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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 Enterovirus 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) replication 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, quantitative 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 (Thoenen 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 Chomnaitree 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;
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 and 1% penicillin/streptomycin (Thermo) at 37 °C in a 5% CO2 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% CO2 atmosphere.

CV-B5 strain 417 (417/JS/CHN/2013, Genbank accession NO. KY309900) 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 strain FY523 [Genbank accession number: EU703812, subtype C4]); Mouse anti-coxsackievirus B3 (CV-B3) serum (immunogen: inactivated CV-B3 strain 112 (DH166/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-B5 and production of wild-type CV-B5 virus

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 reverse 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′-Tcaagaattgcgaaatcactattgcagttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
culture infective dose) of CV-B5 strain 417 at 37 °C for 1 h in 96-well microtiter plates. 100 μL of Vero cells (1 × 10^5/mL) were added and incubated at 37 °C for 7–9 days. Sera were tested with duplication. The neutralization titer was defined as the reciprocal of the highest dilution at which more than 50% of wells showed complete CPE inhibition. Sample with titer ≥8 were considered positive, otherwise were negative.

2.9. Statistical analysis

Experimental data were collected from, at minimum, duplicated samples. Data are here reported as the mean ± standard deviation.
3. Results

3.1. Establishment of CV-B5-luc pseudovirus for single round infection

In our previously reported pseudovirus infection systems for EV-A71 (Chen et al., 2012) and CV-B3 (Chen et al., 2016), structural genes (P1) was replaced with firefly luciferase reporter along with a 2A protease cleavage site; T7 promoter was placed upstream of 5′UTR to initiate transcription of the subgenomic replicon (Fig. 1A). Replicon RNA could be encapsidated by capsid proteins when respective capsid expresser was provided. We first attempted to produce CV-B5 pseudovirus with CV-B5 replicon RNA and CV-B5 capsid; however, CV-B5 pseudovirus encapsidation was not successful using in vitro transcribed CV-B5 replicon RNA. We next tried co-transfection of CV-B5 replicon, CV-B5 capsid expresser, and pcdNA3.0-T7 polymerase; but in vivo transcribed CV-B5 replicon RNA also failed to produce CV-B5 pseudovirus with luciferase reporter (data not shown). Inspired by our finding that CV-A16 could be pseudotyped with EV-A71 replicon RNA and CV-A16 capsid (Chen et al., 2016), we found that CV-B3 replicon RNA instead of EV-A71 replicon RNA could be efficiently encapsidated by CV-B5 capsid to produce infectious CV-B5 pseudovirus (Fig. 1B). This CV-B5 pseudovirus was designated as CVB5p-fluc.

As the capsid of CVB5p-fluc was composed of CV-B5 capsid proteins, the antigenicity of CV-B5 wild type virus was highly preserved on CVB5p-fluc; thus infection of CVB5p-fluc to Hela cell, a CV-B5 permissive cell line, could be inhibited by mouse anti-CV-B5 serum in a dose dependent manner (Fig. 1C). Specificity of CVB5p-fluc was further confirmed by a panel of mouse antisera against other enteroviruses (CV-B3, CV-A6, CV-A16, and EV-A71) as well as hepatitis E virus (HEV) (Fig. 1D).

Taken together, these results showed that CV-B5 could be efficiently pseudotyped with CV-B3 replicon RNA and CV-B5 capsid, and this CV-B5 pseudovirus CVB5p-fluc could be used as a surrogate for wild type virus. Since CVB5p-fluc could only elicit single round infection, it could be an excellent tool for studying viral entry process of CV-B5 as well as the molecular interaction between viral capsid and viral receptors. Besides, CVB5p-fluc could also provide a superior way for anti-CVB5 NtAbs detection, so we next aimed to develop an in vitro neutralization assay based on CVB5p-fluc.

3.2. Optimization of pseudovirus luciferase assay

We first compared relative infectivity of CVB5p-fluc on three common cell lines reported to be permissive to CV-B5 infection and chose RD cell line for assay development (Fig. 2A). Cell number was further optimized for maximal readout (relative light units, RLUs) (Fig. 2B). Further, CV-B5 single round infection system showed superior quantitative measurement of infection events in terms of good linear correspondence between RLUs and the amount of CVB5p-fluc virus input over a broad range (Fig. 2C). In our pilot study, three human plasma samples from healthy adult donors were analyzed using CVB5p-fluc pseudovirus-based neutralization assay, and anti-CV-B5 NtAbs could be detected in these samples at various levels (Fig. 2D).

3.3. Comparison of pseudovirus luciferase assay with traditional CPE based assay

As semi-quantitative CPE assay is traditionally considered as a golden method for the measurement of NtAbs against enteroviruses, we next performed a comparison study and quantified anti-CV-B5 NtAbs in human plasma samples with both pseudovirus assay and CPE assay. NtAbs titers from CPE assay were defined as the reciprocal of the highest dilution at which over 50% of wells showed complete inhibition of CPE (CCID50), sample with titer ≥ 8 were considered positive, otherwise were negative. As for pseudovirus assay, titers were defined as the reciprocal of the dilution at which 50% of the pseudovirus had been neutralized (pNT50), which could be interpolated from neutralization curve after nonlinear regression analysis with Graphpad Prism. Titers from two assays were then statistically analyzed.

207 out of 239 (86.6%) plasma samples were positive in CPE assay, while 190 out of 207 CPE positive samples were also positive in pseudovirus assay. Sensitivity and specificity, as well as the PPV and NPV under our experimental condition are 96.4% and 59.5%, 0.92 and 0.78, respectively. Titers of these 190 plasma samples were used for comparison analysis. Spearman correlation analysis showed that there was good correlation between the results from these two assays ($r^2 = 0.72$, $P < 0.0001$) (Fig. 3A). Bland-Altman method comparison analysis further showed that these two methods were highly consistent. The average of logarithmic difference for quantitative results of the two methods was −0.0709, and the standard deviation was 0.3075 (Fig. 3B).

4. Discussion

We failed in our initial attempt to use CV-B3 replicon RNA to produce a CV-B5 pseudovirus. The instability of CV-B5 replicon RNA in pseudovirus producing cells (HEK-293T) had not been anticipated (data not shown). However, replicon RNA from EV-A71 and CV-B3 were comparatively stable during pseudovirus production. Phylogenetic studies that revealed intra-serotypic recombination in some clinical CV-B5 isolates, suggesting that CV-B5 may have an unstable genomic structure (Hu et al., 2012; Ma et al., 2013; Oberste et al., 2004), it is possible that our genetic modifications of the CV-B5 genome may have further aggravated this putative instability.

Our pseudovirus infection system for CV-B5 represents yet another successful application of the trans-encapsidation strategy to produce an infectious pseudovirus. CV-B3 replicon RNA could be encapsidated with CV-B5 capsid to produce infectious pseudovirus; EV-A71 replicon RNA could not be encapsidated with CV-B5 capsid. We previously reported that EV-A71 replicon RNA could be encapsidated with CV-A16 capsid to produce an infectious CV-A16 pseudovirus, while CV-B3 replicon RNA could not be encapsidated with CA-A16 capsid (Chen et al., 2016). These patterns of compatibility in trans-encapsulation experiments suggested that similar virion assembly mechanisms may be shared between CV-B3 and CV-B5, and between EV-A71 and CV-A16. It is notable that both EV-A71 and coxsackievirus (CV) CV-A16 are group A enteroviruses and are known to share the same viral receptor: human scavenger receptor class B, member 2 (SCARB2) (Yamayoshi et al., 2009). Similarly, CV-B3 and CV-B5 are group B enteroviruses that share a receptor: coxsackievirus-adenovirus receptor (CAR) (Bergelson et al., 1997; Tomko et al., 1997). In light of these relationships, it would be intriguing to classify many enteroviruses accordingly and explore likely major determinants involved in enterovirus assembly. We anticipate that replicon RNA from EV-A71 and CV-B3 could be used as probes to analyze the assembly compatibility among other group A and group B enteroviruses.

In this work, we developed a sensitive NtAbs quantification assay based on this CV-B5 pseudovirus. We found that CV-B3 NtAbs positivity in adults were very high. We compared the pseudovirus assay with CPE assay, which is referred as a gold standard assay for the detection of anti-enterovirus NtAbs. Although improvement on specificity was needed in the future, this pseudovirus based assay provided a superior replacement for anti-CV-B5 NtAbs detection. Development of anti-serum reference standard for CV-B5 would help to optimize this pseudovirus assay, so that proper cutoff value to achieve the highest Youden’s index could be set to produce the best balance of overall...
agreement, sensitivity, and specificity with respect to the reference CPE assay. To date, we had established pseudovirus based NtAbs quantification assay for EV-A71, CV-A16, CV-B3, and CV-B5. Following the successful licensure of EV-A71 vaccine in China, changes in HFMD pathogen spectrum have been noticed and multivalent vaccines against more health threatening enteroviruses co-circulating in HFMD epidemics have been proposed to be ideally for HFMD control (Klein and Chong, 2015; Mao et al., 2016). There are challenges in many aspects of multivalent vaccine development, e.g. selection of potential vaccine strains (Klein and Chong, 2015). Further, safe, convenient and robust bioassays as well as animal models for efficacy evaluation are necessarily required. These aforementioned pseudovirus-based NtAbs quantification assays for HFMD associated enteroviruses could be used for evaluation of vaccine induced humoral immune response. Establishment of reference standards like antiserum standards for measuring vaccine-induced NtAb titers is also an important part for bioassay development.
development (Cooper et al., 2015; Liang et al., 2011). Furthermore, a new EV-A71 infection mouse model had been successfully established based on EV-A71 pseudovirus, which also showed impressive advantages in efficacy evaluation of EV-A71 vaccine (Zhou et al., 2016). Thus, introduction of enterovirus pseudoviruses (e.g., CV-A16, CV-B3 and CV-B5) to mouse infection models would also greatly facilitate the development of multivalent vaccine for HFMD.

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References

Antona, D., Deveque, N., Chomel, J.-J., Leveque, N., Chomel, J.-J., Dubrou, S., Levy-Bruhl, D., Lina, B., 2007. Rapid isolation of enteroviruses. J. Clin. Microbiol. 26, 2576–2580.

Baeck, K., Yeo, S., Lee, B., Park, K., Song, J., Yu, J., Rhee, I., Kim, J., Hwang, S., Choi, Y., Cheon, D., Park, J., 2011. Epidemiology of enterovirus infection in Chungnam Province, South Korea. J. Microbiol. Biotechnol. 21, 1361–1366.

Baek, K., Yeo, S., Lee, B., Park, K., Song, J., Yu, J., Rhee, I., Kim, J., Hwang, S., Choi, Y., Cheon, D., Park, J., 2011. Epidemiology of enterovirus infection in Chungnam Province, South Korea. J. Microbiol. Biotechnol. 21, 1361–1366.

Bergelson, J.M., Cunningham, J.A., Droegert, G., Kurt-Jones, E.A., Krivit, A.H., Hong, J.S., Horwitz, M.S., Crowell, R.L., Finberg, R.W., 1997. Isolation of the common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275, 1320–1323.

Bicho, P., Fernandes, L., Cunha, D., de Moraes, M., Xo, M., Lasso, I., 2015. Coxsackievirus A6: a new emerging pathogen causing hand, foot and mouth disease outbreaks worldwide. Expert Rev. Anti Infect. Ther. 13, 1061–1071.

Chen, P., Song, Z., Qi, Y., Feng, X., Xu, N., Sun, Y., Wu, X., Yao, M., Qiao, Li, Xi, Dong, W., Wan, X., Hu, N., Sheng, X., Liang, Z., Li, W., 2012. Molecular determinants of enterovirus 71 viral entry: clef around GLN-172 on VP1 protein interacts with the variable region on scavenge receptor B2. J. Biol. Chem. 287, 6406–6420.

Chen, P., Tao, Z., Song, Y., Liu, G., Wang, H., Liu, Y., Song, L., Li, Y., Lin, X., Cui, N., Xu, A., 2013. A coxsackievirus B5-associated aseptic meningitis outbreak in shandong province, China in 2009. J. Med. Virol. 85, 483–490.

Chen, P., Wu, X., Yao, Q., Qiao, F., Hua, X., Liao, Z., Gu, F., Li, W., Xu, M., Liang, Z., 2016. A rapid and quantitative assay for measuring neutralizing antibodies of Coxsackievirus B3. J. Virol. Methods 232, 1–7.

Chonnahrntree, T., Ford, C., Sandend, C., Lai, H.L., 1988. Comparison of cell cultures for introduction of enterovirus pseudoviruses (e.g. CV-A16, CV-B3 and CV-B5) to mouse infection models would also greatly facilitate the development of multivalent vaccine for HFMD.

Coligan, J.E., Dunn, W.C., Hartley, J.B., Boyse, E.A., 1990. Lymphocyte Functions Analysis. 1: pp. 7–42. Hum. Vaccines Immunother. 11, 2688–2691.

Plotkin, S.A., 2010. Correlates of protection induced by vaccination. Clin. Vaccine Immunol. 17, 1055–1065.

Sullivan, J.L., Luzuriaga, K., Greenough, T.C., Choe, H., Farzan, M., 2003. Measurement of neutralizing antibody responses for evaluation of enterovirus 71 vaccines. Proc. Natl. Acad. Sci. U. S. A. 100, 2536–2541.

Takavaki, N.P., Wang, H., Nathannatt, S., Dupuis, M., Fusco, H., Hull, R., 2008. Detection and typing of enteroviruses from CSF specimens from patients diagnosed with meningitis/encephalitis. J. Clin. Microbiol. 46, 688–691.

Wan, X., Huang, N., Shen, X., Liang, Z., Li, W., 2012. Molecular determinants of enterovirus 71 viral entry: clef around GLN-172 on VP1 protein interacts with the variable region on scavenge receptor B2. J. Biol. Chem. 287, 6406–6420.

WHO, 2011. A Guide to Clinical Management and Public Health Response for Hand. Foot and Mouth Disease (HFMD). World Health Organization.

Wu, Y., Yeo, A., Phoon, M.C., Tan, E.L., Poh, C.L., Quak, S.H., Chow, V.T., 2010. The introduction of enterovirus 71 and coxsackievirus A strains. Int. J. Infect. Dis.: IJID 14, e1076–e1081.

Yoshida, H., Yasui, Y., Kobayashi, M., Fujii, Y., Tanaka, H., Yamamoto, M., Shimizu, H., 2012. Hand, foot, and mouth disease caused by coxsackievirus A6, Japan, 2011. J. Med. Virol. 84, 1140–1146.