Recent US bluetongue virus serotype 3 isolates found outside of Florida indicate evidence of reassortment with co-circulating endemic serotypes

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Abstract

Since 1999, 11 serotypes of bluetongue virus (BTV) similar to Central American or Caribbean strains have been isolated in the southeastern United States, predominantly in Florida. The majority of the incursive serotypes have remained restricted to the southeastern US. In recent years, BTV serotype 3 (BTV-3) has been isolated in areas increasingly distant from Florida. The current study uses whole genome sequencing of recent and historical BTV-3 isolates from the US, Central America and the Caribbean with additional sequences from GenBank to conduct phylogenetic analyses. The individual segments of the BTV genome were analysed to determine if recent BTV-3 isolates are reassortants containing genomic segments from endemic US serotypes or if they retain a majority of Central American/Caribbean genotypes. The analyses indicate that BTV-3 isolates Mississippi 2006, Arkansas 2008 and Mississippi 2009 are closely related reassortants that contain five to six genomic segments that are of US origin and two to three segments of Central American/Caribbean origin. In contrast, the BTV-3 South Dakota 2012 isolate contains seven genomic segments that are more similar to isolates from Central American and the Caribbean. These different evolutionary histories of the BTV-3 isolates suggest that there are at least two different lineages of BTV-3 that are currently circulating in the US.

INTRODUCTION

Bluetongue virus (BTV) is a non-enveloped, double-stranded RNA virus in the genus Orbivirus, family Reoviridae. The genome consists of ten segments that encode the seven structural (VP1-VP7) and four non-structural proteins (NS1, NS2, NS3/3a). The structural proteins are arranged in three layers comprising the outer capsid (VP2, VP5), the capsid (VP3, VP7) and the inner core (VP1, VP4, VP6) that surround the genomic RNA [1]. The non-structural proteins are responsible for cellular effects such as tubule and viral inclusion body formation (NS1 and NS2 respectively) and viral egress (NS3/3a) [1].

Bluetongue virus is transmitted by several species of biting midge of the genus Culicoides [2, 3]. BTV is the etiological agent of bluetongue disease (BTD), an economically important disease of domestic and wild ruminants. Impacts of BTD on the livestock industry are not limited to the production losses associated with the mortality/morbidity of BTD but also include international restrictions on the trade of animals from areas with BTD or specific BTV serotypes [4, 5].

BTD was first described in South Africa in the early 1900s [6, 7]. Initially, it was believed that BTV would emerge from Africa and devastate the world’s sheep population. However, as additional serotypes of BTV were identified on other continents without the presence of severe disease it was realized that BTV emergence was not a recent event [8–10]. Currently, at least 29 serotypes of BTV exist worldwide [11, 12]. In tropical and subtropical regions that support continuous vector populations and circulation of endemic BTV serotypes disease outbreaks are uncommon [10]. In these areas, outbreaks of disease are generally associated with the introduction of a new serotype often from a neighbouring region.

BTV was first isolated in the United States in the early 1950s, although BTD, known as ‘sore muzzle’, had been

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Abbreviations: BTD, bluetongue disease; BTV, bluetongue virus; CPAE, cattle pulmonary artery endothelial; EHDV, epizootic haemorrhagic disease virus; NI, nucleotide identity; VP, viral protein.

The GenBank accession numbers for the full genome sequences of 27 bluetongue virus isolates are KY091901–KY092170. Two supplementary tables are available with the online version of this article.
Results from the phylogenetic analyses are presented as follows: the outer capsid [segments 2 (VP2) and 6 (VP5)], the capsid [segments 3 (VP3) and 7 (VP7)], the inner virus core [segments 1 (VP1), 4 (VP4) and 9 (VP6)] and non-structural proteins [segments 5 (NS1), 8 (NS2) and 10 (NS3/3a)]. The BTV-3 isolates Mississippi 2006, Arkansas 2008 and Mississippi 2009 (hereafter called the MAM clade) are found to form a distinct, well-supported clade in many of the phylogenetic trees.

The outer capsid
The phylogenetic tree for segment 2, the determinant of serotype, shows sequences grouping according to serotype, as expected (Fig. 1a). All BTV-3 isolates form a single, well-supported clade with 84–86 % nucleotide identity (NI). The MAM clade groups with other BTV-3 sequences from Florida, Central America and the Caribbean, as well as South Dakota 2012. The nearest well-supported relative of the MAM clade for VP2 is BTV-3 Panama 1989. BTV-3 South Dakota 2012 is most closely related to sequences from BTV-3 Martinique 2010 and Barbados 1988 for segment 2 which forms the sister group to the BTV-3 Florida strains. For segment 6, the four isolates of interest cluster together with 95–96 % NI to BTV14 Tobago 1989 (Fig. 1b). In trees for both segments nearly all of the BTV-3 Florida isolates are found in a well-supported clade separate from the recent isolates. This suggests that for the isolates of interest, BTV-3 segments 2 and 6 are the descendants of a Central American/Caribbean BTV-3 ancestor.

The capsid
The geographic origin of the segment 3 (VP3) sequences for the recent BTV-3 isolates is somewhat equivocal due to the majority of BTV-3 sequences grouping together (see Fig. 2a). Here, BTV-3 Mississippi 2006 and Arkansas 2008 form a well-supported clade separate from Mississippi 2009. These two isolates, along with BTV-3 South Dakota 2012 are part of a polytomy that includes nearly all of the US, Central American and Caribbean sequences. This suggests that more sequences are needed to determine the ancestry of segment 3 for Mississippi 2006, Arkansas 2008 and South Dakota 2012. Within this polytomy are two well-supported clades. One contains nearly all of the BTV-2 and BTV-3 Florida isolates with multiple serotypes from Central America and the Caribbean. The other includes multiple isolates of serotypes 10, 11, 13 and 17 from the western US with individual isolates of serotypes 2, 3 and 18 from Central America. BTV-3 Mississippi 2009 is included in this clade and shares 97–98 % NI with BTV-17 Colorado 1962 and BTV-11 isolates from Texas, Kansas and Washington 2011–2013. Analysis of segment 7, Fig. 2b, shows that the majority of BTV-3 isolates cluster together in a large polytomy with several well-supported clades. One of these contains the
MAM clade and BTV-13 US prototype (96 % NI). The closest relative of BTV-3 South Dakota 2012 within this large polytomy is again undetermined. Increased numbers of sequences for segment 7 may aid in the resolution of these relationships. From the presence of large polytomies in the phylogenetic analyses of both segments, only two determinations can be made: BTV-3 Mississippi 2009 segment 3 is of US endemic origin and the MAM clade and South Dakota 2012 segment 7 sequences are of Central American/Caribbean origin.

Inner virus core

The phylogenetic tree for segment 1 shows the MAM clade forming a well-supported sister group to a group of BTV-11 sequences from Texas, Kansas and Washington from 2011 to 2013 (94–97 % NI) and is most closely related (98 % NI) to BTV-9 Honduras 1991. Analysis of segment 4 sequences shows the MAM clade belonging to a clade of US prototype strains for serotypes 10, 13, 11 and 17, BTV-10 from Honduras and a 2011 BTV-11 from Texas (Fig. 3b). BTV-3 South Dakota 2012 is also found to be closely related to isolates of BTV-11 Texas and Kansas 2011–2012 (97 % NI). Analysis of segment 9 sequences places BTV-3 Mississippi 2008 and Arkansas 2008 as the sister group to a large clade of western US isolates of serotypes 10, 11, 13, 14, 15, 16, 17, 22 and 24 (see Fig. 3c). BTV-3 Mississippi 2009 is found within this large clade and is most closely related (97–98 % NI) to US BTV-10 and BTV-13 isolates from California 1989–90. BTV-3

![Fig. 1. Phylogenetic trees of BTV genes encoding the outer capsid structural proteins. (a) Segment 2 (VP2). (b) Segment 6 (VP5). Trees were constructed in Geneious 8.0 using the nucleotide alignment, the neighbour-joining method, Jukes–Cantor distance and 1000 bootstrap pseudo-replicates. Bootstrap values are indicated either above or below the node. An asterisk indicates a bootstrap value of 100. BTV serotype 3 strains of interest are highlighted as follows: Mississippi 2009 (white box with black outline and black text), Mississippi 2006 and Arkansas 2008 (grey box with black text), and South Dakota 2012 (black box with white text).](image-url)
South Dakota 2012 is most closely related to BTV-18 Guatemala (99 % NI) and BTV-14 Guatemala (98 % NI) for segment 9.

Non-structural proteins

The non-structural proteins are encoded by segments 5 (NS1), 8 (NS2) and 10 (NS3/3A). In the phylogenetic tree for segment 5, (Fig. 4a), the four isolates of interest are all found within the same large, well-supported clade that includes US BTV-10, 11, 17, four isolates from Central America and several BTV-3 Florida isolates. BTV-3 Mississippi 2006 and Arkansas 2008, as well as BTV-3 South Dakota 2012 are located within the same clade that consists of isolates of BTV-11 Texas 2011, BTV-10 USA and Honduras and BTV-17 USA. Within this clade, BTV-3 Mississippi 2006 and Arkansas 2008 are most closely related to BTV-17 US 1989 with 98 % NI, while BTV-3 South Dakota 2012 shares a close relationship with BTV-11 Texas 2011 (98 % NI). Although BTV-3 Mississippi 2009 is also included in the same larger group of sequences, it is part of a polytomy and its closest relative is undetermined. The phylogeny for segment 8, shown in Fig. 4b, identifies the MAM clade as the well-supported sister group to all other BTV strains in the study except those from Australia and India. The placement of this clade suggests that the most recent ancestor for these isolates has not been sequenced. BTV-3 South Dakota 2012, in contrast, falls within a clade consisting of BTV-3, BTV-11 and BTV-6 isolates from

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**Fig. 2.** Phylogenetic trees of BTV genes encoding the capsid structural proteins. (a) Segment 3 (VP3). (b) Segment 7 (VP7). Trees were constructed in Geneious 8.0 using the nucleotide alignment, the neighbour-joining method, Jukes–Cantor distance and 1000 bootstrap pseudo-replicates. Bootstrap values are indicated either above or below the node. An asterisk indicates a bootstrap value of 100. BTV serotype 3 strains of interest are highlighted as follows: Mississippi 2009 (white box with black outline and black text), Mississippi 2006 and Arkansas 2008 (grey box with black text), and South Dakota 2012 (black box with white text).
Florida dating from 2001 to 2013. In summary, BTV-3 Mississippi 2006 and Arkansas 2008 have segments 5 and 10 that are of US endemic origin. Determination of US endemic ancestry for BTV-3 Mississippi 2009 can only be made for segment 10. BTV-3 South Dakota 2012 shows a mixture of ancestry for these segments with a US endemic origin for segment 5 and Central American/Caribbean origin for segments 8 and 10.

DISCUSSION

The current study uses whole genome sequencing and phylogenetic analyses of recent and historical isolates from the US, Central America and the Caribbean to determine if recent US BTV-3 isolates are reassortants with endemic US serotypes or if they retain a Central American/Caribbean signature. The analyses show that BTV-3 isolates from Mississippi 2006, Arkansas 2008 and Mississippi 2009 have very similar evolutionary histories that have resulted in the acquisition of a majority of genomic segments of US endemic serotype origin. In contrast, the BTV-3 South Dakota 2012 isolate has a majority of genomic segments that are more similar to BTV-3 sequences from Florida, Central America and the Caribbean. These different histories of the BTV-3 isolates suggest that there are at least two different lineages of reassortant BTV-3 currently circulating in the US (see Table 1).

Early studies of BTV isolates used electropherotypes or the pattern of RNA segments run on a polyacrylamide gel to show that significant variation existed between isolates of the same and different serotypes, as well as within isolates from the same area, outbreak or animal [44, 45]. Oligonucleotide fingerprinting of BTV RNA from US prototype isolates demonstrated that similarities between segment fragments from different serotypes were the result of

Schirzer et al., Journal of General Virology 2018;99:157–168

Fig. 3. Phylogenetic trees of BTV genes encoding the inner virus core proteins. (a) Segment 1 (VP1). (b) Segment 4 (VP4). (c) Segment 9 (VP6). Trees were constructed in Geneious 8.0 using the nucleotide alignment, the neighbour-joining method, Jukes–Cantor distance and 1000 bootstrap pseudo-replicates. Bootstrap values are indicated either above or below the node. An asterisk indicates a bootstrap value of 100. BTV serotype 3 strains of interest are highlighted as follows: Mississippi 2009 (white box with black outline and black text), Mississippi 2006 and Arkansas 2008 (grey box with black text), and South Dakota 2012 (black box with white text).
Reassortment in the field [46, 47]. Further research on field isolates of BTV has shown that reassortment is common where multiple serotypes circulate and/or live attenuated vaccines have been used [47, 48].

Reassortment of segmented viruses in cell culture has been shown to be essentially at random when the parental viruses have equal fitness [43, 49]. In studies where viruses were not matched for fitness, one constellation of viral segments became dominant with a small number of combinations being found at low frequencies [42, 43, 50]. Often when two serotypes were co-infected, only one serotype was found among the progeny [41, 42, 50, 51]. These data suggest that while all segments may be able to reassort, selection for fitness in the animal or vector will determine which reassortants are passed on [43, 52, 53]. In the current study, the BTV-3 Mississippi and Arkansas isolates only retained the BTV-3 segment that confers serotype, segment 2. In the field, previous exposure of livestock to endemic serotypes induces a protective response against subsequent exposures to these serotypes. Since serotype 3 is novel in the US and livestock outside of Florida are naïve, the segments that

![Diagram](image-url)

**Fig. 3.** (cont.)

- EHDV2 Alberta (AM745005)
- EHDV1 New Jersey (NC013404)
- BTV3 Australia 1986 DPP973 (JQ086289)
- BTV3 India 2003 8 (JQ71821)
- BTV9 Honduras 1991 502326
- BTV14 Guatemala 1990 502230
- BTV11 California 2006 06-473906-7
- BTV18 Mississippi 2008 08-370004-2
- BTV3 Arkansas 2008 08-566195
- BTV11 California 2006 06-473906-7
- BTV15 USA prototype (U57976)
- BTV11 USA prototype (U57976)
- BTV10 California 1990 10B902 (U57976)
- BTV10 Arkansas 1990 10B902 (U57976)
- BTV11 Mississippi 2008 08-370004-2
- BTV11 Texas 2011 11-56803-18 (KMS80444)
- BTV11 Texas 2011 129184-11 (KMS80456)
- BTV11 Kansas 2012 120659-12 (KMS80470)
- BTV17 USA prototype (U57976)
- BTV17 USA prototype (U57976)
- BTV11 California 1991 11UC2 (U57976)
- BTV10 California 1990 10UC2 (U57976)
- BTV11 California 1991 11UC2 (U57976)
- BTV10 California 1990 10UC2 (U57976)
- BTV11 Texas 2011 11-56803-18 (KMS80444)
- BTV11 Texas 2011 129184-11 (KMS80456)
- BTV11 Kansas 2012 120659-12 (KMS80470)
- BTV17 USA prototype (U57976)
- BTV17 USA prototype (U57976)
- BTV11 California 1991 11UC2 (U57976)
- BTV10 California 1990 10UC2 (U57976)
- BTV11 California 1991 11UC2 (U57976)
- BTV10 California 1990 10UC2 (U57976)
- BTV11 Texas 2011 11-56803-18 (KMS80444)
- BTV11 Texas 2011 129184-11 (KMS80456)
- BTV11 Kansas 2012 120659-12 (KMS80470)
- BTV17 USA prototype (U57976)
confer serotype may be selected over locally circulating endemic serotypes and may become dominant in the viral progeny.

Segments that code for non-structural proteins tend to be conserved due to negative selection that may be linked to functional constraint of these proteins. However, slight differences in fitness may lead to selection of specific segments. Ramig et al. [50] suggest that reassortant progeny viruses from co-infection of cell cultures contain more segments from the parental virus that was infected at a higher multiplicity of infection. This previous study, however, analysed reassortants by electropherotype. By repeating this former study using current sequencing methods, it may become possible to identify the sequence of parent virus infections in reassortant viruses. In the current study, the differences in evolutionary histories of the BTV-3 Mississippi and Arkansas isolates and the South Dakota isolate may reveal that in South Dakota only BTV-3 was circulating in the affected animals while multiple serotypes were circulating further south. Future studies using the BTV reverse genetics system and sequences from recent BTV-3 and endemic isolates may allow us to tease apart the influence of the different segments on virulence, replication and transmissibility in animals and vectors.

Unfortunately, due to a lack of continual surveillance of BTV serotypes throughout the US, isolations are only made during an outbreak of BTD or when testing for export purposes and usually only serotype is determined at the time. This does not allow us to determine with any precision where and when a virus has undergone reassortment or which parental strains were involved. Whole genome sequencing of new BTV-3 isolates is needed in order to

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**Fig. 4.** Phylogenetic trees of BTV genes encoding the non-structural proteins. (a) Segment 5 (NS1). (b) Segment 8 (NS2). (c) Segment 10 (NS5). Trees were constructed in Geneious 8.0 using the nucleotide alignment, the neighbour-joining method, Jukes–Cantor distance and 1000 bootstrap pseudo-replicates. Bootstrap values are indicated either above or below the node. An asterisk indicates a bootstrap value of 100. BTV serotype 3 strains of interest are highlighted as follows: Mississippi 2009 (white box with black outline and black text), Mississippi 2006 and Arkansas 2008 (grey box with black text), and South Dakota 2012 (black box with white text).
Fig. 4. (cont.)

1. BTV10 USA 1980 10B80Z (AF044379)
   BTV10 USA 1980 10O80Z (AF044378)
   BTV17 Idaho 2003 17US03-13 (EF540902)
   BTV17 Idaho 2003 17US03-15 (EF540922)
   BTV17 Idaho 2003 17US03-16 (EF540923)
   BTV17 Idaho 2003 17US03-21 (EF540926)
   BTV17 Idaho 2004 17US04-29 (EF540932)
   BTV17 Idaho 2003 17US03-29 (EF540931)
   BTV17 Idaho 2003 17US04-27 (EF540930)

2. BTV10 Martinique 2006 10FM06-33 (EF540935)
   BTV2 Martinique 2006 2FM06-32 (EF540934)
   BTV17 Martinique 2006 17FM06-43 (EF540936)

3. BTV10 Georgia 2002 10US02-6 (EF540916)
   BTV10 North Carolina 2002 10US02-9 (EF540917)
   BTV10 Virginia 2002 10US02-7 (EF540915)
   BTV10 Washington 2003 17US03-24 (EF540928)
   BTV10 Idaho 2003 10US03-25 (EF540929)
   BTV17 Idaho 2003 17US03-14 (EF540921)
   BTV17 Idaho 2003 17US03-30 (EF540925)
   BTV17 Idaho 2003 17US03-13 (EF540902)
   BTV17 Idaho 2003 17US03-15 (EF540922)
   BTV17 Idaho 2003 17US03-16 (EF540923)
   BTV17 Idaho 2003 17US03-21 (EF540926)
   BTV17 Idaho 2003 17US03-10 (EF540918)
   BTV17 Idaho 2004 17US04-29 (EF540932)
   BTV17 Idaho 2003 17US03-28 (EF540931)
   BTV17 Idaho 2003 17US04-27 (EF540930)

4. BTV10 USA 1979 10O79X (AF044386)
   BTV11 USA 1981 11C81Z (AF044703)
   BTV11 USA 1981 11O81X (AF044704)
   BTV11 USA 1980 11B80Z (AF044705)
   BTV10 USA 1980 11B80Z (AF044702)
   BTV USA 2009 B09 (GU954426)
   BTV USA 2009 B09 (GU954427)

0.04
continue tracking its evolution and persistence. Monitoring serotypes circulating in vectors, livestock and wild ruminants in areas where BTV-3 has previously been isolated will provide the information necessary to determine if recent isolates are the result of transient incursions from the southeastern US or if BTV-3 will become the next North American endemic serotype.

**METHODS**

A total of 27 bluetongue isolates of multiple serotypes (17 from BTV-3) from the United States, Central America and the Caribbean were sequenced for this study (see Table 2). BTVs not isolated in the United States were obtained from the Inter-American Bluetongue Project and the Onderste- poort Veterinary Institute virus library. Isolates from the United States were obtained from the National Veterinary Services Laboratories or the Arthropod-borne Animal Disease Research Unit reference collection. Viruses were typically isolated in embryonated chicken eggs or cattle pulmonary artery endothelial (CPAE) cells (ATCC CCL-209), followed by one to as many as seven passages in baby hamster kidney (BHK-21) (ATCC CCL-10) or CPAE cells. Total RNA was extracted from cells as previously described [54]. Viral double-stranded RNA was then purified by lithium chloride differential precipitation as described in [55] and subjected to whole genome sequencing using the sequence-independent amplification procedure described by [56] with modifications as described previously [57].

Library preparation and sequencing was performed using the Ion Torrent OneTouch ES, Personal Genome Machine with the Ion Xpress Plus fragmentation library kit, Xpress barcode adapters, Ion library quantitation kit, OneTouch 200 template kit v2, Ion PGM sequencing 400 kit and Ion 314 chip following the protocols recommended by the manufacturer (Life Technologies, Grand Island, NY). Briefly, approximately 1 µg of viral cDNA was fragmented, barcoded and quantitated. Template generation, enrichment and sequencing were performed on the appropriate Ion OneTouch instruments (Life Technologies, Grand Island, NY).

Standard Flow-gram Format files were imported into Geneious 6.0 (Biomatters) for contig creation. Partial contigs were assembled and *BLAST*ed against the NCBI nucleotide database to determine reference sequences that were then used for reference-based assemblies.

**Phylogenetic analysis**

In order to encompass the currently known diversity of isolates from the United States, Central America and Caribbean regions additional BTV segment sequences were downloaded from GenBank. The genome segments from serotypes 1 and 2 of the closely related orbivirus, epizootic haemorrhagic disease virus (EHDV) were used to root the trees. The newly sequenced BTV genomes have been deposited in GenBank, KY091901-KY092170 (Table 2). GenBank accession numbers for all viral segments included in the study are located in Table S2.

Alignments of each BTV segment were made in Geneious 8.0 (www.geneious.com, [58]) using the MAFFT alignment option with default values. The ORF for each segment was determined using the Find ORFs option and the UTRs at the 5’ and 3’ ends were removed.

Phylogenetic trees for each segment were estimated by the neighbour-joining method under the Jukes–Cantor distance model in Geneious 8.0. The homologous segments of EHDV-1 and EHDV-2 were used as outgroups to root the trees. Support values for nodes were estimated by 1000 bootstrap pseudo-replicates. Nodes with bootstrap values less than 70 were considered to be unsupported. Four BTV-3 strains, Mississippi 2006, Arkansas 2008, Mississippi 2009 and South Dakota 2012 were evaluated for potential reassortment events by comparison of nearest neighbours in phylogenetic trees for each individual segment. Reassortment can be demonstrated by the degree of topological divergence of the phylogenetic trees.
| Serotype | Isolate | Year | Location | Segment 1 | Segment 2 | Segment 3 | Segment 4 | Segment 5 | Segment 6 | Segment 7 | Segment 8 | Segment 9 | Segment 10 |
|----------|---------|------|----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| 1        | 502270  | 1990 | El Salvador | KY092117 | KY092118 | KY092120 | KY092119 | KY092121 | KY092122 | KY092123 | KY092124 | KY092125 | KY092126 |
| 2        | 502264  | 1988 | Barbados | KY092135 | KY092137 | KY092139 | KY092141 | KY092143 | KY092144 | KY092145 | KY092146 | KY092147 | KY092148 |
| 3        | 502260  | 1990 | Panama | KY092113 | KY092114 | KY092116 | KY092118 | KY092120 | KY092121 | KY092122 | KY092123 | KY092124 | KY092125 |
| 4        | 502035  | 1989 | Jamaica | KY092130 | KY092132 | KY092134 | KY092136 | KY092138 | KY092140 | KY092142 | KY092144 | KY092146 | KY092148 |
| 5        | 502151  | 1989 | Panama | KY092131 | KY092133 | KY092135 | KY092137 | KY092139 | KY092141 | KY092143 | KY092145 | KY092147 | KY092149 |
| 6        | 601560  | 1997 | South Africa | KY092110 | KY092112 | KY092114 | KY092116 | KY092118 | KY092120 | KY092122 | KY092124 | KY092126 | KY092128 |
| 7        | 502064  | 1990 | Panama | KY092136 | KY092138 | KY092140 | KY092142 | KY092144 | KY092146 | KY092148 | KY092150 | KY092152 | KY092154 |
| 8        | 502056  | 1989 | Trinidad | KY092129 | KY092131 | KY092133 | KY092135 | KY092137 | KY092139 | KY092141 | KY092143 | KY092145 | KY092147 |

Schirzinger et al., Journal of General Virology 2018;99:157–168
congruence between phylogenetic trees of individual genome segments. When all of the segments for a viral strain share the same ancestor–descendent relationships the topologies of the phylogenetic trees of each segment will be very similar or identical. A segment from a co-infecting strain will have different ancestor–descendent relationships and therefore the closest relatives will differ in the phylogenetic tree for that segment.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study does not contain any experiments with human participants or animals performed by any of the authors.

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