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Standardized focus assay protocol for biosafety level four viruses

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ABSTRACT

Working in accordance with biosafety level four practices is highly complex and time-consuming. Therefore, the respective laboratory protocols should be as uniform as possible, simple to perform and straightforward in readout. Here we describe the successful application of a standardized 24-well plate focus assay protocol for the titration of *Zaire ebolavirus* (two isolates), *Marburg virus* (three isolates), *Lassa virus* (two isolates), *Crimean Congo hemorrhagic fever virus* (one isolate), and *tick-borne encephalitis virus* (two isolates). Viral titers are determined based on a simple visual readout. The protocol exhibits high precision, with coefficients of variation for inter-assay variability ranging between 0.05 and 0.21 and those for intraassay variability between 0.08 and 0.23. All reagents required for the test, including primary and secondary antibodies, are commercially available, facilitating the establishment of the protocol in other laboratories.

1. Introduction

Hemorrhagic fever viruses cause severe multisystem syndromes with vascular damage. Due to their high pathogenicity, these viruses are usually classified as biosafety level 4 (BSL-4) agents. Infections with these viruses, including *Zaire ebolavirus* (ZEBOV; genus *Ebolavirus*, family *Filoviridae*), *Marburg marburgvirus* (MARV; genus *Marburgvirus*, family *Filoviridae*), *Lassa virus* (LASV; genus *Arenavirus*, family *Arenaviridae*), and *Crimean Congo hemorrhagic fever virus* (CCHFV; genus *Orthohantavirus*, family *Nairoviridae*) are often severe and life threatening (Brown et al., 2017; Hallam et al., 2018; Shayan et al., 2015).

Far Eastern and Siberian subtype *tick-borne encephalitis viruses* (TBEV-FE, TBEV-Sib; genus *Flavivirus*, family *Flaviviridae*) cause febrile disease with or without neurological involvement (Lindquist, 2014), but may also possess hemorrhagic properties (Ternovoi et al., 2003). In Switzerland, Far Eastern and Siberian subtype TBEV are classified as BSL-4 viruses.

Methods measuring viral infectivity include plaque assay, 50% tissue culture infectious dose ($TCID_{50}$), and focus assay. Whereas quantification by plaque assays and $TCID_{50}$ is only successful for viruses inducing cell death, focus assay utilizes antibody based staining and may therefore detect infected but not necessarily dead cells. While fluorescence-conjugated antibodies have to be visualized under a fluorescence microscope, horseradish peroxidase (HRP) labelled antibodies allow for a direct visual readout. Since working in accordance with BSL-4 practices is highly complex and time-consuming, protocols should be simple to perform, consistent and straightforward in readout. Focus assay protocols for several BSL-4 viruses requiring readout under a fluorescence microscope have been described (Weidmann et al., 2011). However, no methods with direct visual readout using HRP-conjugated antibodies for BSL-4 viruses have been published so far. This manuscript describes a standardized focus assay protocol suitable for the titration of infective particles of ten BSL-4 virus isolates belonging to five different genera. All used reagents, including primary and secondary antibodies, are commercially available.

2. Materials and methods

2.1. Vero and Vero E6 cells

Vero and Vero E6 cells were obtained from the American Type Culture Collection (ATCC CCL-81, ATCC CRL-1586) and cryopreserved

Abbreviations: BSL-4, biosafety level 4; CCHFV, *Crimean Congo hemorrhagic fever virus*; CV, coefficient of variation; FCS, fetal calf serum; FFU, focus forming units; HRP, horseradish peroxidase; LASV, *Lassa virus*; MARV, *Marburg virus*; MEM, minimal essential medium; NEAA, non-essential amino acids; PBS, phosphate-buffered saline; RT, room temperature; TBEV-FE, *tick-borne encephalitis virus* Far Eastern subtype; TBEV-Sib, *tick-borne encephalitis virus* Siberian subtype; $TCID_{50}$, 50% tissue culture infectious dose; ZEBOV, *Zaire Ebolavirus*

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Table 1
List of primary antibodies.

virus isolate	antibody	Concentration / dilution factor for 1 µg / ml	distributor
CCHFV Afg-09-2990	rabbit anti Crimean Congo hemorrhagic fever virus N protein	0.81 mg / ml 1:810	US Biological (Salem, Massachusetts, US)
LASV Josiah, LASV Lib05-1580/121	rabbit anti Lassa virus glycoprotein	0.97 mg / ml 1:970	US Biological
MARV Leiden, MARV Musoke, MARV Popp	rabbit anti Marburg virus glycoprotein	0.739 mg / ml 1:739	US Biological
TBEV-FE Moskva, TBEV-Sib Vasilchenko	rabbit anti Flavivirus group antigen (D1-4G2-4-15)	1 mg / ml 1:1000	Bio-Techne, Minneapolis, Minnesota, US
ZEBOV Gueckedou-C07, ZEBOV Mayinga-76	rabbit anti Ebola virus Zaire, strain Mayinga 1976 GP-RBD	1.07 mg / ml 1:1,070	Sino Biological (Wayne, Pennsylvania, US)

at −196 °C in liquid nitrogen. Individual aliquots were thawed and serially passaged for a maximum of 20 passages. Cells were maintained in Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS), 0.625% L-glutamine 0.5% penicillin-streptomycin, and 0.5% non-essential amino acids (NEAA) (Biochrom, Berlin, Germany) in 150 cm^2 Corning culture flasks (Sigma Aldrich, Basel, Switzerland) at 37 °C without CO_2.

2.2. Virus strains

The viral isolates used for focus assay development were: CCHFV Afg09-2990; LASV Josiah; LASV Lib05-1580/121; MARV Leiden; MARV Musoke; MARV Popp; TBEV-FE Moskva; TBEV-Sib Vasilchenko; ZEBOV Homo sapiens-wt/GIN/2014/Makona-Gueckedou-C07 (ZEBOV Gueckedou-C07); ZEBOV Mayinga-76. Isolates were kindly provided by Stefan Günther and Toni Rieger, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany (CCHFV Afg09-2990, LASV Josiah, LASV Lib05-1580/121, ZEBOV Gueckedou-C07, ZEBOV Mayinga-76); and Daniel Růžek, Veterinary Research Institute and Academy of Scienes of the Czech Republic, Brno, Czech Republic (TBEV-FE Moskva, TBEV-Sib Vasilchenko). All virus isolates were passaged at least twice at Spiez Laboratory in Vero cells (LASV Josiah; LASV Lib05-1580/121; TBEV-FE Moskva, TBEV-Sib Vasilchenko) or Vero E6 cells (CCHFV Afg-09-2990; ZEBOV Gueckedou-C07, ZEBOV Mayinga-76) before being used as virus stocks in this study.

2.3. Focus assay protocol

2.3.1. Preparation of media

The following media are required: a) cell culture medium: MEM supplemented with 10% FCS, 0.625% L-glutamine 0.5% penicillin-streptomycin, and 0.5% NEAA (Biochrom); b) virus dilution medium: identical to cell culture medium, but supplemented with only 2% FCS; c) overlay medium: virus dilution medium supplemented with 1% methylcellulose 90 H G 4000 cP (Sigma Aldrich).

2.3.2. Preparation of Vero and Vero E6 cells (24 well plates)

Cell culture medium was removed from confluent 150 cm^2 Corning culture flasks (Sigma Aldrich). Cells were washed once with 7 ml trypsin (Biochrom), then suspended in 3 ml trypsin and mixed with 7 ml cell culture medium. Cells were counted using trypan blue staining (Sigma-Aldrich) and a Neubauer counting chamber, and a suspension of 180,000 cells / ml was prepared. From this suspension, 1 ml was seeded to each well of a TPP 24 well plate (Sigma Aldrich), and plates were incubated for 24 h at 37 °C without CO_2.

2.3.3. Assay setup

Vero cells were used for setting up the assays for LASV Josiah; LASV Lib05-1580/121; MARV Leiden; MARV Musoke; MARV Popp; TBEV-FE Moskva, and TBEV-Sib Vasilchenko; Vero E6 cells were used for CCHFV Afg-09-2990, ZEBOV Gueckedou-C07, and ZEBOV Mayinga-76 titration. Eleven tenfold serial dilutions of each virus isolate were prepared in virus dilution medium. Cell culture medium was removed from 24 well plates prepared the day before (see Section 2.3.2), and 150 µl of each virus dilution were used to infect a well. Each dilution was assessed in duplicate. Per plate, two wells were used as cell control and treated with 150 µl of virus dilution medium only. Plates were covered with Microsealer B sealing foils (BIO-RAD, Cressier, Switzerland) and incubated for 1 h at 37 °C without CO_2. Then, plates were overlayed with 1 ml overlay medium / well, sealed with Microsealer B sealing foils (BIO-RAD) and incubated for 7 days at 37 °C without CO_2.

2.3.4. Preparation of staining reagents

The following solutions are needed: a) PBS: phosphate buffered saline pH 7.4 without Ca^{2+} and Mg^{2+} (Biochrom); b) PBST: PBS supplemented with 0.05% Tween (Fisher Scientific, Reinach, Switzerland); c) fixation buffer: Roti®Histofix 10% (Carl Roth, Karlsruhe, Germany), i.e. 10% formaldehyde in PBS; d) permeabilization buffer: PBS supplemented with 1% Triton X-100 (Sigma Aldrich); e) blocking buffer: PBST supplemented with 10% FCS (Biochrom) and 4% powdered milk; f) primary antibody solution: primary antibodies directed against the individual viruses (Table 1) diluted to a final concentration of 1 µg / ml in blocking buffer; g) secondary antibody solution: KPL goat anti rabbit HRP (BioConcept, Alschwil, Switzerland) diluted to a final concentration of 1 µg / ml in blocking buffer; h) substrate solution: one tablet (20 mg) of 3-Amino-9-ethylcarbazole (Sigma Aldrich) dissolved in 2.25 ml N, N-Dimethylformamide in glass vessels and topped up with 47.5 ml 0.05 M sodium acetate buffer (Sigma Aldrich) after dissolving and supplemented with 0.05% H_2O_2 (Sigma Aldrich) (H_2O_2 must be added shortly before use).

2.3.5. Focus assay staining

Plates were washed with 1000 µl / well of PBS and fixed for 1 h at room temperature (RT) with 1000 µl / well of fixation buffer. Plates were washed twice with 1000 µl / well of PBST, and cells were permeabilized with permeabilization buffer (500 µl / well) for 5 min. at RT. After two additional washing steps with 500 µl / well of PBST, wells were blocked with 500 µl blocking buffer for 1 h at RT. Then, plates were incubated overnight at 4 °C with 250 µl primary antibody solution per well. The next day, wells were washed four times with 500 µl PBST before 250 µl secondary antibody solution / well were added. Plates were incubated for 1 h at RT, then wells were washed 3 times with 500 µl PBST and twice with 500 µl deionized water. Next, 250 µl substrate solution were added per well, and color was allowed to develop for about 15 min. Finally, substrate solution was removed from all wells, plates were inverted on a light box and foci were counted by hand.

2.3.6. Titer calculation

Wells with foci counts ranging between 10 and 80 were used for titer calculation. Counts from duplicate wells were averaged by arithmetic mean. The average was multiplied by the dilution factor of the

inoculum (e.g. 10, 100) and the factor accounting for the volume of inoculum (6.66 when using 150 µl inoculum volume in a final reaction volume of 1000 µl) to calculate the focus forming units (FFU) per ml of the original virus stock.

2.4. Assessing the effect of primary and secondary antibody concentration

For CCHFV Afg09-2990, LASV Josiah and LASV Lib05-1580/121, the effect of using primary and secondary antibody solutions at concentrations of 0.5 µg / ml and 2 µg / ml instead of 1 µg / ml was assessed. Besides altered antibody concentrations, focus assay staining was done as described in Section 2.3.5.

2.5. Assessing the effect of CO_2 on viral titers

The effect of incubating plates without or with 5% CO_2 was exemplary assessed for CCHFV Afg09-2990, LASV Lib05-1580/121, and ZEBOV Gueckedou-C07. For each virus, two assay plates were simultaneously prepared; one plate was incubated without CO_2, the other was incubated with 5% CO_2. The comparison was done twice to account for the effect of pipetting imprecisions. For plates incubated with 5% CO_2, standard MEM was replaced by MEM supplemented with 1.5 g / l $NaHCO_3$ DMEM (Biochrom), and Microsealer B sealing foils were replaced by gas-permeable tissue culture seals (4titude, Surrey, UK). Focus assay staining and titer calculation were done as described in Sections 2.3.5 and 2.3.6.

2.6. Inter- and intraassay variability

Assay variability was assessed by repeating the assay at three individual days (interassay variability) with two replicates of each dilution (intraassay variability) for CCHFV Afg09-2990, LASV Lib05-1580/121, MARV Musoke, ZEBOV Gueckedou-C07, TBEV-FE Moskva and TBEV-Sib Vasilchenko. Coefficients of variation (CV) for interassay varability were calculated as the standard deviation divided by the mean value of titers (FFU / ml) obtained in the assay repetitions on the four individual days. CV for intraassay variability were defined as the standard deviation divided by the mean value of counted foci for one particular dilution within one assay, and CV for all assays were averaged.

3. Results

3.1. Focus assay staining

Virus titration was successful for all tested virus isolates using the here described standardized focus assay protocol. Although morphology of foci was variable depending on the virus isolate, foci were easily discernible and countable (Fig. 1).

3.2. Effect of primary and secondary antibody concentrations

While using dilutions of primary or secondary antibodies with a final concentration of 0.5 µg / ml impaired staining quality, recognizable as faint staining, using primary or secondary antibodies at concentrations of 2 µg / ml did not significantly improve staining quality compared to 1 µg / ml (detailed results not shown). Therefore, for economic reasons, using primary and secondary antibody solutions at a concentration of 1 µg / ml is reasonable.

3.3. Effect of CO_2 on viral titers

Incubating focus assay plates in incubators with or without 5% CO_2 did not significantly influence the viral titers obtained in the assays. No tendency for higher or lower viral yield could be observed, regardless of the plates being incubated with or without CO_2. Mean titers were as follows: CCHFV Afg09-2990 with CO_2 4.43×10^4 FFU / ml, without CO_2 4.70×10^4 FFU / ml (i.e. +/- 3% from the overall mean); LASV Lib05-1580/121 with CO_2 2.45×10^6 FFU / ml, without CO_2 2.63×10^6 FFU / ml (+/- 4%); ZEBOV Gueckedou-C07 with CO_2 6.43×10^6 FFU / ml (+/- 7%).

3.4. Inter- and intraassay variability

Coefficients of inter- and intraassay variability are shown in Table 2. The standardized focus assay protocol yielded reproducible results, with CV for interassay precision not exceeding 0.21, and CV for intraassay variability not higher than 0.23.

4. Discussion

Plaque assays are frequently used for assessing the infectivity of BSL-4 virus stocks. Published methods include plaque assay protocols for CCHFV, EBOV, LASV and TBEV (De Madrid and Porterfield, 1969; Shepherd et al., 1986; Shurtleff et al., 2016; Tomori et al., 1987) as well as pseudotype assays for CCHFV (Berber et al., 2013). However, plaque assays are often difficult to establish and standardize. Different virus stocks comprise variants that may not perform equivalently in plaque assays. In addition, cell lines can importantly influence the assay, and different isolates may require specific incubation times (Shurtleff et al., 2016). Despite published protocols, robust plaque assays yielding reproducible results for CCHFV, LASV, MARV, and ZEBOV could not be implemented in the author's laboratory so far (data not shown). Therefore, the aim of this work was to establish a standardized and reproducible focus assay protocol being easy to perform and straightforward in readout.

Since focus assays rely on the detection of viral proteins by immunostaining techniques, they offer the unique advantage of detecting viruses not producing cell damage. Regularly, focus assays use secondary antibodies with fluorescent tags (Payne et al., 2006; Weidmann et al., 2011). These assays are typically analyzed after an incubation time of 24–48 h using a fluorescence microscope. However, this readout is often laborious, time-consuming and difficult to interpret. In contrast, the protocol described here relies on HRP-labelled secondary antibodies. This allows for a simple visual readout on a light box, which makes test evaluation easier and faster, countervailing for the prolonged incubation time of 7 days. Colony counting software may be used to minimize the effect of subjectively evaluating focus counts. However, foci produced by all virus isolates used in this study were easily discernible and countable (Fig. 1). Therefore, automatization of focus assay readout is not mandatory with this protocol.

Working in accordance with BSL-4 practices is highly complex and time-consuming. Consequently, protocols suitable for such laboratories should be simple and ideally standardized, not requiring virus- or even isolate-specific adaptations. The focus assay protocol described here is almost universal, with the only changing parameters being the cell line (Vero or Vero E6, both of which are regularly used in most virology laboratories) and the specific primary antibodies (Table 1). Assay setup, incubation conditions and duration as well as the staining protocol and readout were consistent for all virus species used in this study. Compared to plaque assay protocols individualized for specific virus species or even strains, the protocol is straightforward and easy to be implemented.

Precision of the described assay meets the criteria for quantitative microbiological methods (Pharmacopeia U.S., 2007). CVs for interassay variability ranged between 0.06 and 0.21, and those for intraassay variability between 0.08 and 0.23. Given the fact that the focus assay protocol is completely based on manual pipetting and was performed by different laboratory technicians, these values are highly satisfying.

Accuracy of a test is defined as the closeness of test results obtained by the evaluated method compared to the results obtained by a traditional method (Pharmacopeia U.S., 2007). For defining the accuracy of

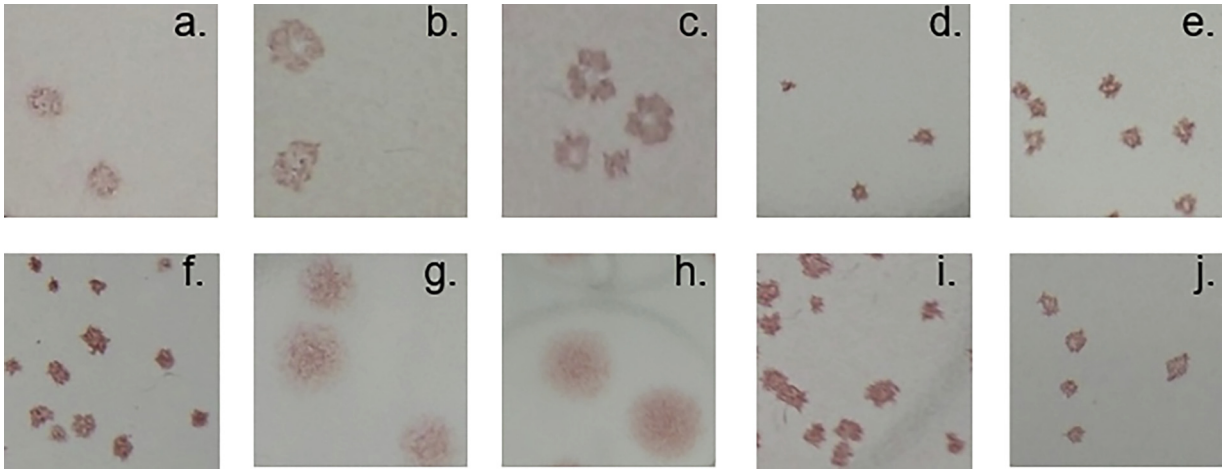

Fig. 1. Focus morphology of all tested isolates. the width of one picture corresponds to 5 mm. a) CCHFV Afg09-2990; b) LASV Josiah; c) LASV Lib05-1580/121; d) MARV Leiden; e) MARV Musoke; f) MARV Popp; g) TBEV-FE Moskva; h) TBEV-Sib Vasilchenko; i) ZEBOV Gueckedou-C07; j) ZEBOV Mayinga-76.

Table 2
Inter- and intraassay variability.

virus isolate	CV interassay variability[a]	CV intraassay variability[a]
CCHFV Afg-09-2990	0.14	0.23
LASV Lib05-1580/121	0.08	0.19
MARV Musoke	0.05	0.11
TBEV-FE Moskva	0.21	0.20
TBEV-Sib Vasilchenko	0.18	0.08
ZEBOV Gueckedou-C07	0.06	0.23

[a] CV, coefficient of variation.

the focus assay protocol, comparing titers against those obtained by plaque assay would have been appropriate. Since not all viruses produce clearly visible cytopathic effect robust plaque assay protocols could not be established in the author's laboratory for all the BSL-4 viruses used in this study. Consequently, a direct comparison of those two methods was not possible. However, due to its ability to detect intracellular viral proteins, the described focus assay protocol is likely to be at least as sensitive as the respective plaque assays. The critical limiting factor of focus assay protocols is their ability to only probe for viral protein subunits, compared to the effect of actual infectious virions on cells as they are detected in plaque assays (Baer and Kehn-Hall, 2014). On the other hand, the focus assay allows visualizing virus replication in cells which are not directly destroyed by the virus.

In addition to measuring viral infectivity, focus assays may be used in focus reduction neutralization tests (FRNT) for quantifying neutralizing antibodies in serum samples

In the author's BSL-4 laboratory, whenever possible, closed culture systems are used due to safety-related considerations. Culture plates are firmly closed with sealing foils and covers and additionally kept in closed containers for all transportation and incubation steps, minimizing the possibility of contaminating air or surfaces outside the biosafety cabinets. However, since incubation of plates with 5% CO_2 is the standard protocol in most laboratories, the effect of this incubation condition on viral titers achieved with the focus assay protocol was evaluated for CCHFV Afg09-2990, LASV Lib05-1580/121, and ZEBOV Gueckedou-C07. No tendency for higher or lower viral yield could be observed, regardless of the plates being incubated with or without CO2. Mean titers were slightly higher with CO_2 for CCHFV Afg09-2990, but vice versa for LASV Lib05-1580/121 and ZEBOV Gueckedou-C07.

5. Conclusion

This manuscript describes a 24-well plate focus assay protocol with simple visual readout with a standardized workflow for ten BSL-4 virus isolates belonging to five different genera. The protocol exhibits high precision, with CVs for interassay variability ranging between 0.05 and 0.21 and those for intraassay variability between 0.08 and 0.23. All reagents are commercially available, facilitating the establishment of the protocol in other laboratories.

Declarations of interest

None.

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