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Short communication

Genetic and antigenic characterization of Bungowannah virus, a novel pestivirus

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ABSTRACT

Bungowannah virus, a possible new species within the genus *Pestivirus*, has been associated with a disease syndrome in pigs characterized by myocarditis with a high incidence of stillbirths. The current analysis of the whole-genome and antigenic properties of this virus confirms its unique identity, and further suggests that this virus is both genetically and antigenically remote from previously recognized pestiviruses. There was no evidence of reactivity with monoclonal antibodies (mAbs) that are generally considered to be pan-reactive with other viruses in the genus, and there was little cross reactivity with polyclonal sera. Subsequently, a set of novel mAbs has been generated which allow detection of Bungowannah virus. The combined data provide convincing evidence that Bungowannah virus is a member of the genus *Pestivirus* and should be officially recognized as a novel virus species.

1. Introduction

The *Pestivirus* genus of the family Flaviviridae includes four recognized species – bovine viral diarrhea virus type 1 and 2 (BVDV-1 and -2), border disease virus (BDV) and classical swine fever virus (CSFV; King et al., 2012). Four further putative species have been proposed: Giraffe virus, Pronghorn virus, a group of viruses variously referred to as HoBi-like or BVDV-3, and Bungowannah virus, which is the subject of the current study (Kirkland et al., 2007; Plowright, 1969; Schirrmeier et al., 2004; Vilcek et al., 2005). In June 2003, in New South Wales, Australia, an outbreak of disease was reported, initially presenting as sudden death in weaner age pigs and later as a marked increase in the birth of stillborn foetuses and elevated preweaning mortalities (McOrist et al., 2004). A novel pestivirus was identified in association with a multifocal non-suppurative myocarditis sometimes with myonecrosis and was named Bungowannah virus (Kirkland et al., 2007). Subsequent field and experimental transmission studies confirmed the role of this virus as a potent fetal pathogen (Finlaison et al., 2009; Finlaison et al., 2010).

Initial phylogenetic analysis based on comparison of 5′ non-translated region (5′NTR), and the N^{pro} and E2 protein coding regions found it to be only distantly related to the 4 recognized pestivirus species or any of the other putative pestivirus species (Kirkland et al., 2007). Selected pestivirus monoclonal antibodies (mAbs), previously reported to be pan-reactive, did not detect Bungowannah virus antigens in infected cell cultures.

In the current study we have generated and compared the whole-genome sequence with that of recognized and putative *Pestivirus* species. In addition, antigenic differences between Bungowannah virus and other pestiviruses have been examined using newly generated mAbs. Collectively these data provide convincing evidence to consider this virus as a new species within the genus *Pestivirus*.

2. Material and methods

2.1. Cell cultures

For initial studies, Bungowannah virus was grown in BVDV-free PK-15 cells (RIE5-1, Collection of Cell Lines in Veterinary Medicine

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(CCLV), Friedrich-Loeffler-Institut, Insel Riems, Germany). BVDV strains were grown in secondary bovine testis (BT) cells, while BDV was grown in primary lamb testis (LT) cells as described previously (Kirkland et al., 2007). Later, for immunofluorescence (IF) studies involving chimeric viruses, diploid bovine oesophageal KOP-R cells (RIE244, CCLV) and porcine SK-6 cells (RIE262, CCLV) were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% BVDV-free fetal calf serum (FCS).

2.2. Viruses

Bungowannah virus grown in PK-15 cells was used at the third passage after isolation from tissues of affected pigs. Viruses selected to represent the recognized pestivirus species were: BVDV-1 (strain NADL or CP7), BVDV-2 (890), BDV (X818), and CSFV (NSW 1966). For testing a panel of Bungowannah virus-specific mAbs the primary Bungowannah virus isolate was propagated two more times in SK-6 cells before use. A chimeric virus (vCP7_E1E2-Bungo) and a bicistronic virus (vCP7_E^{rns}-Bungo_bi) were also included to test the protein specificity of the Bungowannah virus-specific mAbs (see below and also Richter et al., 2011).

2.3. Construction of the chimeric full-length cDNA clone pA/CP7_C-Bungo

The genomic region encoding the Bungowannah virus structural C protein was amplified from a synthetic open reading frame (ORF) Bungo_C-E2mod_pMK-RQ (Geneart AG, Regensburg, Germany). Subsequently, the purified amplicon was used as a megaprimer in a fusion PCR with BVDV CP7 as template (Geiser et al., 2001; Richter et al., 2011; Stech et al., 2008). Primers and details for generation of the recombinant construct are available upon request. *In vitro*-transcription of the mutant pA/CP7_E1-Bungo and RNA transfection by electroporation was performed as described previously (Richter et al., 2011).

2.4. Extraction of viral genome

RNA was extracted from the supernatant of Bungowannah virus-infected PK-15 cells by using a magnetic bead based system (MagMAX^{TM}-96 Viral RNA Isolation Kit AM1836, Ambion, Austin, Texas) in accordance with the manufacturer's instructions. The magnetic beads were handled, washed and the nucleic acid eluted using a magnetic particle handling system (KingFisher 96, Thermo Fisher Scientific, Finland). RNA was stored frozen at −80 °C until use.

2.5. Sequencing of the complete viral genome

In order to complete the Bungowannah virus sequence, fragments containing DNA coding for E^{rns} to p7, p7 to NS3, NS3 to NS5A and NS5A to NS5B were amplified as described previously (Kirkland et al., 2007), using the primers listed in Supplementary Table 1. Sequence data from the 3′NTR was generated by adding a poly (A) tail to the viral RNA, using the A-Plus Poly (A) Polymerase Tailing Kit (EPICENTRE, Madison, Wisconsin, U.S.A.) followed by rapid amplification of cDNA ends (RACE, BD), as described by BD Biosciences (CLONTECH Laboratories, California, U.S.A.) with the following modifications. Hot start PCR (Qiagen, Hilden, Germany) was carried out with primers ER62F and BD Universal primer A mix with an annealing temperature of 65 °C and an extension time of 2 min. The Bungowannah virus-specific primer NS5BF and BD nested Universal Primer A were used for the Hot start nested PCR, with an annealing temperature of 65 °C and an extension time of 2 min. All PCR-products were sequenced as previously described (Kirkland et al., 2007).

2.6. Sequence analysis

The Bungowannah virus genome sequence was assembled using the Sequencher 4.10.1 software (Gene Codes, Michigan). Different segments of the genome were compared using sequences obtained from NCBI GenBank.

Analyses of these sequences were carried out using MEGA version 4 (Tamura et al., 2007). Multiple alignments were completed using Clustal W. Dendrograms were generated using the neighbor-joining method. Percentage of nucleotide and amino acid identity between the viral sequences was determined using the DNADist and ProtDist programs, respectively. Statistical

Table 1a
Length of genome, polyprotein, and NTRs of different pestivirus strains.

Species	Strain	GenBank accession no	Genome length[a] (nucleotides)	Polyprotein[a] (amino acids)	5′NTR (nt)	3′NTR (nt)	References
BVDV-1	NADL	M31182	(12,308)[b]	(3898)	385	226	Collett et al., 1988
	SD-1	M96751	12,308	3898	385	226	Deng and Brock, 1992
	C24V	AF091605	12,310	3898	385	228	
	Osloss	M96687	(12,266)[b]	(3899)	381	185	De Moerlooze et al., 1993
	CP7	AF220247	(12,267)	(3898)	382	188	Becher et al., 2000
BVDV-2	890	U18059	(12,285)	(3897)	385	206	Ridpath and Bolin, 1995
CSFV	Alfort	J04358	12,297	3898	372	228	Meyers et al., 1989
	Brescia	M31768	12,295[b]	3898	372	226	Moormann et al., 1990
	C-strain	Z46258	12,311	3898	373	241	Moormann et al., 1996
BDV	X818	AF037405	12,333	3895	372	273	Becher et al., 1998
Reindeer-1	V60-Krefeld	AF144618	12,318	3895	370	260	Avalos-Ramirez et al., 2001
Giraffe-1	H138	AF144617	(12,308)	(3891)	382	250	Avalos-Ramirez et al., 2001
Pronghorn	Pronghorn	AY781152.3	12,273	3898	369	210	Neill et al., 2014
All Species	Summary		12,266-12,333	3891-3898	369-385	185-273	
Bungowannah virus	Prototype B869	NC_023176	12,649	3918	391	504	This study

Table adapted from Avalos-Ramirez et al. (2001).

[a] Numbers in parentheses indicate the length of the genome or polyprotein without inserted cellular sequences (BVDV-1 NADL, BVDV-1 Osloss, Giraffe-1) or without inserted viral sequences (BVDV-1CP7, BVDV-2 890).

[b] Including sequence deposited in GenBank and additional nt at the 5′ and/or 3′ terminus reported by Brock et al. (1992) (BVDV-1 NADL), and Moormann et al. (1996) (BVDV-1 Osloss, CSFV Brescia).