99.8 | 83.5 | 84.3 | 83.3 | 90.5 | 62.9 | 64.9 | 63.0 | 67.2 | 67.3 | 67.2 |
| VacB3 | 99.0 | 99.6 | 100 | 83.5 | 84.3 | 83.3 | 90.3 | 63.0 | 64.9 | 63.1 | 67.2 | 67.3 | 67.2 |
| VacC | 80.8 | 81.4 | 81.4 | 100 | 98.2 | 89.6 | 83.2 | 62.6 | 63.7 | 62.2 | 66.3 | 66.3 | 66.3 |
| N1/62 | 82.7 | 83.2 | 83.2 | 96.3 | 100 | 90.0 | 83.7 | 62.6 | 63.8 | 62.2 | 66.3 | 66.3 | 66.3 |
| N9/74 | 80.6 | 81.2 | 81.2 | 89.1 | 89.8 | 100 | 84.8 | 62.7 | 65.6 | 62.6 | 67.6 | 67.4 | 67.4 |
| N2/75 | 87.8 | 88.4 | 88.2 | 81.4 | 82.5 | 81.4 | 100 | 62.2 | 63.8 | 61.7 | 66.3 | 66.3 | 66.3 |
| Subgroup 2 | | | | | | | | | | | | | |
| N1/88 | 56.8 | 57.1 | 57.3 | 55.1 | 56.0 | 54.4 | 56.5 | 100 | 78.9 | 95.4 | 61.5 | 61.5 | 61.5 |
| Q3/88 | 60.6 | 60.8 | 60.8 | 59.3 | 59.7 | 59.0 | 61.7 | 73.5 | 100 | 78.6 | 63.5 | 63.5 | 63.4 |
| V18/91 | 56.9 | 57.3 | 57.5 | 54.8 | 55.3 | 53.3 | 55.4 | 92.8 | 72.3 | 100 | 61.5 | 61.5 | 61.5 |
| Isolates | | | | | | | | | | | | | |
| N4/02 | 61.6 | 62.2 | 62.3 | 62.7 | 63.2 | 61.9 | 62.8 | 56.0 | 58.8 | 56.0 | 100 | 99.8 | 99.8 |
| N5/03 | 61.6 | 62.2 | 62.3 | 62.7 | 63.2 | 61.9 | 62.5 | 56.0 | 58.8 | 56.0 | 99.6 | 100 | 99.9 |
| N4/03 | 61.4 | 62.0 | 62.2 | 62.5 | 63.0 | 61.7 | 62.3 | 55.8 | 58.6 | 55.8 | 99.4 | 99.8 | 100 |

**Table 3.** Comparison of nucleotide and amino acid identity for the S1 gene of IBV strains within Australian and European/USA lineages

| Lineage | Nucleotide identity (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | VicS | N1/62 | N1/88 | Q3/88 | N4/02 | N5/03 | M41 | D1446 | 4/91 | D274 | Ark99 | DE072 |
| Amino acid identity (%) | | | | | | | | | | | | |
| Australian | | | | | | | | | | | | |
| VicS | 100 | 84.1 | 62.9 | 64.9 | 66.3 | 66.3 | 83.2 | 57.4 | 78.4 | 81.1 | 80.4 | 57.8 |
| N1/62 | 82.7 | 100 | 62.4 | 63.8 | 65.6 | 65.6 | 81.6 | 58.5 | 78.8 | 80.3 | 80.8 | 58.8 |
| N1/88 | 56.6 | 55.8 | 100 | 78.9 | 62.1 | 62.1 | 63.0 | 55.9 | 63.2 | 63.3 | 63.2 | 54.2 |
| Q3/88 | 60.2 | 59.5 | 73.5 | 100 | 64.3 | 64.3 | 63.7 | 57.4 | 64.1 | 63.7 | 64.3 | 54.3 |
| N4/02 | 61.6 | 63.2 | 56.3 | 58.9 | 100 | 99.8 | 66.5 | 56.9 | 66.8 | 66.8 | 66.9 | 56.5 |
| N5/03 | 61.6 | 63.2 | 56.3 | 58.9 | 99.6 | 100 | 66.5 | 56.9 | 66.9 | 67.0 | 66.9 | 56.5 |
| EU/USA | | | | | | | | | | | | |
| M41 | 78.0 | 80.3 | 55.6 | 55.5 | 60.9 | 61.1 | 100 | 59.4 | 78.5 | 80.2 | 78.7 | 60.0 |
| D1446 | 49.3 | 51.0 | 47.5 | 48.7 | 48.9 | 48.9 | 53.2 | 100 | 58.6 | 58.3 | 59.3 | 65.8 |
| 4/91 | 76.6 | 77.0 | 55.6 | 58.8 | 64.3 | 64.3 | 74.3 | 50.2 | 100 | 78.7 | 77.3 | 57.4 |
| D274 | 79.9 | 79.2 | 56.8 | 58.3 | 64.1 | 64.3 | 77.5 | 50.1 | 78.8 | 100 | 78.6 | 58.4 |
| Ark99 | 79.5 | 81.9 | 55.5 | 58.6 | 63.4 | 63.4 | 75.9 | 51.5 | 75.9 | 78.6 | 100 | 59.0 |
| DE072 | 47.9 | 48.1 | 43.4 | 43.2 | 46.8 | 46.9 | 50.2 | 58.8 | 45.6 | 47.7 | 48.1 | 100 |

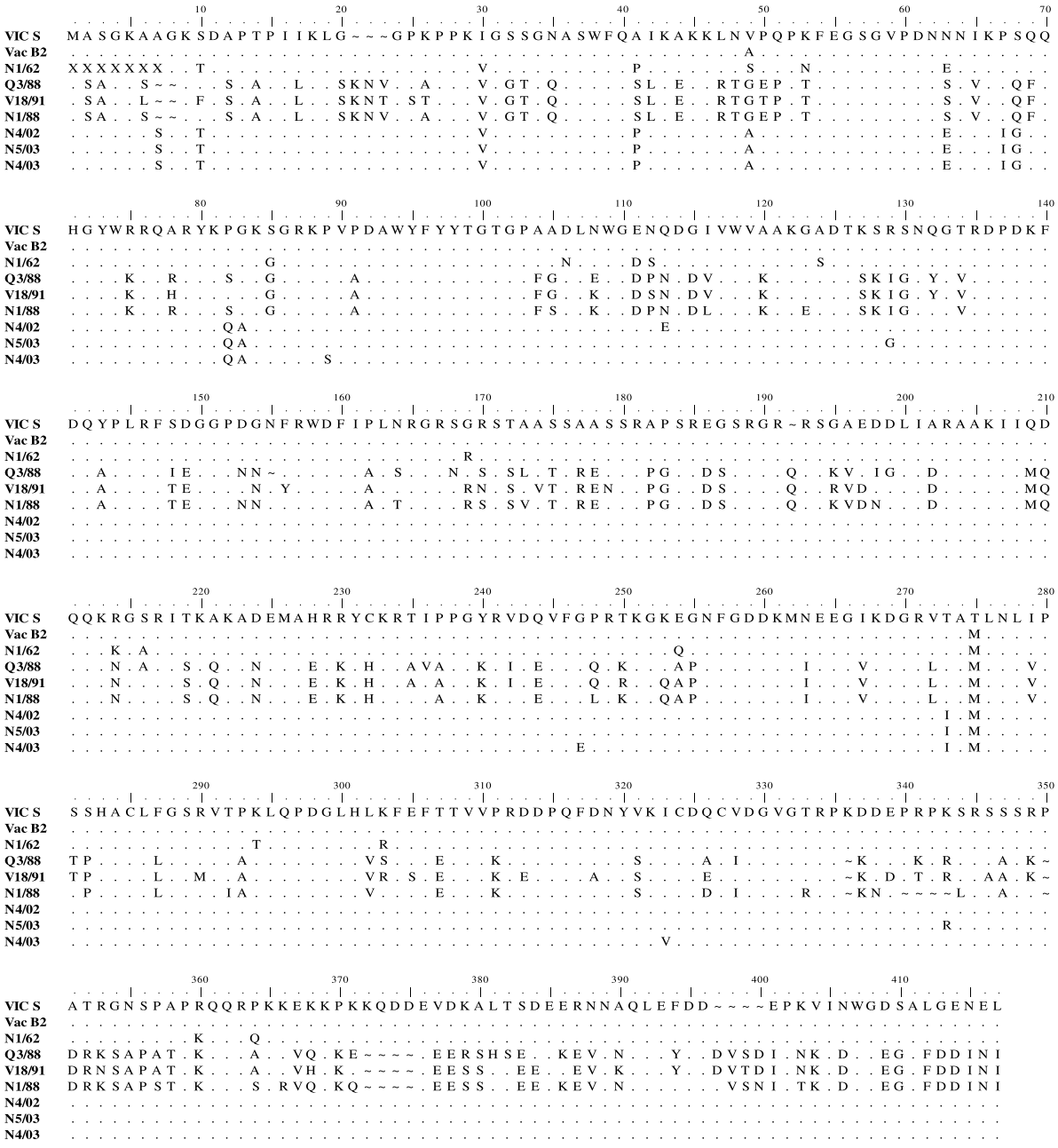



**Fig. 2.** Alignment of deduced amino acid sequences of the N protein of Australian IB vaccines, reference strains and strains isolated in this study. The complete deduced amino acid sequence of N protein of VicS vaccine is shown from amino acid position 1 to 417 [38]. Below this sequence are aligned the deduced amino acid sequences of vaccine VacB2, reference subgroup 1 strain N1/62, subgroup 2 strains N1/88, Q3/88 and V18/91 and IBV strains, N4/02, N5/03 and N4/03 isolated in this study. For all strains only those amino acid residues that differ from VicS are shown. Sequences not obtained are indicated by X

N4/02 and N5/03 with S1 sequences of strains of European/USA lineage is shown in Table 3. The S1 protein of N4/02 and N5/03 differed from the S1 of M41, 4/91, D274 and Ark99 by similar amounts (61–64% identity) as they differed from

**Table 4.** Comparison of nucleotide and amino acid sequences of the N gene of Australian IBV strains

| | Nucleotide identity (%) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | VicS | VacB2 | N1/62 | N9/74 | N2/75 | Q3/88 | V18/91 | N1/88 | N4/02 | N5/03 | N4/03 |
| Amino acid identity (%) | | | | | | | | | | | |
| Subgroup 1 | | | | | | | | | | | |
| VICS | 100 | 99.7 | 92.7 | 90.3 | 92.9 | 63.8 | 64.4 | 64.6 | 97.3 | 97.3 | 97.2 |
| VacB2 | 99.5 | 100 | 92.9 | 90.5 | 93.2 | 64.0 | 64.6 | 64.8 | 97.5 | 97.5 | 97.4 |
| N1/62 | 93.3 | 93.6 | 100 | 91.1 | 93.3 | 63.6 | 64.1 | 63.8 | 92.9 | 92.9 | 92.8 |
| N9/74 | 90.9 | 91.1 | 92.6 | 100 | 90.8 | 62.3 | 64.4 | 62.9 | 90.0 | 90.0 | 89.9 |
| N2/75 | 93.8 | 94.1 | 94.3 | 92.9 | 100 | 63.2 | 63.8 | 64.0 | 93.2 | 93.2 | 93.1 |
| Subgroup 2 | | | | | | | | | | | |
| Q3/88 | 61.1 | 61.3 | 60.6 | 61.8 | 60.9 | 100 | 86.3 | 89.4 | 63.7 | 63.6 | 63.4 |
| V18/91 | 60.9 | 61.1 | 61.1 | 61.8 | 60.1 | 88.0 | 100 | 84.4 | 64.0 | 64.0 | 63.7 |
| N1/88 | 62.0 | 62.2 | 61.2 | 62.2 | 61.0 | 89.2 | 85.8 | 100 | 64.4 | 64.2 | 64.1 |
| Isolates | | | | | | | | | | | |
| N4/02 | 96.8 | 97.3 | 93.1 | 90.2 | 94.1 | 60.6 | 60.4 | 61.5 | 100 | 99.6 | 99.5 |
| N5/03 | 96.5 | 97.0 | 92.9 | 89.9 | 93.8 | 60.9 | 60.6 | 61.5 | 99.2 | 100 | 99.5 |
| N4/03 | 96.3 | 96.8 | 92.6 | 89.7 | 93.6 | 59.9 | 59.7 | 60.8 | 99.0 | 98.7 | 100 |

**Table 5.** Comparison of nucleotide and amino acid sequence of the N gene of IBV strains within the Australian and European/USA lineages

| Lineage | Nucleotide identity (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | VicS | N1/62 | N1/88 | Q3/88 | N4/02 | N5/03 | M41 | D1466 | Ark99 | DE072 |
| Amino acid identity (%) | | | | | | | | | | |
| Australian | | | | | | | | | | |
| VicS | 100 | 92.7 | 64.4 | 63.1 | 97.3 | 97.3 | 89.5 | 87.8 | 89.5 | 88.8 |
| N1/62 | 93.3 | 100 | 63.5 | 62.9 | 92.9 | 92.9 | 88.1 | 87.2 | 88.3 | 87.9 |
| N1/88 | 61.7 | 61.0 | 100 | 89.3 | 64.0 | 63.8 | 63.3 | 64.0 | 63.8 | 63.5 |
| Q3/88 | 60.9 | 60.4 | 89.2 | 100 | 62.9 | 62.8 | 62.9 | 63.7 | 63.5 | 63.0 |
| N4/02 | 96.8 | 93.1 | 61.2 | 60.4 | 100 | 99.6 | 89.4 | 87.8 | 89.5 | 88.9 |
| N5/03 | 96.5 | 92.9 | 61.2 | 60.6 | 99.2 | 100 | 89.4 | 87.8 | 89.5 | 88.9 |
| EU/USA | | | | | | | | | | |
| M41 | 92.1 | 90.4 | 59.8 | 59.2 | 91.6 | 91.4 | 100 | 90.4 | 93.4 | 92.0 |
| D1466 | 91.6 | 90.7 | 61.5 | 60.9 | 91.1 | 90.9 | 91.4 | 100 | 93.0 | 92.9 |
| Ark99 | 92.9 | 90.9 | 61.5 | 60.4 | 92.6 | 92.4 | 93.8 | 93.8 | 100 | 96.3 |
| DE072 | 92.9 | 90.9 | 61.0 | 60.1 | 92.6 | 92.4 | 92.9 | 94.3 | 96.3 | 100 |

Australian subgroup 1 strains VicS and N1/62 (62–63% identity). The S1 proteins of N4/02 and N5/03 also differed from the S1 of D1466 and DE072 (49 and 47% identity, respectively).

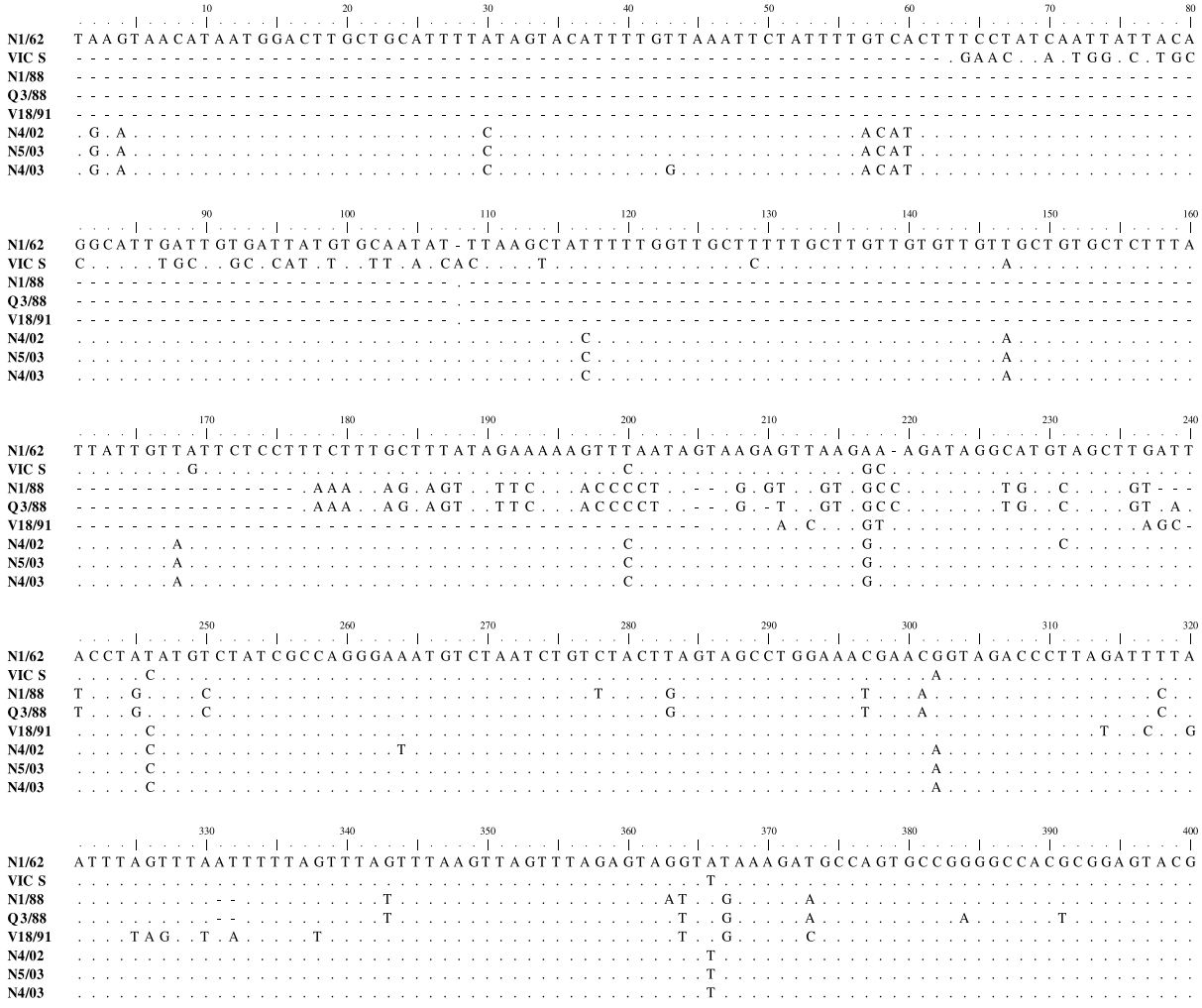
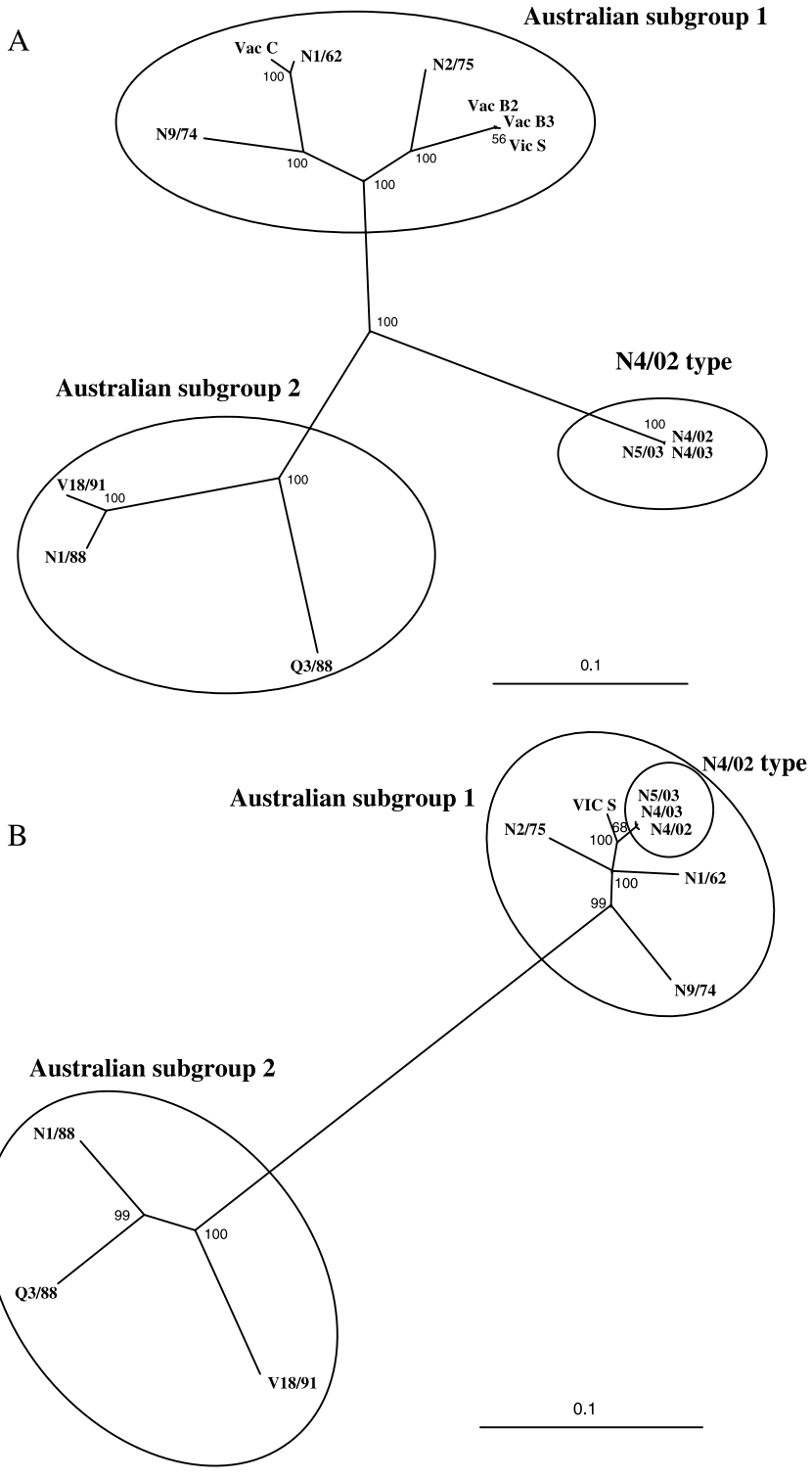



**Fig. 3.** Alignment of nucleotide sequences of the 3′ non-coding region of Australian IBV strains. Nucleotides are numbered from the stop codon for the N gene. For all strains only those residues that differ from N1/62 are shown. Dashes were introduced to align the sequences

**Fig. 4.** Phylogenetic relationship of N4/02, the representative of IBV strains isolated in this study, and Australian vaccine and reference strains. **A** The relationship was based upon nucleotide sequences between nucleotides 1 and 1662 of the S1 gene. **B** The relationship was based upon nucleotide sequences between nucleotides 1 and 1227 of the N gene. The phylogenetic tree was obtained using the neighbor joining method within ClustalX with 1,000 bootstrapping replicas [40]. TreeView was used to display a phylogenetic tree [36]. Branch lengths are proportional to the estimated genetic differences; bar units representing the number of nucleotides substitutions per site. Bootstrap probabilities are expressed as percentages with only those greater than 80% being displayed besides the corresponding node


A
Australian subgroup 1
Vac C
N1/62
N2/75
Vac B2
Vac B3
Vic S
N9/74
100
100
100
56
100
100
N4/02 type
Australian subgroup 2
100
N4/02
N5/03
N4/03
V18/91
100
100
N1/88
Q3/88
0.1
B
N4/02 type
Australian subgroup 1
VIC S
N5/03
N4/03
N4/02
N2/75
68
100
100
N1/62
99
N9/74
Australian subgroup 2
N1/88
99
100
Q3/88
V18/91
0.1

*Sequencing of the N gene and 3′UTR*

Since the Mab ELISA, based primarily on the N protein, indicated that the isolated strains were similar to subgroup 1 strains, but sequence analysis of S1 indicated that they were significantly different, we sequenced the normally conserved N gene and 3′UTR of N4/02, N5/03 and N4/03. The amino acid sequences of the N proteins of N4/02, N5/03 and N4/03 were similar to those of subgroup 1 strain N1/62 and vaccine VicS (Fig. 2). Of the twelve amino acid changes in N4/02, N5/03 and N4/03, ten were at the amino terminal end of the N protein, between residues 7 and 83. The percentage identity at the nucleotide and amino acid levels between the N protein of N4/02 and Australian subgroups 1 and 2 strains are presented in Table 4. As shown, the N protein of N4/02 shared between 90 and 97% amino acid identity with Australian subgroup 1 strains, including vaccine strains. It differed significantly from the N protein of subgroup 2 strains, with which it shared only 60 to 62% identity. The level of amino acid identity between the N proteins of N4/02, N5/03 and IBV strains of the European/USA linage was between 91 and 93% (Table 5).

The nucleotide sequence of the 3′UTR of N4/02, N5/03 and N4/03 are shown in Fig. 3 aligned with subgroup 1 strain N1/62 and VicS vaccine virus as well as subgroup 2 strains Q3/88, N1/88 and V18/91. The 3′UTR of isolated strains was identical in length to the 3′UTR of subgroup 1 strain N1/62. Deletions found previously in subgroup 2 strains N1/88, Q3/88 and V18/91 and vaccine strain VicS were not present in N4/02, N5/03 and N4/03. The nucleotide sequence of the 3′UTR of N4/02, N5/03 and N4/03 was identical to that of VicS from position 140.

*Phylogenetic analysis*

Phylogenetic analysis comparing the nucleotide sequences of N4/02, N4/03 and N5/03 and other Australian strains revealed different clustering depending whether the S1 or N gene was used in the analysis (Fig. 4A and 4B). Based upon the S1 gene, N4/02, N4/03 and N5/03 clustered together in a distinct phylogenetic group well separated from subgroup 1 and subgroup 2 strains (Fig. 4A). In contrast, phylogenetic analysis using the N gene indicated that N4/02, N4/03 and N5/03 belonged to the same cluster as subgroup 1 strains (Fig. 4B).

## Discussion

The IBV strains characterised in this study were shown to be a new genetic type of IBV, not detected previously in Australia. The most prominent feature of this group of viruses was that they had an S1 gene markedly different from all other known IBVs, and an N gene and 3′UTR similar to Australian subgroup 1 strains.

Twelve isolated IBV strains were antigenically and genetically identical to each other, strongly suggesting that a single type of IBV caused the IB outbreak in NSW. The S1 sequence of the representative isolate N4/02 [full designation chicken/Australia/N4/02 [6]] differed significantly from the S1 sequences of other

two genetic types of IBV identified previously in Australia. On average, the S1 protein of N4/02, shared only 62% and 57% amino acid identity with the S1 proteins of subgroup 1 and subgroup 2 strains, respectively. Amino acid changes occurred throughout the S1 protein of N4/02, unlike changes detected in other IBV variants, subgroup 2 strains being an exception, where they tend to cluster in two regions of the S1 protein [9, 10, 26]. There were only seven, small conserved regions. The largest consisted of eight amino acids (position 244 to 251). This region was previously identified as being conserved in all IBV strains [37, 44]. The S1 of N4/02 resembled the S1 of subgroup 2 strains in regard to the extent of amino acid changes, existence of a deletion of five amino acids at position 150 to 154, and locations of the conserved regions. However, a low level of S1 sequence identity with subgroup 2 strains indicated that N4/02 is a new genetic subgroup of IBV.

In contrast to the S1 protein, the N protein and 3′UTR of N4/02, shared high sequence identity ($\geq$90%) with subgroup 1 strains. Sequence analysis of the N protein of N4/02 correlated with the results of the Mab ELISA, which showed that N4/02 was similar to subgroup 1 strains. The Mab antigen ELISA has been used routinely in our laboratory to predict the antigenic type of an isolated IBV, as it is able to discriminate between vaccine viruses, subgroup 1 strains and subgroup 2 strains [18, 21]. Isolation of the N4/02 group of viruses, which by Mab ELISA were predicted to be in subgroup 1, whereas by sequencing of S1 were shown to form a new subgroup, has therefore compromised the predictive value of the Mab ELISA. It has been recognised that differentiation of IBV variants using antigenic typing must be viewed with caution, as the predictive value might not be meaningful or accurate [13].

Phylogenetic analysis based upon nucleotide sequences of the S1 and N genes indicated a different relationship of N4/02 to other Australian IBV strains. Based upon the S1 gene, N4/02 belonged to a separate phylogenetic subgroup within Group 3, distant from subgroups 1 and 2, whereas based on the N gene, N4/02 belonged to the phylogenetic subgroup 1. Genetic analysis of the remaining genes of N4/02 is currently underway (K. Mardani, personal communication) to elucidate further its genetic relatedness to other IBV.

The origin of N4/02 is currently unknown. Mutation, as a mechanism, could not explain the origin of N4/02. If the VicS vaccine, the strain most closely related genetically to N4/02, was an ancestor, then the VicS S1 gene would have had to undergo multiple mutations throughout the entire S1 gene. Such a high mutation rate has not been encountered thus far in IBV, either in field or experimental infections [9, 10]. Accumulation of mutations over time could have given rise to strains that bear some similarity with N4/02, and from which N4/02 could have further evolved by mutations. However, monitoring of broilers for IBV variants over recent years did not detect any such strains (37, J. Ignjatovic and K. Mardani, unpublished results). If recombination was involved in the generation of N4/02, two different viruses could have been the source of the S1 gene and N gene/3′UTR. Such a recombination event is theoretically feasible since regions between the S1 and N genes are involved in recombination [4, 30]. However, while the source

virus for the N gene and 3′UTR might have been a subgroup 1 strain of IBV such as N1/62, the source of the S1 gene is unknown. The origin of two other similar strains, D1466 and DE072 [15, 28], is also unclear. Differences between the S1 gene sequences of the DE072 and D1466 and other IBV strains indicate that they did not originate directly from live vaccine viruses or other common IBV strains [15]. Instead, it is speculated that D1466 and DE072 had the same origin, which is different from other IBVs [31], but that two viruses diverged a long time ago and evolved independently in different geographical locations [30]. Although this might have happened, it is not clear what their origin is and, if they have evolved over time, why strains with some S1 sequence similarity to either D1466 or DE072 had not been detected in either location prior to their sudden emergence.

The origin of three subgroup 2 Australian strains, N1/88, Q3/88 and V18/91, which have markedly different S1 and N genes, has not been determined either. One possibility is that the N4/02 type, as well as subgroup 2 strains, have been introduced into broiler chicks from an unknown source, possibly another avian species. Analysis of more than fifty IBV strains isolated in Australia between 1961 and 2002 did not detect any IBV resembling either subgroup 2 or N4/02 type prior to their emergence in 1988 and 2002, respectively [22; K. Mardani personal communication]. Both subgroup 2 and the N4/02 type of IBV caused outbreaks in vaccinated flocks over a prolonged period of time and required introduction of homologous vaccines for their control (J. Ignjatovic, unpublished observation). Hence, because of their unique genetic, antigenic and immunogenic properties, it is unlikely that precursors to subgroup 2 and N4/02 type of IBV could have circulated in poultry over a prolonged period without being detected.

In the case of N4/02, a recombination event could have occurred between the ancestral N4/02 and a subgroup 1 field strain in an unknown host prior to these viruses being transmitted to chickens. Currently, little is known about the relevance of other types of avian coronaviruses and avian species other than chickens as reservoirs for the epidemiology of IBV [6]. Recently, turkey coronaviruses were shown to be more similar to IBV than to mammalian coronaviruses, where they had been classified for many years [17]. The N proteins and 3′UTR of turkey coronaviruses and IBV share $\geq$90% amino acid and $\geq$78% nucleotide identity, respectively [1, 3, 11, 33]. However, the S1 genes of turkey coronaviruses share only 33% amino acid identity with the S1 protein of IBV [33]. Turkey coronaviruses, however, are not considered recombinants of IBV, but to be a species genetically distinct from IBV [11, 17]. Similarly, it is possible that N2/04 is not a recombinant, but an avian coronavirus originating from another avian species that has crossed into commercial chickens. It has been highlighted previously that the evolution of IBV is a complex, and poorly understood process [23], and this still remains so. The criteria and attributes that differentiate one avian coronavirus from others are currently unclear [6]. Recently, coronaviruses that are similar to IBV have been isolated from pheasants, domestic peafowl and teals [12, 34]. However, coronaviruses isolated from graylag gees, feral pigeons and mallard ducks differed significantly, with some unique genetic features [25].

In conclusion, detection of another genetic type of IBV in Australia further demonstrates the unpredictable nature of IBV evolution and our limited understanding of factors and possible reservoirs involved in the emergence of new IBV variants.

## Acknowledgements

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