Journal of General Virology (2015), **96**, 513–523 **DOI** 10.1099/vir.0.000005

Establishment of a minigenome system for Oropouche virus reveals the S genome segment to be significantly longer than reported previously

Gustavo Olszanski Acrani,[1,2]† Natasha L. Tilston-Lunel,[1,3]† Martin Spiegel,[4] Manfred Weidmann,[4]‡ Meik Dilcher,[4] Daisy Elaine Andrade da Silva,[5] Marcio R. T. Nunes[5] and Richard M. Elliott[1]

Correspondence
Richard M. Elliott
richard.elliott@glasgow.ac.uk

[1]MRC-University of Glasgow Centre for Virus Research, 464 Bearsden Road, Glasgow G61 1QH, Scotland, UK

[2]Department of Cell and Molecular Biology, University of Sao Paulo School of Medicine, 3900, Av. Bandeirantes, Ribeirão Preto, SP 14049-900, Brazil

[3]Biomedical Sciences Research Complex, School of Biology, University of St Andrews, St Andrews KY16 9ST, Scotland, UK

[4]Department of Virology, University Medical Center Göttingen, Kreuzbergring 57, 37075 Göttingen, Germany

[5]Center for Technological Innovation, Instituto Evandro Chagas, Ananindeua, Brazil

Oropouche virus (OROV) is a medically important orthobunyavirus, which causes frequent outbreaks of a febrile illness in the northern parts of Brazil. However, despite being the cause of an estimated half a million human infections since its first isolation in Trinidad in 1955, details of the molecular biology of this tripartite, negative-sense RNA virus remain limited. We have determined the complete nucleotide sequence of the Brazilian prototype strain of OROV, BeAn 19991, and found a number of differences compared with sequences in the database. Most notable were that the S segment contained an additional 204 nt at the 3′ end and that there was a critical nucleotide mismatch at position 9 within the base-paired terminal panhandle structure of each genome segment. In addition, we obtained the complete sequence of the Trinidadian prototype strain TRVL-9760 that showed similar characteristics to the BeAn 19991 strain. By using a T7 RNA polymerase-driven minigenome system, we demonstrated that cDNA clones of the BeAn 19991 L and S segments expressed functional proteins, and also that the newly determined terminal untranslated sequences acted as functional promoters in the minigenome assay. By co-transfecting a cDNA to the viral glycoproteins, virus-like particles were generated that packaged a minigenome and were capable of infecting naive cells.

Received 16 September 2014
Accepted 3 November 2014

INTRODUCTION

Oropouche virus (OROV) is one of the most important arboviruses in Brazil, after dengue virus and yellow fever virus, and was first isolated in 1955 from a febrile patient in Trinidad (Anderson *et al.*, 1961). Subsequently, the virus was isolated in Brazil in 1960 from the blood of a pale-throated three-toed sloth, *Bradypus tridactylus*, at a forest camp-site during construction of the Belém–Brasilia highway, just before the first documented epidemic in Brazil in 1961 (Pinheiro *et al.*, 1962). It is estimated that half a million OROV infections have occurred in >30 outbreaks since the virus became recognized, but it is probable that the actual numbers are much higher as cases may be masked by other febrile illnesses, such as dengue or Mayaro fever, and diseases caused by other orthobunyaviruses, such as Guama virus, that are prevalent in the region (reviewed by Vasconcelos *et al.*, 2011). OROV has also been isolated from various mosquito species (e.g. *Coquillettidia venezuelensis* and *Ochlerotatus serratus*), but during epidemics OROV is transmitted to humans by the biting midge *Culicoides paraensis* (Pinheiro *et al.*, 1981a, b, 1982).

†These authors contributed equally to this work.

‡Present address: Institute of Aquaculture, Pathfoot Building, University of Stirling, Stirling FK9 4LA, Scotland, UK.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the segments of OROV strains BeAn 19991 and TRVL-9760 are KP052850–KP052852 and KP026179–KP026181, respectively.

OROV belongs to the Simbu serogroup of the genus *Orthobunyavirus*. The serogroup also includes a number of veterinary pathogens such as Akabane, Aino, Shuni, Sabo and Douglas viruses, as well the newly emerged Schmallenberg virus (Afonso *et al.*, 2014). OROV is currently the only known human pathogen in the serogroup and recent phylogenetic analysis (Ladner *et al.*, 2014b) places it in a clade separate from the other members. Like all bunyaviruses, the OROV genome consists of three segments of negative-sense ssRNA designated large (L), medium (M) and small (S). The L segment encodes the viral polymerase (L protein), and the M segment encodes the glycoproteins Gn and Gc, along with a non-structural protein called NSm. The S segment encodes the viral nucleocapsid protein (N) and a second non-structural protein (NSs) in overlapping reading frames, although both proteins are translated from the same mRNA (Elliott, 2014; Plyusnin & Elliott, 2011). The terminal sequences at the 3′ and 5′ ends of each segment are complementary, allowing the formation of a panhandle structure that is crucial for genome replication and transcription (Barr *et al.*, 2003; Barr & Wertz, 2004; Kohl *et al.*, 2004).

The epidemiology and genetic variation of OROV has been widely studied, and phylogenetic analysis of numerous partial S segment sequences (mainly N ORF sequences), together with more limited partial sequence data on the M and L segments, suggested the existence of four genotypes (reviewed by Vasconcelos *et al.*, 2011). However, much less is known about the general molecular biology of OROV or virus–host interactions. To facilitate such investigations we intended to develop a reverse genetics system for OROV, as has been reported for other orthobunyaviruses (Elliott, 2012), including two Simbu group viruses: Akabane virus (Ogawa *et al.*, 2007) and Schmallenberg virus (Elliott *et al.*, 2013; Varela *et al.*, 2013). When we produced cDNA clones of the OROV genome segments, we noticed several discrepancies between the viral sequences we obtained and the sequences in the database, notably that the S segment contained an additional 204 nt. The functionality of our cDNA clones was confirmed by establishing minigenome (Blakqori *et al.*, 2003; Weber *et al.*, 2002) and virus-like particle (VLP) (Shi *et al.*, 2007) systems. Our results highlighted the importance of obtaining complete and correct viral sequences, including direct confirmation of the genome termini, in order to establish reverse genetic systems.

RESULTS

Cloning and sequence determination of the genome of OROV strain BeAn 19991

Total RNA was extracted from BHK-21 cells infected with OROV strain BeAn 19991 (prototype Brazilian strain isolated from *B. tridactylus*) and reverse transcribed using random primers. Segment-specific oligonucleotides, based on available complete sequences in GenBank [L, accession number NC_005776.1 (Aquino *et al.*, 2003); M, NC_005775.1 (Wang *et al.*, 2001); and S, NC_005777.1; V. H. Aquino and others, unpublished], were used in PCR (Table 1). Full-length cDNAs were cloned into the T7 RNA polymerase transcription plasmid TVT7R(0,0) (Johnson *et al.*, 2000); the inserts included an extra G residue at their 5′ ends for efficient T7 transcription and the cDNAs were cloned such that T7 polymerase would transcribe anti-genome-sense RNAs, as described previously (Elliott *et al.*, 2013). Descriptions of the sequences in this paper are presented for the antigenome-sense RNA, in the conventional 5′→3′ orientation.

The full-length L segment sequence that we obtained was 6852 nt in length, 6 nt longer than GenBank accession number NC_005776.1. Alignment of our sequence with that of GenBank accession number NC_005776.1 revealed a number of differences in the regions from nt 2405 to 2450 and from nt 2592 to 2617, resulting in amino acid changes in the region from aa 798 to 812 and from aa 860 to 867 (Fig. 1). We verified the sequence of this region by reverse transcription (RT)-PCR amplification of a fragment from nt 2130 to 2980 using specific primers and viral RNA as template. Furthermore, alignment of our sequence with partial sequences of the L segments of OROV strains TRVL-9760, GML-444479 and IQT-1690 (GenBank accession numbers KC759122.1, KC759128.1 and KC759125.1, respectively) revealed that, apart from a few variations at the nucleotide level, the translated amino acid sequence for this region was conserved (Fig. 1). Therefore, we consider the published sequence for the BeAn 19991 L segment contains some errors in this region. In addition, we noted two other amino acid differences: L to F at position 415 and N to D at position 1021. Both of these have been confirmed by independent sequence analysis of our stock of virus, and the F residue at position 415 is also found in the L protein of other strains of OROV (TRVL-9760, GML-444479 and IQT-1690).

The terminal sequences of the L segment UTRs were determined by a 3′ RACE procedure on total infected cell RNA, using oligonucleotides designed to anneal to either the genomic or antigenomic strands. Position 9 of the 5′ UTR was determined as a C residue and the corresponding −9 position in the 3′ UTR was determined as an A residue, resulting in the characteristic mismatch that has been observed in the predicted panhandle structure of other orthobunyavirus genome segments (Kohl *et al.*, 2004). This mismatch is not recorded in the published sequence. Additionally, position 18 at the 5′ end was determined to be a U rather than a C residue, as in the published sequence (Fig. 2).

The full-length M segment was determined to be 4385 nt in length, in agreement with the published sequence. There were a small number of nucleotide variations compared with GenBank accession number NC_005775.1, six of which resulted in amino acid differences: I274F, F587L, K614N, D750G, K981Q and G982S. The sequences encoding these residues were confirmed in independent cDNA clones of the M segment cDNA and also by specific RT-PCR amplification of appropriate regions of the viral RNA. Results from RACE analysis revealed two single nucleotide differences in

Table 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)	Segment/gene	Position
OROLFg	GGGGTACCCGTCTCATATAG**AGTAGTGTGCTCCTATTCCG**	L	1–19
OROL1	GAAGTTAGTTAGATATGTCT	L	3706–3687
OROLRg	GCTCTAGACGTCTCTACCC**AGTAGTGTGCTCCTATTTAG**	L	6833–6852
OROL2	CCCTTGTGAACTCAATGGTA	L	3537–3556
OROMFg	GGGGTACCCGTCTCATATAG**AGTAGTGTGCTACCGGCAACAAACA**	M	1–25
OROMRg	GCTCTAGACGTCTCTACCC**AGTAGTGTGCTACCGACAACAATTT**	M	4508–4484
OROSFg	GGGGTACCCGTCTCATATAG**AGTAGTGTGCTCCACAATTC**	S	1–20
OROSRg	GCTCTAGACGTCTCTACCC**AGTAGTGTGCTCCACTATAT**	S	754–735†
ORODelNSsF	GAGTTCATTTTCAACGA**C**GTACCACAACGGACTACATCTACATTTGATCCGGAGGCAGCATA**C**GT**A**GCATTTGAAGC	delNSs	51–127
ORODelNSsR	GCTTCAAATGC**T**AC**G**TATGCTGCCTCCGGATCAAATGTAGATGTAGTCCGTTGTGGTAC**G**TCGTTGAAAATGAACTC	delNSs	127–51
pTM1-OROVL-F	AAAACACGATAATACC**ATGTCACAACTGTTGCTCAACCAATATCG**	L	44–72
pTM1-OROVL-R	TTAATTAGGCCTCTC**TTAGAAGTCAAATTTGGATTTGCCAGT**	L	6802–6776
pTM1-OROVM-F	AACACGATAATACC**ATGGCGAATTTAATAATTATTTCAATGGTTC**	Glycoprotein	32–62
pTM1-OROVM-R	TTAATTAGGCCTCTC**CTACTTGATTTTCTGCTCCATGGCATATTCTATTTCATGTCTGATT**	Glycoprotein	4294–4249
pTM1-OROVS-F	AAACACGATAATACC**ATGTCAGAGTTCATTTTCAACGATGTACCAC**	N	45–75
pTM1-OROVS-R	TTAATTAGGCCTCTC**CTATATGTCAATTCCGAATTGGCGCAAGAAGTCTCTTGCTGC**	N	740–699
OROVL_anti	ACCTCTCCAAAAATCTCATT	L 5′ UTR	384–365
OROVL_gen	GAACTAGACAATTGTATTCA	L 3′ UTR	6494–6513
OROVM_anti	CTAATATCACATGCTGCTCTACATG	M 5′ UTR	396–372
OROVM_gen	GCACATATCTGTGGGAGAGACAT	M 3′ UTR	3959–3981
OROSlig1	CTTGCGCCAATTCGGAATTGAC	S UTR	713–734
OROSlig2	GGTACATCGTTGAAAATGAAC	S UTR	73–53

*Viral sequences are shown in bold.

†Based on GenBank accession number NC_005777.1.

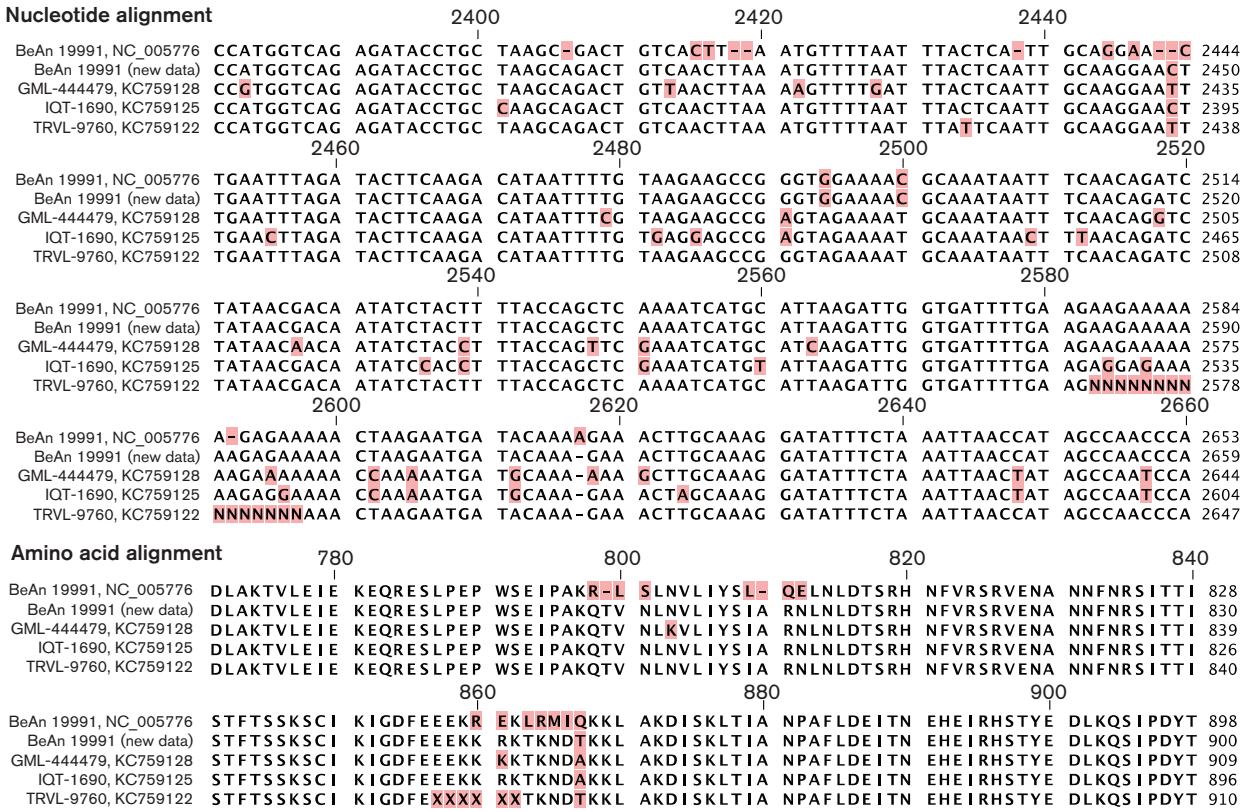

Fig. 1. Alignment of part of the OROV L segment highlighting the differences between the published sequence for the BeAn 19991 strain (GenBank accession number NC_005776) and the sequence obtained in this study (new data), along with three published OROV sequences from different genotypes GML-444479, IQT-1690 and TRVL-9760 (GenBank accession numbers KC759128.1, KC759125.1 and KC759122.1, respectively). The nucleotide alignment is shown in the top panel and the amino acid alignment is shown in the bottom panel. Alignments were performed using CLC Genomics Workbench 6.5.

the 5′ UTR (C at position 9 and A at position 15) and one difference at the 3′ end (U at position 15) compared with the database sequence. Thus, the predicted panhandle has a C/A mismatch at position 9/–9 and a U/A pairing at position 15/–15 (Fig. 2).

The PCR to amplify the S segment surprisingly generated two products of ~750 and 1000 bp (Fig. 3a). After cloning, the sequences of both products were determined. The nucleotide sequence of the smaller fragment was identical to GenBank accession number NC_005777 (V. H. Aquino and others, unpublished) that is described as 'Oropouche virus segment S, complete genome', but no strain designation is given. Saeed *et al.* (2000) reported the complete sequence of the TRVL-9760 strain of OROV also to be 754 nt, although GenBank accession number AF164531 only gives the coding sequence for this strain. In addition, the sequence of the N ORF of the BeAn 19991 was also reported by Saeed *et al.* (2000) (GenBank accession number AF164532) and the amino acid sequence was identical to that that we obtained.

The larger fragment contained an additional 204 nt after the apparent consensus 3′ terminal sequence in the GenBank entry (Fig. 3b).

The DNA products were extracted from the gel and used as templates in further PCR. The shorter template gave rise to a single, similarly sized amplicon, whereas the longer template again generated products ~750 and 1000 bp in length (Fig. 3c). To investigate this observation further, we amplified the S segment of a clinical isolate of OROV (H759025 AMA2080; N. L. Tilston-Lunel, M. R. T. Nunes & R. M. Elliott, unpublished) using the same primers and PCR conditions that were used for BeAn 19991, and again observed two amplified DNA fragments (data not shown). The sequences of both of these amplicons largely matched that of the BeAn 19991 products (data not shown).

Inspection of the 'long' sequence showed that nt 735–752 could allow annealing of the primer used in PCR (Fig. 3d). Thus, binding of the primer to this internal sequence in the S segment would result in a cDNA product with a terminus matching that of the orthobunyavirus consensus sequence, making it appear complete. Using 3′ RACE and RNA ligation methods, we confirmed that the OROV S segment did indeed contain the additional 204 nt at the 3′ end (data not shown). Therefore, the full-length OROV S segment is 958 nt in length.

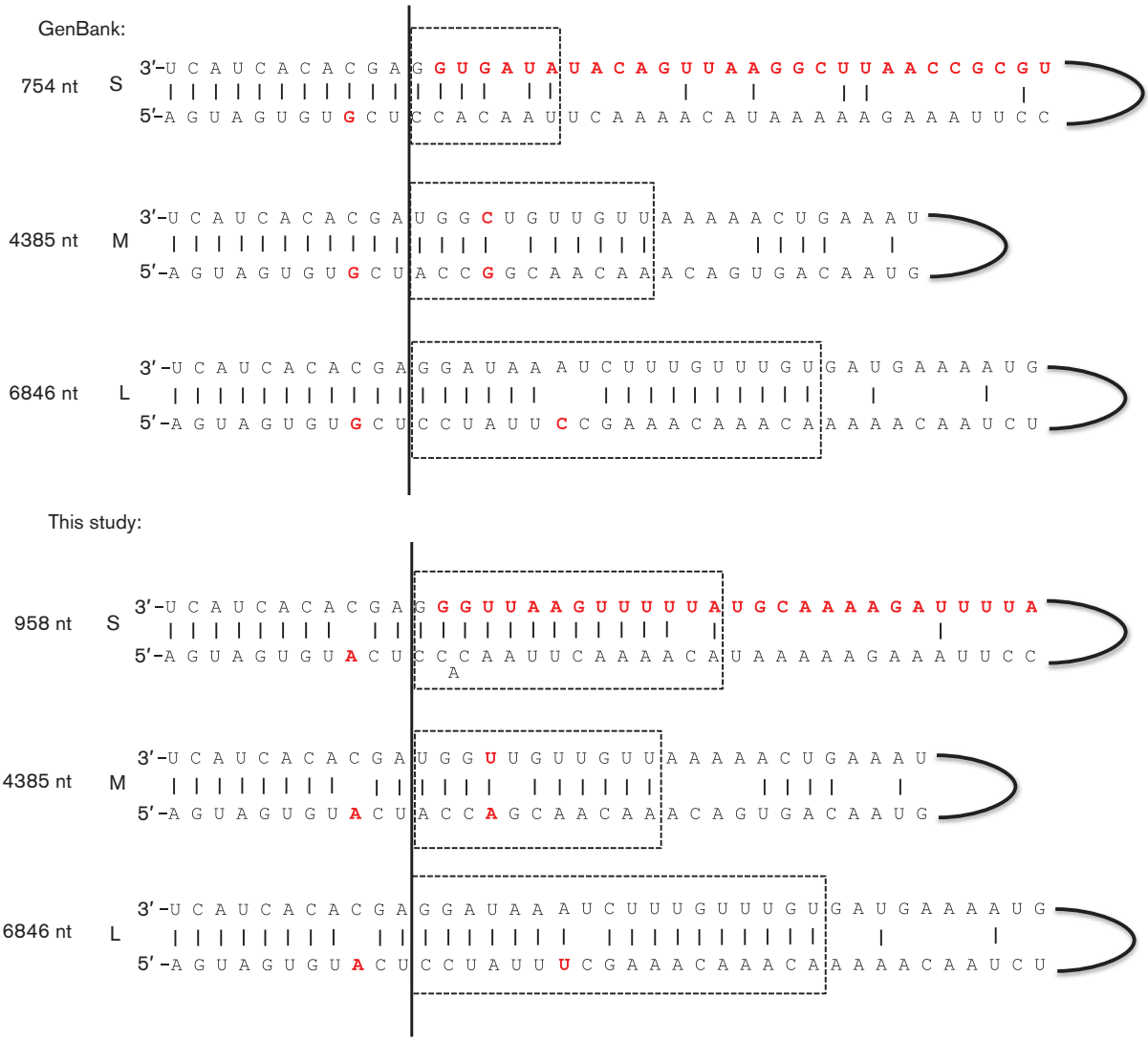

Fig. 2. Comparison of the published and the revised OROV BeAn 19991 UTR sequences shown as a panhandle structure (antigenomic sense). The terminal 11 conserved residues are separated by a vertical line. Differences are highlighted in red.

The corrected sequences of the OROV strain BeAn 19991 genome have been deposited in GenBank with accession numbers KP052850 (L), KP052851 (M) and KP052852 (S).

Sequence determination of the OROV TRVL-9760 strain

Determination of the complete sequence of another strain of OROV, the Trinidadian prototype TRVL-9760, was carried out independently from that of the BeAn 19991 strain. Total RNA was extracted from infected murine type I IFN receptor-deficient (IFNAR$^{-/-}$) cells and reverse transcribed using random hexamer primers. Sequences comprising the L, M and S segment ORFs were amplified by RT-PCR using specific oligonucleotides based on the available sequences (GenBank accession numbers NC_005776.1, NC_005775.1 and NC_005777.1, as described above). Whilst the N ORF sequence was completely amplified in one step, the L and the M segment ORF sequences were amplified as six (L) or three (M) overlapping fragments. The resulting cDNAs were inserted into the TA-vector pCRII and their sequences were determined by Sanger sequencing. In comparison with the BeAn 19991 L ORF sequence (GenBank accession number NC_005776.1), the TRVL-9760 L ORF contained 151 nt exchanges, seven single nucleotide insertions and one single nucleotide deletion. Whilst 134 of the 151 nt exchanges were silent, the nucleotide insertions and deletions which were found from nt 2405 to 2446 and from nt 2592 to 2617 led to several amino acid exchanges and the insertion of two additional amino acids at aa 799 and 810 (Fig. 1). The majority of the amino acid substitutions caused by single nucleotide exchanges were found in the N-terminal half of the L ORF (A136T, M145V, N210S, N273D, Q308K, S313N, I355V, F415L, D442N, T479A, I558M, T640A,

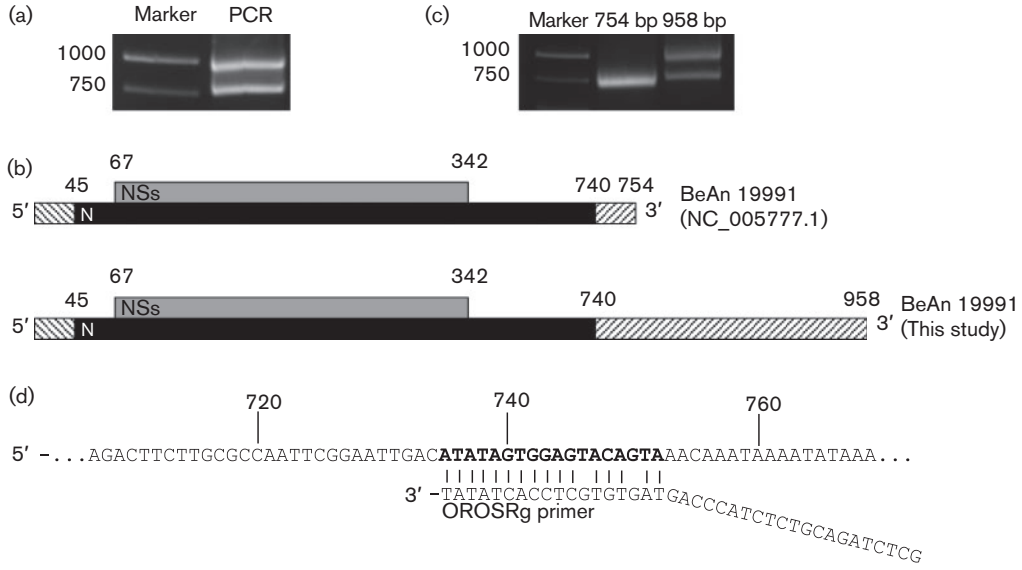

Fig. 3. Analysis of the OROV S segment. (a) Agarose gel electrophoresis of the S segment RT-PCR product. (b) Schematic drawing of the OROV S segment, comparing the published sequence of 754 bp (upper drawing) with the newly determined 958 bp sequence (lower drawing). Black boxes, N ORF; grey boxes, NSs ORF; hatched boxes, UTRs. The sequence is presented in the antigenomic 5′→3′ sense. Numbers indicate nucleotide positions in the sequence. (c) Agarose gel electrophoresis of reamplified DNA products using the 754 and 958 bp PCR products as template. (d) Diagram showing the potential internal binding site (bold) in the OROV S segment. Numbers represent nucleotide positions. OROSRg primer represents the primer sequence that was used in this paper to amplify the S segment.

S921N, L974I and S1021N), whilst only three exchanges were found in the C-terminal half (T1159I, E2056G and R2241K). When compared with the BeAn 19991 M ORF sequence (GenBank accession number NC_005775.1), the TRVL-9760 M ORF showed 100 nt exchanges, with 15 of them leading to amino acid substitutions (S12G, I13V, L67P, A244V, I274F, T463I, A609T, K615N, V732L, D750G, R801K, V846I, S849G, V1241I and M1363I). For the TRVL-9760 N ORF, we detected 13 nt exchanges in comparison to the BeAn 19991 N ORF sequence (GenBank accession number NC_005777.1), but none of these exchanges led to an amino acid substitution. Three of these nucleotide exchanges also affected the overlapping NSs ORF and two of them led to amino acid exchanges (K13R and N74S).

To determine the sequence of the complete L, M and S segments, pyrosequencing was performed. OROV genomic RNA isolated from supernatants of infected murine IFNAR$^{-/-}$ cells was converted to dsDNA by whole-transcriptome amplification, which served as starting material for a shotgun library preparation. After pyrosequencing of the shotgun library, *de novo* assembly with the obtained sequence reads was performed which resulted in sequences for the OROV L, M and S ORFs identical to those obtained by Sanger sequencing. It was not, however, possible to determine the sequences of the non-coding regions by *de novo* assembly. Therefore, an additional reference mapping was performed using the OROV genomic segment sequences from GenBank as reference. With this approach we were able to map the obtained sequence reads to the complete L and the M segment sequences (GenBank accession numbers NC_005776.1 and NC_005775.1). In the case of the S segment, however, it was not possible to map the sequence reads to the 3′ end of the S segment sequence (GenBank accession number NC_005777.1), but mapping was possible for the 5′ non-coding end and the N ORF. We therefore performed another round of reference mapping using an S segment fragment comprising the 5′ end and the N ORF of GenBank accession number NC_005777.1 as reference sequence. Using this approach, the reference mapping resulted in an S segment sequence with an additional 204 nt at the 3′ end.

The complete sequences of the OROV strain TRVL-9760 genome segments have been deposited in GenBank with accession numbers KP026179 (L), KP026180 (M) and KP026181 (S).

Establishment of an OROV minigenome system

Minigenome systems have been described for a number of orthobunyaviruses, and comprise a negative-sense genome analogue encoding a reporter gene that is packaged into ribonucleoprotein complex, transcribed and replicated by co-expressed viral N and L proteins, leading to measurable reporter activity (Elliott, 2012). After confirmation of the nucleotide sequences, the ORFs in each segment are amplified by PCR and subcloned into the pTM1 expression vector (Moss *et al.*, 1990). Minigenome constructs are created by replacing the viral ORF in each segment with the sequence for *Renilla* luciferase and then inverting the insert

in plasmid TVT7R(0,0) (Johnson *et al.*, 2000) so that T7 transcripts would be in the genomic sense (Weber *et al.*, 2001). We first used a minigenome based on the OROV M segment, as studies with Bunyamwera virus (BUNV) showed the M segment minigenome to be the most active (Barr *et al.*, 2003). However, initial attempts using the M segment UTR sequences as reported in GenBank gave low activity over background. When we subsequently obtained the M segment terminal sequences by 3′ RACE analysis and redesigned the minigenome accordingly, with the C/A mismatch at position 9/–9, high levels of luciferase activity were observed, indicating that (i) both N- and L-expressing constructs were functional and (ii) that the M segment UTR sequences determined herein were active promoters. The amounts of transfected N- and L-expressing plasmids were titrated to determine the optimal amounts that gave maximum luciferase activity (data not shown), and the optimized amounts used in all further experiments.

The effects of nucleotide differences in the M segment UTR on minigenome activity are compared in Fig. 4(a). The minigenome with UTR sequences as previously published (9C/G, 15C/G) showed low activity, whereas the minigenome with UTR sequences as determined in our work (9C/A, 15U/A) showed >2000-fold increased activity over background (cells where no L-expressing plasmid was transfected). However, it was not just the mismatch at position 9/–9 that was critical for maximal activity, but also the base-pairing at position 15/–15, as the minigenome with the position 9 C/A mismatch but C/G at position 15/–15 showed only 500-fold increase in activity. Introduction of the U/A pairing was not able to rescue activity when position 9/–9 was C/G and other nucleotide combinations at position 15 were less active than U/A. Taken together, these results highlight the importance of certain residues within the M segment promoter.

The minigenome assay was also used to compare the short and long S segment UTR sequences (Fig. 4b). Minigenome constructs contained the same 5′ UTR and either the 14 nt (as previously published) or 218 nt (as determined herein) long 3′ UTR. The minigenome with the short UTR was inactive, whereas the minigenome with the 218 nt 3′ UTR showed robust luciferase activity. Lastly, we compared L segment-derived minigenomes with either a C or U residue at position 18 in the 5′ UTR. Both minigenomes gave similar high luciferase activity (Fig. 4c).

Together, these results confirmed that the N and L proteins were functional in a minigenome assay, and also that the UTR sequences as determined for the S, M and L segments were functional promoters, and that a base mismatch at position 9/–9 was critical for promoter activity.

VLP production assay

To investigate whether the glycoprotein gene was also functional, a VLP assay was developed. In addition to the M segment minigenome, N- and L-expressing plasmids, cells were also transfected with a plasmid expressing the glycoprotein precursor. Luciferase activity was measured in these donor cells at 24 and 48 h post-transfection (Fig. 5a), and it was noted that there was a significant increase in luciferase activity in cells additionally transfected with the glycoprotein cDNA at 48 h, suggesting spread of VLPs within the culture. The supernatants from transfected cells were harvested at 48 h post-transfection and transferred onto naive BHK-21 cells; luciferase activity in these cells was measured 24 h later. High levels of luciferase activity were recorded in cells exposed to supernatants expressing the glycoproteins (Fig. 5b, L+M) compared with those exposed to supernatants from cells not transfected with the glycoprotein cDNA (Fig. 5b, +L). This is a stringent assay relying only on transcription of the packaged minigenome in the VLP without the need for exogenously supplied viral N and L proteins. Incubation of the supernatant with antibodies to OROV before infection markedly reduced luciferase expression, whereas incubation with an irrelevant antiserum (anti-BUNV serum) had no effect (Fig. 5b). Taken together, these results indicated that the OROV glycoprotein gene cDNA was functional in this VLP assay.

DISCUSSION

A crucial step in developing reverse genetic systems for RNA viruses is obtaining cDNA clones that are representative of the authentic viral genome sequence. As described above, we found a number of sequence differences in our clones derived from the BeAn 19991 strain compared with sequences in the database, including an additional ~200 nt at the 3′ end of the S genome segment, an apparent frame shift in the L segment coding sequence and a critical mismatched nucleotide pair in the terminal panhandle sequence on each segment. These significant differences were confirmed when the complete sequence of the Trinidadian prototype strain TRVL-9760 was also determined.

Early studies comparing orthobunyavirus genome sequences indicated that the terminal 11 nt of each segment exhibited a high degree of conservation, and hence consensus primers based on sequences of Bunyamwera and California serogroup viruses (Dunn *et al.*, 1994; Elliott, 1989a, b; Elliott *et al.*, 1991) have traditionally been used to amplify unknown bunyavirus genomes. However, the actual terminal sequences for the majority of sequences currently available in GenBank have not been verified directly, e.g. by RACE techniques. With regard to the orthobunyavirus 'consensus sequence', there is a single nucleotide difference between the 3′ and 5′ complementary ends such that, using total infected cell RNA as template, mispriming by either primer could occur or a single primer could bind to both genomic and antigenomic RNAs. Indeed, a single primer was used to amplify the OROV M segment (Aquino & Figueiredo, 2004) or the S segments of a range of orthobunyaviruses (Lambert & Lanciotti, 2008). The importance of the terminal sequence has been investigated by minigenome assays for BUNV (Barr & Wertz, 2004; Barr *et al.*, 2003; Dunn *et al.*, 1995; Kohl *et al.*, 2003, 2004) and the

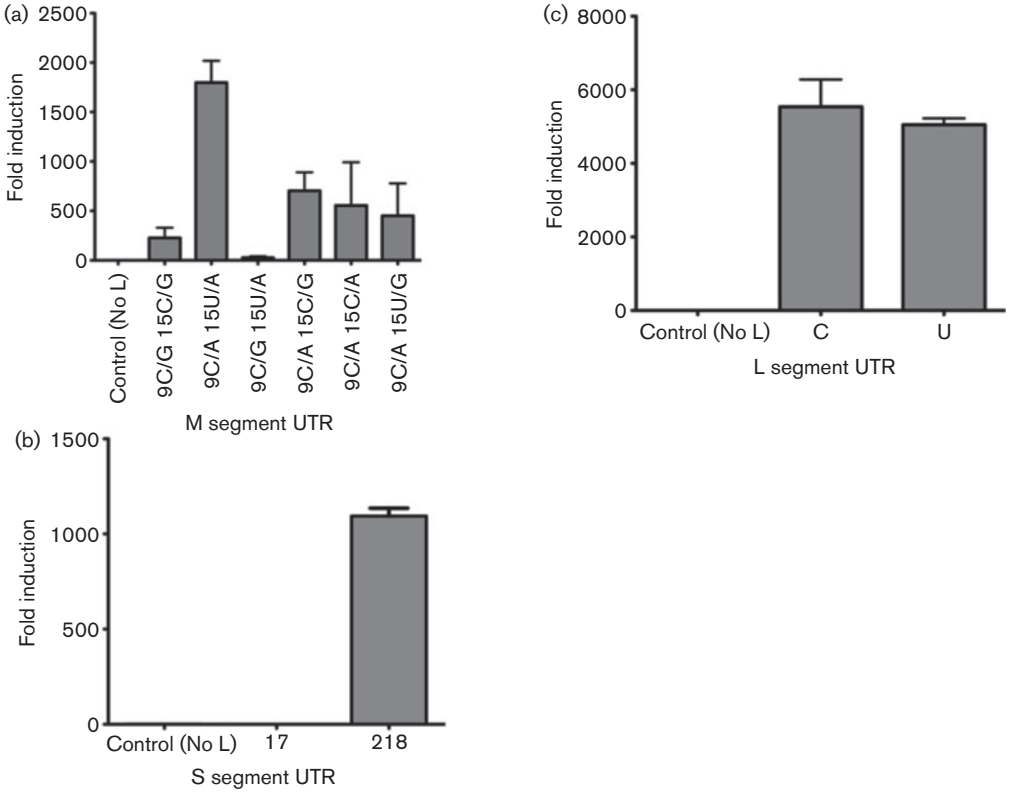

Fig. 4. Minigenome assay. (a) Comparison of M segment-based minigenomes. BSR-T7/5 cells were transfected with 1 μg each pTM1OROV-L and pTM1OROV-N, 0.5 μg M segment minigenome-expressing plasmid, and 100 ng pTM1-FF-Luc; the background control lacked pTM1OROV-L. M segment minigenomes contained different nucleotides at position 9/−9 as indicated. Minigenome activity is expressed as fold induction over the background control. (b) Comparison of S segment minigenomes containing the published (14 nt) or newly defined long (218 nt) 5′ UTR. (c) Comparison of L segment minigenomes containing a C or U at position 18 in the 3′ UTR. Error bars indicate SD.

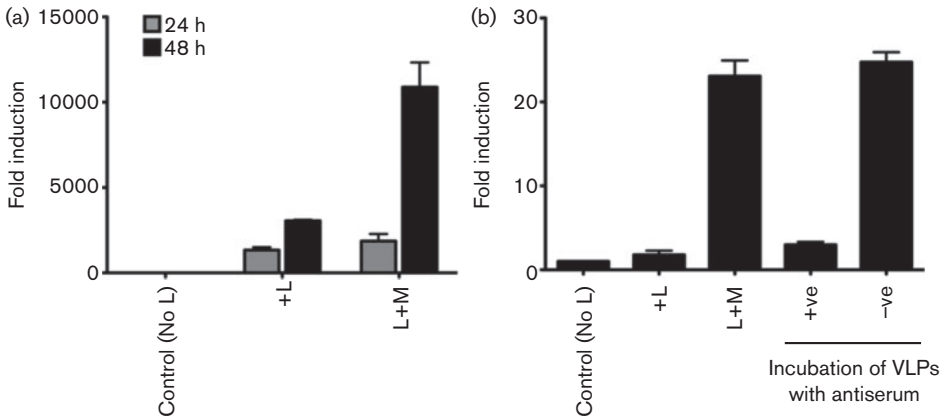

Fig. 5. VLP production assay. BSR-T7/5 cells were transfected with 1 μg each pTM1OROV-L and pTM1OROV-N, 0.5 μg pTM1OROV-M, 0.5 μg M segment minigenome-expressing plasmid, and 100 ng pTM1-FF-Luc; control transfection mixes lacked pTM1OROV-L (No L) or pTM1OROV-M (+L). At 24 or 48 h post-transfection, clarified supernatants were used to infect naive BHK-21 cells, and luciferase activity measured 24 h later. (a) Minigenome activity in transfected BSR-T7/5 cells at 24 or 48 h post-transfection. (b) Minigenome activity in BHK-21 cells infected with supernatants from cells in (a). VLPs were also incubated with anti-OROV antibodies (+ve) or irrelevant antibodies (−ve) before infection of cells as indicated. Error bars indicate SD.

mismatch at position 9/–9 was shown to be crucial for promoter activity (Barr & Wertz, 2005). As more diverse orthobunyavirus genomes have been sequenced, particularly using next-generation sequencing methods (deep sequencing) that are not reliant on specific primers to amplify cDNA, it has become clear that there is more variation in the 'bunyavirus consensus' than observed between Bunyamwera and California serogroup viruses (e.g. Ladner *et al.*, 2014b), highlighting the requirement for direct determination of the terminal sequences. In a similar vein, as the genomes of more members of the genus *Phlebovirus* (another genus in the family *Bunyaviridae*) have been sequenced, it is apparent that the termini also diverge from the 'phlebovirus consensus' (Dilcher *et al.*, 2012a; Elliott & Brennan, 2014; Matsuno *et al.*, 2013).

A recent paper (Ladner *et al.*, 2014a) suggested the standards that should be applied to viral genome sequence determination and we strongly support the recommendations proposed therein.

Saeed *et al.* (2000) reported the first nucleocapsid gene sequences of 28 strains of OROV, including the prototypic Trinidadian OROV isolate TRVL-9760 and the Brazilian isolate BeAn 19991. They determined the complete S segment to be 754 bases and noted the unusually short length of the 3′ UTR, just 14 bases after the translational stop codon, compared with other orthobunyavirus S segments. They employed various experimental procedures to verify the 3′ UTR, including chemical denaturation of the purified viral RNA with methylmercury hydroxide before RT-PCR (in case there was a secondary structure that impeded reverse transcription), and a 5′ RACE procedure using both purified viral RNA and total cellular RNA as starting material (Saeed *et al.*, 2000). All approaches yielded that same short 3′ UTR. Our results indicate that the true length of the S segment is actually 958 nt, which was verified by independent experimental analyses, including deep sequencing of the TRVL-9760 strain. Examination of the correct sequence reveals an internal region highly similar to the terminal sequence that could hybridize with the primer and in our studies resulted in two PCR products. The functionality of the longer 3′ UTR determined in this study was demonstrated in the minigenome assay.

We further confirmed that the sequences of the BeAn 19991 N and L proteins were functional in driving reporter gene expression from minigenomes, and similarly that the determined UTR sequence for all three segments could be used to construct functional minigenomes. Lastly, by co-transfecting a cDNA that expressed the glycoprotein gene, we produced VLPs that were capable of packaging a minigenome and infecting naive cells. Taken together, these data provide strong evidence that the cDNA clones reported in this paper are fully functional and pave the way to establishing a virus rescue system. The availability of such a system will play a crucial role in understanding the molecular biology of this important yet poorly characterized emerging viral zoonosis.

METHODS

Cells and virus. Vero-E6 and murine IFNAR$^{-/-}$ cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10 % FCS. BHK-21 cells were grown in Glasgow minimal essential medium (GMEM; Invitrogen) supplemented with 10 % newborn calf serum and 10 % tryptose phosphate broth (TPB; Invitrogen). BSR-T7/5 cells, which stably express T7 RNA polymerase (Buchholz *et al.*, 1999), were grown in GMEM supplemented with 10 % FCS, 10 % TPB and 1 mg G418 ml^{-1} (Geneticin; Invitrogen).

OROV strain BeAn 19991 was kindly donated by Professor Luiz Tadeu Moraes Figueiredo (University of Sao Paulo School of Medicine, Ribeirão Preto, Brazil) and strain TRVL-9760 was kindly provided by Dr Robert Shope (University of Texas Medical Branch, Galveston, TX, USA). A sample of total infected cell RNA obtained from strain H759025 AMA2080 was provided by Dr Pedro Vasconcelos (Department of Arboviruses and Hemorrhagic Fevers, Evandro Chagas Institute, Ministry of Health, Ananindeua, Brazil).

All experiments with infectious viruses were conducted under Containment Level 3 laboratory conditions.

Cloning of OROV cDNA. OROV was grown in BHK-21 cells at 37 °C, and after 30 h both cells and supernatant were harvested, and RNA extracted using TRIzol reagent (Invitrogen). cDNAs to each segment were synthesized separately, using segment-specific primers for the L and M segments (OROLFg and OROMFg, Table 1), and random primers (Promega) for the S segment, together with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). Each cDNA preparation was used in a segment-specific PCR using the appropriate primer pairs (OROMFg/OROMRg for the M segment and OROSFg/OROSRg for the S segment; Table 1) and KOD Hot Start DNA polymerase (Merck), according to the manufacturer's protocol. The full-length PCR products were cloned into pGEM-T Easy (Promega). After selection of positive clones, the inserts were excised by digestion with *Bsm*BI and ligated into *Bbs*I-linearized plasmid TVT7R(0,0) (Johnson *et al.*, 2000). The L segment cDNA was amplified in two fragments using primer pairs (OROFLg/OROL1 and OROL2/OROLRg; Table 1). The first primer pair amplified nt 1–3706 and the second pair amplified nt 3537–6852, resulting in two PCR products with a 170 bp overlapping region containing a unique *Bsg*I restriction site (nt 3590 in the full-length segment). PCR products were purified from an agarose gel and then cloned into pGEM-T Easy. The inserts were excised by digestion with restriction enzymes *Bsg*I and *Bsm*BI, and the full-length L segment was assembled by ligating both fragments with *Bbs*I-linearized TVT7R(0,0). The cDNA inserts included an extra G residue at their 5′ ends for efficient T7 transcription and the inserts were cloned such that T7 polymerase would transcribe antigenome-sense RNAs. The plasmids were designated pTVTOROVL, pTVTOROVM and pTVTOROVS.

Construction of protein-expressing and minigenome-expressing plasmids. The complete ORFs in the L and M segments were amplified by PCR using specific primers (pTM1 series in Table 1) and the pTVT7 transcription plasmids as templates, and subcloned into expression vector pTM1 (Moss *et al.*, 1990), under the control of the T7 promoter and encephalomyocarditis virus internal ribosome entry site sequence. The constructs were designated pTM1OROV-L and pTM1OROV-M. To generate a plasmid expressing only the N protein, we introduced three point mutations (T68C, T113C and G116A) into pTVTOROVS using primers ORODelNSsF and ORODelNSsR (Table 1), by QuikChange site-directed mutagenesis (Stratagene), prior to PCR amplification of the N ORF. These mutations changed the first and second methionine codons in the NSs ORF into threonine codons, and introduced an in-frame translation stop codon at codon 17; the coding sequence of the overlapping N ORF was unaffected. This plasmid was designated pTM1OROV-N.

The minigenome plasmids were created in three steps. First, the sequence encoding the coding sequence in each pTVT7 clone was deleted by excision PCR, leaving the UTRs intact. These linearized DNAs were then used in an In-Fusion reaction (In-Fusion HD Cloning; Clontech) with PCR-amplified DNA of the *Renilla* luciferase gene. The amplified luciferase gene contained 15 nt extensions homologous to the OROV L, M or S segment UTR sequences in the linearized pTVT7 construct. The UTR–luciferase–UTR sequence was then amplified by PCR using primers containing 15 nt extensions homologous to the T7 terminator (5′ end) and T7 promoter (3′ end). This amplified products were combined with TVT7R(0,0) DNA in an In-Fusion reaction to generate minigenome-expressing plasmids such that in T7 transcripts the *Renilla* luciferase was in the negative sense. These constructs were designated pTVT7OROVSRen(–), pTVT7OROVMRen(–) and pTVT7OROVLRen(–).

Sequencing OROV BeAn 19991 5′ and 3′ termini. As total infected cell RNA contains both genomic and antigenomic segments, 3′ RACE analysis was capable of generating both the 5′ and 3′ terminal sequences using strand-specific primers. Briefly, RNA was polyadenylated (Ambion) for 1 h at 37 °C and then purified using an RNeasy Mini kit (Qiagen). The polyadenylated RNA was then used in a reverse transcription reaction with MMLV reverse transcriptase (Promega) and oligo-d(T) primer, followed by PCR using 3′ PCR anchor primer (Roche) and the appropriate segment specific primer (OROVL_anti/OROVL_gen for the L segment and OROVM_anti/OROVM_gen for the M segment; Table 1) with KOD Hot Start DNA polymerase (Merck). Amplified products were purified on an agarose gel and their nucleotide sequence determined.

To confirm the S segment terminal sequences, total infected cell RNA was first denatured at 90 °C for 3 min and then ligated using T4 RNA ligase (New England Biolabs) for 2 h at 37 °C. The reaction was heat inactivated at 65 °C and purified using an RNeasy Mini kit (Qiagen). cDNA was synthesized using MMLV reverse transcriptase (Promega) and oligonucleotide OROSlig1 (Table 1). PCR was then performed with KOD Hot Start DNA polymerase (Merck) and primers OROSlig1 and OROSlig2 (Table 1). The PCR product was purified on an agarose gel and its nucleotide sequence determined.

Pyrosequencing of the OROV TRVL-9760 strain. OROV TRVL-9760 was grown in IFNAR$^{-/-}$ cells at 37 °C and supernatant was harvested after 48 h. (Preliminary results showed that IFNAR$^{-/-}$ cells gave the highest amounts of genomic RNA in the extracted supernatant compared with Vero-E6 or BHK-21 cells; unpublished observations.) For removal of cell debris, the supernatant was centrifuged at 700 ***g*** for 10 min and at 2800 ***g*** for 5 min, followed by filtration through a 0.2 µm sterile filter. To enrich viral particles, 20 ml cleared supernatant was mixed with 1.48 ml 5 M NaCl and 10.8 ml 30 % PEG8000 in NTE (100 mM NaCl; 10 mM Tris, pH 6.5; 1 mM EDTA), incubated on a shaker for 30 min at 4 °C, and subsequently centrifuged at 6000 ***g*** for 60 min at 4 °C. The virus pellet was resuspended in 500 µl PBS. RNA extraction was performed using PeqGold Trifast (Peqlab). To be able to cover the 3′ terminal parts of the OROV genome segments, 500 ng self-complementary FLAC (full-length amplification of cDNAs) adapters were ligated to 500 ng purified viral RNA as described previously (Dilcher *et al.*, 2012b). To achieve coverage of the 5′ terminal parts, a 5′ RACE RNA adaptor (Ambion) was ligated to the viral RNA after the removal of two phosphate groups via RNA 5′-polyphosphatase. To remove unligated adapters, a subsequent purification step was performed using a CleanAll DNA/RNA Clean-Up and Concentration kit (Norgen Biotek). The concentration of the adaptor-ligated and purified ssRNA was determined by Qant-iT RiboGreen assay (Invitrogen). Then, 60 ng adaptor-ligated viral RNA was amplified and converted to dsDNA using a TransPlex Whole Transciptome Amplification kit (WTA2; Sigma-Aldrich). The newly synthesized dsDNA was purified using a QIAquick PCR Purification kit (Qiagen), and DNA fragments <350 bp were removed using Ampure-XP beads (Agencourt). A sample of 300 ng whole-genome amplified dsDNA was used for Titanium Shotgun Rapid Library Preparation and pyrosequencing on a Genome Sequencer FLX (Roche) as described in the FLX Titanium Protocol (Roche), but omitting the 'DNA fragmentation by nebulization' step. Assembly of the sequenced OROV genome segments was done by means of the Genome Sequencer FLX System software package version 2.3 (GS *De novo* Assembler, GS Reference Mapper) in combination with the commercially available SeqMan Pro version 10.1.1 (DNASTAR, Lasergene).

Minigenome and VLP assays. Subconfluent monolayers of BSR-T7/5 cells were transfected with 1 µg each pTM1OROV-L and pTM1OROV-N, 0.5 µg minigenome-expressing plasmid, and 100 ng pTM1-FF-Luc (Weber *et al.*, 2001). At 24 h post-transfection, *Renilla* and firefly luciferase activities were measured using a Dual-Luciferase Reporter Assay kit (Promega).

To generate VLPs, the M segment minigenome transfection mix was supplemented with 0.5 µg pTM1OROV-M. At 24 and 48 h post-transfection, supernatants were harvested, clarified by centrifugation (4000 r.p.m. for 5 min at 4 °C), digested with benzonase and used to infect BHK-21 cells. *Renilla* activity was measured after 24 h using a Renilla Reporter Assay kit (Promega). To neutralize the VLPs, samples were incubated with hyperimmune mouse ascetic fluid to OROV or with anti-BUNV rabbit antiserum for 1 h at room temperature before infecting BHK-21 cells.

ACKNOWLEDGEMENTS

This work was supported by a Wellcome Trust Senior Investigator Award to R. M. E. (099220), a Medical Research Council postgraduate studentship to N. L. T.-L., a FAPESP-Sao Paulo Research Foundation fellowship to G. O. A. (2013/02798-0, CNPQ) and a CAPES-National Council for the Improvement of Higher Education (Brazil) scholarship to D. E. A. S. (3851/10-9).

REFERENCES

Afonso, A., Abrahantes, J. C., Conraths, F., Veldhuis, A., Elbers, A., Roberts, H., Van der Stede, Y., Méroc, E., Gache, K. & Richardson, J. (2014). The Schmallenberg virus epidemic in Europe – 2011–2013. *Prev Vet Med* **116**, 391–403.

Anderson, C. R., Spence, L., Downs, W. G. & Aitken, T. H. (1961). Oropouche virus: a new human disease agent from Trinidad, West Indies. *Am J Trop Med Hyg* **10**, 574–578.

Aquino, V. H. & Figueiredo, L. T. (2004). Linear amplification followed by single primer polymerase chain reaction to amplify unknown DNA fragments: complete nucleotide sequence of Oropouche virus M RNA segment. *J Virol Methods* **115**, 51–57.

Aquino, V. H., Moreli, M. L. & Moraes Figueiredo, L. T. (2003). Analysis of oropouche virus L protein amino acid sequence showed the presence of an additional conserved region that could harbour an important role for the polymerase activity. *Arch Virol* **148**, 19–28.

Barr, J. N. & Wertz, G. W. (2004). Bunyamwera bunyavirus RNA synthesis requires cooperation of 3′- and 5′-terminal sequences. *J Virol* **78**, 1129–1138.

Barr, J. N. & Wertz, G. W. (2005). Role of the conserved nucleotide mismatch within 3′- and 5′-terminal regions of Bunyamwera virus in signaling transcription. *J Virol* **79**, 3586–3594.

Barr, J. N., Elliott, R. M., Dunn, E. F. & Wertz, G. W. (2003). Segment-specific terminal sequences of Bunyamwera bunyavirus regulate genome replication. *Virology* **311**, 326–338.

Blakqori, G., Kochs, G., Haller, O. & Weber, F. (2003). Functional L polymerase of La Crosse virus allows *in vivo* reconstitution of recombinant nucleocapsids. *J Gen Virol* **84**, 1207–1214.

Buchholz, U. J., Finke, S. & Conzelmann, K. K. (1999). Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J Virol* **73**, 251–259.

Dilcher, M., Alves, M. J., Finkeisen, D., Hufert, F. & Weidmann, M. (2012a). Genetic characterization of Bhanja virus and Palma virus, two tick-borne phleboviruses. *Virus Genes* **45**, 311–315.

Dilcher, M., Hasib, L., Lechner, M., Wieseke, N., Middendorf, M., Marz, M., Koch, A., Spiegel, M., Dobler, G. & other authors (2012b). Genetic characterization of Tribeč virus and Kemerovo virus, two tick-transmitted human-pathogenic Orbiviruses. *Virology* **423**, 68–76.

Dunn, E. F., Pritlove, D. C. & Elliott, R. M. (1994). The S RNA genome segments of Batai, Cache Valley, Guaroa, Kairi, Lumbo, Main Drain and Northway bunyaviruses: sequence determination and analysis. *J Gen Virol* **75**, 597–608.

Dunn, E. F., Pritlove, D. C., Jin, H. & Elliott, R. M. (1995). Transcription of a recombinant bunyavirus RNA template by transiently expressed bunyavirus proteins. *Virology* **211**, 133–143.

Elliott, R. M. (1989a). Nucleotide sequence analysis of the large (L) genomic RNA segment of Bunyamwera virus, the prototype of the family *Bunyaviridae*. *Virology* **173**, 426–436.

Elliott, R. M. (1989b). Nucleotide sequence analysis of the small (S) RNA segment of Bunyamwera virus, the prototype of the family *Bunyaviridae*. *J Gen Virol* **70**, 1281–1285.

Elliott, R. M. (2012). Bunyavirus reverse genetics and applications to studying interactions with host cells. In *Reverse Genetics of RNA Viruses: Applications and Perspectives*, pp. 200–223. Edited by A. Bridgen. Chichester: Wiley.

Elliott, R. M. (2014). Orthobunyaviruses: recent genetic and structural insights. *Nat Rev Microbiol* **12**, 673–685.

Elliott, R. M. & Brennan, B. (2014). Emerging phleboviruses. *Curr Opin Virol* **5**, 50–57.

Elliott, R. M., Schmaljohn, C. S. & Collett, M. S. (1991). *Bunyaviridae* genome structure and gene expression. *Curr Top Microbiol Immunol* **169**, 91–141.

Elliott, R. M., Blakqori, G., van Knippenberg, I. C., Koudriakova, E., Li, P., McLees, A., Shi, X. & Szemiel, A. M. (2013). Establishment of a reverse genetics system for Schmallenberg virus, a newly emerged orthobunyavirus in Europe. *J Gen Virol* **94**, 851–859.

Johnson, K. N., Zeddam, J. L. & Ball, L. A. (2000). Characterization and construction of functional cDNA clones of Pariacoto virus, the first Alphanodavirus isolated outside Australasia. *J Virol* **74**, 5123–5132.

Kohl, A., Bridgen, A., Dunn, E., Barr, J. N. & Elliott, R. M. (2003). Effects of a point mutation in the 3′ end of the S genome segment of naturally occurring and engineered Bunyamwera viruses. *J Gen Virol* **84**, 789–793.

Kohl, A., Dunn, E. F., Lowen, A. C. & Elliott, R. M. (2004). Complementarity, sequence and structural elements within the 3′ and 5′ non-coding regions of the Bunyamwera orthobunyavirus S segment determine promoter strength. *J Gen Virol* **85**, 3269–3278.

Ladner, J. T., Beitzel, B., Chain, P. S., Davenport, M. G., Donaldson, E. F., Frieman, M., Kugelman, J. R., Kuhn, J. H., O'Rear, J. & other authors (2014a). Standards for sequencing viral genomes in the era of high-throughput sequencing. *MBio* **5**, e01360–e14.

Ladner, J. T., Savji, N., Lofts, L., Travassos da Rosa, A., Wiley, M. R., Gestole, M. C., Rosen, G. E., Guzman, H., Vasconcelos, P. F. & other authors (2014b). Genomic and phylogenetic characterization of viruses included in the Manzanilla and Oropouche species complexes of the genus *Orthobunyavirus*, family *Bunyaviridae*. *J Gen Virol* **95**, 1055–1066.

Lambert, A. J. & Lanciotti, R. S. (2008). Molecular characterization of medically important viruses of the genus *Orthobunyavirus*. *J Gen Virol* **89**, 2580–2585.

Matsuno, K., Weisend, C., Travassos da Rosa, A. P., Anzick, S. L., Dahlstrom, E., Porcella, S. F., Dorward, D. W., Yu, X. J., Tesh, R. B. & Ebihara, H. (2013). Characterization of the Bhanja serogroup viruses (*Bunyaviridae*): a novel species of the genus *Phlebovirus* and its relationship with other emerging tick-borne phleboviruses. *J Virol* **87**, 3719–3728.

Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W. A. & Fuerst, T. R. (1990). New mammalian expression vectors. *Nature* **348**, 91–92.

Ogawa, Y., Sugiura, K., Kato, K., Tohya, Y. & Akashi, H. (2007). Rescue of Akabane virus (family *Bunyaviridae*) entirely from cloned cDNAs by using RNA polymerase I. *J Gen Virol* **88**, 3385–3390.

Pinheiro, F. P., Pinheiro, M., Bensabath, G., Causey, O. R. & Shope, R. E. (1962). [Oropouche virus epidemic in Bélem]. *Rev Serv Esp Saude Pub* **12**, 15–23 (in Portugese).

Pinheiro, F. P., Hoch, A. L., Gomes, M. L. & Roberts, D. R. (1981a). Oropouche virus. IV. Laboratory transmission by *Culicoides paraensis*. *Am J Trop Med Hyg* **30**, 172–176.

Pinheiro, F. P., Travassos da Rosa, A. P., Travassos da Rosa, J. F., Ishak, R., Freitas, R. B., Gomes, M. L., LeDuc, J. W. & Oliva, O. F. (1981b). Oropouche virus. I. A review of clinical, epidemiological, and ecological findings. *Am J Trop Med Hyg* **30**, 149–160.

Pinheiro, F. P., Travassos da Rosa, A. P., Gomes, M. L., LeDuc, J. W. & Hoch, A. L. (1982). Transmission of Oropouche virus from man to hamster by the midge *Culicoides paraensis*. *Science* **215**, 1251–1253.

Plyusnin, A. & Elliott, R. M. (editors) (2011). *Bunyaviridae. Molecular and Cellular Biology*. Norfolk: Caister Academic Press.

Saeed, M. F., Wang, H., Nunes, M., Vasconcelos, P. F., Weaver, S. C., Shope, R. E., Watts, D. M., Tesh, R. B. & Barrett, A. D. (2000). Nucleotide sequences and phylogeny of the nucleocapsid gene of Oropouche virus. *J Gen Virol* **81**, 743–748.

Shi, X., Kohl, A., Li, P. & Elliott, R. M. (2007). Role of the cytoplasmic tail domains of Bunyamwera orthobunyavirus glycoproteins Gn and Gc in virus assembly and morphogenesis. *J Virol* **81**, 10151–10160.

Varela, M., Schnettler, E., Caporale, M., Murgia, C., Barry, G., McFarlane, M., McGregor, E., Piras, I. M., Shaw, A. & other authors (2013). Schmallenberg virus pathogenesis, tropism and interaction with the innate immune system of the host. *PLoS Pathog* **9**, e1003133.

Vasconcelos, H. B., Nunes, M. R., Casseb, L. M., Carvalho, V. L., Pinto da Silva, E. V., Silva, M., Casseb, S. M. & Vasconcelos, P. F. (2011). Molecular epidemiology of Oropouche virus, Brazil. *Emerg Infect Dis* **17**, 800–806.

Wang, H., Beasley, D. W., Li, L., Holbrook, M. R. & Barrett, A. D. (2001). Nucleotide sequence and deduced amino acid sequence of the medium RNA segment of Oropouche, a Simbu serogroup virus: comparison with the middle RNA of Bunyamwera and California serogroup viruses. *Virus Res* **73**, 153–162.

Weber, F., Dunn, E. F., Bridgen, A. & Elliott, R. M. (2001). The Bunyamwera virus nonstructural protein NSs inhibits viral RNA synthesis in a minireplicon system. *Virology* **281**, 67–74.

Weber, F., Bridgen, A., Fazakerley, J. K., Streitenfeld, H., Kessler, N., Randall, R. E. & Elliott, R. M. (2002). Bunyamwera bunyavirus nonstructural protein NSs counteracts the induction of alpha/beta interferon. *J Virol* **76**, 7949–7955.