Journal of Virological Methods 260 (2018) 34–40

Contents lists available at ScienceDirect

Journal of Virological Methods

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

An optimized high-throughput fluorescence plate reader-based RSV neutralization assay

Yong-Peng Sun[a,1], Wei Zhang[b,1], Qin-Jian Zhao[a], Jian-Li Cao[b], Lu-Jing Zhang[a], Jiang-Yan Xiang[b], Jun-Yu Si[b], Xue Lin[a], Li Chen[a], Zi-Zheng Zheng[a,*], Ning-Shao Xia[a,b,**]

[a] *State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Public Health, Xiamen University, Xiamen, Fujian 361002, PR China*

[b] *State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Public Health School of Life Sciences, Xiamen University, Xiamen, Fujian 361002, PR China*

A R T I C L E I N F O

Keywords:
Fluorescence plate reader
Flow cytometry
RSV
Neutralization assay

A B S T R A C T

A licensed vaccine for respiratory syncytial virus (RSV) has yet to be developed, and a reliable and repeatable neutralizing assay is indispensable for vaccine development. Here, we demonstrated an optimized high-throughput RSV neutralization assay that utilizes a fluorescence plate reader (reader) as a substitute for flow cytometry to detect fluorescent signals in RSV-A2 mKate-infected cells. Furthermore, this study tested the influence of virus input and infectivity on the neutralizing assay and highlighted critical factors (together with a suggested protocol) for obtaining stable data using this assay.

1. Introduction

Respiratory syncytial virus (RSV) was first isolated in 1955 and is an enveloped, non-segmented, negative-sense, single-stranded RNA virus that is a major, widespread pathogen causing severe lower respiratory tract disease in infants under 2 years of age (Glezen et al., 1981) and elderly adults (Falsey et al., 2005; Walsh et al., 2004). RSV infection inflicts a heavy financial burden on national governments (Nair et al., 2010; Hall et al., 2009). Although a safe and effective vaccine has not been developed for RSV, several vaccine candidates are undergoing clinical or pre-clinical research. The current major methods for RSV vaccine research include anti-F IgG detection, a palivizumab-competitive antibody (PCA) assay (Smith et al., 2012) and neutralization assays (Anderson et al., 1985; Zielinska et al., 2005; Chen et al., 2010). An RSV neutralization assay should comprehensively reflect the level of an immunogen-induced antibody response, which is critical for evaluating the immunogenicity of vaccine candidates. Although anti-RSV neutralization assays exist in multiple formats, two predominant methods are the classical plaque reduction neutralization assay (or microneutralization) (Anderson et al., 1985; Zielinska et al., 2005) and neutralization assays based on viral recombination with a fluorescent reporter gene (Chen et al., 2010). Although the plaque reduction neutralization assay is common, it is time-consuming and cannot be used for high-throughput detection. In addition, the accuracy of this neutralization evaluation system is significantly dependent on the use of a strict multiplicity of infection (MOI) and the number of viral plaques. Chen et al. (2010) developed an RSV neutralization assay based on viral recombination with a fluorescent reporter gene, which displayed high sensitivity, specificity, repeatability and greatly shortened the experimental cycle. However, the detection time and throughput of this method were still subject to the detection speed of flow cytometry. Here, we attempted to use a fluorescence plate reader instead of flow cytometry and investigated the factors that correlated with assay stability.

Abbreviations: RSV, Respiratory syncytial virus; PCA, Palivizumab-competitive antibody; mKate, Monomeric Katushka fluorescent protein; mAb, Monoclonal antibody; MOI, Multiplicity of infection; PBS, Phosphate buffered saline; PBS-T, PBS containing 0.05% Tween 20; qPCR, Quantitative polymerase chain reaction; pre-F, Prefusion; post-F, Postfusion; FCM, Flow cytometry; CV, Coefficient of variation

* Corresponding authors.

** Corresponding author at: State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Public Health School of Life Sciences, Xiamen University, Xiamen, Fujian 361002, PR China.

E-mail addresses: 451464997@qq.com (Y.-P. Sun), aaronzhang@stu.xmu.edu.cn (W. Zhang), zhaoqinjian@xmu.edu.cn (Q.-J. Zhao), caojianli@stu.xmu.edu.cn (J.-L. Cao), 104534737@qq.com (L.-J. Zhang), 1767537307@qq.com (J.-Y. Xiang), 21920122202736@stu.xmu.edu.cn (J.-Y. Si), 634991471@qq.com (X. Lin), 542773483@qq.com (L. Chen), zhengzizheng@xmu.edu.cn (Z.-Z. Zheng), nsxia@xmu.edu.cn (N.-S. Xia).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.jviromet.2018.07.004
Received 5 December 2017; Received in revised form 4 July 2018; Accepted 8 July 2018
Available online 09 July 2018
0166-0934/ © 2018 Elsevier B.V. All rights reserved.

2. Materials and methods

2.1. Virus and cells

Viral stocks of RSV strain A2 carrying the monomeric Katushka fluorescent protein (RSV-A2 mKate) were prepared in HEp-2 cells cultured with 10% minimum essential medium (MEM). RSV-A2 mKate was constructed based on RSV L19-mKate which was kindly provided by Martin Moore at Emory University and published by Hotard et al (Hotard et al., 2012). T175 flasks of cells at 80–90% confluence were infected with RSV-A2 mKate master stock (MOI = 0.3), sealed and incubated on a shaker for 1 h at room temperature. Fifty milliliters of 10% MEM was then added to the flasks, which were incubated at 37 °C. When substantial syncytia had formed, cells were scraped from the flasks and transferred to 50-mL centrifugation tubes for ultrasonication, followed by centrifugation at 4 °C and 2500 rpm for 20 min. The supernatant containing virus was divided equally into dram vials, frozen quickly in an alcohol–dry ice complex and stored at −80 °C. The viral stock titer used for the experiments was 1×10^8 pfu/mL, which was titrated by RSV plaque assay as previously described (Devincenzo, 2004).

2.2. Monoclonal antibody and serum

5C4 (RSV F mAb, binding site Ø) (McLellan et al., 2013a, b; Zhao et al., 2017), 11H8 (RSV N mAb, unknown binding site) and irrelevant mAb (anti-HEV mAb 9F7, binding antigenic site C6 on E2s domain) (Tang et al., 2015; Zhao et al., 2015) were produced via a standard murine mAb preparation protocol in our laboratory. RSV mAb motavizumab (binding site II) (McLellan et al., 2010) and 1129 (binding site II) (Johnson et al., 1997) were prepared using a eukaryotic expression system. 40 adult sera from healthy donors, age ranging from 20 to 84, were randomly selected. Age and sex showed random distribution. Clinical serum samples from 44 children below 5 years of age were collected, who were hospitalized with RSV infection (n = 16) or with other respiratory virus infection (n = 28) during the acute phase in Xiamen maternal and child health hospital, China, in April 2018. Mouse sera were derived from mice infected by RSV. All sera before performance were inactivated at 56 °C for 30 min to avoid complement dependent cytotoxicity.

2.3. Fluorescence plate reader-based RSV neutralization assay

Ten microliters of antibody or serum was mixed with 90 μL of cell culture medium. Then, the mixture was serially diluted 5-fold 10 times. Serial dilutions of samples were mixed with an equal volume of RSV-A2 mKate in 96-well plates and incubated at 37 °C for 1 h. Then, 100 μL of sample-virus mixture was added to each well of a 96-well plate seeded with 5×10^4 cells in a 100-μL volume per well and then incubated with HEp-2 cells at 37 °C for 24 h. Then, the cells were washed once with phosphate buffered saline (PBS) and fixed with 1% glutaraldehyde for 15 min. The fluorescent intensity of each well was measured by using a fluorescence plate reader (SpectraMax Paradigm, Molecular Devices, Sunnyvale, CA, USA) at 588-nm excitation and 633-nm emission. Fluorescent reading data were analyzed by non-linear fitting (GraphPad Prism, GraphPad Software Inc., San Diego, CA, USA) such that the software output curve fit $\log IC_{50}$ and corresponding IC_{50} values. The "Analysis" module of GraphPad Prism was used to normalize these data. Smallest and largest value in each data set are respectively defined as 0% and 100%, and then neutralization curves were fitted with the nonlinear regression.

2.4. Flow cytometry neutralization assay

A flow cytometry-based RSV neutralization assay was performed by using a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) as previously reported (Chen et al., 2010). Briefly, serial dilutions of samples were added to an equal volume of virus in 96-well plates and incubated at 37 °C for 1 h. Then, 100 μL of sample-virus mixture was transferred into each well in 96-well plates seeded with HEp-2 cells. After incubation for 24 h, the cells were digested with pancreatin to prepare single-cell suspensions and then fixed with 0.5% paraformaldehyde before flow cytometry.

2.5. Dot blotting

One hundred microliters of RSV-A2 mKate was transferred onto nitrocellulose membranes by using a vacuum blotter (Bio-Rad, Richmond, CA, USA). Membranes were blocked with 5% (wt/vol) skim milk for 1 h and then incubated with primary mAbs (RSV F mAbs 5C4, 1129 and anti-HEV mAb 9F7) diluted 1:500 in PBS for 1 h at room temperature. After three washes, the membranes were further incubated with goat anti-mouse IgG-FITC (Sigma-Aldrich, St. Louis, Missouri, USA) at a dilution of 1:3000 for 1 h. The membranes were scanned using a Gel Doc™ EZ System imager (Bio-Rad, Richmond, CA, USA) at 488-nm excitation.

2.6. Western blotting

RSV-A2 mKate mixed with sample loading buffer was boiled for 10 min and then separated by SDS-PAGE. Separated viral proteins were transferred onto nitrocellulose membranes (Whatman PLC, UK). The membranes were blocked for 1 h with 5% skim milk in PBS and washed in PBS containing 0.05% Tween 20 (PBS-T). The nitrocellulose membranes were then incubated with primary Ab (RSV F mAb motavizumab and RSV N mAb 11H8) for 1 h. Blots were washed three times with PBS-T and incubated with HRP-conjugated goat anti-mouse or human IgG Ab (1:8000, Abcam, Cambridge, UK). After five washes with PBS-T, immunoreactive bands were developed using a chemiluminescent substrate, luminol enhancer solution and stable peroxide solution (1:1, Thermo Fisher Scientific, San Jose, CA, USA) and detected using ImageQuant LAS 4000.

2.7. Quantitative real-time PCR

Viral RNA was extracted from 200 μL of RSV-A2 mKate incubated for various amounts of time at 4 °C using a commercial kit (Qiagen, Germany). qPCR was performed using a probe against the N protein gene of RSV (forward primer: 5′-CTC AAT TTC CTC ACT TCT G-3′; reverse primer: 5′-CTT GAT TCC TCG GTG TAC CTC TGT-3′; probe: 5′-(FAM) TCC CAT TAT GCC TAG GCC AGC AGC A(BHQ1)-3′). The pMD18-T-N plasmid was constructed and served as the qPCR standard. Viral RNA was amplified in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The threshold fluorescence level was set automatically by the software. The threshold cycle (Ct) value and the nucleic acid level were determined automatically for each sample.

3. Results

3.1. Comparison of a fluorescence plate reader-based RSV neutralization assay and a flow cytometry-based RSV neutralization assay

To compare the use of a fluorescence plate reader to collect the fluorescent intensity versus the use of flow cytometry to detect the rate of positively infected cells, the sensitivity and correlation of these two neutralization assays were first compared by detecting HEp-2 cells infected with RSV-A2 mKate at MOIs ranging from 0.25 to 128. The plate reader showed a similar sensitivity to that of flow cytometry. A linear regression analysis demonstrated a significant correlation between the fluorescence intensity from the plate reader and the positive infection rate from flow cytometry at MOIs from 0.25 to 128 ($R^2 = 0.9737$), particularly at MOIs from 1 to 16 ($R^2 = 0.9785$) (Fig. 1A). Furthermore,

Download English Version:

https://daneshyari.com/en/article/8746982

Download Persian Version:

https://daneshyari.com/article/8746982

Daneshyari.com