Genetic and biological characterization of a densovirus isolate that affects dengue virus infection

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Brevidensoviruses have an encapsidated, single-stranded DNA genome that predominantly has a negative polarity. In recent years, they have received particular attention due to their potential role in the biological control of pathogenic arboviruses and to their unnoticed presence in cell cultures as contaminants. In addition, brevidensoviruses may also be useful as viral vectors. This study describes the first genetic and biological characterization of a mosquito densovirus that was isolated in Brazil; moreover, we examined the phylogenetic relationship between this isolate and the other brevidensoviruses. We further demonstrate that this densovirus has the potential to be used to biologically control dengue virus (DENV) infection with in vitro co-infection experiments. The present study provides evidence that this densovirus isolate is a fast-spreading virus that affects cell growth and DENV infection.

Key words: densovirus - *Brevidensovirus* - biological control - dengue virus

Over the past several decades, a new set of viruses that are non-pathogenic to humans have been isolated from mosquito vectors, such as *Aedes* sp. (Kittayapong et al. 1999, Jousset et al. 2000). Arbovirology researchers are particularly interested in these viruses because of their potential use as biological controls and expression vectors (Afanasiev et al. 1994, Carlson et al. 2006, Wei et al. 2006). In this study, we identified and molecularly characterised a mosquito densovirus (MDV) that belongs to the *Brevidensovirus* genus of the Densovirinae sub-family, which belongs to the Parvoviridae family (Bergoin & Tijssen 2000). MDVs are non-enveloped viruses that have a single-stranded DNA genome of approximately 4 kb and predominantly negative polarity (85%), packaged within the capsid (Afanasiev et al. 1994). Viruses belonging to this genus have three major open reading frames in the positive polarity strand, which encode for non-structural (NS1 and NS2) and structural proteins (VP1 and VP2). These genomic sequences possess self-complementary palindromic sequences at their 5' and 3' ends, which are essential for the replication and encapsidation of their genetic material (Afanasiev et al. 1994). These sequences differ between the various brevidensoviruses in terms of their length and sequence pairing generates a Y or T shaped structure (Bergoin & Tijssen 2000). These viruses are thought to be non-pathogenic to humans, but they show varying degrees of pathogenicity in mosquitoes (O'Neill et al. 1995, Kittayapong et al. 1999, Carlson et al. 2006, Suchman et al. 2006).

In this paper, we isolated and characterised a MDV that was a contaminant in C6/36 cell cultures infected with yellow fever virus. Our results from in vitro co-infection assays with this MDV and a recent clinical isolate of dengue virus (DENV) indicate that the MDV interfered with dengue viral morphogenesis.

MATERIALS AND METHODS

Cells and viruses - The *Aedes albopictus* cell line (Igarashi 1978) (C6/36, ATCC, CRL-1660) was cultured in Leibovitz's medium (L-15, Gibco/Invitrogen, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS) (Gibco/Invitrogen, Grand Island, NY, USA), 0.26% tryptose (Sigma-Aldrich, St. Louis, MO, USA) and 25 μg/mL gentamicin (Gibco/Invitrogen, Grand Island, NY, USA) at 28ºC. Kidney epithelial cells from *Cercopithecus aethiops* (Vero E6, ATCC, CRL-1586) were maintained at 37ºC with 5% CO_2 in Iscove's Modified Dulbecco's medium (Gibco/Invitrogen, Grand Island, NY, EUA) supplemented with 5% FBS and 25 μg/mL gentamicin.

The MDV stocks, which are referred to as BR/07 MDV, were prepared by infecting C6/36 cell cultures.

BR DEN3 290-02, which is a recent clinical isolate of DENV serotype 3 (DENV-3), was used for the co-infection experiments (GenBank accession EF629369) (Nogueira et al. 2008). The viral stock that was used for the experiments was the supernatant from the fifth passage of the C6/36 cell cultures.

The YF17DD virus was kindly provided by Dr Ricardo Galler [Biomanguinhos, Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro]; the viral supernatants used in the experiments were prepared through serial passage in Vero cells.

Total nucleic acid extraction - The total cellular nucleic acid content was extracted from the cells by phenol extraction as follows. The cells were lysed by three cycles of freeze/thaw. The cell debris was removed by centrifu-

gation (10 min at 400 g) and sodium dodecyl sulfate and β-mercaptoethanol were added to the supernatant at a final concentration of 1%. Following incubation for 3 min at 72ºC, an equal volume of phenol:chlorophorm:isoamyl-alcohol (25:24:1) was added. The aqueous phase was removed and re-extracted with an equal volume of chlorophorm:isoamyl-alcohol (24:1). The nucleic acids were precipitated with 10% 3 M sodium acetate, pH 5.3, and 2.5 volumes of ethanol; the extracted nucleic acids were finally resuspended in nuclease-free water.

Panchip - The nucleic acids that were extracted from the C6/36 cell line were randomly amplified and hybridized to a pan-viral DNA microarray, as described by Wang et al. (2003).

Transmission electron microscopy - For routine transmission electron microscopy, the cells were fixed for 1 h in 2.5% glutaraldehyde and diluted in 0.1 M cacodylate buffer, pH 7.2. The samples were washed twice with the same buffer and subsequently fixed in 1% OsO_4, 0.8% $KFe(CN)_6$ and 5 mM $CaCl_2$ diluted in 0.1 M cacodylate buffer. After fixation, the cells were washed, dehydrated in a graded series of acetone solutions and embedded in Epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate before analysis with a Zeiss 900 transmission electron microscope.

MDV isolation - BR/07 MDV was initially identified in a cell culture supernatant sample that was also infected with yellow fever virus (BR/01). A neutralization assay with yellow fever virus polyclonal antibodies was performed to isolate the MDV. The sample was serially diluted (10^{-1}-10^{-7}) in L-15 medium supplemented with 0.26% tryptose and 25 µg/mL gentamicin to a final volume of 500 µL. A yellow fever polyclonal antibody

(50 µL) was then added to each dilution and the mixture was incubated for 2 h at 37ºC. Following the incubation period, each mixture was used to infect 3.5 x 10^5 C6/36 cells. After incubation for 1 h at 28ºC, the inoculum was discarded and replaced with fresh medium. Five days after infection, the total cellular nucleic acid content was extracted from the cells.

Yellow fever reverse transcription-polymerase chain reaction (RT-PCR) and MDV PCR - Yellow fever virus (BR/01) infection of the samples was assessed with reverse transcription of the total nucleic acid extract. An ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) with random hexamers (Invitrogen, Carlsbad, CA, USA) was used according to manufacturer's instructions. The synthesised cDNA and the total nucleic acid were tested with specific yellow fever and MDV primers, respectively, in a PCR reaction with recombinant Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The primers used for the PCR amplification were YF21, YF34, DNV3F and DNV3R (Table I). Positive and negative samples were included for each reaction.

MDV genome amplification and sequencing - Whole genome PCR amplification was used to genetically characterise BR/07 MDV. We used the following high-fidelity enzymes according to the manufacturer's instructions: Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), QIAGEN® LongRange PCR System (QIAGEN, Valencia, CA, USA) and TripleMaster® PCR System (Eppendorf, Westbury, NY, USA). The primers were initially designed based on the *Ae. albopictus* densovirus sequence (GenBank accession NC_004285) and subsequently from the BR/07

TABLE I
Primers used in reverse transcription-polymerase chain reaction (PCR), PCR and sequencing

Primer	Sequence 5' → 3'	Position
DNV1F	TGTTGGGAGCATGACGCACAGT	375-396[a]
DNV1R	GGTTCTCTGTCTGCGTCTGCGATGAACA	3072-3045[a]
DNV2F	TCGTGATACGGATACTCCAAGATACAG	43-69[a]
DNV3F	AATCGAGAAACAGCATACTACACATTCGT	1134-1162[a]
DNV3R	TTTATTTCCATAGATATTGACTGTTTCGAT	1457-1428[a]
DNV4F	TATAAGTCCATATTCCATATAAGAAATATTATT	10-42[a]
DNV4R	ACGGTTGATATACACGTTCCATCA	913-890[a]
DNV5F	GGAGAAGACAACAGCAAAACAGCAA	1860-1884[a]
DNV5R	CTGGTGTTATCATTGGTTCTTCAAATAGGA	2163-2134[a]
DNV6F	TACTTGGACTTCAACTACGTATCAAATCA	2783-2811[a]
DNV6R	TTCTGAGGTGGAATACGGAGGTGGA	3791-3767[a]
DNV7F	CACAACCACAAATTCCAGACGAAACC	3438-3463[a]
DNV7R	CAACGCAGACTGAATTCATCACTCGAT	327-301[a]
DNV8R	GGCTCAATGAGAGTTGGAGGACGACT	2437-2412[a]
YF21	CACATATCCTAGGTATTGTAAGCC	9193-9170[b]
YF34	GCCAATGAGGCTGTCCAAGACC	8903-8924[b]

a: GenBank accession: GU452720; *b*: GenBank accession: DQ100292.

MDV nucleotide sequence as it became available. The primer sequences are listed in Table I. To amplify the 5' and 3' ends of BR/07 MDV, the total nucleic acid extract from the supernatant of infected cell cultures was denatured at 95°C for 5 min, ligated with T4 RNA ligase (New England Biolabs, Ipswich, MA, USA) at 37°C for 30 min and incubated at 16°C for 16 h. The ligated product was purified by phenol extraction as previously described. The purified DNA was used as the template for a PCR reaction with the DNV7F and DNV7R primers. The PCR product was purified with a High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Alemanha) and was inserted into a pGEM®-T-Easy vector (Promega, Madison, WI, USA) for nucleotide sequencing.

The nucleotide sequencing was performed with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, IA, USA) in an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, IA, USA). Alternatively, the PCR products were sequenced by Macrogen Inc (Seoul, Korea). The sequences were assembled with the phred/Phrap/consed software package (www.phrap.org) (Ewing et al. 1998, Ewing & Green 1998, Gordon et al. 1998, 2001). The final consensus sequence was deposited in GenBank under the accession GU452720.

Secondary structure prediction for the 5' and 3' ends - The DNA mfold server (Zuker 2003) (www.bioinfo.rpi. edu/applications/mfold/cgi-bin/dna-form1.cgi) was used to predict the secondary structure of the BR/07 MDV 5' and 3' ends. For comparison, the same procedure was used to predict the secondary structure of *Aedes densonucleosis* virus (GenBank accession M37899), which has previously been described (Afanasiev et al. 1991). The default settings were used for all of the analyses.

Phylogenetic analysis - Bioinformatic searches for similar sequences were performed online with the National Center for Biotechnology Information Blast server. Based upon the results, we assembled a reference panel of densovirus sequences from GenBank (Table II). Sequences ranging from the start codon of the open reading frame (ORF) on the 5' end to the stop codon of the ORF on the 3' end were aligned using the ClustalW algorithm (Thompson et al. 1994); distance calculations and phylogenetic analysis were performed using Molecular Evolutionary Genetics Analysis version 4 (Tamura et al. 2007). The pairwise distance comparisons were based on the proportion of nucleotide differences between the sequences and phylogenies were estimated by neighbour-joining (Saitou & Nei 1987) using the maximum composite likelihood distance (Tamura & Kumar 2002). Support for the nodes was evaluated with 1,000 bootstrap pseudoreplicates (Felsenstein 1985).

Polyclonal and monoclonal antibody production - Adult BALB/c mice were immunised five times with viable BR/07 MDV virus particles obtained from the supernatant of C6/36 infected cell cultures. Prior to immunisation, a pre-immune serum sample was collected. On day zero, the mice were immunised intraperitoneally with a 1:1 mixture (200 μL) of virus and Freund's complete adjuvant (Sigma-Aldrich, St. Louis,

MO, USA). On days seven, 14 and 21, the mice were immunised intraperitoneally with a 1:1 mixture (200 μL) of virus and Alu-Gel-S (SERVA Electrophoresis, Heidelberg, Germany). Finally, on day 28, the mice were immunised intravenously and intraperitoneally with virus only (100 μL/route). Three days after the last immunisation, a post-immune serum sample and the spleen were collected. The serum was evaluated with an indirect immunofluorescence assay (IFA) with infected cell cultures to validate its use as a source of a polyclonal antibody (Supplementary data). The spleen was processed for monoclonal antibody production as described by Mazzarotto et al. (2009). Our protocol was approved by the Ethical Committee on Animal Experiments of Fiocruz (LW-26/09).

MDV concentration - Viral stock titration was done by quantitative PCR as described by Ledermann et al. (2004). DNA from the viral supernatants was purified with an AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen Biosciences, Union City, CA, USA) and quantified in an ABI 7500 thermocycler/fluorescence reader with a TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, IA, USA). Standard curves were constructed using serial 10-fold dilutions (10^{10}-10^1 genome copies) of the plasmid T&A cloning vector (Real

TABLE II

Densovirus sequences used in phylogenetic analysis

GenBank accession	Identification
EF579760	*Culex pipiens pallens densovirus* strain XJ057
EF579763	*Cux. pipiens pallens densovirus* strain XJ0511
EF579762	*Cux. pipiens pallens densovirus* strain XJ059
EF579764	*Cux. pipiens pallens densovirus* strain XJ0545
EF579770	*Cux. pipiens pallens densovirus* strain YN05169
EF579758	*Cux. pipiens pallens densovirus* strain GZWN2
EF579759	*Cux. pipiens pallens densovirus* strain GZWN5
EF579761	*Cux. pipiens pallens densovirus* strain XJ058
EF579769	*Cux. pipiens pallens densovirus* strain YN05159
EF579765	*Cux. pipiens pallens densovirus* strain YN0569
EF579768	*Cux. pipiens pallens densovirus* strain YN05152
EF579767	*Cux. pipiens pallens densovirus* strain YN05150
EF579757	*Cux. pipiens pallens densovirus* strain GZWN1
EF579766	*Cux. pipiens pallens densovirus* strain YN05145
EF579771	*Cux. pipiens pallens densovirus* strain YN05217
EF579756	*Cux. pipiens pallens densovirus* strain JZ-16
EU233812	*Anopheles gambiae densonucleosis* virus
NC_011317	*An. gambiae densonucleosis* virus
M37899	*Aedes densonucleosis* virus
AY095351	*Aedes albopictus* C6/36 cell densovirus
AY310877	*Ae. albopictus* C6/36 cell densovirus
AY605055	*Haemagogus equinus* densovirus
NC_004285	*Ae. albopictus* densovirus
X74945	*Ae. albopictus* parvovirus

Biotech Corporation, Banqiao, Taiwan) with the PCR insert, which was cloned according to the manufacturer's instructions. The plasmid molecular weight was calculated with the sequence manipulation software available at http://www.bioinformatics.org/sms2/dna_mw.html. The plasmid was purified with a Wizard Plus SV Miniprep kit (Promega, Madison, WI, USA), the concentration was estimated with a Nano Drop ND-1,000 Spectrophotometer (Nano Drop technologies, Wilmington, DE, USA) and the number of copies was calculated according to Ke et al. (2006). The number of copies was calculated by taking into account that our target consists of single-stranded DNA, although double-stranded DNA was used for the standard curve.

Cell growth and cell-cycle kinetics - Six-well plates were seeded with 4×10^4 C6/36 cells/cm^2 and incubated for 24 h; the cells were subsequently infected with a multiplicity of four genomes/cell [4 multiplicity of genome (MOG)] of BR/07 MDV or mock infected. At 24, 48 and 72 h post-infection, the cells were detached, stained with trypan blue and the number of viable cells was counted with a Neubauer chamber. The same conditions were used to evaluate the cell cycle stage via flow cytometry, as described by Fried et al. (1978).

Co-infection experiments - C6/36 cell cultures were infected with a multiplicity of infection (MOI) of one or 10 of DENV-3 (BR DEN3 290-02), four or 0.04 MOG of BR/07 MDV or a mixture of these two viruses. Non-infected cultures were used as the negative control (mock). The extent of infection was evaluated at 24, 48 and 72 h with flow cytometry in a BD FACSCalibur™ Flow Cytometer (BD Biosciences, San Jose, CA, USA), as well as by titration of the DENV particles from the cell culture supernatants. The titration was performed using the focus immunodetection technique (Desprès et al. 1993); flow cytometry was performed as previously described by Bordignon et al. (2002). The results were analysed with the FlowJo flow cytometry analysis software (Tree-Star, Inc, Ashland, OR, USA).

Replicon experiments - C6/36 cell cultures prepared and infected with BR/07 MDV (3 MOG or 0.03 MOG), as described above, were subsequently transfected with the BR DEN3 290-02 replicon (Mosimann et al. 2010). Five days post-infection/transfection, the monolayer was fixed and stained for immunofluorescence analysis with either anti-MDV or anti-DENV-3 polyclonal antibodies and the percentage of positive cells was estimated.

Virus spread experiments - The C6/36 cells were seeded at a density of 5×10^4 cells/cm^2 and incubated for 48 h; the cells were subsequently infected with 0.6 or 0.006 MOG of BR/07 MDV (or a mock infection for the control). After incubation for 1 h at 28ºC, the inoculum was removed and cells were washed with phosphate buffered saline (PBS) buffer before replenishment with cell medium. At various time points (0, 1, 3, 5, 7, 9, 11 h and 24 h), the cells were either detached and immunolabeled for flow cytometry analysis or fixed and permeabilized with a 1:1 solution of methanol:acetone at -20ºC for at least 2 h for an indirect immunofluorescence assay.

IFA - The C6/36 cell monolayers that were previously fixed and permeabilised were incubated with mouse anti-MDV polyclonal antibodies at 37ºC for 45 min, washed three times with PBS and incubated for 45 min at 37ºC with goat anti-mouse conjugated to fluorescein isothiocyanate diluted 1:100 in PBS containing 0.3% (v/v) of 10 µg/mL Evan's blue. The cells were subsequently incubated at room temperature for 5 min with 1 mg/mL 4'-6-diamidine-2-phenyl indole, washed five times with PBS buffer and, once dry, were overlaid with a solution of PBS and 10% (v/v) glycerol.

The immunofluorescence images were captured with a Nikon Eclipse TE300 microscope (Nikon, Tokyo, Japan) attached to a CoolSNAP™-Pro$_{cf}$ camera (Media Cybernetics, Bethesda, MD, EUA). The images were visualised and edited using the Image-Pro® PLUS v.4.5.1.29 software (Media Cybernetics, Bethesda, MD, EUA).

Statistics - The results are expressed as the mean ± standard deviation of the data. Differences between the groups were analysed with a one-way or two-way analysis of variance, followed by a Bonferroni post-test. A p value < 0.05 was considered significant.

RESULTS

The BR/07 MDV was initially detected in the supernatant of C6/36 cells infected with YFV (BR/01) that had been sent to our laboratory for research purposes (Supplementary data). Because of the atypical cytopathic effect that was observed in the C6/36 cell cultures, we suspected that there was additional microorganism contamination. A putative contaminating virus was identified by electron microscopyt, which showed viral particles of various sizes within the same cell. Hybridization of randomly amplified nucleic acids from the culture to a pan-viral DNA microarray yielded strong hybridisation for multiple oligonucleotide probes from known densoviruses. Viral identification was later confirmed by PCR using primers for conserved regions of the *Brevidensovirus* genome, which were retrieved from the public data banks (Supplementary data).

The BR/07 MDV was subsequently isolated by eliminating the yellow fever virus, which was also present in the sample, by neutralization. BR/07 MDV isolation was confirmed by RT-PCR and PCR with yellow fever and MDV-specific primers, respectively (Supplementary data).

Genomic sequencing indicated that the BR/07 MDV isolate contains three major ORFs, which is consistent with previous descriptions of other viruses within this genus. The left ORF is 2,550 nucleotides (nt), the mid ORF is 1,089 nt and the right ORF 1,077 nt (Fig. 1A). Nucleotide sequence alignments indicate that BR/07 MDV is closely related to a densovirus that was previously isolated from *Aedes aegypti* (GenBank accession M37899). When the two predicted coding sequences were compared for these two viruses, we found 101 nucleotide substitutions and 38 amino acid replacements for BR/07 MDV. The relationship between these viruses was confirmed by a neighbour-joining phylogeny, which grouped them together with 100% bootstrap support (Fig. 1B).

Due to the limited amount of sequence data available for the 5' and 3' ends of these viruses, it was difficult to predict the first and last nt of the BR/07 MDV genome. However, the BR/07 MDV end sequences that were defined by the genome assembly display the predicted "Y"-shaped structure, similar to the predicted structure for *Ae. densonucleosis* virus, although with longer arms (Supplementary data). The arms formed by the 5' and 3' end of the BR/07 MDV isolate were 108 and 164 nt long, respectively, whereas the analogous structures for *Ae. densonucleosis* virus are 102 and 146 nt long.

Our initial biological characterization of the BR/07 MDV isolate involved assessment of the cell growth and cell cycle kinetics. Preliminary observations indicated that infection with this virus affected cell growth. Infected C6/36 cell growth was significantly inhibited relative to the non-infected cell growth (Fig. 2A). Cell cycle kinetic experiments showed that the infected cells were arrested in the G2 phase (Fig. 2B).

Our next step was to quantify the viral inoculum. We tried several titration protocols for the BR/07 MDV isolate, including the $TCID_{50}$ protocol described by Jousset et al. (1993), or the focus immunodetection technique

(Desprès et al. 1993), which were both unsuccessful. The number of genome copies was quantified through quantitative PCR as described by Ledermann et al. (2004).

The observations made while attempting to titrate the virus suggest that the BR/07 MDV infection spread in the culture within a few hours. To evaluate the rate of viral spread, an experiment was performed with two different virus concentrations (0.6 MOG and 0.006 MOG). The results of these experiments (Fig. 3A) clearly show that the BR/07 MDV infection spread rapidly. At an MOG of 0.6, we were able to detect protein synthesis in the infected cells as early as 3 h post-infection (white arrows) and by 7 h post-infection, the whole monolayer was infected. This process was delayed with 0.006 MOG, and the whole monolayer was infected by 9 h post-infection. These IFA results are consistent with the flow cytometry data (Fig. 3B).

Several previous studies (Jousset et al. 1993, Carlson et al. 2006, Suchman et al. 2006, Wei et al. 2006) have shown that MDV can be used as a biological control for arboviral infection. Based on these observations, we evaluated the capacity of BR/07 MDV to affect the in vitro infection time course with a clinical isolate of dengue virus. Co-infection experiments were performed with either 4 MOG or 0.04 MOG of BR/07 MDV in combination with DENV-3 at a MOI of one or 10. We found that there was significant inhibition of DENV infection, which was measured by the percentage of infected cells, irrespective of the MOI of DENV used when the cells were co-infected at 0.04 MOG of BR/07 MDV (Fig. 4A, B). In contrast, there was no difference in the percentage of DENV infection in cells co-infected at 4 MOG of BR/07 MDV and cells infected exclusively with DENV-3. Titration of DENV in the supernatants of the cultures infected with 4 MOG of
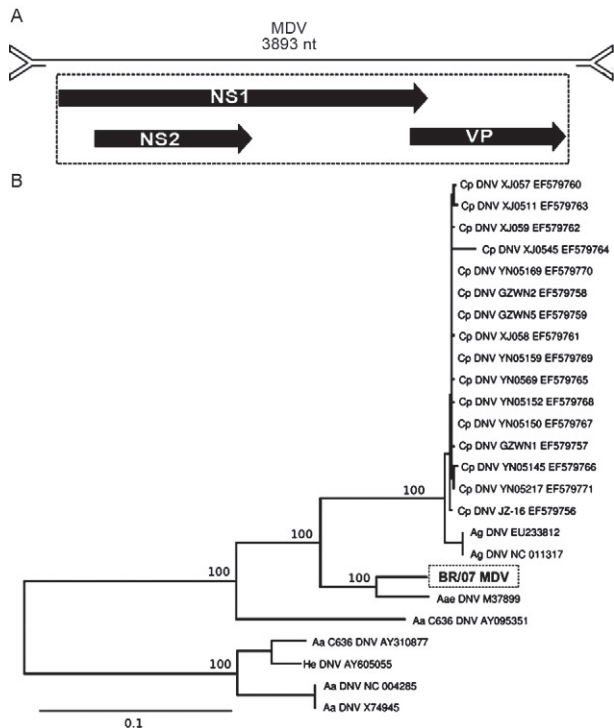

Fig. 1A: schematic representation of the BR/07 mosquito densovirus (MDV) viral isolate genome (above) and its open reading frames (below) (drawn with Vector NTI software from Vector NTI Advance 10.3.1 Software package, Invitrogen, Carlsbad, CA, USA); B: phylogenetic tree of viruses belonging to the *Brevidensovirus* genus. The tree was generated based on the region contained within the dashed box in A. Aa: *Aedes albopictus*; Aae: *Aedes aegypti*; Ag: *Anopheles gambiae*; Cp: *Culex pipiens*; He: *Haemagogus equinus*; NS: non-structural; VP: structural proteins.
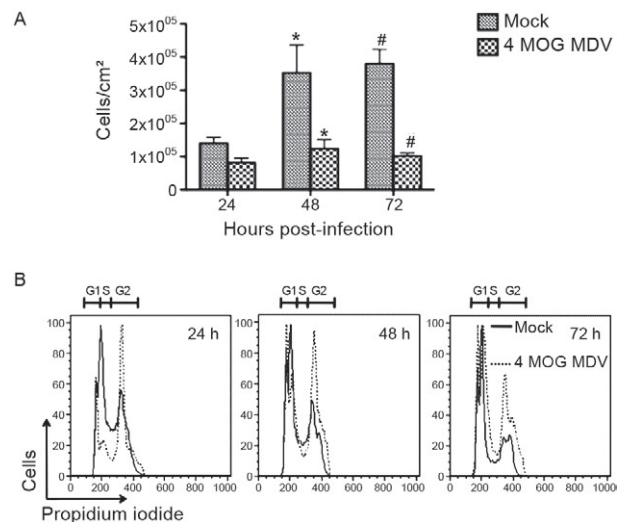

Fig. 2: cell growth and cell-cycle kinetics. A: cell growth. Results are expressed as the average number of cells/cm^2 ± standard deviation of two experiments with two biological replicates each (*: p < 0.01; #: p < 0.001); B: cell-cycle kinetics. Results are from one representative of two experiments performed. MOG: multiplicity of genome; MDV: mosquito densovirus.

BR/07 MDV (Fig. 4C) typically had lower values at 24 h and 48 h post-infection, although these observations were not statistically significant. At 72 h post-infection, a significant decrease in the DENV-3 viral titre was observed when the cells were co-infected with BR/07 MDV at both MOIs tested (4 MOG and 0.04 MOG).

To determine whether BR/07 MDV infection interfered with DENV-3 replication, we transfected MDV-infected C6/36 cells with a DENV-3 replicon (BR DEN3 290-02 replicon) and estimated the number of anti-DENV-3 reactive cells at five days post-infection/transfection. The results suggest that (Supplementary data) there was no significant difference in the percentage of anti-DENV-3 reactive cells for the MDV infected and non-infected cells that were subsequently transfected with the DENV-3 replicon.

DISCUSSION

The main purpose of this work was to genetically and biologically characterise a new *Brevidensovirus* isolate (BR/07 MDV), as well as to assess its ability to affect DENV morphogenesis. BR/07 MDV was first detected in a C6/36 cell culture infected with an YFV sample (BR/01) that was sent to our laboratory for research purposes. The atypical YFV phenotype in the mosquito cell culture suggested the presence of a contaminating co-infecting microorganism. Electron microscopy, microarray hybridisation, PCR and genome sequencing identified this microorganism as a MDV.

Based on the previously published data (Gorziglia et al. 1980, Jousset et al. 1993, O'Neill et al. 1995, Chen et al. 2004), we hypothesize that the cell line initially used in the YFV isolation, most likely a mosquito cell line, may have been contaminated. Unfortunately, because the cell culture supernatant sample was sent seven years ago for research purposes, there is no way to track the infection origin.
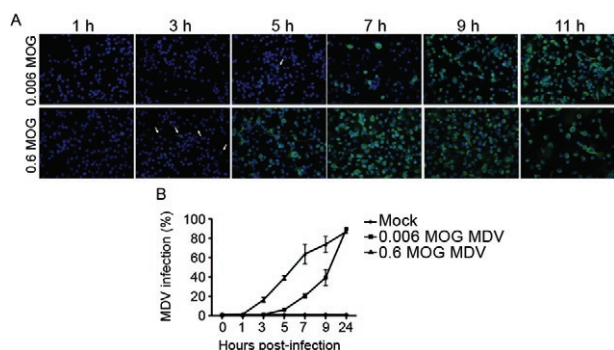

Fig. 3: virus spread experiments. A: indirect immunofluorescence assay of C6/36 cultures infected at 0.006 or 0.6 multiplicity of genomes (MOG) of BR/07 mosquito densovirus (MDV). Cells were fixed and incubated with anti-MDV polyclonal antibody followed by anti-mouse conjugated to fluorescein isothiocyanate. Cell nuclei were stained with 4'-6-diamidine-2-phenyl indole. Images were obtained with a 400X magnification factor; B: flow cytometry results of C6/36 cultures infected at 0.006 or 0.6 MOG of BR/07 MDV. For flow cytometry, cells were immunolabeled with anti-MDV polyclonal antibody followed by anti-mouse conjugated to phycoeritrin. Results are expressed as means ± standard deviation of three experiments.

Thus, we suggest that researchers who work with arbovirus isolation from mosquitoes should check their cell lines periodically for MDV infection. The potential of these viruses to pass unnoticed in cell cultures is due to their ability to establish persistent infections that are not associated with any obvious cytopathic effect (Gorziglia et al. 1980, Jousset et al. 1993, O'Neill et al. 1995).

Phylogenetic analysis grouped BR/07 MDV with *Ae. aegypti* densovirus, which was supported by high bootstrap values (Fig. 1). This could be due to the fact that the viral isolate reported in this article was clearly present as a cell culture contaminant, without means of tracing its real origin. Similar results were described by Chen et al. (2004).

Previous findings by Paterson et al. (2005) suggest that the expression of *Ae. aegypti* densonucleosis virus NS1 in C6/36 cells causes the cells to halt in the G2 phase. Consistent with this, we saw that BR/07 MDV affected *Ae. albopictus* cell growth (Fig. 2A) and appeared to cause cell cycle arrest in G2 (Fig. 2B). Cell cycle arrest has also been previously described by Op De Beeck and Caillet-Fauquet (1997) in a study with another parvovirus, the Minute virus of mice. This study showed that NS1 interferes with cellular DNA replication and induces chromatin damage. Similarly, Oleksiewicz and Alexandersen (1997) saw that infection of Crandell feline kidney cells with Aleutian mink disease parvovirus induced cell-cycle arrest.

Previous studies have also suggested that MDV reduces the severity of DENV infection (Burivong et al. 2004). Thus, we tested the ability of MDV to act as a biological control for DENV infection in vitro with co-infection experiments. We observed significantly lower levels of cell infection and a lower virus titre in C6/36 cells that were co-infected with BR/07 MDV and DENV-3 compared to cells infected with DENV-3 alone. However, the discrepancy observed between the DENV titre results (Fig. 4C) and the percentage of infected cells determined with flow cytometry (Fig. 4A, B) remains unclear. Although co-infection at 4 MOG of BR/07 MDV did not significantly inhibit dengue infection (Fig. 4A, B), DENV-3 viral progeny generation was significantly inhibited by BR/07 MDV at both MOG tested 72 h post co-infection (Fig. 4C). Therefore, the effect of BR/07 MDV on DENV infection does not appear to be related to viral entry into the cells or genome replication/translation but may be due to changes in viral assembly or budding. Replicons are especially well suited to study viral replication once they have all the elements needed for the replication of the viral genetic material in cells, but they do not encode the functional structural proteins and consequently are not able to generate new viral particles (Jones et al. 2005). We therefore used a DENV-3 replicon (Mosimann et al. 2010) in co-infection experiments with BR/07 MDV as described above to confirm our findings. DENV-3 replication was not significantly affected by MDV infection (3 MOG or 0.03 MOG) (Supplementary data). However, because the percentage of transfected cells was low, the possibility that MDV infection interferes with DENV-3 replication cannot be completely ruled out.
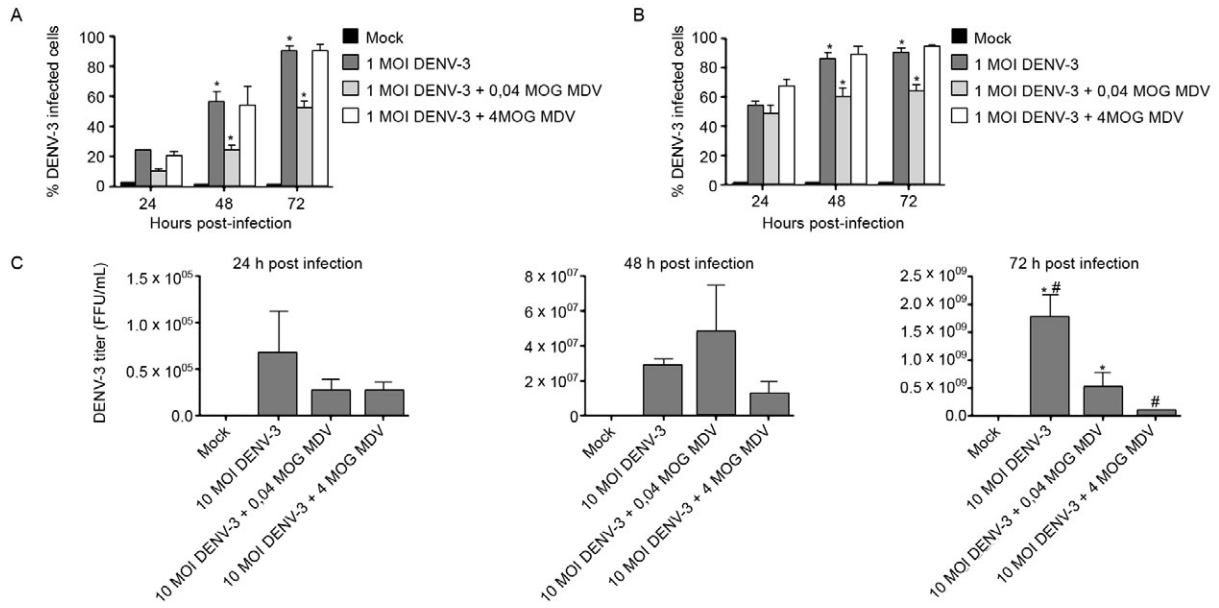
Fig. 4: co-infection experiments. A: flow cytometry on cells co-infected with one multiplicity of infection (MOI) of dengue virus-3 (DENV-3) and 0.04 multiplicity of genomes (MOG) or 4 MOG of BR/07 mosquito densovirus (MDV); B: flow cytometry on cells co-infected with 10 MOI DENV-3 and 0.04 MOG or 4 MOG of MDV; C: DENV-3 titers in the supernatants of co-infected cultures (#/*: p < 0.001). Results are expressed as means ± standard deviation of three experiments. FFU: focus forming units.

Wei et al. (2006) obtained promising results for the use of MDV as a biological control. Specifically, they observed that DENV-2 infection was 100-fold decreased in MDV-co-infected *Ae. albopictus* mosquitoes, compared to mosquitoes infected with DENV-2 alone. Using a different approach, Burivong et al. (2004) showed that C6/36 cells that were persistently infected with *Ae. albopictus* densovirus had a less severe infection, although they were not protected from infection itself. Significantly decreased DENV-2 viral particle production was observed in cells that were persistently infected with MDV at 72 h and 96 h but was not observed at 120 h post-infection. However, the use of different co-infection protocols makes it difficult to compare the previous findings with our own.

Viruses that belong to the *Densovirus* genus display certain features that make them suitable for use as biological controls. They are non-enveloped viruses that can survive for long periods of time in nature. They also act as larvicides and can be vertically transmitted to mosquito offspring. Additionally, infection in adult mosquitoes has been associated with a decreased lifespan (Jousset et al. 1993, Carlson et al. 2006, Suchman et al. 2006). Because these viruses possess a small genome, they may also prove useful as expression vectors, although this remains to be confirmed (Afanasiev et al. 1994). They may also be useful for immunisation strategies, where sequences that are able to interfere with either the replication of clinically important arboviruses or the reproduction of mosquito vectors could be inserted into the MDV genome (Carlson et al. 2006). However, such possibilities must be addressed carefully, because previous findings have suggested that arthropod cells can have balanced, persistent infections with two or three heterologous viruses (Burivong et al. 2004, Kanthong et al. 2008, 2010).

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