TITLE:

Pseudotyped viruses as a molecular tool to monitor humoral immune responses against SARS-CoV-2 via neutralization assay

Production and Characterization of SARS-CoV-2 Spike Pseudotyped Lentiviruses for Studying Host-Virus Interactions and Serum Neutralization

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SUMMARY:

Pseudotyped viruses (PVs) are replication-defective virions that are used to study host-virus interactions under safer conditions than handling authentic viruses. Presented here is a detailed protocol that shows how SARS-CoV-2 PVs can be used to test the neutralizing ability of patients' serum after COVID-19 vaccination.

ABSTRACT:

Pseudotyped viruses (PVs) are molecular tools that can be used to study host-virus interactions and to test the neutralizing ability of serum samples, in addition to their better-known use in gene therapy for the delivery of a gene of interest. PVs are replication defective because the viral genome is divided into different plasmids that are not incorporated into the PVs. This safe and versatile system allows the use of PVs in biosafety level 2 laboratories. Here, we present a general methodology to produce lentiviral PVs based on three plasmids as mentioned here: (1) the backbone plasmid carrying the reporter gene needed to monitor the infection; (2) the packaging plasmid carrying the genes for all the structural proteins needed to generate the PVs; (3) the envelope surface glycoprotein expression plasmid that determines virus tropism and mediates viral entry into the host cell. In this work, SARS-CoV-2 Spike is the envelope glycoprotein used for the production of non-replicative SARS-CoV-2 pseudotyped lentiviruses.
Briefly, packaging cells (HEK293T) were co-transfected with the three different plasmids using standard methods. After 48 h, the supernatant containing the PVs was harvested, filtered, and stored at -80 °C. The infectivity of SARS-CoV-2 PVs was tested by studying the expression of the reporter gene (luciferase) in a target cell line 48 h after infection. The higher the value for relative luminescence units (RLUs), the higher the infection/transduction rate. Furthermore, the infectious PVs were added to the serially diluted serum samples to study the neutralization process of pseudoviruses' entry into target cells, measured as the reduction in RLU intensity: lower values corresponding to high neutralizing activity.

INTRODUCTION:

Pseudotyped viruses (PVs) are molecular tools used in microbiology to study host-virus and pathogen-pathogen interactions[1–4]. PVs consist of an inner part, the viral core that protects the viral genome, and an outer part, the envelope glycoproteins on the surface of the virus that defines the tropism[5]. A pseudovirus is replication-incompetent in the target cell because it does not contain all the genetic information to generate new viral particles. This combination of peculiar features makes PVs a safe alternative to a wildtype virus. Wildtype viruses, on the other hand, are highly pathogenic and cannot be used in BSL 2 laboratories for analysis[6].

The infectivity of PVs can be monitored by the presence of a reporter gene, usually coding for a fluorescent protein (GFP, RFP, YFP) or an enzyme that produces chemiluminescent products (luciferase). This is contained in one of the plasmids used for PV production and incorporated in the genome of the pseudovirus[7].

Several types of PV cores currently exist, including lentiviral-derived particles based on the HIV-1 genome. The great advantage of HIV-1-based PVs over other platforms is their intrinsic integration process in the target cell genome[8]. Although HIV-1 is a highly contagious virus and is the causative agent of AIDS, these lentiviral vectors are safe to use because of the extensive optimization steps over the years. Optimal safety conditions were achieved with the introduction of 2nd-generation lentiviral vectors, in which viral genes were depleted without influencing transduction capabilities[9]. The 3rd and 4th generations contributed to the increased safety of lentiviral vector handling with the further splitting of the viral genome into separate plasmids[10, 11]. The latest generations of PVs are generally employed to produce lentiviral vectors for gene therapy.

PVs can be used to study interactions between viruses and host cells, during both the production and the infection phases. PVs are especially employed in pseudovirus neutralization assays (PVNA). PVNAs are widely validated to assess the neutralization potential of serum or plasma by targeting the viral glycoprotein on the PV's envelope[12,13]. Neutralization activity is expressed as the inhibitory concentration 50 (IC50) which is defined as the dilution of serum/plasma that blocks 50% of viral particle entry[14]. In this protocol, we described the set-up of a PVNA to test the antibody activity against Severe Acute Respiratory Syndrome – Coronavirus 2 (SARS-CoV-2) in sera collected before and after receiving a booster vaccine dose.

PROTOCOL:

The present protocol has been approved by and follows the guidelines of the Ethical Committee of the University of Verona (approval protocol number 1538). Informed written consent was obtained from the human subjects participating in the study. Whole blood samples were collected from healthcare worker (HCW) volunteers who were in the process of receiving anti-SARS-CoV-2 vaccines. These samples were collected in plastic tubes containing anticoagulants for the subsequent isolation of serum[15].

All the following processes must be performed in a Class-2 biological hood, working under sterile conditions. Virus handling must be performed with care, and all waste products must be neutralized in a diluted bleach solution. An overview of the protocol is displayed in **Figure 1**.

[place **Figure 1** here]

1. SARS-CoV-2 PVs production and infectivity test

1.1. Seed 5 x 10^5 HEK293T cells in complete Dulbecco's Modified Eagle Medium (DMEM, high-glucose, 10% foetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin) in a 6-well plate (6WP) to reach a suitable cell density compatible with the transfection reagent used. In the case of performing transfection with polyehtylenimine (PEI) (prepare the reagent following the manufacturer instructions), ensure that the cells reach 40-60% density on the day of transfection (step 1.3). Keep the cells in a humidified incubator at 37 °C and 5% CO_2.

1.2. Prior to transfection, replace the spent cell medium with fresh medium without antibiotics (DMEM, high-glucose, 10% FBS, 1% L-glutamine) to achieve higher transfection efficiency.

NOTE: The day after seeding, HEK293T cells are ready to be transfected.

1.3. Transfect adherent HEK293T cells with a suitable transfection reagent according to the manufacturer's instructions. If using PEI, prepare 2 mixes and follow the steps below.

1.3.1. To prepare mix A add 500 ng of pCMV-dR8.91 packaging plasmid[16], 750 ng of pCSFLW reporter plasmid[16] and 450 ng SARS-CoV-2 Spike expressing plasmid in 100 µL of reduced serum medium: 100.

1.3.2. To prepare mix B will add 17.5 µL of PEI (concentration: 1 mg/mL) to 100 µL of reduced serum medium.

1.3.3. Allow both mixes to incubate at room temperature (RT) for 5 min. Next, mix the contents of both tubes together by adding the PEI mix B into DNA mix A.

1.3.4. Incubate the tube for 20-30 min at RT. Flick the tube gently every 3-4 min to enhance the mixing. Finally, add the mixture to the HEK293T cells.

1.4. 16-20 h after the transfection, replace the culture medium with fresh, complete DMEM. Incubate at 37 °C and 5% CO_2, to allow for the production of PVs by transfected cells.

1.5. 72 hours after the transfection, harvest the supernatant containing PVs. Then centrifuge at 1600 x *g* for 7 min at room temperature to remove cell debris and dead cells, and filter it through a 0.45 µm cellulose acetate filter.

1.6. OPTIONAL STEP: To increase the final yeald of PV titre, perform multiple transfections, pool the cell media containing PVs and concentrate it using concentrating tubes.

1.7. Proceed directly with the next steps ("PVs titration", section 2) or aliquot the PV-containing medium in suitable tubes to store at -80 °C until use. Prepare an additional aliquot (400-500 µL) to be used for titration.

NOTE: Making multiple aliquots will guarantee reproducibility between experiments by avoiding excessive thaw-freeze cycles.

2. PVs titration

2.1. Use the fresh PV-containing medium for the next steps or thaw the testing aliquot (step 1.7) to perform the titration of the new viral stock. Freezing aliquots of the same PV stock will guarantee reproducibility.

2.2. Add 50 µL of complete DMEM (or complete medium compatible with the target cell line in usage) in all the wells of a 96 well-plate (96WP) necessary to test in duplicate the PV stock, leaving row "A" empty. Add 100 µL of PVs stock to row "A". Based on the number of preparations to be tested, leave one column without virus as a "cell only" control (**Figure 2**).

2.3. Pipette 50 µL from row A to row B and repeat this process up to row G to obtain serial dilutions of the initial stock. Discard the excess volume from the last row.

2.4. Detach cells using trypsin + ethylenediaminetetraacetic acid 1x (EDTA) in Dulbecco's phosphate buffer saline 1x (DPBS 1x), after removing the spent medium and washing cells with DPBS 1x twice. Prepare cells to a density of 4 x 10^5 cells/mL.

NOTE: In this protocol, PVs infection was tested on the susceptible cell line HEK293T/ACE2; such cells were derived from HEK293T which were transduced using a lentiviral vector to express ACE2 receptor.

2.5. Add 50 µL of the cell suspension into each well to ensure a cell count of 2 x 10^4 cells per well.

2.6. Incubate at 37 °C and 5% CO_2, for 48 h.

2.7. After the incubation, perform the Luciferase assay to obtain the reading as per the manufacturer's instructions. Add 100 µL of the luciferase reagent to the wells and incubate in the dark at RT for 2 min. Move the content of each well to a black 96WP (compatible with the available plate reader) and read the plates in a 96WP reader.

NOTE: The luminometer used for Luciferase readout will produce a spreadsheet file with the raw, unprocessed data that will be used for downstream analysis (in this case, an Excel file). The virus' infectivity will be expressed as relative luminescence units (RLU) (described in paragraph 4.1).

[place **Figure 2** here]

3. Neutralization assay

3.1. Thaw patients' sera on ice. Inactivate serum samples by incubating them at 56 °C for 30 min.

3.2. In a 96WP, add 50 µL of the fresh, complete DMEM (or complete medium compatible with the target cell line used) in each of the following wells: from row B (columns 1-10) to row H (columns 1-10). Put 95 µL of the fresh, complete DMEM in row A (columns 1-10). Add 50 µL and 100 µL of complete DMEM into the wells of columns 11 and 12, respectively. These will be the infected (virus control, or VC) and uninfected (cell only, or CC) controls, respectively (**Figure 3**).

3.3. Add 5 µL of heat-inactivated serum/plasma samples in row A (columns 1-10). Each sample will be in duplicate. With a multichannel pipette, mix the samples in the first row and move 50 µL of medium containing serum from row A to row B. Repeat this process up to the last row (**Figure 3**). Discard the remaining 50 µL.

3.4. Thaw the necessary number of PVs' aliquots and dilute to ≥ 10^4 RLU/mL. Add 50 µL of the diluted PV-containing medium to each well (from column 1 to column 11) using a multichannel pipette to reach a 1:1 dilution of heat inactivated serum/plasma to virus. Incubate at 37 °C and

5% CO_2, for 1 h to allow the antibodies in the serum samples to bind to the SARS-CoV-2 spike protein on the PVs.

3.5. Prepare at least 5 mL suspension of susceptible cells (HEK293T/ACE2) at a cell density of 4 x 10^5 cells/mL. Add 50 µL of the cell suspension to each well and incubate at 37 °C and 5% CO_2, for 48 h.

3.6. After the incubation, perform the luciferase assay reading according to the manufacturer's instructions, as described in step 2.7.

NOTE: The luminometer used for luciferase readout will produce a spreadsheet file (in this case, .xlsx) with the raw, unprocessed data that will be used for downstream analysis (the Luciferase assay file).

[place **Figure 3** here]

4. Titration analysis

4.1. On the Luciferase assay file, assign the names/titles to the corresponding samples.

4.2. Multiply the RLU measure by the dilution factors (from the top to the bottom of the grid: 20x, 40x, 80x, 160x, 320x, 640x, 1,280x, 2,560x) to obtain RLU/mL. If different dilution factors are used, change the multiplication factors accordingly.

4.3. Calculate the average RLU/mL for each PV preparation.

5. PVs neutralization assay analysis

5.1. On the Luciferase assay spreadsheet file (in this case, .xlsx), assign the corresponding titles to the tested samples. Enter the dilution factor of the sample (40s, 80x, 160x, 320x, 640x, 1,280x, 2,560x, 5,120x). Calculate the Log10 of the dilution factors.

5.2. Calculate the average RLU of uninfected and infected control (**Figure 3**, columns 11 and 12 respectively). These values will be useful for the normalization in step 5.5.

5.3. Open a new document for data analysis. Select **X/Y analysis**, input X as **Numbers** and Y as **Enter 2 replicate values in side-by-side sub-columns**.

5.4. Enter Log10 (dilution) values as X numbers. Enter the duplicate RLU of the samples.

5.5. Go to **Analyze** > **Normalize** > **Flag all** the samples on the same sheet. Input the average VC and CC values in **How is 0% defined?** and **How is 100% defined?**, respectively. Click **OK**.

5.6. On the Normalized data sheet, go to **Analyze** > **XY analyses** > **Nonlinear analyses (curve fit)**. Flag all the samples and click **OK**. In **Dose-response – Inhibition**, select **log(inhibitor) vs normalized response – variable slope**.

5.7. Under **Constrain**, change **HillSlope** to **Must be less than 0**.

5.8. Under **Output**, flag **Create summary table and graph**. Click on **OK** to obtain the final analyses. A working sheet with a template for the analysis is provided in Supplementary material.

REPRESENTATIVE RESULTS:

This protocol describes the production of SARS-CoV-2 PVs and a downstream application of these PVs to analyze the neutralization activity of serum/plasma of subjects receiving anti-COVID-19 vaccination[17]. Furthermore, this protocol can be applied to produce pseudotypes of each SARS-CoV-2 variant of concern (VOC) to test the evolution of the neutralizing response. Despite this protocol facilitating the study of humoral immune response after COVID-19 vaccination, it can be adapted to easily test the neutralization of different sera/plasma, against different viruses[13,18,19].

Figure 4A represents the increment of the dilution of serum (Log(dilution)) corresponding to the increase of the RLU signal. Thus, the higher the dilution of the sample, the less blocked the virus entry is (**Figure 4A**). This is further expressed as percentage of neutralization (**Figure 4B**).

The IC50 result shows the neutralization capacity of a single vaccine serum over time. In the example reported in **Figure 4C**, , the subject developed a strong humoral activity against the virus at four weeks after vaccination; however, after 16 weeks the IC50 is similar to the one prior to vaccine administration. In this case, the PVNA showed the loss of neutralization potential over time.

[place **Figure 4** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Graphical representation of a neutralization assay. (**A**) PV production, (**B**) PV titration and (**C**) neutralization assay**.** All the procedures are performed in a class-2 biological hood, under sterile conditions. Titration step (B) needs to be performed to standardize the infectivity levels of PVs before use in the neutralization assay (C). This figure was created with BioRender.

Figure 2: Representative layout of a 96 well plate for PVs titration. A fixed volume of PVs-containing supernatant is added to row A, columns 1-11, and serially diluted. The last column is left as the "cell only" control. This figure was created with BioRender.

Figure 3: Plate representation based on serum dilution. Bright red corresponds to a higher quantity of serum, and bright blue lane (column 11) corresponds to infected cell control (VC, virus control). Light blue lane (column 12) corresponds to uninfected cells (CC, cell control). This figure was created with BioRender.

Figure 4: Representative results of PVNA. (**A**) Infectivity (RLU, and (**B**) percentage of neutralization are shown at week 0 (W0, before the vaccination); W4 (four weeks after vaccination); W16 (sixteen weeks after W0). (**C**) IC50 values at the same time points.

DISCUSSION:

Although using a wildtype virus simulates the actual infection, lentiviral PVs are a safer option to study the mechanisms associated with viral entry and infection without the strict safety requirements necessary to work with pathogenic viruses[4,20,21]. PVs are composed of a replication-defective viral core surrounded by the surface envelope glycoprotein of a pathogenic virus which is the objective of the study.

HIV-1-based PVs are one of the most widely used platforms and these have been employed in this protocol for the production of SARS-CoV-2 pseudoviral particles. The reporter gene can be different as per the use of the PVs; in this case, the choice of the luciferase reporter gene provides an easy, fast, and sensitive readout of the infectivity of the produced PVs.

PVs based on lentiviruses are widely applied to study anti-HIV-1 humoral response[22]. The PV technology was instantly applied during the recent COVID-19 pandemic, caused by SARS-CoV-2. SARS-CoV-2 is a highly pathogenic human *Betacoronavirus*, identified for the first time in China (Wu Han) which became rapidly pandemic, causing more than 6 million deaths worldwide[23,24]. Because of the validation of vaccine strategies, the pandemic has been largely controlled; nonetheless, in most vulnerable people, such as cancer patients or people living with HIV, it does still pose a risk[25–27]. In this context, there is still a need for validated assays to monitor the anti-vaccine humoral response in terms of neutralizing activity. In this article we have described a simple protocol that can be easily be performed in laboratories with no access to category-3 containment. Furthermore, the PV platform is a versatile system to study different SARS-COV-2 virus variants. Indeed, by changing the envelope-expressing plasmid with different spikes, it is possible to generate PVs of SARS-CoV-2 new variants or of any other coronaviruses[28]. These virus portfolios can be used to assess the reactivity of vaccine-induced humoral response against the different variants of concern[15, 29–32]. This information can guide the generation of new and more effective vaccines.

Three major obstacles could be encoutered while following this protocol, concerning transfection conditions, titration failure and/or neutralization assay. First, the packaging cells may not be sufficiently confluent at the time of transfection. This may be due to the lack of nutrients. Ensure that step 1.1. is properly followed. Otherwise, perform seeding in the morning of the day before transfection and transfect the packaging cells later the next day to increase the growth time. A recurring problem is also the potential contamination of the cell medium between transfection and medium replacement the next day. In this case, repeat the procedure by increasing the sterilization procedure before use when working under the BSL2 hood, or include antibiotics to avoid unwanted contaminations. Second, an undetected luciferase signal may occur, that can be attributed to various stages of PVs production or to the characteristics of the target cell line. Plasmids should be extracted with endotoxin-free kits. The transfection step is critical for the

outcome of the protocol. PEI reagent must be prepared at the correct concentration of 1 mg/mL. Gently flicking the tube during the preparation of transfection mixes enhances the formation of DNA-PEI complexes. To verify that the cells have been transfected correctly, it is recommended to perform the luciferase assay immediately to the harvested cells. In addition, include a control virus envelope glycoprotein such as VSV: VSV-PVs give strong RLU signals on human cells lines. Moreover, it is necessary to mention that the target cell line must express the receptor, which is easily verified via western blot or flow cytometry.

This method has been previously optimized[16] with respect to the experimental conditions including the selection of the transfection reagent, the determination of the ratios between the different plasmids needed for the generation of the PV and the selection of the target cell lines, the use of luciferase as reporter genes. Nonetheless, each laboratory will need to validate the proposed methods according to the available equipment. For example, (step 2.7) requires the addition of 100 µL of Luciferase substrate as suggested by the producer: this is optimal for the readout of the luciferase assay with the plate reader that is currently available. On the other hand, other laboratories that are equipped with a different plate reader, may adapt the protocol using different luciferase substrates or volume of the reagent[33]. Futhermore, other authors have proposed the use of the green fluorescent protein (GFP) as reporter gene instead of the luciferase. This could be considered if a laboratory is fully equipped for GFP readout but not luciferase[34,35].

To conclude, PVs are a flexible and straightforward system that allows quantifying the infection by using a simple detection method. It represents a cost-effective approach which is more accessible for many research groups and allows avoiding the use of pathogenic viruses that require a biosafety level 3 laboratory[21]. The use of PVs represents a well-characterized and safe approach to study the antibody-mediated neutralization in individuals that experienced SARS-CoV-2 infection and/or vaccination.

ACKNOWLEDGMENTS:
We aknowledge the contribution of the health-care workers volunteers. This project was supported by the Department of Excellence 2023/2027, MUR, Italy.

DISCLOSURES:
The authors declare to have no conflict of interest.

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