Veterinary Microbiology 197 (2016) 27–38

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Full genome analysis of Australian infectious bronchitis viruses suggests frequent recombination events between vaccine strains and multiple phylogenetically distant avian coronaviruses of unknown origin

José A. Quinteros[a,*], Sang-Won Lee[a,c], Philip F. Markham[a], Amir H. Noormohammadi[b], Carol A. Hartley[a], Alistair R. Legione[a], Mauricio J.C. Coppo[a], Paola K. Vaz[a], Glenn F. Browning[a]

[a] Asia-Pacific Centre for Animal Health, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, 3010, Victoria, Australia
[b] Asia-Pacific Centre for Animal Health, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Werribee, 3030, Victoria, Australia
[c] College of Veterinary Medicine, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 143-701, Republic of Korea, Republic of Korea

ARTICLE INFO

Article history:
Received 1 September 2016
Received in revised form 3 November 2016
Accepted 4 November 2016

Keywords:
Infectious bronchitis virus
Recombinants
Unknown avian coronaviruses
Full genome sequences
Vaccine recombinants
Recombination

ABSTRACT

Australian strains of infectious bronchitis virus (IBV) have been evolving independently for many years, with control achieved by vaccination with local attenuated strains. Previous studies have documented the emergence of recombinants over the last 20 years, with the most recent one, Ck/Aus/N1/08, detected in 2008. These recombinants did not appear to be controlled by the vaccines currently in use.

In this study we sequenced the complete genomes of three emergent Australian strains of IBV (IBV/Ck/Aus/N1/88, IBV/Ck/Aus/N1/03 and IBV/Ck/Aus/N1/08) and a previously incompletely characterised vaccine strain, IBV/Ck/Aus/Armidale, and compared them to the genome of the vaccine strain VicS. We detected multiple recombination events throughout the genome between wild type viruses and the vaccine strains in all three emergent isolates. Moreover, we found that strain N1/88 was not entirely exogenous, as was previously hypothesised. Rather, it originated from a recombination event involving the VicS vaccine strain. The S glycoprotein genes of N1/88 and N1/03 were known to be genetically distinct from previously characterised circulating strains and from each other, and the original donors of these genes remains unknown. The S1 glycoprotein gene of N1/88, a subgroup 2 strain, shares a high nucleotide identity with the sequence of the S1 gene of the recent isolate N1/08. As the subgroup 2 strains have not been isolated for at least 20 years, it appears likely that an unknown avian coronavirus that was the donor of the S1 glycoprotein sequence of N1/88 in the 1980s is still recombining with IBV strains in the field.

1. Introduction

Infectious bronchitis (IB) is a disease mainly affecting chickens that is caused by the infectious bronchitis virus (IBV), a member of the genus Gammacoronavirus in the family Coronaviridae (International Committee on Taxonomy of Viruses, 2015), with a single stranded positive sense RNA genome approximately 27.6 kb in length (Jackwood and de Witt, 2013). It causes significant losses in meat and egg producing chickens due to its effects on growth rates, egg production and quality, and mortality in infected flocks. There is no effective treatment, and vaccination with attenuated vaccine strains is the major tool used to prevent the losses in production caused by IBV in Australia (Chousalkar et al., 2009). Inactivated vaccines are used outside Australia, normally as part of vaccination programs in combination with live attenuated vaccines, particularly in layer and breeder flocks.

There is considerable variation in the virulence and tropism of IBVs, and new strains frequently emerge, in part due to point mutations, deletions and insertions. However, one of the most

* Corresponding author at: Veterinary Preclinical Centre, Corner of Flemington Road and Park Drive, Parkville, 3010, Victoria, Australia.
E-mail address: jose.quinteros@unimelb.edu.au (J.A. Quinteros).

http://dx.doi.org/10.1016/j.vetmic.2016.11.003
0378-1135/

important mechanisms underlying the emergence of new strains is genomic recombination (Jia et al., 1995). The emergent virulent strain Ark DPI appears to have originated from recombination between four different IBV strains (Ammayappan et al., 2008). Additionally, the emergence of new strains of IBV in the USA has been reported to have arisen from recombination events between the Massachusetts (Mass), Conn and Holte strains and field strains (Thor et al., 2011).

In Australia, IBV strains have been classified into three subgroups based on the nucleotide sequences of the genes encoding their S1 protein (Ignjatovic et al., 2006): subgroup 1, which includes vaccine or vaccine-related strains; subgroup 2, a relatively new group that emerged at the end of the 1980s and the beginning of the 1990s; and subgroup 3, which includes more recently isolated strains that have been shown to be derived from recombination between subgroup 1 and 2 strains (Mardani et al., 2010).

There have been several episodes of re-emergence of IB as a clinical problem in Australian chicken flocks, and it has been suspected that the emergent viruses may have originated from recombination events. The origin of subgroup 2 strains has remained unknown, but as the S1 gene of this subgroup shares less than 64% nucleotide (nt) sequence identity with those of subgroup 1 (vaccine related) strains (Sapats et al., 1996), it has been assumed that they may have arisen from a coronavirus exogenous to chickens (Mardani et al., 2008). The subgroup 3 strains, which were first isolated in 2002–2003, have an S1 gene that differs considerably from those of subgroup 1 and 2 strains, with nucleotide identities of 61–63% and 56–59%, respectively (Ignjatovic et al., 2006). Phylogenetic and similarity plot analyses of the genomic region encoding the structural genes have suggested that this subgroup emerged as a result of recombination between subgroup 1 and 2 strains (Ignjatovic et al., 2006; Mardani et al., 2010). The strain Ck/Aus/N1/08 (N1/08) was isolated from a flock of chickens with clinical signs of respiratory disease that had previously been vaccinated against IB. Analysis of the section of the genome encoding the structural proteins found evidence of recombination in the S1 gene, with the subgroup 2 strain N1/88 and a subgroup 3 strain as possible parental viruses (Hewson et al., 2014).

Although there is evidence of involvement of recombination within the structural and accessory genes in the emergence of new strains of IBV in Australia, little is known about the genomic region that contains the polymerase genes. Here, we sequenced the complete genomes of subgroup 1, 2 and 3 strains in order to detect potential recombination hot spots in the polymerase genes, and to investigate the potential involvement of recombination in these regions in the emergence of novel IBV strains.

2. Materials and methods

2.1. Virus strains and culture

Four different Australian strains of IBV were selected for this study: one from subgroup 1 (Armidale A3), a vaccine strain from subtype (subtype C) distinct from the most commonly used vaccine strain VicS (subtype B), as assessed by virus neutralisation, (Wadey and Faragher, 1981), one from subgroup 2 (IBV/Ck/Aus/N1/88, or N1/88) (Arvidson et al., 1991), one from subgroup 3 (IBV/Ck/Aus/N1/03, or N1/03) (Ignjatovic et al., 2006), and a relatively recent recombinant strain IBV/Ck/Aus/N1/08, or N1/08 (Hewson et al., 2014).

Viral culture was performed by inoculating the allantoic cavity of 8- to 9-day-old embryonated specific pathogen-free chicken eggs. After 48 h incubation, the allantoic fluid (AF) was aseptically collected. The AF extracted from the embryonated eggs was clarified and the virus purified as described previously (Lougovskaia et al., 2002), with minor modifications. Briefly, the AF was clarified by centrifugation at 2,500 × *g* for 20 min. The supernatant was then centrifuged at 100,000 × *g* for 2 h at 4 °C. Viral pellets were then resuspended in 200 μL of Tris-buffered saline (TBS) (pH 7.4), and this viral suspension was layered over a 30% to 55% continuous sucrose gradient in TBS. The gradient was centrifuged at 100,000 × *g* for 4 h at 4 °C. The virus band was resuspended in TBS and the viral particles pelleted by centrifugation at 90,000 × *g* for 1 h and the pellet resuspended in 250 μL of TBS. The genomic RNA was extracted using RNeasy kits (Qiagen), following the manufacturer's instructions for RNA extraction from cell cultures and tissues, with minor modifications (Asia Pacific Centre of Animal Health protocol for RNA extraction from allantoic fluid). In summary, the initial denaturation was performed by mixing 100 μL of the viral pellet with 400 μL of lysis buffer plus 5 μL of β-mercaptoethanol, and the mixture was incubated at 4 °C overnight. A 300 μL volume of 70% ethanol was added to the lysate. For the steps with washing buffers, the time and speed were changed from 15 to 30 s, and from 8000 to 10,000 × *g*. The final centrifugation at full speed was performed for 2 min instead of 1 min, and the speed during the elution step was modified from 8000 to 10,000 × *g*.

2.2. Complete genome sequencing

RNA was fragmented using RNase III (Life Technologies) and purified using magnetic beads. To construct the libraries, the RNA was reverse transcribed and the cDNA purified and amplified following the manufacturer's protocols, and then sequenced (Quinteros et al., 2015). Sequencing was performed using the PGM Ion Torrent™ platform (Life Technologies), with a 314 chip and the 200-base sequencing Ion OneTouch Kit v2. All the sequencing protocols were performed at the Monash Health Translation Precinct (MHTP) medical genomics facility, within the Monash Institute of Medical Research, Victoria, Australia. All reads were compared to chicken ribosomal RNA and mitochondrial genome sequences, and the matching reads were discarded. The remaining reads were mapped using the genome sequences of the Beaudette strain of IBV, two IBV vaccine strains from the United States (strains Conn46 1996 and Massachusetts; Genbank accession numbers FJ904716 and GQ504724, respectively), and two strains from China (SAIBK and SC021202; Genbank accession numbers DQ288927 and EU714029, respectively) as reference sequences, using Geneious version 6.1.4 (Biomatters). Because of the high level of sequence diversity of the S gene, the reads were also mapped using the previously determined sequences of the entire structural protein gene region (from the S glycoprotein to the 3′ end) of the Australian strains Armidale, N1/08, N1/88, VicS-del and VicS (accession numbers DQ490205, JN176213, DQ490207, JN983807 and JN176213, respectively), and the sequences of the S and N genes of N1/03 (accession numbers FJ235186 and FJ235194, respectively). All gaps and ambiguous sequences were resolved by Sanger sequencing of PCR products using BigDye® Terminator v3.1 (Applied Biosystems) kits with specific primers designed using Primer 3 (Untergasser et al., 2012). The list of primers are available upon request.

In order to confirm the sequences generated using this approach, a second assembly method was used. The reads were assembled *de novo*, using Geneious version 8.1.8 (Biomatters), allowing a maximum gaps per read of 10% and a maximum gap size of 2 nucleotides. The minimum overlap allowed was 75 nucleotides, with at least 95% identity in the overlapping region. The maximum proportion of mismatches per read allowed was 10%, with an ambiguity of 2 bases or less. All contigs were then mapped to the reference sequence, using at least 4 different levels