incidence of anti-JEV IgG examined. The positive rate for anti-JE IgG was 29.58% (210/710). The positive rate in samples taken during the post-epidemic season (November to December) was 92.48% (664/718).

2.2.3. Flavivirus surveillance in mosquitoes

To examine incidence of flaviviruses in mosquitoes, 2,763 mosquitoes were captured between January 2005 and December 2006. The mosquitoes were classified into 4 genera and 8 species as follows; *Aedes (A.) albopictus* (32.75%), *Culex (C.) fatigans* (26.06%), *C. tritaeniorhynchus* (25.30%), *C. Sitiens* (7.75%), *Anopheles Sinensis* (3.84%), *Armigeres subalbatus* (3.18%), and others (1.12%). Mosquito suspensions were then prepared for virus isolation. Seven out of 132 suspensions showed CPEs in treated cells. Culture supernatants were then collected and the cellular RNAs were extracted and applied to standard RT-PCRs using pan-flavivirus primers and the specific primers for JEV, DENV-1 to -4, and WNV. However, we could not detect any flavivirus RNAs in the samples (data not shown). Real-time TaqMan RT-PCRs were performed to detect JEV, DV and WNV RNA in the mosquito suspensions and their culture supernatants using TaqMan primers (Table 2). We detected JEV RNA in only the *C. Sitiens* mosquito pool (Fig. 3). Further experimental information is needed for this virus.

3. Discussion

3.1. DF epidemiology in China

DF is an infectious disease common in Guangdong Province, China. DF epidemics have been seen in China for the last 20 years, though the outbreaks have occurred mainly in three or four provinces; Guangdong,

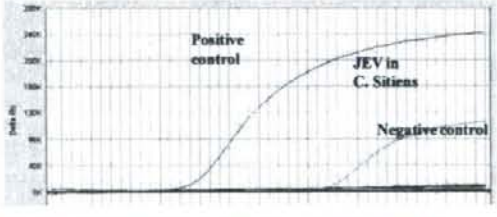

Fig. 3. Amplification of JEV RNA in *C. Sitiens* by real-time RT-PCRs
JEV RNA in *C. Sitiens* was amplified by real-time RT-PCRs. To detect JEV, DENV, and WNV RNA in mosquitoes, RNAs were extracted from the mosquito suspensions and TaqMan RT-PCRs were performed using amplifying PCR probes and TaqMan MGB probes (Table 2).

Hainan, Guangxi, and Yunnan. However, most of the recent outbreaks have occurred in Guangdong province (Table 1). According to reports from the infectious disease monitoring system of Guangdong province, 1,430 cases of DF were reported from Guangdong province between 2005 and 2007, while 1,628 cases were reported for the whole of China for the same period. An outbreak was recoded in the urban area of Guangzhou city and Shantou city in 2006, with 765 and 177 cases reported, respectively (Table 2). The reason why recent outbreaks have been localized in Guangdong province must be clarified and preparations for future DF/DHFS outbreaks in China undertaken.

3.2. Human JE epidemiology in Guangdong province

In 2007, a JE outbreak occurred in Zhanjiang city and Zhuhai city with 205 and 129 cases reported, respectively. JE has been prevalent in other cities of Guangdong province since the May of 2003 [10] and 384 of reported cases (82.03%) had no immunization history. These figures indicate the need for the establishment of an effective vaccination program to control the disease in Guangdong province.

3.3. Pig JE epidemiology in Guangdong province

In Asia, pigs as well as birds are thought to be important natural hosts and reservoirs of JEV. Although they do not manifest the disease, they produce viruses at a high titer in their circulating blood and thereby infect mosquitoes. As these animals are often kept close to human dwellings, they serve as amplifying or bridging hosts that can transmit the virus to humans. Therefore, the detection of antibodies against JEV in pigs may reflect the incidence of natural JEV infection and allow future human JE epidemics to be predicted. Although the positive rate for anti-JEV antibodies was less than 50% during the pre-epidemic season, the positive rate was around 100% during the post-epidemic season in 2007. Therefore, JEV is still circulating in the natural environment in Zhuhai and human and pig vaccination programs against JE could not be neglected.

3.4. Mosquito surveillance in Guangdong province

Guangdong province is located in a subtropical region, where the temperature and rainfall are higher than in other regions of China. Considering recent climate and environment changes, it is likely that the *Aedes spp.* and *Culex spp.* populations will increase in this region and effective insecticides, such as *N, N-Diethyl-m-toluamide* (DEET), are needed to control the disease vectors.

3.5. Risk factors of flaviviruses in Guangdong province

3.5.1. Mosquito vectors

Haoyan *et al.* reported that DENV-2 replicated in *A. albopictus* mosquitoes and bats in the endemic areas in Hainan Island [11]. Shiqing *et al.* [12] and Liping *et al.* [13] also reported that DENV-2 was isolated from

A. albopictus mosquitoes in Fujian and Guizhou provinces. However, there has been no report of the isolation of DENV from mosquitoes in Guandong province, which experiences frequent DF outbreaks, although DENV has been isolated from a patient's serum [14].

While *Culex spp.* and *Aedes spp.* together with *C. tritaeniorhynchus* mosquitoes play a role in the transmission of JEV [15], Hailin *et al.* [16] reported that JEV was isolated from *A. albopicus, A. vexans, A. lineatopennis*, and *A. assamensis* [16]. Vythilingam *et al.* reported on the isolation of JEV from 2 pools of *C. sitiens* in Malaysia [17] and Weng *et al.* [18]. also reported that JEV was detected in 1 pool of *C. sitiens* by RT-PCR targeted the viral NS5 gene in Taipei City, Taiwan [18]. In this study, we detected JEV RNA in one pool of *C. Sitiens* using real-time RT-PCR (Fig 3). The results indicated that the JEV could naturally infect *C. Sitiens* and that *C. Sitiens* may serve as a vector for the transmission of JEV. Further study needs to confirm this hypothesis.

3.5.2. Environment for human in Guangdong province

In recent years, many cities in Guangdong province are undergoing major construction due to the recent economic development in China. Many people are employed in temporary jobs, and the health and hygiene conditions for the people are not at all good. DF mostly occurs in people unable to take the necessary preventative medication. Tourism, foreign exchange, and increased domestic travel have also assisted in the spread of the diseases. Additionally, some cases were detected among emigrants who had come into the province. Although dengue is listed by the People's Republic of China on the Prevention and Control of Infectious Diseases as a notifiable infectious disease, its prevalence had been limited to one or two provinces, and there has been no reporting of its incidence due to a lack of knowledge in local health workers. Additionally, some cases were misdiagnosed with typhoid or influenza.

3.6. Strategy for the prevention and control of flaviviruses in Guangdong province

There is still no antiviral agent for the treatment of JE. JE immunization of both humans and its amplifying hosts, such as pigs, is a fundamental control tool. China has successfully implemented a vaccination program with impressive results. JE immunization of all children under 10 years of age was started in China in 1968. To control JE epidemics, vaccination for JE is carried out in epidemic areas. And unimmunized children are examined to generate sufficient neutral antibodies against JEV and provided JE vaccines in other areas. However, vaccination for JE has not been effectively carried out in some rural areas of Guangdong province. Therefore, the vaccination coverage for children is still low in these areas and no reliable immunity barrier has been established in China.

Pigs are normally slaughtered at 6 to 8 months of age. In most of the pig farms in Guangdong province, vaccination should be provided to prevent JEV infection in breeding sows.

There is no specific treatment for DEN nor are there any currently available vaccines for DENV infection. The only way to prevent DEN transmission is to the control the vector mosquitoes and to establish an effective surveillance system for the disease based on reliable laboratory diagnostic protocols and health information systems. Additionally, emergency response capacity should be established to control outbreaks of flavivirus infections with appropriate medication.

Acknowledgements

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7 Differential sero-diagnosis of flaviviruses using subviral particles and virus-like particles

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Abstract

Flaviviruses affect the mosquitoes-, ticks-, and non vector-bore viral diseases in human or animals. In Far East Asian countries, Japanese encephalitis (JE) and dengue fever/hemorrhagic fever syndrome are endemic every summer season. Although West Nile (WN) fever/encephalitis is not yet endemic in Japan, small numbers of imported cases have been reported recently. Generally, the anti-serum from the affected humans or animals with flaviviruses, which belong to the same sero-typing group, is highly cross-reactive with the

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other viral antigens. Because JE virus (JEV) and WN virus (WNV) belong to the JEV sero-typing group of Flaviviridae, it is difficult to differentiate between JEV and WNV infections using standard diagnostic tests. In this research review, we provide an brief overview of recent developments in the differential sero-diagnostic protocols of flaviviruses, and propose our new protocols for flavivirus infection, especially JEV and WNV infection, using diagnostic tools produced through the application of flavivirus reverse-genetics.

Introduction

Flaviviruses, such as Yellow fever virus [1], Dengue viruses (DENV) [2], Japanese encephalitis virus (JEV) [3], and West Nile virus (WNV), are known to cause diseases that affect both humans and animals. The viruses are transmitted by mosquitoes, ticks, or non-vector pathways from animals to animals, or from animals to humans [4, 5]. Some flavivirus infections are zoonotic in nature. The flaviviruses in human pathogens are classified by sero-typing antigenic complexes of flaviviruses [6]. Within the sero-typing group, an anti-serum of one virus is often cross-reactive with the viral antigens of other viruses within the group [6]. Because JEV and WNV belong to the same sero-typing group of flaviviruses, the JEV sero-typing group, differentiation between JEV and WNV infections has been thought to be difficult. Although JEV has historically caused endemic infections in Japan [7, 8], WN fever/encephalitis has not, except for small numbers of imported cases [9, 10]. However, we now need to prepare against the emergence of WNV infections in Japan and other Asian countries through the establishment of detection, protection, and prevention protocols for WNV infection.

Flaviviruses are enveloped, positive-strand RNA viruses with a genome size of around 11 kilo-bases (kb) [11, 12]. The RNA encodes three structural proteins, Core (C), premature membrane (pr and M), and envelope (E) proteins, and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [11, 12]. Viruses begin the infection cycle by attachment to the surface of susceptible host cells through the host receptor proteins of the viruses [11, 12]. Viral E protein has the most important role in this step [12], and most of viral epitopes for viral neutralizing antibodies are located within the E protein [13]. Therefore, the anti-E antibodies have been chosen as the target for viral diagnosis in many protocols [14-16]. However, the cross-reactivity of the antibodies against flavivirus proteins limits the test specificity [17]. Anti-flavivirus immunoglobulin G (IgG) cross-reacts with other viral antigens larger than IgM. Therefore IgM-based assays are more specific than IgG-based assays

[18, 19]. As the principal serological protocols for flavivirus diagnosis, hemagglutination inhibition (HI) test [20], indirect immunofluorescence assay (IFA) [21], and plaque reduction neutralization test (PRNT) have been recently developed [22]. Enzyme-linked immunosorbent assay (ELISA) has also been developed for the diagnosis of flavivirus infections [15, 16 18, 19]. More recently, three types of ELISA system have been developed for flavivirus diagnosis; IgG capture ELISA [15, 16], IgM antibody-captured ELISA (MAC-ELISA) [18, 19, 23], and epitope-blocking ELISA [24]. Additionally, original new protocols for the detection of anti-viral antibodies have been developed; for example, anti-IgG optical fiber immunoassay, in which biosensors and chemiluminescence are used [25], and another system based on covalently coupled recombinant E protein on fluorescent polystyrene microspheres [26 27].

Recent advances in flavivirus reverse-genetic systems have been applied to flavivirus diagnosis [28-30], and most studies on flavivirus diagnosis using flavivirus reverse genetics report advantages associated with the use of empty particles, subviral particles (SvPs), often referred to as virus-like particles, though we use the term "SvPs" in this review, for the test antigens [31-36]. SvPs have been used in many diagnostic tests, particularly IgG- or IgM-captured ELISA tests [31-33, 35]. Here, we also report our newly developed differential sero-diagnostic protocols for WNV and JEV using SvPs.

Virus-like particles (VLPs) have been shown to be applicable to neutralizing tests in place of live viruses [37]. VLPs are composed of viral enveloped shells and viral replicons within the shells [38]. Replicons are self-replicating viral RNA from which most of the virus structural protein genes are deleted [39]. Therefore, VLPs are thought to be one-round infectious particles. If we could perform a neutralizing test using VLPs instead of a plaque reduction of neutralization test (PRNT) using live viruses for the differential diagnosis of flaviviruses, there would be no need for the use of bio-safety level (BSL) 3 facilities when performing WNV diagnostic tests. Diagnosis would, therefore, be safer and more convenient using VLPs in place of live viruses.

1. Differential sero-diagnosis of flaviviruses using viral empty particles (Subviral particles, SvPs)

1.1. Flavivirus sero-diagnosis

Natural viral materials, such as native viral antigens, have been traditionally used for flavivirus diagnosis [22] following extraction from the virus-infected animals [40] or cells [41]. Within the last couple of decades, recombinant viral protein expression systems have been developed for many kinds of cell systems including bacterial cell systems [42], insect cells-baculovirus systems [43], and mammalian cells-vaccinia virus systems [15]. The methodology has also been applied to flavivirus diagnosis. The viral E protein of flaviviruses is a good target for these applications. However, the recombinant proteins sometime demonstrate unexpected features such as post-translational modifications and variations in antigenic properties from the native proteins. Therefore, more natural forms of viral proteins should be used as test antigens for disease diagnosis. One approach to resolving this problem is the use of SvPs as test antigens.

1.2. Generation of flavivirus SvPs

We examined the possibility of using flavivirus SvPs for differential sero-diagnostic tests. We constructed the expression vectors of flavivirus SvPs by the following procedure (Fig.1A). Viral RNA was extracted from

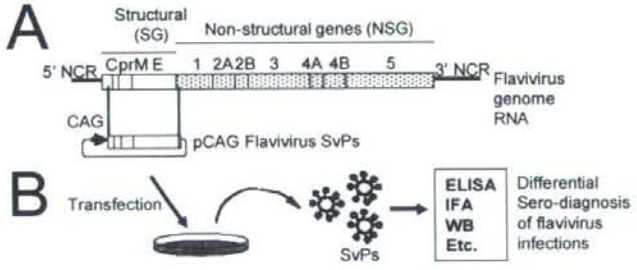

Fig. 1. Construction and generation of flavivirus SvPs

(A) The construction procedure of flavivirus SvPs is shown schematically. Flavivirus genome RNA is shown from the 5' non-coding region (NCR) to the 3' NCR. Viral structural protein genes (SG), C, prM and E, viral non-structural protein genes (NSG), NS1 (1), NS2A (2A), NS2B (2B), NS3 (3), NS4A (4A), NS4B (4B), and NS5 (5) are shown in the upper figure. The sequence of the C-terminal signal peptide sequence within the C, the prM, and the E genes was PCR-amplified and inserted into the pCAGGS plasmid (referred to as pCAG Flavivirus SvPs). A CAG promoter sequence is shown as a closed arrow head. (B) Generation procedure of the flavivirus SvPs is schematically shown. pCAG Flavivirus SvPs were transfected into HEC293T cells. At 48 to 72 hr.p.t., SvPs were recovered from the culture supernatant. The differential sero-diagnosis of the flaviviruses was then performed using SvPs by ELISA, IFA, and WB tests.

the virus particles and its cDNA was synthesized using a random 9-mer as a reverse transcription (RT) primer. The sequences of the C-terminal of the C protein gene, which is thought to contain the signal peptide, the prM gene, and the E protein gene were then amplified by polymerase chain reactions (PCR). Subsequently, the cDNAs were inserted into a pCAGGS expression plasmid [44] in the sense direction (pCAG WNV SvPs for the individual WNV SvPs expression plasmid or pCAG JEV SvPs for the JEV SvPs expression plasmid) (Fig.1A). The pCAG Flavivirus SvPs were then transfected into human kidney cell line (HEC293T) cells. At 48 to 72 hours post transfection (hr.p.t.), SvPs were recovered from the supernatant of the transfected cells (Fig. 1B).

1.3. Flavivirus differential sero-diagnosis using SvPs

Many researchers have tried to differentiate flavivirus infections serologically [24, 31-36]. However, the specificities of the tests are problematic for accurate flavivirus differential sero-diagnosis [17]. To differentiate anti-WNV and anti-JEV antiserum for the differential sero-diagnosis of WNV and JEV infections, we examined three diagnostic tests, IFA, ELISA, and Western blotting (WB) analyses, using SvPs as test antigens (unpublished data). Our results showed that the anti-serum of WNV could be differentiated from that of JEV using SvPs as test antigens. To provide a more practical protocol, we are planning to establish a new protocol for a differential diagnostic system for flaviviruses using dot-blot or slot-blot analysis (Fig. 2). Briefly, WNV- and JEV-SvPs are prepared as

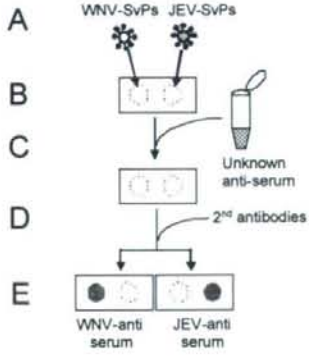

Fig. 2. A new protocol for the differential sero-diagnosis of WNV and JEV

WNV and JEV SvPs are generated as shown in Fig. 1B (A). WNV and JEV SvPs are blotted on a membrane (B) and react with an unknown anti-serum (serum sample) (C). Subsequently, the membrane are reacted with secondary antibodies conjugated with some detector enzymes (D). Finally, the infected viruses are determined (E).

shown in Fig. 1 (Fig.2A). Equal amounts of each type of SvPs are blotted onto a membrane (Fig. 2B). The anti-serum sample, for which we want to determine the infecting pathogen of the patient or animal, is reacted with the test antigens on the membrane (Fig. 2C), and then the membrane is reacted with the secondary antibody to detect the bound primary antibody on the membrane (Figs. 2D and 2E).

2. Differential sero-diagnosis of Flavivirus infection using virus-like particles (VLPs)

2.1. Flavivirus neutralization test

With regard to the serological differentiation of flavivirus infections, PRNT is the most effective protocol available for flavivirus differential sero-diagnosis [22]. However, this test requires the use of live viruses. As WNV belongs to the physiological level 3 (P3) virus class, researchers and test-examiners must use highly regulated BSL3 facilities. Pierson *et al.* examined the possibility of using flavivirus VLPs for diagnosis [37]. They established an effective VLP-generating protocol and employed VLPs for flavivirus diagnosis. We also examined the applicability of using VLPs in place of live viruses in modified neutralizing tests. Additionally, we examined the possibility of using this protocol for the differential sero-diagnosis of WNV and JEV.

2.2. Generation of flavivirus VLPs encoding a fluorescent protein gene

To generate flavivirus VLPs, we constructed a WNV replicon encoding a red fluorescent protein, DsRed, gene as an expression marker (Fig. 3A) [45]. Flavivirus structural protein-expression plasmids as a source of particle shell proteins were also constructed (Fig. 3B). The DsRed gene was amplified by PCR using the pDsRed2 C1 plasmid (CLONTECH) as a template. The amplified DsRed gene was then inserted into the plasmid, pUC19(Δ*Sph*I)repWNV, at the site where most of the flavivirus structural gene (SG) was deleted (repWNV/F, Fig. 3A) [45]. The repWNV/F was then *in vitro*-transcribed using SP6 RNA polymerase (Ambion). The entire sequence of the flavivirus SG was amplified by RT-PCR. RT was performed using viral RNA extracted from WNV particles as a template and a random 9-mer as an RT primer. The PCR product was inserted into a pCAG mammalian expression vector (pCAG Flavivirus/SG). The replicon was transfected into baby hamster kidney (BHK-21) cells (ATCC). After 6 to 24 hr.p.t. of the replicon, pCAG Flavivirus/SG was transfected serially

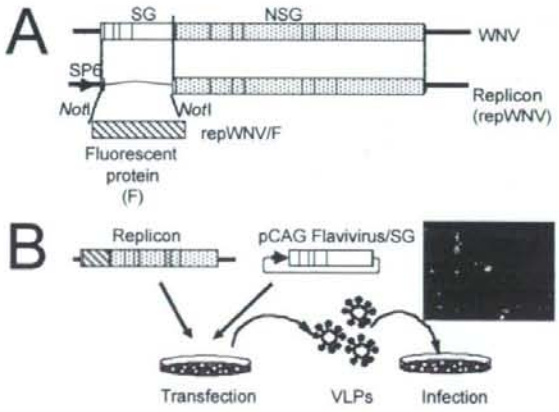

Fig. 3. Construction and generation of a WNV replicon encoding a fluorescent protein gene

(A) The construction procedure of the WNV and reporter gene-expressing replicons is schematically represented. WNV genome RNA is shown in the uppermost figure. A self-replicating WNV RNA (replicon, repWNV) was constructed by the deletion of the almost entire sequence of SG from the WNV genome RNA. The restriction enzyme, NotI, site was introduced into the replicon cDNA at the SG deletion site as shown in the figure. A fluorescent protein (F) gene was introduced into the replicon at the NotI site as a reporter gene (referred to as repWNV/F). (B) The generation procedure of the replicon is schematically represented. The repWNV/F was co-transfected with the WNV SG-expressing vector, pCAG Flavivirus SG, into BHK-21 cells. The VLPs were then recovered from the culture medium of the transfected cells. The cells image in B shows the fluorescence of the reporter red fluorescent protein, DsRed, which was expressed within the cells in which the replicon was replicated.

into the cells (Fig. 3B). The VLPs were released from the cells co-expressing the WNV replicon and viral structural proteins, and recovered from the supernatant of the cells. The VLP titer was determined by a modified plaque assay protocol. The cells expressing DsRed protein translated from the replicon were counted and the VLPs titers of the cell supernatants were determined (Fig. 3B panel). However, VLP could not be passaged on cells using the supernatant of the first VLP-infected cells (data not shown). Therefore, VLP has only one round-infectious property as suggested previously [28].

2.3. Generation of flavivirus VLPs encoding a secreted alkaline phosphatase (SEAP) gene

We constructed a replicon that encoded a secreted alkaline phosphatase (SEAP) gene as a reporter gene. The SEAP-expressing replicon (repWNV/SEAP) was transcribed *in vitro* using SP6 RNA polymerase (Fig. 4A). The repWNV/SEAP was then transfected into BHK-21 cells and pCAG Flavivirus/SG was serially transfected into the cells at 6 to 24 hr.p.t. (Fig. 4B). We examined the relationship between infectious dose and SEAP expression by VLP infection (Fig. 4C). Vero cells were infected with 0, 50, or 100μl of VLPs and the time course of the secretion of SEAP from the cells into the supernatant was measured and plotted in Fig. 4C. These results showed that infection with the VLPs proceeded in a similar manner to that of the native virus.

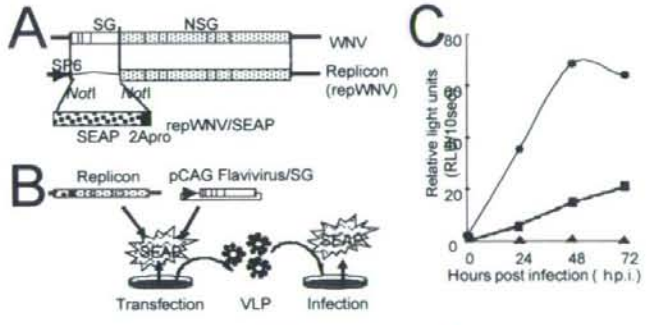

Fig. 4. Construction of a WNV replicon encoding a secreted alkaline phosphatase (SEAP) gene and generation of a WNV/VLP expressing SEAP

(A) The construction procedure of the SEAP-expressing replicon is schematically represented. WNV genome RNA is shown in the uppermost figure. The secreted alkaline phosphatase (SEAP) gene was introduced into the replicon at the NotI site as a reporter gene (repWNV/SEAP). SG; structural protein genes. NSG; non-structural protein genes, SP6; SP6 RNA polymerase promoter sequence, 2Apro; FMDV 2A proteinase. (B) The generation of a VLP encoding SEAP is schematically represented. repWNV/SEAP was co-transfected with the WNV SG-expressing vector, pCAG Flavivirus/SG, into BHK-21 cells. The VLPs were recovered from the culture medium of the transfected cells at 48 to 72 hr.p.t. (C) Time courses of SEAP expression from the cells infected with various volumes of VLPs, 0μl (-_-), 50μl (-_-), and 200μl (-_-), were plotted. Indicated volumes of SEAP-expressing VLPs in Vero cells. SEAP activity in the medium at each time point was analyzed and plotted (C).

2.4. Characterization of WNV replicons encoding various reporter protein genes

To select the best WNV replicon construct for the efficient generation VLPs, we constructed WNV replicons encoding various reporter protein genes. The construction of these reporter gene-expressing replicons is summarized in Fig. 5A, and their replication and packaging efficiency in the transfected BHK-21 cells are shown in Fig. 5B. First, we constructed and synthesized a series of AcGFP-expressing replicons (repWNV/AcGFP, repWNV/AcGFP/2Apro, and repWNV/IRES/AcGFP). The sequence of the AcGFP gene was inserted into the WNV genome RNA at the site at which almost the entire SG sequence was deleted (repWNV/AcGFP). The sequence of the AcGFP gene and the additional sequence at the 3'-end of Food and Mouse Disease virus (FMDV) 2A proteinase sequence were then inserted into the same site of the AcGFP gene insertion of repWNV/AcGFP (repWNV/AcGFP/2Apro). The sequence of AcGFP gene was also inserted into the most 5' end region of the 3' non-coding sequence (NCS) of the viral genome RNA. AcGFP expression from this construct was driven by the internal ribosomal entry site (IRES) (srepWNV/IRES/AcGFP). Unfortunately the packaging efficiencies of all replicons encoding AcGFP into VLPs were not high enough for our purpose (Fig. 5). We also constructed the replicon encoding the hMGFP gene (Promega) (repWNV/hMGFP) and examined the replication and packaging efficiency into VLPs (Fig. 5). This packaging efficiency of this

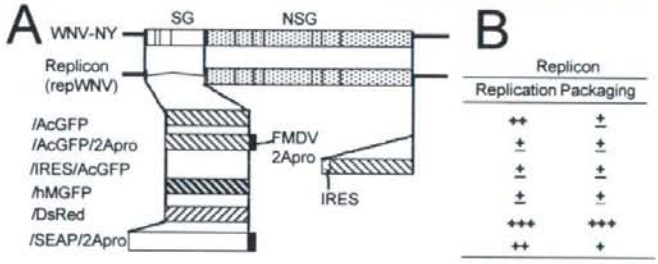

Fig. 5. Summary of the replication and packaging efficiencies of reporter gene-expressing WNV replicons

(A) Six WNV replicons, repWNV/AcGFP, repWNV/AcGFP/2Apro, repWNV/IRES/AcGFP, repWNV/hMGFP, repWNV/DsRed, and repWNV/SEAP/2Apro, are schematically represented. (B) The replication and packaging efficiencies of each WNV replicon in the transfected BHK-21 cells are shown. Replication level and packaging efficiency into VLPs were graded and are shown as ±, +, ++, or +++.

construct into VLPs was also unsuitable. We next examined a replicon expressing a red fluorescence protein, DsRed, (CLONTECH) (repWNV/DsRed, Figs. 3 and 5). The repWNV/DsRed replicon showed good replication in the transfected cells and was efficiently packaged into VLPs. We also examined a SEAP-expressing replicon (repWNV/SEAP/2Apro, Figs. 4 and 5). The 2Apro gene was inserted into just downstream of the SEAP gene to be processed the protein exactly at the downstream of the 2Apro, and to released from the cells. The repWNV/SEAP/2Apro replicated well compared to the GFP-expressing replicons in the transfected cells. However, the packaging efficiency of repWNV/SEAP/2Apro into VLPs was less than that of repWNV/DsRed. We cannot here answer why some replicons replicated well, but others did not, or why some replicons were more efficiently packaged into VLPs than others. One reason of these variations might be that some sequences inserted into the replicons affected their replication and packaging efficiency into VLPs. Further experimental data regarding the factors influencing virus replication and its genome packaging into viral particles are required.

2-5. Flavivirus differential sero-diagnosis using VLPs

Wong *et al.* reported that the WNV infection could be differentiated from dengue and St. Louis encephalitis virus infections by VLP-neutralizing test using its antiserum [46]. To perform the differential sero-diagnosis of WNV and JEV by a VLP-neutralizing test such as viral PRNT, two kinds of VLPs using the repWNV/DsRed construct as replicons were generated;

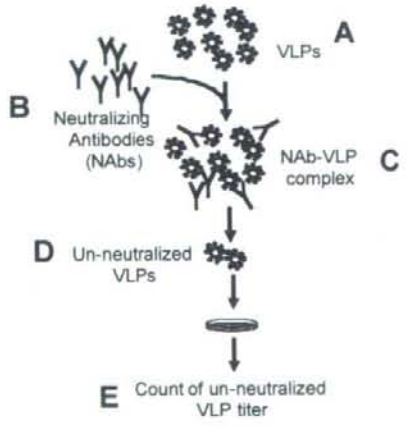

Fig. 6. Procedure for the VLP-neutralizing test

First, VLPs (A) are mixed with a serum sample containing neutralizing antibodies (NAbs) (B). Next, a NAbs-VLP complex was formed (C). The un-neutralized VLPs infect susceptible cells; for example, Vero cells (D). The infectious titer of the un-neutralizing VLPs is measured by counting the cells expressing reporter proteins (E). The NAb titer is then calculated using the reduction rate of the infectious VLPs.

one replicon was enveloped by WNV-shell (WNV/VLP), and the other by JEV-shell (JEV/VLP). First, we confirmed the packaging efficiency of repWNV/DsRed into the WNV- and JEV-shells and found that both envelopes could efficiently package repWNV/DsRed (data not shown). Then, using WNV/VLP and JEV/VLP, we developed the VLP-neutralizing test shown in Fig. 6. As this protocol was more specific, more convenient and safer for test examiners than other protocols in which live viruses were used, this VLP-neutralizing test is thought to afford a valuable new protocol for the differential sero-diagnosis of flaviviruses.

3. Summary

The difficulties facing flavivirus serology are 1) the need for highly regulated BSL3 facilities when using live viruses as some of flaviviruses belong to the P3 virus class, and 2) the high cross-reactivity of its antiserum to the other viral antigens among the same flavivirus sero-typing group. Therefore, we need a diagnostic protocol that can differentiate between infecting pathogens efficiently and that is safe and convenient for test examiners. Here propose a couple of newly developed protocols for the differential sero-diagnosis of WNV and JEV infections. These protocols use flavivirus SvPs or VLPs in place of live viruses as test antigens. The advantages of these protocols are that they are both "safer" and "more convenient" compared to other flavivirus diagnostic protocols as there is no need for BSL3 facilities if SvPs or VLPs are used in place of live viruses. The antigenecity of SvPs and VLPs are very similar to that of the native live viruses, and the "specificity" of these tests, particularly the VLP-neutralizing test, is thought to be sufficiently high. Therefore, these methods appear to afford a suitable protocol for application to the differential sero-diagnosis of flaviviruses.

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