Generation of recombinant Oropouche orthobunyaviruses lacking the non-structural proteins NSm or NSs

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Running title: Oropouche virus rescue

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Abstract

Oropouche virus (OROV) is a midge-borne human pathogen with a geographic distribution in South America. OROV was first isolated in 1955 and since then is known to cause recurring outbreaks of a dengue-like illness in the Amazonian regions of Brazil. OROV however, remains amongst one of the poorly understood emerging viral zoonosis. Here we describe the successful rescue of infectious OROV entirely from cDNA copies of its genome and generation of OROV mutant viruses lacking either the NSm or the NSs coding regions. Characterisation of the recombinant viruses carried out in vitro demonstrated that the NSs protein of OROV is an IFN antagonist as in other NSs-encoding bunyaviruses. Additionally, we demonstrate the importance of the nine C-terminal amino acids of OROV NSs in IFN antagonistic activity. OROV was also found to be sensitive to IFN-α when cells were pre-treated, however the virus was still capable of replicating at doses as high as 10,000 U/ml of IFN-α in contrast to the family prototype BUNV. We found that the
OROV lacking a complete NSm protein displayed characteristics similar to the wild-type virus, suggesting that the NSm protein is dispensable for virus replication in mammalian and mosquito cell-lines that were tested.

**Importance**

Oropouche virus (OROV) is a public health threat in Central and South America where it causes periodic outbreaks of dengue-like illness. In Brazil, OROV is the second most frequent cause of arboviral febrile illness after dengue virus and with the current rates of urban expansion more cases of this emerging viral zoonosis could occur. To better understand the molecular biology of OROV we have successfully rescued the virus along with mutants. We have established that the C-terminus of the NSs protein is important in interferon antagonism and that the NSm protein is dispensable for virus replication in cell-culture. The tools described in this paper are important in terms of understanding this important yet neglected human pathogen.

**Introduction**

Bunyaviruses form a large group of single stranded negative-sense RNA viruses consisting of important human and veterinary pathogens such as the recently emerged severe fever with thrombocytopenia syndrome virus (SFTSV) and Schmallenberg virus (SBV). The family is divided into genera *Hantavirus, Nairovirus, Phlebovirus, Tospovirus* and the largest genus *Orthobunyavirus*, to which Oropouche virus (OROV) belongs (1, 2). OROV causes an acute febrile illness in humans with signs and symptoms of fever, headache, malaise, myalgia, arthralgia, photophobia, nausea, vomiting, dizziness, and in some cases encephalitis and meningitis. Symptoms last between 2 to 7 days, with some patients reporting a recurrence of these symptoms (3-10). OROV is transmitted amongst humans via the biting midge *Culicoides paraensis*, and is maintained in the wild by circulating in nonhuman primates such as the pale-throated three-toed sloth (*Bradypus tridactylus*) and the black-tufted marmoset (*Callithrix penicillata*), though the vector/s remain largely unknown (3-5, 10-12). Laboratory experiments and epidemiological surveys have reported that mosquitoes *Aedes serratus, Aedes scapularis, Aedes albopictus, Culex fatigans, Culex quiquefaciatus, Coquillettidia venezuelensis* and *Psorophora ferox* are susceptible to OROV infection (13-16). Neutralizing antibodies against
OROV have also been detected in both wild and domestic birds (10, 14, 15), leading to speculation that birds could be carriers of the virus (Personal communication, Professor Alan Barrett, University of Texas Medical Branch).

Oropouche fever (OROF) outbreaks have mainly been reported in Brazil’s Amazonian cities. OROV however, was first recorded in Trinidad, West Indies in 1955 (13). In Brazil the virus was isolated in 1960 from a dead sloth found near one of the Belem-Brasilia highway construction sites. The following year in 1961 in Belem city 11,000 people were reported ill in what became the first OROF outbreak (17). Between 1961 and 2009 over 30 OROF outbreaks have been recorded with an estimated 500,000 cases (13, 17, 18). Outside of Brazil OROF was reported for the first time in Panama in 1989 and Peru in 1992. The geographic distribution of OROV today includes Brazil, Panama, Peru and Argentina. Serological evidence suggests that the virus may also be circulating in Ecuador and Bolivia, and in nonhuman primates in Colombia (18-24). However, without a differential surveillance system to distinguish infections with similar clinical symptoms such as OROV, dengue, chikungunya and Mayaro fevers the exact epidemiology of OROV in Central and South America remains unclear. OROV reassortant viruses have also been isolated in Peru, Venezuela and outside the epidemic zone within Brazil (25-27).

The lack of a reverse genetics system has, until now, limited research on OROV at a molecular level. In order to address this issue we previously reported the establishment of a minigenome and virus-like particle production assay for OROV (28). In the present paper we report the recovery of infectious OROV, entirely from cDNA plasmids. Like all bunyaviruses, OROV contains a tri-partite RNA genome with a Large (L) segment that encodes the viral RNA-dependent RNA polymerase, a Medium (M) segment that encodes the viral glycoproteins Gn and Gc and a Small (S) segment that encodes the nucleocapsid (N) protein. OROV also encodes two non-structural proteins, NSm, which is a co-translationally cleaved product formed along with Gn and Gc from the M segment, and NSs, which is encoded from a downstream AUG site on the same mRNA transcript as the N protein. The rescue system described in this paper is based on a T7 RNA polymerase-driven plasmid system (29). Using this we have successfully recovered wild-type OROV, along with mutant viruses lacking the NSm or NSs proteins. We report here the characterization
of these recombinant viruses in cultured cells, as a way to contribute to the understanding of this important, yet poorly understood emerging viral zoonosis.

Methods and Materials

Cells and viruses

A549 (human alveolar adenocarcinoma epithelial cells), A549/BVDV-NPro (A549 cells that express bovine viral diarrhea virus NPro protein), A549/V (derived from A549, and express simian virus 5 V protein), CPT-Tert (sheep choroid plexus cells), DF-1 (chicken embryo fibroblasts), HeLa (human cervix adenocarcinoma epithelial cells), LLC-MK2 (*Macaca mulatta* kidney epithelial cells), MDCK (canine kidney epithelial cells), MRK101 (grey red-backed vole kidney cells), QT-35 (Japanese quail fibrosarcoma cells), Vero-E6 (African green monkey kidney cells) and 2fTGH (human epithelial fibrosarcoma cells) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS). BHK-21 (baby hamster kidney fibroblasts) cells were grown in Glasgow minimal essential medium (GMEM; Invitrogen) supplemented with 10% newborn calf serum (NCS) and 10% tryptose phosphate broth (TPB; Invitrogen). BSR-T7/5 cells, which stably express T7 RNA polymerase (30) were grown in GMEM supplemented with 10% FBS, 10% TPB and 1 mg/ml G418 (Geneticin; Invitrogen). All mammalian cell lines were grown at 37°C with 5% CO₂.

Mosquito cells U4.4 (*Aedes albopictus* neonatal larvae cells) were grown in Leibovitz 15 medium (Gibco) supplemented with 10% FCS and 10% TPB, whilst Aag-2 cells (derived from *Aedes aegypti* neonatal larvae) were grown in Schneider’s *Drosophila* medium with L-glutamine (Gibco) supplemented with 10% FBS. Both cell-lines were maintained at 28°C.

OROV strain BeAn19991 was kindly donated by Professor Luiz Tadeu Moraes Figueiredo (University of Sao Paulo School of Medicine, Ribeirao Preto, Brazil). OROV isolate BeH759025 was kindly provided by Professor Pedro Vasconcelos (Department of Arboviruses and Hemorrhagic Fevers, Evandro Chagas Institute, Ministry of Health, Ananindeua, Brazil).
All experiments with infectious viruses were conducted under BSL-3 conditions.

**Plasmids**

Plasmids pTVTOROVL, pTVTOROVM and pTVTOROVS used for OROV rescue have previously been described (28). pTVTOROVdelNSm was created from pTVTOROVM by excision PCR using 5’ phosphorylated primers to delete nucleotides (nt) 1039 to 1476 of the M segment, hence removing the entire NSm ORF after the first transmembrane domain up to the third transmembrane domain, whilst maintaining the predicted cleavage site that resides between NSm and Gc. pTVTOROVdelNSs and pTVTOROV246NSs were generated by QuikChange site-directed mutagenesis on pTVTOROVS to insert stop codons at specific regions in order to generate viruses with truncated NSs proteins. These plasmids contain mutations at cDNA nt position 116 for pTVTOROVdelNSs and 313 for pTVTOROV246NSs. The N ORF remains unchanged. pTVTOROV2080S was generated by amplifying the full length S segment of clinical isolate BeH759025 (25). Primers and cloning strategy were the same as for pTVTOROVS (28). All PCR reactions were carried out using KOD Hot Start DNA polymerase (Merck). Products were gel purified (Promega) and where needed ligated using T4 DNA Ligase (Promega), as per manufacturer’s protocol. Plasmids were confirmed by nucleotide sequencing (Source Bioscience). Primers used are listed in Table 1.

**Generation of recombinant OROV from cDNA**

Recombinant OROV viruses were generated by transfecting BSR-T7/5 cells (1.5×10^5 cells/ml) with 1 µg of pTVTOROVL, pTVTOROVM (or pTVTOROVdelNSm) and pTVTOROVS (or pTVTOROV2080S) using 3 µl transfection reagent TransIT-LT1 (Mirus Bio LLC) per µg of DNA. Replacing the wt S segment (pTVTOROVS) with 1.5 µg of pTVTOROVdelNSs or pTVTRO246NSs, mutant NSs viruses were generated. Supernatants were harvested 7 days post transfection (p.t). Rescue outcome was assessed by plaque assay on BHK-21 cells. All recovered viruses were grown in Vero E6 cells and genome segments amplified by RT-PCR for sequence determination (Sanger sequencing).

**Plaque assay**
Viruses were titrated on BHK-21 cells seeded at a density of 3×10^5 cells per ml in a 12-well plate, while virus phenotype assays (in various cells) were carried out in six-well plates. Cell monolayers were infected with either 150 μl (12-well plate) or 200 μl (six-well plate) of virus diluted in PBS/2% NCS. Cells were then overlaid using 0.6% Avicell (FMC) in 2X MEM/2% NCS. 3 days p.i cells were fixed with 4% formaldehyde and stained using crystal violet to visualize plaques.

**Virus propagation**

Working stocks of recombinant and wt viruses were grown in Vero E6 cells at a multiplicity of infection (MOI) of 0.001. Cells were maintained at 37°C/5% CO₂ until visible cytopathic effect (CPE) was observed. Virus-containing supernatant was clarified by low-speed centrifugation and aliquots stored at -80°C. Recombinant viruses used here were at passage 2. All the introduced mutations were confirmed by amplifying the segment in question by RT-PCR, followed by Sanger sequencing with primers covering the region of interest.

**RT-PCR**

Virion RNA of each passaged virus was extracted using the RNeasy Mini Kit (QIAGEN) as per manufacturer's protocol. RT-PCR was then carried out using segment specific forward or reverse primers (28), with M-MLV Reverse Transcriptase (Promega). PCR was carried out using KOD Hot Start DNA polymerase (Merck) and the amplified products were agarose gel purified (Promega), as per manufacturer's protocol. Specific regions of each segment were then sequenced via Sanger sequencing (Source Bioscience).

**Viral infection**

Cells were infected with viruses at the desired MOI for 1 hour at 37°C. Cell monolayer was washed three times using PBS and then complete growth medium added. At desired time points virus was harvested or cell lysates collected. Viral titers were determined by plaque assay on BHK-21 cells for 3 days.

**Western blot**

Cell lysates were prepared in lysis buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 200 mM DTT, 0.2% bromophenol blue, and 25 U/ml Benzonase; Novagen)
and proteins were then separated on a 4-12% gradient NuPAGE Bis-Tris gel (Invitrogen), at 180 V for 50 minutes. Proteins were transferred to a nitrocellulose membrane (Amersham) and a semi-dry transfer performed using the Trans-Blot® Turbo™ Transfer System (Bio-Rad) at 10 V for 50 minutes. Membranes were then blocked for 1 h in 5% milk/PBS 0.1% (v/v) Tween-20 and incubated in primary antibody overnight at 4°C. Secondary antibody was added for 1 h at 37 °C. Proteins were then visualized using SuperSignal WestPico chemiluminescent substrate (Pierce) followed by exposure on a Bio-Rad ChemiDoc imager. Information for primary antibodies include: BUNV anti-N-Rb (1:5000; (31)), anti-MxA (1:500; catalogue no. sc-50509, Santa Cruz Biotech), anti-pSTAT1 (1:750; catalogue no. 9167S; Cell signaling), anti-STAT1 (1:750; catalogue no. 9172, Cell signaling) and anti-tubulin monoclonal antibody (1:5000; catalogue no. T5168, Sigma). OROV anti-N polyclonal rabbit antibody (1:1000; GenScript) was a kind gift from Professor Massimo Palmarini (MRC-University of Glasgow Centre for Virus Research). HRP-coupled secondary anti-rabbit (catalogue no. A0545; Sigma) and anti-mouse (catalogue no. A4416; Sigma) were used at 1:5000.

**Metabolic radiolabelling**

Vero E6 cells were grown in 12-well plates and infected at an MOI of 3 of each virus and at indicated time points supernatant was removed and cells starved in methionine/cysteine-free DMEM at 37 °C for 30 min. Cells were then washed and labeled with 10μCi per well of [35 S]-EasyTag™ EXPRESS mixed in methionine/cysteine-free DMEM for 2 hours at 37 °C. Cells were then lysed in 150 μl of lysis buffer (100 mM Tris-Cl [pH 6.8], 4% SDS, 20% glycerol, 200 mM DTT, 0.2% bromophenol blue, and 25 U/ml Benzonase; Novagen) and proteins separated by SDS-PAGE. Gels were fixed and dried and then labeled proteins were visualized by phosphorimaging (Storm840 Phosphorimager; Molecular Dynamics).

**Biological IFN production assay**

A549 cells were grown in 12-well plates and infected at an MOI of 1 of each virus. 24 h post infection (p.i) supernatant was harvested and treated with UV light (8 W, 254 nm, 2 cm distance) for 4 mins with shaking after 2 mins to inactivate any virus. Cells were lysed in 150 μl of lysis buffer (100 mM Tris-Cl [pH 6.8], 4% SDS, 20% glycerol, 200 mM DTT, 0.2% bromophenol blue, and 25 U/ml Benzonase; Novagen)
to check viral N and STAT1 protein levels. Phosphorylated STAT1 and MxA production in each sample were also probed. A549/BVDV-NPro cells grown in a 96-well plate were then treated with the UV-inactivated supernatant for 24 h. These cells were then infected with interferon-sensitive encephalomyocarditis virus (EMCV; 0.05 PFU/cell) and incubated for three days at 37 °C. Cells were fixed in 4% formaldehyde and stained with crystal violet to visualize CPE.

IFN-α sensitivity assay

Vero E6 at 1.5×10^5 cells/ml were treated either pre- or post- viral infection with various concentrations of universal type 1 IFN-α (catalogue no. 11200, Lot no. 5283; Stratech Scientific). Cells were infected with virus at a desired MOI in PBS /2%FBS for 1 h at 37°C. Cells were washed three times in PBS before replacing media with/without IFN-α. Cells were incubated at 37°C for 48 h before harvesting and determining virus yield by plaque assay on BHK-21 cells. For plaque assays confluent Vero E6 cells were pre-treated with IFN-α for 24 h before titrating the virus. At 4 days p.i cells were fixed in 4% formaldehyde and stained with crystal violet. IFN-α was maintained in the media and Avicell overlay for the duration of infection.

Results:

Recovery of wild-type OROV strain BeAn 19991

OROV is a negative-sense virus and previously (28) we described the cloning of full-length antigenomic sense cDNA copies of its L, M and S segments into the T7 RNA polymerase-driven plasmid backbone pTVT7R(0,0) (32). This plasmid contains a single G residue immediately downstream of the T7 promoter sequence to aid efficient transcription. cDNA copies of OROV genome segments were cloned into pTVT7R in the antigenomic sense (FIG 1A). To recover infectious OROV BSR-T7/5 cells (30) were transfected with 1 µg of the pTVTOROVL, pTVTOROVM and pTVTOROVS plasmids. Supernatant was harvested 7 days p.t. once CPE was visible and success of the rescue attempt was determined by titration of infectious virus by plaque assay. The rescue of OROV was easily reproducible, yielding titers of 2.0×10^7, 4.5×10^6 and 2.3×10^7 PFU/ml in three independent experiments. As a control, BSR-T7/5 cells transfected with only pTVTOROVM and pTVTOROVS did not give rise to infectious virus. To test the authenticity of the recombinant OROV
(rOROV), permissive Vero E6 cells were infected with the rescue supernatant and cell extracts used for Western-blotting (data not shown). Furthermore, rOROV infected Vero E6 cells were fixed and stained at 24 h p.i. using a polyclonal anti-OROV antibody (a kind gift from Professor Luiz Tadeu Moraes Figueiredo, University of Sao Paulo School of Medicine, Brazil). Substantial amounts of cytoplasmic OROV protein were detectable in the infected cells (data not shown) further confirming the successful recovery of infectious OROV.

The growth kinetics and plaque phenotype of rOROV was similar to that of the authentic wild-type (wt) virus (FIG 1B-D). All experiments from this point on were carried out with rOROV.

**Growth of recombinant OROV in mammalian cell-lines**

The growth properties of rOROV were tested in Vero E6 cells at MOIs ranging from 0.0001 to 1 PFU/cell. Previous work from our group has demonstrated that some viruses show a better fitness in certain cell types and at different MOIs possibly due to the efficiency at which defective-interfering particles are generated (33, 34). rOROV grows to similar titers by 48 h p.i at all MOIs tested (FIG 1E), and in a wide range of cell-lines derived from several species (MOI 0.001; FIG 1F), similar to other bunyaviruses (33, 34). Lower titers were obtained in human cell-lines 2fTGH and HeLa compared to A549 cells. Lower virus titers were also obtained from CPT-tert, QT-35 and MRK101 cells, however due to the specific aims of the current study these observations were not investigated further. rOROV forms plaques on rodent, monkey, human and sheep cell-lines that were investigated (FIG 1G). At 3 days p.i in BHK-21 cells rOROV plaques were larger than in the other cell-lines, and on A549 cells the plaques were harder to visualise (FIG 1G). Based on these results, BHK-21 cells were chosen for virus titration, whilst Vero E6 and A549 cell-lines were chosen for the purpose of initial characterisation of all recombinant viruses in this study. Vero E6 and BHK-21 cells both lack fully functional IFN systems (35-37), whilst A549 cells are IFN competent (38).

**Generation of OROV mutants**

Using our newly established OROV rescue system we generated OROV mutant viruses as described below.
1. NSm deletion

A mutant OROV lacking the entire NSm ORF from the M segment was generated. This was done by deleting the entire NSm coding region immediately after the first NSm transmembrane domain (TMD) and predicted cleavage site up to the third TMD, leaving the predicted cleavage site of the Gc protein intact (FIG 2A). These sites were predicted using the TMHMM Server v. 2.0 and SignalP 4.1 Server algorithms (www.cbs.dtu.dk) based on work done by Dr. Xiaohong Shi (MRC-University of Glasgow, Centre for Virus Research) for the characterization of orthobunyavirus M segments (Xiaohong Shi and Richard M. Elliott, manuscript submitted). Primers delNSmOROVF/delNSmOROVR (Table 1) were designed to bind to positions 1475 – 1498 and 1036 – 1013 of the M segment respectively. This allowed an excision PCR to be performed thereby deleting the entire NSm region, but leaving the first TMD site, so as not to alter the position of the Gc protein in the endoplasmic reticulum and Golgi, during folding. To rescue rOROVdelNSm virus BSR-T7/5 cells were transfected with 1 µg of pTVTOROVL, pTVTOROVS and pTVTOROVdelNSm plasmids. At 7 days p.t infectious virus particles were recovered, titrated and sequenced (Source Bioscience) to confirm the mutation.

2. NSs mutants

The following step was the creation of the NSs mutant viruses. As NSs lies in an over-lapping reading frame within the N ORF, the positions at which mutations could be introduced were limited. The NSs ORF of OROV has four in-frame methionines, therefore, in an attempt to abrogate NSs transcription the NSs start codon was left in place and instead a translational stop codon was inserted in-frame immediately after the second methionine at amino acid 17 (FIG 2B; 2. rOROVdelNSs). At the nt level this is at position 115 and changes TGG (W) to TAG (stop), resulting in a 48 nt NSs ORF. The reason for this approach was because previous work done on BUNV revealed that when the start codon of NSs was removed the virus was still capable of producing an NSs protein from a downstream methionine (39). The strategy used in this study for OROV is similar to the one used to create a SBV mutant lacking NSs (33). In addition to this, a C-terminal truncated NSs was also engineered. This was generated by introducing a stop codon at nt position 313 changing TTA (L) to TAA (stop). This results in a 246 nt NSs ORF and a protein sequence of 82 aa compared
to wt NSs which is 92 aa (FIG 2B; 1. rOROV246NSs). Primers used in generating the plasmids are in Table 1. In order to rescue the NSs mutants (named, rOROVdelNSs and rOROV246NSs) BSR-T7/5 cells were transfected with 1 µg of pTVTOROVL, pTVTOROVm and 1.5 µg of the mutant S segment (pTVTOROdelNSs or pTVTORO246NSs). At 7 days p.t infectious virus particles were recovered, titrated and the entire NSs OROF was sequenced (Source Bioscience) to confirm mutations.

3. S-segment mutant

In our previous work (25) we report the isolation and sequencing of OROV clinical isolates that differ from the prototype strain (BeAn19991) in the S segment, as they lack 11 nts at position 781 to 791 in the 3’ UTR. The encoded NSs ORF of these viruses also contain a tandem AUG translation start codon created by a C-U variation at position 332, and a Gln to Arg change in the NSs ORF at position 89 (FIG 2C; rOROV2080S). To test whether these variations altered the in vitro growth properties of the rescued virus, a cDNA plasmid (designated as pTVTOROV2080S) containing the S segment of clinical isolate BeH759025 (GenBank accession number KP691614; (25)) was generated using the same cloning strategy as pTVTOROVS (28). In order to rescue this S-segment mutant (named rOROV2080S) BSR-T7/5 cells were transfected with plasmids pTVTOROVL, pTVTOROVm and pTVTORO2080S (1 µg each). At 7 days p.t infectious virus particles were recovered, titrated and the entire S segment sequenced (Source Bioscience).

All mutant viruses in this study were passaged three times at low MOI in Vero E6 cells and sequenced. Introduced mutations were maintained confirming the stability of these viruses. Subsequent experiments utilised viruses from passage two.

**Growth properties of recombinant viruses in mammalian cell-lines and their effect on host-protein synthesis**

Growth kinetics of rOROV, rOROVdelNSm, rOROVdelNSs, rOROV246NSs and rOROV2080S were compared in Vero E6 cells at MOI 0.1. Viruses rOROV, rOROVdelNSm and rOROV2080S replicate with similar efficiency, however mutants rOROVdelNSs and rOROV246NSs appear attenuated and reach titers that are one log lower than rOROV (FIG 2D). Western blotting analysis revealed higher amounts
of N protein from rOROVdelNSs at earlier time-points suggesting a possible increased efficiency of the virus to translate N (FIG 2F). Plaque morphology of the recombinant viruses were then compared on BHK-21 cells. rOROV, rOROVdelNSm and rOROV2080S produce plaques with a round morphology and are clear and similar to each other. The plaques of viruses rOROVdelNSs and rOROV246NSs on the other hand are smaller with corrugated and ill defined borders (FIG 2E).

To investigate whether the recombinant viruses caused inhibition of host-cell protein synthesis Vero E6 cells were infected at MOI 3 and at 12, 24 and 48 h p.i cells were radiolabelled with [35S]methionine. Cell extracts were analysed by SDS-PAGE. rOROV, rOROVdelNSm, rOROV2080S, as well as the rOROVdelNSs and rOROV246NSs demonstrated an ability to cause a shut-off of host translation by 24 h p.i (FIG 3). It was also observed that the latter two viruses compared to the others produced noticeably more N protein at this time point. This result also confirmed that the mutant viruses rOROVdelNSm and rOROVdelNSs do not express NSm and NSs proteins respectively, and that the rOROV246NSs virus expresses a truncated version of NSs (FIG 3).

As rOROV2080S behaves similar to rOROV and rOROV246NSs similar to rOROVdelNSs in terms of *in vitro* replication kinetics, only viruses rOROV, rOROVdelNSm and rOROVdelNSs were focussed on for growth comparison in IFN-competent A549 cells. rOROV and rOROVdelNSm grew with similar kinetics reaching comparable titers, whereas rOROVdelNSs growth appeared more restrictive and at 48 h the viral titers were almost two logs lower than that of rOROV and rOROVdelNSm viruses (FIG 4A). Western blot analysis of N expression showed lower amounts of protein in the rOROVdelNSs-infected cells (FIG 4B). Next, the growth of rOROV, rOROVdelNSm and rOROVdelNSs were compared in A549 cells to their growth in IFN-incompetent A549/V cells. These cells express the V protein of parainfluenza type-5 virus thereby blocking type I IFN signalling via STAT1 degradation (40). Cells were infected at MOI 0.001 and titers measured at 48 h p.i. Cells were also infected with BUNV or a BUNV mutant lacking the NSs protein (rBUNVdelNSs2) for comparison (41, 42). All viruses grew to higher titers in the IFN-incompetent cell-line, similar to BUNV. rOROVdelNSs titers were over one log higher in A549/V cells compared to A549 cells, although this difference was not as high as
with rBUNVdelNSs2 (FIG 4C). Western blot for N confirmed lower levels of expression in A549 cells infected with rOROVdelNSs and rBUNVdelNSs2, corresponding with the yield assay (FIG 4D).

**OROV NSs protein inhibits type I IFN production in A549 cells**

We measured IFN production in A549 cells in response to infection with rOROV, rOROVdelNSm, rOROV2080S, rOROVdelNSs or rOROV246NSs viruses at an MOI of 1. For comparison we also infected cells with BUNV or rBUNVdelNSs2. At 24 h p.i the media from infected monolayers was collected, infectious virus inactivated by UV treatment, and the amount of IFN present measured in a biological protection assay as described (43). As expected no IFN was produced from mock or BUNV infected cells, and rBUNVdelNSs2-infected cells produced considerable amounts of IFN. Whilst rOROV, rOROVdelNSm and rOROV2080S induced small amounts of IFN, rOROVdelNSs induced high amounts (FIG 5A). rOROV246NSs virus that lacks only nine aa at the NSs protein C-terminus, induced IFN to the same extent as rOROVdelNSs (FIG 5A and B). Next, we used western-blotting to probe the A549 cell extracts for STAT1, phosphorylated STAT1 (pSTAT1) and the Interferon Stimulated Gene (ISG) protein MxA. pSTAT1 and MxA expression were detected in cells infected with rOROVdelNSs and rOROV246NSs, but not in cells infected with rOROV, rOROV2080S and rOROVdelNSm (FIG 5C) confirming that OROV NSs is an IFN antagonist.

**OROV is less sensitive to IFN-α treatment than BUNV**

BUNV replication was previously shown to be highly sensitivity to IFN-α (44). To test if OROV was equally sensitive Vero E6 cells (which cannot produce but can respond to IFN (37)) were treated with increasing doses of universal type-1 IFN-α (0, 10, 100, 1000 and 10,000U/ml), either pre (-24 or -2 h) or post (+2 or +24 h) infection. Cells were infected with BUNV or OROV at an MOI of 0.01 and IFN-α was maintained in the media throughout the infection period. At 48 h p.i the amount of infectious virus in the culture media was estimated by plaque assays. Whilst both viruses showed sensitivity to IFN, OROV was clearly less sensitive than BUNV (Fig 6A). For example, pre-treating cells with 10,000 units of IFN-α either 2 or 24 h pre-infection completely inhibited BUNV replication, as did treating cells with 10,000 units at 2 h p.i. In
contrast, there was only a 1 to 2 log reduction in the titres of OROV in cells pre-
treated for 2 h with 10,000 units of IFN-α prior to infection, and a 3 log reduction in
cells pre-treated for 24 h. Furthermore, whilst pre-treating cells with 1000 units of
IFN-α for 24 h pre-infection completely inhibited BUNV, there was only a 2 log
reduction in cells infected with OROV (FIG 6A). We have repeated the experiment
with rOROV using 10,000 U/ml of IFN and at MOIs 0.001 and 0.01. At 24 or 48 h p.i
at both MOIs rOROV replication was not completely inhibited, as observed with
BUNV, with titers decreased by 2 to 3 logs compared to untreated cells (data not
shown). Viruses rOROVdelNSm, rOROV2080S, rOROVdelINSs and rOROV246NSs
demonstrate a similar sensitivity to IFN-α as rOROV (FIG 6B), indicating that the
increased resistance of OROV to IFN-α compared to BUNV is not due to expression
of a functional NSs protein. Next, we investigated the plaque morphology on pre-
treated Vero E6 cells for rOROV and rOROVdelINSs in comparison to BUNV and
rBUNVdelINSs2. 1000 U/ml of IFN-α was maintained in the overlay during the
infection period. No BUNV or rBUNVdelINSs plaques were observed when the
plaques assays were performed in the presence of IFN-α. In contrast, rOROV and
rOROVdelINSs plaques were observed in the presence of IFN-α, although they were
considerably smaller than those on untreated cells (FIG 6D). Taken together, these
results demonstrate that in the tested cells and with the moi of virus used, OROV is
sensitive to IFN-α in a dose-dependent manner, however is significantly more
resistant than BUNV. Furthermore, the NSs protein is not responsible for this
increased resistance.

Replication of recombinant viruses in mosquito cell-lines
We have also compared the growth kinetics of rOROV, rOROVdelINSm and
rOROVdelINSs viruses in mosquito cell-lines U4.4 (Aedes albopictus) and Aag2
(Aedes aegypti). Interestingly, and unlike the situation in mammalian cells,
rOROVdelINSs grows to similar levels as rOROV (FIG 7 A-D). In both U4.4 and Aag2
it appears that rOROVdelINSm grows to slightly higher titers than the other viruses.
Investigating this further is beyond the scope of the current study.

Discussion
In this paper we describe the successful recovery of OROV in cultured cells entirely from cloned cDNAs. OROV is a midge-borne orthobunyavirus that causes a febrile illness in the South American human population. The virus has caused over half a million infections, and though not fatal, its dengue-like symptoms can persist for weeks and in a few cases can progress into more severe symptoms such as meningitis and encephalitis (18). OROV is closely related to SBV, another Simbu virus also spread by biting midges from the genus *Culicoides*. SBV causes severe fetal malformations in ruminants in Europe and has not been known to infect humans. Using the previously described reverse genetics system for SBV (33, 45) and our newly established OROV rescue system we can begin to understand the basis for pathogenicity in humans and study reasons for such a species-barrier. In addition to this, we can also begin to study the M segment variations that are found amongst Oropouche species. OROV M segment reassortants Iquitos and Madre de Dios viruses can cause disease in humans (26, 27), whilst Perdoes virus and the more divergent Jatobal virus have only been isolated from nonhuman primates (*Callithrix penicillata*) and the South American coati (*Nasua nasua*), respectively (25, 46). It is interesting that members of the Oropouche species display a broad phylogenetic diversity predominately due to the M segment. The variations observed in the M segments of these viruses could have resulted from either genomic reassortment or extensive adaptation to different hosts and habitats. Using the OROV reverse genetics system established in this study it would now be possible to study in detail these differences in terms of pathogenesis, virulence outcome and host-range of these viruses. This work would contribute to understanding the evolution of Clade A Simbu serogroup viruses within South America.

The recombinant OROV (rOROV) that we have generated replicates similar to the authentic virus (wtOROV) reaching titers of $10^7$ PFU/ml (FIG 1B). Using this system, mutant viruses lacking either the NSm or the NSs protein were also generated. Only some bunyaviruses encode these proteins, and until now the exact role played by the NSm protein in orthobunyavirus infections remains unclear. Work on BUNV NSm demonstrated that the protein can localise to the Golgi efficiently on its own (47) and may play a role in viral assembly (48). In RVFV, the NSm protein is important for infection in mosquitoes by allowing the virus to cross the midgut barrier (49, 50). Similarly, in tospoviruses the NSm protein has been shown to be important for virus
cell-to-cell spread (51-53). Results from our work indicate that for OROV the NSm protein is dispensable for virus replication in cultured cells, as rOROVdelNSm grows and replicates similar to the rOROV virus (FIG 2D, E; FIG 4; FIG 7). We have previously (25) discussed the sequence similarity of the M segment genes between different OROV reassortants, and noted that the NSm region of the M polyprotein of all these viruses is highly conserved when compared to the Gn and Gc glycoproteins, which could indicate that this portion of the polyprotein is less prone to mutation due to a common, yet unknown, selective pressure. Future work could include performing mutations on the NSm coding sequence and monitor for effects on virus replication in more relevant primary cell-lines and in vivo models, such as insects. Similarly, the rOROV2080S mutant generated here would also require in vivo characterization in order to determine if the S segment difference observed between OROV isolates offers any advantage over the prototype BeAn19991 S segment, as the current study was not sufficient to determine this.

As with other NSs-encoding bunyaviruses OROV NSs protein is an IFN antagonist and by deleting the NSs ORF, OROV induces high levels of IFN and thus induces STAT1 phosphorylation and MxA expression (FIG 5, rOROVdelNSs). Interestingly, the C-terminal truncated NSs mutant was also incapable of inhibiting type I IFN production (FIG 5, rOROV246NSs). Work on BUNV and RVFV has demonstrated that NSs inhibits IFN-β activation downstream of transcriptional activation through disruption of the DNA-dependent RNA polymerase II (RNAPII) activity (54-56). BUNV NSs interacts with subunit MED8 of the RNAPII regulatory module (57) preventing Ser2 phosphorylation and hence prevents elongation and 3'-end processing of the nascent mRNA transcript (58-60). This was initially thought to be due to an interaction of BUNV NSs C-terminus (aa 83 – 91) with MED8, however a BUNV NSs mutant lacking an N-terminus of 21 amino acids is also unable to degrade RNAPII, indicating that both the C- and the N- terminus are important for BUNV NSs function (39, 57, 61). The BUNV MED8 binding domain was mapped to a C-terminal amino acid motif ‘LPS’, which is conserved in orthobunyavirus NSs proteins (57), and interestingly OROV C-terminal mutant rOROV246NSs also lacks a similar motif ‘LPC’ (Supplementary Figure A). This ‘LPC’ motif is conserved amongst only the Clade A viruses in the Simbu serogroup (Supplementary Figure B). Whether the inability of rOROV246NSs to inhibit IFN production is due to its lack of the MED8
binding domain will be investigated in follow-up studies. LACV and SBV NSs function as IFN antagonists by targeting RNAPII for degradation by the proteasome (62-64). Mutations to the C-terminus of SBV NSs have also been shown to affect the protein’s ability to degrade RNAPII (64). In the phlebovirus RVFV the NSs protein interacts with subunits of the general transcription factor TFIIH, which also has a role in RNAPI transcription (65). SFTSV NSs forms viral inclusion bodies in the cytoplasm and uses these to capture kinases TBK1 and I KKε, and proteins STAT1 and STAT2 (66, 67). Recently a study comparing 6-week old C57BL/6 mice knockout mutants demonstrated that mitochondrial antiviral-signaling protein (MAVS) activation plays a crucial role in type 1 IFN signalling during OROV infection (68), it would be interesting to see how the rOROVdelNSs and rOROV246NSs mutants replicate in such in vivo systems. Interestingly both rOROVdelNSs and rOROV246NSs are attenuated in Vero E6 (FIG 2D) and in BHK-21 (FIG 2E) cells, both of which lack fully functional IFN systems, and from radiolabelling experiments rOROVdelNSs is capable of causing translational shut-off in Vero E6 cells (FIG 3). Using OROV reverse genetics system we can now begin to study protein-protein interactions and investigate the role of the NSs protein further.

This study also observed that whilst OROV is sensitive to IFN-α, to see maximal effects cells have to be treated for 24 h prior to infection (FIG 6A, rOROV). In contrast BUNV is highly sensitive to IFN-α (FIG 6A). These findings are consistent with previously published work demonstrating a resistance of OROV to the antiviral effects of IFN-α both in vivo and in vitro in comparison to other pathogenic orthobunyaviruses (69). The reasons for the differences in relative sensitivity of OROV and BUNV to IFN-α are currently under investigation, but may, for example, be due to the differential effects of certain ISGs on these viruses, or on the ability of OROV to more rapidly switch off host cell gene expression than BUNV. Whatever the reason, the increased resistance of OROV to IFN-α is not due to expression of the NSs protein as rOROVdelNSs shows similar sensitivity to IFN-α as OROV.

In conclusion, our present work has shown that we are able to generate infectious OROV entirely from cDNA and that similar to other bunyaviruses OROV NSs is an IFN antagonist. We have also demonstrated that the NSm protein appears to be
non-essential for virus replication in cultured cells that were tested. The work we have presented here will now enable us to study OROV in more detail in order to establish the molecular details involved in viral replication and pathogenesis, and potentially generate attenuated vaccine strains. The work we present here is an important move forward towards understanding this important yet neglected human pathogen.

Acknowledgements

We thank Xiaohong Shi for advice on creating rOROVdelNSm and Massimo Palmarini for OROV-N antibody and a batch of IFN-α. We thank Stephen Welch for reviewing the manuscript and Mark Tilston for proof reading it.

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Dedication

This paper is dedicated to the memory of Professor Richard M. Elliott who will forever hold a special place in our hearts.

Thank you for being a friend, father figure and a mentor.

References


34. **Brennan B, Welch SR, Elliott RM.** 2014. The Consequences of Reconfiguring the Ambisense S Genome Segment of Rift Valley Fever Virus on Viral Replication in Mammalian and Mosquito Cells and for Genome Packaging. PLoS pathogens **10**:e1003922.


Figure Legends

FIG 1. Characterisation of recombinant OROV. (A) RT-PCR products derived from the L, M and S segments of OROV strain BeAN19991. Amplified products were separated on a 1% agarose gel along with a 1 kb marker (Promega). Products were cloned into plasmids containing a T7-RNA polymerase promoter and a hepatitis delta ribozyme as shown in the schematic below. (B) Growth properties of wild-type (wt) and recombinant (r) OROV in Vero E6 cells. Cells were infected at MOI 0.1. At indicated time points samples were harvested and titers determined by plaque assay on BHK-21 cells. Graph is a representative experiment. (C) Western blot showing N protein synthesis from the growth curve (A). Tubulin was probed as a loading control. (D) Comparison of plaque phenotypes of wtOROV and rOROV. A plaque assay was carried out on BHK-21 cells and at 3 days p.i cells were fixed and stained with crystal violet. (E) Effect of different MOI on rOROV yields in Vero E6 cells. Infected cells were harvested 48 h p.i and titrated on BHK-21 cells. Graph is presented for a representative experiment. (F) Comparison of rOROV growth in various cell-lines. Indicated cells were infected at an MOI of 0.001 and at 48 h p.i harvested and titrated on BHK-21 cells. Bars represent range from two experiments. (G) Comparison of rOROV plaque phenotypes on BHK-21, Vero E6, A549 and CPT-Tert cells. Infected cells were fixed and stained with crystal violet at 3 days p.i.

FIG 2. Creation of OROV mutant viruses. (A) Schematic of the M segment showing Gn, NSm and Gc regions. The arrows depict where cleavage occurs. The patterned box indicates the signal peptide and the black boxes represent transmembrane domains. Nucleotides 1036 to 1475 were deleted in order to generate delNSm M segment. (B) S segment products N and NSs. NSs is coded from an overlapping reading frame with N. Schematic shows how NSs mutants differ from wt. rOROV246NSs has a stop codon (asterisk) placed at nucleotide (nt) position 314 of S segment cDNA changing TTA to TAA thereby deleting the last 8 aa. rOROVdelNSs has a stop codon at cDNA nt position 116 changing TGG to TAG so
that a stop codon is generated immediately after the second start codon (methionine, M). Numbers are amino acid lengths. (C) rOROV2080S S segment in comparison to wt/rOROV S segment. Numbers are nt positions. Arrows show where changes occur. First two positions generate a variation in the NSs OROF. Black highlights are the UTR regions. (D) Growth properties of recombinant viruses in Vero E6 cells. Cells were infected at an MOI of 0.1. Samples were harvested at indicated time points and titrated on BHK-21 cells. Presented graph is a representative experiment. (E) Plaque phenotype of recombinant viruses in BHK-21 cells. A plaque assay was carried out and at 3 days p.i cells were fixed and stained with crystal violet. (F) N production in recombinant viruses. Cell lysates from growth curve (D) were probed for OROV-N and Tubulin.

FIG 3. Host-cell protein shut-off. Vero E6 cells were infected with recombinant viruses rOROV, rOROVdelNSm, rOROV2080S, rOROVdelNSs, rOROV246NSs or mock infected. Cells were infected at an MOI of 3 and incubated at 37 °C. At indicated time-points the cells were labeled with [35S]methionine for 2 h. Cells lysates were then separated by SDS-PAGE. Arrows indicate the position of viral proteins Gc, N, NSm and NSs.

FIG 4. Growth properties of recombinant viruses in A549 cells. (A) Growth kinetics of rOROV, rOROVdelNSm and rOROVdelNSs in A549 cells at an MOI of 0.1. At indicated time-points samples were harvested and viral titers determined by plaque assay on BHK-21 cells. Graph presented for one representative experiment. (B) Western-blot for cell lysates from growth curve (A). Lysates were separated by SDS-PAGE and probed for OROV N and Tubulin. (C) Comparison of growth properties in A549 and A549/V cells. Cells were infected at an MOI of 0.001 with indicated viruses. 48 h p.i viral titers were determined by plaque assay. BUNV was used for comparison. Bars indicate ±SD (n=3; Students t-test was performed; ** = p < 0.01). (D) Western-blot analysis for (C). Cells lysates were probed for viral N protein. Tubulin was probed as loading control.

FIG 5. Biological interferon production assay. A549 cells were infected at MOI 1 with BUNV, rBUNdelNSs2, rOROV, rOROVdelNSm, rOROV2080S, rOROVdelNSs, rOROV246NSs or mock infected. Supernatant was harvested at 24 h p.i and cell
extracts separated by SDS-PAGE. (A) UV-inactivated supernatant was used to pre-
treat A549-N pro cells prior to infection with EMCV. At 3 days p.i cells were fixed and
stained with crystal violet. (B) Graph calculated from (A) and represents relative IFN
units (RIU) expressed as 2N where N is the number of 2-fold dilution that offered
protection. (C) Cells extracts were probed for OROV N, STAT1, pSTAT1 and MxA.
Tubulin was probed as a loading control.

FIG 6. Sensitivity of OROV to IFN-α treatment. (A) IFN-α sensitivity test. Vero E6
cells were treated with an increasing concentration of IFN-α (0, 10, 100, 1000,
10000) either before (-) or after (+) infection. Cells were infected with BUNV or
rOROV at MOI 0.01. 48 h p.i supernatant was harvested and viral titers determined
by plaque assay on BHK-21 cells. Graphs are from a representative experiment. (B)
Vero E6 cells were treated with 10,000 U/ml of IFN-α 24 h prior to infection with
indicated viruses; at an MOI of 0.001. Samples were harvested at 48 h p.i and viral
titers determined by plaque assay on BHK-21 cells. Bars represent range from two
experiments. (D) Vero E6 cells were treated (1000 U/ml) or untreated (0 U/ml) with
IFN-α 24 h prior to infection. A plaque assay for BUNV, rBUNVdelNSs2, OROV or
rOROVdelNSs viruses was performed. 4 days p.i cells were fixed and stained with
crystal violet.

FIG 7. Growth kinetics in mosquito cells. Cells were infected with rOROV,
rOROVdelNSm or rOROVdelNSs at MOI 0.1. At indicated time-points samples were
harvested and viral titers determined by plaque assay. Presented graphs are
representative experiments. Cell extracts were separated by SDS-PAGE and probed
for viral N and Tubulin. (A) Replication in U4.4 cells. (B) Replication in Aag2 cells.
(C) N production in U4.4 cells. (D) N production in Aag2 cells.
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FIG 1

A

B

C

D

E

F

G

Cell lines

Vero E6, moi: 0.1

Virus Titer (Log₁₀ PFU/ml)

Time post infection (h)

Tubulin

Tubulin

Human

monkey

rodent

fowl

sheep

canine

A549

2TGH

HeLa

Vero E6

LLC-MK2

BHk-21

MRK101

DF-1

QT-35

CPT-Tert

MDCK

wtOROV

rOROV

wtOROV

rOROV

BHK-21

Vero E6

A549

CPT-Tert
**FIG 2**

A. Schematic representation of the ORF7 domain of OROV showing the signal peptide, transmembrane domain, and cleavage site.

B. Graph showing the virus titer (Log$_{10}$ PFU/ml) over time post-infection (h) for Vero E6 cells infected with different strains of OROV.

C. Schematic representation of the ORF7 domain of OROV showing the positions of the stop codons.

D. Schematic representation of the ORF7 domain of OROV showing the positions of the mutations.

E. Representative images showing the plaque morphology of different strains of OROV on Vero E6 cells.

F. Western blot analysis showing the expression of tubulin N protein in cells infected with different strains of OROV at 6, 12, 18, 24, and 48 h post-infection.
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**Notes:**
- Gc
- N
- NSm
- NSs
Fig 5

**A**

EMCV - + 2-fold dilution

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**B**

Relative IFN units (RIU)

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**FIG 5**
FIG 7

A

![Graph A](image)

- Virus Titer (Log$_{10}$ PFU/ml)
- Time post-infection (h)
- U4.4, moi: 0.1
- rOROV
- rOROVdelINSm
- rOROVdelINSs

B

![Graph B](image)

- Virus Titer (Log$_{10}$ PFU/ml)
- Time post-infection (h)
- Aag2, moi: 0.1
- rOROV
- rOROVdelINSm
- rOROVdelINSs

C

![Images C](image)

- Tubulin
- h
- 24 48 72 96
- rOROV
- rOROVdelINSm
- rOROVdelINSs

D

![Images D](image)

- Tubulin
- h
- 24 48 72 96
- rOROV
- rOROVdelINSm
- rOROVdelINSs

FIG 7