Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods 246 (2017) 21–26

Contents lists available at ScienceDirect

Journal of Virological Methods

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

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

Pan Chen[a,b], Xing Wu[b], Yao Su[b], Xiaotian Hao[b], Qunying Mao[b], Zhenglun Liang[b,*]

[a] Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Beijing 100050, China
[b] National Institutes for Food and Drug Control, Beijing 100050, China

ARTICLE INFO

Keywords:
Coxsackievirus B5
Neutralizing antibody
Pseudovirus
Single round infection

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;

* Corresponding author at: National Institutes for Food and Drug Control, No. 2 Tiantan Xili, Dongchen, Beijing, 100050 China.
E-mail address: lzhenglun@126.com (Z. Liang).

http://dx.doi.org/10.1016/j.jviromet.2017.04.005
Received 3 January 2017; Received in revised form 15 April 2017; Accepted 16 April 2017
Available online 20 April 2017

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

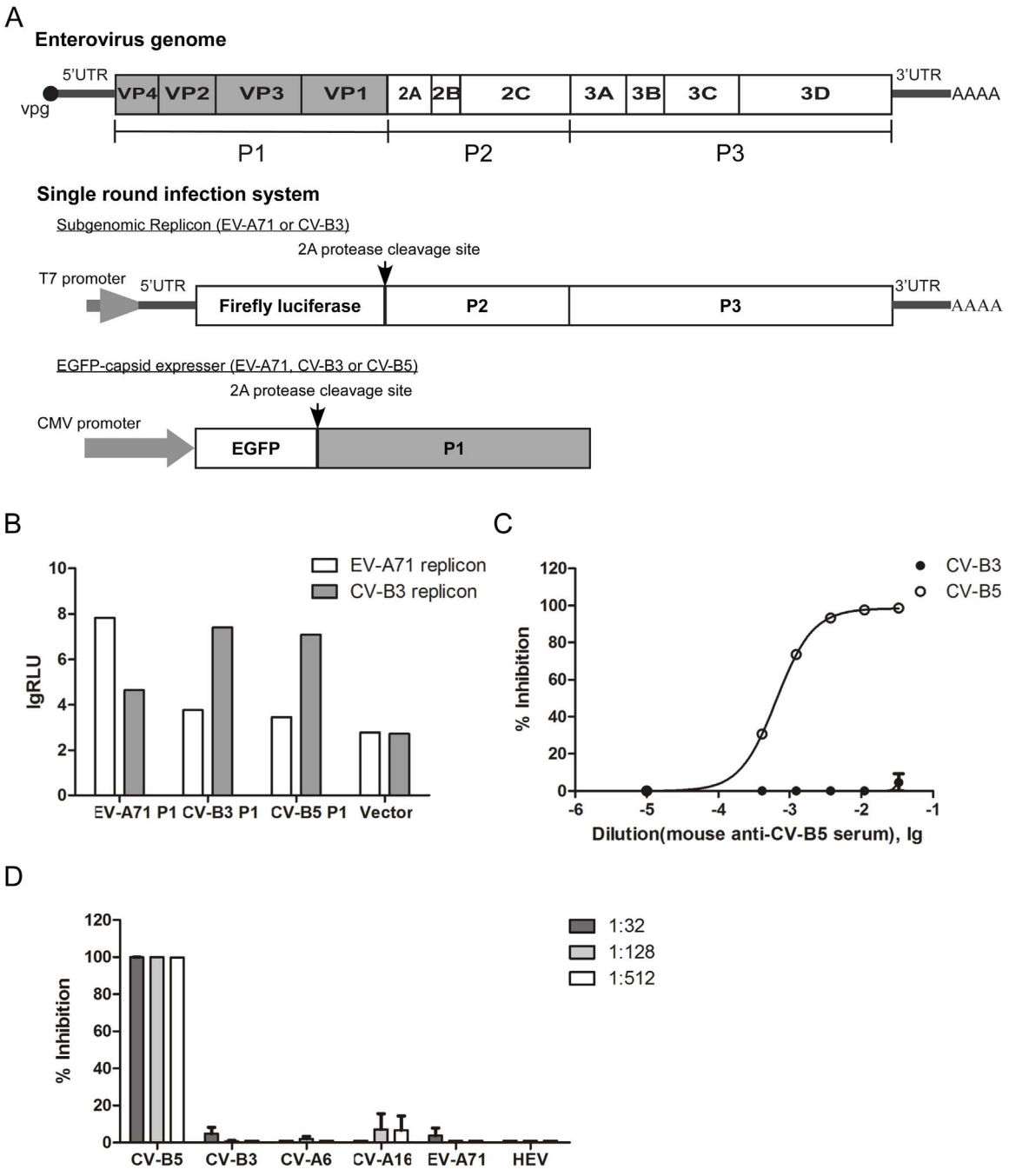

Fig. 1. Generation and verification of CVB5-luc pseudovirus. (A) Schematic diagram showing enteroviruse genome and the pseudovirus encapsidation strategy for CV-B5. Subgenomic replicons and capsid expressers of EV-A71 and CV-B3 were described previously. Briefly, replicon was produced by replacing P1 region with a firefly luciferase reporter gene in the full length genome and a T7 promoter was placed at the 5′ end for transcription initiation; capsid expresser was used to express all the capsid proteins (P1) *in trans*, the *EGFP* gene was inserted upstream of P1 separated by EV71 2A self cleavage site (-AITTL-). CV-B5 capsid expresser was constructed accordingly. (B) Trans-encapsidation compatibility between subgenomic replicons and capsid expressers. Subgenomic replicons (EV-A71 and CV-B3), capsid expressers (EV-A71, CV-B3, and CV-B5) and pcDNA3.0-T7 polymerase were co-transfected into HEK-293T cells. Supernatant samples were collected at 48 h post transfection. Hela cells seeded on 96-well plate were incubated with 20 μL of supernatant samples. Luciferase activity was measured at 16 h post infection. (C) Infection of CV-B5 pseudovirus could be neutralized by mouse anti-CV-B5 serum. 50 μL of diluted CV-B3 and CV-B5 pseudoviruses were mixed with an equal volume of serial diluted mouse anti-CV-B5 serum in duplicates, respectively. After incubation at 37 °C for 1 h, 100 μL of Hela cells (2×10^5/mL) was added and incubated at a CO_2 incubator. Luciferase activity was measured at 16 h post infection. Nonlinear regression was performed with Graphpad Prism. (D) Specificity of CV-B5 pseudovirus. 50 μL of diluted CV-B5 pseudoviruses were mixed with an equal volume of diluted mouse anti-CV-B5, CV-B3, CV-A6, EV-A71, and HEV serum samples in duplicates, respectively. After incubation at 37 °C for 1 h, 100 μL of Hela cells (2×10^5/mL) was added and incubated at CO_2 incubator. Luciferase activity was measured at 16 h post infection.

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

(SD). The nonlinear regression, Spearman correlation analysis, and Bland-Altman method comparison analysis were performed with GraphPad Prism software.

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 CVB5-luc 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 us CV-B5 replicon RNAto 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 an 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-encapsidation experiments suggested that similar virion assembly mechanisms may be shared between CV-B3 and CV-B5, and between EV-A71 and CV-A16. However, it seems likely that the trans-encapsidation mechanism adopted by CV-B3 and CV-B5 is distinct from that adopted by 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 NtAb 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

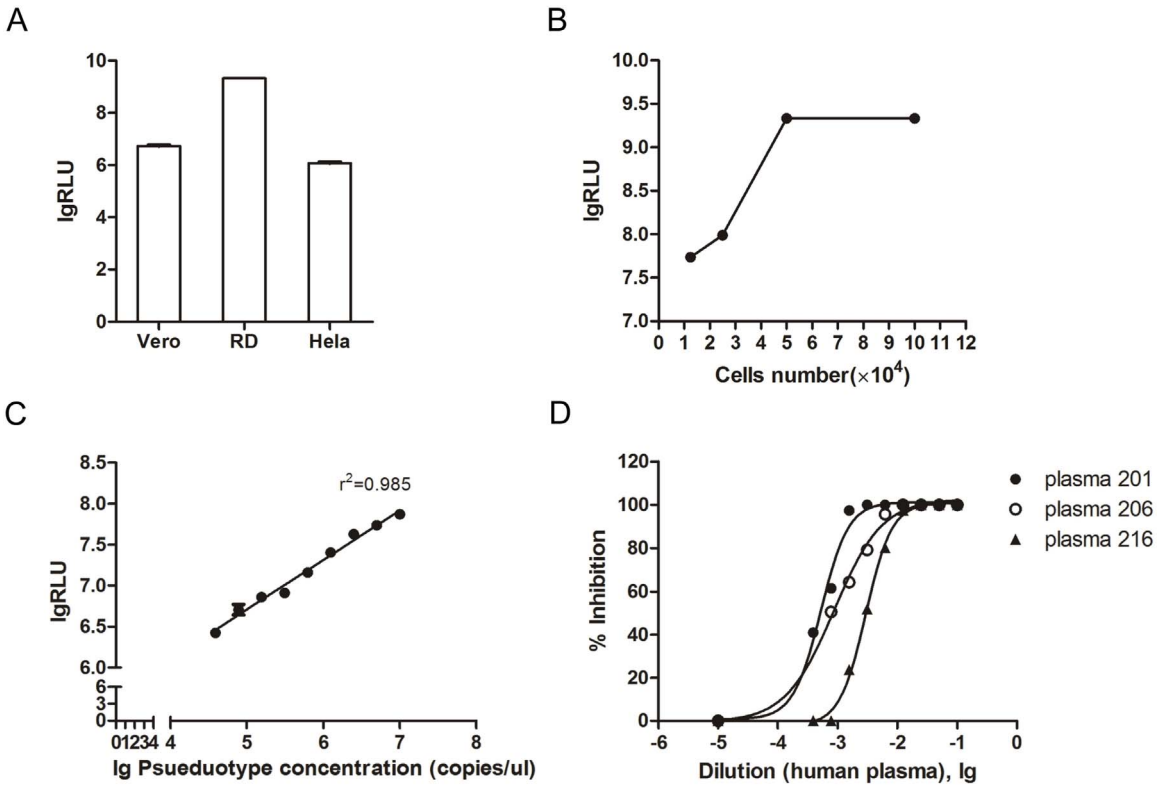

Fig. 2. Optimization of CVB5p-fluc pseudovirus luciferase assay. (A) The infectivity of CVB5p-fluc on different susceptible cell lines. 2×10^4 cells were infected with 20 μL of CVB5p-fluc and luciferase activity was measured at 16 h post infection. (B) Cell number optimization. 10 μL of CVB5p-fluc was used to infect RD cells with increasing cell density. Each data point represents the average of eight replicates. Luciferase activity was measured at 16 h post infection. (C) Linearity of the infection of CVB5p-fluc. 5×10^5 RD cells were incubated with serially diluted CVB5p-fluc (from 2×10^3 to 4×10^7 per μL). Luciferase activities were measured at 16 h post infection. Each data point represents the average of eight replicates. Linear regression analysis was performed using Graph Pad Prism. (D) Neutralization of the infection of CVB5p-fluc with human plasma samples. Equal volume of CVB5p-fluc and serial dilutions of human plasma samples were incubated in duplicates at 37 °C for 1 h, the mixed samples were then added to 5×10^5 RD cells. Luciferase activity was measured at 16 h post infection. Nonlinear regression was performed with Graphpad Prism.

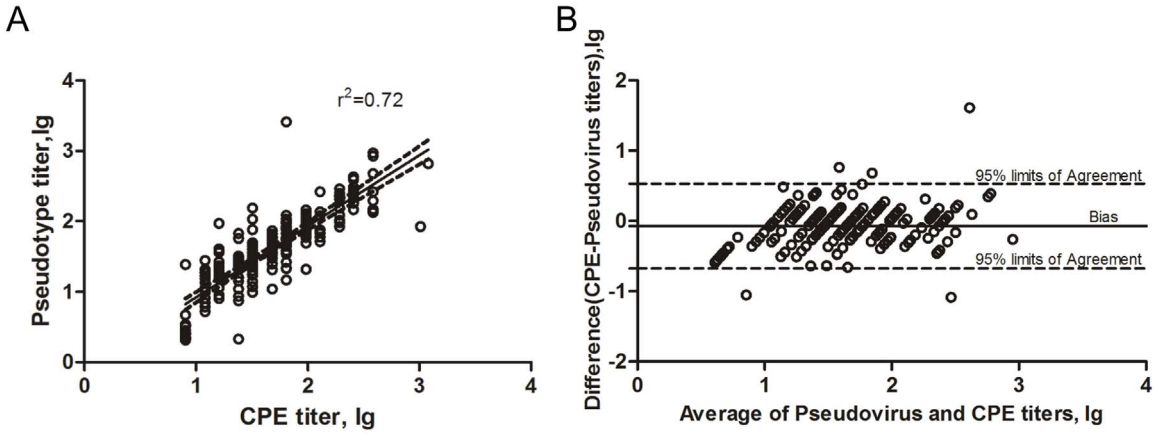

Fig. 3. Correlation analysis of CVB5p-fluc pseudovirus based neutralization assay with CPE assay (A) Spearman correlation analysis of anti-CV-B5 NtAbs titers in human plasma samples measured both by CPE assay and pseudovirus assay. Plasma samples from 190 health adult donors were considered positive in both assays. Data analysis was performed using GraphPad Prism. The solid line represents the linear regression curve. ($r^2 = 0.72$, $p < 0.0001$). Each hollow dot represents an anti-CV-B5 seropositive donor. (B) Bland-Altman method comparison analysis of CVB5 NtAbs titers in human plasma samples measured both by CPE assay and pseudovirus assay. The solid line represented the mean value, while dashed lines represented the 95% confidence limits. Each hollow dot represents an anti-CV-B5seropositive donor.

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 (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 enteroviruse 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.

Acknowledgement

This work was supported by National High Technology Research and Development Program 〈"863" program, No. 2012AA02A402〉 from the Ministry of Science and Technology of the People's Republic of China.

References

Antona, D., Leveque, N., Chomel, J.J., Dubrou, S., Levy-Bruhl, D., Lina, B., 2007. Surveillance of enteroviruses in France, 2000–2004. Eur. J. Clin. Microbiol. Infect. Dis. 26, 403–412.
Baek, K., Yeo, S., Lee, B., Park, K., Song, J., Yu, J., Rheem, I., Kim, J., Hwang, S., Choi, Y., Cheon, D., Park, J., 2011. Epidemics of enterovirus infection in Chungnam Korea, 2008 and 2009. Virol. J. 8, 297.
Bergelson, J.M., Cunningham, J.A., Droguett, G., Kurt-Jones, E.A., Krithivas, A., Hong, J.S., Horwitz, M.S., Crowell, R.L., Finberg, R.W., 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275, 1320–1323.
Bian, L., Wang, Y., Yao, X., Mao, Q., Xu, M., Liang, Z., 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, X., Mao, Q., Li, X., Dong, W., Wan, X., Huang, N., Shen, X., Liang, Z., Li, W., 2012. Molecular determinants of enterovirus 71 viral entry: cleft around GLN-172 on VP1 protein interacts with variable region on scavenge receptor B 2. 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, 483–489.
Chen, P., Wu, X., Mao, Q., Gao, F., Hao, X., Bian, L., Zhu, 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.
Chonmaitree, T., Ford, C., Sanders, C., Lucia, H.L., 1988. Comparison of cell cultures for rapid isolation of enteroviruses. J. Clin. Microbiol. 26, 2576–2580.
Cooper, G., Mao, Q., Crawt, L., Wang, Y., Dougall, T., Rigsby, P., Liang, Z., Xu, M., Minor, P., Wang, J., Martin, J., 2015. Report on the WHO Collaborative Study to Establish the 1 St International Standard for Anti-EV71 Serum. WHOpp. 39.
Dos Santos, G.P., Skraba, I., Oliveira, D., Lima, A.A., de Melo, M.M., Kmetzsch, C.I., da Costa, E.V., da Silva, E.E., 2006. Enterovirus meningitis in Brazil, 1998–2003. J. Med. Virol. 78, 98–104.
Fujimoto, T., Iizuka, S., Enomoto, M., Abe, K., Yamashita, K., Hanaoka, N., Okabe, N., 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. Emerg. Infect. Dis. 18, 337–339.
Gao, F., Bian, L.L., Mao, Q.Y., Chen, P., Yao, X., Li, J.X., Zhu, F.C., Liang, Z.L., 2016a. An epidemic of coxsackievirus B3 infection in infants and children in Jiangsu Province, China: a prospective cohort study. Arch. Virol. 161, 1945–1947.
Gao, F., Mao, Q.Y., Chen, P., Bian, L.L., Yao, X., Li, J.X., Zhu, F.C., Liang, Z.L., 2016b. Seroepidemiology of Coxsackievirus A6, Coxsackievirus A16, and Enterovirus 71 infections in infants and children: a prospective cohort study in Jiangsu, China. J. Infect. 73, 509–512.
Grumbach, I.M., Heim, A., Pring-Akerblom, P., Vonhof, S., Hein, W.J., Muller, G., Figulla, H.R., 1999. Adenoviruses and enteroviruses as pathogens in myocarditis and dilated cardiomyopathy. Acta Cardiol. 54, 83–88.
Han, J.F., Jiang, T., Fan, X.L., Yang, L.M., Yu, M., Cao, R.Y., Wang, J.Z., Qin, E.D., Qin, C.F., 2012. Recombination of human coxsackievirus B5 in hand, foot, and mouth disease patients, China. Emerg. Infect. Dis. 18, 351–353.
Hao, X., Chen, P., Mao, Q., Shao, J., Lin, H., Luo, Z., Wu, X., Liang, Z., 2016. Establishment of a Pseudovirus Luciferase Assay for the detection of coxsackievirus A16 neutralizing antibody. Chin. J. Viral Dis. 6–11.
Hu, Y.F., Zhao, R., Xue, Y., Yang, F., Jin, Q., 2012. Full genome sequence of a novel coxsackievirus B5 strain isolated from neurological hand, foot, and mouth disease patients in China. J. Virol. 86, 11408–11409.
Ji, H., Li, L., Liu, Y., Ge, H., Wang, X., Hu, J., Wu, B., Fu, J., Zhang, Z., Chen, X., Zhang, M., Ding, Q., Xu, W., Tang, F., Zhou, M., Wang, H., Zhu, F., 2012. Seroepidemiology of human enterovirus71 and coxsackievirusA16 in Jiangsu province, China. Virol. J. 9, 248.
Jin, P., Li, J., Zhang, X., Meng, F., Zhou, Y., Yao, X., Gan, Z., Zhu, F., 2016. Validation and evaluation of serological correlates of protection for inactivated enterovirus 71 vaccine in children aged 6–35 months. Hum. Vaccines Immunother. 12, 916–921.
Klein, M., Chong, P., 2015. Is a multivalent hand, foot, and mouth disease vaccine feasible? Hum. Vaccines Immunother. 11, 2688–2704.
Kopecka, H., Brown, B., Pallansch, M., 1995. Genotypic variation in coxsackievirus B5 isolates from three different outbreaks in the United States. Virus Res. 38, 125–136.
Kumar, A., Shukla, D., Kumar, R., Idris, M.Z., Misra, U.K., Dhole, T.N., 2011. An epidemic of encephalitis associated with human enterovirus B in Uttar Pradesh, India, 2008. J. Clin. Virol. 51, 142–145.
Lee, S.T., Ki, C.S., Lee, N.Y., 2007. Molecular characterization of enteroviruses isolated from patients with aseptic meningitis in Korea, 2005. Arch. Virol. 152, 963–970.
Li, W., Moore, M.J., Vasilieva, N., Sui, J., Wong, S.K., Berne, M.A., Somasundaran, M., Sullivan, J.L., Luzuriaga, K., Greenough, T.C., Choe, H., Farzan, M., 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 426, 450–454.
Liang, Z., Mao, Q., Gao, Q., Li, X., Dong, C., Yu, X., Yao, X., Li, F., Yin, W., Li, Q., Shen, X., Wang, J., 2011. Establishing China's national standards of antigen content and neutralizing antibody responses for evaluation of enterovirus 71 (EV71) vaccines. Vaccine 29, 9668–9674.
Lu, Q.B., Zhang, X.A., Wo, Y., Xu, H.M., Li, X.J., Wang, X.J., Ding, S.J., Chen, X.D., He, C., Liu, L.J., Li, H., Yang, H., Li, T.Y., Liu, W., Cao, W.C., 2012. Circulation of coxsackievirus A10 and A6 in hand-foot-mouth disease in China, 2009–2011. PLoS One 7, e52073.
Ma, H., Huang, X., Kang, K., Li, X., Tang, X., Ren, Y., Wang, Y., Zhao, G., Xu, B., 2013. Recombination in human coxsackievirus B5 strains that caused an outbreak of viral encephalitis in Henan, China. Arch. Virol. 158, 2169–2173.
Mao, Q., Wang, Y., Bian, L., Xu, M., Liang, Z., 2016. EV-A71 vaccine licensure: a first step for multivalent enterovirus vaccine to control HFMD and other severe diseases. Emerg. Microbes Infect. 5, e75.
Marier, R., Rodriguez, W., Chloupek, R.J., Brandt, C.D., Kim, H.W., Baltimore, R.S., Parker, C.L., Artenstein, M.S., 1975. Coxsackievirus B5 infection and aseptic meningitis in neonates and children. Am. J. Dis. Child. 129, 321–325.
Montefiori, D.C., et al., 2005. Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. Curr. Protoc. Immunol. 11 /edited by John E. Coligan. [et al.] Chapter, Unit 12.
Oberste, M.S., Penaranda, S., Pallansch, M.A., 2004. RNA recombination plays a major role in genomic change during circulation of coxsackie B viruses. J. Virol. 78, 2948–2955.
Osterback, R., Vuorinen, T., Linna, M., Susi, P., Hyypia, T., Waris, M., 2009. Coxsackievirus A6 and hand foot, and mouth disease, Finland. Emerg. Infect. Dis. 15, 1485–1488.
Papa, A., Dumaidi, K., Franzidou, F., Antoniadis, A., 2006. Genetic variation of coxsackie virus B5 strains associated with aseptic meningitis in Greece. Clin. Microbiol. Infect. 12, 688–691.
Plotkin, S.A., 2010. Correlates of protection induced by vaccination. Clin. Vaccine Immunol.: CVI 17, 1055–1065.
Sui, J., Li, W., Murakami, A., Tamin, A., Matthews, L.J., Wong, S.K., Moore, M.J., Tallarico, A.S., Olurinde, M., Choe, H., Anderson, L.J., Bellini, W.J., Farzan, M., Marasco, W.A., 2004. Potent neutralization of severe acute respiratory syndrome (SARS) coronavirus by a human mAb to S1 protein that blocks receptor association. Proc. Natl. Acad. Sci. U. S. A. 101, 2536–2541.
Tavakoli, N.P., Wang, H., Nattanmai, S., Dupuis, M., Fusco, H., Hull, R., 2008. Detection and typing of enteroviruses from CSF specimens from patients diagnosed with meningitis/encephalitis. J. Clin. Virol. 43, 207–211.
Thoelen, I., Lemey, P., Van Der Donck, I., Beuselinck, K., Lindberg, A.M., Van Ranst, M., 2003. Molecular typing and epidemiology of enteroviruses identified from an outbreak of aseptic meningitis in Belgium during the summer of 2000. J. Med. Virol. 70, 420–429.
Tomko, R.P., Xu, R., Philipson, L., 1997. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc. Natl. Acad. Sci. U. S. A. 94, 3352–3356.
Trallero, G., Avellon, A., Otero, A., De Miguel, T., Perez, C., Rabella, N., Rubio, G., Echevarria, J.E., Cabrerizo, M., 2010. Enteroviruses in Spain over the decade 1998–2007: virological and epidemiological studies. J. Clin. Virol. 47, 170–176.
Tsai, C., Caillet, C., Hu, H., Zhou, F., Ding, H., Zhang, G., Zhou, B., Wang, S., Lu, S., Buchy, P., Deubel, V., Vogel, F.R., Zhou, P., 2009. Measurement of neutralizing antibody responses against H5N1 clades in immunized mice and ferrets using pseudotypes expressing influenza hemagglutinin and neuraminidase. Vaccine 27, 6777–6790.
WHO, 2011. A Guide to Clinical Management and Public Health Response for Hand. Foot and Mouth Disease (HFMD).
Wang, Y., Feng, Z., Yang, Y., Self, S., Gao, Y., Longini, I.M., Wakefield, J., Zhang, J., Wang, L., Chen, X., Yao, L., Stanaway, J.D., Wang, Z., Yang, W., 2011. Hand, foot, and mouth disease in China: patterns of spread and transmissibility. Epidemiology 22, 781–792.
Wu, Y., Yeo, A., Phoon, M.C., Tan, E.L., Poh, C.L., Quak, S.H., Chow, V.T., 2010. The largest outbreak of hand; foot and mouth disease in Singapore in 2008: the role of enterovirus 71 and coxsackievirus A strains. Int. J. Infect. Dis.: IJID 14, e1076–81.
Wu, X., Mao, Q., Yao, X., Chen, P., Chen, X., Shao, J., Gao, F., Yu, X., Zhu, F., Li, R., Li, W., Liang, Z., Wang, J., Lu, F., 2013a. Development and evaluation of a pseudovirus-luciferase assay for rapid and quantitative detection of neutralizing antibodies against enterovirus 71. PLoS One 8, e64116.
Wu, Z., Du, J., Zhang, T., Xue, Y., Yang, F., Jin, Q., 2013b. Recombinant human coxsackievirus B3 from children with acute myocarditis in China. J. Clin. Microbiol. 51, 3083–3086.
Yamayoshi, S., Yamashita, Y., Li, J., Hanagata, N., Minowa, T., Takemura, T., Koike, S., 2009. Scavenger receptor B2 is a cellular receptor for enterovirus 71. Nat. Med. 15, 798–801.
Yen, F.B., Chang, L.Y., Kao, C.L., Lee, P.I., Chen, C.M., Lee, C.Y., Shao, P.L., Wang, S.C., Lu, C.Y., Huang, L.M., 2009. Coxsackieviruses infection in northern Taiwan–epidemiology and clinical characteristics. J. Microbiol. Immunol. Infect. = Wei mian yu gan ran za zhi 42, 38–46.
Zhou, S., Liu, Q., Wu, X., Chen, P., Guo, Y., Liu, S., Liang, Z., Fan, C., Wang, Y., 2016. A safe and sensitive enterovirus A71 infection model based on human SCARB2 knock-in mice. Vaccine 34, 2729–2736.