Development of a neutralization assay based on the pseudotyped chikungunya virus of a Korean isolate

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Introduction

Chikungunya virus (CHIKV) belongs to the Alphavirus genus of Togaviridae family and is an arbovirus that can be transmitted by mosquitoes such as Aedes aegypti and Aedes albopictus. Its infection causes chikungunya fever, characterized by a high fever, rashes, headaches, and severe joint pain that can last for several months or years. CHIKV is considered a re-emerging human pathogen and has caused millions of cases of infection around the world (Schwartz and Albert, 2010; Thiboutot et al., 2010). As a tropical disease, CHIKV epidemic has mostly been reported in regions near and including the Indian Ocean, Pacific Islands, and the Americas (Morrison, 2014). However, global warming and international transportation have increased the risk of spreading CHIKV infections to non-endemic regions, including South Korea, where travel-associated or imported cases of CHIKV infection have been reported (Cha et al., 2013; Hwang and Lee, 2015; Yeom, 2017). In addition, there may be a potential risk of local transmission of CHIKV, because the habitat of Aedes albopictus has been increased significantly in South Korea (Chang et al., 2018).

The complete genome of CHIKV is a linear, positive-sense, single-stranded RNA with 11,805 nucleotides that encodes two polyproteins (Schwartz and Albert, 2010; Morrison, 2014). One polyprotein consists of the nonstructural proteins nsP1, nsP2, nsP3, and nsP4 that are responsible for viral genome replication and viral gene transcription in infected cells. The other polyprotein consists of the capsid, E3, E2, 6K, and E1 structural proteins that are responsible for virion formation. The translated structural polyprotein is autocatalytically processed into capsid protein C, E1, E6K, and p62. The capsid protein C is essential for viral genomic RNA encapsidation, whereas the glycoproteins E1 and p62 interact to form heterodimers that subsequently oligomerize into a viral spike in the endoplasmic reticulum (Jose et al., 2009; Li et al., 2010). The glycoprotein p62 is essential for viral budding, and the glycoprotein E1 is responsible for membrane fusion during viral infection (Brehin et al., 2008; Kuo et al., 2012). Although CHIKV glycoprotein E3 is released after budding and not associated with the mature virion, glycoprotein E3 facilitates E1-p62 heterodimerization and prevents the exposure of the E1 fusion loops from premature fusogenic activity (Mulvey and Brown, 1995; Carleton et al., 1997; Solignat et al., 2009; Voss et al., 2010; Snyder and Muk-
hopadhyay, 2012; Yap et al., 2017). In addition, the E2 protein has been shown to be a major epitope for neutralizing antibodies (Voss et al., 2010).

Despite the increasing global concerns about the worldwide re-emergence of CHIKV, there is no approved antiviral treatment for CHIKV infection or vaccine against CHIKV (Jain et al., 2008; Tharmarajah et al., 2017). Multiple technology platforms have been applied to develop CHIKV vaccines using live attenuated viruses, inactivated viruses, recombinant viruses, chimeric alphaviruses, or virus-like particles (VLP), although such vaccines are still in various stages of the clinical or pre-clinical processes (Weaver et al., 2012; Powers, 2018). In addition, vaccine efficacy is often evaluated using the conventional plaque reduction neutralization test (PRNT) (Azami et al., 2016). PRNT determines the neutralizing antibody titer of a test sample by counting the number of plaques formed by cytopathic effects of viral infection. However, it is difficult to handle live, biohazardous CHIKV because its use requires a biosafety level-3 (BSL-3) facility, therefore an alternative assay to evaluate vaccine efficacy may facilitate vaccine development.

In this study, we aimed to develop a pseudovirus-based neutralization assay (PBA) using KNIH/2009/77, a Korean isolate of CHIKV (Cha et al., 2013). A CHIKV-pseudotyped virus (CHIKVpseud) expressing the structural polyprotein of CHIKV, was constructed using a lentiviral vector, which expressed both luciferase and green fluorescence protein (GFP) reporters. Validation and comparisons of PBA results with those from conventional PRNT indicate that the PBA using CHIKVpseud may be a safe, rapid, and reliable neutralization assay for evaluating the neutralizing activity of anti-sera against CHIKV.

Materials and Methods

Cells, viruses, antibodies, and human sera

Vero, HeLa, BHK-21, and HEK293T cells were cultured in complete Dulbecco’s modified Eagle’s medium (HyClone) containing 10% fetal bovine serum (FBS; HyClone) and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (HyClone). KNIH/2009/77, the Korean isolated strain of CHIKV, was provided by the National Culture Collection for Pathogens, Korea. A neutralizing antibody against CHIKV E2 protein (anti-E2; chk-265 from Absolute Antibody Ltd.) was used for plaque reduction neutralization tests (PRNTs) and pseudovirus-based neutralization assays (PBNA) (Pal et al., 2013), while a neutralizing antibody against the spike protein of the Middle East Respiratory Syndrome coronavirus (MERS-CoV) (Sino Biological Inc., Cat: 40069-R273) was used as a control for PBNA specificity. Human sera were obtained from a study titled “Burden of dengue infection in children and adults of Ouagadougou, Burkina Faso” with informed consent of the subjects. The subjects authorized researchers for the utilization of the left-over blood samples to identify other pathogens to study disease transmission in this area. This additional study was approved by the Institutional Review Board of International Vaccine Institute (IVI IRB protocol number: 2014-008).

Sequencing and phylogenetic analysis of structural proteins

KNIH/2009/77 infected BHK-21 cells were harvested with TRI reagent (MRC). RNA was extracted using a chloroform extraction method. The cDNAs were synthesized using the RevertAid first-strand cDNA synthesis kit (Invitrogen) with oligo dT primers. The synthesized cDNA was used as template DNA for PCR amplification of the structural polyprotein coding region, which was then cloned into the pCMV2-FLAG vector (pCMV2-FLAG-CHIKVst) using the following primers: F: 5’-CTTGCCGGCGGATGGTGTCAC-3’ and R: 5’-TATATGGATCCCTAGTGCCGCTGTAAGCGACACGC-3’. Underlined letters indicate the NotI and BamHI restriction enzyme sites used for cloning. To sequence the cloned structural polyprotein, additional primers targeting the Capsid gene (5’-GCCATCCCAGTTATGCGCTGGTG3’) and the E2 gene (5’-CAGTATAACTCCCCCTCTGTCGTCGCT-3’) were used and the sequence submission can be found in GenBank. The nucleotide sequences of structural polyprotein coding genes from 16 CHIKV isolates were selected for phylogenetic analysis (Table 1). Amino acid sequences were aligned by the Clustal Omega multiple sequence alignment program. The neighbor-joining algorithm and the maximum composite likelihood model were used for tree construction by MEGA 7 software (Tamura et al., 2011). The reliability of the analysis was evaluated by a bootstrap test with 2,000 replicates.

Production of pseudotyped CHIKV

The expression of structural proteins of CHIKV from pCMV2-FLAG-CHIKVst was tested. The plasmid was transfected into HEK293T cells using polyethyleneimine (Fukumoto et al., 2010). At 24 h post-transfection, cells were harvested and analyzed by western blot as described below. To produce CHIKVpseud, HEK293T cells were transfected with pCMV2-FLAG-CHIKVst, psPAX2 (a gift from Dr. Seungmin Hwang, The University of Chicago, IL, USA), and Luc2P pLVX-IRES-ZsGreen1, which is able to express two reporter genes, luciferase and GFP (a gift from Dr. Nam-Hyuk Cho, Seoul Na-

| Table 1. CHIKV strains used in phylogenetic analysis |
|----------------|-----------------|-----------------|
| Strain          | Geographic origin | Sampling year |
| ROSS            | Tanzania          | 1953            |
| PM2951         | Senegal           | 1966            |
| 18211            | South America     | 1976            |
| HD 180760       | Senegal           | 2005            |
| La Reunion       | La Reunion        | 2006            |
| MY/06/37348     | Malaysia          | 2006            |
| SL-CK1          | Sri Lanka         | 2007            |
| 0706aTw         | Taiwan            | 2007            |
| SGHEICHDD 122508 | Singapore        | 2008            |
| MY/08/065       | Malaysia          | 2008            |
| Mal2008         | Malaysia          | 2008            |
| TN06410         | India             | 2010            |
| VE53 20        | Trinidad and Tobago | 2014         |
| IND-73-MH5      | India             | Unknown         |
| IND-63-WB1      | India             | Unknown         |
| AF15561        | Thailand          | Unknown         |
| KNIH/2009/77    | Korea             | 2009            |
Phylogenetic analysis of CHIKV strains based on the whole structural polyprotein sequence

Although not frequent, travel-associated cases of CHIKV infection have recently been increased and detected in 5% of patients with dengue-like symptoms (Cha et al., 2013; Hwang and Lee, 2015; Yeom, 2017). KNIH/2009/77 was isolated from such a patient and deposited to the National Culture Collection for Pathogens (Cha et al., 2013). To further perform a genetic analysis of KNIH/2009/77, we amplified the structural polyprotein coding region from cDNA of KNIH/2009/77 using a reverse transcription system. The amplified structural polyprotein DNA of 3.7 kb in length infected Vero cells. After 4 days of incubation, cells were fixed and stained with 2% crystal violet in 20% ethanol. Plaques were counted to calculate the inhibitory concentration of the antibody against CHIKV. The neutralization curve and the effective concentration of 50% neutralization (LogIC50) were calculated using GraphPad Prism (GraphPad).

Pseudovirus-Based Neutralization Assay (PBNA) using CHIKV-pseudo

PBNA experiments were done in triplicate. For the PBNA, three-fold serial dilutions of the anti-E2 antibody were mixed with CHIKV-pseudo solution at an estimated titer of 100 GFP unit/well and then incubated at 4°C for 1 h. All antibody dilution was performed with DMEM containing 5% heat-inactivated FBS. Then, a HeLa cell monolayer was infected with the CHIKV-pseudo/antibody mixtures and incubated at 37°C for 4 h. Infected cells were cultered in complete media for additional 2 days. Cells were then harvested and analyzed by a luciferase activity using the luciferase reporter assay system (Promega) following manufacturer’s instructions. The neutralization curve and the effective concentration for 50% neutralization (LogIC50) were calculated using GraphPad Prism.

Results

Phylogenetic analysis of KNIH/2009/77 based on the whole structural polyprotein sequence

Although not frequent, travel-associated cases of CHIKV infection have recently been increased and detected in 5% of patients with dengue-like symptoms (Cha et al., 2013; Hwang and Lee, 2015; Yeom, 2017). KNIH/2009/77 was isolated from such a patient and deposited to the National Culture Collection for Pathogens (Cha et al., 2013). To further perform a genetic analysis of KNIH/2009/77, we amplified the structural polyprotein coding region from cDNA of KNIH/2009/77 using a reverse transcription system. The amplified structural polyprotein DNA of 3.7 kb in length

Fig. 1. Phylogenetic analysis of CHIKV strains based on the structure protein sequences. Phylogenetic relationships were inferred using the Neighbor-Joining method with whole structural polyprotein of CHIKV. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method. The position of the Korean Isolate, KNIH/2009/77, is indicated by bold letters and an asterisk. The names of clustered genotype strains are indicated. ECSA: East/ Central/South African.
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We have established a reliable plaque reduction neutralization test (PRNT) using KNIH/2009/77. However, because of the potential limitations of handling CHIKV in laboratories owing to its biohazard risk level, we set out to develop an alternative pseudovirus-based neutralization assay (PBNA) that may replace the conventional PRNT. A pseudotyped virus of KNIH/2009/77 (CHIKVpseudo) was designed to express the full-length structural polyprotein rather than individual structural proteins to enhance its infectivity (Hu et al., 2014).

A schematic diagram of the structural polyprotein expression plasmid of KNIH/2009/77 (pCMV2-FLAG-CHIKVst) is shown in Fig. 2A. To produce CHIKVpseudo, pCMV2-FLAG-CHIKVst was transfected into HEK293T cells with a lentivirus-based dual reporter vector (Luc2P-pLVX-IRES-ZsGreen1) and a packaging vector of lentivirus (psPAX2) (Fig. 2B). FLAG-tagged capsid protein was detected in HEK293T cells infected with CHIKVpseudo using anti-FLAG antibody (Fig. 2C). The expression of glycoprotein E2 was

Fig. 2. Construction of a pseudotyped virus expressing the structural polyprotein of CHIKV (CHIKVpseudo). (A) A schematic diagram of the KNIH/2009/77 structural polyprotein from pCMV2-FLAG-CHIKVst. (B) The CHIKVpseudo construction scheme. (C) Expression of CHIKV proteins from the CHIKVpseudo-infected or KNIH/2009/77-infected cells. At 48 h post-infection by CHIKVpseudo (100 GFP unit/well) or KNIH/2009/77 (MOI=0.05), HEK293T cells were harvested and analyzed by western blot for CHIKV E2 and FLAG-Capsid expression. (D) GFP expression from CHIKVpseudo infected cells. HEK293T cells were infected with CHIKVpseudo then 2 days later GFP signal was examined. Lentivirus with VSV-G was used as control. Scale bar: 200 μm. (E) Luciferase activity from CHIKVpseudo infected cells. HeLa cells were infected with CHIKVpseudo then, 2 days later, cells were harvested for luciferase reporter assays. Lentivirus with VSV-G was used as control.
Fig. 3. Neutralization assays of anti-E2 antibody using CHIKVpseudo. (A) PBNA using CHIKVpseudo was performed with three-fold serial dilutions of anti-E2 antibody solution, starting from the concentration of 24 μg/ml. Luciferase activity was measured at 48 h post-infection of CHIKVpseudo. In parallel, PRNT with KNIH/2009/77 was performed with three-fold serial dilutions of anti-E2 antibody solution. The plaque numbers were counted at 4 days post-infection. The effective concentration of 50% inhibition (Log IC\textsubscript{50}) of the PBNA or PRNT was calculated using GraphPad Prism. (A) The calculated Log IC\textsubscript{50} of the PBNA for the anti-E2 antibody with CHIKVpseudo. (B) Validation report of the PRNT. The average of calculated Log IC\textsubscript{50} ± standard deviation is indicated. The PBNA of anti-E2 antibody solution using CHIKVpseudo was assessed for its precision, which is determined by the coefficient of variation (CV) and linearity, which is indicated by a regression coefficient (R\textsuperscript{2}). Each value was compared with the validation report of the plaque reduction neutralization test (PRNT) with KNIH/2009/77. Calculated values were obtained from five independent experiments of PBNA using different batches of pseudovirus and three independent experiments of PRNT.

Fig. 4. Linear regression analysis of the neutralization assay with CHIKVpseudo. PBNA using CHIKVpseudo was performed with three-fold serial dilutions of anti-E2 antibody solution, starting from the concentration of 24 μg/ml. Luciferase activity was measured at 48 h post-infection of CHIKVpseudo. In parallel, PRNT with KNIH/2009/77 was performed with three-fold serial dilutions of anti-E2 antibody solution. The plaque numbers were counted at 4 days post-infection. The neutralization assay of anti-E2 antibody solution using CHIKVpseudo (A) and the PRNT (B) were assessed for linearity, indicated by the regression coefficient (R\textsuperscript{2}). The calculated values of average R\textsuperscript{2} ± standard deviation were obtained from three independent experiments.

also observed in CHIKVpseudo-transduced cells, as in KNIH/2009/77-infected cells using anti-E2 antibody. CHIKVpseudo-transduced cells also showed expression of both the GFP and firefly luciferase dual reporter genes, allowing us to quantify the infectivity of CHIKVpseudo (Fig. 2D and E). Although both HEK293T cells and HeLa cells resulted in high efficiency in transduction of CHIKVpseudo, we set to use HeLa cells for PBNA, because HeLa cells were more durable for the process of PBNA, providing more reliable values (data not shown).

Precision of PBNA with anti-E2 using CHIKVpseudo

To check whether CHIKVpseudo can be utilized for a neutralization assay, we assessed the neutralizing activity of an antibody against the structural protein E2 (anti-E2), ranging the concentrations from 24 μg/ml to 1.2 ng/ml, as produced from CHIKVpseudo infection. First, the concentration of antibody required to inhibit CHIKVpseudo infection by 50% (IC\textsubscript{50}) was calculated. CHIKVpseudo infection levels
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were decreased by the anti-E2 in a dose-dependent manner and the LogIC50 of anti-E2 was estimated to be 5.808 (pg/ml) (Fig. 3A). We next compared the results of the neutralization assay using CHIKVpseudo with the conventional PRNT for KNIH/2009/77 to validate our results. The neutralizing activity of the same anti-E2 antibody was tested with PRNT for infection with KNIH/2009/77 and each calculated value was obtained from 3 independent experiments. PBNAs using anti-E2 and CHIKVpseudo elicited an average LogIC50 value of 5.85 ± 0.09 pg/ml while a PRNT with KNIH/2009/77 yielded a LogIC50 of 5.68 ± 0.06 pg/ml. We then used the estimated values of LogIC50 from those 3 independent experiments to determine assay precision by calculating the coefficient of variation (CV) for each assay. Neutralization assays with CHIKVpseudo showed a CV value of 1.09%, which is similar to the PRNT CV of 0.98%, indicating that PBNA is a sufficiently precise neutralization assay.

Linearity of PBNA with anti-E2 using CHIKVpseudo

When we used serial three-fold dilutions of the anti-E2 antibody, starting from the concentrations of 24 μg/ml in our PBNA, the linear regression analyses of the neutralizing activity showed linearity in the range of 5% to 80% infectivity reduction (Fig. 4). PBNAs with CHIKVpseudo yielded an average regression coefficient (R²) value of 0.96 from 3 independent PBNAs (Fig. 4A). Similarly, conventional PRNTs yielded an average R² value of 0.95 (Fig. 4B). These results thus indicate that for both the neutralization assay with CHIKVpseudo and the PRNT, there is a significant correlation between the level of anti-E2 and the inhibition of CHIKVpseudo infectivity.

Specificity of PBNA with anti-E2 using CHIKVpseudo

To evaluate the specificity of PBNAs for anti-E2 targeting of CHIKV, we compared the neutralization ability of anti-E2 and an antibody against the spike protein of MERS-CoV (anti-spike), an unrelated protein. Serial three-fold dilutions of the antibody solution, starting from the concentrations of 24 μg/ml were incubated with 100 GFP unit of CHIKVpseudo per dilution and added to the cells for infection. Results from these infections indicated that the anti-E2 antibody inhibits CHIKVpseudo in a dose-dependent manner. However, the anti-spike antibody did not show dose-dependent inhibition (Fig. 5), indicating that CHIKVpseudo infection was specifically inhibited by the antibody against the CHIKV protein E2.

Neutralization of CHIKVpseudo by CHIKV-infected human sera

As previously reported, pseudotyped virus can be applied in detection of neutralizing antibodies in infected patients (Hu et al., 2014; Noranate et al., 2014). For this purpose, we tested the neutralization of CHIKVpseudo with serum samples from 5 CHIKV-positive individuals. These sera were only positive for CHIKV, but negative for all other mosquito-borne viruses including dengue virus (DENV), zika virus, yellow fever virus, West Nile virus, and Japanese encephalitis virus, as confirmed in ELISA (data not shown). Serum samples that were negative for all abovementioned mosquito-borne viruses were also used as negative controls (CHIKV-negative). Four out of five serum samples from CHIKV-positive individuals neutralized CHIKVpseudo in a dilution-dependent manner to various degrees, while none of serum samples from CHIKV-negative controls did (Fig. 6). This result indicates that CHIKVpseudo can be applied to measure the neutralizing activity of clinical samples.

Discussion

CHIKV, a member of the Alphavirus genus of the Togaviridae family, is a tropical disease-causing virus that is transmitted by mosquitoes. Global warming and frequent international travel have increased the risk of mosquito-borne diseases globally, and CHIKV is no exception. Over millions of CHIKV infection cases have been reported in more than 50 countries, suggesting that CHIKV has re-emerged as a global pathogen.
(Morrison, 2014). There are three strains of CHIKV based on their partial E1 sequences: East/Central/South African (ECSA), West African, and Asian (Powers et al., 2000; Rezza et al., 2007; Volk et al., 2010). Each strain has been associated with regional epidemics, but the ECSA strain caused an extensive nationwide outbreak in ectopic areas such as Malaysia in 2008 (Sam et al., 2012; Morrison, 2014). Recently in South Korea, the cases of various mosquito-borne viral infections have increased, and CHIKV infections have been detected in 5% of patients with dengue-like symptoms, suggesting that surveillance and prevention programs for CHIKV are required (Cha et al., 2013; Hwang and Lee, 2015; Yeom, 2017). In this study, we identified the whole sequence of the structural polyprotein of a CHIKV isolated in Korea, KN1H/2009/77, using reverse transcription. In addition, similar to a previous report analyzing the E1 sequence, we submitted our structural polyprotein sequence to an NCBI-BLAST search and phylogenetic analysis and confirmed that the KN1H/2009/77 isolate is clustered within ECSA genotype of CHIKV (Cha et al., 2013).

To evaluate the titer of neutralizing antibodies in a tested serum, the PRNT has been conventionally used in laboratories. Although it is considered a gold standard for neutralization assays, the use of hazardous live CHIKV and the requirement of a BSL-3 facility have impeded research for treatment of diseases caused by this virus. In addition, it takes a relatively long time (about 4–5 days) to visualize and count the plaques for the PRNT. To overcome these limitations, alternative neutralization assays using non-infectious virus replicon particles (VRPs) or pseudotyped viruses have been developed (Gläsker et al., 2013; Kishishita et al., 2013; Hu et al., 2014; Lee et al., 2014). Here, we adopted the strategy of expressing the entire structural polyprotein of CHIKV to generate the pseudotyped virus form of KN1H/2009/77 (CHIKVpseudo) for the enhanced infectivity compared to a virion expressing either an individual E1 or E2 protein (Hu et al., 2014). In addition to increased safety, the PBNA using CHIKVpseudo takes only 2 days to evaluate the neutralizing activity of a tested antibody and makes it easy to quantify the infectivity level using the luciferase activity of infected cells. Furthermore, CHIKVpseudo has additional advantages when compared to other pseudotyped virus systems. First, CHIKVpseudo expresses a FLAG-tagged capsid protein that can be easily detected by a commercially available antibody against FLAG, so that one does not need to generate or use a specific antibody against CHIKV proteins to check expression. Second, we used a dual reporter gene construct that expresses both GFP and luciferase in an infected cell. Many studies using pseudotyped viruses or VLPs often titrate viral infectivity using time- and labor-intensive methods, like measuring the viral genome copy number or virion protein concentration, as is the case for the HIV-1 protein p24 (Salvador et al., 2009; Gläsker et al., 2013; Kishishita et al., 2013; Wichit et al., 2017). Compared to these cases, CHIKVpseudo can be more easily titrated by measuring the GFP signal or luciferase activity. Since GFP is expressed only in infected cells, GFP-positive foci can serve as infectious centers similar to the plaques formed by infectious CHIKV. In addition, we validated the PBNA using CHIKVpseudo in terms of precision, linearity, and specificity by comparison with the conventional PRNT. The calculated IC50 and validation values were comparable in both assays despite differences in cell types and incubation times. These results indicate that PBNA with CHIKVpseudo can be successfully employed in a rapid neutralization assay with improved safety compared to PRNTs. Furthermore, the PBNA results with clinical samples from CHIKV-infected patients indicate that our CHIKV pseudo system can be applied to evaluate the neutralizing activities of human sera. In agreement of our results, a recent study reported an application of pseudotyped CHIKV to assess CHIKV DNA-vaccine candidates in their ability to raise neutralizing antibodies (Wu et al., 2017). Likewise, CHIKVpseudo can be used for assessment of vaccine efficacy.

Taken together, we have identified the whole structural polyprotein sequence of a Korean isolated CHIKV and its phylogenetic position. Using this sequence, we constructed CHIKVpseudo containing the entire structural polyprotein and developed a pseudovirus-based neutralization assay for CHIKV with improved safety and speed. Validation reports of our PBNA also support its reliability as a neutralization test compared to conventional PRNTs. Clinical samples can be also used for our PBNA to measure the neutralizing activity against CHIKV. These results indicate a potential use of CHIKVpseudo to detect neutralizing antibodies for vaccine efficacy tests as well as during clinical diagnosis.

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