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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.

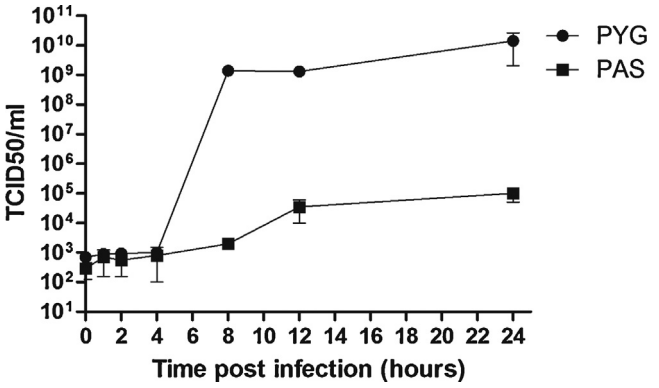

Fig. 1. *One-step growth curve of APMV in PYG and PAS solutions.* APMV growth was evaluated in *A. castellanii* cultivated in PYG medium or Page's Amoeba Saline (PAS) solution. APMV one-step growth curves were constructed for both conditions using flasks (25 cm^2) of amoebae at an MOI of 10 $TCID_{50}$/per 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

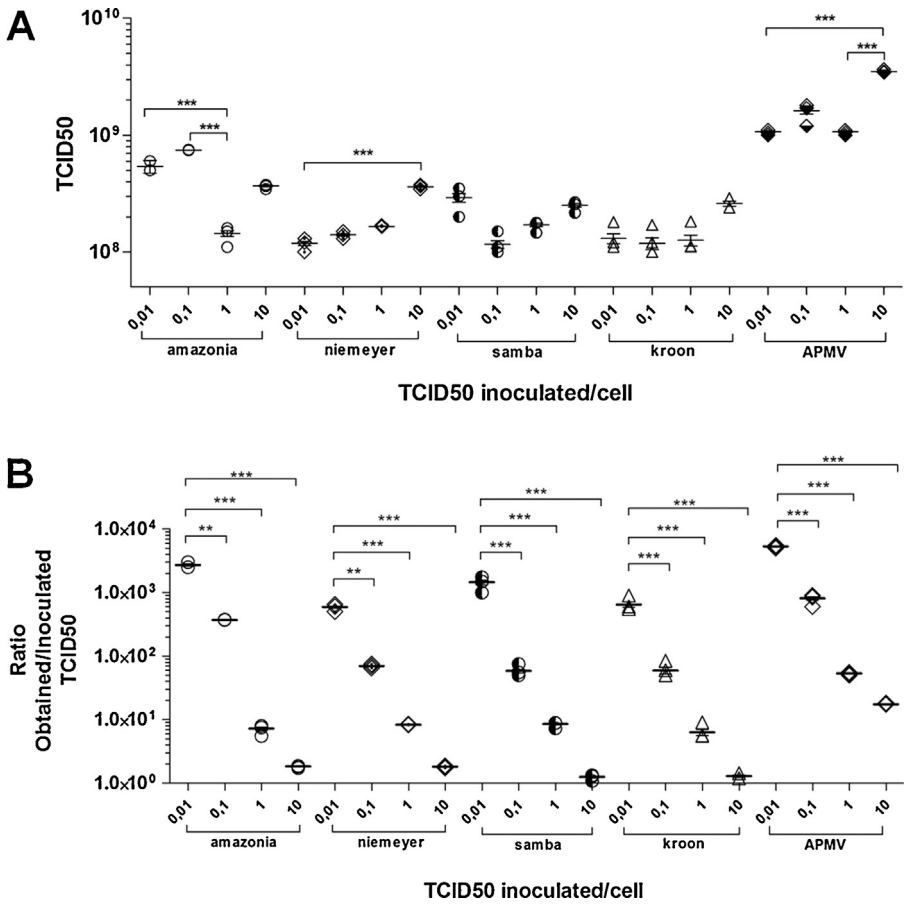

Fig. 2. (A) Infectious particle production of the mimivirus isolates at different MOIs. Flasks (125 cm^2) containing 20,000,000 fresh *A. castellanii* were infected with amazonia, niemeyer, samba, kroon and APMV isolates at MOIs of 0.01, 0.1, 1 and 10 ($TCID_{50}$ per cell). The flasks were kept at 32 °C for 72 h and then titered. (B) TCID50 obtained per TCID50 of inoculated mimivirus strains at different MOIs. The data obtained above were divided based on inoculum values to highlight the amount of produced $TCID_{50}$ at each MOI for each viral strain. ** or *** mean $p < 0.01$ and 0.001, respectively.

normalization control targeting amoebal 18S rDNA (primers: 5′TCCAAT TTTCTGCCACCGAA3′ and 5′ATCATTACCCTAGTCCTC-GCGC3′).

2.5. Statistical analysis

Statistical analyses were performed using the Minitab14 and GraphPad Prism software. The significance analysis was performed by comparing the average of the obtained results within a given virus isolate (all MOIs). The values were submitted in different combinations to one-way ANOVA tests and Bonferroni post-tests (95% confidence intervals). Differences between groups were considered significant when the p-values were smaller than 0.05.

3. Results

3.1. Infectious particle production at different MOIs

To evaluate the production of infectious mimivirus particles at different MOIs ($TCID_{50}$ per cell), two distinct analyses were performed: (I) quantification of infectious particles ($TCID_{50}$) after virus growth and purification and (II) calculation of the ratio between the "obtained $TCID_{50}$ per inoculated $TCID_{50}$" for each isolate and MOI. In general, the viruses produced similar amounts of infectious particles (between 10^8 and 10^9 $TCID_{50}$), except for APMV, whose titers reached averages higher than 10^{10} $TCID_{50}$ (Fig. 2A). When analyzing groups within each virus isolate, different MOIs produced similar $TCID_{50}$ values. Significant differences could only be observed between some MOIs of amazonia, niemeyer and APMV (Fig. 2A). However, calculation of the ratios between the obtained $TCID_{50}$ per inoculated $TCID_{50}$ revealed that low MOIs produced higher proportions of infectious particles than high MOIs. At an MOI of 0.01, amazonia, niemeyer, samba, kroon and APMV produced ratios of approximately 3×10^3, 5×10^2, 10^3, 5.5×10^2 and 5×10^3, respectively (Fig. 2B). In contrast, at high MOIs, these ratios did not reach 10^2 $TCID_{50}$. Statistical analysis demonstrated that an MOI of 0.01 was significantly more productive than any other MOI, regardless of the viral isolate (Fig. 2B). Additionally, if the inoculum was mathematically removed from the infectious particle results, an MOI of 0.01 was more productive than higher MOIs.

3.2. Total particle production

In contrast to the infectious particle results, total viral particle counting revealed a higher level of variability among MOIs for each isolate (Fig. 3). The statistical comparison between an MOI of 0.01 and the other MOIs revealed a significant difference for all analyzed viruses. However, although these values were not linearly proportional, it was possible to observe a direct relationship between MOI and total particles (Fig. 3). Most of the MOIs of amazonia, niemeyer and APMV showed total particle counting values between 10^{10} and 10^{11}, while the other isolates showed particle counting values between 10^9 and 3×10^{10} (Fig. 3). However, it is important to highlight that if the inoculum was mathematically removed from the total particle values, it was possible to observe similar amounts of total particles among all analyzed MOIs ($p > 0.01$).

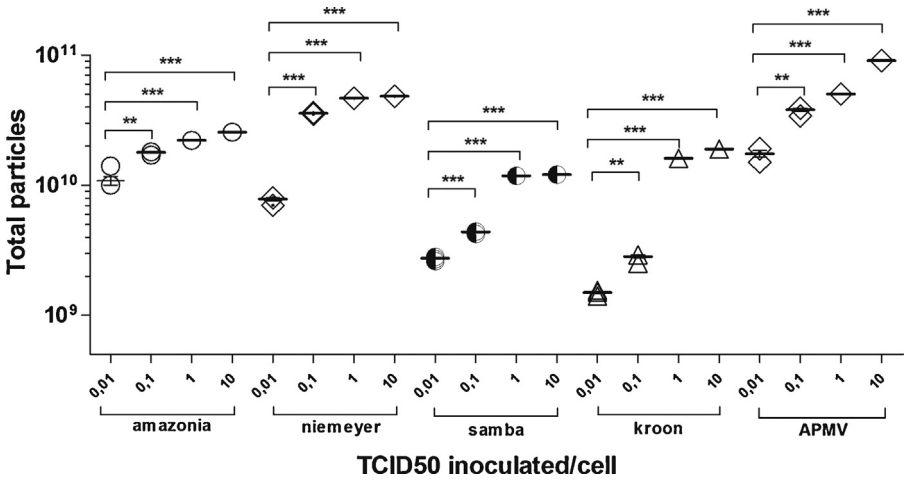

Fig. 3. Total viral particle production of the mimivirus isolates at different MOIs. Purified viruses were inoculated (10 μL) on a thin layer of 1% agarose. After drying of the virus drop, all virus particles within the dried drop borders were counted using an optical microscope (Olympus CK2, Japan) at a magnification of 1000×. Total viral particle quantification was performed by counting the total number of particles and adjusting that value to the total volume of purified viruses. ** or *** mean $p < 0.01$ and 0.001, respectively.

Table 1
Ratio between total particles/obtained TCID50—mean values (standard deviation).

	M.O.I. (TCID50 inoculated/cell)			
Isolate	0.01	0.1	1	10
Amazonia	20.27 (3.25)	23.99 (0.59)	155.50 (15.19)*	169.56 (2.57)
Niemeyer	66.64 (3.27)	254.81 (13.75)*	281.52 (13.64)	232.47 (12.12)
Samba	9.80 (2.40)	38.30 (5.45)*	69.65 (6.25)*	48.50 (14.80)
Kroon	11.81 (2.70)	24.85 (4.89)*	132.21 (14.74)*	123.61 (7.49)
APMV	16.33 (2.53)	24.01 (4.90)*	46.80 (2.02)*	39.93 (4.25)
Average	24.97	73.19*	153.0*	122.81

* Statistically significant in comparison to immediately lower viral concentration

In light of the infectious particle production results, these data may be used to determine the proportion of defective mimivirus particles that are produced during the viral replication cycle in the different tested conditions. Because the mimiviruses were titered using the TCID method, we determined this proportion by calculating the ratio between the total particles and obtained $TCID_{50}$. Table 1 shows that an MOI of 0.01 had the lowest value of the total particles per $TCID_{50}$, regardless of the viral isolate, and ranged from 9.80 to 66.64 total particles per each obtained $TCID_{50}$. Considering the MOIs of 0.01, 0.1 and 1, a non-linear relationship between MOI and defective particles was observed (Table 1). Interestingly, considerable differences could be observed in the defective particle ratios of the different isolates.

3.3. Quantification of viral genome production

Viral genome production was quantified using two different methods: (I) DNA extraction and spectrophotometry quantification, which revealed the amount of extracted DNA in micrograms and (II) real-time PCR targeting the viral helicase gene, which permitted the quantification of viral genome copies. Among the evaluated parameters, the measurement of viral genome production by spectrophotometry showed more variable data, both within and among isolate groups (Fig. 4). However, despite this variation, the genomic quantification data followed a similar profile to that observed for total particle analysis (Fig. 4). At an MOI of 0.01, the DNA recovery ranged from 1.6 μg (niemeyer) to 3.2 μg (APMV) per flask. At MOIs of 0.1, 1 and 10, the average DNA recoveries were 4.59, 9.23 and 10.5 μg per flask, respectively (Fig. 4). qPCR quantification also showed a non-linear relationship between MOI and genomic copies (Fig. 5). Considering MOIs of 0.01, 0.1, 1 and 10, the total averages (considering all isolates) of viral genome copies per flask were approximately 8.9×10^9, 2.1×10^{10}, 3×10^{10} and 4×10^{10}, respectively (Fig. 5). However, similar to the total particle assays, if the inoculum was mathematically removed from the presented viral DNA data, similar amounts of viral DNA (spectrophotometry and helicase copies) at all analyzed MOIs was observed ($p > 0.08$).

4. Discussion

Mimivirology is an open field for basic and applied studies. Since their discovery (La Scola et al., 2003), giant viruses have drawn the attention of virologists, evolutionists, clinicians and others (Boyer et al., 2009; Arslan et al., 2011; Desnues et al., 2012; Yoosuf et al., 2012; Philippe, 2013; Legendre et al., 2014). To improve research involving the characterization of these viruses as well as elucidate their clinical and environmental significance, it is necessary, for example, to test and determine the best conditions for viral isolation and production. Analyzing growth conditions, obtaining purified virus at high titers as well as searching for fast, efficient and cheap viral production strategies are strategic points in the scientific routine that are directly linked to the biology of viruses (Mutsafi et al., 2013; Kuznetsov et al., 2014) but require a standardization process. In this context, the study of the biology of viruses is an ally to the choice of the best method for viral infection and growth with the potential to reduce cost and effort.

The definition of MOI for virus cultivation is useful for several methodological procedures, such as the production of viral stocks (virological assays), viral antigens (serological purposes), genomic DNA purification (for sequencing), and other applications (Condit, 2007). The cost of viral production also includes the input of

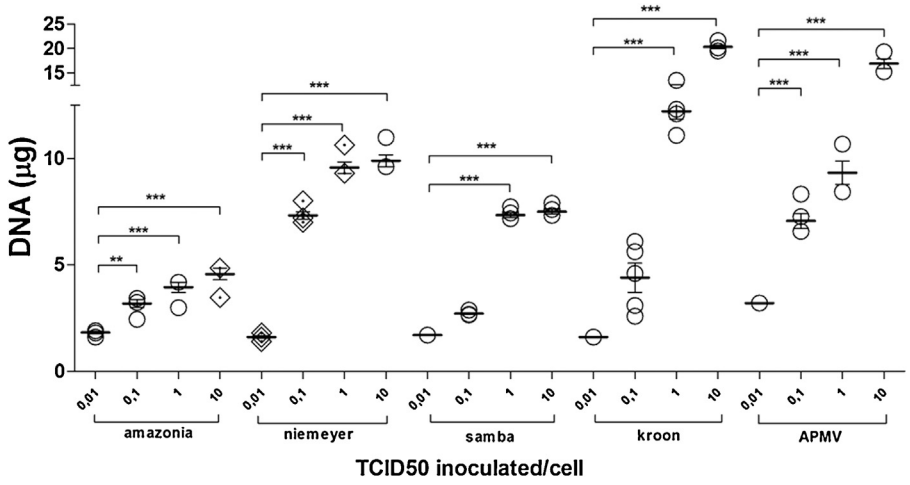

Fig. 4. DNA recovery after the production of mimivirus strains at different MOIs. Purified viruses were submitted to DNA extraction, resuspended in DNase-free water and quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA). ** or *** mean $p < 0.01$ and 0.001, respectively.

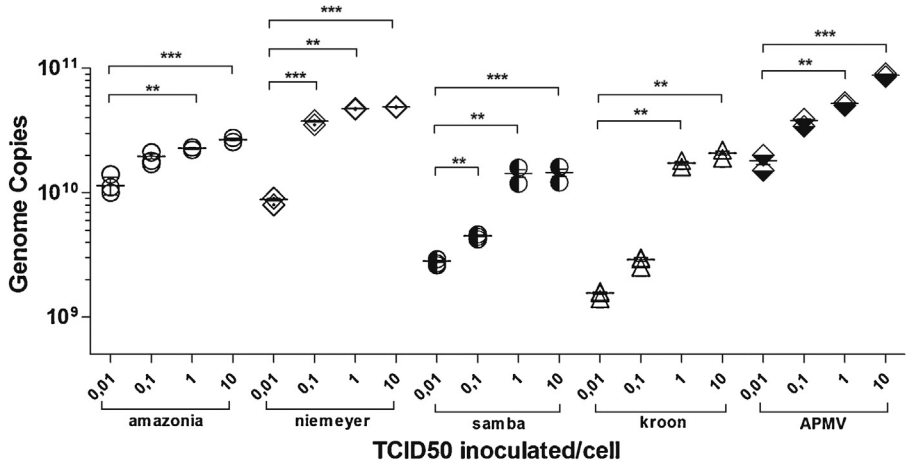

Fig. 5. Quantification of the genomes of the mimivirus isolates at different MOIs. The genomic units were quantified based on a standard curve of the helicase gene. Fifty nanograms of *A. castellanii* DNA was spiked into samples that were previously submitted to a PCI extraction step and used as a normalization control targeting amoebal 18S rDNA.

inoculum, which is indirectly related to the cost-benefit of production. Therefore, during a viral production process, the viral load that is obtained in the end as well as the initial viral input is important (Condit, 2007).

In this context, the presented data suggest that an MOI of 0.01 is the most useful for mimivirus growth. Although some isolates showed higher titers at high MOIs (Fig. 2A), the $TCID_{50}$ that was obtained per inoculated $TCID_{50}$ highlighted that more infectious particles were produced from a single particle at low MOIs (Fig. 2B). A possible explanation for this is that a low percent of cells are promptly infected at low MOIs; therefore, several cycles likely occur over time (Condit, 2007). In contrast, at high MOIs, most cells are concomitantly infected, resulting in one-step growth. Similar results were described for poxvirus production (Staib et al., 2004; Smallwood et al., 2010). MOIs lower than 0.01 were also tested, but the results showed lower virus production values, likely due to an unfavorable balance between viral input and assay time/dynamics (data not shown). This theory is reinforced if the innoculum is mathematically removed.

Regarding the production of genomic DNA, which can be important for sequencing, the results indicated a high recovery of viral DNA at higher MOIs (Fig. 5), although qPCR quantification did not show a linear relationship (Fig. 5). Because high MOIs may be related to a higher rate of defective particle formation (including incomplete genomes and/or particles) (Condit, 2007), one might speculate that genomic mutations may be increased in the pool of genome units that are obtained from this growth condition. Although this could be an issue when obtaining genome information, we believe that next-generation sequencing platforms might bypass this problem during contig assembly because mutations likely follow a random pattern (Mundry et al., 2012). Nevertheless, new studies are necessary to clarify these questions for the production of mimiviruses as well as other viruses. To date, most of the sequenced mimivirus genomes that were obtained in the last decade usually required approximately twenty 125-cm^2 flasks for completion (Raoult et al., 2004).

Taken together, the presented data provide, for the first time, information on the production of five mimivirus isolates at distinct MOIs and suggests that low MOIs are efficient for infectious particle production. As mentioned, *Acathamoeba* is the best-known model for mimivirus growth in the laboratory. However, we believe that future studies might expand the viral host range and possibly introduce new elements into viral production.

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