**Development of a Consensus Microarray Method for Identification of Some Highly Pathogenic Viruses**

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Some highly pathogenic viruses, such as Chikungunya virus, Japanese encephalitis virus, Yellow fever virus, Dengue virus, Hanta virus, SARS-CoV, and H5N1 avian influenza virus can cause severe infectious diseases. However, the consensus method for detecting these viruses has not been well established. A rapid and sensitive microarray approach for detection of these viruses and a panel of specific probes covering nine genera and 16 virus species were designed. 70-mer oligonucleotides were used at the genus level and 50-mer oligonucleotides were at the species level, respectively. To decrease the interference of the host genome in hybridization, the consensus genus primers were designed and used to reverse transcribe only virus genome. The synthesis of the second strand was carried out with a random primer sequence (5'-GTTTCCCAGTAGGTCTCNNNNN-3'). The amplified products were labeled and processed for microarray analyses. This microarray-based method used the highly conserved consensus primers to synthesize specifically the virus cDNA and could identify effectively Chikungunya virus, Japanese encephalitis virus, Yellow fever virus, Dengue virus, Tick borne encephalitis virus, and H5N1 avian influenza virus. Using this method, one unknown virus isolated from pig brain in Shanxi Province, China was identified. This method may have an important potential application for the diagnosis of virus infection.

**INTRODUCTION**

It is important to develop accurate methods for detecting and identifying the highly pathogenic viruses which can cause severe diseases. These viruses include Eastern equine encephalitis virus (EEE), Western equine encephalitis virus (WEE) and Chikungunya virus (CHIK) which belong to genus *Alphavirus*; Japanese encephalitis virus (JEV), Yellow fever virus (YFV), and Dengue virus (DEN) which belong to genus *Flavivirus*; and others such as Hanta virus, H5N1 avian influenza virus, Rift Valley virus (Rift) and SARS-CoV. Many of these viruses have been considered as potential biological warfare agents. Therefore, accurate detection and identification of these pathogens is a prerequisite for effective control of their transmission. In recent years, a wide range of detection methods such as electron microscopy, ELISA, PCR (or real-time PCR) and IFA have been developed and are used currently in laboratory diagnosis. However, all these methods are designed to detect a single or just a few targets. When unknown pathogens are presented, multiple diagnostic assays have to be performed in parallel, which is a time-consuming process. Microarray, which is high throughput and can detect multiple targets on a single chip, is a promising diagnostic tool for detection of pathogens [Elnifro et al., 2000; David et al., 2002; Kessler et al., 2004; Oh et al., 2004; Jehanara et al., 2005; Kostrzynska et al., 2006].

In this study, a novel microarray method was developed for detecting 16 highly pathogenic viral pathogens. The oligonucleotide probes at both genus and species levels were designed to identify these viruses. By using a consensus protocol, a number of viruses were able to be identified with 70-mer genus and 50-mer species probes.

**MATERIALS AND METHODS**

**Virus Culture**

CHIK was inoculated in BHK cells; JEV, YFV, DEN, and Tick-borne encephalitis virus (TBE) were inoculated in C6/36 cells; H5N1 virus was obtained from the
spleen of infected Balb-c mice; an unknown virus isolated from pig brain (from Shanxi province, China) was inoculated in BHK-21 cells. All viruses were cultured in DMEM medium supplemented with 10% FCS and antibiotics. The infections were allowed to proceed until the onset of cytopathic effects (CPE). RNAs were extracted from the culture supernatants. Viral cultures and Balb-c mice injection with H5N1 were carried out in Biosafety Level-3 (BSL-3) and Animal Biosafety Level-3 (ABS3) laboratories, respectively.

**Probe and Consensus Primer Design**

Table I lists the viruses included in this study. The virus sequences covered by the array were obtained from the database of fully sequenced viral genomes in GenBank.

For initial screening of viruses, probes were designed at the genus level. In order to cover viruses as many as possible of the genus probes, all viral sequences were aligned with the ClustalX program (version 1.83) to select the most conserved regions of each sequenced viral genome. The sequences which were 70 nt long and shared more than 50% sequence identity among all the members of a given genus were selected as genus probes.

Similarly, for virus probes at the species level, all the sequences of the same viral species were obtained from GenBank and aligned by ClustalX. After the conserved sequences were identified, the probes were designed with the Array designer software (version 4.0). Through sequence alignment analysis, each probe of a significant homology among different viral genomes within a specific species was longer than 30 nt. Then, the BLASTN program was used to select those probes that shared less than 50% sequence identity with other viral species [Kane et al., 2000; Chang and Peck, 2003]. Finally, a panel of specific probes including 16 species probes including 16 species was selected, with five 50 nt probes for each viral species.

Reverse transcription primers were designed to synthesize specific viral sequences out of the mixed RNA in the host genome. All the primers were designed based on the genus consensus sequences; and one common tag region was included (5'-GTTTCCCAGTAGGTCTC-3') at the 5' end, which was used for PCR amplification. In total, 11 reverse transcription primers to cover nine virus genera were designed, with two for Hanta virus, two for Influenza virus, and one for each of the other genera.

All oligonucleotides were suspended in 3 x SSC at a concentration of 50 pmol/L and printed on aldehyde glass slides. In addition, the oligonucleotide probe (5'-cctcggagagacatagtgtcgggaagctgtacacgg-aattgccaggacgaccggctctc-3') in the conserved region of hepatitis C virus (HCV) genome was selected as a positive control and printed at the top and bottom of the chip for microarray scanning. Specific PCR primers for a 181 bp product which was complementary to this region were also designed. Sequences of HCV-specific primers were HCV1 5'-AGTGTGTCAGCCCTCCAG-3' and HCV2 5'-GCCTTTCGGGACCCCAACTCTC-3'.

**RNA Extraction and cDNA Synthesis**

RNAs from infected and uninfected viral cell cultures were isolated with RNeasy mini kit (Qiagen, Inc., Valencia, CA). Viral cDNA was obtained by using genus-specific primer pools. The 11 primers for reverse transcription were divided into three groups: group A contained primers of CCFV-M, Hanta L, Hanta M, and Alpha; group B contained primers of Flavi, Nipah, H5, and N1; and group C contained primers of Rift-M, Lassa, and SARS. Reverse transcription was performed in three separated reaction tubes. Each tube contained one group of primers, 1 x reaction buffer, 2.5 U RNase inhibitor, 2.5 U AMV (Takara, Inc., Dalian City, Shiga, Japan), 0.5 mM deoxynucleoside triphosphates (dNTPs), 5 μL template; and RNAase-free water was added to a final volume of 10 μL. The reaction was performed at 42°C for 1 hr. The second-strand cDNA synthesis was carried out with Sequenase (New England Biolabs, Inc., Beverly, MA) by PrimerA (5'-GTTTCCCAGTAGGTCTC-3'). The tag sequence in primer A was the same as that in the genus primers to make it possible for subsequent PCR amplification of the viral genome with the anchor primer B (5'-GTTTCCCAGTAGGTCTC-3').

**Random PCR Amplification of the Virus Genome**

Following reverse transcription, 10 ng cDNA was taken as template and primer B (5'-GTTTCCCAGTAGGTCTC-3') was used for PCR amplification in the

| Genus   | Species                     | Primer                                      |
|---------|-----------------------------|---------------------------------------------|
| Alphavirus | CHIK, WEE, EEE              | Alpha: 5–3 GTTTCCCAGTAGGTCTCCTCCCACAT       |
| Flavivirus | TBE, DEN, YFV, JEV         | Flavi: 5–3 GTTTCCCAGTAGGTCTCCTCCCACATCAGTT |
| Hantavirus | Hanta virus                 | HANTA-L: 5–3 GTTTCCCAGTAGGTCTCCTCAGG       |
| Phlebovirus | Rift valley virus          | HANTA-M: 5–3 GTTTCCCAGTAGGTCTCCTAGTGA       |
| Nairovirus | Crimean-Congo hemorrhagic fever virus | RIFT-M: 5–3 GTTTCCCAGTAGGTCTCCTCATTAGTA |
| Arenavirus | Lassa, Junin, Machupo      | CCFV-M: 5–3 GTTTCCCAGTAGGTCTCCTACTGCAATT |
| Coronavirus | SARS-CoV                   | Lassa: 5–3 GTTTCCCAGTAGGTCTCCTCAGGAAG     |
| Influenza A | H5N1                       | SARS: 5–3 GTTTCCCAGTAGGTCTCCTCAAAATCTTCGCA |
| Henipavirus | Nipah virus                | H5N1: 5–3 GTTTCCCAGTAGGTCTCCTCAAAAAATCTTCGCA |
|          |                             | N1: 5–3 GTTTCCCAGTAGGTCTCCTCAGGAAGGCAAC |
|          |                             | Nipah: 5–3 GTTTCCCAGTAGGTCTCAGGGAGGCAGGC   |
presence of aminoally-dUTP. The reaction conditions consisted of a 5 min denaturation step at 94°C, followed by 30 cycles of 94°C for 30 sec, 56°C for 45 sec, and 72°C for 60 sec, and a final extension step at 72°C for 10 min. RNA extraction, cDNA synthesis and PCR amplification of the host cells without viral inoculation were also carried out simultaneously as a negative control [Bohlander et al., 1992; David et al., 2002; Sengupta et al., 2003]. Specific RT-PCR to amplify targeting nucleic acids from HCV plasmids (kindly provided by Dr. Dong from CDC, China) was performed as positive control.

**Hybridization of Labeled PCR Products to Arrays**

After pre-hybridization of the aldehyde-activated silylated microscope slides (CEL Associates, Inc., Houston, TX) with pre-hybridization solution (5 × SSC, 0.1% SDS, 0.1% BSA), PCR products amplified from viral supernatants and from HCV plasmid were mixed together and coupled with Cy3 (Amersham, Inc., London, UK) for labeling. Meanwhile, PCR products from the host cells without infection were coupled with Cy5 (Amersham, Inc.). The resulting labeled products were purified with PCR Purification Kit (Qiagen, Inc.) according to the manufacturer's protocol. The purified mixture was denatured for 5 min at 95°C, cooled on ice for 3–5 min, and then kept on ice until hybridization were performed. Hybridization was conducted by adding 1 μg of purified mixture to hybridization buffer to a final volume of 40 μl. The hybridization buffer contained 5 × SSC, 0.1% sodium dodecyl sulfate (SDS) and 50% formamide. The arrays were hybridized at 42°C for 3 hr followed by three washes at room temperature (5 min in 2 × SSC and 0.2% SDS, 5 min in 0.2 × SSC and 0.2% SDS, and 5 min in 0.075 × SSC) [Ryabinin et al., 2006; Lin et al., 2007].

**Microarray Scan and Data Analysis**

After hybridization, the arrays were analyzed with a ScanArray Gx PLUS microarray scanner (Perkin Elmer Instruments, Boston, MA) at a wavelength of 532 nm for Cy3 and a wavelength of 635 nm for Cy5, with a PMT (photomultiplier tube) gain between 55 and 70. The data was analyzed by the Genepix Pro software. The Cy3 intensity of each viral probe was plotted with a continuous linear color scale. The Cy3 intensity of each viral probe was plotted with a continuous linear color scale. The Cy3 intensity of each viral probe was plotted with a continuous linear color scale. The Cy3 intensity of each viral probe was plotted with a continuous linear color scale.

**RESULTS**

**Oligonucleotide and Primer Design**

To maximize the spectrum of detectable viruses, oligonucleotide probes at the genus level were designed. Oligonucleotide probes specific to each viral genus were designed based on the highly conserved sequences among all the members of a given genus. Sequence similarity between individual 70 nt probes and each viral species in the same genus was assessed by the nucleotide identity score in BLAST. In total, 94 probes were designed to cover nine genera. Similarly, oligonucleotide probes at the species level were selected from the conserved regions of each species. The specificity of oligonucleotides was optimized by excluding probes that could cross-hybridize with other viral species. A total of 80 oligonucleotide probes covering 16 viral species were obtained, each of which was 50 mer. As the viruses included in this assay were all RNA viruses, the primers derived from the genus consensus sequences could specifically lead to the synthesis of viral DNAs from the mixture of RNA sample.

Primer sequences were listed in Table I, and oligonucleotide probes at both genus and species levels were listed in supplemental file 1.

**RT-PCR**

Before hybridization, the viral RNAs were synthesized into double-stranded cDNA and then amplified by random PCR. At the reverse transcription step, 11 primers for nine viral genera were divided into three groups. The reverse transcriptions of first-strand cDNA from each viral sample were performed in parallel in three tubes, each of which contained a group of primers, respectively. Given one virus species, only the reaction tube with the matched primers could specifically initiate the cDNA synthesis of the virus. Through this step, the viral genome could be specifically amplified from a RNA sample mixture. As shown in Figure 1, the PCR products of CHIK and YFV demonstrated the effectiveness of this method. CHIK belongs to genus Alpha, while YFV belongs to genus Flavivirus. Therefore, the CHIK and YFV genomes were specifically synthesized and amplified in the reaction tubes containing the primers of CHIK and YFV, respectively.

![Electrophoresis of randomly amplified products from cultured supernatants of CHIK virus (A) and YFV (B). Lane 1: DL2000 DNA Marker; lane 2: PCR products of group A containing the primers for CCFV-M, Hanta L, Hanta M, and Alpha; lane 3: PCR products of group B containing primers for Flavi, Nipah, H5, and N1; and lane 4: PCR products of group C containing primers for Rift-M, Lassa and SARS. To decrease the interference of host genome in hybridization, 11 consensus genus primers for the viruses were designed and divided into three groups to initiate reverse transcription reaction of each virus sample. After the reverse transcription reaction at 42°C for 1 hr, the synthesis of the second strand was carried out with random primer (5'-GTTTCCCATGAGTCTCCNNN-3'). Then, the products of the three groups were taken as template to amplify into a large amount by using the anchor primer B (5'-GTTTCCCATGAGTCT-3').](image)
groups A and B, respectively. As expected and shown in Figure 1A, when CHIK was used as the template, the amount of PCR products in lane 2 from the reaction tube with group A primers was significantly higher than those of lanes 3 and 4, which contained the primers of groups B and C, respectively. Similarly, as shown in Figure 1B, when YFV was used as the template, the amount of PCR products in lane 3 from the tube with group B primers was significantly higher than those of lane 2 and lane 4, which contained the primers of groups A and C, respectively.

**Detection of Viruses From Cell Culture**

Validation of this microarray was performed by using RNAs extracted from supernatants of viral infected cell cultures. The tested viruses included YFV, CHIK, TBE, DEN, JEV, and H5N1. In all cases, a two-color competitive hybridization was conducted to compare fluorescence-labeled PCR products from virally infected cells with those from uninfected cells. Primary microarray data were converted to a color visualization scheme where the Cy3 intensity was plotted as a linear red color scale (Fig. 2A). The hybridization results from viral infections were demonstrated in Figure 2 B–F, which indicated the success detection of this microarray [Ryabinin et al., 2006].

**Detection of H5N1 From Inoculated Balb-C Mice**

The RNAs were extracted from the spleen of Balb-C mice inoculated with high pathogenic H5N1 influenza virus. After reverse transcription and random PCR amplification, the labeled nucleic acids were hybridized with the microarray. Figure 2G showed the success detection of H5N1 virus from the infected mice.

**Detection of an Isolated Unknown Virus**

An unknown virus was isolated from a pig brain (collected in Shanxi province in July, 2006) and was inoculated in BHK21 cells. RNAs were extracted and amplified by the above-mentioned procedure for subsequent DNA microarray hybridization.

Figure 2H showed the hybridization patterns of PCR products derived from the unknown virus. Strong signals were detected with the Flavivirus genus probes and with the JEV species probes, which suggested that this unknown virus should belong to JEV species. The specific RT-PCR and sequence analysis further confirmed this result (data not shown).

**DISCUSSION**

Traditional viral detection techniques such as in vitro viral cultures, immunologic assays, and PCR [Sengupta et al., 2003] can identify only one or a few specific viral targets in a single test. When a patient presents symptoms of unknown etiology, multiple diagnostic assays have to be performed in parallel, which is a time-consuming and labor-intensive process. DNA microarray has the advantage of high throughput, and therefore it can detect many pathogens at the same time. In this study, a microarray for detecting of some highly pathogenic viruses was developed and validated by using probes at both genus and species levels.

Oligonucleotides microarray has been used widely for monitoring and analyzing viral pathogens [David et al., 2003; Sengupta et al., 2003; Kessler et al., 2004; Jehanara et al., 2005; Chou et al., 2006]. Nucleic acid amplification of viral genomes needs to be performed...
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before hybridization in a DNA microarray. Generic [Coiras et al., 2003], multiplex [Casas and García-Ochoa, 1999; Calvario et al., 2002] and random PCR procedures [Bohlander et al., 1992; Elnifro et al., 2000; David et al., 2002] have been employed for viral genome amplification. Generic PCR is designed to amplify a target sequence from a group of related viruses (e.g., those from the same genus or family). Multiplex PCR involves the combination of several primer sets targeting different sequence regions in one amplification reaction. Viral pathogens amplified by these two methods are limited to small numbers, such as viruses in a genus, or neurological viruses. A random PCR procedure is used to detect a large number of viral pathogens, including unknown or unrecognized viruses [David et al., 2002]. However, this strategy requires complicated computational analysis of hybridization results due to the interference of the host genome. In this study, a panel of consensus primers was designed in order to increase the specificity of reverse transcription and reduce the interference of the host genome from subsequent random PCR procedure. Moreover, by using three groups of reverse transcription primers, this microarray could significantly increase the spectrum of detecting viruses.

It is known that long oligonucleotide probes can tolerate sequence mismatches; while short oligonucleotide probes are more sensitive to single-base mismatches. Therefore, longer probes (70 mer) was designed at the genus level in this study because of their broad screen capacity and shorter probes (50 mer) were used at the species level because of their specific detection capacity.

The principle for designing probes at the genus level or the species level is to search for conserved sequences that can hybridize to all the viruses in a given genus or species, but not to the viruses in other genera or species [Albrecht et al., 2006; Lin et al., 2007]. In general, 60–80% global sequence similarity between two sequences can cause substantial cross hybridization [Kane et al., 2000; Chang and Peck, 2003; Urisman et al., 2005; Albrecht et al., 2006]. Therefore, for genus probes, in order to cover as many viruses as possible in a genus, a 35-nt BLASTN sequence identity match were used. For species probes, in order to improve the detection accuracy, a 50 nt long oligonucleotide was selected whose sequence similarity was lower than 25 nt compared with other viral species.

In this study, different probes targeting the same virus yielded different signal intensities. This may due to the failure of random PCR amplification for some sequences. Four to fifteen probes for each viral genus and five probes for each viral species were designed for this microarray. True positive result was accepted only when two or more than two genus probes and species probes presented as positive signals in hybridization.

Our previous microarray analysis showed that the hybridization of random amplified PCR products of the virus genome without any purification can cause a high rate of cross reaction and give the false positive signals. This may result from the contamination of RNA from host cells. In order to increase the hybridization specificity, the genus primers were designed for reverse transcription of the viral RNAs, which could specifically initiate the virus cDNA synthesis. This step may greatly reduce the random amplification of host-cell genes. Indeed, little cross hybridization between viruses was observed, demonstrating the effectiveness of this method.

In summary, a consensus approach was developed for detection of some highly pathogenic viruses, including CHIK, JEV, YFV, DEN, TBE, and H5N1 avian influenza virus. This microarray was used successfully to identify an unknown virus isolated from a pig brain. This unknown pathogen was identified as Japanese encephalitis virus by the microarray and the result was confirmed by specific RT-PCR and sequence analysis. All these results indicate that this microarray method is an effective tool for detecting these highly pathogenic viruses.

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