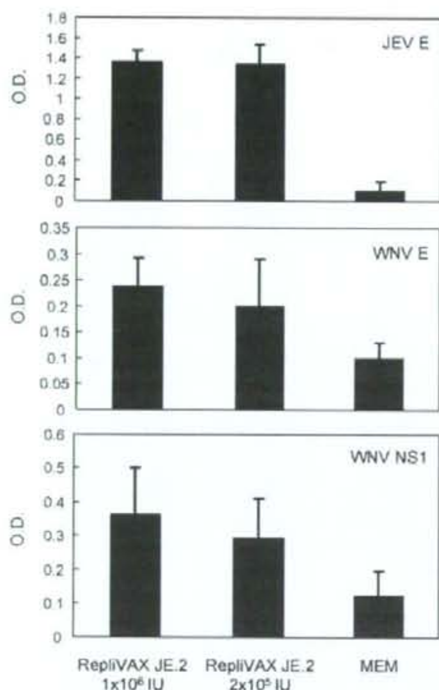

Figure 6 Serum ELISA antibody levels detected in vaccinated hamsters. Antibody levels to the JEV E, WNV E, and WNV NS1 proteins were detected in hamster sera collected at 21 days post-vaccination with the indicated preparations. The bars show the mean O.D. (450 nm) values obtained with sera from individual animals and the extended bars show the standard deviation among these samples.

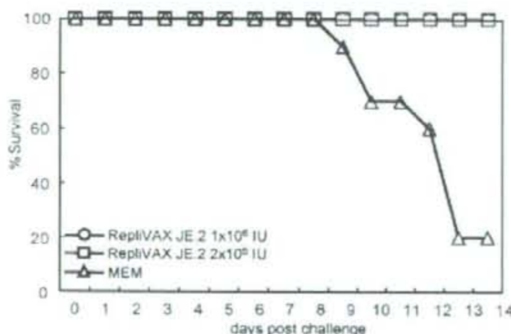

Figure 7 Survival data following WNV challenge of vaccinated hamsters. At 28 days post-vaccination, hamsters were challenged with 1×10^7 ffu of WNV NY99 strain, and survival was monitored for 14 days. Open circle, open square, open triangle indicated hamsters inoculated with 1×10^6 ffu of RepliVAX JE.2, 2×10^5 IU of RepliVAX JE.2 and MEM, respectively.

completely protected animals from WN encephalitis (Fig. 7) demonstrating a significant increase in survival relative to the control ($p < 0.001$) for both doses tested. Furthermore, none of the RepliVAX JE-immunized hamsters showed any manifestations of disease during the post-challenge observation period. All hamsters that survived infection developed high titers of neutralizing antibodies against WNV by 21 days post-challenge (data not shown).

Thus immunization with RepliVAX JE.2 induced high levels of JEV-specific neutralizing antibodies in hamsters, and induced protective immunity against WN encephalitis.

Discussion

In the present study, we developed and tested a novel JE chimeric pseudoinfectious virus vaccine, RepliVAX JE. The results of our investigations demonstrate that this platform for vaccine development is amenable to the chimerization strategy used to engineer live-attenuated flavivirus vaccines by others [14,33–37]. Although chimerization is known to be a good way to attenuate a pathogenic virus, live-attenuated flavivirus chimeras are infectious and can spread in vaccinated hosts, producing several concerns that do not apply to our non-spreading RepliVAX chimeras. First, there are concerns about the potential virulence of the backbone of some chimeras, notably the yellow fever virus 17D strain (YFV-17D), which has recently been associated with a large number of adverse events [38], and which is not acceptable for use in infants, pregnant women, or the immunocompromised [39]. Second, there is a concern that addition of the "foreign" prM-E cassette in the context of existing LAV can contribute to virulence of the chimeric product, as has been shown in studies comparing YFV-17D-JEV chimera created from prM/E genes of two different strains of JEV [40]. There is also a concern that chimerization may be responsible, in part, for the attenuation of the chimeras [41], making reversion to virulence unpredictable. Finally, LAV preparation suffers from the fact that viruses selected to grow better in cell culture may lose the *in vitro* phenotypes associated with lack of virulence in man (e.g., small plaque, temperature sensitivity) complicating production, and producing substrains with altered virulence, as was observed in YFV-17D vaccine generation in the latter half of the 20th century [42].

The fact that RepliVAX cannot spread in vaccinated hosts permits it to be engineered to grow better in cultures of C-expressing cells, without altering its virulence. In this manuscript (and in Widman et al. [23]), we demonstrated that better-growing variants selected in C-expressing cells remained unable to spread in the absence of the trans-expressed C protein. In the accompanying paper by Widman et al., we rigorously demonstrated that repeated blind passage of RepliVAX WN in the packaging cells used to propagate it did not produce a recombinant virus capable of growing in normal cells [23]. In this manuscript, we used blind passage (discussed further below) to produce a better-growing version of RepliVAX JE, and during that process we never detected any recombinant virus generation. Following the blind passage of RepliVAX JE on BHK(VEErep/Pac-Ubi-C*) cells, a mutation which allowed RepliVAX JE to grow better in these cells was selected. This mutation produced an

amino acid change three amino acids downstream of the NS2B/NS3 cleavage site that cleaves the WNV C protein from the signal peptide for prM. A similar, but different mutation was observed upon blind passage of RepliVAX WN, as well as a second mutation near the signalase cleavage site at the start of prM which also enhanced growth [23]. Interestingly, all JEV strains have an N at this position, but we had expected that the parental structure, QKKR/GGKT, would be the optimal one to have in the context of a chimeric RepliVAX, since the NS2B/NS3 proteinase that cleaves it is the WNV proteinase. The fact that release of E protein from RepliVAX JE.2-infected cells was enhanced relative to the parental RepliVAX JE, suggests that this mutation increases availability of prM/E heterodimers for particle formation. This supposition is consistent with previous reports demonstrating that mutation in the signal sequence of prM can increase production of SVP from flavivirus genomes [21,43]. In one case, these mutations were introduced to increase the immunogenicity of flavivirus RNA vaccines carrying a C deletion, so a role in infectious particle production was not studied [21]. However, in the other case, the increased prM cleavage activity resulted in reduced production of infectious virions [43]. In our study, the mutation we found in the NS2B/NS3 cleavage site enhanced production of both SVPs and infectious particles. Moreover, when this mutation was introduced into our live JE WN chimeric virus, the growth kinetics of the mutant were indistinguishable from the parental chimera (data not shown). Taken together, our data suggest that this mutation downstream of the NS2B/NS3 cleavage site appears to play an important role in particle formation, especially in trans-complementation. As discussed above, a similar finding was also found with RepliVAX WN [23] and both findings are consistent with studies showing that manipulation of the cleavage sites in this region of the flavivirus polyprotein can alter packaging [43–45].

In a mouse model, no significant differences in immunogenicity between RepliVAX JE and RepliVAX JE.2 were observed, although antibody levels against JEV E and WNV NS1 proteins induced by RepliVAX JE.2 were higher than those elicited by RepliVAX JE. Nevertheless, RepliVAX JE.2 would have an advantage over RepliVAX JE at the level of the vaccine preparation because RepliVAX JE.2 grows much better *in vitro* and its enhanced yield is likely to reduce manufacturing costs. Previous studies have demonstrated that JEV-specific Neut-titers of greater than 1:10 can protect mice from a lethal JEV challenge [46]. A single dose of 4×10^4 IU of RepliVAX JE.2 induced a Neut-titer of 1:40 and protected 100% of mice from lethal JEV challenge in our studies. This is an important finding, since data from a large-scale clinical trial has also indicated that a Neut-titer in man of 1:10 is efficacious [47], and thus the ability of RepliVAX to induce protective immunity by a single immunization with such a low dose of vaccine suggests that it could be developed into an economically viable vaccine.

Studies in a second animal model for flavivirus infection demonstrate that RepliVAX JE-inoculated hamsters developed Neut-titers even greater than those observed in mice. Interestingly, RepliVAX JE.2-inoculated hamsters were also able to develop detectable neutralizing antibodies against WNV. In both humans and mice, it has been reported that vaccination against JE could induce neutralizing antibodies against WNV [48,49], however, another report failed to detect neutralizing antibodies against WNV in JE-vaccinated humans [50]. One recent comparison of cross-protective immunity among JE-WN serogroup flaviviruses demonstrated that immunization with a JE LAV vaccine could induce protective immunity against WNV in hamsters [31]. In the present study we took advantage of this model, and demonstrated 100% protection against WNV disease in our RepliVAX JE.2-immunized hamsters.

In the case of several (but not all) of these previous reports, the majority of the cross-protective antibodies were likely to be the result of cross-neutralizing antibodies that recognized the E protein of the challenge virus. However, it is possible that some immunity in our RepliVAX JE.2-vaccinated hamsters was due to the immunity to WNV NS1 protein, since several studies have demonstrated that NS1 immunity can protect against flavivirus diseases [51–55]. Furthermore it has been reported that mice inoculated with a chimeric virus expressing dengue virus prM and E in a JEV backbone (encoding the JEV NS1 protein) were partially protected from lethal JEV challenge [56]. Thus, it is unclear if the protection we observed against WNV challenge in the RepliVAX JE-vaccinated hamsters was due to cross-neutralization of the WNV challenge virus or immunity to WNV NS1 (encoded in its entirety by RepliVAX JE). The contribution of immunity of NS1 and E to protection against flavivirus disease is now being investigated in detail, but it seems likely that immunity to both proteins, as well as a strong T-cell response contribute to the efficacy of RepliVAX.

In conclusion, a single dose of RepliVAX JE.2 was able to elicit both anti-NS1 and virus-neutralizing antibodies in mice and hamsters, and provide protection from lethal disease. These properties, as well as the ability to produce RepliVAX JE (and its parent) like an LAV in stable cell lines (see Ref. [23]), indicate that the RepliVAX platform could be suitable for development of useful vaccines for a variety of flavivirus diseases.

Acknowledgements

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Japanese Encephalitis Viral Infection Remains Common in Japan

To the Editor:

Japanese encephalitis (JE) virus is a flavivirus that causes about 15,000 deaths in Asia every year. Mosquitoes and swine play important roles in the spread of JE virus in the paradomestic environment, the former as a vector and the latter as an amplifier. In the 1950s, approximately 5000 cases, about 20% fatal, were reported yearly in Japan, but recently fewer than 10 cases are reported each year.

Reasons for the marked decrease in JE in Japan include decreases in the number of rice fields which are habitats of vector mosquitoes (mostly *Culex tritaeniorhynchus*), subsequent decreases in the mosquito population, and increased distance between the habitats of people and swine. However, urban residents are still bitten by mosquitoes during the summer, with the exception of northern Japan. Furthermore, 50–100% of Japanese pigs younger than 8 months have antibodies against the JE virus in summer and autumn, indicating that most Japanese pigs can still be JE virus producers.

The extremely low reported incidence of JE in human patients in Japan, despite the high prevalence of antibodies against the JE virus in swine and continued mosquito bites in humans, constitutes a paradoxical situation. To explain this paradox, we proposed a hypothesis that human JE viral infections still occur, but produce only mild symptoms. To test this hypothesis, we measured titers of the nonstructural protein-1 (NS1) antibody,[1] which increases only with natural JE viral infection, and titers of the HI antibody, which increases with both vaccination and JE viral natural infection, in blood samples obtained from 50 volunteers and patients, aged 1–88 years, at Eijudo Clinic, located in an urban area of eastern To-

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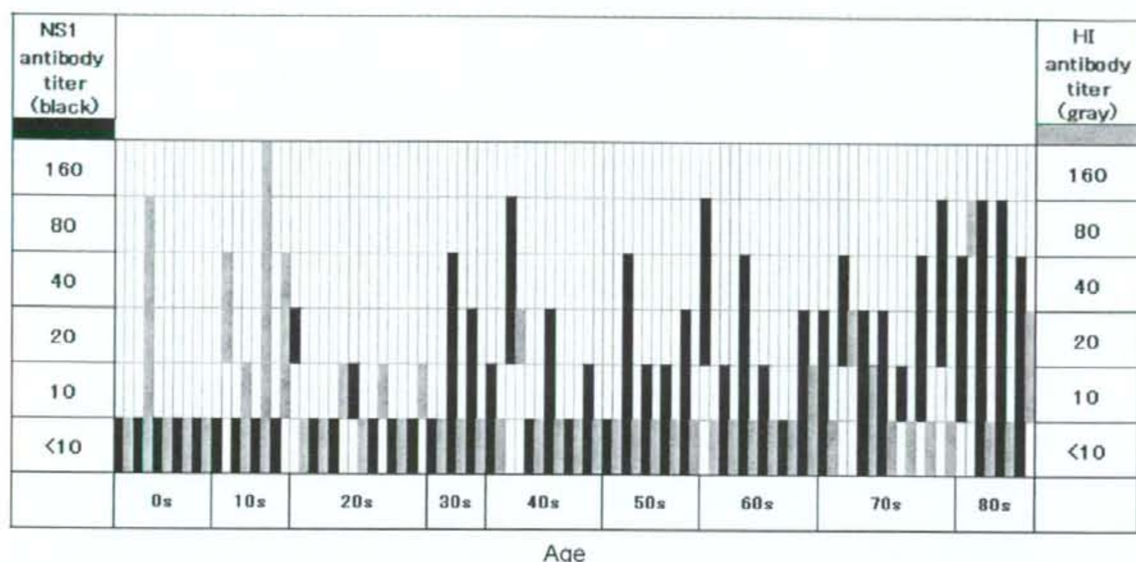

FIGURE 1. Range of NS1 and HI antibody titers against Japanese encephalitis. The black bars indicate changes in NS1 antibody titers in each serum series, and the grey bars show changes in HI antibody concentrations. The adjacent bars are results for a single person. Sera collected from each person for 2–3 years.

kyo. Blood samples were collected for 2–3 years from each subject.

Results are shown in Figure 1. The black bars indicate changes in NS1 antibody titers in each serum series, and the gray bars show changes in HI antibody concentration. The adjacent bars are results for a single person. The bars are arranged horizontally by subject age. The NS1 titers indicate that the incidence of JE viral infection has increased with subject age, whereas HI titers, which reflect vaccination, have slowly fallen with subject age. Two of 50 subjects showed rapid and marked increases in the NS1 titer. One was a 57-year-old woman who took a 3-day trip from June 30 through July 2, 2002, to Okinawa, a southern Japanese island where 3 American soldiers contracted JE with severe sequelae several years ago.[2] The NS1 titer on June 21 was <1:10 but by July 21 had increased to 1:20 and remained high for 1 year. During this time the subject exhibited no symptoms. Another subject was a 85-year-old woman who had remained at home for several years. The NS1 titer was <1:10 on July 8, 2002, but the titers on July 15 and September 8, 2002, were 1:80. From July 8 through 15 she had a low-grade fever, but no infectious focus was identified.

We have shown rising NS1 antibody titers with subject age in a series of blood samples obtained at a clinic in eastern Tokyo and described 2 subjects with rapidly increasing NS1 titers. These results and a previous report[3,4] suggest that human JE viral infection remains prevalent in Japan, although symptoms can be mild or absent. Therefore, vaccination against JE remains extremely important, although the Japanese Government halted strong recommendation of routine JE vaccination in 2005.[5]

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Short Communication

Vero Cells Infected with Vaccinia Viruses Expressing Japanese Encephalitis Virus Envelope Protein Induce Polykaryocyte Formation under Neutral Conditions

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SUMMARY: Flavivirus membranes fuse with cellular membranes by low pH-induced mechanisms. Although Japanese encephalitis virus (JEV) has similar mechanisms, fusion induced under neutral conditions has been observed. We report herein polykaryocyte formation using Vero cells infected with recombinant vaccinia viruses, vP829 expressing JEV premembrane (prM) and envelope (E), or vP555 expressing prM, E, and the nonstructural proteins NS1 and NS2A. Polykaryocytes were detected under pH 7.0 as early as 9 h after infection. Higher fusion indices were shown with vP555 than vP829. A monoclonal antibody to E suppressed vP829/vP555-induced polykaryocyte formation. Polykaryocytes were also formed under alkaline conditions (pH 8.0).

Enveloped viruses have a mechanism of membrane fusion to transport their genomes into host cells in the early phase of infection. Flavivirus fusion mechanisms have been extensively studied using tick-borne encephalitis virus (TBEV) (1) and other viruses (2,3). The low pH-induced oligomerization process of the envelope (E) protein is critical to acquiring fusion activity in flaviviruses. Japanese encephalitis virus (JEV), a member of the genus *Flavivirus*, has been considered to have a fusion mechanism similar to those of other flaviviruses. Specifically, polykaryocyte formation is induced by infection with JEV (fusion-from-within [FFWI]) (4-6) or virus adsorption followed by exposure to low pH (fusion-from-without [FFWO]) (6-8). However, electron microscopy studies have indicated that JEV enters cultured cells by fusion with plasma membranes without receptor-mediated endocytosis (9). The present paper reports polykaryocyte formation under neutral conditions, which was observed in Vero cells infected with recombinant vaccinia viruses expressing JEV premembrane (prM) and E (designated vP829) or prM, E, and the nonstructural proteins NS1 and NS2A (designated vP555) (10).

Vero cells were infected with vP829 or vP555, as well as their parent vaccinia virus vP410, at a multiplicity of infection (m.o.i.) of 2 PFU/cell, incubated at pH 7.0 and stained with Giemsa 16 h after infection (Fig. 1A). While infection with vP410 caused rounding and aggregation of cells as a normal cytopathic effect by vaccinia virus, infection with vP829 or vP555 induced formation of relatively large polykaryocytes. The polykaryocytes that developed in vP829-infected Vero cells were round in shape, whereas those in vP555-infected cells were elongated. The time courses of fusion indices for 6-22 h following infection (Fig. 1B) indicated that the increase in fusion indices was initiated 9 h after infection and continued until 12 or 16 h after infection. vP555 induced higher levels of polykaryocyte formation than did vP829: significant differences were shown between fusion indices obtained from vP555- and vP829-infected cells 12 and 19 h ($P < 0.05$) or 22 h ($P < 0.001$) after infection. The polykaryocyte formation induced by infection with vP829 or vP555 was inhibited by the addition of purified IgG fractions of monoclonal antibodies specific for E (JE-10B4; for both vP829 and vP555) or NS1 (JE-2D5; only for vP555) to the medium at a final concentration of 50 μg/ml (Fig. 1C). JE-10B4, which was generated in a standard protocol from mice immunized with Triton X100-treated virion fractions purified from JEV-infected Vero cell culture fluid, had neutralizing and hemagglutination-inhibiting activities (data not shown). To further investigate polykaryocyte formation under nonacidic conditions, we next investigated whether the induction of Vero cell fusion by infection with vP829 or vP555 would occur under a condition of pH 8.0. For this experiment, Vero cells were maintained at pH 8.0, as well as pH 7.0, for 16 h after infection. As shown in Fig. 1D, pH 8.0 induced even higher levels of polykaryocyte formation than pH 7.0 in both vP829- or vP555-infected cells.

Polykaryocyte formation induced by JEV has been reported using mosquito culture cells in both FFWI and FFWO systems (4-8). The only exception is that FFWI was observed in a particular clone of mammalian BHK cells (BHK-21-528) (5). On the other hand, neither FFWI nor FFWO has been achieved using a mammalian cell line, Vero (6,7). In the present study, Vero cells formed polykaryocytes when prM and E were produced by using a vaccinia virus expression system. It is speculated that a certain alternation in membrane nature triggered by vaccinia virus infection may have allowed polykaryocyte formation in Vero cells. Importantly, although JEV induced cell fusion basically under acidic conditions in mosquito cells, polykaryocyte formation in Vero cells was induced under pH 7.0, representing one particular aspect of the fusion phenomenon caused by JEV E protein. Although details were not investigated in the present study,

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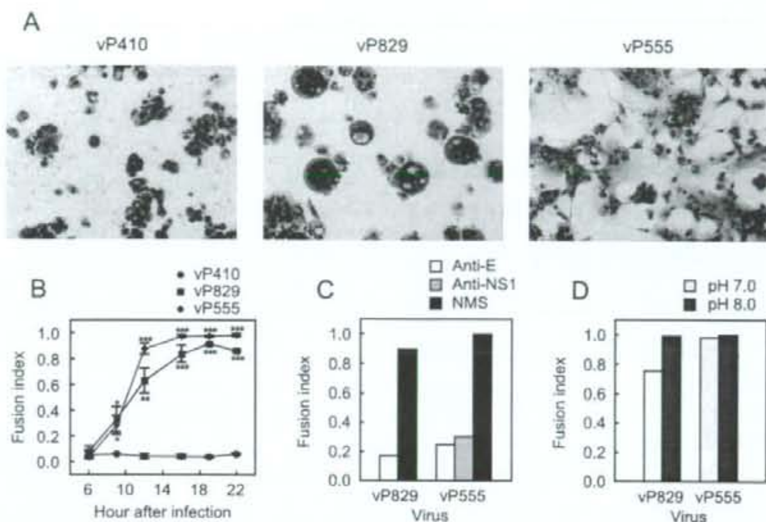

Fig. 1. Polykaryocyte formation induced in vP829- or vP555-infected Vero cells under neutral conditions. Vero cells were infected with vP829 or vP555, as well as vP410 as a control, at a multiplicity of infection (m.o.i.) of 2 PFU/cell. Cells were then incubated in Eagle's minimal essential medium (MEM) supplemented with 0.1% bovine serum albumin, kanamycin at 60 μg/ml, and 10 mM HEPES buffer (pH 7.0), at 37°C in a humidified atmosphere of 5% CO_2-95% air. Six to 22 h after infection, cells were stained with 5% Giemsa's solution (Merck, Darmstadt, Germany). The numbers of cells and nuclei in 5 different fields per sample were counted under a microscope. Fusion indices were calculated by a formula 1-C/N, where N and C are the numbers of nuclei and cells, respectively (7). (A) Photographs of Vero cells 16 h after infection with vP410, vP829 or vP555. (B) Time courses of fusion indices for Vero cells infected with vP410 (circles), vP829 (squares), and vP555 (diamonds). Vertical bars represent standard deviations of data obtained by two separate experiments. Asterisks indicate significant differences between fusion indices shown by vP410 and vP829 or vP555: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Inhibition of vP829/vP555-induced Vero cell fusion by monoclonal antibodies to JEV E (Anti-E; JE-10B4; open bars) or NS1 (Anti-NS1; JE-2D5 [11]; gray bars). IgG fractions of mouse ascites containing monoclonal antibodies were added to the medium at a final concentration of 50 μg/ml after infection with vP829 or vP555. As a control, normal mouse serum (NMS; closed bars) was used in place of monoclonal antibodies. Fusion indices obtained 16 h after infections were shown. (D) Fusion indices of Vero cells which were maintained at pH 8.0 (dark gray bars), as well as pH 7.0 (light gray bars), for 16 h after infection with vP829 or vP555. For adjusting the medium to pH 8.0, 10 mM Tris-HCl buffer was used.

NS1 may play some role in polykaryocyte formation of Vero cells under neutral conditions, since vP555 produced higher fusion indices than vP829.

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Isolation and Molecular Characterization of Banna Virus from Mosquitoes, Vietnam

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We isolated and characterized a Banna virus from mosquitoes in Vietnam; 5 strains were isolated from field-caught mosquitoes at various locations; Banna virus was previously isolated from encephalitis patients in Yunnan, China, in 1987. Together, these findings suggest widespread distribution of this virus throughout Southeast Asia.

Banna virus (BAV) is a 12-segment, double-stranded RNA virus that is classified in the family *Reoviridae* and the genus *Seadornavirus* (*1*). BAV was first isolated from encephalitis patients in southern China, Yunnan Province, Xishuangbanna Prefecture, in 1987 (*2*). In addition, antigenetically BAV-consistent viruses were isolated from pigs, cattle, and humans in Yunnan Province and from the sera of febrile patients in Xinjiang Province (*3*). Therefore, BAV is suspected to be a pathogen of vertebrates and an encephalitis pathogen for humans. BAV was also isolated from mosquitoes in China (*3*–*5*) and Indonesia (*6*), prompting an alert about a pathogenic arbovirus in these countries. Until now, no mammalian cell line has been reported to propagate BAV, and experimental infection with BAV has not resulted in clinical encephalitis in mice (*7*). However, another species of *Seadornavirus*, the Liao Ning virus, has been propagated in various mammalian cell lines. Although this virus has caused hemorrhaging in mice, it has not been associated with the development of encephalitis (*7*).

The pathogenicity and precise distribution of *Seadornavirus* spp., including BAV, are not well understood, and the scope and impact of BAV infection in Asia require further investigation. We report the circulation of BAV in *Culex* spp. mosquitoes in Vietnam and demonstrate that 2 new phylogenetically distinct types of BAVs are co-circulating. These findings suggest that this virus is widely distributed throughout Southeast Asia.

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The Study

Mosquito samples were collected in Vietnam in 2002. Each species was separated and pooled, and each mosquito pool was then homogenized and centrifuged. The supernatant was filtrated through a 0.22-µm filter and then inoculated onto a monolayer C6/36 mosquito cell culture and subsequently incubated at 28°C for 7 days. Virus amplification in C6/36 cells was repeated twice, and the culture fluids were stored at –80°C for further analysis. RNA was extracted from the second cell culture fluid by TRIZOL LS Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed by using Superscript III Reverse Transcriptase (Invitrogen) and random hexamers, after RNA denaturation at a temperature of 95°C for 5 minutes in the presence of 15% dimethyl sulfoxide. PCR was conducted by using TaKaRa LA Taq DNA polymerase (Takara Bio, Inc., Otsu, Japan) with BAV targeting primers (for segment 5, 5′-CAGCTGCAGTGGTTATTGGA-3′ and 5′ACCGTGCATCTTAACCCTTG-3′; for segment 8, 5′-TTGCAGTCGCTGAGCTTTTA-3′ and 5′-CGCATTTGATCGTATGCTTG-3′). These targeting primers were designed from sequences of BAV strains from China and Java (GenBank accession nos. AF134519–AF134527,AY549307–AY549309,AF052024–AF052035, AY568287–AY568290,NC_004211,NC_004217-NC_004221, NC_004200-NC_004204, NC_004198, and AF052008–AF052013). The amplified cDNAs were sequenced in the 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The genomic sequences of all 12 genome segments for the 5 strains were determined (GenBank accession nos. EU265673–EU265727, EU312980).

The sequences were translated to protein sequences by using the Transeq program of the EMBOSS package (*8*), version 5.00. The protein sequences were aligned with the T-COFFEE program version 5.05 (*9*) with Chinese and Javanese BAVs. Nucleotide sequences were then aligned, on the basis of protein alignment, with the Tranalign program of the EMBOSS package. Maximum likelihood phylogenetic trees were constructed from the protein-guided alignments of nucleotide sequences with DNAML software of the PHYLIP package (*10*), version 3.67.

Five BAV strains were isolated in Ha Tay and Quang Bing Provinces (Figure 1) in Vietnam from *Culex annulus* and *Cx. tritaeniorhunchus* (Table 1). Phylogenetic analysis showed diversity within the phylogenetic clustering of each segment (Table 2). Segments 7 and 9 formed 5 independent clusters, segment 12 formed 3 clusters, and the remaining segments formed 4 clusters. Phylogenetic trees for some representative segments (segments 2, 6, 9, and

Figure 1. Mosquito collection sites in Vietnam.

12) are shown in Figure 2. Javanese strains formed a single cluster for segments 1, 2, 6, 8, 10, and 12. For segments 7, 9, and 11, however, they diverged into 2 clusters, I and II (Figure 2, panel C). The Vietnamese strains were distributed within 2 independent clusters for all 12 segments. Segments 11 and 12 of the 2 Vietnamese strains 02VN178b and 02VN018b were included in cluster III with Chinese BAVs. Segments 6 and 9 of isolate 02VN178b belonged to cluster V, although segments 1, 2, 3, 4, and 8 belonged to cluster IV. Moreover, segments 5 and 7 of isolate 02VN178b and segments 8 and 11 of isolate 02VN009b showed a mixed electrogram pattern of clusters V and IV or III.

Conclusions

We report isolation of BAV in Vietnam. The nucleotide sequences of the Vietnamese isolates' genomic RNA segments diverged into 2 phylogenetically distant clusters. Our data indicate that 2 BAV populations exist in the country and that both evolved independently. Two strains were clearly different from Chinese and Javanese BAVs (02VN078b and 02VN180b). Two other strains (02VN018b and 02VN178b) were phylogenetically close to Chinese BAVs when 2 shorter segments, segments 11 and 12, were analyzed but not when the remaining 10, longer segments were considered. This finding implies that a recent reassortment event has occurred and that segments of Chinese or Vietnamese strains were replaced. In addition, although some Vietnamese strains were phylogenetically distant, they could produce a viable progeny by reassortment of segments 5, 6, 7, 8, 9, and 11. This finding is clearly illustrated by the diversity in the phylogenetic clustering pattern and the mixed electrogram pattern of these segments (Table 2).

Analysis of segment 12 showed that cluster III (Table 2) contained 2 Vietnamese strains (02VN178b and 02VN018b) and the Chinese strains AF052030 and YN-6 (isolated in Yunnan Province) and BJ95-75 (isolated in Beijing). This finding suggests the possibility of wide circulation of BAVs containing segment 12 type III not only in Southeast Asia but also in East Asia.

A regionwide genotype shift of Japanese encephalitis virus (JEV), which is also carried by *Culex* spp. mosquitoes, from genotype III to genotype I, was witnessed in East Asia during the 1990s (*11,12*). These results and phylogenetic analysis of old JEV strains in Japan and Southeast Asia (*11*) strongly suggested that JEV could be transferred frequently from Southeast Asia to East Asia. Similarly, BAVs with segment 12 belonging to cluster type III were also distributed in both Southeast and East Asia. Therefore, we can speculate that BAVs are actively circulating within the Asian continent. To achieve a better understanding of the distribution dynamics of BAVs in Asia, more isolates are needed.

In Vietnam, a seasonal increase of viral encephalitis and meningitis cases is reported during the rainy season every year. Among these cases, 50%–70% are diagnosed as Japanese encephalitis, while the rest of them remain idiopathic (P.T. Nga et al., unpub. data). A clear association between BAV and human diseases, as well as the preva-

Table 1. Mosquito samples collected in Vietnam, 2002

Sample	Location	Month collected	Origin	No. mosquitoes
02VN009b	Ha Tay	Jan	*Culex annulus*	25
02VN018b	Quang Binh	Mar	*Cx. annulus*	170
02VN078b	Ha Tay	May	*Cx. tritaeniorhunchus*	100
02VN178b	Quang Binh	Aug	*Cx. tritaeniorhunchus*	102
02VN180b	Quang Binh	Aug	*Cx. tritaeniorhunchus*	83

Table 2. Phylogenetic clustering of Banna virus genomic RNA segments*

Strain	Isolation		Segments											
	Location	Year	1	2	3	4	5	6	7	8	9	10	11	12
Length (bp)			3747	3048	2400	2038	1716	1671	1136	1119	1101	977	867	862
Aligned area			990–3227	211–2541	108–2183	570–1748	110–760	106–1386	145–981	129–913	597–872	86–751	75–609	44–664
JKT-6423	Java, Indonesia	1980	I	I	I	I	I	I	I	I	I	I	I	I
JKT-6969	Java, Indonesia	1981	I	I	NA	NA	NA	I	II	I	II	I	II	I
JKT-7043	Java, Indonesia	1981	I	I	NA	NA	NA	I	II	I	II	I	II	I
Chinese	Yunnan, China	1987	III	III	III	III	III	III	III	III	III	III	III	III
YN-6	Yunnan, China	2000	NA	NA	NA	NA	NA	NA	NA	NA	III	NA	NA	III
BJ95-75	Beijing, China	1995	NA	NA	NA	NA	NA	NA	NA	NA	III	NA	NA	III
02VN018b	Quang Binh, Vietnam	2002	IV	IV	IV	IV	IV	IV	IV	IV	IV	IV	III	III
02VN178b	Quang Binh, Vietnam	2002	IV	IV	IV	IV	IV/V	V	IV/V	IV	V	IV	III	III
02VN009b	Ha Tay, Vietnam	2002	V	V	V	V	V	V	V	IV/V	V	V	V/ III	V
02VN078b	Ha Tay, Vietnam	2002	V	V	V	V	V	V	V	V	V	V	V	V
02VN180b	Quang Binh, Vietnam	2002	V	V	V	V	V	V	V	V	V	V	V	V

*Length, length of each segment in the reference sequence of Banna virus (BAV) from RefSeq (NC_004198, NC_004200–NC_004204, NC_004211, NC_004217–NC_004221); aligned area, the fragment used to make the alignment and phylogenetic tree. The numbers are set following the sequences of strain JHT-6423, which is the reference strain in National Center for Biotechnology Information Reference Sequences. I, the cluster including the Javanese JKT-6423 strain; II, the cluster specific to the JKT-6969 and JKT-7043 strains; III, the cluster including Chinese BAVs; IV, the cluster including the Vietnamese 02VN018b strain; V, the cluster including the Vietnamese 02VN180b strain. NA, not available.

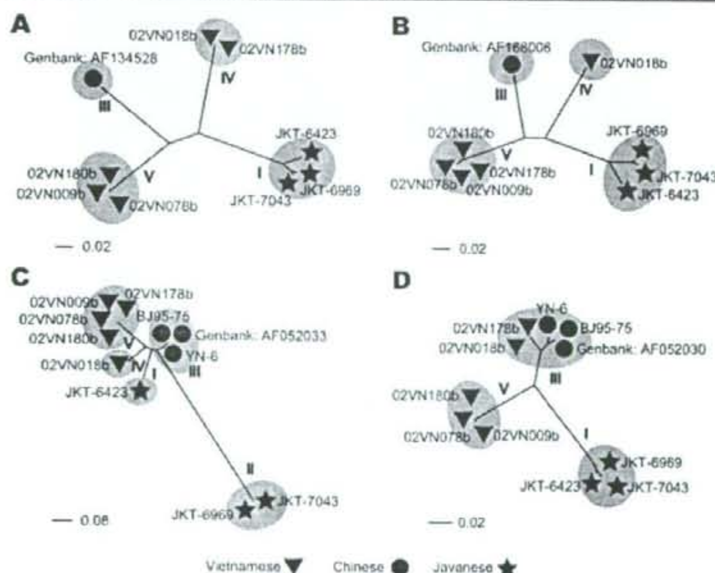

Figure 2. Phylogenetic trees of representative segments of Banna virus genomic RNA. A) Segment 2, encoding VP2, inner-layer coat protein. B) Segment 6, VP6, nonstructural protein, function is unknown. C) Segment 9, VP9, outer-layer attachment protein. D) Segment 12, VP12, dsRNA-binding protein. Clusters were numbered according to the clustering type classification presented in Table 2.

lence of BAV infection in humans, has not yet been established in Vietnam. This topic deserves further study. We also believe that BAV is a good candidate for the differential diagnosis of viral encephalitis and meningitis cases of unknown origin in tropical and subtropical Asia, where *Culex* mosquitoes are abundant.

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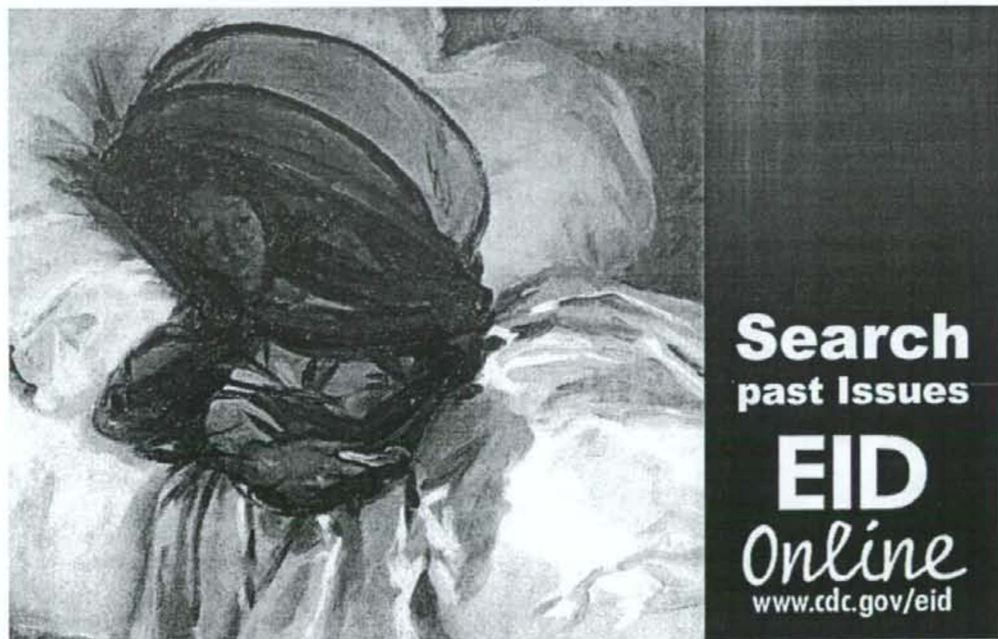

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Dengue Virus, Nepal

To the Editor: *Dengue virus* belongs to the genus *Flavivirus*, family *Flaviviridae*. It has 4 serotypes: dengue virus type 1 (DENV-1), dengue virus type 2 (DENV-2), dengue virus type 3 (DENV-3), and dengue virus type 4 (DENV-4). Dengue virus is maintained in a cycle between humans and *Aedes aegypti*, domestic day-biting mosquitoes. Dengue virus induces clinical illness, which ranges from a nonspecific viral syndrome (dengue fever [DF]) to severe and fatal hemorrhagic disease (dengue hemorrhagic fever [DHF]). DF/DHF occurs primarily in tropical and subtropical areas of the world. Domestic dengue virus infection occurs in >100 countries; >2.5 billion persons live in these areas. Approximately 100 million cases of DF, 500,000 cases of DHF, and several thousand deaths occur annually worldwide (*1*). During the past decades, dengue virus has emerged in southern Asia; DF/DHF epidemics have occurred in Bhutan, India, Maldives, Bangladesh, and Pakistan (*2–4*).

From August through November 2006, the number of febrile patients increased in 4 major hospitals in the Terai region of Nepal: Nepalgunj Medical College, Bheri Zonal Hospital in Nepalgunj, Tribhuban Hospital in Dang, and Narayani subregional hospital in Birgunj. Patients with severe symptoms were referred to Sukraraj Tropical and Infectious Disease Hospital, Kathmandu, for diagnosis and treatment. The clinical features in most patients were consistent with signs of DF, but some patients showed signs (high fever, rash, ecchymosis, epistaxis, positive tourniquet test, liver dysfunction, and thrombocytopenia [platelet count <100,000/mm^3]) consistent with the World Health Organization (WHO) definition of DHF. Ascites and plural effusion developed in 2 patients. Blood specimens were collected from all patients at the time of admission to the local hospitals. Particle agglutination (PA) assay (Pentax Ltd, Tokyo, Japan) (*5*) and immunoglobulin (Ig) M–capture ELISA (Dengue/JE IgM Combo ELISA kit, Panbio Ltd, Brisbane, Queensland, Australia) were performed. Dengue virus–specific IgM was detected in 11 patients who had fever, headache, and rash (Table). Each of these patients had negative

Table. Clinical and laboratory data for 11 patients admitted to hospitals and diagnosed with dengue fever or dengue hemorrhagic fever, Nepal, 2006*

Patient age, y/Sex	Month admitted	Location	Initial diagnosis	Travel history	Clinical signs and symptoms	Selected laboratory and other test results
20/M	Sep	Kathmandu	DF	Yes	Fever, headache, nausea	Hb 15.4 g/dL; TLC 10,500/mm^3; Plt 185,000/mm^3; blood culture for salmonellae negative; ALT 38 IU/L
27/F	Sep	Bardiya	Viral fever	No	Fever, headache, vomiting	TLC 5,600/mm^3; blood culture for salmonellae negative
3/M	Sep	Salayan	Encephalitis	No	Fever, vomiting, convulsions	Widal negative; TLC 4,700/mm^3
13/M	Oct	Sindhuli	Typhoid fever	No	Fever, headache	Widal negative; TLC 4,500/mm^3; blood culture for salmonellae negative; *Brucella* antigen negative; chest radiograph normal
22/M	Oct	Birgunj	DHF	No	Fever, headache, vomiting, ascites	Bil 0.8 mg/dL; ALT 80 IU/L; Plt 22,000/mm^3; chest radiograph normal
55/F	Oct	Dang	DF	No	Fever, headache, muscular pain	Plt 51,000/mm^3; TLC 7,600/mm^3; MP negative; ESR 20 mm/h; Bil 0.7 mg/dL
22/F	Oct	Birgunj	Viral fever	No	Fever, headache, body ache	*Brucella* negative; Widal negative; TLC 5,600/mm^3
13/M	Nov	Dang	DF	No	Fever, headache, rashes	Plt 95,000/mm^3; TLC 4,700/mm^3; Hb 13.1 g%; Bil 0.8mg/dL; ALT 26 IU/L
35/F	Nov	Birgunj	DHF	No	Fever, headache, bruises; tourniquet: positive	Bil 0.81mg/dL; Plt 31,000/mm^3; PT 2 min 30 s (control 14)
40/M	Nov	Birgunj	DF	No	Fever, headache, rashes	ALT 127IU/L; Plt 110,000 /mm^3; PCV 38.8%;TLC 5,500/mm^3; ultrasonography liver size, 16.8 cm
42/M	Nov	Dang	DF	No	Fever, headache, rashes	Bil 0.7 mg/dL; Widal test negative; TLC 6,800/mm^3; Plt 164,000/mm^3

*Blood specimens were collected at time of hospital admission. Diagnosis was confirmed by using immunoglobulin M–capture ELISA. DF, dengue fever; Hb, hemoglobulin;TLC, total leukocyte count; Plt, platelets; ALT, alanine aminotransferase; DHF, dengue hemorrhagic fever Bil, bilirubin; MP, malaria parasites; ESR, erythrocyte sedimentation rate; PT, prothrombin time; PCV, packed cell volume.

results for Japanese encephalitis virus–specific IgM. Of the 11 patients, 10 had no history of travel to India or other dengue-endemic countries. DF or DHF was initially diagnosed for 7 patients, and viral encephalitis, typhoid fever, or viral fever was diagnosed for others without serologic tests. Reverse transcription–PCR and virus isolation were performed at Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan, but the dengue virus genome was not detected, and no virus was isolated, likely because sample collection was delayed and the sample was transported to Japan in a deteriorated condition.

DF/DHF have been considered to be a possible public health threat to Nepal because DF/DHF epidemics have occurred recently in India and Pakistan, which reported several thousand cases and >100 deaths (*6*). The first DF case in Nepal was reported in 2004 (*7*). Further, the first DENV-2 strain of Nepal origin was isolated from a Japanese traveler who visited Nepal and in which DF developed after the patient returned to Japan. The isolated DENV-2 (GenBank accession no. AB194882) was 98% homologous with DENV-2 isolated in India (*8*). The prevalence of dengue virus antibody was reported to be 10.4% in the southwestern region of Nepal (*9*). These reports suggest that dengue virus has been circulating in Nepal for several years. Thus, DF/DHF has likely been misdiagnosed and illness caused by dengue virus underestimated in Nepal. In contrast, Japanese encephalitis has been a public health problem in southwestern region of Nepal, and large epidemics have occurred almost every year since 1978 (*10*). Nepal has no dengue surveillance programs, and health professionals do not usually consider dengue as a differential diagnosis.

The emergence occurred in the lowland Terai belt region, which borders the state of Bihar, India. The *Aedes* mosquito is known to persist in this region. The emerging DENV-2 is likely to have been introduced into Nepal from India.

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Human Tuberculosis Caused by *Mycobacterium bovis*, Taiwan

To the Editor: *Mycobacterium bovis* is one of the causative agents of tuberculosis (TB) in humans and animals. Drinking unpasteurized milk, eating undercooked meat, and close contact with infected animals are the main sources of infection for humans. Currently, 119 *M. bovis* spoligotypes are contained in the fourth international spoligotyping database (SpolDB 4) and are categorized into 3 main sublineages corresponding to ST prototypes 482, 683, and 479 (*1*).

Although an *M. bovis* surveillance program for farm animals has been implemented by the Taiwan Council of Agriculture, no surveillance system exists for human TB cases caused by *M. bovis*. To monitor the epidemiology of *M. bovis* in domestic animals, a regular tuberculin skin test (TST) is compulsory for cattle and sheep and optional for deer in Taiwan (*2*). In 2005, screening of *Mycobacterium* spp. infections by TST was performed for 111,412 cattle and 73,396 caprint and ovine herds, of which 188 (0.17%) and 148 (0.2%), respectively, were positive (*2*). We used spacer oligonucleotide typing (spoligotyping) and

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REVIEW

Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases

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SUMMARY

Loop mediated isothermal amplification (LAMP) is a powerful innovative gene amplification technique emerging as a simple rapid diagnostic tool for early detection and identification of microbial diseases. The whole procedure is very simple and rapid wherein the amplification can be completed in less than 1 h under isothermal conditions employing a set of six specially designed primers spanning eight distinct sequences of a target gene, by incubating all the reagents in a single tube. Gene amplification products can be detected by agarose gel electrophoresis as well as by real-time monitoring in an inexpensive turbidimeter. Gene copy number can also be quantified with the help of a standard curve generated from different concentrations of gene copy number plotted against time of positivity with the help of a real-time turbidimeter. Alternatively, gene amplification can be visualised by the naked eye either as turbidity or in the form of a colour change when SYBR Green I, a fluorescent dsDNA intercalating dye, is employed. LAMP does not require a thermal cycler and can be performed simply with a heating block and/or water bath. Considering the advantages of rapid amplification, simple operation and easy detection, LAMP has potential applications for clinical diagnosis as well as surveillance of infectious diseases in developing countries without requiring sophisticated equipment or skilled personnel. Copyright © 2008 John Wiley & Sons, Ltd.

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INTRODUCTION

Nucleic acid amplification is a valuable tool for the diagnosis of infectious diseases. Several amplification methods are available including PCR, nucleic acid sequence based amplification (NASBA), self-sustained sequence replication (3SR) and strand displacement amplification (SDA). Among these, PCR is the most widely used in various forms such as reverse transcription PCR (RT-PCR), nested PCR and multiplex PCR.

THE NEED FOR BETTER MOLECULAR DIAGNOSTIC TOOLS

These PCR-based methods require either high precision instruments for amplification or elaborate methods for detection of the amplified products. In addition, they are often cumbersome to adapt for routine clinical use especially in peripheral health care settings and private clinics. In addition, PCR has several intrinsic disadvantages, such as the requirement for thermal cycling, and time consuming post-PCR analysis, thereby potentially leading to laboratory contamination.

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Abbreviations used
3SR, self-sustained sequence replication; B3, backward outer primer; BIP, backward internal primer; BLP, backward loop primer; ΔG, free energy; DNA, deoxyribose nucleic acid; F3, forward outer primer; FIP, forward internal primer; FLP, forward loop primer; FMDV, foot and mouth disease virus; g-POCT, gene point-of-care testing; HPLC, high performance liquid chromatography; LAMP, Loop mediated isothermal amplification; NASBA, nucleic acid sequence based amplification; PEI, Polyethylenimine; POCT, point-of-care testing; RT-LAMP, reverse transcription loop mediated isothermal amplification; RT-PCR, reverse transcription polymerase chain reaction; SDA, strand displacement amplification; SNP, single nucleotide polymorphism; Tm, melting temperature; Tp, time of positivity; UV, ultra violet.

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The development of real-time PCR has brought true quantitation of target nucleic acids out of the pure research laboratory and into the diagnostic laboratory, by combining PCR amplification with fluorescent-labelled virus specific probes able to detect amplified DNA during the amplification reaction. The fluorescent chemistry coupled with advanced optical detectors makes it more sensitive than conventional gel-based PCR. Several real-time PCR assays have been developed to address the need for reliable detection systems for early infection and quantification of virus load in the acute phase of illness. Real-time PCR assays used for quantitative RT-PCR combine the best attributes of both relative and competitive (end-point) RT-PCR in that they are accurate, precise, capable of high throughput and relatively easy to perform. In addition, real-time PCR automates the laborious process of amplification by quantitating reaction products for each sample in every cycle. Data analysis, including standard curve generation and copy number calculation, is performed automatically.

The real-time assays have many advantages over conventional PCR methods, including rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity and easy standardisation [1]. However, all these nucleic acid amplification methods have several intrinsic disadvantages of requiring either a high precision instrument for amplification or an elaborate complicated method for detection of amplified products. Real-time PCR requires an instrumentation platform that consists of a thermal cycler, computer, optics for fluorescence excitation and emission collection and data acquisition and analysis software. Real-time PCR machines are expensive and thus are not within purchasing reach of laboratories in developing countries. More cost-effective, sensitive and real-time based assays are, therefore needed to complement the existing PCR-based assay systems. The present review describes a new generation of novel gene amplification technique that is gaining popularity among researchers due to its simple operation, rapid reaction and easy detection.

LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP): NEW GENERATION OF GENE AMPLIFICATION ASSAY

'LAMP' is characterised by the use of six different primers specifically designed to recognise eight distinct regions on the target gene. The amplification proceeds at a constant temperature using strand displacement reaction. Amplification and detection of a gene can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 63°C) [2]. Compared to PCR and real-time PCR, LAMP has the advantages of reaction simplicity and higher amplification efficiency. The LAMP reaction also yields large amounts of by-product, pyrophosphate ion, leading to a white precipitate of magnesium pyrophosphate in the reaction mixture. Since the increase in turbidity of the reaction mixture according to the production of precipitate correlates with the amount of DNA synthesised, real-time monitoring of the LAMP reaction can be achieved by real-time measurement of turbidity [3].

Design of LAMP primers

Design of a highly sensitive and specific primer set is crucial for performing LAMP amplification. The target selection for primer design can be accomplished by using the Primer Explore [LAMP primer designing support software program, Net laboratory, Japan, http://venus.netlaboratory.com] after considering the base composition, GC content and the formation of secondary structures. The primer set for LAMP amplification includes a set of six primers comprising two outer, two internal and two loop primers that recognise eight distinct regions on the target sequence. The two outer primers were described as forward outer primer (F3) and backward outer primer (B3) and have a role in strand displacement during the non-cyclic step only. The internal primers were described as forward internal primer (FIP) and backward internal primer (BIP) having both sense and antisense sequence in such a way that it helps in the formation of a loop. Further, two loop primers viz; forward loop primer (FLP) and backward loop primer (BLP) were designed to accelerate the amplification reaction by binding to additional sites that are not accessed by internal primers.

The design of the above mentioned six types of primers are based on the following eight distinct regions of the target gene: the F3c, F2c, F1c and FLP regions at the 3′ side and the B1, B2, B3 and BLP regions at the 5′ side (see Figure 1). FIP

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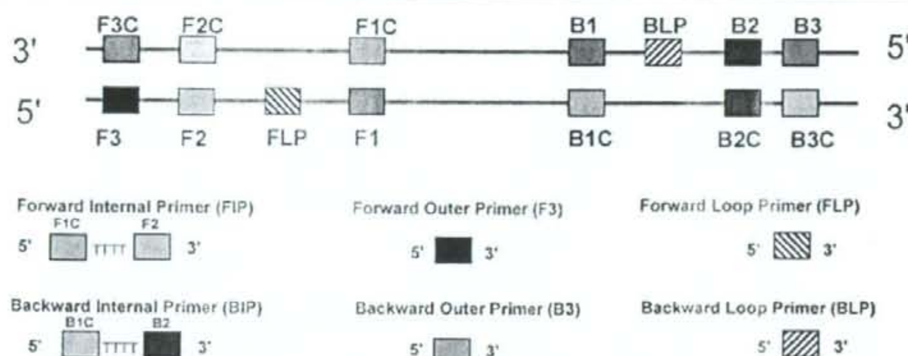

Figure 1. Schematic representation of primer design for RT-LAMP assay showing the position of the six primers spanning the target gene

consists of the F2 region (at the 3′ end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5′ end. Forward outer primer (F3) consists of the F3 region that is complementary to the F3c region. BIP consists of the B2 region (at the 3′ end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5′ end. Backward outer primer (B3) consists of the B3 region that is complementary to the B3c region. FIP consists of a complementary sequence of F1 and a sense sequence of F2. BIP consists of a complementary sequence of B1 and a sense sequence of B2 (Figure 1). FIP and BIP were high performance liquid chromatography (HPLC) purified primers. The FLP and BLP primers were composed of the sequences that are complementary to the sequence between F1&F2 and B1&B2 regions respectively [2].

In addition, the following criteria needs to be considered critically for getting an ideal LAMP primer set having excellent combination of sensitivity and specificity. The GC content of the primers should be about 50–60% in the case of GC rich and about 40–50% for AT rich. The primers should be designed so as not to easily form secondary structures. 3′ end sequence should not be AT rich or complementary to other primers. The distance between 5′ end of F2 and B2 should be 120–180 bp, and the distance between F2 and F3 as well as B2 and B3 should be 0–20 bp. The distance for loop forming regions (5′ of F2 to 3′ of F1, 5′ of B2 to 3′ of B1) should be 40–60 bp. The melting temperature (Tm) for primer regions should be about 60–65°C in the case of GC rich and about 55–60°C for AT rich. The stability of primer end should be established based on the change in free energy (ΔG) calculated 6 bp from the following end regions which should be less than −4 kcal/mol, 5′ end of F1c/B1c and 3′ end of F2/B2 as well as F3/B3. If restriction enzyme sites exist on the target sequence, except the primer regions, they can be used to confirm the amplified products.

Principle of LAMP amplification

The chemistry of LAMP amplification is based on the principle of auto cyclic strand displacement reaction being performed at a constant temperature using a DNA polymerase. There are two steps of LAMP amplification comprising non-cyclic and cyclic steps [4].

Non-cyclic step

In the non-cyclic step (see Figure 2A), there is the formation of DNA with stem-loops at each end that serve as the starting structure for the amplification by LAMP cycling. Because double stranded DNA is in the condition of dynamic equilibrium at the temperature around 65°C, one of the LAMP primers can anneal to the complimentary sequence of double stranded target DNA, then initiates DNA synthesis using the DNA polymerase with strand displacement activity, displacing and releasing a single stranded DNA [2,4].

With the LAMP method, unlike with PCR, there is no need for heat denaturation of the double stranded DNA into a single strand. Through the activity of DNA polymerase with strand displace-

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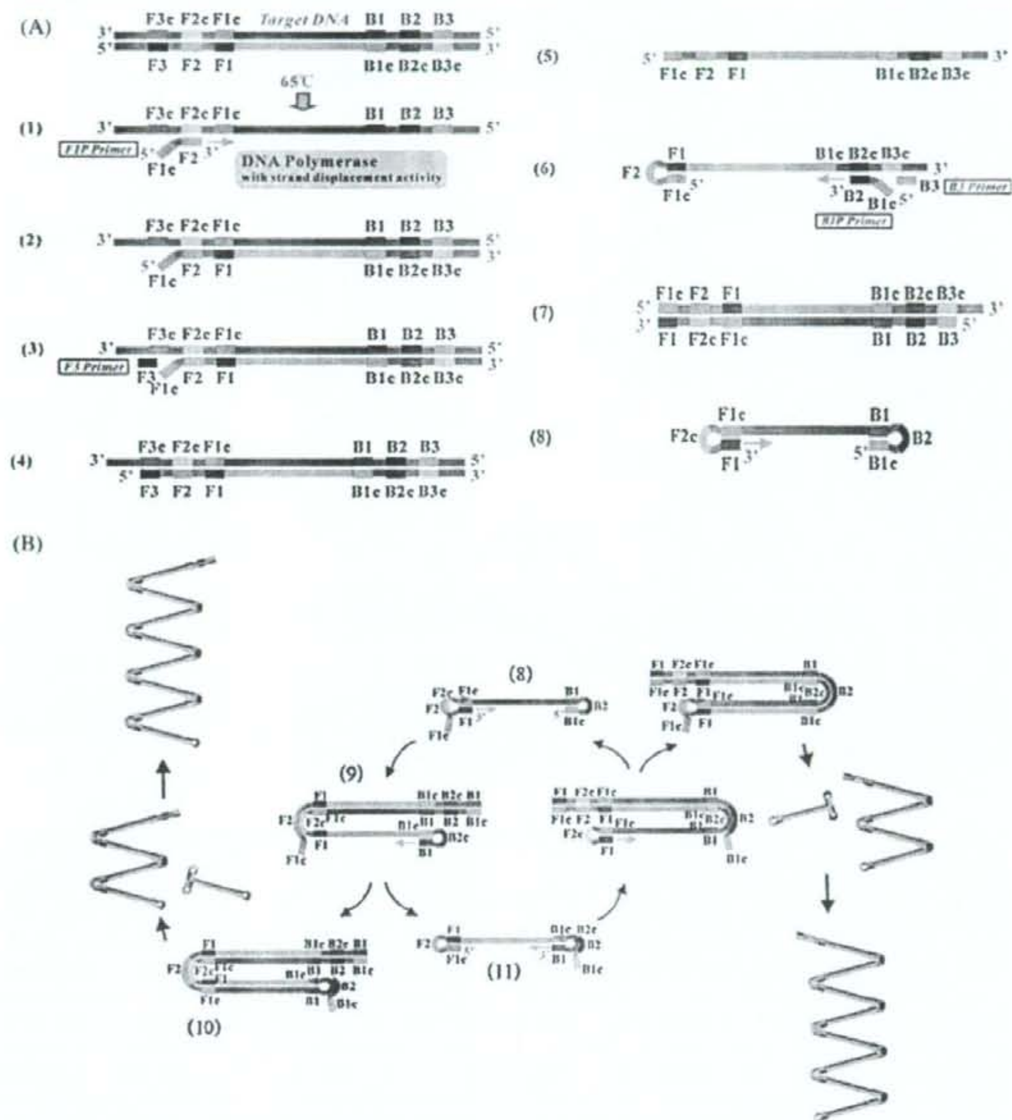

Figure 2A. Principles of Lamp amplification. Non-Cyclic Step [1--8]: generation of stem loop DNA with dumbbell-shaped structure at both ends that is ready to enter into cyclic amplification step. Initially, the strand displacement activity of *Bst* DNA polymerase helps in synthesis of a complementary DNA strand, starting with FIP. The outer primer (F3) then displaces the FIP-linked complementary strand, which forms a stem-loop structure at the 5′ end. This serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The final product is a structure with stem-loops at each end. Copyright ©, 2005, Eiken Chemical Co. Ltd., Japan. (B) Principles of LAMP amplification. Cyclic Amplification Step [9--11]: exponential amplification of original dumbbell-shaped stem-loop DNA employing internal primers. The product is the differently sized structures consisting of alternately inverted repeats of the target sequence on the same strand, giving a cauliflower-like structure. Copyright ©, 2005, Eiken Chemical Co. Ltd., Japan

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(C)

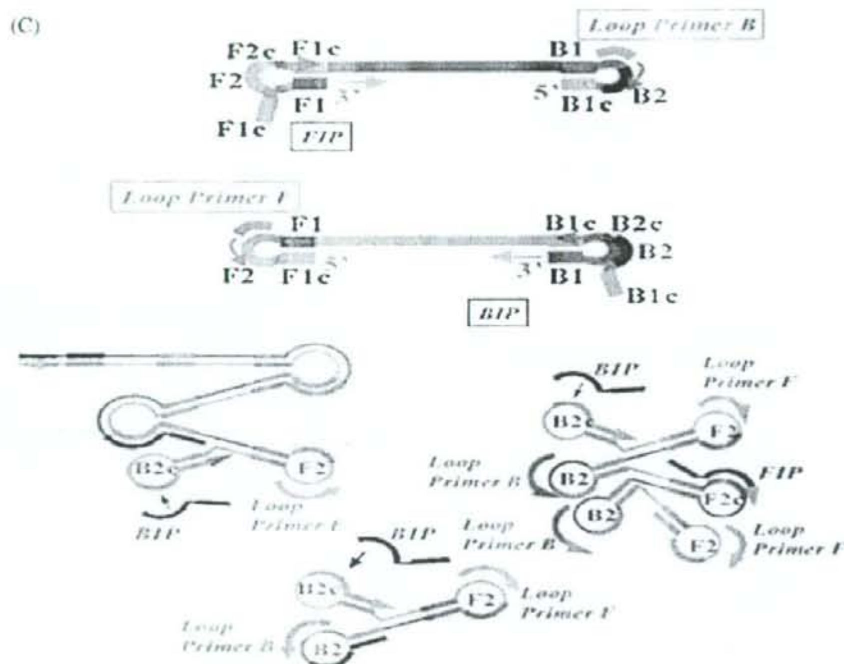

Figure 2C. Principles of LAMP amplification. Accelerated LAMP Amplification using loop primers. Loop primers are complementary to the single stranded loop region. They provide additional starting sites for DNA synthesis and accelerate the amplification thereby reducing the reaction time to less than 30 min. Copyright ©, 2005, Eiken Chemical Co. Ltd., Japan

ment activity, a DNA strand complementary to the template DNA is synthesised, starting from the 3′ end of the F2 region of the FIP. The F3 primer anneals to the F3c region, outside of FIP, on the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand. A double strand is formed from the DNA strand synthesised from the F3 primer and the template DNA strand. The FIP-linked complementary strand is released as a single strand because of the displacement by the DNA strand synthesised from the F3 primer. Then, this released single strand forms a stem-loop structure at the 5′ end because of the complementary F1c and F1 regions. This single strand DNA in turn serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The BIP anneals to the DNA strand produced by the above step. Starting from the 3′ end of the BIP, synthesis of complementary DNA takes place. Through this process, the DNA reverts from a loop structure into a linear structure. The B3 primer anneals to the outside of the BIP and then, through the activity of the DNA polymerase and starting at the 3′ end, the DNA synthesised from the BIP is displaced and released as a single strand before DNA synthesis from the B3 primer. The BIP-linked complementary strand displaced forms a structure with stem-loops at each end, which looks like a dumbbell structure. This dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. This structure serves as the starting structure for exponential amplification.

Cyclic amplification

In subsequent LAMP cycling (see Figure 2B) one internal primer hybridises to the loop on the product and initiates displacement DNA synthesis,

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yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice as long. Briefly the FIP anneals to the single stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesised strand. This released single strand forms a stem-loop structure at the 3′ end because of complementary B1c and B1 regions. Then, starting from the 3′ end of the B1 region, DNA synthesis starts using self-structure as a template, and releases FIP-linked complementary strand. The released single strand then forms a dumbbell-like structure as both ends have complementary F1–F1c and B1c–B1 regions, respectively. Furthermore, BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand. As a result of this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed. The cycling reaction continues leading to accumulation of 10^9 copies of target in less than an hour. The final products are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand (Figure 2B).

LAMP amplification can also be accomplished with the two outer (F3 and B3) and two internal primers (FIP and BIP) but by using the two loop primers (FLP and BLP), the amplification is accelerated thereby reducing the amplification time [5]. The investigation on how loop primers affect amplification time (original method: no loop primer; rapid method: with loop primers) revealed that the time required for amplification with loop primers is one-third to one-half of that without loop primer. With the use of loop primers, amplification can be achieved within 30 min (Figure 2C).

Assay protocol for RT-LAMP amplification

The LAMP reaction is usually carried out in a total 25 µl reaction volume containing 50 pmol each of the primers FIP and BIP, 5 pmol each of the outer primers F3 and B3, 25 pmol each of loop primers FLP and BLP in a 2× reaction mixture having 20 mM Tris-HCl pH8.8, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 10 mM KCl, 1.4 mM dNTPs, 0.8 M betaine, 0.1% Tween20, 8 units of the *Bst* DNA polymerase and 2 µl of DNA template. Positive and negative controls should be included in each run, and all precautions to prevent cross-contamination should be observed. The optimum temperature for the LAMP reaction is 63°C, which is optimum for the activity of *Bst* DNA polymerase.

The amplification of RNA template is accomplished through Reverse Transcription-Loop-mediated Isothermal Amplification (RT-LAMP) assay by employing RT for reverse transcription step in addition to the *Bst* DNA polymerase. RT-LAMP method can synthesise cDNA molecules from template RNA and apply LAMP technology to amplify and detect them. As the template is an RNA sample, in addition to the reagents of DNA amplification (primers, DNA polymerase with strand displacement activity, substrates, etc.), RT is added to the reaction mixture. After mixing and incubating at a constant temperature between 60–65°C, amplification and detection can be carried out in a single step.

Monitoring of RT-LAMP amplification

Real-time monitoring

The real-time monitoring of LAMP amplification can be accomplished through spectrophotometric analysis with the help of loop amp real-time turbidimeter (LA-200, Teramecs, Japan) that records the turbidity in the form of O.D. at 400 nm every 6 s (Figures 3 and 4A). The turbidimeter is relatively inexpensive as compared to the real-time PCR machine. Turbidity is the unique phenomenon associated with LAMP amplification and it is attributed to the higher amplification efficiency of LAMP reaction. In order to observe turbidity in the form of a white precipitate, a DNA yield of ≥4 µg is required so as to push the pyrophosphate ion concentration above 0.5 ppm. LAMP reaction produces a DNA yield of ≥10 µg compared to 0.2 µg in PCR in 25 µl reaction scale. In addition, the low amount of pyrophosphate ion formed during PCR gets hydrolysed to phosphate due to the high temperature of >94°C. Thus turbidity is the easiest way of monitoring gene amplification by LAMP method [6].

Agarose gel analysis

Following incubation at 63°C for 30 min, 10 µl aliquot of LAMP amplified products are electrophoresed on 3% NuSieve 3:1 agarose gel (BMA,

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