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Bungowannah virus in the affected pig population: a retrospective genetic analysis

Anja Dalmann[1] · Kerstin Wernike[1] · Ilona Reimann[1] · Deborah S. Finlaison[2] · Peter D. Kirkland[2] · Martin Beer[1]

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Abstract

Bungowannah virus, which belongs to the genus *Pestivirus* within the family *Flaviviridae*, has been associated with myocarditis and a high incidence of stillbirths in pigs. In 2003, the virus was initially detected in a large pig farming complex on two separate sites in New South Wales, Australia. Until now, it has not been detected at other locations. Despite a program of depopulation and disinfection, the virus could be only eradicated from one of the affected farm complexes, the Bungowannah unit, but became endemic on the second complex, the Corowa unit. In the present study, the genetic variability of virus isolates collected between 2003 and 2014 in the endemically infected population has been retrospectively investigated. Phylogenetic analysis carried out based on sequences of the E2 and NS5B coding regions and the full-length open-reading frame revealed that the isolates from the different farm sites are closely related, but that samples collected between 2010 and 2014 at the Corowa farm site clustered in a different branch of the phylogenetic tree. Since 2010, a high-genetic stability of this RNA virus within the Corowa farm complex, probably due to an effective adaptation of the virus to the affected pig population, could be observed.

Keywords Bungowannah virus · Pestivirus · Sequence analysis · Virus evolution · Molecular epidemiology

Introduction

There are currently four officially recognized pestivirus species within the family *Flaviviridae*, namely Bovine viral diarrhea virus (BVDV) types 1 and 2, Classical swine fever virus (CSFV), and Border disease virus (BDV) [1]. These classical members of the genus *Pestivirus* were initially described in the 18th or in the middle of the 19th century [2–5], infect cloven-hoofed animals, and are important pathogens of livestock. During the last decade, however, the known phylogenetic and host diversity of the genus *Pestivirus* expanded considerably (reviewed in [6]). An example of a novel porcine pestivirus is the Australian Bungowannah virus, which was at the time of its description the most divergent pestivirus. Bungowannah virus was identified following an outbreak of stillbirths and sudden death accompanied by cardiomyopathy with signs of cardiac failure in neonatal piglets within the largest integrated pig farm in New South Wales, Australia [7, 8]. The virus initially spread through both of the large farm complexes whereby surviving pigs developed immunity. Thereafter, only sporadic clinical cases were reported, but Bungowannah virus remained endemic in both affected farm complexes. Subsequently, complete depopulation and disinfection led to the eradication from one farm (Bungowannah farm site). On the second farm (Corowa farm complex), however, similar attempts to eradicate the virus were unsuccessful. After depopulation, disinfection and repopulation of two units with naïve animals there was a re-introduction of Bungowannah virus to those units, again causing severe reproductive losses [9]. It was considered that the virus had been reintroduced from one of the remaining infected units potentially during the disinfection process of that unit. Since this reintroduction, Bungowannah virus established an endemic status in the Corowa farm complex. Until now, the virus has not been reported from any other farm, region or country [10].

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✉ Martin Beer
martin.beer@fli.de

[1] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald - Insel Riems, Germany

[2] Virology Laboratory, Elizabeth Macarthur Agriculture Institute, Woodbridge Rd, Menangle, NSW 2568, Australia

The genome of all currently recognized pestiviruses is organized similarly, the single-stranded positive-sense RNA genome encodes four structural (C, E^{rns}, E1, E2) and at least eight nonstructural proteins (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) in one large open-reading frame (ORF) flanked by 5′ and 3′ non-translated regions. The resulting polyprotein is co- and post-translationally processed by cellular and viral proteases into the individual proteins [11]. E2, which induces as a main immunogen neutralizing antibodies in infected hosts [12], shows a high variability and, therefore, it is an ideal target for genotyping and phylogenetic studies of pestiviruses. In addition to E2, further genome regions such as 5′NTR, N^{pro} and NS5B are frequently used for genetic typing of pestiviruses [13–19]. For more precise results it is also possible to sequence full-length genomes, which reflects the genetic variability between virus strains more accurately, but is also very time-consuming and not suitable for high-throughput sample analysis [20–22].

Here, we investigated Bungowannah virus isolates obtained from both affected farm complexes between 2003 and 2014 based on their E2, NS5B and full-length protein coding sequences.

Materials and methods

Bungowannah viruses were isolated from samples obtained from affected fetuses or stillborn piglets between 2003 and 2014 in porcine kidney cells (PK-15) as described previously [7]. The sample material of isolate B-C843 originated from the Bungowannah farm site and all other samples (C-03/2007-5, C-03/2007-9, C-E570, C-E997, C-E889, C-H482, C-H151, C-H153, C-H624) from the Corowa farm complex. All virus isolates were passaged 2–4 times in PK-15 cells (Table 1). Total viral RNA was extracted from infected cells or the original fluid sample (C-03/2007-5 and C-03/2007-9) using the QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) and reverse transcription (RT)-PCR was done using the Qiagen® OneStep RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A series of overlapping RT-PCRs were used to amplify the E2 and NS5B encoding region of all isolates and the complete protein-coding region of the isolates C-03/2007-9, C-H482 and C-E570. Primer sequences are available upon request. The amplified PCR fragments were separated on agarose gels, visualized by staining with ethidium bromide, and subsequently excised and purified by using the QIAquick® Gel Extraction kit (Qiagen, Hilden, Germany). Sequencing reactions of both strands were carried out with the primers used for amplification and the BigDye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). Nucleotide sequences were read with an automatic sequencer (3130 Genetic Analyzer, Applied Biosystems, Foster City, USA). For analysis of the obtained nucleotide sequences, assembly of the complete ORF sequences, to prepare alignments of the generated sequences, and to calculate the number and percentages of nucleotide and amino acid substitutions Geneious software (Version 10.2.3., Biomatters Ltd., Auckland, New Zealand) was used. The sequences generated in the present study were submitted to NCBI GenBank (Accession Numbers MH712512 to MH712531).

For phylogenetic characterization, neighbor-joining (NJ) trees were generated using the Tamura-3 parameter model with 1000 bootstrapping replicates as implemented in the MEGA 7 software [23]. The published full-length sequence of the first Bungowannah virus isolate (GenBank Accession Number NC_023176) and full-length sequences of selected classical and atypical pestiviruses were used for comparison (CSFV Accession Number Z46258; BDV KF925348; BVDV-1 AJ133738; BVDV-2 KR093034; BVDV-3, Bovine viral diarrhea virus 3 (BVDV-3, syn. HoBi virus) AB871953; Pronghorn antelope pestivirus NC024018;

Table 1 Field-collected samples and virus isolates investigated in the present study

Sample ID	Collection date	Origin	Sample material for virus isolation	No. passages PK-15 cells
B-C843	21.07.2003	Bungowannah farm site	Stillborn piglets	3
C-03/2007-5	06.08.2003	Corowa farm complex	Body fluid	0
C-03/2007-9	06.08.2003	Corowa farm complex	Body fluid	0
C-E570	29.09.2010	Corowa farm complex	Stillborn piglets	4
C-E889	08.06.2011	Corowa farm complex	Stillborn piglets	4
C-E997	29.08.2011	Corowa farm complex	Pooled fetal/placental tissue	2
C-H151	24.07.2013	Corowa farm complex	Pooled fetal/placental tissue	4
C-H153	24.07.2013	Corowa farm complex	Pooled fetal/placental tissue	4
C-H482	09.01.2014	Corowa farm complex	Pooled fetal/placental tissue	4
C-H624	05.03.2014	Corowa farm complex	Pooled fetal/placental tissue	4

Linda virus KY436034; Norway rat pestivirus NC025677; Atypical porcine pestivirus KR011347).

Results

Sequencing and phylogenetic analysis of E2 and partial NS5B genomic regions

The complete E2 nucleotide sequences from all investigated isolates were generated and compared to the reference sequence (NC_023176); some non-synonymous mutations were observed in every case. In the isolate B-C843 from the Bungowannah farm site and the early samples (C-03/2007-5; C-03/2007-9) from the Corowa farm site, only one to four nt differences could be detected leading to aa substitutions in either none (C-03/2007-5) or one case (B-C843: K214E; C-03/2007-9: V48E). In contrast, isolates obtained since 2010 from the Corowa farm complex showed a higher number of nucleotide exchanges within the E2 genomic region compared to the reference isolate (Fig. 1). The substitution on nucleotide position 801 (T- > C) could be identified in the E2 sequence of all isolates (Fig. 1, depicted in red). However, the late Corowa-derived sequences showed only few differences between each other. As a result, the Bungowannah isolate B-C843, the Corowa samples C-03/2007-5 and C-03/2007-9 and the reference sequence grouped together in the E2-based phylogenetic tree. The late Corowa-derived isolates C-E570, C-E889, C-E997, C-H151, C-H153, C-H482 and C-H624 clustered on a different branch of the NJ tree (Fig. 2a).

The phylogenetic tree based on the highly conserved NS5B genome region supported the finding of distinct clusters. The sample from the Bungowannah farm site (B-C843) and the early samples from the Corowa farm site were closely related to the first Bungowannah isolate NC_023176, whereas the late isolates from the Corowa farm site also cluster in a separate group in the NS5B-based tree (Fig. 2b).

Sequence comparison and phylogenetic analysis of full-length ORF sequences

The complete coding sequences of the Corowa-derived sample C-03/2007-9, which was collected in 2003, of an isolate from the Corowa farm site from the year 2010 (C-E570), and of one of the most recent virus isolates (C-H482, year 2014) were generated. The isolate C-03/2007-9 exhibited 27 nucleotide differences (21 silent, 6 nonsynonymous), C-E570 showed 210 (153 silent, 57 nonsynonymous) and C-H482 showed 236 nucleotide differences (173 silent, 63 nonsynonymous), respectively, resulting in a nucleotide identity of 99.8% (C-03/2007-9), 98.2% (C-E570) and 98% (C-H482) to the reference sequence, which originated from a virus present in the Bungowannah farm site. However, both of the late Corowa-derived isolates showed a notably higher similarity of 99.4% to each other, although the collection dates differed by several years.

When comparing the sequences of the individual genome regions, the E2 protein-encoding region showed the highest differences, followed by the NS4 and NS5 genomic regions. Accordingly, phylogenetic analysis of the full-length ORF confirmed the results determined by analysis of the E2 and NS5B genome regions, respectively (Fig. 2c).

In the phylogenetic tree, that is based on the full-length sequences and contains, besides Bungowannah virus, representative classical and atypical pestivirus strains, the first Bungowannah virus isolates of the different farm units cluster together. In contrast, the isolates originating from the Corowa farm site since 2010 build a different cluster. However, they can be clearly distinguished from further pestiviruses, where Linda virus, which was detected in piglets in Austria in 2015 [24], represents the most closely related virus.

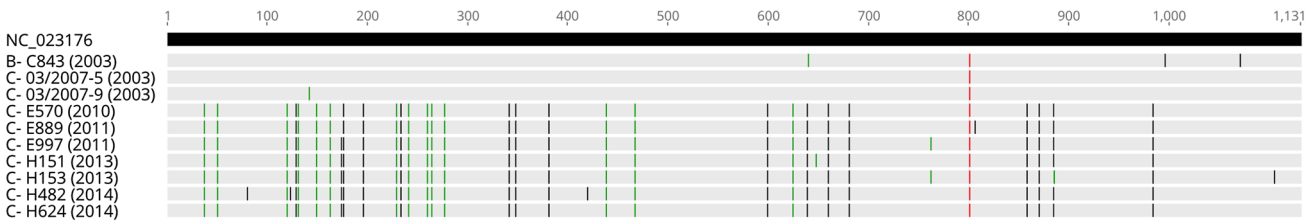
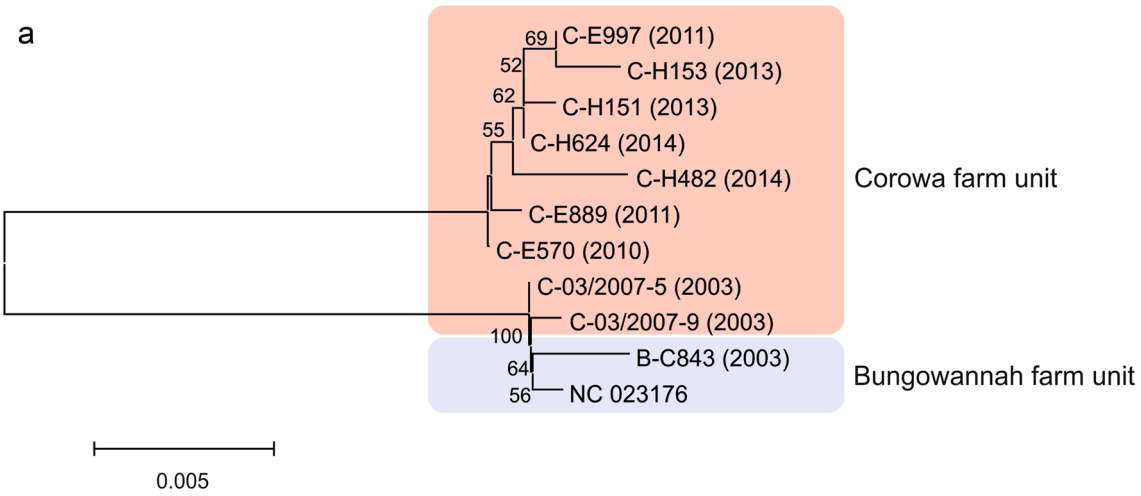
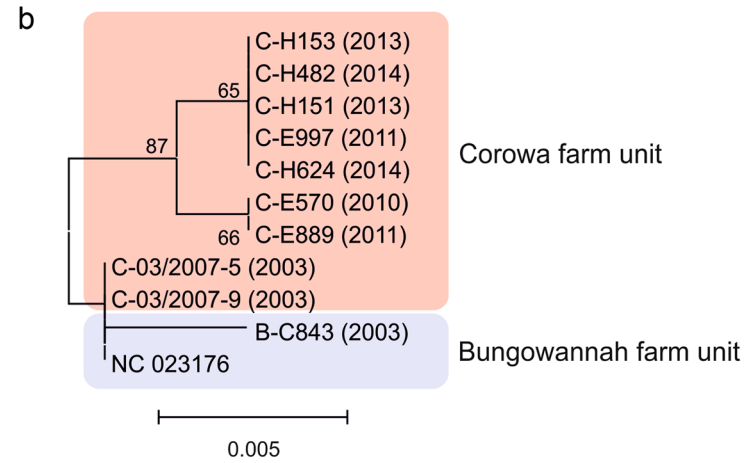
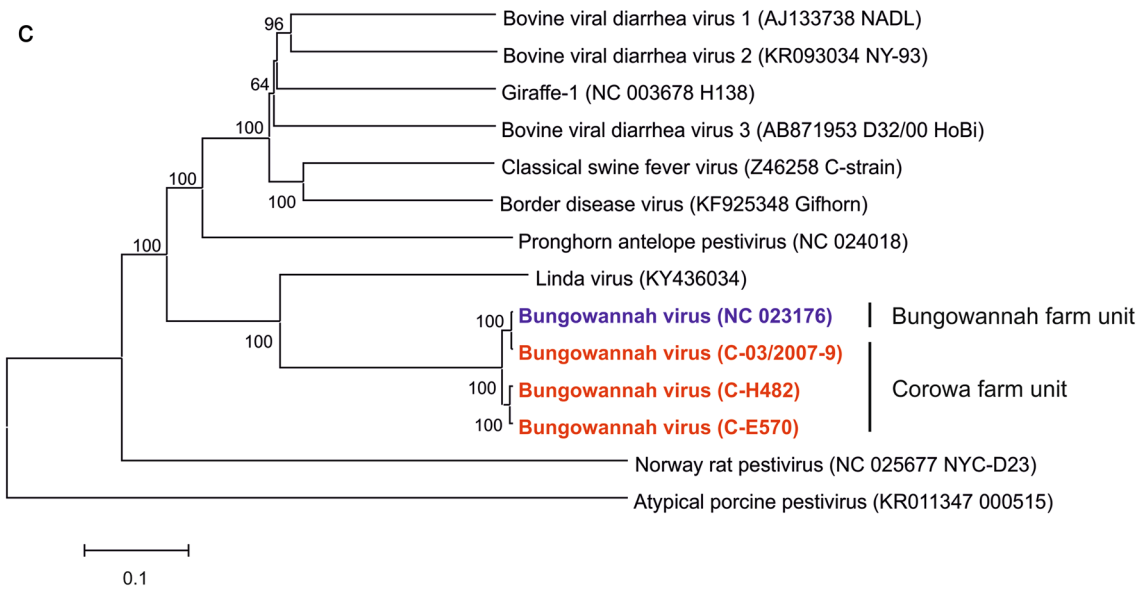


Fig. 1 Comparison of E2 sequences of Bungowannah virus isolates to the reference strain (NC_023176). The thick horizontal black line represents the nucleotide positions of the reference E2 sequence. The vertical black lines indicate the nucleotide differences to the original isolate and vertical green lines indicate nucleotide mutations that led to amino acid substitutions. The substitution that was observed in every newly generated sequence is shown in red


a
69
C-E997 (2011)
52
C-H153 (2013)
62
C-H151 (2013)
55
C-H624 (2014)
C-H482 (2014)
C-E889 (2011)
C-E570 (2010)
C-03/2007-5 (2003)
C-03/2007-9 (2003)
100
64
B-C843 (2003)
56
NC 023176
Corowa farm unit
Bungowannah farm unit
0.005


b
C-H153 (2013)
C-H482 (2014)
65
C-H151 (2013)
87
C-E997 (2011)
C-H624 (2014)
C-E570 (2010)
66
C-E889 (2011)
C-03/2007-5 (2003)
C-03/2007-9 (2003)
B-C843 (2003)
NC 023176
Corowa farm unit
Bungowannah farm unit
0.005


c
96
Bovine viral diarrhea virus 1 (AJ133738 NADL)
Bovine viral diarrhea virus 2 (KR093034 NY-93)
64
Giraffe-1 (NC 003678 H138)
100
Bovine viral diarrhea virus 3 (AB871953 D32/00 HoBi)
100
Classical swine fever virus (Z46258 C-strain)
100
Border disease virus (KF925348 Gifhorn)
100
Pronghorn antelope pestivirus (NC 024018)
Linda virus (KY436034)
100
Bungowannah virus (NC 023176)
100
Bungowannah virus (C-03/2007-9)
100
Bungowannah virus (C-H482)
100
Bungowannah virus (C-E570)
Norway rat pestivirus (NC 025677 NYC-D23)
Atypical porcine pestivirus (KR011347 000515)
Bungowannah farm unit
Corowa farm unit
0.1

◂**Fig. 2** Phylogenetic analysis of the E2 protein (**a**), partial region of NS5B (**b**), and the complete ORF (**c**) of the newly characterized Bungowannah virus isolates and the corresponding reference strain (NC_023176) by neighbor-joining method based on the Tamura 3-parameter model. Statistical support for nodes was obtained by bootstrapping (1000 replicates); only values ≥ 50% are shown. The scale bar indicates nucleotide substitutions per site. Sequences highlighted in blue originate from the Bungowannah farm complex and sequences highlighted in red originate from the Corowa farm complex

Discussion

Since its first appearance in 2003, Bungowannah virus spread only through the affected Australian company complex which consists of two large farms on different sites, the Bungowannah farm site and the Corowa farm site. After the initial outbreak, the occurrence of disease declined, however, despite disinfection and depopulation measures, the virus could be only eradicated from the Bungowannah farm site, but became endemic in the Corowa farm site [7]. Here, we retrospectively investigated the genetics of Bungowannah viruses in this endemically infected farm complex.

Different genome regions have been recommended for phylogenetic and diagnostic investigations of pestivirus isolates, among them the E2-encoding and short NS5B-encoding regions [13, 25, 26], which were also used in the present study for Bungowannah isolates obtained between 2003 and 2014. Because of the limitation of sequencing of small fragments [17, 27] for the comparisons of highly similar virus strains, the full-length ORF sequences of four representative isolates were analyzed as well. In general, the E2, NS5B and full-length sequences provided the same result, i.e., that the isolates from the different farm sites are closely related, but the early isolates from both complexes and the late Corowa isolates clustered on separate branches of the tree with Linda virus being the most closely related virus. Thus, the aforementioned individual proteins represent also in our study suitable targets for phylogenetic analyses.

Unfortunately, the virus evolution within the first affected pig farm complex could not be evaluated because the virus was eradicated from this complex shortly after its emergence [7]. However, the initial isolate from the second affected farm site showed a high similarity to the first sequence obtained from the Bungowannah farm site, which correlates with the transfer of this virus from the Bungowannah to the Corowa farm site, either as a result of the movement of pigs, people or vehicles [28], to establish a second focus of infection.

Interestingly, the isolates obtained since 2010 differed markedly from both sequences and formed their own cluster. Since there were unfortunately no virus isolates available from 2004 to 2009, two main different scenarios could be possible reasons for the observed clustering: (i) reintroduction of the virus to the Corowa farm complex from an unknown external source—as it occurred initially in the Bungowannah farm unit—and after the original Bungowannah virus has disappeared; or (ii) the initially introduced virus further adapted to the local pig population over the years until the endemic stage was reached.

However, the genetic stability subsequently found in viruses isolated from the Corowa complex between 2010 and 2014 is surprisingly high. This genetic stability, particularly of the E2 protein, is the more surprising since the virus was subjected to significant immune pressure arising from the high antibody prevalence on the affected farm. Since the usual mutation rate of RNA viruses roughly range between 10^{-6} and 10^{-4} substitution per nucleotide per cell infection [29, 30], a much lower identity of viruses isolated over a period of 3.5 years might be expected. The high genetic stability, which was observed despite the large interval between the first and last sampling and the presumed large number of animal passages (the Corowa site has approximately 250,000 pigs at any time point), could indicate a very effective adaptation and genetic optimization of the virus to the local pig population over the long period of time. For further pestiviruses, it has been described that persistent infections induced by transplacental transmission of the virus to a not yet immunocompetent fetus could affect the genetic diversity of the virus in a way that there is a selection against mutants that might break tolerance [31, 32]. Whether this mechanism also plays a role in the epidemiology of Bungowannah virus should be the topic of further investigations.

In addition, future studies will compare the initial Bungowannah virus and its Corowa farm unit variant in regard to further optimization to the pig host, and will also focus on analyzing the genetic differences of the two newly described Bungowannah virus clusters.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The samples for virus isolation were taken by the responsible farm veterinarian in the context of the health-monitoring program of the farm complexes.

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