

Naturally occurring recombination between distant strains of infectious bronchitis virus

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Abstract New variants of infectious bronchitis virus (IBV) have emerged in Australia despite its geographical isolation and intensive vaccination programs. In the present study, the 3' terminal 7.2 kb of the genome of a recently isolated variant of IBV (N1/03) was sequenced and compared with the sequences of classical and novel strains of IBV, the two main groups of these viruses in Australia. The comparison revealed that recombination between classical and novel IBVs was responsible for the emergence of the new variant. It was concluded that novel IBVs, which have not been detected since 1993, and which are phylogenically more distant from classical IBVs than turkey coronaviruses, might still be circulating and contributing to the evolution of IBV in Australia.

Introduction

Infectious bronchitis virus (IBV), the prototype of the family *Coronaviridae*, is an important pathogen in chickens and infects the respiratory tract, kidneys and oviduct, causing reduced performance, reduced egg quality and quantity, increased susceptibility to infection with other pathogens and increased mortality [4]. The genome of IBV

is single-stranded, positive-sense RNA of about 27.6 kb, encoding four structural proteins, including the spike glycoprotein (S), the membrane glycoprotein (M), the phosphorylated nucleocapsid protein (N) and the small membrane protein (E).

New serotypes and genotypes of IBV emerge frequently in different parts of the world [1, 6, 9, 10, 15, 18, 30, 31]. A number of factors, including mutation and recombination, and the widespread use of live attenuated vaccines, play an important role in increasing the number of new genetic variants [3, 8, 15, 16, 28]. Recombination and mutation are two major forces in coronavirus evolution, and polymerase jumping during coinfection may promote recombination events between coronaviruses [22]. Molecular characterization using sequencing and phylogenetic analysis are effective methods for detection and characterization of recombination events among RNA viruses [29].

N1/62, a nephropathogenic strain of IBV, was the first isolate of IBV obtained in Australia, in 1962 [7]. Since 1962, a number of distinct strains of IBV have been isolated, including a group of strains that were found to have no epitopes in common with other IBV strains on either their N or M proteins [12]. In a study on the pathogenicity of Australian strains of IBV, a change was detected in the prevalence of IBV strains, from highly nephropathogenic strains in the 1960s and 1970s to strains predominantly pathogenic for the respiratory tract in the 1980s and early 1990s, indicating that IBV strains in Australia have undergone a significant change since 1962 [13].

IBV strains in Australia have been classified into two groups, classical and novel [21], based on their genotypes. The novel IBVs are phylogenetically distant from the classical IBVs, and in fact, classical IBVs and turkey coronaviruses are more similar to each other than they are to the novel IBVs. Recently, the isolation of a new variant

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of IBV in Australia, from broiler farms located in New South Wales, that had low S1 gene identity to those of classical and novel IBVs but N gene and 3' untranslated region sequences similar to those of classical IBVs, suggested the emergence of a new subgroup of IBVs in Australia [14]. In this study, the new variant of IBV that circulated in NSW during 2002 and 2003 was further analysed by comparing the 3' terminal 7.2 kb of its genome (all the structural protein genes) with those of the other two IBV groups in Australia.

Materials and methods

IBV strain

The IBV strain N1/03 used in this study has been described previously [14].

Extraction of RNA

Virus propagated in allantoic fluid was used for RNA extraction. Extraction of RNA was performed using an RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Approximately 50 µl of allantoic fluid was used for each extraction, and RNA was eluted in 30 µl of elution buffer. The extracted RNA was used as template in a reverse transcription reaction.

Synthesis of cDNA

For synthesis of cDNA, 5 µl of extracted RNA was denatured at 100°C for 1 min, cooled by placing on ice for 5 min, and then mixed with 20 µl of reaction buffer containing 10 µl of diethylpyrocarbonate-treated water, 0.5 µM oligo (dT) (Promega, Madison, WI, USA), 0.65 U of RNAGuard (Amersham Pharmacia Biotech, Sydney, Australia), 50 µM each of dATP, dTTP, dGTP and dCTP, 5 µl of 5× reaction buffer (Promega), and 50 U of Moloney murine leukaemia virus reverse transcriptase (Promega). The reaction mixture was incubated at 42°C for 1 h and subsequently incubated at 100°C for 5 min to inactivate the reverse transcriptase. The resultant cDNA was immediately used or stored at -70°C for later use.

Polymerase chain reaction (PCR)

For the amplification reaction, two primers, POLY-F1 (GATTGTGCATGGTGGACAATG) and UTR-R1 (CTGTACCCTCGATCGTACTC), binding to the 3' end of the polymerase gene (nucleotides 20,070–20,090, Beaudette strain, GenBank accession number NC_001451) and the 3' untranslated region (nucleotides 27,489–27,508, Beaudette

strain) of the IBV genome, respectively, were used to amplify a 7.2-kb fragment of the IBV genome that contains all of the genes for the structural proteins. The PCR reaction was carried out in 50-µl mixtures containing 50 µM each of dATP, dTTP, dGTP and dCTP, 0.5 µM of each primer, 5 µl of 10 × High Fidelity PCR buffer (Invitrogen, Carlsbad, CA, USA), 2 mM magnesium sulfate, 1.5 U Platinum *Taq* High Fidelity DNA polymerase (Invitrogen) and 5 µl of cDNA as template. Amplification was performed using 35 cycles of incubation at 94°C for 30 s, 57°C for 30 s and 68°C for 8 min, with a final extension at 68°C for 10 min. The resultant PCR products were separated in a 0.8% agarose gel, and the gel was photographed. PCR product was purified using a PCR purification kit (Ultra Clean PCR Clean-Up, Mo Bio Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions.

DNA sequencing

The 3' 7.2-kb PCR product from IBV strain N1/03 was cloned in the pGEM-T plasmid using a pGEM-T Vector System Cloning Kit (Promega). The cloned PCR product was sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and the reaction products were sent to the Australian Genomic Research Facility, Walter and Eliza Hall Institute of Medical Research, Melbourne, for analysis on an ABI Prism 3100 Genetic Analyzer.

Sequence analysis

Sequences of different genes of N1/03 were aligned with the same genes from other Australian IBVs and the Beaudette strain (Table 1) using ClustalW [11]. Evolutionary relationships between Australian IBV genes were inferred using the neighbour-joining method [24]. The evolutionary distances were computed using the Maximum Composite Likelihood method [26] and measured as the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA 4 [27].

Similarity and breakpoint analysis

The 3'-terminal 7.2 kb of the genomes of the representative classical and novel Australian IBV strains were aligned with the sequences from the same region of the N1/03 isolate, and the multiple alignment results were introduced into SimPlot version 3.5.1 to identify likely recombination sites [19]. Bootscanning analysis was performed in Simplot to identify and map the putative recombinant IBV genome [25]. This program was also used to find phylogenetically informative sites, as described previously by Robertson et al. [23].

Table 1 IBV sequences used in this study

Strain	Accession number
Vic S	DQ490221
Armidale	DQ490205
A6	DQ490213
N1-62	DQ490206
V2-71	DQ490216
Q1-73	DQ490209
N2-75	DQ490208
Q1-76	DQ490210
Q1-99	DQ490211
V3-02	DQ490217
Beaudette	NC_001451
N1-88	DQ490207
Q3-88	DQ490212
V18-91	DQ490220

Statistical analysis

Statistical analysis of the distribution of phylogenetically informative sites was performed using Minitab version 15 (Minitab Inc, US). A two-proportion comparison was performed, and a *P* value of less than 0.05 was considered significant.

Results

Amplification, sequencing and analysis of the 3' 7.2-kb region of N1/03

The 3' 7.2 kb of the N1/03 genome was amplified, cloned and sequenced successfully and compared with the same region of a number of classical and novel strains. Phylogenetic trees were constructed for each gene.

Analysis of the S gene sequences of Australian IBV strains showed that, among classical strains, they differed by 3–12.5% and, among novel strains, by 3.2–16.4%. The classical and novel IBVs differed by 36.9–39.9%, indicating a major difference between the S genes of these two groups of IBVs. Interestingly, the S1 gene sequence of strain N1/03 differed from those of both classical and novel IBVs by 29.2–32%. Phylogenetic analysis of the S gene grouped N1/03 in a separate cluster from classical and novel IBVs (Fig. 1).

E gene sequences of classical strains differed by 0–9.8%, and those of novel strains by 24–27%. The difference between classical and novel IBVs ranged from 66.5 to 78.8%. More interestingly, the E gene sequence of N1/03 was very similar to those of the classical strains (differences of 3.8–8.9%) and very different from those of novel

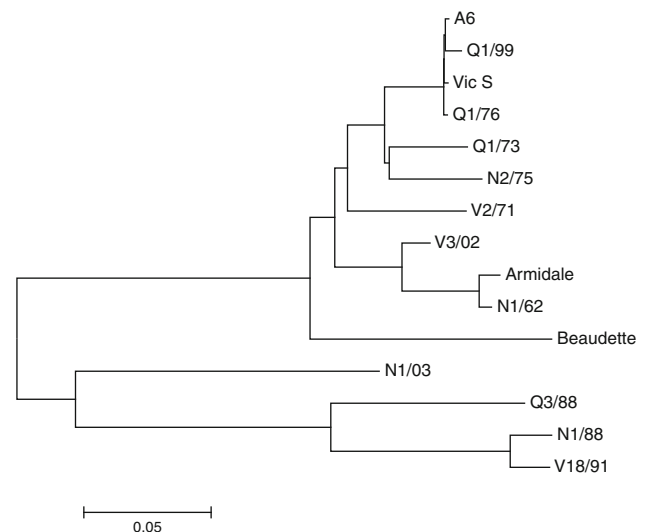


Fig. 1 Evolutionary relationships between S genes of Australian IBV strains inferred using the neighbour-joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are given in the number of base substitutions per site

IBVs (differences of 72.2 to 73.4%). In the E gene phylogenetic tree, N1/03 clustered with the classical IBVs (Fig. 2).

M gene sequences of classical strains differed by 0–10.4%, and those of novel IBVs differed by 3–3.6%. The M gene sequences of classical and novel IBVs differed by 54.2–60.9%. The N1/03 M gene clustered with the classical strains (differences of 2.4–9.8%) (Fig. 3).

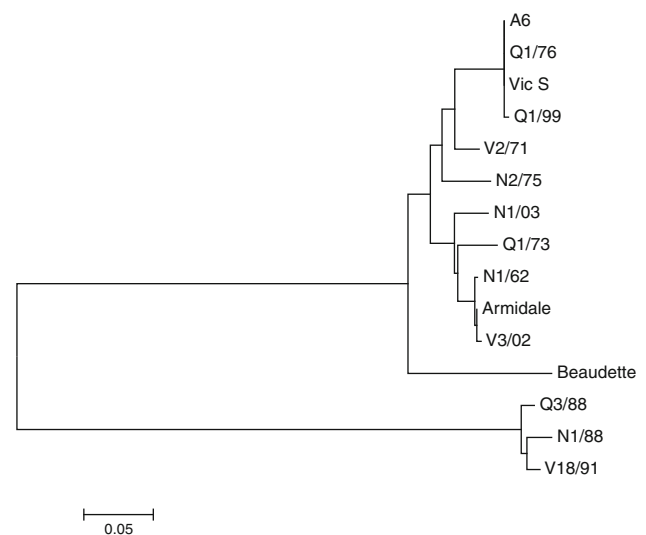


Fig. 2 Evolutionary relationships between E genes of Australian IBV strains inferred using the neighbour-joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are given in the number of base substitutions per site

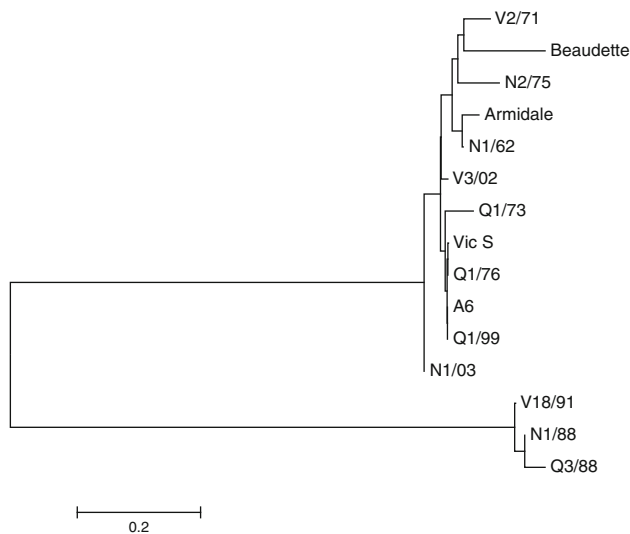


Fig. 3 Evolutionary relationships between M genes of Australian IBV strains inferred using the neighbour-joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are given in the number of base substitutions per site

The N gene of classical IBVs differed by 0.2–8.3%, and those of novel IBVs differed by 9.9–16.4%. The N gene sequences of classical and novel IBVs differed by 50.7–54.4%. The N gene of N1/03 was more similar to the N genes of the classical IBVs (differences of 2.4–7.7%) than of novel IBVs (differences of 54.3–55.6%) and clustered with the classical strains in phylogenetic analyses (Fig. 4).

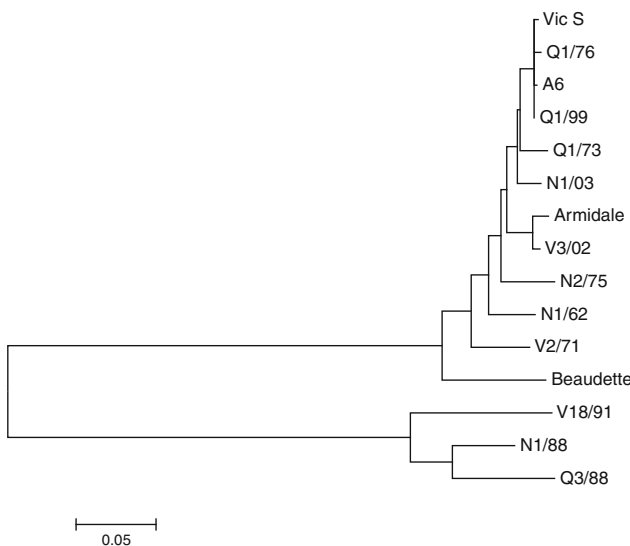


Fig. 4 Evolutionary relationships between N genes of Australian IBV strains inferred using the neighbour-joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are given in the number of base substitutions per site

Similarity plot and bootscanning analysis of the 3'-terminal 7.2 kb of IBV viruses

To identify the region likely to have been involved in recombination in N1/03, similarity plots and bootscan analysis were performed using strains VicS and N1/88 as representatives of the two main groups of IBV in Australia and the Armidale strain as a query. In both the similarity plots and the bootscan graph, N1/03 had greater similarity to N1/88 (a novel IBV strain) in its S gene region (almost 3 kb), while the remainder of the 3'-terminal 7.2-kb region had greater similarity to VicS (a classical IBV strain) (Fig. 5, 6). Data from phylogenetic analyses also suggested that N1/03 had sequence similarity to both main groups of Australian IBVs. These findings suggested that there had been a recombination event with a crossover point at the end of the S gene (base number 3000) of N1/03 (Fig. 5, 6).

To obtain a precise picture of this possible crossover point, the distribution of phylogenetically informative sites along the 3'-terminal 7.2-kb sequences of N1/88 and VicS were examined. Using Simplot, 456 informative sites were found that could support one of three possible trees. For almost 90% of the S gene (3 kb), 365 informative sites were found, and 84% of these informative sites supported a tree clustering N1/03 with the novel IBV strain N1/88. In contrast, only 8% of the informative sites supported each of the other two possible trees in this region ($P < 0.0005$). For the rest of sequences (3,000–7,200 bp), 95 informative sites were found, with 60% supporting the tree clustering N1/03 with VicS and only 14% supporting the tree clustering N1/03 with N1/88 ($P < 0.05$). This indicated that N1/03 clustered with the classical IBV strain VicS throughout the 3'-terminal 4.2 kb of its genome and confirmed that a recombination event had occurred at the end of the S gene region (at around position 3000) and that N1/03 is a recombinant virus that emerged from recombination between these two groups of IBVs in Australia.

Discussion

New IBV isolates have been reported in recent years in Australia and have been classified as subgroup 3 viruses. The emergence of these new isolates was considered to be unexplained, as was the earlier emergence of subgroup 2, or novel viruses. N1/03 is one of these new viruses, which have been isolated from broiler farms using one of the four commercial IBV vaccines [14]. This study was undertaken to explore the mechanism behind the emergence of the subgroup 3 IBVs in Australia.

Based on phylogenetic analysis of the complete 3' 7.2-kb region of the genome, N1/03 was distinct from both classical and novel IBVs. However, the S gene sequence of

Fig. 5 Similarity plot analysis of Australian IBVs. The y-axis shows the percentage similarity within a sliding window of 200 bp centered on the position plotted, with a step size between plots of 20 bp. IBV strains VicS and N1/88 were compared to N1/03, and Armidale was used as the query strain. The genomic map is shown at the top of the plot

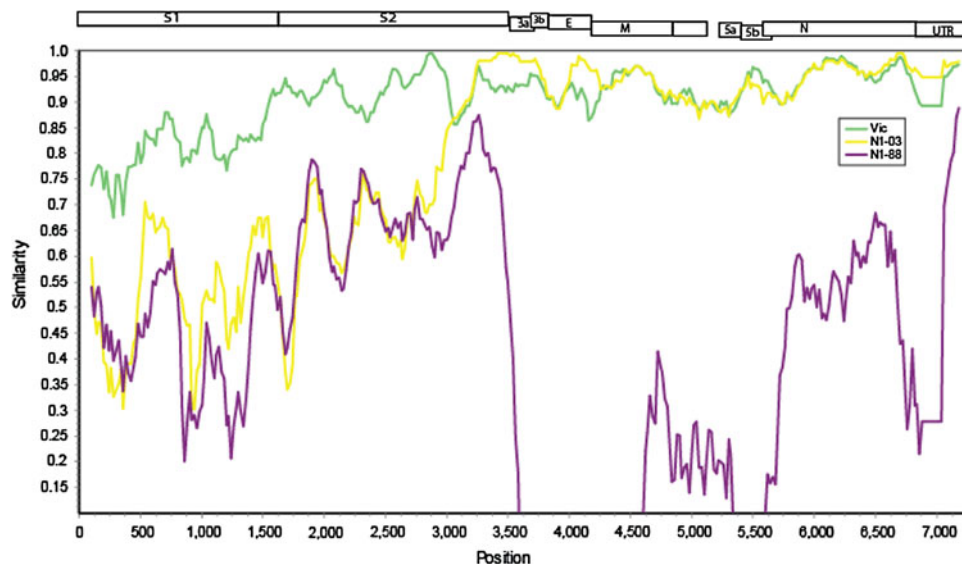
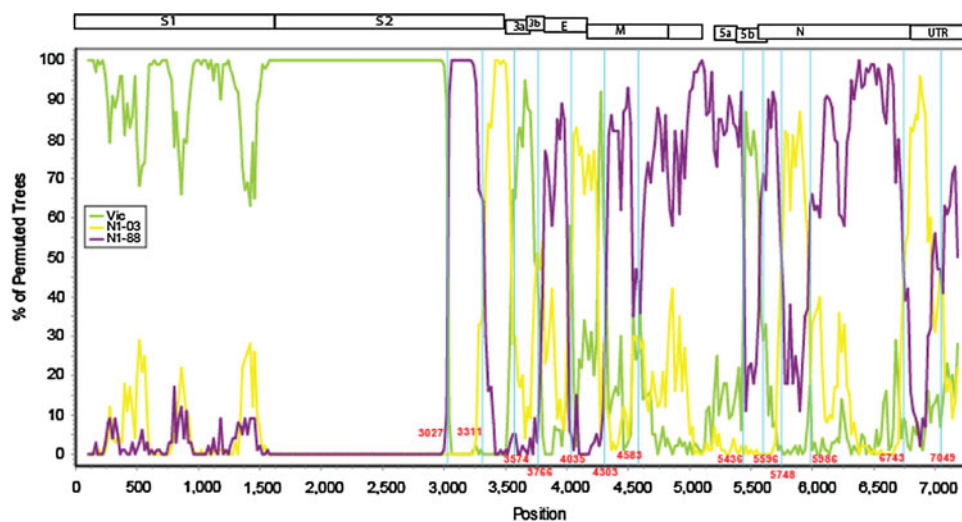


Fig. 6 Bootscanning analysis of the 3' terminal 7.2-kb of the genomes of Australian IBVs. The y-axis shows the percentage of permuted trees using a sliding window of 200 bp wide centered on the position plotted, with a step size between plots of 20 bp. IBV strains VicS and N1/88 were compared to N1/03, with Armidale used as the query strain. The genomic map is shown at the top of the plot. The vertical lines are the breakpoints and the numbers at the bottom of these lines are their positions in the sequence



N1/03 was equally different from both the classical and novel IBVs, while the rest of the 3'-terminal 7.2-kb region of the genome clustered closely with those of the classical IBVs and was very different from those of the novel IBVs. The similarity of the N1/03 E, M and N genes to those of classical IBVs suggested that this isolate was closely related to the most commonly used Australian IBV vaccine strains. As these strains have been used for a long time in Australia, it is possible that they have contributed to the emergence of variant IBVs in the field. The S gene of N1/03 clustered more closely with the novel IBVs, and similarity plotting and bootscan analysis confirmed the close relationship between the S genes of N1/03 and the novel IBVs and suggested that the crossover event occurred near the 3' end of this gene.

These analyses provide convincing evidence that recombination has occurred between classical and novel IBVs, leading to emergence of variant IBVs in the field.

Novel IBVs have not been isolated since 1992, but this finding suggests that novel IBVs are still circulating in the field in Australia. The failure to detect them since 1992 may be a result of the relatively slow growth of these viruses [20]. These results confirm the hypothesis of Ignjatovic et al. [14] that recombination may have played a role in the emergence of this new variant of IBV in Australia.

Recombination between IBVs and its role in emergence of new IBV variants has been reported previously [2, 15, 17, 21] and may occur at multiple sites [15, 17], although crossover events occur more frequently at the 3' end of the S gene [5, 15, 21], as seen in this study. However, it is particularly notable here because of the very significant phylogenetic distance between the novel and classical Australian strains of IBV. Indeed, the novel strains of IBV are more distinct from the classical strains than is turkey coronavirus, and they lack several of the smaller non-structural genes found in classical strains.

In conclusion, this study showed that the recombination was involved in the emergence of the new variant of IBV in Australia and that a rapid and reliable technique is needed to determine whether the novel IBVs are still circulating in poultry farms. It would be appropriate to sequence and analyse the polymerase genes of classical, novel and new variant strains of IBV to obtain further information about the relationships between the different Australian IBVs.

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