47．輸血後感染症検査でHCVコア抗原又はHCV抗体又はHCV-RNAが陽性であった症例で、上記以外の症例についてお聞きしま
それらの症例が上記に含まれなかった理由を以下の中からお選び下さい（複数回答可）

【上記以外の症例】

輸血後感染症検査において HCVコア抗原又はHCV抗体又はHCV-RNA陽性であった症例のうち、以下のa.、b.を除く症例

a. 輸血前感染症検査でHCV抗体陽性 and/or HCVコア抗原 and/or HCV-RNA陽性であった症例

b. 輸血前感染症検査でHCV抗体、HCVコア抗原、HCV-RNA が陰性で、献血者の保管検体の個別NAT検査でHCVが証

（以下該当する項目をすべて囲んでください）

①輸血前感染症検査を行っていなかった
②輸血前検体保存を行っていなかった
③院内感染が原因であった
④性交渉が原因であった
⑤不明
⑥その他　→「その他」の場合、詳細を記入してください。

記入欄

48．輸血後感染症検査でHCVコア抗原、HCV抗体、HCV-RNA陽性症例に関する症例調査にご協力いただけますか（個人を特定
輸血によるウイルス感染症の実態を把握するために必須の調査です。是非ともご協力下さい。

（以下該当する項目を一つ○で囲んでください）

①はい　→「①はい」の場合、下欄に施設名、所属、担当者名をお書き下さい。後日調査票をお送りします。
②いいえ

貴施設名	
ご所属	
ご芳名	

6) 輸血とHIV感染

49．過去1年間(2007年)に輸血後感染症検査でHIV抗体又はHIV-RNAが陽性であった症例はありますか
(以下該当する項目を一つ○で囲んでください)
①あり
②なし　→「②なし」の場合、設問54にお進み下さい。
③把握していない　→「③把握していない」の場合、設問54にお進み下さい。
ありの場合は症例数をご記入下さい
[　　]例

50．輸血後感染症検査でHIV抗体又はHIV-RNAが陽性であった症例で、輸血前からHIV感染者と分かっていた症例はありますか
【輸血前からHIV感染症例】
輸血後感染症検査においてHIV抗体又はHIV-RNA陽性であった症例のうち、
輸血前感染症検査、又は輸血前保管検体の検査でHIV抗体 and/or HIV-RNA陽性であった症例
(以下該当する項目を一つ○で囲んでください)
①あり
②なし
ありの場合は症例数をご記入下さい
[　　]例

51．輸血後感染症検査でHIV抗体又はHIV-RNAが陽性であった症例で、輸血によるHIV感染症伝播が証明された症例はありますか
【HIV感染症伝播が証明された症例】
輸血後感染症検査において HIV抗体又はHIV-RNA陽性であった症例のうち、輸血前感染症検査でHIV抗体 and/or HIV-
献血者の保管検体の個別NAT検査でHIVが証明された症例
(以下該当する項目を一つ○で囲んでください)
①あり
②なし
ありの場合は症例数をご記入下さい
[　　]例

52．輸血後感染症検査でHIV抗体又はHIV-RNAが陽性であった症例で、上記以外の症例についてお聞きします
それらの症例が上記に含まれなかった理由を以下の中からお選び下さい(複数回答可)

【上記以外の症例】
輸血後感染症検査において HIV抗体又はHIV-RNA陽性であった症例のうち、
a. 輸血前感染症検査、又は輸血前保管検体の検査でHIV抗体 and/or HIV-RNA陽性であった症例
b. 輸血前感染症検査でHIV抗体、HIV-RNA が陰性で、日赤で行った輸血された血液の個別NAT検査でHIVが証明された
を除く症例

(以下該当する項目をすべて囲んでください)

①輸血前感染症検査を行っていなかった
②輸血前検体保存を行っていなかった
③院内感染が原因であった
④性交渉が原因であった
⑤不明
⑥その他　→「その他」の場合、詳細を記入してください。

記入欄

53．輸血後感染症検査でHIV抗体又はHIV-RNA陽性であった症例の調査にご協力いただけますか(個人を特定できない調査です

①はい　→「①はい」の場合、下欄に施設名、所属、担当者名をお書き下さい。後日調査票をお送りします。
②いいえ

貴施設名	
ご所属	
ご芳名	

7) その他

54．輸血前感染症検査と輸血後感染症検査のあり方についてお聞きします

①輸血前感染症検査、輸血前検体保存、輸血後感染症検査を現状のまま行う

②輸血前感染症検査は省略し、輸血前検体保存と輸血後感染症検査を行う

③輸血後感染症検査のみ行う

④全て行わない

⑤その他　→「その他」の場合、詳細を記入してください。

記入欄

55　日本におけるヘモビジランスシステム（輸血患者の感染症伝播や副作用の全国的監視体制）の構築について、ご意見やアイデアがあればお書き下さい。

記入欄

輸血後感染症検査を含めた日本のヘモビジランス体制を確立するため、ご協力をお願いすることがありますので、差し支えなければ、貴施設名、ご所属、ご回答者氏名をお書き下さい。

貴施設名	
ご所属	
ご芳名	

ご協力ありがとうございました。

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	頁	出版年
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Short communication

Rapid determination of viral RNA sequences in mosquitoes collected in the field

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Abstract

A method for rapid determination of viral RNA sequences (RDV) was applied to homogenates of *Aedes aegypti* collected in Thailand in an area in which dengue fever (dengue hemorrhagic fever) is endemic, using the mosquito cell line C6/36. Nucleic acid sequences of dengue virus type 4 and cell fusing agent virus were detected. This RDV method has the potential to become a standard method for detection of both known and newly emerging, unknown mosquito-borne viruses.

Keywords: Rapid determination; Dengue virus type 4; Cell fusing agent virus; *Aedes aegypti*

Dengue viruses (DENV) cause dengue fever, the most important mosquito-borne viral disease, and these viruses pose a major public health problem in tropical and subtropical areas (Guzman and Kouri, 2002). *Aedes aegypti* is the primary and most effective epidemic vector of DENV (Gubler, 1998). Virological surveillance of mosquitoes naturally infected with DENV, using a reverse transcriptase-polymerase chain reaction (RT-PCR), is thought to be a fast and effective predictive method for detecting dengue outbreaks (Urdaneta et al., 2005). However, it is possible for mosquitoes to also carry viruses that are unknown or that cannot currently be identified by RT-PCR based on known viral nucleic acid sequences. Therefore, a system for rapid nucleic acid sequence determination is necessary to identify newly emerging mosquito-borne viruses.

Recently, a method was developed to determine rapidly the RNA of viruses (RDV) that can determine the nucleotide sequence of viral RNA without a specific primer (Mizutani et al., 2007). It was also possible to detect mosquito-borne viruses, such as West Nile virus, Japanese encephalitis virus, and DENV-2, from culture supernatants (Mizutani et al., 2007). The RDV method can detect at least 10^4 copies of *in vitro* synthesized RNA (unpublished data). In this study, the RDV method was used as a virological surveillance tool on *Ae. aegypti* collected in the field in Thailand.

Ae. aegypti specimens (93 adult females) were collected around houses of patients diagnosed clinically with dengue fever in Jomthong District, Bangkok, Thailand, in July 2006. Each mosquito was placed in a 0.5 ml tube and was homogenated

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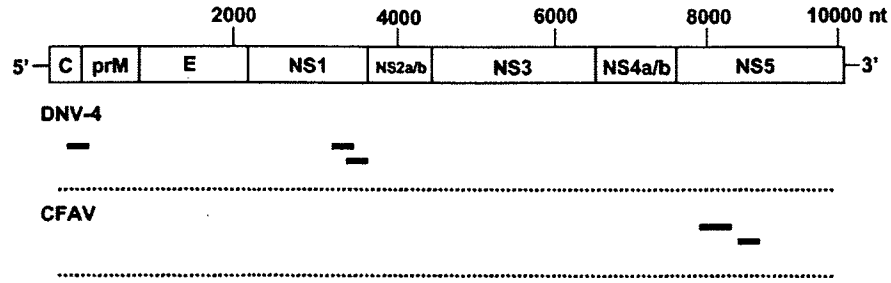

Fig. 1. Location of PCR fragments of viral cDNAs amplified using the RDV method. Amplified PCR fragments were directly sequenced and mapped in the flavivirus genome.

using a pellet mixer in 100 μl of minimum essential medium (MEM) with 4% fetal bovine serum (FBS). The homogenates were centrifuged at 2500 rpm for 10 min at 4 °C, and 50 μl of the supernatant was used. RT-PCR was performed using the OneStep RT-PCR enzyme mix (Gibco-BRL, Cergy Pontoise, France) and primers designed to detect all four serotypes of DENV. Eight of the 93 homogenates were DENV-positive. Four DENV-positive homogenates numbered 1, 2, 3 and 4 were filtrated using a Millex-GX filter (Millipore Corp., Bedford, Massachusetts), and the filtrate was added to a C6/36 cell culture (7×10^5 cells) in a 24-well plate. Total volume was brought to 1 ml per well by adding MEM with 2% FBS, and was incubated at 28 °C in a CO_2 incubator. After 9 days, cytopathic effects were observed in the cells exposed to DENV-positive homogenate. The culture supernatants were collected and centrifuged at 2500 rpm for 10 min at 4 °C. Then, 50 μl of the supernatant was used for cDNA synthesis.

The four cDNAs were mixed for the following RDV method. The protocol for the RDV method is described in detail elsewhere (Mizutani et al., 2007). The nucleic acid sequences from 16 fragments were used to search for homological sequences using BlastN. Three fragments were found to be highly homologous with DENV-4, and two fragments were found to be homologous with cell fusing agent virus (CFAV) (Fig. 1). CFAV belongs to flavivirus that does not have a vertebrate host (Stollar and Thomas, 1975). CFAV replicates in *Aedes* mosquitoes and in mosquito cells. No antigenic cross-reaction was found between CFAV and other member of flavivirus (Cammisa-Parks et al., 1992). The nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under accession nos. AB300619–AB300621 (DENV-4) and AB300622–AB300623 (CFAV). This result shows that nucleic acid sequences of DENV-4 and CFAV can be detected using the RDV method without using specific primers. To determine which homogenates contained these viruses, the four cDNAs were pre-amplified individually using a GenomiPhi V2 kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to manufacturer's protocol. PCR was performed using KOD-Plus (Toyobo Co. Ltd., Osaka, Japan). Primers for detecting DENV-2 and DENV-4 are described by Morita et al. (1994). Three primer sets for detecting CFAV were designed to be at the NS4A region of CFAV based on accession number NC_001564. DENV-4, but not DENV-2, was detected in all four of the cDNA samples, whereas CFAV was only detected in the cDNA of homogenate 1, but not in homogenates 2, 3, and 4 (data not shown), indicating that both DENV-4 and CFAV co-infected the mosquito of homogenate 1. Integration of part of the CFAV genome in C6/36 genomic DNA has been reported (Crochu et al., 2004). However, CFAV was not detected from RNA and DNA extracted from two different C6/36 cell stocks using RT-PCR (data not shown). Although it cannot be ruled out that the CFAV genome was integrated in the genome of the C6/36 cells below detection level, these results strongly suggest that CFAV nucleic acid sequences found by the RDV method originated from the mosquito of homogenate 1.

It was demonstrated that the RDV method is able to detect DENV and CFAV without using specific primers for amplification. There have been a number of mosquito-borne outbreaks of flaviviruses recently, and birds are also important vectors for viruses such as West Nile virus. Recently, direct determination of avian viral RNA sequences was demonstrated in allantoic fluids inoculated with a test specimen using the RDV method (Sakai et al., 2007). The RDV method therefore has the potential to become a standard method for the detection of both known and newly emerging, unknown avian-borne and mosquito-borne viruses.

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Rapid Genome Sequencing of RNA Viruses

Tetsuya Mizutani,* Daiji Endoh,† Michiko Okamoto,‡ Kazuya Shirato,* Hiroyuki Shimizu,* Minetaro Arita,* Shuetsu Fukushi,* Masayuki Saijo,* Kouji, Sakai,* Chang Kweng Lim,* Mikako Ito,* Reiko Nerome,* Tomohiko Takasaki,* Koji Ishii,* Tetsuro Suzuki,* Ichiro Kurane,* Shigeru Morikawa,* and Hidekazu Nishimura‡

We developed a system for rapid determination of viral RNA sequences whereby genomic sequence is obtained from cultured virus isolates without subcloning into plasmid vectors. This method affords new opportunities to address the challenges of unknown or untypeable emerging viruses.

Over the past few years, global migration has led to emerging infectious diseases that pose substantial risks to public health. To prevent potential outbreaks, early detection of infectious pathogens is necessary. In particular, the recent outbreak of severe acute respiratory syndrome (SARS) provided important lessons on how unknown viruses should be detected rapidly. Thus, a standardized and qualified system is required for rapid nucleic acid sequence determination for newly emerging viruses.

Recently, we developed a new method for detecting RNA viruses. This method, based on cDNA representational difference analysis (cDNA RDA), uses 96 hexanucleotides that are not suitable for priming ribosomal RNAs but that normally prime most of the genome of an RNA virus as primers for reverse transcription in cDNA RDA (*1*). However, the RDA method with a cloning step requires at least 1 week for the determination of the nucleic acid sequence.

The Method

Our new system for rapid determination of viral RNA sequence (RDV) uses whole-genome amplification and direct sequencing techniques (Figure 1). The RDV method comprises 6 procedures: 1) effective destruction of cellular RNA and DNA for semipurification of viral particles, 2) effective elimination of DNA fragments by using a prefiltration column system and elution of small amounts of RNA, 3) effective synthesis of first- and second-strand cDNAs, 4) construction and amplification of a cDNA library, 5) construction of a second cDNA library, and 6) direct sequencing using optimized primers. The RDV method enables a broad range of partial nucleotide sequences within the entire viral RNA genome to be obtained within 2 days without cloning into plasmids.

To eliminate contaminating cellular RNA and DNA from the samples, 0.001 μg of RNase A (Qiagen, Hilden, Germany) and 1 μL (2 U) of Turbo DNA-free DNase I (Ambion, Austin, TX, USA) with 1× Turbo DNA-free buffer were incubated at 37°C for 30 min under conditions that prevented destruction of viral RNA in the viral particles. The RNA in the viral particles was then extracted within 30 min by using a total RNA isolation mini kit (Agilent Technologies Inc., Palo Alto, CA, USA). We confirmed that DNA was effectively eliminated by this RNA extraction kit.

In accordance with the Invitrogen manual, cDNA was synthesized, by using random hexamers (Takara Bio Inc., Kyoto, Japan) and Superscript III (Invitrogen, Carlsbad, CA, USA) lacking RNase H activity, at 50°C for 1 h. Then 60 U of RNase H (Takara Bio Inc.) added before synthesis of second-strand cDNA at 50°C for 1 h. In accordance with the manual, a whole genome amplification system (WGA; Sigma-Aldrich, Saint Louis, MO, USA), which was developed for amplification of genomic DNA, was used to amplify viral double-stranded cDNA. This process was

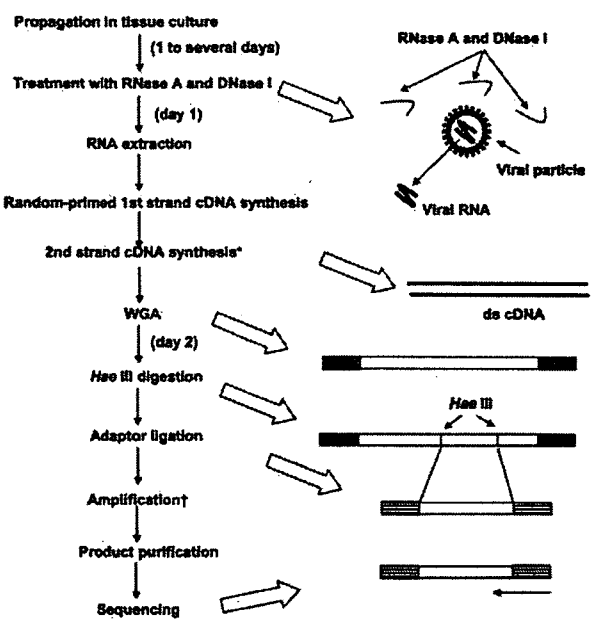

Figure 1. Overall scheme of the rapid determination of viral RNA sequence method. *By adding RNase H; WGA, whole genome amplification; †With specially designed primer sets as shown in Figure 2.

*National Institute of Infectious Diseases, Tokyo, Japan; †Rakuno Gakuen University, Ebetsu, Japan; and ‡Sendai Medical Center, Sendai, Japan

performed within 90 min. Instead of the Taq polymerase recommended in the kit, we used 1.25 U of AmpliTaq Gold LD (Applied Biosystems, Foster City, CA, USA) to obtain a high yield of the PCR products. Primers were provided in the WGA kit, but no information regarding their sequences was obtained. The reaction mixture was heated at 95°C for 9 min (for activation of AmpliTaq Gold), followed by 70 cycles of amplification using Mastercycler (Eppendorf AG, Hamburg, Germany). Each PCR cycle consisted of annealing at 68°C for 1 min, primer extension at 72°C for 5 min, and denaturation at 94°C for 1 min.

The 1st cDNA library was digested with 40 U of *Hae*III (Takara Bio Inc.) at 37°C for 30 min. DNA was purified by using the MonoFas DNA isolation system (GL Science, Tokyo, Japan), and a blunt *EcoR*I-*Not*I-*Bam*HI adaptor (10 pmol; Takara Bio Inc.) was ligated at 16°C for 30 min by using DNA Ligation Kit, Mighty Mix (Takara Bio Inc.). The second cDNA library was amplified by PCR with specially designed primer sets in which 6 nucleotides composed of CC (*Hae*III-digested sequence) and 4 variable nucleotides were added to the 3′ end of the adaptor sequence (Figure 2). For example, 1 primer set was as follows: forward primer, H1–1: 5′-AATTCGGCGGCCGCG GATCC*CC*GGGG-3′; reverse primer H9–3: 5′-AATT CGGCGGCCGCGGATCC*CC*AGGA-3′ (the adaptor sequence is underlined, and the *Hae*III-digested sequence is shown in italics) (Figure 2).

We always used >12 primer sets and 0.83 μmol of each primer per cDNA library. PCR was performed with AmpliTaq Gold Master Mix (Applied Biosystems). The reaction mixture was heated at 95°C for 12 min, followed by 70 cycles of amplification. Each PCR cycle consisted of annealing and primer extension at 72°C for 30 s and denaturation at 94°C for 30 s. A single band was consistently obtained in ≈50% of the reactions. DNA was purified from the PCR by using MonoFas. Occasionally, we purified DNA fragments from the gels when >2 bands were detected. Direct sequencing was performed with the forward primer, reverse primer, or both.

When the number of viral particles in the sample was high, we omitted the RNase A and DNase I treatments and used the RNeasy Mini Kit (Qiagen) for RNA extraction. We occasionally used a whole transcriptome amplification kit (Rubicon Genomics Inc, Ann Arbor, MI, USA) instead of the WGA kit because both kits yielded similar amplification results.

In preliminary studies that used referential RNA viruses, we attempted to determine the nucleic acid sequences of SARS coronavirus, mouse hepatitis virus, West Nile virus, Japanese encephalitis virus, and dengue virus type 2 in culture supernatants (10–100 μL) by using the RDV method. The percentages of positive fragments (number of fragments containing viral nucleic acid/total number of sequenced fragments) in the reactions for detection of these 5 viruses were 60% (3/5), 45% (5/11), 100% (12/12), 50% (5/10), and 40% (4/10), respectively. As a clinical application, a throat swab specimen from a patient with fever and upper respiratory infection was characterized. Although the specimen exhibited enterovirus-like cytopathic effect by inoculation into HEF and GMK cells when cell culture system for virus isolation was used (*2*), extracted RNA from the supernatant of the cells showed no amplification by reverse transcription–PCR (RT-PCR) when 1 of the conventional primer sets for human enteroviruses was used (*3*,*4*). In the cell culture supernatant analysis by the RDV method, the specimen exhibited amplification of the partial nucleotide sequences of coxsackie A14 virus (nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under accession nos. AB275848–AB275853). Thus, the RDV method could detect unidentified cytopathic-effect agents such as enterovirus that could not be detected by RT-PCR when the conventional primer set for enteroviruses was used.

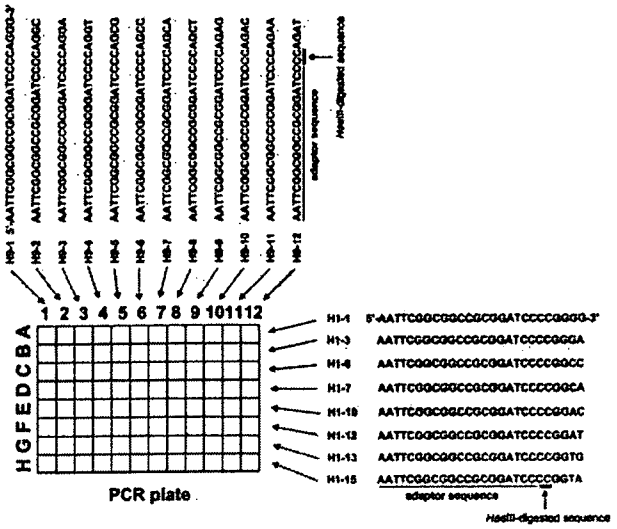

Figure 2. Primers used in rapid determination of viral RNA sequence method.

Conclusions

The RDV method is a rapid method for the direct determination of viral RNA sequences without using the cDNA cloning procedure. The limitations of the RDV method are the requirement for cell culture isolate and the large number of steps. However, RDV would be useful for species-independent detection of RNA viruses including unknown or untypeable emerging RNA viruses. Furthermore, with minor modifications, this method would also be applicable to the detection of DNA viruses and bacteria.

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