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Rapid identification of flavivirus using the polymerase chain reaction

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Summary

A rapid and accurate detection and identification system was developed for flaviviruses that makes use of reverse transcription-polymerase chain reaction (RT-PCR). A primer pair (YF-1 and YF-3), which corresponds to the highly conserved sequence at 3′ noncoding region among flaviviruses, was useful for identification of mosquito-borne flaviviruses. Nine sets of species specific primer pairs were also selected to identify and distinguish species, i.e., yellow fever, West Nile, Murray Valley encephalitis, Japanese encephalitis, St. Louis encephalitis and dengue type 1 to 4 viruses.

This method required only two hours for completion using infected culture fluid, thus facilitating rapid identification of mosquito-borne flavivirus species.

RT-PCR; Flavivirus; Universal primer; Virus identification

Introduction

The genus flavivirus in family Flaviviridae is a single-stranded, positive-polarity RNA virus (Rice et al.,1985) and includes at least 66 species. 29 of these are associated with human disease and are transmitted mainly by mosquitoes (Porterfield, 1980). Dengue virus (DEN), yellow fever virus (YF), and Japanese encephalitis virus (JE), for example, are highly pathogenic in humans and their epidemics are significant public health problem in tropical countries (Halstead et al., 1980; Umenai et al., 1985; Monath, 1986).

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The viruses are often isolated from brain specimens, cerebrospinal fluid, peripheral blood, and field-caught mosquitoes using suckling mouse brain (Work, 1964), cultured mosquito cells (Igarashi, 1978) or laboratory-colonized mosquitoes (Kubersky and Rosen, 1977). Identification of the isolated virus is important for clinical and epidemiological investigation.

Flaviviruses are widely cross-reactive (Porterfield; 1980, de Madrid and Porterfield, 1974; Calisher et al., 1989), and the identification of their field isolates was not easy until the development of species-specific monoclonal antibodies (mAbs) (Henchal et al., 1982; Peiris et al., 1982; Schlesinger et al., 1983; Roehrig et al., 1983; Kobayashi et al., 1984; Kimura-Kuroda and Yasui, 1986; Broom et al., 1987). This assay has limitations, however, in that the sense mutations affecting monoclonal reactive epitopes can result in negative detection or identification of virus isolates.

In recent years, the polymerase chain reaction (PCR) method (Saiki et al., 1988) has been used widely in rapid and specific detection of viruses such as the human immunodeficiency virus (Ou et al., 1988; Rayfield et al., 1988), hepatitis B virus (Kaneko et al., 1989), hepatitis C virus (Shibata et al., 1990), and cytomegalovirus (Demmler et al., 1988). We previously reported the identification of dengue virus serotypes using PCR following reverse transcription (RT-PCR) on infected culture fluid without RNA extraction (Morita et al., 1991).

In the present study, using the sequence data of several flavivirus species, we designed flavi-universal primer pair and flavi-species specific primer pairs in full consideration of nucleotide similarity and devised a strategy for rapid identification of flavivirus by direct RT-PCR without RNA purification steps using virus infected cell culture fluid.

Material and Methods

Virus strains

The flavivirus strains used in this study were YF (17D vaccine strain), West Nile virus (WN: Eg101 strain), Murray Valley encephalitis virus (MVE: prototype), JE (JaOArS982 strain), St. Louis encephalitis (SLE: prototype), type 1 DEN (D1: Hawaiian strain), type 2 DEN (D2: New Guinea B strain), type 3 DEN (D3: H87 strain) and type 4 DEN (D4 H241 strain). The viruses were inoculated once to C6/36 cells (Igarashi, 1978) in tube culture. The infected fluids were harvested 1 week after incubation at 28°C and stored at −80°C until use.

Sequence-handling software

All sequence selections, searches, comparisons and confirmations after sequencing were performed with a DNASIS-MAC Version 1.0 software system (Hitachi, 1991).

TABLE I

Sequence of synthetic oligonucleotide primers.

Primer code	Sequence (5' to 3')	Product (bp)	References
YF-1	GGTCTCCTCTAACCTCTAG		Rice et al., 1987
YF-3	GAGTGGATGACCACGGAAGACATGC		
YF-S10	GTAACAGTTACATCGCTGAG	278	Rice et al., 1987
YF-C10	AGCAAGAAACATGTCCACCA		
WN-S3	CACAGCGGGCTTTACTATCT	229	Castle et al., 1985, Wengler et al., 1985
WM-C3	CATTTCCAGCAGCTAGGACC		
MVE-S	GTCAGACGTTTCTACGGTGT	273	Dalgarno et al., 1986
MVE-C	AGAATAATTTCCATGACTGG		
JE-NS3-1S	AGAGCGGGGAAAAAGGTCAT	142	Sumiyoshi et al., 1987
JE-NS3-4R	TTTCACGCTCTTTCTACAGT		
SLE-S	GATGTATATCACATACACCG	233	Trent et al., 1987
SLE-C	GAATGAACCAATGGAATCGT		
D1-S	GGACTGCGTATGGAGTTTTG	470	Mason et al., 1987
D1-C	ATGGGTTGTGGCCTAATCAT		
D2-S	GTTCCTCTGCAAACACTCCA	210	Hahn et al., 1988, Deubel et al., 1988
D2-C	GTGTTATTTTGATTTCCTTG		
D3-S1	GTGCTTACACAGCCCTATTT	300	Osatomi and Sumiyoshi, 1990
D3-C1	TCCATTCTCCCAAGCGCCTG		
D4-S	CATTATGGCTGTGTTGTTT	378	Mackow et al., 1987, Zhao et al., 1986
D4-C	CTTCATCCTGCTTCACTTCT		

Primers

All primers were synthesized with an Applied Biosystems DNA synthesizer (model 392) and checked for purity by ion-exchange gel chromatography (Genpack, Waters). When more than 1% of incomplete length oligomer was observed, the primer was discarded and the sequence was synthesized anew. The nucleotide sequence and reference information of the primers are listed in Table 1, and the locations of YF-1, YF-3 within a few flavivirus genomes are illustrated in Fig. 1.

Probes

For hybridization to confirm the RT-PCR product, each flavivirus species-specific cDNA probe was prepared with TA Cloning System (Invitrogen). Amplified RT-PCR products using species-specific primer pairs were ligated with pCR vector (Invitrogen), and the recombinant pCR plasmids were sequenced by the method of Sanger et al. (1977). Each flavivirus species-specific cDNA was cut from the recombinant pCR plasmids with restriction enzyme of vector's polylinker site. The cloned flavivirus species-specific cDNA fragments were labeled with biotin using a Bio-Nick Labeling System (BRL).

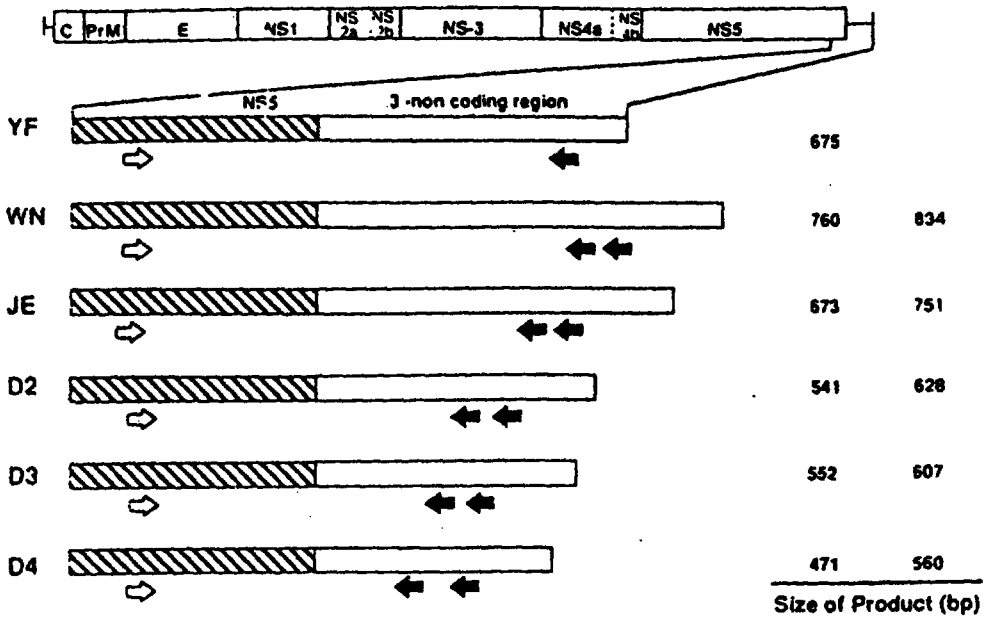


Fig. 1. Location of flavi-universal primer pair in flavivirus genomes. Open arrows indicate YF-3, closed arrows indicate YF-1. The size of products (bp) are given.

Rapid Reverse Transcription (RT)-PCR

5 μl of infected culture fluid was incubated with an equal volume of detergent mix (1% Nonidet P-40, 10 U of RNase inhibitor (Takara Co., Kyoto, Japan) in phosphate-buffered saline (−)) in a 0.5 ml Eppendorf-type tube for 1 min at room temperature. This was followed by addition of 90 μl of RT-PCR mix (100 pmol of each primer, 0.2 mM deoxynucleoside triphosphate, 10 mM Tris/HCl (pH 8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg of bovine serum albumin per ml, 0.1% sodium cholate, 0.1% Triton X-100, 10 U of reverse transcriptase (Life Science Inc., UK) and 2 U of thermostable Tth DNA polymerase (Toyobo Co., Osaka, Japan). The reaction mixture was covered by 2 drops of mineral oil, and the tube was incubated for 10 min at 53°C for 1st strand cDNA synthesis by reverse transcriptase reaction. PCR amplification was achieved in 25 cycles using the following steps: denaturation at 94°C for 60 s, annealing at 53°C for 60 s, and extension at 72°C for 60 s using a thermal cycler (Iwaki Co., Tokyo, Japan).

Analysis of amplified DNA

After cycling, 5 μl of PCR product was resolved on a 3% NuSieve 3:1 Agarose gel (FMC) in TAE buffer (40 mM Tris/acetate (pH 8.0), 1 mM EDTA). Gels were stained with ethidium bromide, and amplified DNA bands were visualized by UV light transillumination.

For dot-blot analysis, a 10 μl portion of RT-PCR mixture was denatured in 0.25 M NaOH at room temperature for 5 min and then immobilized on

Hybond-N membrane (Amersham) with 10 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate) using a BIO-DOT apparatus (Bio-Rad). After rinsing, blots were air dried and irradiated with UV light (256 nm) for 3 min. Prehybridization was performed in Hybridization buffer (Amersham) containing 45% formamide at 42°C for 1 h. After 1 h, each biotin-labeled flavivirus specific cDNA probe was added to the prehybridization buffer. After overnight incubation at 42°C, positive signals were determined according to the instructions of the manufacturer using the BluGENE Nonradioactive Nucleic Acid Detection System (BRL).

For sequencing of direct PCR products after RT-PCR reaction, 50 μl of PCR products were separated from the primers and dNTPs by filtration through a Quick Spin Columns (Boehringer) according to the instructions of the manufacturer (Boehringer). The eluted material was precipitated with ethanol and dried in vacuum centrifuge and was dissolved in an appropriate volume of water immediately before use. Sequencing was performed using the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems INC.). Briefly, a 20-μl mixture was prepared to contain 3.2 pmol of primer (each complementary PCR primer), 1 μg of PCR product, 4, μl of 5 × sequence buffer, each 1 μl DyeDeoxy N terminator (dideoxynucleoside triphosphate fluorescent terminator), and 4 U *Taq* DNA polymerase, and the reaction mixture was overlaid with one drop of mineral oil. Immediately after placing the reaction tubes in a thermal cycler (Techne, Ltd), the following thermal cycling was started: 96°C, 15 s; 50°C 1 s; 60°C, 4 min for a total of 25 cycles. Then, the reaction mixture was purified with Quick Spin Column and dried in vacuum centrifuge. Precipitate was resuspended in 3 μl of loading solution (5:1 mixture of deionized formamid: 50 mM EDTA) and the sample was heated to 95°C for 2 min and loaded onto an Applied Biosystems 373A DNA Sequencer according to the instructions of the manufacturer (Applied Biosystems Inc.).

Results

Detection of mosquito-borne flavivirus genomic RNA with flavi-universal primer

Using the highly conserved sequences in the 3′ untranslated region (UTR) of mosquito-borne flavivirus genome (Rice et al., 1988), flavi-universal primer pair (primer code YF-1, YF-3) was selected for maximum homology with 6 species of flaviviruses, YF, MVE, JE, D2, D3, D4 which have already been sequenced for their 3′ UTR. The nucleotide sequences of flavi-universal primer pair are shown in Fig. 2 together with the number of mismatches with sequenced flavivirus species. Since the primer code YF-1 was expected to anneal with 2 conserved sequences near the 3′-end except YF genomic RNA, 2 amplified DNA bands were observed with the expected size on the agarose gel for the remaining 9 flaviviruses (Fig. 3). Because this RT-PCR could detect mosquito-born flaviviruses such as SLE, D1, MVE for which the 3′-UTR

YF-1 primer*	GGTCTCCTCTAACCTCTAG	5' Location	Mismatches
YF	----------	10709	0
WN	---G------	10787	1
JE	----------	10750	0
D2	----------G----	10496	1
D3	----------A----	10472	1
D4	----------G----	10419	1
YF-1 primer**	GGTCTCCTCTAACCTCTAG		
WN	----------	10861	0
JE	----------	10828	0
D2	----------	10583	0
D3	--------A---A------	10557	2
D4	----------	10508	0
YF-3 primer	GAGTGGATGACCACGGAAGACATGC		
YF	----------	10052	0
WN	--A--------G----------	10045	2
JE	----------	10095	0
D2	--A--------G----------	9973	2
D3	C----------T--A----------	9968	3
D4	C----------T-----T----	9966	3

Fig. 2. Alignment of the flavi-universal primer pair with the corresponding sequences of other flaviviruses. The symbol (–) represents identical bases: mismatched bases are indicated by the characters. The 5' nucleotide position of the sequences and number of mismatches are given. YF-1 primer was designed to anneal with 2 position except for YF virus: * shows 5' side annealing position, ** shows 3' side annealing position.

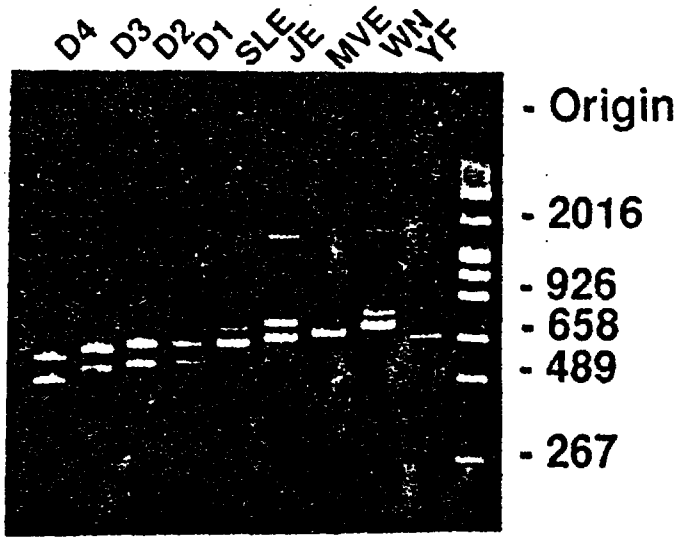


Fig. 3. Utility of universal primers for amplification of different flaviviruses. Each flavivirus species was amplified by RT-PCR using flavi-universal primer pair and the product was visualized by ethidium bromide staining after agarose gel electrophoresis.

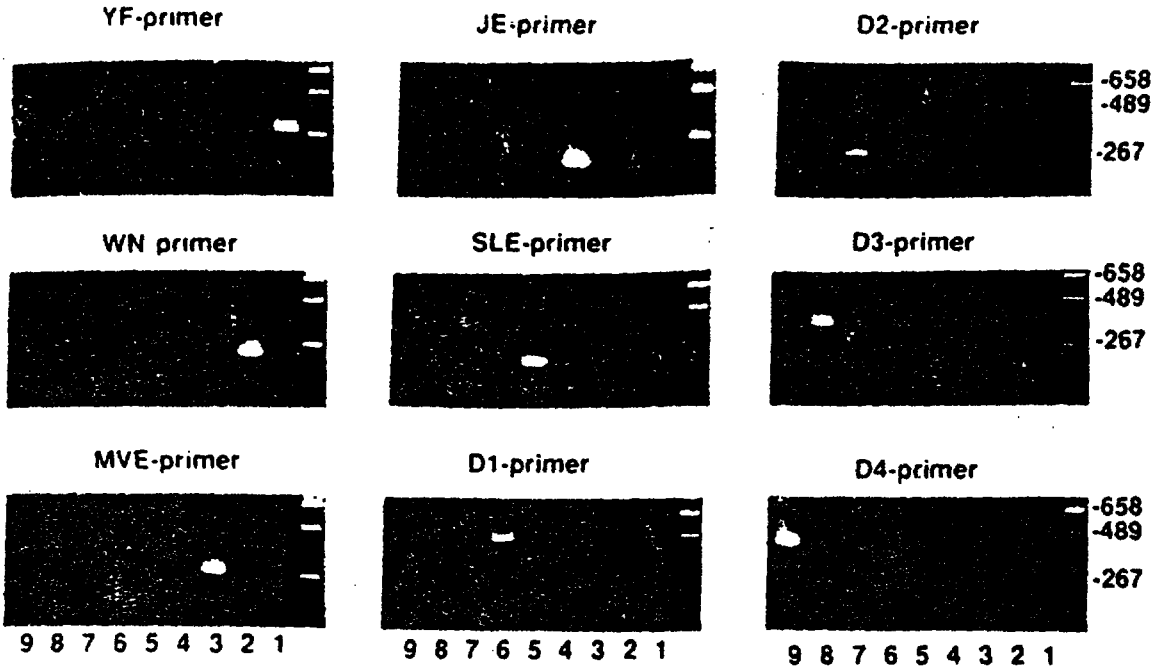


Fig. 4. Identification and specificity of each species-specific primer pair to amplify flavivirus by RT-PCR. Each flavivirus infected fluid (5×10^5 to 6×10^7 PFU/ml) was amplified with 9 kinds of species-specific primer pairs and the amplified product was detected in ethidium bromide-stained agarose gel electrophoresis.

sequence has not yet been reported, the result indicated that these viruses possess a conserved sequence similar to that of other mosquito-born flaviviruses.

Detection of each flavivirus species using species specific primers

In order to identify the viral-species, the nucleotide sequence of flavivirus species-specific primer pairs was selected from published data by computer analysis. A computer search showed that the homology of the complementary primers was less than 4 base pairs to the heterologous flavivirus RNA sequences. Each primer could amplify only its homologous virus genome, and no detectable DNA fragment was amplified by heterologous primers for any of the flavivirus species (Fig. 4). The specificity of the RT-PCR product was confirmed by dot blotting using species-specific cDNA probes, since each species-specific cDNA probe reacted only with the homologous virus species product (Fig. 5). The specificity of the RT-PCR product was further confirmed by complementary primer direct sequencing. As an example of this experiment, nucleotide sequence of D1-specific amplified PCR product (Hawaiian strain) was shown to compare to the published D1 sequence (Nauru Island strain) (Fig. 6). There were only 13 base mismatches in the sequenced region of 304 nucleotide, showing that the method can detect strain difference.

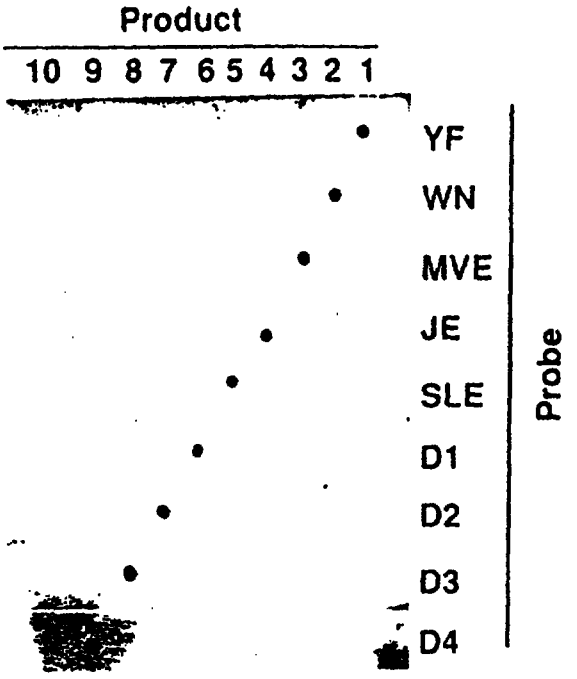


Fig. 5. Dot blot hybridization of flavivirus species-specific RT-PCR product with cloned cDNA probes of each flavivirus. Code number of the product. 1: Yellow Fever virus. 2: West Nile virus. 3: Murray Valley encephalitis virus. 4: Japanese encephalitis virus. 5: St. Louis encephalitis virus. 6: Dengue type 1 virus. 7: Dengue type 2 virus. 8: Dengue type 3 virus. 9: Dengue type 4 virus. 10: each homologous primer pair (50 pmol each).

Discussion

This study describes a simple method for the identification of flavivirus group and species from infected C6/36 cell culture fluids by direct RT-PCR. Classical hemagglutination inhibition and neutralization tests have been used for these purposes, but both are cumbersome and time-consuming. The identification and detection of the virus isolates have been facilitated by the application of MAbs. Recently, however, we observed that three D1 virus strains from dengue hemorrhagic fever patient in Thailand did not react with a D1-specific MAb 15F3 (Henchal et al., 1982), although they reacted with polyclonal anti-dengue and anti-D1 MAb 1F1 (kindly supplied by Drs. B. Innes and A. Nisalak). These three isolates were identified as D1 by RT-PCR described in this paper (Maneekarn, in preparation). The results suggest that one particular MAb may not be able to detect such mutants with altered amino acid sequence of the relevant epitope. Using the PCR method, it is possible to identify the virus isolates with target sequence of the primers within approximately 20% nucleotide sequence variation (Lee et al., 1988). Moreover, the PCR method may be applicable to pathological specimens from which antigen detection by MCA may be difficult.

Although identification of flavivirus or dengue virus genome by RT-PCR has been reported (Deubel et al., 1990; Henchal et al., 1991; Eldadah et al.,

```
        2349                          2373
NAU     CAGTTGGCATGGTCACGCTGTACCTAGGAGTCATGGTTCAGGCGGACTCG
        ************************************************
HAW     CAGTTGGCATGGTCACGCTGTACCTAGGAGTCATGGTTCAGGCGGACTCG
        1                             25
        2399                          2423
NAU     GGATGTGTAATCAACTGGAAAGGCAGAGAACTCAAATGTGGAAGCGGCAT
        **********  ********************************  *****
HAW     GGATGTGTAATTAACTGGAAAGGCAGAGAACTCAAATGTGGAAGTGGCAT
        51                            75
        2449                          2473
NAU     TTTTGTCACCAATGAAGTCCACACCTGGACAGAGCAATATAAATTCCAGG
        ****************** *****  ********  ***************
HAW     TTTTGTCACCAATGAAGTTCACACTTGGACAGAACAATATAAATTCCAGG
        101                           125
        2499                          2523
NAU     CCGACTCCCCTAAGAGACTATCAGCGGCCATTGGGAAGGCATGGGAGGAG
        ********************** *  *********  *************
HAW     CCGACTCCCCTAAGAGACTATCACCACCCATTGGGAGGGCATGGGAGGAG
        151                           175
        2549                          2573
NAU     GGTGTGTGTGGAATTCGATCAGCCACGCGTCTCGAGAACATCATGTGGAA
        ********************  ****************************
HAW     GGTGTGTGTGGAATTCGATCACCCACGCGTCTCGAGAACATCATGTGGAA
        200                           225
        2599                          2623
NAU     GCAAATATCAAATGAACTAAACCACATCTTACTTGAAAATGACATGAAAT
        ***  ******  *****  ********************************
HAW     GCAGATATCACATGAATTAAACCACATCTTACTTGAAAATGACATGAAAT
        251                           275
           2652
NAU     TTAC
        ****
HAW     TTAC
           304
```

Fig. 6. Alignment of the nucleotide sequences of RT-PCR amplified product (304 nucleotides) from D1 infected fluid (Hawaiian strain) and the published D1 Nauru Island strain. Nauru Island nucleotide positions are numbered according to Mason et al., 1987. NAU: Dengue type 1 Nauru Island strain, HAW: Dengue type 1 Hawaiian strain.

1991; Laille et al., 1991; Lanciotti et al., 1992), our system allows identification of the flaviviral genomes without RNA extraction using a single reaction tube and mosquito-borne flavivirus universal primer pair. The reactivity of our flavivirus universal primer, YF-1 and YF-3, with SLE, D1, MVE indicated that the target sequences of these primers were well conserved among mosquito-borne flaviviruses and probably possess an important biological function in the replication process.

The present results can be utilized systematically for the identification of unknown flavivirus isolates. The relevant virus specimen should first be screened with the YF-1 and YF-3 primer pair to obtain a rough estimate of the viral species by the number and size of the amplified product on agarose gel electrophoresis. Secondly, viral species should be confirmed by RT-PCR using species-specific primers. Hybridization with enzyme-labeled species-specific probe can be used as a confirmatory test. Our method is also suitable for rough

quantitative estimation of flavivirus RNA in the studies of viral replication, inhibition or interference.

Although Southern blotting, dot blotting or restriction enzyme cleavage have been used for the confirmation of PCR product, direct sequencing of RT-PCR product using the automatic DNA sequencer can serve as an alternative confirmatory method that can be completed within 2 days. This direct sequencing method is a valuable way to obtain sequence information required to elucidate the molecular epidemiology of the viruses.

Recently, our RT-PCR protocol was utilized for the rapid diagnosis of acute hepatitis C virus (HCV) infection (Kurihara et al., 1992). HCV appears to be distantly related to the flavivirus family (Choo et al., 1989), although the HCV genome does not react to flavi-universal YF-1 and YF-3 primer pair.

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Cosmid library of the turkey herpesvirus genome constructed from nanogram quantities of viral DNA associated with an excess of cellular DNA

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Summary

A protocol was designed for the rapid and efficient construction of cosmid libraries from cell-associated viral genomes available in very low quantities. Purification of viral DNA from cellular DNA was unnecessary. The vast excess of cellular DNA compensated for the limited amount of available viral DNA, enabling titration of the restriction endonuclease partial digest. A cosmid library of the turkey herpesvirus DNA genome was constructed from 1.5 micrograms of cellular DNA containing approximately 6 nanograms of viral DNA.

Cosmid library; Recombinant DNA; Herpesviruses; Cloning

Introduction

Traditional methods of constructing cosmid libraries from very large viral genomes involved isolating DNA from purified virion and generating DNA fragments in the 30 to 50 kbp size range either by shearing or partial restriction endonuclease digestion. The DNA fragments within the 30–50 kilobase pair (kbp) size range were purified from other fragment sizes by sucrose gradient centrifugation or gel electrophoresis before cloning into the cosmid vector. One difficulty with this approach was the requirement for large quantities of DNA

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