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Development of a pseudovirus based assay for measuring neutralizing antibodies against coxsackievirus B5

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ABSTRACT

Coxsackievirus B5 (CV-B5), an important Coxsackie B virus from genus *Enteroviruse* within the family *Picornaviridae*, has also been isolated from Hand, Foot, and Mouth Disease (HFMD) patients, and often associated with neurological manifestations. In this study, we found out that Coxsackievirus B3 (CV-B3) replicon RNA could be encapsidated with CV-B5 capsid to assemble infectious CV-B5 pseudovirus. We then utilized this single round infection system of CV-B5 to develop a neutralizing antibody quantification assay. This pseudovirus neutralization assay showed superiority in biosafety, sensibility, quantitativity, efficiency and high throughput, and would facilitate the epidemiological studies and vaccine development of CV-B5.

1. Introduction

Coxsackievirus B5 (CV-B5) is one of the most prominent serotypes of Coxsackie B viruses which belong to the genus *Enterovirus* within the family *Picornaviridae*. CV-B5 infection in human is often associated with serious neurological symptoms like aseptic meningitis. Although less common than neurological symptoms, both cardiomyopathy and diabetes have also been reported for some CV-B5 infection cases (Grumbach et al., 1999; Marier et al., 1975). Meningitis cases caused by CV-B5 have been reported in many countries, including the United States (Kopecka et al., 1995; Tavakoli et al., 2008), Belgium (Thoelen et al., 2003), Greece (Papa et al., 2006), France (Antona et al., 2007), Spain (Trallero et al., 2010), Brazil (Dos Santos et al., 2006), Korea (Baek et al., 2011; Lee et al., 2007), and India (Kumar et al., 2011), among others. In China, sporadic CV-B5 infection cases are frequent, and CV-B5 outbreaks have also been reported (Chen et al., 2013; Yen et al., 2009).

Although enterovirus 71(EV-A71) and coxsackievirus A16 (CV-A16) have been first recognized as the major pathogens responsible for Hand, Foot, and Mouth Disease (HFMD) outbreaks (WHO, 2011), several other enteroviruses have also been isolated from patients since HFMD was listed as a notifiable disease in the national surveillance systems of many countries: coxsackievirus B5 (CV-B5) (Han et al., 2012; Hu et al., 2012); coxsackievirus A6 (CV-A6) (Bian et al., 2015; Fujimoto et al., 2012; Osterback et al., 2009); coxsackievirus A10 (CV-A10) (Lu et al., 2012); coxsackievirus B3 (CV-B3) (Gao et al., 2016a; Wu et al., 2013b) (Fujimoto et al., 2012; Wang et al., 2011; Wu et al., 2010). CV-B5 infection with neurological manifestation is now recognized as a serious threat for HFMD control and efforts are underway to develop prophylactic vaccines for CV-B5 (Klein and Chong, 2015).

It has been shown many times that the detection methods used to monitor neutralizing antibodies (NtAbs) titers can predict the *in vivo* protection efficacy of vaccines(Plotkin, 2010) (Jin et al., 2016). Further, data acquired via these detection methods have helped generate predictive insights in epidemiological studies (*e.g.* historical infections and population immunity) (Gao et al., 2016b; Ji et al., 2012). Current conventional practices for anti-enterovirus NtAbs titer measurement employ microtiter plate neutralization assays based on the inhibitory effect of these NtAbs on the cytopathic effect (CPE) in cells (Chonmaitree et al., 1988). Although these methods have been in use for a long time, there are biosafety concerns about their use, and these assays have other major shortcomings. They are only semi-quantitative, labor-intensive, and time-consuming which requires at least 5–7 days before results can be obtained. These weaknesses have highlighted the need to develop superior alternative methods for the quantification of NtAbs titers.

Genetically modified pseudoviruses expressing luciferase reporter have been used to measure NtAbs. These pseudovirus-based neutralization assays offer superior biosafety and improved sensitivity performance over assays with wild type viruses. It is thus not surprising that pseudovirus assays are increasingly popular and are now used to measure NtAbs titers against Human Immunodeficiency virus (HIV) (Montefiori et al., 2005), Influenza virus (Tsai et al., 2009), and Severe Acute Respiratory Syndrome coronavirus (SARS-coV) (Li et al., 2003;

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Sui et al., 2004). In the past, our research group has been focused on HFMD associated enteroviruses. We have to date developed infectious pseudoviruses which do not cause CPE effect on cells and established safe and efficient pseudovirus-based NtAbs detection methods for enterovirus 71(EV-A71) (Wu et al., 2013a), coxsackievirus A16 (CV-A16) (Hao et al., 2016), and coxsackievirus B3 (CV-B3) (Chen et al., 2016). Here, we successfully established a robust pseudovirus infection system for CV-B5 and subsequently developed a pseudovirus-based neutralization assay for the measurement of anti-CV-B5 NtAbs titers. Direct comparison of traditional CPE-based microtiter plate neutralization assays for CV-B5 against our new pseudovirus-based assay revealed that our new method is superior in terms of biosafety, detection limit, and quantitative performance. Our new method should thus be viewed as a desirable replacement for previously-standard CPE assays for the quantification of anti-CV-B5 NtAbs titers.

2. Materials and methods

2.1. Cell lines, virus and antisera

Human embryonic kidney (HEK)-293T cells were maintained in Dulbecco's modified essential media (DMEM) (Thermo) containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Thermo) at 37 °C in a 5% CO_2 atmosphere. Human rhabdomyosarcoma (RD) cells, Vero cells and Hela cell were maintained in Minimum Essential Media (MEM) (Thermo) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere.

CV-B5 strain 417 (417/JS/CHN/2013, Genbank accession NO. KY303900) was isolated from a three-year-old patient diagnosed with herpangina in Pizhou city in China.

The following antisera were generated via immunization of mice: Mouse anti-CVA16 serum (immunogen: inactivated CVA16 virus strain G10); Mouse anti-EV-A71 serum (immunogen: inactivated EV71 virus FY523 (Genbank accession number. EU703812, subtype C4)); Mouse anti-coxsackievirus B3 (CV-B3) serum (immunogen: inactivated CV-B3 strain 112 (DH16G/JS/2012, Genbank accession number. KP036480)); Mouse anti-Hepatitis E virus (HEV) (immunogen: HEV vaccine Hecolin® produced by Xiamen Innovax Biotech Co.,Ltd); Mouse anti-CV-A6 serum (immunogen: CV-A6 strain TW-2007-00141 which was kindly provided by Dr. Ningshao Xia (GenBank accession number: KR706309)); Mouse anti-CV-B5 (immunogen: inactivated CV-B5 strain 417)

2.2. Human plasma samples

Plasma samples were collected from 234 healthy adult donors at blood centers established by Hualan Biological Engineering Inc., approved by local Ethical Review Boards. Written informed consent was obtained from each participant.

2.3. Cloning of full-length CV-B5and production of wild-type CV-B5virus

5×10^6 Hela cells were infected with CV-B5 (417) with an M.O.I (Multiplicity of infection) of 0.1, total RNA was isolated with Trizol reagent when 80–90% cells had rounded up, and was reversed transcribed using SuperScript® III RT (Thermo). Full length cDNA of CV-B5 was amplified using Phusion high-fidelity DNA polymerase (New England Biolabs) with two primers:CVB5-NotI-T7-5′UTR-F(5′-TcaagaattgcggccgcgtaatacgactcactataggTTTTAAAACAGGCTGTGGG-3′) and CVB5-SalI-polyA-3′UTR-R (5′-CATGAGAATTGTCGACTTTTTTTTT TTTTTTTTTTTTTTTT-3′). The amplification product was then inserted into vector pSVA using an In-Fusion® HD Cloning Kit (Clontech) and was designated as pSVA-CVB5. The T7 promoter sequence was added to the 5′ end of cDNA prepared from the viral genome. The sequence of full length CV-B5 cDNA on pSVA-CVB5 was verified by Sanger sequencing.

Infectious wild type CV-B5 virus was rescued by co-transfection of Hela cells with pSVA-CVB5 and a plasmid expressing T7 polymerase (pcDNA3.0-T7 polymerse). Briefly, equal amounts of these two plasmids were co-transfected using jetPRIME® (Polyplus) into Hela cells grown to 90–100% confluence. Supernatant samples containing infectious CV-B5 virus were collected when 80–90% of co-transfected cells showed CPE. After debris clarification by centrifugation, CV-B5 was stored at −80 °C in aliquots.

2.4. CV-B5 capsid expresser

The CV-B5 capsid gene was amplified from pSVA-CVB5, and the *EGFP* gene was inserted upstream of the CV-B5 capsid gene with a 2A protease self cleavage site (AITTL).The EGFP reporter was used for monitoring both transfection efficiency and the expression level of the four viral structural genes.

2.5. Pseudovirus production

Pseudovirus was produced by co-transfection of replicon plasmid (pEV71-replicon-fluc or pCVB3-replicon-fluc), capsid expresser (EV-A71 capsid expresser, CV-B3 capsid expresser or CV-B5 capsid expresser), and pcDNA3.0A-T7 polymerase. Briefly, the three plasmids were mixed at a 1:1:1 ratio and were then reverse transfected into HEK-293T cells at 80% confluence with jetPRIME® (Polyplus). At 48 h post capsid transfection, virions were harvested from supernatants and from cell lysates following 2 freeze-thaw cycles; after debris clarification by centrifugation, samples were stored at −80 °C in aliquots

2.6. Quantification of CV-B3 (Nancy)-luc pseudovirus with qPCR

10 μL of pseudovirus supernatant was initially treated with 1U of DNase I (New England Biolabs) to remove plasmid residue contamination. Viral RNA was then extracted with a QIAamp Viral RNA Mini Kit (Qiagen). cDNA was synthesized with a PrimeScript RT Reagent Kit (Takara). Genome copy equivalents were quantified by qPCR using primers targeting firefly luciferase reporter gene (SYBR Premix Ex Taq II, Perfect Real Time) (Takara) (qLuc-F: 5′-caaatacgatttatctaatttacacga-3′; qLuc-R: 5′-ccggtatccagatccacaac-3′).

2.7. Neutralization assay with CV-B5 pseudovirus

Plasma samples were heat-treated at 56 °C for 30 min to inactivate complements. Plasma samples, in two-fold serial dilutions ranging from 1:8 to 1:1024, were mixed with an equal volume of diluted CV-B5 pseudovirus (50 μL). After incubation at 37 °C for 1 h, 100 μL of an RD single cell suspension (5×10^5/mL) was added and incubated in a 5% CO_2 incubator for 16 h. Next, the incubation medium was discarded and cells were lyzed in 50 μL of 1 × passive lysis buffer (Promega) with two freeze-thaw cycles. Luciferase activity, reported in relative light units (RLUs), was measured according to the user's manual for the luciferase assay system (Berthold). The viral inhibition ratio was calculated as: $[1- (RLU_{serum/plasma}-RLU_{background})/(RLU_{viruscontrol}-RLU_{background})] \times 100$. The titer of NtAbs was defined as the reciprocal of the dilution at which 50% of the pseudovirus had been neutralized (pNT50).

2.8. Microtiter plate neutralization assay based on CPE

As previously described (Wu et al., 2013a), two-fold serial diluted sera (starting from 1:8) were mixed with an equal volume (50 μL) of virus working solution containing 100 TCID50/well (50% of tissue