Vaccine 37 (2019) 6962–6969

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Defining the multiplicity and time of infection for the production of Zaire Ebola virus-like particles in the insect cell-baculovirus expression system

Ana Ruth Pastor [a,1], Gonzalo González-Domínguez [a,b,1], Marco A. Díaz-Salinas [c], Octavio T. Ramírez [a], Laura A. Palomares [a,*]

[a] Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Ave. Universidad 2001, Cuernavaca, Morelos 62210, Mexico
[b] Facultad de Farmacia, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, Mexico
[c] Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Ave. Universidad 2001, Cuernavaca, Morelos 62210, Mexico

ARTICLE INFO

Article history:
Available online 28 June 2019

Keywords:
Ebola virus disease
Virus-like particles
ZEBOV-VLP
Design of experiments

ABSTRACT

The Ebola virus disease is a public health challenge. To date, the only available treatments are medical support or the emergency administration of experimental drugs. The absence of licensed vaccines against Ebola virus impedes the prevention of infection. Vaccines based on recombinant virus-like particles (VLP) are a promising alternative. The Zaire Ebola virus serotype (ZEBOV) is the most aggressive with the highest mortality rates. Production of ZEBOV-VLP has been accomplished in mammalian and insect cells by the recombinant coexpression of three structural proteins, the glycoprotein (GP), the matrix structural protein VP40, and the nucleocapsid protein (NP). However, specific conditions to manipulate protein concentrations and improve assembly into VLP have not been determined to date. Here, we used a design of experiments (DoE) approach to determine the best MOI and TOI for three recombinant baculoviruses: bac-GP, bac-VP40 and bac-NP, each coding for one of the main structural proteins of ZEBOV. We identified two conditions where the simultaneous expression of the three recombinant proteins was observed. Interestingly, a temporal and stoichiometric interplay between the three structural proteins was observed. VP40 was required for the correct assembly of ZEBOV-VLP. High NP concentrations reduced the accumulation of GP, which has been reported to be necessary for inducing a protective immune response. Electron microscopy showed that the ZEBOV-VLP produced were morphologically similar to the native virus micrographs previously reported in the literature. A strategy for producing ZEBOV in insect cells, which consists in using a high MOI of bac-VP40 and bac-GP, and reducing expression of NP, either by delaying infection or reducing the MOI of bac-NP, was the most adequate for the production of VLP.

1. Introduction

The Ebola virus disease continues to be a challenge to human health. In particular, the Zaire Ebola virus (ZEBOV) is the most aggressive serotype and has the highest mortality rates. Clinical trials have shown the efficacy of several vaccines, among them a vectored vaccine based on chimpanzee adenovirus [1] and a vesicular stomatitis virus (VSV) chimeric vaccine [2]. These vaccines are very promising. However, both vaccines are live virus vaccines that may be safe when administered to healthy individuals, but that may pose risks to immunodeficient patients, malnourished children and other fragile populations. Therefore, the development of a recombinant, protein-based vaccine with very high purity and low adverse effects is desirable. A vaccine with such characteristics would be used not for emergency situations, but to provide long-lasting protection before an outbreak starts. An alternative that has shown to be safe and efficacious is the use of virus-like

Abbreviations: DoE, design of experiments; GP, ZEBOV glycoprotein; hpi, hours post first infection; MOI, multiplicity of infection; NP, ZEBOV nucleoprotein; pfu, plaque forming units; TEM, transmission electron microscopy; TOI, time of infection; VLP, virus-like particles; ZEBOV, Zaire serotype Ebola virus.

* Corresponding author.
E-mail addresses: rpastor@ibt.unam.mx (A.R. Pastor), gonzalo030793@gmail.com (G. González-Domínguez), marcoadiazs@gmail.com (M.A. Díaz-Salinas), tonatiuh@ibt.unam.mx (O.T. Ramírez), laura@ibt.unam.mx (L.A. Palomares).
[1] Both authors contributed equally.

https://doi.org/10.1016/j.vaccine.2019.06.029
0264-410X/

particles (VLPs), which are obtained when the structural proteins of a virus are recombinantly expressed and assembled into structures similar to the native virus [3,4].

Ebola virus is an enveloped filamentous negative-stranded RNA virus formed by seven structural proteins [5]. The three most abundant structural proteins are VP40, the Ebola virus glycoprotein GP, and the nucleoprotein (NP); with a content of each protein in the capsid of approximately 37.7%, 4.7% and 17%, for VP40, GP and NP, respectively. VP40, the most abundant viral protein, has a molecular weight of 36 kDa. It promotes assembly of the viral capsid by interacting with host proteins, and its recombinant expression results in the formation of filamentous particles [6]. VP40 interacts with the cell membrane to promote virus release from the host cell. Coexpression of VP40 with GP results in the formation of filamentous enveloped structures with GP incorporated into them, and a morphology similar to that of the native virus [7]. These particles induce higher humoral and cellular protective responses than particles formed only by VP40, highlighting the importance of GP for a potential vaccine [8]. In the native virus, NP forms the viral nucleocapsid that protects genomic RNA. NP assembles into helical tubular structures covered by the envelope that contains VP40 and GP [9]. It has been shown that coexpression of GP or NP with VP40 increases budding of VLP by 40% [10]. Interestingly, the composition of the ZEBOV VLP not only determines their immunogenicity but also their productivity by promoting budding.

Based on information from previous reports, we decided that an Ebola VLP vaccine candidate should include NP, GP, and VP40. NP will increase VLP budding and provide stability, GP will induce neutralizing humoral and cellular responses, and VP40 will form the VLP as the main structural protein. The insect cell-baculovirus expression system was selected to produce ZEBOV VLP because of its high productivity, versatility and the efficient coexpression of several proteins, which has been previously reported. These characteristics make the baculovirus expression system particularly suitable for the production of complex VLPs [3,4,11]. ZEBOV VLPs produced by insect cells have been shown to be as immunogenic as VLPs produced by mammalian cells [12,13,14]. However, a potential disadvantage of the baculovirus expression system is the need to generate large volumes of baculovirus stocks for large scale manufacturing, which can increase the appearance of defective baculovirus particles upon repeated passaging [11]. Low multiplicities of infection (MOI) would then be preferred for recombinant protein production, but the use of low MOI is challenging for the production of VLPs formed by several proteins, as only a fraction of the population is infected upon virus addition, and secondary infection is needed for the infection of all cells in the culture [11,15–17].

It has been documented that the efficient production of complex VLPs requires a fine-tuning of the concentration of each structural protein, to maximize VLP assembly and reduce waste [3,4,15,16]. Previous studies of Ebola VLP production have not considered the effect of manipulating the concentration of each structural protein on VLP composition and yield. Here, we utilized a multifactorial experimental design to determine the impact of multiplicity (MOI) and time (TOI) of infection of three baculoviruses coding for the three ZEBOV structural proteins, NP, GP and VP40, on cell growth kinetics and the production of each protein. Previously, it has been demonstrated that manipulation of MOI and TOI is a powerful tool to improve protein assembly and production of VLP [15–17]. The use of a design of experiments (DoE) allowed us to evaluate a wide range of conditions, and to statistically determine the relevance of both factors on production of each VLP component, in contrast to previous qualitative studies. We identified the conditions that resulted in the extracellular presence of the three proteins. The formation of VLP at the identified conditions was evaluated, and produced VLP were characterized. The results presented here are useful for the design of production strategies of Ebola VLP.

2. Materials and methods

2.1. Cell line and culture medium

Sf9 (*Spodoptera frugiperda*) insect cells were routinely subcultured to 0.6×10^6 cells/mL every 3–4 days in 250 mL shake flasks with 50 mL of working volume, maintained at 27 °C and 110 rpm using serum-free *Protein Sciences Formulary Medium* (PSFM) (SAFC Biosciences™, Cat 67916 – 200L3216) for protein production, and *SF900II* (Gibco, Cat 10902-104) for baculovirus amplification and titration.

2.2. Baculovirus amplification and titration

Recombinant baculovirus containing: (i) *gp* gene (named bac-GP), (ii) *vp40* gene (bac-VP40) and (iii) *np* gene (bac-NP) of ZEBOV were designed by Dr. Carlos F. Arias and Dr. Susana López (IBt-UNAM), and constructed using the Bac to Bac system (Invitrogen). Baculovirus stocks were amplified in *Sf9* cells infected at 1.5×10^6 cells/mL at a MOI of 0.1 plaque forming units (pfu)/cell. When the cell viability reached 30–40%, cultures were harvested and centrifuged at 2000*g* for 10 min at 4 °C. The pellet was discarded and the supernatant was stored at 4 °C until further use. Baculovirus titers were determined using a cell viability MTT assay [18].

2.3. Design of experiments (DoE) and coinfected cultures

To identify the effects of MOI and TOI on recombinant ZEBOV protein production and VLP assembly, a multi factorial design was generated using the software Statgraphics Centurion XVI.II. Two levels of MOI, 0.1 and 5 pfu/cell, and of time of infection (TOI), simultaneous infection or consecutive infection (addition of one baculovirus at 6 h post the first infection (hpi)), were set as factors, resulting in 32 unique experiments listed in Table 1. Responses were cell growth and presence of each recombinant protein. Results were analyzed by analyses of variance (ANOVA), with a significance of $p < 0.05$.

For infection, *Sf9* cells were seeded at 1×10^6 cells/mL in 50 mL of PSFM using 250 mL shake flasks and infected with the three baculoviruses when cultures reached 2×10^6 cells/mL, as described in Table 1. Samples from each experiment were collected every 12 h and centrifuged at 2000*g* for 10 min at 4 °C. Pellets were stored at −20 °C and supernatants at 4 °C for further analysis. Coinfected cultures were harvested when cell viability was between 60 and 50%. Additionally, cultures were infected individually with each baculovirus to generate material to be used as positive controls in blots (data not shown).

2.4. SDS-PAGE analysis and Western blotting

Total protein was quantified using the Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Cat 500-0006) in 96 well plates, using bovine serum albumin as standard. Aliquots containing 10 μg of total protein were diluted with reducing Laemmli sample buffer (10% w/v SDS; 5% w/v bromophenol blue, 40% v/v glycerol, 1% v/v β-mercaptoethanol, 200 mM Tris-HCl pH 6.8) and denatured by heating at 99 °C for 10 min. Proteins were separated under reducing conditions in 10% bis-acrylamide gels at 150 V for 55 min and visualized using Coomassie blue staining. VP40 and GP proteins were analyzed by Western blot, and NP was analyzed by Dot blot, as the antibody used did not recognize denatured protein. Proteins were transferred to PVDF membranes (Merck Millipore,

Table 1
Conditions and responses of the 32 experiments performed to evaluate the effect of MOI and TOI of bac-VP40, bac-GP and bac-NP on production of each protein. Delayed infection indicates what baculovirus was added at 6 hpi. A dash indicates that all baculoviruses were added at 0 hpi. Responses are shown in the last three columns, and refer to the presence of each protein in culture supernatants at 60 hpi. Relative scores denote the absence (0), low (1) or high (2) relative abundance of each protein in blots.

Experiment	MOI, pfu/cell			TOI, delayed infection	Responses, presence of each protein		
	bac-VP40	bac-GP	bac-NP		VP40	GP	NP
1	0.1	5	5	bac-VP40	0	0	1
2	5	5	0.1	bac-NP	0	1	0
3	5	0.1	5	–	2	0	1
4	5	5	5	–	0	2	2
5	0.1	0.1	5	bac-GP	0	0	2
6	5	0.1	5	bac-GP	1	0	2
7	0.1	0.1	5	bac-NP	0	0	1
8	0.1	0.1	0.1	bac-NP	0	0	0
9	0.1	0.1	0.1	bac-GP	1	0	2
10	5	0.1	0.1	–	2	0	0
11	5	0.1	5	bac-VP40	0	0	2
12	0.1	0.1	5	–	0	0	1
13	0.1	0.1	0.1	–	1	0	0
14	5	5	0.1	bac-VP40	0	2	0
15	5	0.1	5	bac-NP	2	0	1
16	5	5	0.1	–	1	2	1
17	5	5	5	bac-NP	2	2	1
18	0.1	5	5	–	0	0	1
19	0.1	5	0.1	bac-GP	0	2	2
20	5	0.1	0.1	bac-NP	1	0	0
21	5	0.1	0.1	bac-GP	2	0	2
22	5	5	5	bac-VP40	0	0	1
23	5	5	0.1	bac-GP	2	0	1
24	0.1	5	5	bac-GP	0	0	0
25	0.1	5	0.1	–	0	2	0
26	0.1	0.1	0.1	bac-VP40	0	0	0
27	0.1	5	0.1	bac-VP40	0	1	0
28	0.1	5	5	bac-NP	0	2	0
29	5	5	5	bac-GP	2	0	2
30	5	0.1	0.1	bac-VP40	0	0	0
31	0.1	5	0.1	bac-NP	0	2	0
32	0.1	0.1	5	bac-VP40	0	0	2

Cat # IPVH304F0). All membranes were temporarily stained with Ponceau red to confirm that the same amount of protein was loaded into each lane. For protein detection, three polyclonal antibodies were used: (i) Rabbit anti-EBOV GP (IBT Bioservices, Cat # 0301-015), (ii) Rabbit anti-EBOV NP (IBT Bioservices, Cat # 0301-012), (iii) Rabbit anti-ZEBOV VP40 (Gentaur, Cat # 0301-010). As a secondary antibody, an anti-rabbit IgG conjugated with horseradish peroxidase was used at a 1:5000 dilution (Santa Cruz Biotechnology, Cat # sc-2004). Protein detection was performed with a chemiluminescence detection system (Millipore Luminata Forte, Cat WBLUF0500) using a ChemiDoc (BioRad) image acquisition system. Negative controls (infected insect cells with an irrelevant baculovirus) were included in all blots to discard the presence of unspecific detection by antibodies. The same amount of standardized positive controls samples was included in each gel, to allow comparison between blots (see supplementary information). The standardized positive controls were recombinant VP40, GP and NP with His-tags produced in *E. coli* and purified. Purified proteins were generously provided by Alejandro Olvera (IBt-UNAM). The relative abundance of each protein was scored as follows: absent was given a value of 0, present at a low relative concentration was given a value of 1, and a strong signal was given a value of 2. Scores at 48 hpi or 60 hpi, for low MOI cultures, were used for the statistical analysis of the multifactorial matrix (Table 1).

2.5. VLP purification by discontinuous density gradient ultracentrifugation

Some experiments were repeated at a larger scale (500 mL shake flasks with 100 mL of working volume) to purify VLP. Cultures were harvested at 48 hpi, and VLP were recovered by density gradients. Briefly, each culture was centrifuged at 2000*g* for 10 min at 4 °C. Pellets were discarded and supernatants were concentrated 10× by ultrafiltration using a SpinX-UF concentrator 30 KDa (Corning, Cat # 431489). Discontinuous iodixanol (Sigma-Aldrich, Cat # D1556) 5–30% (w/v) density gradients were used. Visible bands were collected. Recovered bands were concentrated by ultrafiltration to a protein concentration of 0.5 μg/μL and analyzed by immunoblotting, as described previously. The density of collected VLP was estimated based on the iodixanol concentration in the fraction where VLP were recovered, as described in http://www.axis-shield-density-gradient-media.com/C49.pdf (accessed on Dec 2018). The presence of VP40, GP, and NP in each band was analyzed by SDS-PAGE followed by Western blot and Dot blot.

2.6. Characterization of VLP by transmission electron microscopy (TEM)

Negative staining TEM was used to analyze the shape and size of purified ZEBOV-VLP. Briefly, 10 μL of purified VLP samples were fixed for 2 min on copper grids coated with Formvar-carbon (Electron Microscopy Sciences, Cat CF200-Cu). Grids were washed with 10 μL filtered water and then stained with 1% (v/v) uranyl acetate for 2 min and washed with 10 μL of filtered water. Grids were left to air dry. Finally, samples were observed in a Zeiss Libra 120 transmission electron microscope at 70 kV (Unidad de Microscopía Electrónica, IBT-UNAM).

3. Results and discussion

3.1. Growth kinetics and accumulation of ZEBOV proteins in culture supernatants

Growth kinetics of 16 of the cultures described in Table 1 are shown in Fig. 1. As expected, cultures infected with a lower MOI resulted in longer culture times and also higher cell densities (Fig. 1A), whereas when cultures were infected with at least one baculovirus per cell at a high MOI, no significant growth was observed and culture time was shorter (Fig. 1B–D). In all cultures, cell viability remained high until 36 hpi, and later decreased until harvest. For comparison, kinetics of individually infected cultures can be found in the supplementary information. Immunoblot analyses of cell pellets and supernatants showed that recombinant proteins were mostly undetectable in cell pellets, and when detected in pellets, products of proteolysis were observed (Supplementary information). It is possible that intracellular proteins did not assemble into VLPs and thus, were unstable.

In contrast, the three structural ZEBOV proteins were observed in culture supernatants, depending on the infection conditions used (Table 1, Fig. 2). At high culture viability, proteins in the supernatant are expected to be assembled into VLPs, but they can also be found when they are integrated into the baculovirus envelope or in extracellular vesicles. Assembly probably protected proteins from proteolysis, as has been previously reported [3,9,19]. Protein profiles in culture supernatants observed by immunoblotting shows that depending on the coinfection conditions, a temporal and stoichiometric interplay between structural proteins existed. The results obtained are summarized in Table 1. Illustrative results, showing the temporal evolution of proteins in supernatants are shown in Fig. 2. In general, proteins were detected as early as 24 hpi. At low MOIs, the maximum protein concentration was reached around 60 hpi, while at high MOIs the maximum was detected at 48 hpi. Thereafter, the concentration of recombinant proteins remained relatively constant. Fig. 2A shows the results of cultures that were coinfected at low MOIs of the three baculoviruses, either simultaneously or delayed. In general, cultures infected at a low MOI resulted in small or undetectable amounts of the corresponding recombinant protein in the culture supernatants. In contrast, when a high MOI of 5 pfu/cell was used for all baculoviruses (experiments 4, 17, 22 and 29, Fig. 2B), higher concentrations of the recombinant proteins were observed. At a simultaneous MOI of 5 pfu/cell of each baculovirus, VP40 was not observed in the culture supernatant (experiment 4), but it was detected when infection with bac-NP was delayed (experiment 17). This suggests that overexpression of NP somehow inhibits the interaction of VP40 proteins with host proteins, a step necessary for assembly of this matrix protein [6]. It should be considered that previous reports show that a postponed infection does not result in the later expression of the gene coded by the delayed baculovirus, as at the time that the second baculovirus enters the cell, baculovirus protein expression has already been initiated by the initial infection [15,16,20]. Therefore, the expected effect is a reduced expression of the gene coded by the delayed baculovirus.

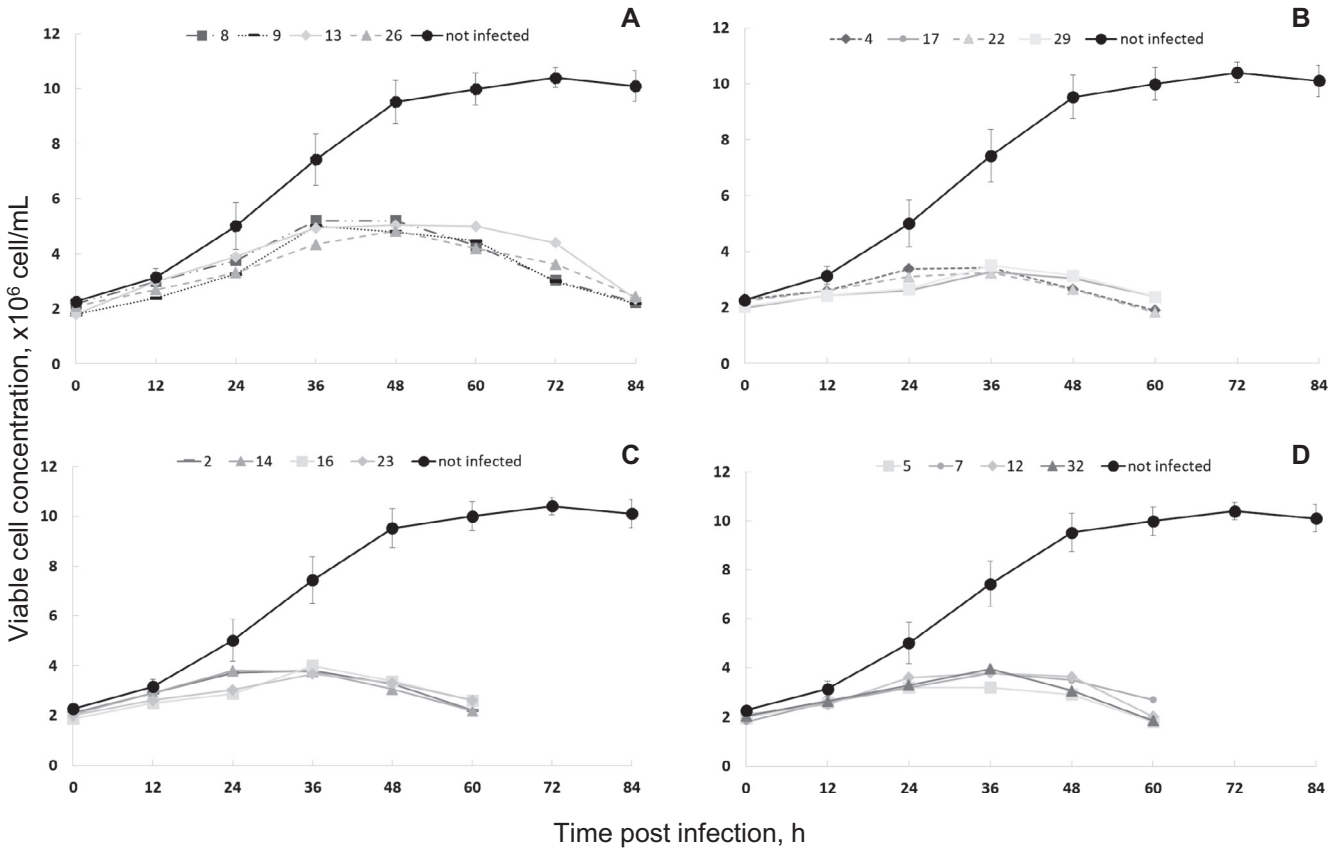

Fig. 1. Growth kinetics of *Sf*9 insect cell cultures co-infected with three recombinant baculoviruses: bac-VP40, bac-GP and bac-NP. Cultures were infected at 2×10^6 cel/mL using different MOI and TOI, as described in Table 1. A, Experiments 8, 9, 13 and 26 coinfected at low MOI; B, Experiments 4, 17, 22, and 29 coinfected at high MOI; C, Experiments 2, 14, 16 and 23 coinfected with two baculoviruses at high MOI and third one at low MOI; and D, Experiments 5, 7, 12 and 32 coinfected with one baculovirus at high MOI and two at low MOI. Numbers represent experiments described in the DoE (Table 1). As a control, *Sf*9 cells were seeded in the same condition as experimental shaker flasks without being infected. Error bars represent the standard deviation of 4 independent uninfected experiments.

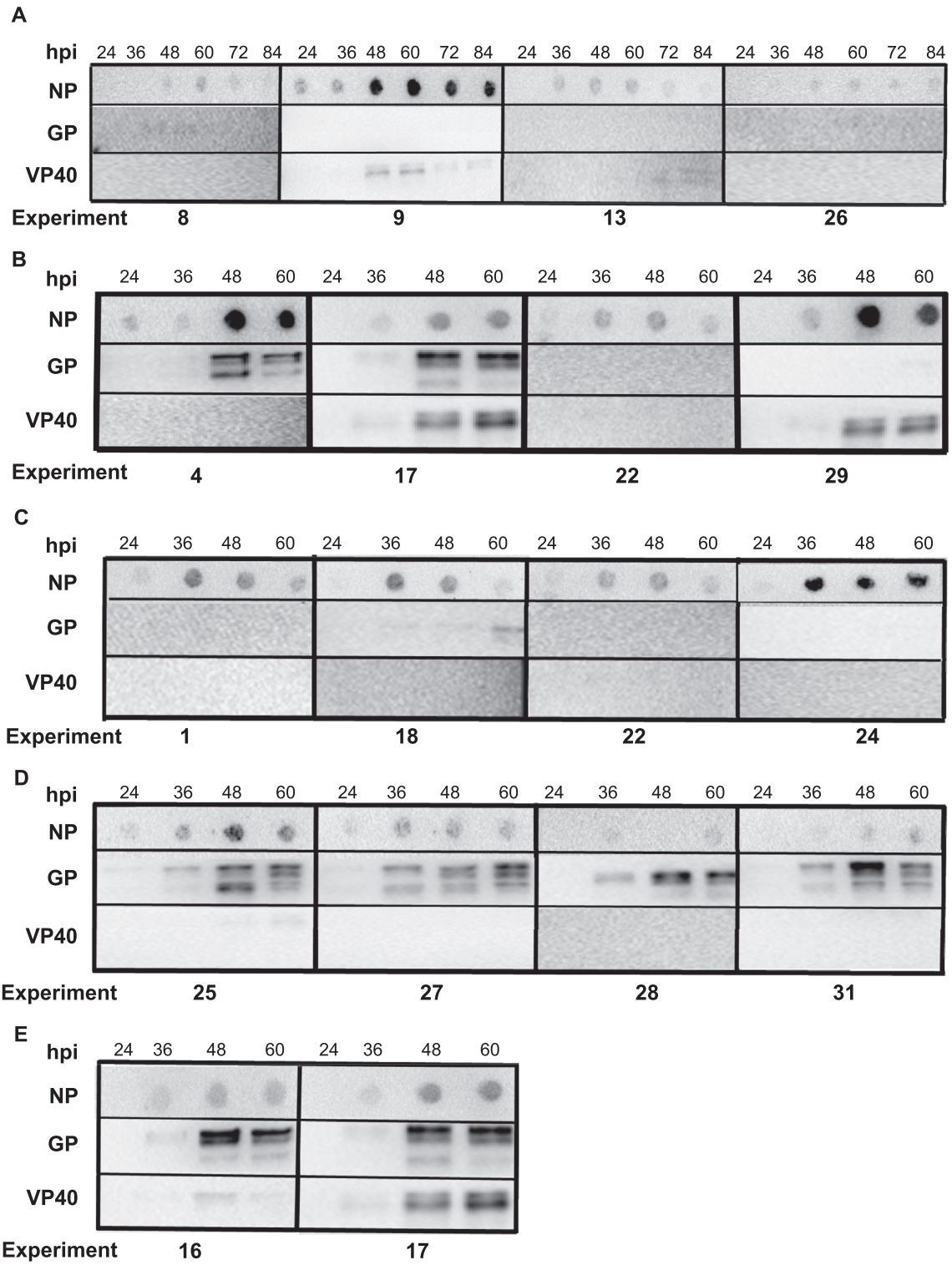

Fig. 2. Immunodetection of ZEBOV proteins at different time points in culture supernatants. Expression of GP and VP40 were analyzed by WB, while expression of NP was followed by dot blot. Samples were collected every 12 h starting at 24 hpi. A, experiments infected at low MOI (experiment 8, the three baculoviruses at MOI of 0.1 pfu/cell, delayed bac-NP; experiment 9, the three baculoviruses at MOI of 0.1 pfu/cell, delayed bac-GP; experiment 13, simultaneous infection with the three baculoviruses at MOI of 0.1 pfu/cell; experiment 26, the three baculoviruses at MOI of 0.1 pfu/cell, delayed bac-VP40). B, experiments infected at high MOI (experiment 4, simultaneous infection at MOI of 5 pfu/cell; experiment 17, the three baculoviruses at 5 pfu/cell, delayed bac-NP; experiment 22, the three baculoviruses at MOI of 5 pfu/cell, delayed bac-VP40; experiment 29, the three baculoviruses at MOI of 5 pfu/cell, delayed bac-GP). C, example of experiments where bac-NP was added at high MOI (experiment 1, bac-VP40 at 0.1 pfu/cell, bac-GP and bac-NP at 5 pfu/cell, delayed bac-VP40; experiment 18, simultaneous infection with bac-VP40 at 0.1 pfu/cell and bac-GP and bac-NP at 5 pfu/cell; experiment 22, the three baculoviruses at 5 pfu/cell, delayed bac-VP40; experiment 24, bac-VP40 at 0.1 pfu/cell, bac-GP and bac-NP at 5 pfu/cell, delayed bac-GP). D, experiments infected at MOI 5 of bac-GP and 0.1 pfu/cell of bac-VP40 (experiment 25, bac-VP40 and bac-NP at 0.1 pfu/cell, bac-GP at 5 pfu/cell; experiment 27, bac-VP40 and bac-NP at 0.1 pfu/cell, bac-GP at 5 pfu/cell, bac-VP40 delayed; experiment 28, bac-VP40 at 0.1 pfu/cell, bac-GP and bac-NP at 0.1 pfu/cell, delayed bac-NP; experiment 31, bac-VP40 and bac-NP at 0.1 pfu/cell, bac-GP at 5 pfu/cell, bac-NP delayed). E, Experiments 16 (bac-VP40 and bac-GP at 5 pfu/cell, bac-NP at 0.1 pfu/cell) and 17 (the three baculoviruses at 5 pfu/cell, delayed bac-NP).

Interestingly, also the accumulation of GP in the supernatant was reduced with NP overaccumulation. Delaying infection with a baculovirus decreased the concentration of the recombinant protein expressed by that particular virus, as has been previously observed [16]. Delaying infection with bac-VP40 reduced the accumulation of both VP40 and GP in the culture supernatant, confirming that VP40 is needed for the budding of GP during the formation of VLP (experiment 22). Remarkably, delaying infection with bac-GP resulted in a high concentration of VP40 in the culture supernatant (experiment 29, Fig. 2B), even when the concentration of NP was high. Our results contrast with the observations of Licata et al. [10], who observe a cooperative role of NP and GP in budding of VP40 in HEK 293T cells. It is possible that baculovirus infection and baculovirus proteins also interfere with ZEBOV VLP budding in the BEVS.

Comparison of some of the cultures infected with bac-NP at high MOIs (Fig. 2C) shows that overexpression of NP importantly diminished the presence of VP40 and GP in culture supernatants. It can be seen that a low MOI of bac-VP40 or delaying its addition resulted in undetectable concentrations of VP40. Interestingly, both VP40 and GP were observed in supernatants of cultures individually infected at 0.1 pfu/cell of either bac-VP40 or bac-GP, indicating that the presence of the other two proteins interfered with their expression or budding from cells (data not shown). In cultures infected at a MOI of bac-GP of 5 and of bac-VP40 of 0.1 pfu/cell (Fig. 2D), no presence of VP40 was observed in the supernatant, and no VLP formation is expected. Interestingly, in all cultures where NP was detected at high concentrations, a low concentration of either GP or VP40 was observed. Only in two conditions the three ZEBOV structural proteins were detected, experiments 16 and 17 (Fig. 2E). Both were infected with high MOIs of bac-VP40 and bac-GP, and expression of the NP gene was decreased either by a low MOI or by a delayed infection, demonstrating again that an interplay between the three proteins and the budding of ZEBOV VLPs to the supernatant exists.

3.2. Statistical analysis

Maximum cell concentrations were used as responses to determine the effect of MOI and TOI on cell growth. As expected, a high MOI of all three baculovirus significantly decreased cell growth, with p values of 0.0016, 0.0276 and 0.0001 for bac-VP40, bac-GP and bac-NP, respectively. TOI, which refers to either simultaneous or delayed infection, did not have a significant effect on cell growth. Scores of relative protein abundance at 60 hpi, shown in Table 1, were used to determine by ANOVA the effects of MOI and TOI on recombinant protein accumulation in culture supernatants. MOI had a significant impact on the production of each recombinant protein. For the accumulation of VP40 in the supernatant, the only significant factor was the MOI of bac-VP40, which had a positive effect, with a p-value of 0.0062. No significant interaction with the MOI of the other two baculoviruses for the accumulation of VP40 in the culture supernatant was observed. Similarly to VP40, the MOI of bac-GP had a positive effect on GP accumulation in the supernatant ($p = 0.0001$), but the MOI of bac-NP had a negative effect ($p = 0.0147$). Interaction of both factors was significant ($p = 0.0151$), with a negative effect on GP presence in the supernatant. How NP interacts with GP to reduce its concentration in the culture supernatant remains to be investigated. For NP, only the MOI of bac-NP had a statistically significant positive effect ($p = 0.0465$). However, although not significant, a positive interaction between the MOI of bac-VP40 and the MOIs of the three baculoviruses on the concentration of NP was observed. It should be considered that NP was quantified differently than the other proteins, possibly making the effect of this interaction non-significant due to experimental variation. Interestingly, according to the ANOVA results, TOI (delayed infection) did not have a significant effect on the concentration of recombinant proteins in the culture supernatant. The results from the statistical analysis coincide with the empirical observations of Western blots, that showed that for the presence of the three proteins in the culture supernatants, an indication of VLP formation, high MOIs of bac-VP40 and bac-GP and a low MOI of bac-NP should be used.

3.3. Characterization of ZEBOV-VLP

Conditions of experiments 16 and 17, where the three ZEBOV structural proteins were observed (Fig. 2E), were selected for the production and characterization of ZEBOV-VLP. 100 mL cultures were infected, and supernatants were recovered. Immunoblots of supernatants from cultures at both conditions show the presence of the three structural proteins, as observed in the DoE experiments (Fig. 3). VLPs were recovered by iodixanol gradients. Three bands were visible in gradients (data not shown). They were recovered and characterized. F1 was collected from the 6% w/v iodixanol zone (density 1.03 g/mL), F2 was collected from the 12% w/v iodixanol zone (density 1.06 g/mL), and F3 was collected from the 18% w/v iodixanol zone (density 1.09 g/mL). Under the conditions of experiment 16 (high MOIs of bac-VP40 and bac-GP and a low MOI for bac-NP), only F2 and F3 were observed. Experiment 16 had a lower VP40 content than experiment 17, probably explaining why F1 did not form. F2 and F3 contained a high amount of the three ZEBOV structural proteins (Fig. 3A), and their density was within the range reported by Johnson et al. [9] for Ebola virus-like particles containing the three structural proteins. A lower abundance of NP was observed in F3, in comparison with F2. For

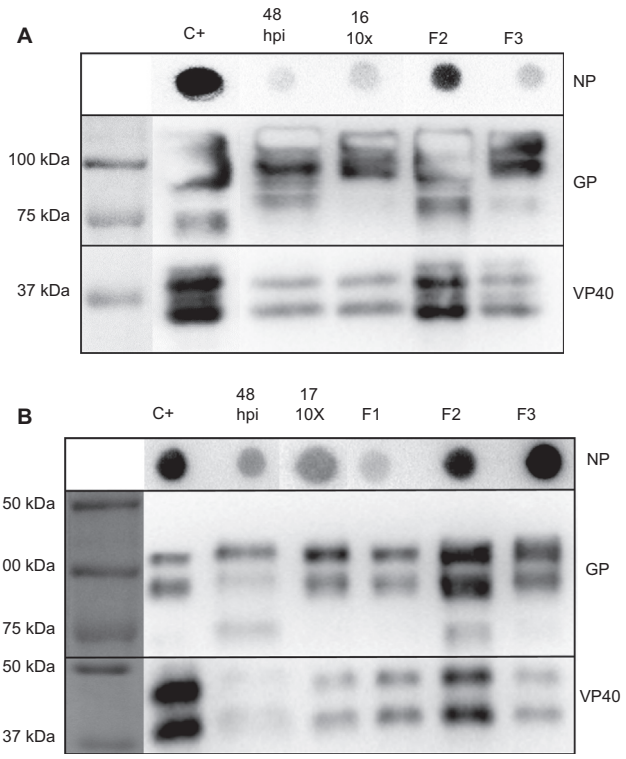

Fig. 3. Analysis of fractions obtained by density gradient ultracentrifugation. A, Experiment 16. B, Experiment 17. C+, positive control, *Sf9* cultures were infected individually with each baculovirus and used as positive controls for each recombinant protein; 48 hpi, supernatants at the time of harvest; supernatant concentrated 10x. F1, fraction 1 (6% w/v iodixanol), F2, fraction 2 (12% w/v iodixanol), F3, fraction 3 (18% v/v iodixanol).

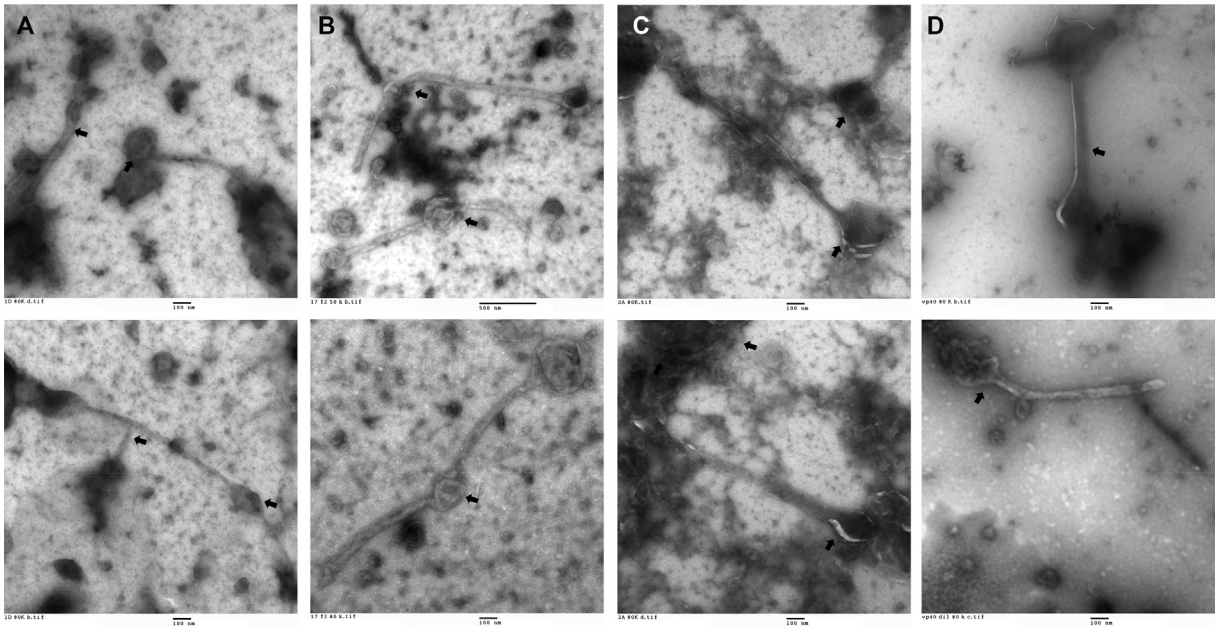

Fig. 4. Transmission electron microscopy of VLP recovered from ultracentrifuged iodixanol gradients. In all cases, materials from F2 are shown. A and B, Conditions of experiments 16 and 17, respectively. C, Conditions of experiment 29, in which GP was not detected; D, Culture infected only with bac-VP40. Images were obtained in a Zeiss Libra 120 transmission electron microscope at a magnification of 80,000× at 70 kV. Arrows point to VLPs.

experiment 17, the three bands were present. The three bands contained the three proteins, but NP was more abundant as the iodixanol concentration increased. A lower content of VP40 was observed in F3, in comparison with F1 and F2. For comparison, a culture with conditions of experiment 29, where GP was not observed, was also subjected to gradient ultracentrifugation. A similar protein profile as that obtained in smaller flasks was observed (data not shown), and F1, F2 and F3 were recovered. Also, a culture infected only with bac-VP40 was analyzed. A very faint band was obtained in F1, while abundant material was recovered in F2 and F3 (data not shown).

Material recovered from each band was analyzed by TEM. No distinguishable particles were observed in material from F1 or F3 of any of the experiments. It may appear surprising that VLPs were absent in these bands even when the structural ZEBOV proteins were present. This can be explained by the presence of the recombinant proteins in extracellular vesicles or extracellular debris, which had densities that could be recovered by gradients. Fig. 4A and B show micrographs of F2 obtained from experiments 16 and 17, respectively. Filamentous VLPs with differences in size, shape, and abundance were observed in these samples. Numerous assembled VLPs with an average length of 1230 ± 343 nm, and an average diameter of 46 ± 10.3 nm (Fig. 4A and B) were observed. These structures were similar in size and shape to those found in previous reports [14]. For experiment 29, where no GP was detected, similar filamentous particles were observed, with no difference in length or diameter to the particles obtained in experiments 16 and 17 (Fig. 4C). The culture expressing only VP40 also resulted in filamentous particles (Fig. 4D), confirming previous reports that this protein is the only one necessary for the formation of VLPs [10]. Further experiments are required to test the ability of these VLPs to trigger an immune response or to stimulate the neutralizing antibody response and to evaluate their potential as a vaccine.

4. Conclusions

Using a design of experiments approach, we investigated for the first time in the baculovirus expression system the effect of multiplicity and time of infection on cell growth and the extracellular accumulation of three ZEBOV structural proteins that form VLPs, VP40, GP, and NP. This approach allowed us to determine in a quantitative and statistically significant manner the effect of production conditions on the accumulation of ZEBOV structural proteins. The conditions that resulted in the extracellular accumulation of the three proteins also resulted in the presence of VLPs. It was found that high MOIs significantly ($p < 0.05$) decreased cell growth and increased recombinant protein concentrations, while the delayed addition of a baculovirus did not significantly affect recombinant protein concentrations, according to the ANOVA analysis. It should be noted that the empirical observation of the results of experiment 4, 17, 22 and 29 shows that delaying infection with bac-NP increased both the concentrations of GP40 and/or VP40, and that delaying infection with bac-GP or bac-VP40 also reduced the presence of both proteins.

Interestingly, a negative effect of the MOI of bac-NP that interacted with the MOI of bac-GP over GP extracellular accumulation was observed, indicating that for the presence of GP in ZEBOV VLPs, the concentration of NP should be low. ZEBOV VLPs with size and morphology similar to that previously reported was observed in the conditions that resulted in the extracellular presence of the three structural proteins. VLPs were also observed in conditions that did not result in the detection of the three structural proteins in the supernatant. Such incomplete VLP may have lower stability or immunogenicity than their complete counterparts. It was determined that either MOI or TOI are tools that can be used to manipulate the expression of ZEBOV recombinant proteins and the composition of VLPs. In particular, reduced expression of NP, either by using a low MOI or delaying infection with bac-NP, ensures that GP is present in VLPs. This is relevant as previous reports have shown that GP is required for inducing protective humoral and cellular responses towards ZEBOV infection.

Acknowledgements

This project was partially funded by CONACyT P-364. The funding agency had no involvement in the execution of the research or

preparation of this article. Technical assistance by Vanessa Hernández, Guadalupe Zavala, Martha Contreras, and the Unidad de Microscopía Electrónica IBT-UNAM is acknowledged. We thank Alejandro Olvera for providing the purified recombinant EBOV proteins that were used as standards.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.06.029.

References

[1] Ledgerwood JE, DeZure AD, Stanley DA, Coates EE, Novik L, Enama ME, et al. Chimpanzee adenovirus vector Ebola vaccine. N Engl J Med 2017;376:928–38.
[2] Regules JA, Beigel JH, Paolino KM, Voell J, Castellano AR, Hu Z, et al. A recombinant vesicular stomatitis virus Ebola vaccine. New Eng J Med 2017;376:330–41.
[3] Palomares LA. Ramírez OT Challenges for the production of virus-like particles in insect cells: the case of rotavirus-like particles. Biochem Eng J 2009;45:158–67.
[4] Fernandes F, Teixeira AP, Carinhas N, Carrondo MJT, Alves PM. Insect cells as a production platform of complex virus-like particles. Expert Rev Vaccines 2013;12:225–36.
[5] Jun SR, Leuze MR, Nookaew I, Uberbacher EC, Land M, Zhang Q, et al. *Ebolavirus* comparative genomics. FEMS Microbiol Rev 2015;39:764–78.
[6] Jasenosky LD, Neumann G, Lukashevich I, Kawaoka Y. Ebola virus VP40-induced particle formation and association with the lipid bilayer. J Virol 2001;75:5205–14.
[7] Noda T, Sagara H, Suzuki E, Takada A, Kida H, Kawaoka Y. Ebola virus VP40 drives the formation of virus-like filamentous particles along with GP. J Virol 2002;76:4855–65.
[8] Wahl-Jensen V, Kurz SK, Hazelton PR, Schnittler HJ, Ströher U, Burton DR, et al. Role of Ebola virus secreted glycoproteins and virus-like particles in activation of human macrophages. J Virol 2005;79:2413–9.
[9] Johnson RF, Bell P, Harty RN. Effect of Ebola virus glycoproteins GP, NP and VP35 on VP40 VLP morphology. Virol J 2006;3:31.
[10] Licata JM, Johnson RF, Han Z, Harty RN. Contribution of Ebola virus glycoprotein, nucleoprotein, and VP24 to budding of VP40 virus-like particles. J Virol 2004:7344–51.
[11] Palomares LA, Realpe M, Ramírez OT. An overview of cell culture engineering for the insect cell-baculovirus expression vector system (BEVS). In: Al-Rubeai M, editor. Cell Engineering. Vol. 9 Animal Cell Culture. Switzerland: Springer International; 2015. p. 501–19.
[12] Sun Y, Carrion R, Ye L, Wen Z, Ro YT, Brasky K, et al. Protection against lethal challenge by Ebola virus-like particles produced in insect cells. Virol 2009;383:12–21.
[13] Warfield KL, Posten NA, Swenson DL, Olinger GG, Esposito D, Gillette WK, et al. Filovirus-like particles produced in insect cells: Immunogenicity and protection in rodents. J Infect Dis 2007;196:S421–9.
[14] Ye L, Lin J, Sun Y, Bennouna S, Lo M, Wu Q, et al. Ebola virus-like particles produced in insect cells exhibit dendritic cell stimulating activity and induce neutralizing antibodies. Virol 2006;35:260–70.
[15] Aucoin MG, Perrier M. Kamen AA Production of adeno-associated viral vectors in insect cells using triple infection: optimization of baculovirus concentration ratios. Biotechnol Bioeng 2006;95:1081–92.
[16] Palomares LA, López S, Ramírez OT. Strategies for manipulating the relative concentration of recombinant rotavirus structural proteins produced by insect cells. Biotechnol Bioeng 2002;78:635–44.
[17] Tsao EI, Mason MR, Cacciuttolo MA, Bowen SH, Folena-Wasserman G. Production of parvovirus B19 vaccine in insect cells coinfected with double baculoviruses. Biotechnol Bioeng 1996;49:130–8.
[18] Mena JA, Ramírez OT, Palomares LA. Titration of non-occluded baculovirus using a cell viability assay. Biotechniques 2003;34:260–4.
[19] Martin B, Hoenen T, Canard B, Decroly E. Filovirus proteins for antiviral drug discovery: a structure/function of analysis of surface glycoproteins and virus entry. Antiviral Res 2016;135:1–14.
[20] Mena JA, Ramírez OT, Palomares LA. Population kinetics during simultaneous infection of insect cells with two different recombinant baculoviruses for the production of rotavirus-like particles. BMC Biotech 2007;7:39.