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Growing a giant: Evaluation of the virological parameters for mimivirus production

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A B S T R A C T

Acanthamoeba polyphaga mimivirus (APMV) was described in 2003, and due to its unique structural and genetic complexity, the viral family *Mimiviridae* was created. APMV prompted the creation of an open field of study on the function of hundreds of never-before-seen open reading frames (ORFs) and their roles in virus–host interactions. In recent years, several giant viruses have been isolated from different environments and specimens. Although the scientific community has experienced a remarkable advancement in the comprehension of the mimivirus replication cycle in the last years, few studies have been devoted to the investigation of the methodological features and conditions for mimivirus cultivation. In this work, conditions for the cultivation of mimivirus isolates were investigated to obtain relevant information about the production of infectious particles, total viral particles and viral DNA. The results suggest that low viral doses are more efficient for the production of infectious particles, yielding up to 5000 $TCID_{50}$ for each inoculated $TCID_{50}$. Besides methodological information, these data also reveal, for the first time, the ratio between total and infectious particles (in $TCID_{50}$) that are produced during mimivirus cultivation in laboratory conditions. All of this information can be used as a worldwide guide for the production of mimiviruses and can help prompt mimivirological studies in different fields.

1. Introduction

Acanthamoeba polyphaga mimivirus (APMV), the prototype of the family *Mimiviridae*, was discovered in a hospital water-cooling system in Bradford, England during an outbreak of pneumonia (La Scola et al., 2003). APMV is an amoeba-associated virus with unique features, including a ~1.2 megabase (Mb), double-stranded DNA genome, a >700-nm-diameter particle and capsid-associated fibers (La Scola et al., 2003; Raoult et al., 2004). APMV prompted the creation of an open field of study on the function of hundreds of never-before-seen open reading frames (ORFs) and their roles in virus–host interactions (Raoult et al., 2004). Following the discovery of APMV, an increasing number of giant viruses were described (Boyer et al., 2009; Arslan et al., 2011; Desnues et al., 2012; Yoosuf et al., 2012; Philippe, 2013; Legendre et al., 2014), raising new questions about viral evolution, ecology and pathogenesis and bringing more researchers to this amazing virology field.

Acanthamoeba is believed to be the natural host of *Mimiviridae* (La Scola et al., 2003), although there is also evidence of mimivirus replication in vertebrate phagocytes (Ghigo et al., 2008; Silva et al., 2013) and detection of viral genomes in vertebrate samples (LaScola et al., 2005; Dornas et al., 2014). Therefore, the cultivation of APMV and other giant viruses in the laboratory is usually performed in amoebae of the *Acanthamoeba* genus (La Scola et al., 2003). Mimiviruses, similar to other large DNA viruses, replicate in the cytoplasm of amoebae (Mutsafi et al., 2013), producing large amounts of particles a few hours after infection. Viral morphogenesis takes place in "volcano-like" viral factories, which contain most of the elements necessary for the assembly of the structurally complex viral particles (Mutsafi et al., 2010). A few hours after the formation of virions, it is possible to visualize the lysis of amoebae cells. Although the scientific community has experienced a remarkable advancement in the comprehension of mimivirus replication cycles in the last years (Mutsafi et al., 2013; Kuznetsov et al., 2014), few studies have been devoted to the investigation of methodological features and conditions for optimal mimivirus cultivation.

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In this work, these conditions were investigated to obtain relevant information concerning the production of infectious particles, total particles and viral DNA. Besides methodological information, the data presented here also reveal, for the first time, the ratio between the total and infectious particles (in $TCID_{50}$) that are produced during mimivirus cultivation under laboratory conditions. All of this information can be used as a guide for the production of mimiviruses and can help prompt mimivirological studies in different fields.

2. Materials and methods

2.1. Viral stock preparation

Five viral isolates were used in this study: APMV, which is the mimivirus prototype that was isolated from a hospital water-cooling system in Bradford, England (La Scola et al., 2003; Raoult et al., 2004), and four mimivirus isolates that were obtained from rivers and lakes in Brazil: amazonia, niemeyer, samba and kroon (unpublished data). The viruses were initially grown by amoebae infection as previously described (La Scola et al., 2003; Campos et al., 2012) at a multiplicity of infection (MOI) of 0.1. Briefly, *Acanthamoeba castellanii* ATCC 30234 were grown in 75-cm^2 cell culture flasks (Nunc, US) in PYG medium supplemented with 7% fetal calf serum (FCS, Cultilab, Brazil), 25 mg/mL fungizone (amphotericin B, Cristalia, São Paulo, Brazil), 500 U/mL penicillin and 50 mg/mL gentamicin (Schering-Plough, Brazil). After reaching confluence, the amoebae were infected with each virus isolate and incubated at 32 °C until the appearance of a cytopathic effect. Mimivirus-rich supernatants from the infected amoeba were collected, aliquoted and stored at −80 °C. To determine the virus titer, the viruses were serially diluted, and multiple replicate samples of each dilution were inoculated into *A. castellanii* ATCC 30234 monolayers. After 72–96 h of incubation, the amoebae were analyzed to determine whether infection had taken place. Based on these data, the virus titers were ascertained by determining the precise dilution required for infection of 50% of the wells using the Reed–Muench (1938) method. These titer values were used to calculate the MOI, in $TCID_{50}$ per cell, of the experiments described below.

2.2. Analysis of viral production: Amoeba infection at different MOIs ($TCID_{50}$ per cell)

Before viral production at different MOIs was evaluated, the growth of APMV was evaluated in *A. castellanii* cultivated in PYG medium or Page's Amoeba Saline (PAS) solution to select an optimal amoeba medium/solution for mimivirus growth. Therefore, APMV one-step-growth curves were constructed for flasks (25 cm^2) of amoebae at a MOI of 10 $TCID_{50}$/per cell in both conditions (Condit, 2007). Sixty minutes after infection, the inoculum was removed from the amoebae, and the infected amoebae were harvested after 0, 2, 4, 8, 12 and 24 h, frozen, thawed and then titered as described. Because this assay revealed that APMV growth yielded approximately 6 logarithmical units of amoebae cultivated in PYG medium (Fig. 1), all subsequent experiments were performed in amoebae grown in PYG.

To analyze viral production at different MOIs, 125 cm^2 flasks containing 20,000,000 fresh *A. castellanii* were infected with amazonia, niemeyer, samba, kroon and APMV at MOIs of 0.01, 0.1, 1 and 10 $TCID_{50}$ per cell, and the flasks were kept at 32 °C for 72 h. After this time, the cell lysates were collected and submitted to purification as previously described (La Scola et al., 2003-modified). Briefly, APMV-rich supernatants from the infected amoebae were filtered through a 1.2 μm filter to remove amoebal debris. The viruses were then ultracentrifuged in a sucrose cushion (24%), suspended in PBS and submitted to virus titration, total particle counting and DNA extraction/qPCR. To simulate the exact viral production procedure, the viral inoculum was not removed from the infected cells; therefore, all of the obtained data were presented as the sum of the initial inoculum plus the viral progeny. All assays described in this work were independently performed at least five times.

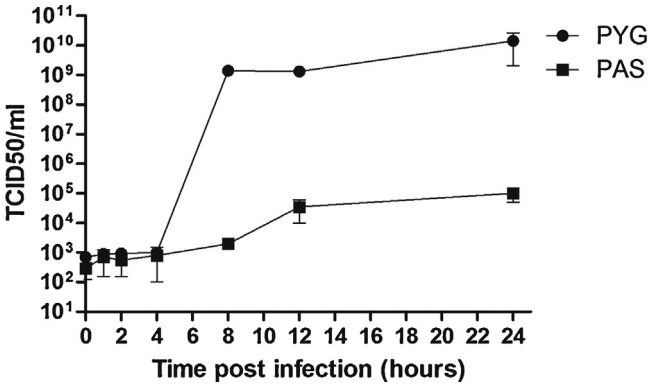

Fig. 1. *One-step growth curve of APMV in PYG and PAS solutions.* APMV growth was evaluated in *A. castellanii* cultivated in PYG medium or Page's Amoeba Saline (PAS) solution. APMV one-step growth curves were constructed for both conditions using flasks (25 cm^2) of amoebae at an MOI of 10 $TCID_{50}$/per cell. Sixty minutes after infection, the inoculum was removed from the amoebae, and the infected amoebae were harvested after different time points and titered.

2.3. Quantification of total mimivirus particles

Because mimiviruses can be visualized using optical microscopy, the quantification of total viral particles was performed by optical microscopy without staining. Purified viruses were diluted appropriately and inoculated (10 μL) on a thin layer of 1% agarose. After drying the virus drop, all viral particles within the dried drop borders were counted using an optical microscope (Olympus CK2, Japan) at a magnification of 1000X. Total viral particle quantification was performed by adjusting the total number of counted particles to the total volume of purified viruses.

2.4. DNA extraction and qPCR

To evaluate the production of viral genomic DNA (important for sequencing and other molecular procedures), purified viruses were submitted to DNA extraction. To eliminate amoebae or viral genome fragments outside capsids, 10 μL of each purified virus was treated with DNase before extraction (New England Biolabs, UK). Then, the viral capsids were heated for 60 min at 70 °C and submitted to a phenol-chloroform-isoamyl alcohol (PCI) (25:24:1) DNA extraction protocol (Sambrook and Russell, 1989). The extracted DNA was resuspended in DNase-free water and quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA).

After DNA quantification, the genomic viral copies were also estimated using real-time PCR targeting the conserved mimivirus helicase gene (primers: 5′ACCTGATCCACATCCCATAACTAAA3′ and 5′GGCCTCATCAACAAATGGTTTCT3′). Real-time PCR was performed using the commercial Power SYBr Green mix (Applied Biosystems, USA), primers (4 mM each) and 1 μL of sample in a 10 μL final volume. All reactions were performed using a Step One thermocycler with the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60° for 15 s, followed by a dissociation step (specific Tm = 73 °C). The quantification of genomic units was performed based on a standard curve of the helicase gene (pGEM-T, Promega, USA). Fifty nanograms of *A. castellanii* DNA was previously spiked into all samples at the PCI extraction step and used as a