Improvement of the Specificity of a Pan-Viral Microarray by Using Genus-Specific Oligonucleotides and Reduction of Interference by Host Genomes

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Rapid detection of viral pathogens is crucial for antiviral therapy. High-density 60–70-mer oligonucleotide microarrays have been explored for broad detection of many viruses. However, relatively low specificity and the complex analytical processes are the major limitations when pan-viral oligonucleotide microarrays are used to detect viral pathogens. In this study, genus-specific oligonucleotides were used as probes and modified sample preparations were carried out to improve the specificity and accuracy of the pan-viral oligonucleotide microarray. Genus-specific 63-mer oligonucleotide probes were used for screening human pathogenic RNA viruses. A total of 628 oligonucleotide probes covering 32 RNA viral genera from 14 viral families were used. The number of oligonucleotide probes was decreased to simplify the analytical process of hybridization and to minimize cross-hybridization. Host genomes were removed by DNase I/RNase T1 digestion before viral nucleic acid extraction, and non-ribosomal hexanucleotides were used for reverse transcription to minimize interference of host genomes. Cultured viruses were used for microarray validation. The microarray was validated by cultured isolates that belonged to five viral genera. By using DNase I/RNase T1 digestion before viral nucleic acid extraction and non-ribosomal hexanucleotides for reverse transcription, the specificity of the microarray was improved. Furthermore, the analytical process of hybridization results was simplified. The specificity of pan-viral microarray could be improved by using genus-specific oligonucleotides as probes and by using non-ribosomal hexanucleotides for reverse transcription. Combined with subsequent degenerate reverse transcriptase-polymerase chain reaction and sequencing processes, this improved genus-specific oligonucleotides microarray provides a relatively flexible strategy for diagnosis of RNA virus diseases. J. Med. Virol. 83:1624–1630, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: oligonucleotide microarray; virus; screening; detection

INTRODUCTION

More than 70% of human infectious diseases are caused by viruses. Among human pathogenic viruses, >80% are RNA viruses [Morse, 1994]. Therefore, rapid detection of these pathogens is crucial for initiation of antiviral therapy and prevention of virus spread.

Enzyme-linked immunosorbent assay (ELISA), reverse transcriptase-polymerase chain reaction (RT-PCR), and real-time RT-PCR are the most common and widely used methods for routine screening of viral pathogens. However, the main limitation of these techniques is that the maximum number of viruses detectable in a single assay is relatively small. DNA
The Specificity of a Pan-Viral Microarray

The specificity of oligonucleotide microarrays to screen human pathogenic RNA viruses was explored. By using oligonucleotide probes only at the genus level, the numbers of oligonucleotide probes printed on arrays was decreased dramatically, which in turn, reduces hybridization specificity and detection sensitivity. Thousands of oligonucleotide probes printed on arrays also make the analysis of hybridization unwieldy.

In the present study, strategies for improving the specificity of oligonucleotide microarrays to screen human pathogenic RNA viruses were explored. By using oligonucleotide probes only at the genus level, the numbers of oligonucleotide probes printed on arrays was decreased dramatically, which in turn, made the analytical process more straightforward. To improve the specificity and accuracy of the microarray, the host genomic DNA and RNA were removed before viral RNA extraction [Chou et al., 2006]. Furthermore, to reduce the interference of host genomes on microarray hybridization, non-ribosomal hexanucleotides were used for reverse transcription. All the known genomic-size viral sequences include non-ribosomal hexanucleotides [Allander et al., 2005], therefore, non-ribosomal hexanucleotides can reverse transcribe viral RNA more efficiently than ribosomal RNA of host cells. Finally, the specificity of the microarray was validated by using cultured viruses from different genera.

MATERIALS AND METHODS

Cultured Viruses and the Unknown Sample

Six cultured viral isolates that belonged to five genera were used in this study for validation. Dengue virus 2 (D2-43) and yellow fever virus (17D) belong to Flavivirus, rabies virus (CGX89-1) belongs to Lyssavirus, Hantaan virus (H8205) belongs to Hantavirus, H3N2 influenza virus (H3-263) belongs to Influenza virus A, and Sindbis virus (HRSP) belongs to Alphavirus. Hantaan virus and YFV 17D infection were induced in Vero E6 cells; rabies virus and Sindbis virus infection in BHK21 cells; Dengue virus infection in C6/36 cells; and H3N2 virus infection in MDCK cells. All of the cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. Hepatitis C virus (HCV) plasmid that contained the whole genome sequence of HCV was constructed and kindly provided by Dr. Weijun Chen from the Institute of Beijing Genome. To validate the microarray, supernatants were collected after the onset of cytopathic effect (CPE) or after culturing for three passages. An unknown cerebrospinal fluid (CSF) sample collected from a patient suspected of having encephalitis syndrome (from Shanxi Province, China) was inoculated and propagated in BHK21 cells.

Primer Design and Microarray Preparation

According to the database of the eighth report of the International Committee on Taxonomy of Viruses (ICTV) submitted in 2005 [Fauquet et al., 2005] (http://phene.cpmc.columbia.edu/ICTV/index.htm) and the taxonomy database of NCBI (http://www.ncbi.nlm.gov/Taxonomy/Browser/Viruses), all pathogenic RNA viruses were selected including double-stranded RNA viruses, single-stranded (ssRNA) negative-strand viruses, and ssRNA positive-strand viruses. A total of 32 RNA viral genera from 14 viral families were included in this study. Viral genome sequences were downloaded from Genbank. To select the most conserved regions, viral sequences in each genus were aligned by software ClustalX (version 1.83). Arraydesigner 4.0 was employed for designing the oligonucleotide probes. Some probes published by other groups [Wang et al., 2002, 2003; Chou et al., 2006] were adopted and modified for this study. Generally, 10–30 oligonucleotides of 63-mer were designed and selected to cover all the isolates in each genus, and in total, 628 viral oligonucleotide probes were included. Tm value of these oligonucleotides was 75 ± 5°C, which was necessary to ensure that all the oligonucleotides had similar hybridization dynamics. By sequence alignment analysis, the significant homology of each oligonucleotide sequence with the sequences in the same genus was >25 nt. Then, the probes were optimized by pairwise BLASTN program to select those sharing <50% sequence similarity with other viral genera. In addition, the oligonucleotide probe (5′-ggagagccatgtgtgggtcct-3′) in the conserved region of HCV genome was selected as a positive control.

Oligonucleotide probes were suspended in 5× SSC (10× SSC: 87.6 g/L NaCl and 44.1 g/L sodium citrate, pH 7.0) at a final concentration of 40 μmol/L. The probes were printed on aldehyde-activated sylated microscope slides (CEL Associates, Pearland, TX). The specificity of a microarray has gained much attention in detecting pathogens due to its high-throughput and the explosive growth of pathogen genomic sequences available in public databases. In recent years, low-density microarrays with short oligonucleotide probes (such as 20–30 mer that are sensitive to single-base mismatches) have been used to detect or identify subtypes of known viruses [Sengupta et al., 2003; Boriskin et al., 2004; Korimbocus et al., 2005; Jääskeläinen et al., 2006]. High-density microarray with long oligonucleotide probes (such as 60–70 mer) have been explored to detect a large number of viruses and identify unknown or unsequenced members of existing viral families [Wang et al., 2002, 2003; Palacios et al., 2007]. However, relatively low specificity and sensitivity and complex analytical processes are the major limitations when pan-viral oligonucleotide arrays are used to detect viral pathogens [Chou et al., 2006]. One reason for low sensitivity and low specificity is the low level of viral nucleic acids compared with that of the host genome within the sample preparation. As nucleic acids from unknown viruses can only be amplified with sequence-independent PCR methods before microarray hybridization, the low level of viral nucleic acids in a sample make it probable that the vast majority of amplicons produced by random amplification do not contain the target sequences of interest, which in turn, reduces hybridization specificity and detection sensitivity. Thousands of oligonucleotide probes printed on arrays also make the analysis of hybridization unwieldy.

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TX) by SpotArray 24 Microarray Printing System (PerkinElmer Instruments, Boston, MA). Each probe was printed in triplicate. Printed arrays were dried at room temperature overnight, and then they were blocked with 0.25% NaBH₄ at 37°C before hybridization.

**Sample Preparation and PCR Amplification**

Cultured viral supernatants and grinded clinical samples were centrifuged at 13,400×g for 5 min, and then the supernatants were filtrated with a 0.22-μm filter. Genomic DNA was removed by DNase I/RNase T1 digestion at 37°C for 2 hr before RNA extraction. RNA was extracted by RNeasy Mini Kit (QIAGEN, Chatsworth, CA) according to the manufacturer’s instructions. First-strand reverse transcription was initiated with Superscript II (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. A mixture of non-ribosomal hexanucleotides linked to a specific primer sequence. The mixture contained 96 non-ribosomal hexanucleotides [Endoh et al., 2005]; each linked to a specific primer sequence. The mixture was then purified with QIAquick PCR Purification Kit. Purified random PCR products and purified together with a 1:9 mixture of non-ribosomal hexanucleotide mixture as primer. Finally, cDNA was amplified in the presence of aminoallyl-dUTP.

- **Microarray Hybridization, Scanning, and Data Analysis**

The aldehyde-activated silylated microscope slides were pre-hybridized with pre-hybridization solution (5× SSC, 0.1% SDS, 0.5 μg/μl salmon sperm DNA). This mixture was applied to the array and incubated at 42°C in a humidified chamber overnight. The arrays were washed at 42°C with 1× SSC that contained 0.1% SDS and 0.1× SSC that contained 0.1% SDS for 5 min, respectively. Finally, the arrays were dried and scanned with a ScanArray Gx instrument (PerkinElmer) that was equipped with a 532-nm laser for Cy3 and a 635-nm laser for Cy5. The scanning results were analyzed by GenePix Pro5.0 software (PerkinElmer), and the Cy3 or Cy5 average intensity of the probes in each viral genus was plotted with a continuous linear color scale by TreeView software. The Cy3 intensity of >2,000 and the ratio of Cy3/Cy5 >2 were used as the criteria for positive identification.

**Microarray Sensitivity and Specificity**

Viral titers of Japanese encephalitis virus (JEV) were measured with plaque forming assay. A total of 1.13 × 10⁴ PFU of JEV was used to determine the microarray sensitivity. The JEV viral stock was 10-fold serially diluted and hybridized with the array. An JEV sequence was detected by this array, which was isolated from CSF of patients with encephalitis syndrome and propagated in BHK21 cells with DMEM that contained 5% fetal bovine serum (FBS). The viral cultures and the isolated sample were pretreated with DNase I/RNase T1. RNA was extracted from 0.25 ml supernatant from each sample and reversely transcribed with the non-ribosomal hexanucleotide mixture as primer. Finally, cDNA was amplified at random by PCR. The hybridization results from pretreated and non-pretreated isolates were analyzed for comparison of the detection specificity.

**RESULTS**

**Validation With Cultured Viruses**

To test the effect of DNase I/RNase T1 digestion and non-ribosomal hexanucleotide mixture during reverse transcription, the hybridization results of pretreated and non-pretreated viral cultures were compared. The non-pretreated RNA was extracted directly from 0.25 ml viral supernatant without DNase I/RNase T1 digestion, and it was transcribed reversely with the random hexamers. A total of six cultured viruses were used for comparison and validation of the microarray. The tested results confirmed that these viral isolates were successfully identified at the genus levels after DNase I/RNase T1 digestion and non-ribosomal hexanucleotide reverse transcription (Fig. 1). The hybridization results of non-pretreated viruses were difficult to interpret due to cross-hybridization (data not shown). The number of oligonucleotide probes decreased dramatically compared to other pan-viral microarrays [Wang et al.,...
2002, 2003; Palacios et al., 2007], therefore, analysis of the hybridization results was simplified significantly because of the reduction in intensive computation. Although non-specific hybridization could not be avoided completely, the positive results could easily be judged by TreeView-analyzed graphs. Furthermore, the specificity of the assays using pretreated cultures was improved significantly compared with non-pretreated cultures (Fig. 2). From the hybridization imaging and TreeView-analyzed graphs, non-specific hybridization was decreased significantly. To evaluate the sensitivity of the microarray, 1.13 × 10^4 PFU of JEV was diluted serially 10-fold and preceded microarray hybridization. The lowest detectable limit of JEV dilution was 1:100, which indicated that the sensitivity of this array for cultured JEV was 113 PFU.

Analysis of Unknown Sample From Patients With Encephalitis Syndrome

An outbreak of encephalitis in Shanxi Province, China, resulted in 66 cases of encephalitis from July 13 to August 17, 2006, and 19 were fatal (29%). Whole blood and CSF were collected from patients with encephalitis syndrome, along with swine brain tissues. Human CSF was inoculated into BHK21 cell line and propagated in DMEM that contained 5% FBS. The onset of CPE was observed after 72 hr incubation. The cultured supernatant was then tested by the RNA genus-specific oligonucleotide microarray for virus screening. Figure 3a shows that the isolated virus belonged to the genus Flavivirus. Furthermore, the specific RT-PCR for JEV was performed for those collected samples. A pair of specific primers was designed from the conserved NS1 region as follows: 5'-GTGCCATTGACATCACAAG-3' (2487–2506, forward) and 5'-TCTGGGAACCCGTACGGGAC-3' (2677–2697, reverse). Figure 3b shows that a 211-bp PCR product was obtained from human CSF and swine brain tissues. Subsequently, the PCR product was sequenced and the result confirmed that the isolated virus belonged to genotype I of JEV, which shared 99% nucleotide identity with the strain JEV_SW_Mie_40_2004-Japan-2004 (GenBank accession number: AB241118.1) isolated in Japan.

DISCUSSION

Pan-viral microarray that employed long oligonucleotide probes (60- or 70-mers) was explored to detect a broad range of viral pathogens. Due to the tolerance of long oligonucleotides to sequence mismatches, these arrays have been used to detect many known viruses and to identify unknown or unsequenced members of existing viral families. Derisi's group first designed 1,600 viral oligonucleotides (70-mers) that represented about 140 viruses in 2002 [Wang et al., 2002], and the number was increased to 10,000, which covered about 1,000 viruses in 2003 [Wang et al., 2003]. They identified the novel SARS coronavirus and confirmed that the combination of array
hybridization followed by direct viral sequence recovery can serve as a general strategy. With this strategy, novel viruses and emerging infectious disease can be rapidly identified and characterized. Lipkin’s group have developed GreeneChipVr version 1.0 that contains 9,477 probes for all vertebrate viruses in the integrated ICTV/NCBI database (1,710 species) [Palacios et al., 2007]. These studies have highlighted the potential of pan-viral microarrays for detection of many known and unknown viral isolate. To increase the specificity, Chou et al. [2006] have developed a new method to design probes using genus and species probe sets to facilitate a cross-examination strategy. The identity of a virus is established by the concordant results for the genus- and species-specific probes. However, too many oligonucleotides make the

Fig. 2. a: Detection of Sindbis virus belongs to Alphavirus by genus-specific RNA viral oligonucleotide microarray with or without sample pretreatment. The hybridization patterns were manifested by using computer-assisted false two-color imaging. Virus infected culture was labeled with Cy5 and non-infected culture was labeled with Cy3 fluorescent molecules. Oligonucleotide probes of one genus were printed in different blocks. Sample pretreatment with DNaseI/RNaseT1 digestion and non-ribosomal hexanucleotides reverse transcription (A) greatly improved the specificity of hybridization than non-pretreatment (B). b: Detection of Hantaan virus belongs to Hantavirus (B, C) and Rabies virus belongs to Lyssavirus (D, E) by genus-specific RNA viral oligonucleotide microarray with or without sample pretreatment. The hybridization intensities were analyzed by GenePix 5.0 and graphed by Treeview software. The average intensity of oligonucleotides in each genus from the microarray is depicted as a vertical stripe. The horizontal distance of each genus is generated by numbers of oligonucleotides in each genera. (A) Indication of different genera (due to the limited space, some viral genera are not displayed); (B, D) hybridization results with sample pretreatment; (C, E) hybridization results without sample pretreatment. (Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com)
analytical program rather complicated. Furthermore, over-printed oligonucleotides on array obviously increase the frequency of cross-hybridization. For parallel screening of a broad spectrum of viruses with fewer probes, 63-mer genus-specific oligonucleotides were employed. By only designing genus-specific oligonucleotide probes, the number of probes decreased significantly to 628 from thousands. The reduction of oligonucleotide probes simplified the analysis of the complex hybridization results, and it also decreased the interference of cross-hybridization. This also made microarrays more flexible for clinical use. Once it is known to which genera the detected viruses belong, further degenerate or specific RT-PCR can proceed. The species can then be identified by sequencing the PCR product or using other more specific protocols.

Compared with the host genomes, the relatively low number of viral nucleic acids is another drawback of microarrays for broad virus screening. The over-representation of the host genome in the total nucleic acid population is a major factor that interferes with microarray analysis. In pan-microbial microarray detection, unknown pathogens are amplified with sequence-independent PCR methods, such as random-primed PCR [Froussard, 1992; Vora et al., 2004; Allander et al., 2005] and sequence-independent single primer amplification (SISPA) [Reyes and Kim, 1991; Linnen et al., 1996; Ambrose and Clewley, 2006]. Both methods are efficient but non-selectively amplify all DNA in the sample. Inevitably, the host genomes are abundantly amplified due to their high representation within total nucleic acids during sample preparations, either from viral cultures or virus-infected clinical specimens. To improve the detection specificity of microarrays, DNase I/RNase T1 digestion was used before RNA extraction, and reversely transcribed RNA with non-ribosomal hexanucleotide mixed primers. As viral nucleic acids are generally protected from DNase degradation by the more stable protein capsids, and sometimes also by a lipid envelope [Allander et al., 2001], DNase I/RNase T1 treatment can effectively remove the host genomes. A large quantity of rRNA from host genomes reduces the efficiency of reverse transcription of viral RNA, therefore, the adoption of non-ribosomal hexanucleotides as primers can enhance the reverse transcription of viral RNA. Daiji et al. [2005] have confirmed that sequences of selected non-ribosomal hexanucleotides are normally present in known viral sequences. Based on this finding, reverse transcription was performed by using these 96 non-ribosomal hexanucleotides; each of which was linked to a specific tag sequence as the primer. This can further decrease the interference of host genomes.

By using the cultured viruses and unknown viral isolate, the specificity and accuracy of the genus-specific RNA viral microarray were validated. The results confirmed the feasibility of this microarray method. The genus-specific oligonucleotide microarray hybridization combined with degenerate or specific RT-PCR, followed by sequencing analysis, could serve as a feasible strategy for the rapid identification and characterization of RNA viruses. As with unknown viral isolate, once it is known to which genus the unknown isolate belongs, subsequent specific RT-PCR and sequencing identification are relatively easy to perform.

CONCLUSIONS

In conclusion, by reducing the number of oligonucleotide probes, the analytical process was simplified, and cross-hybridization of pan-vial microarrays was minimized for broad detection of human RNA viruses. Using DNase I/RNase T1 digestion before RNA extraction.
extraction and non-ribosomal hexanucleotides as primers for reverse transcription, the specificity and accuracy of microarrays were improved through reducing the interference of host genomes. This microarray can be used for screening of cultured viruses and clinical viral isolates. Compared with the complex viral species microarray, this platform is relatively cost effective and flexible.

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